306318674

Abstract

Nuclear Magnetic Resonance Studies of Modified Eukaryotic Cytochromes c

Thesis submitted for the Degree of Doctor of Philosophy

by Andrew Philip Boswell

Trinity Term 1981

Wolfson College.

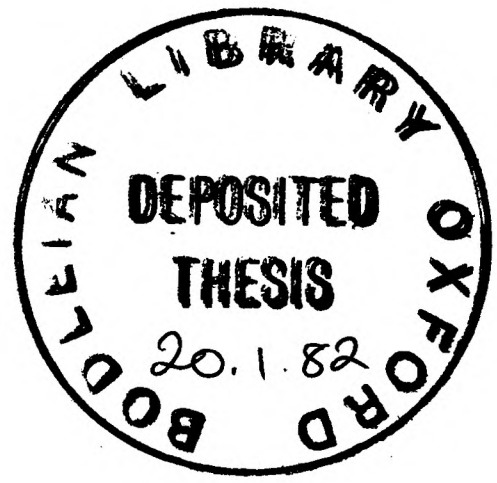
The central theme of this thesis is a study of the structural changes accompaning chemical modification and denaturation of eukaryotic cytochrome c as characterised by ^{1}H nuclear magnetic resonance (n.m.r.) spectroscopy. First, however, it was necessary to obtain and confirm assignments for individual resonances; this was achieved by a novel method of cross assignment between ferricytochrome c and ferrocytochrome c and by double resonance techniques. A variety of perturbations were caused to native cytochromes c, which ranged in degree from the elevation of ~~temperature~~ temperature for ferrocytochrome c to the complete denaturation of the protein with urea or methanol. Modification at single sites both on the surface (e.g. Met 65, Tyr 74) and in the core (e.g. Tyr 67) of the molecule were found to cause only small local effects to the structure, although the dynamic features of the molecules were altered. One single site modification, the breaking of the iron - sulphur cross linking bond, caused considerable disruption to one side of the molecule, although hydrophobic domains in the other side were preserved; this state of the molecule is analogous to the penultimate state in the refolding pathway. Modification of all the charged lysine residues caused small changes to the surface structure of the molecule, though the complete reversal of the

charges in maleyl cytochrome c produced a species which unfolded reversibly from a native configuration with the increase of temperature. The unfolding of the protein is virtually identical with both methanol and urea, but the pathways are shown to differ for the oxidised and reduced proteins.

NUCLEAR MAGNETIC RESONANCE STUDIES OF MODIFIED EUKARYOTIC CYTOCHROMES C

A thesis submitted in partial
fulfilment of the requirements
for the Degree of
Doctor of Philosophy
in the
University of Oxford
by

Andrew Philip Boswell.



Wolfson College
Oxford

Trinity Term
1981

To my parents and to Sally

A C K N O W L E D G E M E N T S

I thank my supervisor, Prof. R.J.P. Williams F.R.S., for the opportunity to carry out the work presented in this thesis, and for his enthusiam, encouragement, and continual help during its course.

I also wish to express my thanks to Dr. G.R. Moore, who taught me how to use n.m.r. spectroscopy for the study of proteins and has given much encouragement and help at all hours of day and night. Much of the work presented in this thesis continues from Geoff's earlier work and I have benefitted greatly in discussions with him from his immense knowledge of the cytochrome c molecule and its ^{1}H n.m.r. spectra.

Many colleagues have helped with the contribution of materials and time. I am specially indebted to Drs. C.J.A. Wallace, D.E. Harris, P. Boon and Prof. R.E. Offord for supplying modified cytochromes c, and to Dr. W. Taylor for building a 'Lab Quip' model of cytochrome c.

I have benefitted greatly from valuable discussions with many people, and the following in particular; Prof. R.J.P. Williams, Drs. G.R. Moore, C.J.A. Wallace, D.E. Harris, F.M. Inagaki, and P. Sadler and Messrs. S. Mann, N.D. Pluck, N.J. Clayden, C.G.S. Eley, M.N. Robinson, and I.Ismail.

Finally, I thank the Science Research Council and the Medical Research Council for providing me with financial support throughout the course of this work.

C O N T E N T S

1. Introduction 1

References 4

2. Brief literature review of the states and structures of eukaryotic cytochrome c 6

2.1. Introduction 6

2.2. The structure of the native states of ferricytochrome c (State III) and ferrocytochrome c (State II) 6

2.2.1. The oxidation state conformation change 6

2.2.2. The structure of eukaryotic cytochrome c 7

2.3. The pH dependent states of ferricytochrome c 9

2.3.1. Ferricytochrome c 9

2.3.2. The alkaline states of ferricytochrome c 12

2.3.3. The nature of the alkaline states of ferricytochrome c 12

2.3.4. The acidic states of ferricytochrome c 15

2.3.5. Ferrocytochrome c 16

References 18

3. ^{1}H n.m.r. assignments and structural studies of native eukaryotic cytochrome c 21

3.1. Introduction 21

3.2.1. The ^{1}H n.m.r. spectrum of a protein and its assignment 21

3.2.2. The previous assignments of ^{1}H n.m.r. resonances of horse cytochrome c 22

3.3.1. Cross assignments of ^{1}H n.m.r. resonances by redox titrations in mixtures of horse ferricytochrome c in rapid electron exchange 29

3.3.2. The electron exchange rate during the redox titration of horse cytochrome c 36

3.4. Assignments for ferrocytochrome c using double irradiation techniques 39

3.5. Studies of lamprey cytochrome c and the assignment of Phe 65 57

3.6.1. Structural studies close to Ile 57 in cytochrome c: Introduction 64

3.6.2. The temperature dependence of the NOEs between neighbouring protons close to Ile 57 67

3.6.3. NOEs observed from Ile 57 in cytochrome c other than horse ferrocytochrome c 75

3.6.4. The pH dependence of Ile 57 in ferrocytochrome c at low pH; the pH titration of lamprey ferrocytochrome c 75

3.6.5. The pH dependence of Ile 57 in ferrocytochrome c at high pH; the pH titration of some modified cytochrome c 77

3.6.6. Summary of structural studies of the region close to Ile 57 82

References 83

4. Singly modified cytochromes c 86

4.1. Introduction 86

4.1.1. The method of chemical modification 86

4.1.2. The method of protein semisynthesis 88

4.2. Modification at methionine 65 in cytochrome c: Introduction 89

4.2.1. $(Hse)^{65}$ horse ferrocytochrome c and $\left[Met(Cm)^{65}\right]$ tuna ferrocytochrome c 90

4.2.2. $(Hse)^{65}$ horse ferricytochrome c and $\left[Met(Cm)^{65}\right]$ tuna ferricytochrome c 100

4.2.3. The binding of platinum complexes to tuna cytochrome c 108

4.2.4. The chemical shift of the S-CH_3 resonance in platinum complexes of N-acetyl methionine and methionine 65 in cytochrome c 115

4.2.5. $(Leu)^{67}$, $(Hse)^{65}$ horse cytochrome c and $(Leu)^{74}$, $(Hse)^{65}$ horse cytochrome c 120

4.3. Monodeamidated cytochromes c 125

References 136

5. Studies of cytochromes c fully modified at positively charged lysine residues 140

5.1. Introduction 140

5.2. The solution structures of cytochrome c and four fully lysine modified derivatives 142

5.3. Maleyl cytochrome c 153

5.4. The pH titrations of N -acetimidylated cytochrome c 160

References 166

6. ^1H n.m.r. studies of derivatives of cytochrome c with Met 80 replaced as sixth ligand to the haem group 168

6.1. Introduction 168

6.2.1. The ^1H n.m.r. spectra of ferricytochrome c-CN and Cm-ferricytochrome c-CN: Introduction 171

6.2.2. A brief comparison of the spectra of ferricytochrome c-CN and Cm-ferricytochrome c-CN 172

6.2.3. The spectrum of ferricytochrome c-CN 175

6.3.1. The study of the unstable species ferrocytochrome c-CN: Introduction 188

6.3.2. The recording of a spectrum of ferrocytochrome c-CN 189

6.3.3. The spectrum of ferrocytochrome c-CN 190

6.4.1. Studies of Cm-ferrocytochrome c-CN: Comparison with ferrocytochrome c-CN 197

6.4.2. The spectrum of Cm-ferrocytochrome c-CN 200

References 209

7. Studies of the unfolding of cytochromes c 211

7.1. Introduction 211

7.2. A brief resume of the theories of protein folding and the dynamic structure of proteins 212

7.3. ^{1}H n.m.r. studies of the denaturation of cytochrome c 217

7.3.1. The methanol titration of ferricytochrome c 218

7.3.2. The methanol titration of ferrocytochrome c 221

7.3.3. The comparison of the methanol denaturations of ferricytochrome c and of ferrocytochrome c 227

7.3.4. The urea titrations of ferricytochrome c 231

7.3.5. The urea denaturation of ferrocytochrome c 239

7.3.6. The denaturation pathways of cytochrome c: Summary 240

References 241

8. Summary and Conclusion: Overview of chemically modified cytochromes c described in this thesis 245

References 255

CHAPTER ONE

Introduction

This thesis describes an in depth study of the effect of chemical modifications and denaturing conditions upon a small globular protein molecule, eukaryotic cytochrome c (Mol. Wt. = 13.000 daltons), using high resolution ^{1}H nuclear magnetic resonance*. Cytochrome c was chosen because;

i) several X-ray crystallographic studies of its three dimensional structure have been carried out for the protein in both oxidation states and from several species (1,2,3,4). The latest study is at 1.8Å and 1.5Å resolution for tuna ferricytochrome c and ferrocytochrome c respectively (5), and thus the structure of the protein in crystals is well defined.

ii) the ^{1}H n.m.r. spectrum of the protein in both oxidation states and under a variety of conditions is well defined (6,7,8) and many assignments for individual protons have been obtained (9,10,11). Furthermore, the solution structure determined by ^{1}H n.m.r. and the crystal structure have been shown to be consistent (12), and ^{1}H n.m.r. has also provided information on the dynamic structure of the molecule (13,14).

iii) the protein contains elements of structural rigidity (as defined by Williams (15)). The previous ^{1}H n.m.r. work shows that the interior of the protein is 'more closely related to rigid models constructed from X-ray diffraction data than any other protein yet examined (15)' and that the protein shows no significant expansion with increased temperature up to its denaturation transtion (16). This rigidity, combined with the close folding of the protein chain around its haem 'template' (17) provides an ideal molecular framework upon which

* henceforth, n.m.r.

to determine the extent of the perturbations induced to such a structure by modifications at single and multiple sites.

iv) a large variety of modifications have been carried out upon the protein, which are well documented (for a review of such work up to 1976, see 18) and the protein has been the subject of protein semisynthetic projects in several laboratories (19,20,21,22).

^{1}H n.m.r. spectroscopy is a most useful technique for studying the effect of modification upon the conformation of such a small protein in solution, provided that many protons in the molecule are assigned to resonances in the spectrum and can be observed individually when the protein is placed in a variety of conditions, including after modification. The first task in the thesis is the assignment of resonances, which is a very time consuming process. The majority of the cytochrome c resonance assignments used are from Moore and Williams' and previous author's work (9,10) but further assignments and confirmation of previously tentative assignments are given here. In Sections 3.1. and 3.2. assignments obtained by nuclear Overhauser effects, sequence substitutions and the study of the ^{1}H n.m.r. spectrum of mixtures of ferricytochrome c and ferrocytochrome c in fast exchange are described. These provide a basis for the discussion of results presented in later chapters. Some assignments have also originated from the study of the chemically modified proteins themselves, and these are described in the appropriate chapters.

If a globular protein, such as cytochrome c, is defined to be native only under very specific conditions, namely when dissolved in H_2O (or ^2H_2O), at pH 7, at 25°C, the concept of modification can be extended to changes of the properties of the medium; for example, a change in solvent or a change in temperature. Using such a broad terminology the following types of protein modification can occur and are the basis of the bulk of this thesis:

a) the change of one of the bulk properties of the medium e.g. the gradual

change of the solvent structure, or the elevation of the temperature. The gradual alteration of the solvent is effectively a gradual modification to the surface of the protein and can lead eventually to the disruption of the whole structure. Titrations of cytochrome c with methanol and urea are described in Chapter 7. The temperature dependence of the ^{1}H n.m.r. spectrum of eukaryotic cytochrome c has been thoroughly studied (13,16) and is extended in this thesis by the study of the temperature dependence of nuclear Overhauser effects between resonances from protons in one region of the protein structure (Section 3.3).

b) the change of the primary sequence of the protein. This can arise from sequence changes between different species of cytochrome c, or by chemical modification of the side chains of amino acid residues, either by direct means or protein semisynthesis. A chemical modification may be at a single site or at many sites in the protein structure, and may leave the charge of the protein unaltered or drastically altered, as in the case of fully maleylating all 19 lysine residues in horse cytochrome c (see Section 5.3.). Examples of all these types of modification appear in this thesis.

c) the change of the bulk properties of the medium and the primary sequence of the protein (i.e. both a) and b)). This arises, when the pH is altered and the exact primary sequence of the protein is altered by loss or acceptance of protons. Several pH titrations are described in this thesis.

d) The change of tertiary structure of the protein without a change in primary sequence. This is afforded in cytochrome c by the breaking of one of the cross linking bonds of the protein, the iron-sulphur bond at the coordination centre(17), by the addition of cyanide or another strong ligand to the haem iron. Such complexes of the protein are studied in Chapter 6.

References: Chapter One

1. Dickerson, R.E., Takano, T., Eisenberg, D., Kallai, O.B., Samson, L., Cooper, A., and Margoliash, E. (1971), J.Biol.Chem., 246, 1511.

2. Swanson, R., Trus, B.L., Mandel, N., Mandel, G., Kallai, O.B., and Dickerson, R.E. (1977), J.Biol.Chem., 252, 759.

3. Takano, T., Trus, B.L., Mandel, N., Mandel, G., Kallai, O.B., Swanson, R., and Dickerson, R.E. (1977), J.Biol.Chem., 252, 776.

4. Tanaka, N., Yamane, T., Tsukihara, T., Ashida, T., and Kakudo, M. (1975), J.Biochem. (Tokyo), 77, 147.

5. Takano, T., and Dickerson, R.E. (1980), Proc.Nat.Acad.Sci.,

6. Wuthrich, K. (1969), Proc.Natl.Acad.Sci., 63, 1071.

7. Mc. Donald, C.C., and Phillips, W.D. (1973), Biochemistry, 12, 3170.

8. Moore, G.R., and Williams, R.J.P. (1975), FEBS Lett. 53, 334.

9. Campbell, I.D., Dobson, C.M., Moore, G.R., Perkins, S.J., and Williams, R.J.P. (1976), FEBS Lett. 70, 96.

10. Moore, G.R., and Williams, R.J.P. (1980), Eur.J.Biochem. 103, 493.

11. Moore, G.R., and Williams, R.J.P. (1980), Eur.J.Biochem. 103, 503.

12. Moore, G.R., and Williams, R.J.P. (1980), Eur.J.Biochem. 103, 533.

13. Moore, G.R., and Williams, R.J.P. (1980), Eur.J.Biochem. 103, 513.

14. Keller, R.M., and Wuthrich, K. (1981), accepted for publication.

15. Williams, R.J.P. (1979), Biol.Rev. 54, 389.

16. Moore, G.R., and Williams, R.J.P. (1980), Eur.J.Biochem. 103, 523.

17. Dickerson, R.E., and Timkovitch, R. (1975), in The Enzymes (Boyer, P.,ed) 3rd edn., vol. 11, pp. 397 - 547, Academic Press, New York.

18. Ferguson-Miller, S., Brautigan, D.L., and Margoliash, E. (1976), in The Porphyrins (Dolphin, D., ed.)

19. Corradin, G., and Harbury, H.A. (1974),Biochem.Biophys.Res.Comm., 61, 1400.

20. Boon, P.J., Tesser, G.I., and Nivard, R.J.F. (1979),Proc.Natl.Acad.Sci., 76, 61.

21. Nix, P.T., and Warme, P.K. (1979),Biochem.Biophys.Acta., 578, 413.

22. Wallace, C.J.A., and Offord, R.E. (1979), Biochem. J. 179, 169.

CHAPTER TWO

Brief literature review of the states and structures of eukaryotic cytochrome c

2.1. Introduction

Respiratory cytochrome c from the mitochondrion is perhaps the best known and certainly the most intensively studied of all electron transport proteins. It is small (Mol. Wt. = 13,000 daltons) and is easily extracted from ruptured mitochondria, in contrast to other protein components of the electron transport chain which are larger and generally membrane bound. It, also, has very great stability in its native state under *in vitro* conditions. It was discovered during the spectroscopic studies of MacMunn in 1887 (1), but neglected until its virtual rediscovery by Keilin in 1925 (2). Since then, it has been studied by Theorell and the Stockholm school (3) and in countless other laboratories (4,5,6). Amino acids sequences of proteins have been determined to date for over 100 plants, animals and microorganisms. This chapter provides a brief review of literature concerning: i) the structures of the protein in its native oxidised and reduced states, and ii) the pH dependent states of the oxidised and reduced proteins.

2.2. The structure of the native states of ferricytochrome c (State III) and ferrocytochrome c (State II)

2.2.1. The oxidation state conformation change

For many years one of the main questions asked about cytochrome c was 'is there a conformation change upon change of oxidation state?' (e.g. see 6). Recently this question has been resolved by three independent investigations, and these will be outlined briefly before the discussion proceeds to a description of the structure.

i) One of the only significant differences between the ^{1}H n.m.r. spectra

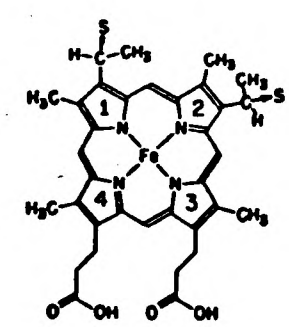
of horse ferrocytochrome c and cobalticytochrome c (a diamagnetic model for ferricytochrome c) is the chemical shift position of the δCH_3 group of Ile 57 (7). The same resonance is the only assigned resonance that does not shift towards its diamagnetic position with increasing temperature in the spectrum of ferricytochrome c (8). Both these results imply a conformational change close to Ile 57 upon the change of oxidation state.
ii) The ε-amino groups of Lys 39, 53 and 55 were the only ones (out of a total of 19 lysines) to show an oxidation state dependence for their reactivity with acetic anhydride - they were significantly less reactive in ferrocytochrome c (9). These lysines are close to Ile 57 (10).
iii) The latest x-ray structure (10) shows that there is a slight displacement of the peptide backbone between residues 39 and 55 upon an oxidation state change in crystals of tuna cytochrome c.

All these results are consistent and show that there is a very minor conformation change on the back surface of the molecule upon the change of oxidation state. The size of the conformation change is not sufficient to require separate descriptions of the whole structures of ferricytochrome c and ferrocytochrome c. The conformation change may also be considered as a change upon modification of the protein - i.e. modification by electron addition to the Fe(III) atom of ferricytochrome c.

2.2.2. The structure of eukaryotic cytochrome c

The protein consists of a haem c unit attached to a single polypeptide chain of 103 to 113 amino acids by two (or in exceptional cases, one) covalent thioether bridges; the points of attachment are two evolutionary conserved cysteine residues. The protein chain is attached to the haem

Haem c, with the conventional numbering of the pyrrole rings.



by two further bonds in the native protein, namely those of the fifth and sixth ligands to the iron atom; these ligands were finally established as histidine 18 (4) and methionine 80 (11,12) respectively. Thus there are four cross linking bonds in the internal space of the protein and these contribute to the stability of the protein. The weakest of these bonds is the iron - sulphur (Met 80) bond, which is weaker in the oxidised protein than in the reduced protein, and which is displaced easily by other ligands, either from the protein (as in the alkaline state of ferricytochrome c, see Section 2.3.3.) or from the external environment (e.g. OH^- or CN^-). The presence of this ~~band~~ bond can be determined by the presence of a charge - transfer band at 695 nm. in the absorption spectrum of ferricytochrome c (13) or the observation of proton resonances from the methionine methyl protons at known chemical shift positions in the ^1H n.m.r. spectra of ferricytochrome c and ferrocytochrome c (14).

The static fold of the peptide backbone and its orientation to the haem group, as determined by X-ray crystallography, is shown in Fig. 2.1. ^1H n.m.r. results show that the protein expands very little with increase of temperature (15) and indeed that the main fold is so tight in the interior of the protein that several aromatic side chains are effectively immobilised on an n.m.r. timescale at 27°C (16), having extremely high activation energies for ring flipping (over 100 kJ mol^{-1}) (16). There are three regions of the protein, where the protein fold is so tight that motion is hindered and these are in hydrophobic domains centred around the haem group (e.g. Tyr 67) and the aromatic rings of Phe 46 and Tyr 48 and of Phe 10 and Tyr 97 (in horse cytochrome c). The ^1H n.m.r. spectra show that the remaining aromatic rings of the protein are not hindered, and in one part of the molecule around Tyr 74 and Ile 57 on the back surface+ there is a significant, but very localised, temperature dependent displacement of the relative positions of some amino acid side chains. This

+, the front surface of the molecule is defined to contain the haem cleft.

is more fully discussed in Section 3.3.1.

These dynamic aspects of the protein are illustrated in Fig. 2.2. This diagram is based upon considering cytochrome c as a typical 'oil drop model' globular protein (18) with charged and hydrophilic residues on its surface and uncharged and hydrophobic residues filling the internal space. The model is extended so that the hydrophobic core is divided into zones or domains which possess different dynamic properties (this is consistent with the more detailed account of the theory of protein structure given in Section 7.2.); as discussed above there are three main regions of hindered motion in cytochrome c. The effect of protein modification to both the static and dynamic features of the molecule will be discussed in later sections of this thesis.

2.3. The pH dependent states of ferricytochrome c and ferrocytochrome c

2.3.1. Ferricytochrome c

In 1941 Theorell proposed five distinct pH dependent equilibrium conformational states of ferricytochrome c (19), which are shown in the equilibrium:

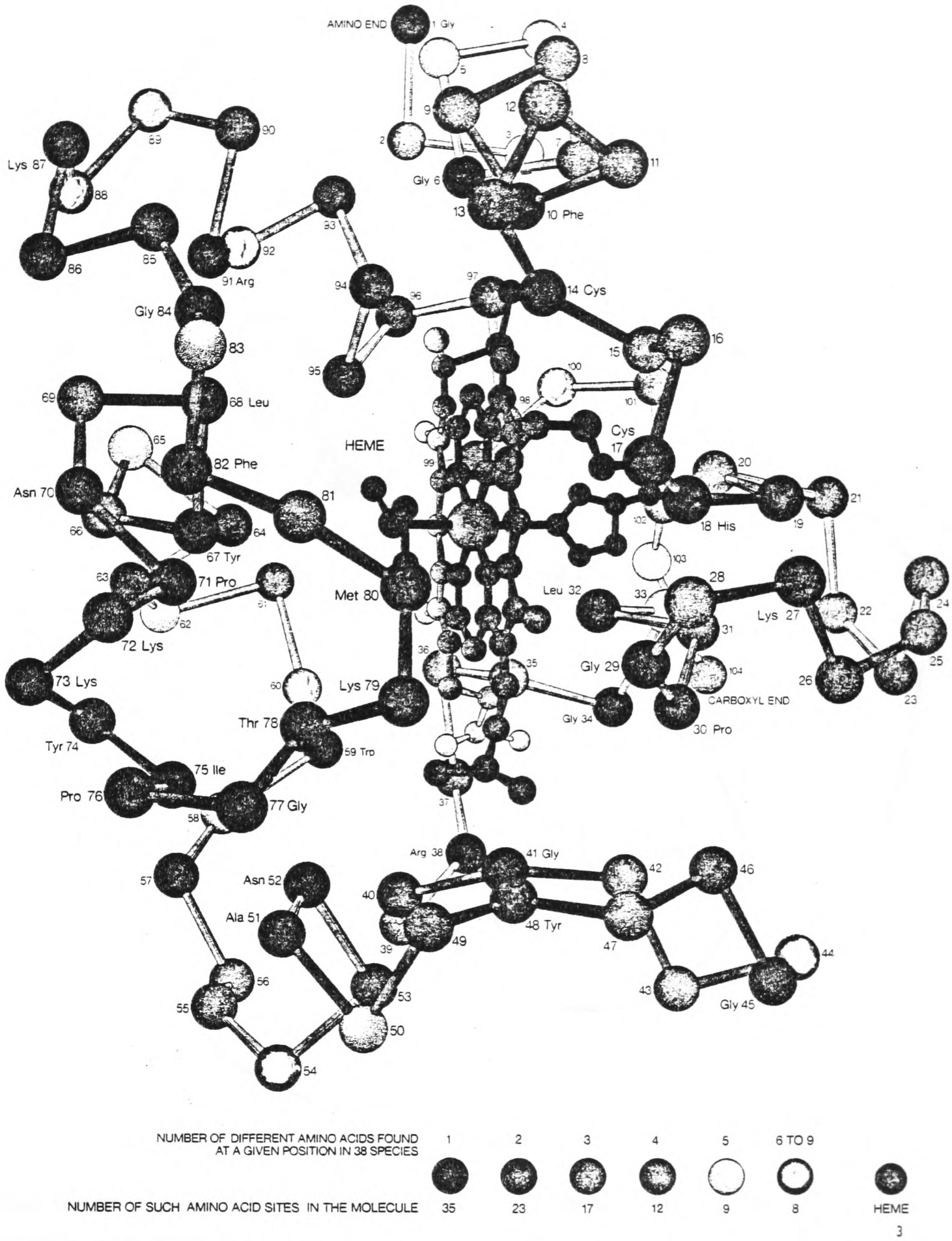
$$\mathrm{I} \underset{0.42}{\rightleftharpoons} \mathrm{II} \underset{2.5}{\rightleftharpoons} \mathrm{III} \underset{9.2}{\rightleftharpoons} \mathrm{IV} \underset{12.76}{\rightleftharpoons} \mathrm{V}$$

These were determined from the pH dependence of the absorption spectrum of the protein. State III in this equilibrium is taken to be the native and physiologically active state of ferricytochrome c. In this section, it is proposed that there are at least seven distinct equilibrium conformational states, that can be defined in terms of the physicochemical data now available for ferricytochrome c, and that these may be written as follows:

$$\mathrm{I} \underset{0.42}{\rightleftharpoons} \mathrm{II} \underset{2.5}{\rightleftharpoons} \mathrm{III} \underset{9.2}{\rightleftharpoons} \mathrm{IV} \underset{11.0}{\rightleftharpoons} \mathrm{V} \underset{12.2}{\rightleftharpoons} \mathrm{VI} \underset{13.5}{\rightleftharpoons} \mathrm{VII}$$

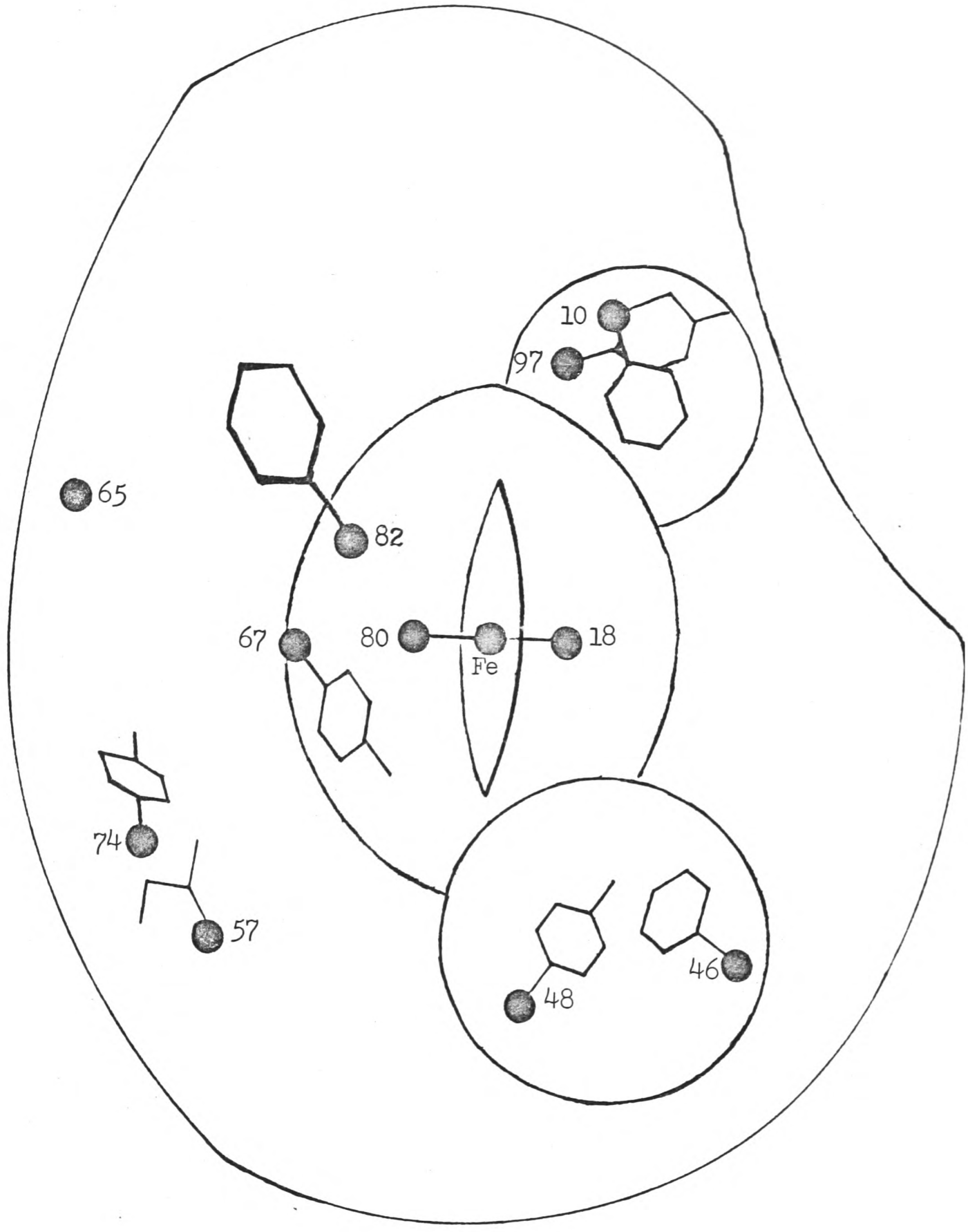
State III remains the native state. As stated in Chapter 1 a pH titration may be considered to give rise to other states of a protein that are protein modifications. The alkaline states will be described first.

Fig. 2.1.



The peptide backbone skeleton of eukaryotic cytochrome c, as viewed from the front of the molecule (from Dickerson, R.E. (1972), Scientific American). The shading of the circles indicates the frequency of variation of amino acid residues at individual positions among 38 species (see key); amino acid types are only given for residues which are invariant within the 38 species.

Fig 2.2.



A sketch of horse cytochrome c viewed from the front (the figure is on the same scale and from the same viewpoint as Fig. 2.1., for comparison). The figure is designed to show the three regions of known hindered motion in the molecule; the haem crevice (e.g. Tyr 67), and the two hydrophobic domains containing Phe 46/Tyr 48 and Phe 10/Tyr 97. Regions outside those marked have not been found to have hindered motion on a ^{1}H n.m.r. timescale and examples of well characterised residues are given (e.g. Ile 57, Met 65 Tyr 74 and Phe 82); a further residue of this category is Phe 36 on the back surface of the molecule behind the haem crevice (not marked).

2.3.2. The alkaline states of ferricytochrome c

Theorell and Akeson (19) detected two changes in the absorption spectra of ferricytochrome c between pH 7 and 14. The first is the loss of the native 695 nm. band with a pK of 9.3 and the second is the shift of the Soret band from 408 nm. to 412 nm. with a pK of 12.76. The first pK at 9.3 is commonly known as the alkaline isomerisation of ferricytochrome c. Morishima et al (20) have reported a detailed ^{1}H n.m.r. study of ferricytochrome c at pH values of 7 to 12.5 and their results indicate pKs at ca. 9.2, ca. 11.0 and ca. 12.2 between species of ferricytochrome c that are differentiable on the basis of their high field hyperfine shifted haem resonances. All these species possess low spin ferric ion as determined by absorption spectra and ^{1}H n.m.r. A resonance Raman study (21) indicates a further pK at 13.5, and shows that the III to IV and V to VI transitions are very temperature dependent.

2.3.3. The nature of the alkaline states of ferricytochrome c

The nature of state IV ferricytochrome c and in particular its sixth ligand has been a subject of much controversy in the literature (6 and refs. therein), and in this section this and the other alkaline states are discussed.

Firstly, there is threefold evidence that the III to IV transition involves the deligation of the native sixth ligand, the sulphur atom of Met 80:

i) The transition involves the loss of the 695 nm. band, which is assigned to a charge transfer from the sulphur ligand to the ferric ion.

ii) The Met 80 methyl resonance in the ^{1}H n.m.r. spectrum of ferricytochrome c is lost with a pK of 9.2 (22) as pH is increased. When the Met 80 methyl is enriched with ^{13}C, the ^{13}C n.m.r. spectra of ferricytochrome c only possess a resonance from this methyl above a pK of 9.2, at a chemical shift position and linewidth that suggests that the group is not close to the haem group (23). At lower pH values the resonance is not observed

due to being broadened because of its proximity to the ferric ion.

iii) The ^{1}H n.m.r. spectra of state IV ferricytochrome c at pH 9.9 and of carboxymethyl$^+$- ferricytochrome c between pH 7 and 9.9 are virtually identical. The resonances from the haem methyl groups are at identical chemical shift positions in the downfield region of the spectrum for both species and this indicates that the haem coordination is almost certainly identical in both cases (20). As Met 80 can not ligate the haem in the latter species, due to being modified by a carboxymethyl group, it follows that it is not coordinated in State IV ferricytochrome c.

Secondly, the sixth ligand in states IV and V is the same, because;

i) no change in the absorption spectrum of ferricytochrome c is seen between pH 9.2 and 12.2 (19).

ii) the pK at 11.0 in the ^{1}H n.m.r. spectrum causes only a small change to the haem resonances indicating a similar or identical ligand in states IV and V (20,22).

iii) No frequency shifts in the resonance Raman spectrum of ferricytochrome c that are significant of ligand exchange are observed between pH 9.2 and 12.0 (21).

Thus the IV to V transition corresponds to a change in the polypeptide chain and not in the coordination site.

Thirdly, the sixth ligand in state VI is most likely to be hydroxide, on the basis of e.p.r. (24) and spectrophotometric (19) studies. Lastly, state VII is most likely to possess hydroxide as both fifth and sixth ligands to the haem (i.e. His 18 is replaced by hydroxide ion at pH 13.5).

The above points suggest assignments for the sixth ligand in all alkaline states of ferricytochrome c except states IV and V. We now return to state IV (and state V, which has the same sixth ligand as state IV) and the following points are made:

+ cytochrome c in which both Met 65 and Met 80 are carboxymethylated. the compound is discussed further in Chapter 6.

i) Evidence that the state III to IV transition is not a simple one step reaction came from two results. Firstly, ferricytochrome c is reduced by ascorbate at alkaline pH in a biphasic reaction consisting of a fast bimolecular reaction followed by a slow first order process (25). Secondly, the rates of oxidation and reduction of cytochrome c in the following system

$$Fe(CN)_6^{4-} + \text{cyt } c^{III} \rightleftharpoons Fe(CN)_6^{3-} + \text{cyt } c^{II}$$

are pH independent in the range pH 7.0 to 9.4 (26), although the redox potential drops from +0.26V at pH 7 to +0.12V at pH 10 with a pK of 9.3 (27). Schejter showed by equilibrium studies and subtle kinetic studies of the disappearance/appearance of the 695 nm. band and the release/uptake of hydrogen ions that the following mechanism is operative (28) at pH values below pH 10 (29):

$$\underset{(\text{695 nm. band present})}{III = HC} \xrightleftharpoons{pK_H=11} H^+ + \underset{(\text{no 695 nm. band})}{B} \xrightleftharpoons[0.05s^{-1}]{6.0s^{-1}} A = IV \quad +$$

i.e. deprotonation occurs first (with a pK of 11) forming a metastable state B, which rearranges (with a half life, $t_{\frac{1}{2}} = 0.16$ s) to A or IV by the deligation of Met 80. Note that the equilibrium B to A has an equilibrium constant of ca. 100 and that the overall pK as determined by the products of the individual constants is ca. 9.0 close to the values from equilibrium studies. The most important point from this is that the group that deprotonates in the III to IV transition has a pK of ca. 11, not ca. 9, and is either a tyrosine (pK = 10), an arginine (pK = 12) or a lysine (pK = 11) (30).

ii) Bosshard (31) reports the pK of Lys 79 to be 11.3 and 10.2 in states III and IV respectively and that Lys 79 is buried in state IV.

iii) Two surface interactions are important in stabilising the haem crevice of ferricytochrome c; 'the top haem crevice bond', a salt bridge between Lys 13 and Glu 90, and 'the bottom haem crevice bond', a hydrogen bond

+ Schejter's terminology is used here.

between the amino group of Lys 79 and the backbone carbonyl of residue 47 (varies with species, Thr in horse) (42,10). If either bond is destabilised or broken, and particular the 'bottom' bond, the pK for the III to IV transition is lowered (42).

iv) Results from ^{1}H n.m.r. (20) and from e.p.r. measurements (24) indicate the sixth ligand in state IV to be a nitrogenous base.

v) There is much evidence, reviewed thoroughly in ref. 4, that suggests that the sixth ligand in state IV is a lysine. Many authors suggest Lys 79.

The following interpretation of all the above data is given here. Evidence from points ii), iii) and v) show that Lys 79 is involved in the III to IV transition. The deprotonation step HC to B is the deprotonation of Lys 79, which breaks the 'bottom haem crevice bond' and destabilises the haem crevice in state B. Then state B rearranges on the Met 80 side of the haem group to produce state A or IV. The ligand in A is a lysine and most likely Lys 79, although this remains to be proven; Lys 72 has also been suggested (32).

The state III to IV transition is of interest to the general theme of this thesis - modifications of cytochrome c. State B may be considered as a single site modification of state HC; namely the native state (III) with Lys 79 deprotonated. This modification causes a drastic destabilisation, and hence change of structure, to the protein.

2.3.4. The acidic states of ferricytochrome c

Many authors have noted that below pH 2.5 the spin state of ferricytochrome c changes from low spin (state III) to high spin (state II) concomitantly with the appearance of a band at 620 nm. in the absorption spectrum (19,33). There is a second transition at pH 0.42 to a completely denatured high spin form (state I). The state III to II transition apparently results from the ionisation of His 18 (34). There is an unfolding of the polypeptide chain at the pK of 2.5 following by further gradual unfolding as the pH is lowered to 0.42. A recent stopped flow

circular dichroism study of the state III to II transition used a pH jump from pH 6.0 to pH 1.6 to observe that the kinetics occur by two steps;

i) the low spin to high spin transition occurs in less than 2 ms.

ii) a slower step of the unfolding of the polypeptide chain (35). The fifth and sixth ligands in state II are from the bulk medium and are usually H_2O or Cl^- (depending on the NaCl concentration) (6).

2.3.5. Ferrocytochrome c

Ferrocytochrome c is much more stable than its oxidised counterpart and in accord with this the pH range of its physiologically active conformation is greater. The physical and spectroscopic properties of the protein have been studied under a variety of conditions (36,37) and at 25°C it can exist in three pH dependent states (6), as shown in the following equilibria:

$$\text{I} \underset{4.2\ (27^\circ\text{C})}{\overset{4.4\ (57^\circ\text{C})}{\rightleftharpoons}} \text{II} \underset{12.7(57^\circ\text{C})}{\overset{11.6(57^\circ\text{C})}{\rightleftharpoons}} \text{III}$$

Moore (38) has made a comprehensive study of the equilibria and the nature of the groups that ionise in the state II conformation between pH 4 and pH 12, by ^1H n.m.r. at various temperatures and the pKs quoted are from his work. Some aspects of Moore's work are continued in this study, and so this work, and particularly that relevant to this thesis, is summarised below:

i) Very few proton resonances are perturbed until a point immediately prior to the denaturation, which occurs over a very narrow pH range. This indicates a cooperative transition in both cases.

ii) When titrating towards the II to I transition the only resonances that are affected are those of His 26, Ile 57 and Trp 59. His 26 is in a hydrophobic pocket of the molecule and is hydrogen bonded to Pro 44 (39). This interaction lowers its pK and is important in stabilising the structures of state III ferricytochrome c and state II ferrocytochrome c. It

ionises just before denaturation, as the carboxyl groups in the protein ionise, and hence its ionisation is probably the result rather than cause of denaturation (38). The shifts observed to Ile 57 and Trp 59 are the basis of further experiments described in Chapter 3 of this thesis.

iii) In state II ferrocytochrome c one ionisation is observed between pH 6.0 and pH 9.0, which is that of His 33 with a pK of 6.5 (27°C). This residue ionises in ferricytochrome c with a pK of 6.4 (27°C) (39). It is a surface residue, but not close to the proposed oxidase and reductase binding sites (40,41), so its ionisation should not affect the physiological reaction.

iv) A number of resonances shift above pH 9.5 (27°C and 57°C) in ferrocytochrome c. These are all due to surface residues and mostly reflect the ionisations of lysine residues that perturb the surface conformation. Between pH 9.5 and pH 12.7 (57°C) a downfield shift of 0.43 ppm is observed on the δCH_3 resonance of Ile 57 - this effect is further investigated in chapter 3.

v) Some 'new' aromatic resonances appear above pH 12. These are studied further in chapter 5, where a pH titration is described for fully N^{ε}- acetimidylated ferrocytochrome c.

vi) It is interesting to note that the effects described in iv) and v) start above pH 9.5, which is the pH above which ferrocytochrome c become autoxidisable and reactive towards CO and CN^- (36). This implies that the changes observed in the ^1H n.m.r. spectra between pH 9.5 and pH 12.5 lead to a loosening of the peptide chain around the haem group, so the haem crevice is more easily penetrated by small ligands.

vii) The process that occurs in both the state II to I and state II to III transitions is the deligation of Met 80. The sixth ligand is probably H_2O and OH^- in states I and III respectively (6,38), though the exact nature of either state is not known.

References: Chapter Two

1. MacMunn, C.A. (1887), J. Physiol., 8, 51.

2. Keilin, D. (1966), 'The History of Cell Respiration and Cytochrome', Cambridge University Press.

3. Åkeson, Å., and Ehrenberg, A. (1972), editors 'Structure and function of Oxidation and Reduction Enzymes', Pergamon Press, Oxford.

4. Margoliash, E., and Schejter, A. (1966), Adv.Protein Chem., 21, 113.

5. Lemberg, R., and Barrett, J. (1973), 'Cytochromes', Academic Press, New York.

6. Dickerson, R.E., and Timkovitch, R. (1975), In 'The Enzymes' (P.D.Boyer, editor), Third Edition, Vol. XI, pp. 397 - 547.

7. Moore, G.R., Williams, R.J.P., Chien, J.C.W., and Dickinson, L.C. (1980), J.Inorg.Biochem., 12, 1.

8. Moore, G.R., and Williams, R.J.P. (1980), Eur.J.Biochem. 103, 523.

9. Bosshard, H.R., and Zurrer, M. (1980), J.Biol.Chem., in press.

10. Takano, T., and Dickerson, R.E. (1980), Proc.Natl.Acad.Sci., in press.

11. McDonald., C.C., Phillips, W.D., and Vonogradov, S.N. (1969), Biochem.Biophys.Res.Comm., 36, 44.

12. Wuthrich, K. (1970), Struct.Bonding, 8, 53.

13. Schecter, E., and Saludjian, P. (1967), Biopolymers, 5, 788.

14. Moore, G.R., and Williams, R.J.P. (1980), Eur.J.Biochem. 103, 503.

15. Moore, G.R., and Williams, R.J.P. (1980), Eur.J.Biochem, 103, 523.

16. Campbell, I.D., Dobson, C.M., Moore, G.R., Perkins, S.J., and Williams, R.J.P. (1976), FEBS Lett. 70, 96.

17. Keller, R.M., and Wuthrich, K. (1981), Biochim.Biophys.Acta., in press.

18. Mahler, H.R., and Cordes, E.H. (1971), 'Biological Chemistry', 2nd edition, Harper and Row, New York.

19. Theorell, H., and Åkeson, Å. (1941), J.Amer.Chem.Soc. 63, 1812.

20. Morishima, I., Ogawa, S., Yonezawa, T., and Iizuka, T. (1977), Biochim.Biophys.Acta, 495, 287.

21. Kihara, H., Hon-mani, K., and Kitagawa, T. (1978), Biochim.Biophys.Acta, 532, 337.

22. Gupta, R.K., and Koenig, S.H. (1971), Biochem.Biophys. Res.Comm., 45, 1134.

23. Schejter, A., Ianir, A., Vig, I., and Cohen, J.S. (1978), J.Biol.Chem., 253, 3768.

24. Brautigan, D.L., Feinberg, B.A., Hoffman, B.M., Margoliash, E., Peisach, J., and Blumberg, W.E. (1977), J.Biol.Chem., 252, 574.

25. Greenwood, C., and Palmer, P. (1965), J.Biol.Chem., 240, 3660.

26. Brandt, K.G., Parks, P.C., Czerlinski, G.H. and Hess, G.P. (1966), J.Biol.Chem., 241, 4180.

27. Rodkey, F.L., and Ball, E.G. (1950), J.Biol.Chem., 182, 17.

28. Davis, L.A., Schejter, A., and Hess, G.P. (1974), J.Biol.Chem., 249, 2624.

29. Kihara, H., Saigo, S., Nakatani, H., Hiromi, K., Ikeda-Saito, M., and Iizuka, I. (1976), Biochim.Biophys.Acta., 430, 225.

30. Greenstein, J.P., and Winitz, M. (1961), 'Chemistry of Amino Acids' volume 1, John Wiley and Sons, Inc., New York.

31. Bechtold, R., and Bosshard, H.R. (1981), in press.

32. Smith, H.T., and Millett, F. (1980), Biochemistry

33. Greenwood, C., and Wilson, M.T. (1971), Eur.J.Biochem. 22, 5.

34. Babul, J., and Stellwagen, E. (1972), Biochemistry 11, 1195.

35. Hasumi, H. (1980), Biochim.Biophys.Acta, 626, 265.

36. Butt, W.D., and Keilin, D. (1962), Proc. Roy. Soc., B156, 429.

37. Margoliash, E., and Schejter, A. (1966), Adv.Protein.Chem., 21, 113.

38. Moore, G.R. (1978),D.Phil thesis, Oxford.

39. Cohen, J.S., and Hayes, M.B. (1974), J.Biol.Chem., 249, 5472.

40. Ferguson-Miller, S., Brautigan, D.L., and Margoliash, E. (1978), J.Biol.Chem., 253, 149.

41. Ahmed, A.J., Smith, H.T., Smith, M.B., and Millett, F.S. (1978), Biochemistry, 17, 2479.

42. Osheroff, N., Borden, D., Koppenol, W.H., and Margoliash, E. (1980), J.Biol.Chem., 255, 1689.

CHAPTER THREE

^{1}H n.m.r. assignments and structural studies of native eukaryotic cytochrome c

3.1. Introduction

One of the necessary steps in the work described in this thesis is to extend the assignments of individual proton resonances for ferricytochrome c and ferrocytochrome c. In the first half (Sections 3.2. to 3.5.) of this chapter several new assignments are obtained, and a novel method that cross assigns resonances from one oxidation state to the other is described. In the second half (Section 3.6.) of this chapter structural studies using nuclear Overhauser enhancement (NOE) effects and pH titrations are described for one specific region of the protein close to Ile 57.

3.2.1. The ^{1}H n.m.r. spectrum of a protein and its assignment

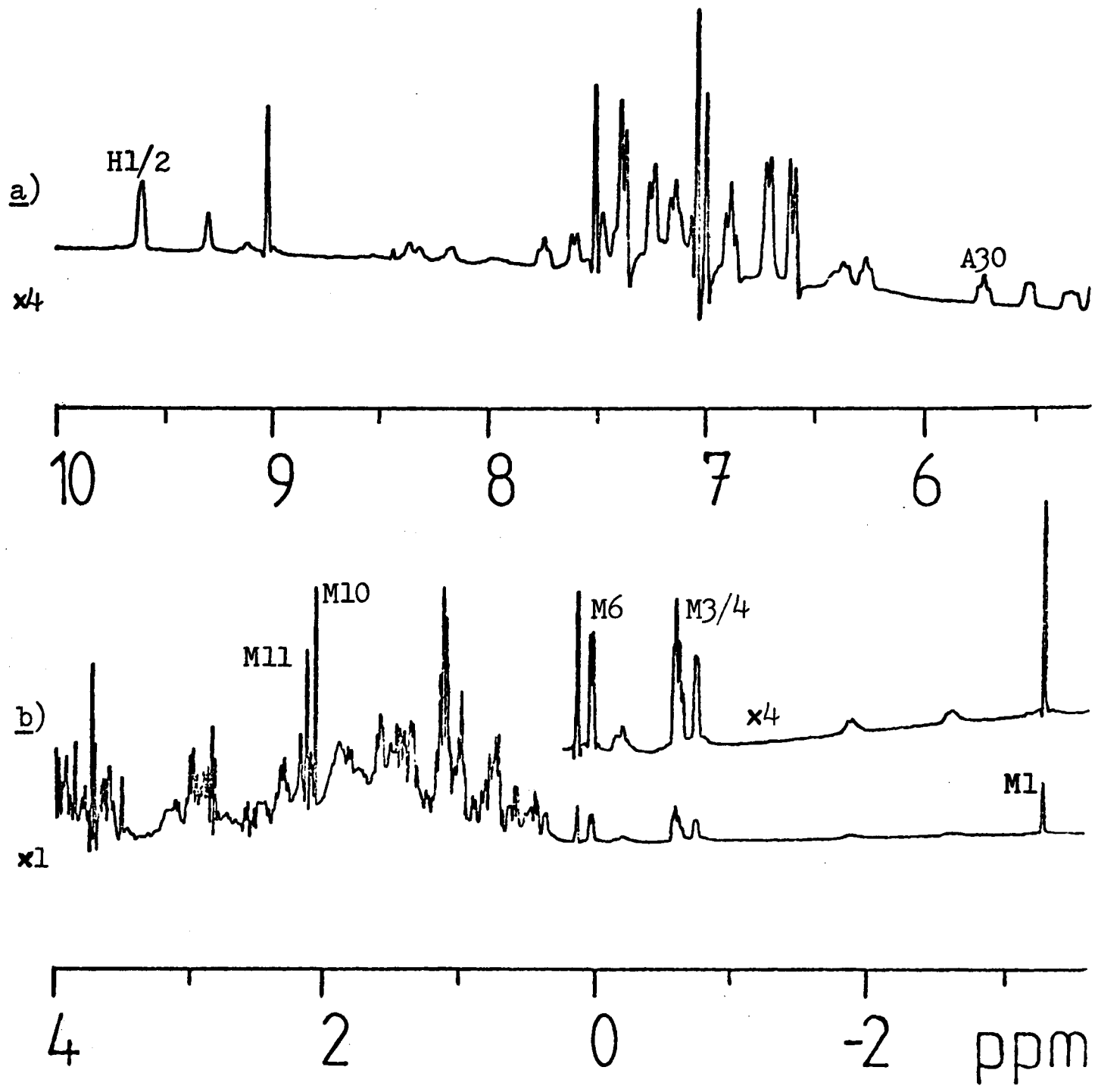
A high resolution ^{1}H n.m.r. spectrum of a protein is usually recorded using a sample of the protein dissolved in ^2H_2O at the required pH (unadjusted for deuterated solvent (1)). In this solvent most of the exchangeable -NH protons from the peptide backbone will exchange for deuterons, and non-exchangeable protons of the amino acid side chains and any prosthetic group (e.g. haem c) will remain (for horse cytochrome c there is a total of 679 such protons). Resolution enhancement techniques, as the multiplication of the free induction decay by a Gaussian function (2) prior to Fourier transformation, are applied to the naked spectrum to resolve out individual resonances and their coupling patterns. The spectrum then obtained consists of two main groups of resonances either side of the ^{2}HOH peak at ca. 4.80 ppm; aromatic protons between 5 and 10 ppm, and aliphatic protons between -5 and 5 ppm. Despite the resolution enhance-

ment techniques many of the resonances may still overlap due to accidental degeneracy of the protons in the protein. Fig. 3.1. shows the spectrum of ferrocytochrome c at 27°C and at pH 5.3 illustrating these two regions of the spectrum. For a native folded protein the resonances of an individual group will suffer shifts from its primary position (that in a small peptide) (3) due mainly to ring current fields (4); this is called a secondary shift and can provide valuable structural information. But first, the resonances must be assigned to individual nuclei within the protein. First stage assignment identifies a group of mutually coupled resonances by spin decoupling experiments and on the basis of their unique coupling pattern indicates a type (e.g. two coupled two proton doublets in the aromatic region is unique to a tyrosine residue) or types (e.g. a three proton doublet coupled to a one proton quartet in the aliphatic region is unique to either an alanine or threonine) of amino acid residue. In regions of overlapping resonances the decoupling is not always clear and decoupling difference spectra (5) and spin echo double resonance (6) techniques may be used. Second stage assignments identifies the set of resonances to a particular amino acid out of the total sequence of the protein (e.g. to Tyr 74 in ferrocytochrome c, there are 3 other tyrosines in the molecule). A variety of methods and arguments are used, but as many examples of these appear in Section 3.4. further discussion is deferred until then (7).

3.2.2. The previous assignments of ^{1}H n.m.r. resonances of horse cytochrome c

Before the work of this thesis was initiated many resonances in ferricytochrome c and ferrocytochrome c had been assigned (8,9,10). A resonance nomenclature scheme has been devised and is followed throughout this work (9). Resonances are designated as follows; non-haem aromatic

Fig. 3.1.



The spectrum of horse ferrocytochrome c, at 27°C and pH 5.3, illustrating the two regions of a high resolution protein ^{1}H n.m.r. spectrum; a) downfield, and b) upfield of the ^{2}HOH resonance at ca. 4.8 ppm. The resolution of the spectrum is enhanced by the multiplication of the free induction decay by a Gaussian function prior to Fourier transformation (2). The relative intensities of the individual plots are marked on the diagram. Some resonances are marked according to the resonance nomenclature scheme introduced in Section 3.2.2.

resonances, A1 - An, starting with the furthest downfield resonance; non haem methyl resonances, M1 - Mn, starting with the furthest upfield methyl resonance; non haem aliphatic (non methyl) resonances, C1 - Cn, starting with the furthest upfield resonance; haem resonances, H1 - Hn, starting with the furthest downfield resonance. The scheme is identical for ferricytochrome c except that an asterisk is added to each designate e.g. $M^{*}4$ (see Fig. 3.1. for examples).

The assignments of resonances in horse cytochrome c, known before this work, are tabulated in Table 3.1. and result mostly from the work of Dr. G. Moore (9,10). In order to illustrate primary and secondary assignment procedures some aspects of the logic used to obtain these assignments will now be discussed.

There is only one tryptophan residue in horse cytochrome c and it is at position 59. Its aromatic side chain will give rise to five one proton resonances; a singlet, two doublets and two triplets. The singlet was assigned in both oxidation states as the remaining aromatic singlet, after the singlets due to histidine residues have been found and assigned (11 and see Section 3.3.1.). For ferricytochrome c irradiation of $A^{*}16$ caused $A^{*}15$ to decouple from a triplet to a doublet and $A^{*}6$ to decouple from a doublet to a singlet. Similarly irradiation at $A^{*}15$ caused $A^{*}16$ to decouple from a triplet to a doublet and $A^{*}7$ to decouple from a triplet to a doublet. Such a coupling pattern is unique to a Trp residue (its first stage assignment) in a protein and these resonances were assigned to Trp 59 (the second stage assignment) in ferricytochrome c. Due to the symmetry of the benzenoid ring of tryptophan these resonances can not be assigned to individual protons on the basis of these results alone; the assignment to individual protons is given in Section 3.3.1.

When there are several residues of one type found in a protein, a

Table 3.1.

Previous ^{1}H n.m.r. assignments of horse cytochrome c

Assignments are given for the proteins at 57°C and pH 5.3 unless stated otherwise

Assignment	Ferricytochrome c Resonance designate	and chemical shift	Ferrocytochrome c Resonance designate	and chemical shift
His 18 C-2			A32	0.50
His 18 C-4			A33	0.13
His 26 C-2	A*4	7.62	A8	7.52
His 26 C-4	A*9	7.01	A18	7.06
His 33 C-2	A*1	8.63	A1	8.55
His 33 C-4	A*3	7.69	A6	7.55
Trp 59 C-2	A*11	6.86	A19	6.99
Trp 59 C-4/7	A*7	7.37	A5	7.60
Trp 59 C-5/6	A*15	6.54	A24	6.70
Trp 59 C-6/5	A*16	6.31	A30	5.74
Trp 59 C-7/4	A*6	7.57	A16	7.09
Phe 10/82 o-			A17+	7.10
Phe 10/82 m-			A25+	6.70
Phe 10/82 p-	A*2	8.26	A27	6.34
Phe 36 o-	A*8	7.23	A9	7.40
Phe 36 m-	A*14	6.64	A20	6.89
Phe 36 p-	A*13	6.76	A15	7.10
Phe 82/10 o-	A*18	6.20	A23	6.71
Phe 82/10 m-	A*18	6.20	A10	7.40
Phe 82/10 p-			A13	7.20

Table 3.1.(cont.)

Phe 46 o-			A2	8.00
Phe 46 o-			A7	7.53 } at 27°C
Phe 46 m-				7.50 } at 27°C
Phe 46 m-				6.91 } at 27°C
Phe 46 o-			A4	7.75
Phe 46 m-			A12	7.22
Phe 46 p-			A3	7.75 (also at 27° C)
Tyr 74 o-	A*2	7.62	A11	7.22
Tyr 74 m-	A*12	6.82	A26	6.64

The complete assignments for Tyr 48 and Tyr 97 were obtained after the writing of this thesis, an extra page is given for these results at the end of this Table.

N-acetyl CH_3	M*20	1.97	M10	2.07
Met 65 CH_3	M*19	1.94	M11	2.11
Met 80 βCH			C2	-2.58
Met 80 βCH			C4	-0.19
Met 80 γCH	C*1	-24.9	C1	-3.74
Met 80 γCH	C*2	-22.2	C3	-1.87
Met 80 CH_3	M*1	-21.0	M1	-3.28
Ala 15 αCH	C*4	5.63		
Ala 15 CH_3	M*22	2.07		
Leu 32 βCH				0.52
Leu 32 CH_3			M2	-0.76
Leu 32 CH_3			M3	-0.60

Table 3.1.(cont.)

Thr 47 CH_3	M*17	0.74	M8	1.13
Ile 57 γCH				0.52
Ile 57 γ CH				0.90
Ile 57 δCH_3	M*6	-0.18	M4	-0.43
Thr 89 CH_3	M*18	1.23	M9	1.36
Meso αCH			H3	9.32
Meso βCH			H2	9.59
Meso γCH			H1	9.62
Meso δCH			H4	9.04
Ringmethyl 1	H*8	7.3	H9	3.49
Ringmethyl 3	H*2	30.4	H7	3.88
Ringmethyl 5	H*6	11.0	H8	3.60
Ringmethyl 8	H*1	32.4	H11	2.21
Thioether CH bridge 2			H6	5.27
Thioether CH_3 bridge 2	H*10	-1.54	H12	1.53
Thioether CH bridge 4			H5	6.34
Thioether CH_3 bridge 4	H*9+	3.1	H10	2.57
Propionate 6 βCH	H*4+	14.3		
Propionate 6 βCH	H*7+	8.2		
Propionate 7 βCH	H*3+	19.0		
Propionate 7 βCH	H*5+	11.5		

+, not observed below 47°C;

+, the assignments of the haem resonances ~~is~~ are given at 27°C.

Table 3.1. (cont.)

Tyr 97 A	A14	7.19	at 15°C
Tyr 97 B	A22	6.74	
Tyr 97 C	A28	6.62	
Tyr 97 D	A31	5.55	
Tyr 97 AB	A21	6.78	at 97°C
Tyr 97 CD	A29	6.18	
Tyr 48 A		5.28	only seen at 15°C and below
Tyr 48 B		7.30	
Tyr 48 C		6.43	
Tyr 48 D		7.07	

The resonances now assigned to Tyr 97 were previously assigned to Tyr 48 (9). The reassignment of these resonances to Tyr 97 comes from the work of Wuthrich (31), and is now confirmed with new assignments for Tyr 48 (Moore, G.R., and Eley, C.G.S., personal communication).

sequence substitution of one of the residues in the protein from a different species may aid assignment, and particularly if there are no other substitutions in the same region of the protein structure. Such a substitution may be considered a type of protein modification. For example, tuna ferrocytochrome c has Tyr 46 where horse ferrocytochrome c has Phe 46, and the spectrum of the former does not contain resonances A3, A4 and A12 which possess a coupling pattern unique to a phenylalanine residue and are found in the spectrum of the latter. This is sufficient data to assign these resonances to Phe 46 in horse ferrocytochrome c.

Other resonances were assigned to individual groups by a combination of sequence comparisons, the effects of broadening reagents (specific to resonances of one or more surface residue), the pH and temperature dependence of resonances, analysis of effects due to the haem group (diamagnetic ring currents and paramagnetic pseudocontact shifts) and aromatic rings in the protein based on calculations using the X-ray coordinates (12)(9,10). Some examples of these types of assignments appear in the text of Section 3.3.1. Apart from the sequence comparisons these methods are indirect and often so circumstantial that only tentative assignments were made (11). One of the aims of this work is to confirm these assignments and provide further assignments by more direct methods. An added advantage for tentative assignments obtained by broadening reagents, pH and temperature dependence studies is that once the assignments are confirmed by direct methods the original circumstantial data becomes valid in its own context and can provide useful structural information. In chapter 4, 'direct' assignments are obtained by studies of chemically modified cytochromes c. In this chapter, assignments are obtained by a novel method of direct cross assignment, nuclear Overhauser effects between neighbouring nuclei and further sequence comparisons.

3.3.1. Cross assignments of ^1H n.m.r. resonances by redox titrations in mixtures of horse ferricytochrome c and ferrocytochrome c in rapid electron exchange

Cytochrome c exists in two oxidation states and in a mixture of the two species electron transfer between them can be demonstrated (13). The ^1H n.m.r. spectrum of a mixed solution of horse ferricytochrome c and ferrocytochrome c (5×10^{-3}M in each species, a typical concentration for n.m.r. studies) at 27°C is a superposition of the spectra of the individual species, because the self exchange rate is 'slow' on an n.m.r. timescale (see Appendix). The rate under these conditions is sufficiently rapid for saturation transfer experiments to be carried out on the proton resonances and this method can provide some cross assignments between the two species (9,14). If the electron exchange rate could be increased sufficiently some of the resonances would go into 'fast' exhange (see Appendix), where their chemical shift value is the weighted average of their chemical shifts in ferricytochrome c and ferrocytochrome c, and thus the resonances would be able to be titrated from the spectrum of one species to that of the other. This was achieved for horse cytochrome c by the addition of iron hexacyanides as redox mediators (their use for this purpose has been previously documented (15)) at elevated temperatures. Under the conditions of the experiments only resonances with small differences in chemical shift between oxidation states (ca. <1 ppm.) were placed in fast exchange.

The redox titrations were carried out as follows: spectra were recorded of a solution of 5×10^{-3}M ferricytochrome c and 5×10^{-3}M $K_3Fe(CN)_6$ in ^2H_2O at pH 7 both before and after the addition of aliquots of a solution of 0.2M sodium ascorbate in ^2H_2O at pH 7. The titration was carried out at 57°C and was continued until a spectrum of fully reduced cytochrome c was obtained. A concentrated solution of sodium ascorbate was used so

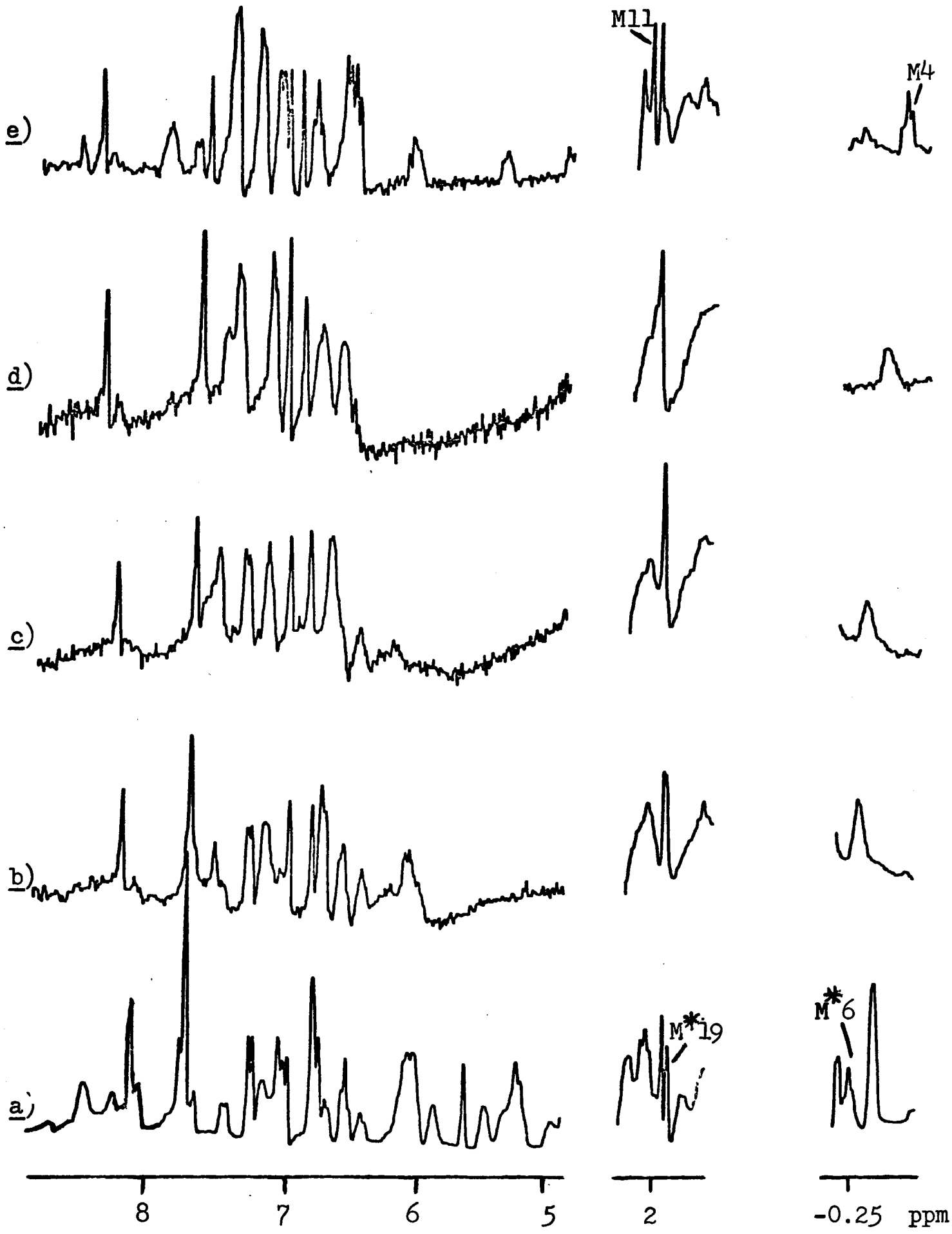
as to cause minimal dilution of the sample (which in turn would reduce the electron exchange rate), and the additions were made so as to titrate the cytochrome c in twelve equal steps.

Fig. 3.2. shows selected spectral regions of five of the total of twelve spectra for the redox titration of horse cytochrome c at 57°C and pH 7. Many resonances in these regions are in fast exchange, for example, the five singlet resonances of His 26, His 33 and Trp 59. The shifts to these resonances were used to calibrate the ordinate of a graph of chemical shift versus mole fraction of oxidised cytochrome c , which was used to correlate several resonances which overlapped with others at stages during the course of the titration. The correlations, so obtained, are summarised in Table 3.2. Several of these cross assignments will be discussed in turn and their usefulness to the general ^{1}H n.m.r. studies of eukaryotic cytochromes c will be explained.

a) The three one proton singlets of His 26 and Trp 59

In the aromatic regions of both ferricytochrome c and ferrocytochrome c five one proton singlets are found. A total of seven such resonances are expected, but two from His 18, one of the haem ligands, are severely shifted from this spectral region in both species. The five remaining that are observed are then due to His 26, His 33 and Trp 59 (16), and the two due to His 33 are assigned by comparison to tuna cytochrome c which has Trp 33 in this position. Of the remaining three resonances in each oxidation state A*4, A*9, A8 and A18 are perturbed by the addition of the resonance broadening reagents Cu^{++}, $Cr(CN)_6^{3-}$ and $Cr(oxalate)_3^{3-}$, while A*11 and A19 are not affected (11). The primary resonance positions of C-2 and C-4 protons of histidine in alkaline solution occur at ca. 7.0 ppm and ca. 7.5 ppm respectively (3) and thus the following tentative assignments are made; A*11 and A19 to Trp 59 C-2, A*4 and A8 to His 26 C-2, and A*9 and A18 to His 26 C-4. The cross pairing of these resonances from each oxid-

Fig. 3.2.



Selected spectral regions from five spectra recorded during the redox titration of horse cytochrome c (in the presence of iron hexacyanides) at pH 7 and 57°C, a) 0% reduced, b) 20% reduced, c) 30% reduced, d) 60% reduced, and e) 100% reduced.

Table 3.2.

Cross assignment correlations for horse cytochrome c obtained by redox titration in mixtures in fast exchange

Assignments are given for the proteins at pH 7 and 57°C

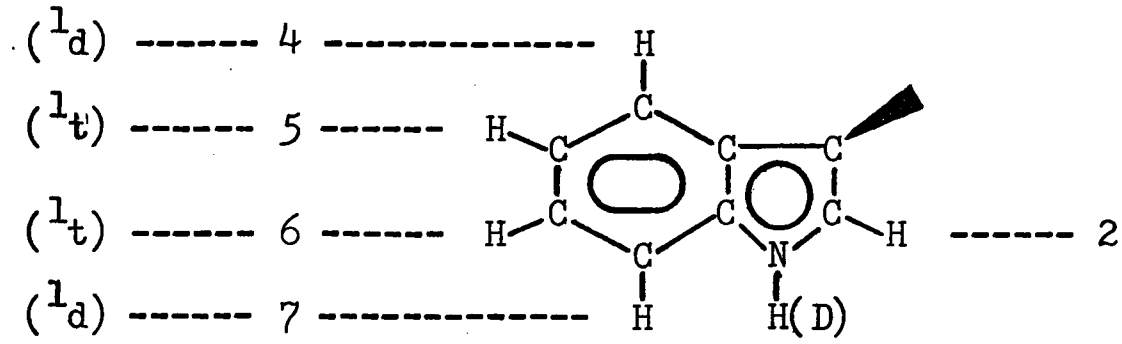
Ferricytochrome c		Ferrocytochrome c		Assignment
Designate	Chemical shift	Designate	Chemical shift	
A^*1	8.62	A1	8.55	His 33 C-2
A^*3	7.69	A6	7.55	His 33 C-4
A^*4	7.62	A8	7.52	His 26 C-2
A^*9	7.01	A18	7.06	His 26 C-4
A^*11	6.86	A19	6.99	Trp 59 C-2
A^*6	7.57	A16	7.10	Trp 59 C-7
A^*16	6.31	A30	5.74	Trp 59 C-6
A^*15	6.54	A24	6.71	Trp 59 C-5
A^*7	7.37	A5	7.60	Trp 59 C-4
A^*5	7.62	A11	7.22	Tyr 74 ortho
A^*12	6.82	A26	6.64	Tyr 74 meta
A^*18	6.20	A23	6.71	Phe 82 ortho +
A^*18	6.20	A10	7.40	Phe 82 meta +
A^*8	7.23	A9	7.40	Phe 36 ortho
A^*14	6.64	A20	6.89	Phe 36 meta
A^*13	6.76	A15	7.10	Phe 36 para
M^*6	-0.18	M4	-0.43	Ile 57 δCH_3
M^*19	1.94	M11	2.11	Met 65 CH_3
M^*20	1.97	M10	2.07	N-acetyl CH_3

+, Second stage assignment of these resonances to Phe 82 is given in Section 3.4.a).

ation state is directly proved by the redox titration (Fig 3.2.), and although the C-2/C-4 assignments of the histidine remain indirectly obtained it would be coincidental if these resonances are shifted by ca. 0.5/ca. -0.5 ppm respectively from their primary positions in both ferricytochrome c and ferrocytochrome c.

b) The benzenoid resonances of Trp 59

In Section 3.2.2., the coupling pattern of Trp 59 in ferricytochrome c was described, however no unique assignments to individual protons could be given on the basis of the spin decoupling data (e.g. is A 7 from the C-4 or C-7 proton?, see Table 3.1.). This is a consequence of the inherent symmetry of the coupling patterns of the resonances of the protons around the benzenoid ring, which is illustrated below:



The proton resonances of a tryptophan residue

Recently Moore (personal communication) has observed and assigned the N-H proton of Trp 59 at 10.06 ppm in horse ferrocytochrome c. An automatic NOE difference spectrum with irradiation at 10.06 ppm and a blank part of the spectrum contains A5 and A19 and no other Trp 59 resonances. This gives the assignment of A5 to the C-4 proton which is adjacent to the N-H proton, which is in turn adjacent to the C-2 proton, previously assigned to A19. The other three benzenoid resonances are now uniquely assigned using the coupling patterns and a process of elimination. Likewise, the cross assignment of one of these resonances to that in ferricytochrome c will

provide the complete assignment of the four benzenoid protons in this oxidation state. Such a correlation was suggested between A30 and A*16 by cross saturation in a mixture of ferricytochrome c and ferrocytochrome c at 27°C together with experiments to check that the effects observed did not arise from NOEs in either oxidation state (16). However the cross saturation difference spectra were difficult to interpret with certainty due to poor signal to noise; the redox titration shows that this cross assignment is, indeed, correct and combined with the NOE experiment described above provides firm evidence for the assignments of all the Trp 59 aromatic CH resonances in both oxidation states.

c) Two methyl singlets

In both the spectra of ferricytochrome c and ferrocytochrome c, two sharp singlets are found in the region around 2 ppm, M*19, M*20, and M10, M11 respectively (see Fig. 3.2.). It can be clearly seen in Fig. 3.2. that these resonances 'cross over' during the redox titration and that between 40% and 70% ferricytrochrome c they overlap as one resonance. Thus M*19 cross assigns to M11 and similarly M*20 to M10; further assignment of these resonances is deferred until Section 4.2.

d) The Ile 57 δCH_3 resonance

The only proton groups that give rise to three proton triplet resonances in the ^1H n.m.r. spectra of proteins are the δCH_3 protons of an isoleucine residue (7). In horse ferrocytrochrome c such a triplet resonance is resolved at -0.43 ppm (at 57°C), and is the only resonance in the diamagnetic protein to suffer a large temperature dependent shift (10), which will be discussed further in Section 3.6.1. Sequence comparisons and ring current calculations were used to assign this resonance to the δCH_3 protons of Ile 57 (17,10). The latest X-ray coordinates (18) show that these protons are ca. 6.5 Å from Trp 59 and ca. 3.5 Å from Tyr 74,

while ring current field calculations show that both these rings give the resonance upfield ring current shifts which place it in the 'upfield methyl window' region of the spectrum (19). The assignment has now been confirmed by other methods (e.g. NOEs from the protons of Tyr 74, see Section 3.6.2., and the disappearance of the resonance from the spectrum of $(Hse)^{65}$, $(Leu)^{74}$ ferrocytochrome c, see Section 4.2.5.) and it is crucial to much of the work presented later in this thesis. The redox titration of this resonance (M4) can be seen in Fig. 3.2. and the corresponding resonance in ferricytochrome c is $M^{*}6$. The assignment of $M^{*}6$ to the δCH_3 protons of Ile 57 is important because: (i) much data has been obtained for the resonances M4 and $M^{*}6$, which indicate that Ile 57 is in a region of the protein that is more flexible than others (20,21), is different between the two oxidation states (21) and is a major antigenic determinant (22). Further discussion of these points is deferred until Section 3.6.1. (ii) M4 is the only methyl triplet resolved in horse ferrocytochrome c although there are six isoleucines present in the molecule. However, Eley (23) has studied the region upfield of 0.50 ppm in the spectra of ferricytochromes c from several species and found that several triplets can be clearly demonstrated by recording Carr-Purcell A spectra (24). Once $M^{*}6$ is assigned to Ile 57, the following further assignments of Ile δCH_3 protons can be made using the species differences and by a process of elimination; Ile 95 (-0.09 ppm, lamprey; -0.03 ppm, horse; -0.06 ppm, kangaroo; and -0.09 ppm, rabbit) and Ile 35 or Ile 58 (-0.29 ppm, kangaroo).

So far the discussion has concerned resonances that are in fast exchange and can be followed throughout the redox titration. Some resonances are in intermediate exchange and as the titration proceeds these shift towards their position in the other oxidation state and broaden out of the spectrum. These resonances do not provide any firm data, but can provide

useful clues to their position in the other oxidation state. For example, in a mixture of ferricytochrome c and ferrocytochrome c at 27°C, irradiation at A*18 (6.2 ppm) causes partial saturation to A10 (7.40 ppm) and to A23 (6.73 ppm), which can be shown to be the meta and ortho resonances of a phenylalanine by spin decoupling. However, irradiation at A10 or A23 causes no effect at A*18; this effect most likely arises because the resonance(s) at A*18 have spin - lattice relaxation times that are very short (11). This experiment suggests that both ortho and meta resonances of the phenylalanine lie under A*18 in the spectrum of ferricytochrome c. In the redox titration one resonance can be titrated from A*18 to A23, while a further resonance shifts downfield from A*18 in the early steps of the titration and broadens out. This is not conclusive evidence, but it does support the reasoning given above for a further phenylalanine resonance under A*18 that cross assigns to A10 as it shifts in this direction before it broadens out into intermediate exchange. It should be theoretically possible to predict where such a resonance that broadens out will reappear by its linebroadening as a function of the mole fraction of ferricytochrome c This is not practically possible as linewidths and mole fractions can not be determined accurately enough from the data.

3.3.2. The electron exchange rate during the redox titration of horse cytochrome c

The main purpose of the redox titration described in the last section was to obtain ^{1}H n.m.r. assignments between the different oxidation states of horse cytochrome c. It is of value to calculate the electron exchange rate as mediated by iron hexacyanides and compare it to the rate without the presence of mediators, and this is the concern of this section.

The linewidth of a resonance in fast exchange between two sites A and B is given by (further equations are given in the Appendix):

$$\left(\frac{1}{T_2}\right)_{obs} = \left(\frac{f_A}{T_{2A}}\right) + \left(\frac{f_B}{T_{2B}}\right) + \frac{4\pi^2 . f_A . f_B . (\Delta_o)^2}{k_1 + k_{-1}} \qquad (1)$$

where f_A and f_B are the mole fractions of A and B; $1/T_{2A}$ and $1/T_{2B}$ are the linewidths of the resonances in A and B; Δ_o is the chemical shift difference between the resonances in A and B. The equilibrium is

$$A \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} B \qquad (2)$$

and when $f_A = f_B = 0.5$, the equation (1) reduces to:

$$\left(\frac{1}{T_2}\right)_{50\%} - \frac{1}{2}\left[\left(\frac{1}{T_{2A}}\right) + \left(\frac{1}{T_{2B}}\right)\right] = \frac{(\pi . \Delta_o)^2}{2k_1} \qquad (3)$$

For the cytochrome c system the electron transfer is bimolecular and equation 2 should be rewritten as

$$\text{cyt } c^{III} + \text{cyt } c^{II} \rightleftharpoons \text{cyt } c^{II'} + \text{cyt } c^{III}$$

and hence at $f_A = f_B = 0.5$,

$$\left[\frac{\text{cyt c}}{2}\right] = \left[\text{cyt } c^{II}\right] = \left[\text{cyt } c^{III}\right]$$

and $$k_1 = k_{-1} = k_{ex} . \left[\text{cyt } c^{III}\right] = k_{ex} . \left[\text{cyt } c^{II}\right] = \frac{k_{ex} . \left[\text{cyt c}\right]}{2} \qquad (4)$$

Note that in the redox titration described hexacyanide concentration equals cytochrome c concentration, and since it has been observed in this work and in previous work (15) that maximal exchange is achieved with 1:1 concentrations with no further enhancement with further hexacyanide there is no need to take into account the concentration of the iron hexacyanide.

Data for calculating the exchange rate was obtained from two

resonances that could be followed through the whole redox titration and that did not overlap with other resonances at any stage of the titration. These two resonances were M*6/M4 from Ile 57 and A*8/A9 from Phe 36. $(1/T_2)_{obs}$ was measured as the linewidth at half height of the resonances in a spectrum to which no linebroadening function had been applied prior to Fourier transformation of the f.i.d.+ f_A values (or mole fraction of oxidised species) were taken from those calculated from the calibration graph described in the last section. The values of $(1/T_2)_{obs}$ were plotted against f_A and values of $(1/T_2)_{50\%}$ were obtained from the graph at $f_A = 0.5$ for both resonances. The values obtained were used in equations 2 and 4, where cyt c $= 5\times10^{-3}$M. The table below shows this analysis of the data.

Parameter	Units	Phe 36 A 8/A9	Ile 57 M 6/M4	
Δ_o	Hz	39.0	64.6	
$\left(\frac{1}{T_2}\right)_{50\%}$	Hz	20.7	32.3	(measured graphically)
$\left(\frac{1}{T_2}\right)_{red}$	Hz	14.0	12.2	
$\left(\frac{1}{T_2}\right)_{ox}$	Hz	16.5	20.1	
k_1	s^{-1}	461.2	407.0	
k_{ex}	$mol^{-1}s^{-1}$	1.84×10^5	1.64×10^5	

Thus the exchange rate at 57°C between ferricytochrome c and ferrocytochrome c in the presence of iron hexacyanides is calculated to be k_{ex} (average) $= 1.74\times10^5$ $mol^{-1}s^{-1}$.

+ f.i.d., free induction decay (see Appendix)

This value is enhanced by a factor of ca. 30 over that obtained without iron hexacyanide mediators (13.14), and is close to that predicted by a simple collision theory which has as its main assumption that electron exchange can occur through about 3% of the surface area of the cytochrome c molecule.

3.4. Assignments for ferrocytochrome c using double irradiation techniques

This section concerns assignments obtained by two double resonance techniques; spin-spin decoupling (25) and truncated nuclear Overhauser effects (26), henceforth NOEs.

The former involves the irradiation of a coupled proton resonance at the same time as collecting the f.i.d. (called time shared irradiated), which causes the collapse of coupling that is due to the protons of the irradiated resonance on any other proton resonance. In a crowded spectral region a difference spectrum between the spectrum with irradiation on the resonance and a spectrum with the irradiation in a blank part of the spectrum may be used to see the decoupling effects. The second method uses a gated pulse (of 0.5 seconds typically and in the experiments described here) to irradiate a resonance immediately prior to the collection of the f.i.d. The protons of the irradiated resonance are saturated and the spin lattice relaxation of any neighbouring protons is perturbed by dipolar coupling between the different protons, which leads to a change in intensity to the resonances of these protons (this is described further in the Appendix). In the case of molecules where spin diffusion (27) is relatively insignificant, as in ferrocytochrome c under the experimental conditions used here, the magnitude of the intensity change, or NOE, is proportional to the inverse sixth power of the distance between the protons whose resonances are saturated and those whose resonances are

perturbed. It is now routine procedure to record an automatic NOE difference spectrum, where eight scans are collected with the irradiation on the resonance and eight scans with the irradiation in a blank part of the spectrum; the second set of scans is subtracted from the first set and the cycle is repeated (e.g. for 64 cycles giving a final f.i.d. of 1024 scans). Resonances that appear in the difference spectrum are those that receive an NOE from the irradiated protons and are related to those in a blank spectrum of the sample at the same temperature. The data obtained in this experiment can be used to find the resonances of protons in neighbouring parts of the molecule, and analysed quantitively (28) or semi-quantitively as in Section 3.6.2. to provide structural information concerning part of the molecule.

This section is divided into subsections concerning the assignment of individual groups of resonances. All the experiments were carried out on a standard n.m.r. sample of horse ferrocytochrome c, unbuffered at pH 5.3 and under Argon.

a) The phenylalanine resonances of cytochrome c

Table 3.3. shows the first stage assignments of all the phenylalanine resonances that have been found in the ^{1}H n.m.r. spectrum of horse ferricytochrome c and ferrocytochrome c (the residues that have no resonances listed are undergoing an exchange process that broadens their resonances out of the spectrum (11)) based on spin decoupling and saturation transfer experiments (11) and the redox titration of horse cytochrome c (Section 3.3.1.). The sixth column of the table shows the assignments made prior to this work (9). The assignment of Phe 46 in ferrocytochrome c has already been described (Section 3.2.2.), and of the remaining phenylalanines Phe 36 is far from the haem group and its resonances are not expected to be oxidation state dependent, and on this basis A9, A20, A15, A*8, A*14 and A*13 are

Table 3.3.

Summary of assignments for phenylalanine residues in horse cytochrome c

First stage assignment	Ferricytochrome c Designate	and chemical shift	Ferrocytochrome c Designate	and chemical shift	Previous second stage assignment	Present second stage assignment
ortho	A*18	6.2	A23	6.71		
meta	A*18	6.2	A10	7.40	Phe 10	Phe 82
para			A13	7.20		
ortho	A*8	7.23	A9	7.40		
meta	A*14	6.64	A20	6.89	Phe 36	Phe 36
para	A*13	6.76	A15	7.10		
ortho			A4	7.80		
meta			A12	7.22	Phe 46	Phe 46
para			A3	7.80		
ortho			A17	7.10		
meta			A25	6.70	Phe 82	Phe 10
para	A*2	8.26	A27	6.34		

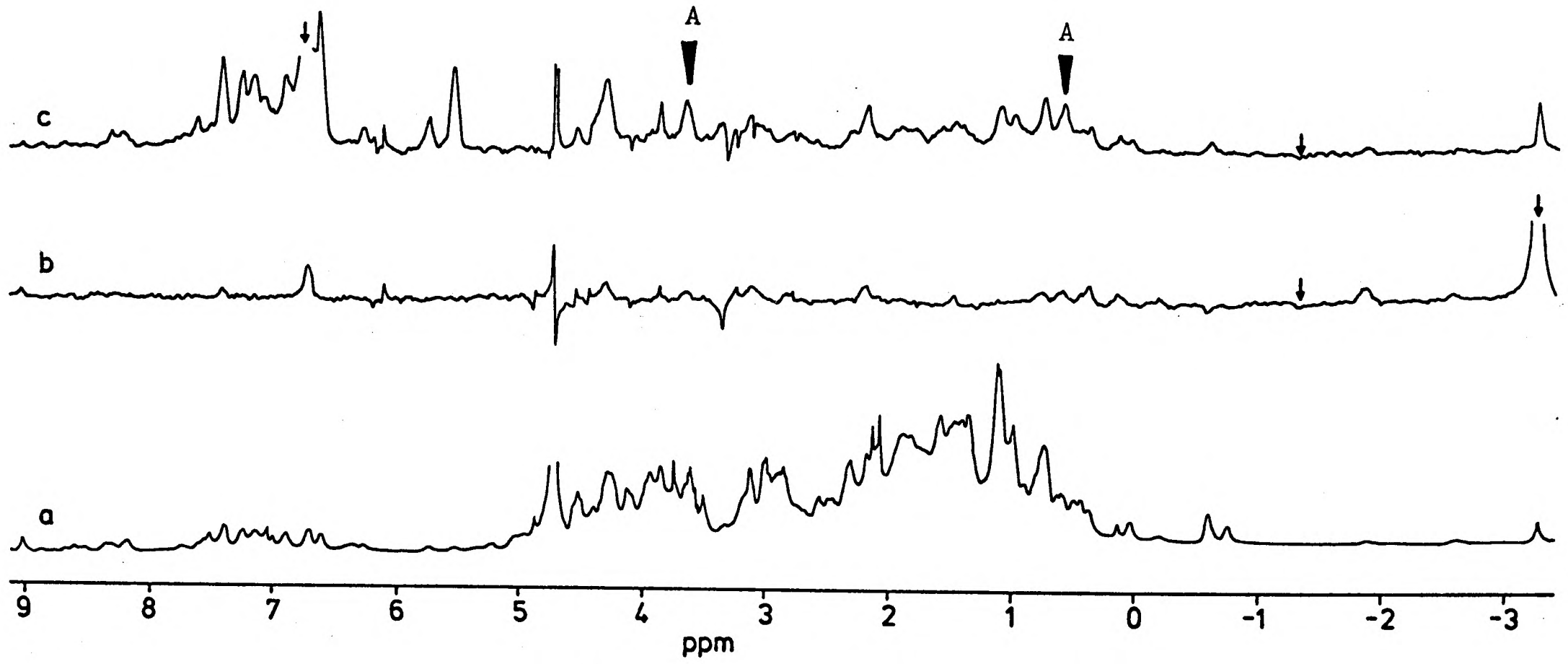
Previous assignments are from ref. 9; not all the phenylalanine resonances of ferricytochrome c are observed.

assigned to it. The remaining sets of resonances , which are due to Phe 10 and Phe 82, can not be uniquely assigned without further experimental work. However, they were tentatively assigned previously on the basis of a simple model for the haem ring current shifts to the protons of Phe 82, which predicted the shifts to the individual protons to be in the order para > meta > ortho , which is that found for the resonances A17, A25 and A27.

Fig. 3.3. shows the spectrum of ferrocytochrome c and the automatic NOE difference spectra obtained upon irradiating at -3.28 ppm and 6.7 ppm The resonance at -3.28 ppm. is firmly assigned to the methyl group of the axial ligand Met 80 (10) and its saturation causes an NOE to A23 (6.71 ppm) and A10 (7.40 ppm). Similarly saturation of A23 causes an NOE to the methyl resonance of Met 80. There are very few other resonances in either NOE difference spectrum, which demonstrates that spin diffusion is insignificant under the conditions of these experiments. Fig. 3.4. shows the relative positions of the haem group, Met 80 and the four phenylalanine residues in horse cytochrome c and it can be seen that Phe 82 is ca. 4Å and Phe 10 is ca. 11Å from the methyl group of Met 80 (18). Thus the NOE data described above shows that A10 and A23 are resonances from Phe 82 since this residue is the closest phenylalanine to Met 80. The corrected assignments resulting from the consequent reassignment of (Phe 82 and) Phe 10 are given in the seventh column of Table 3.3.

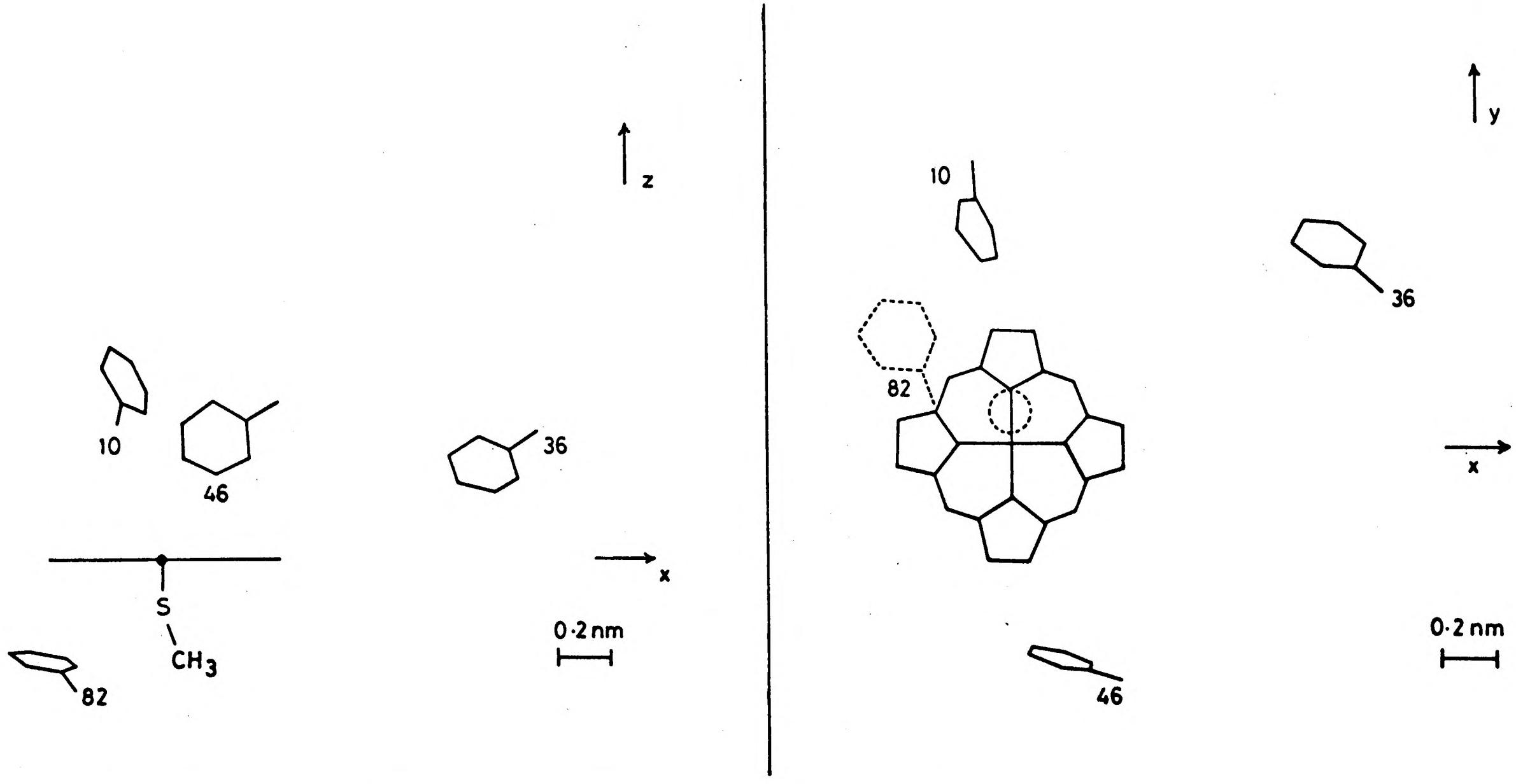
Using a more sophisticated model for the haem ring currents Perkins predicts (19) that the order of the secondary shifts to the resonances of Phe 10 is para > meta > ortho, which is that found on the basis of the reassignment. However, the order predicted for Phe 82 is different to that found experimentally, and illustrates along with the poor agreement between predicted and observed shifts for the firmly assigned Phe 46

Fig. 3.3.



Spectra of horse ferrocytochrome c in $^{2}H_{2}O$ at pH 5.3 and 30°C, a) the normal spectrum, b) NOE difference spectrum with alternate irradiations at -1.3 ppm and -3.28 ppm, and c) NOE difference spectrum with alternate irradiations at -1.3 ppm and 6.7 ppm. The arrows in the diagram indicate the points of irradiation. Two resonances marked A are due to Ala 83 and are discussed further in Section 3.4.c.

Fig 3.4.



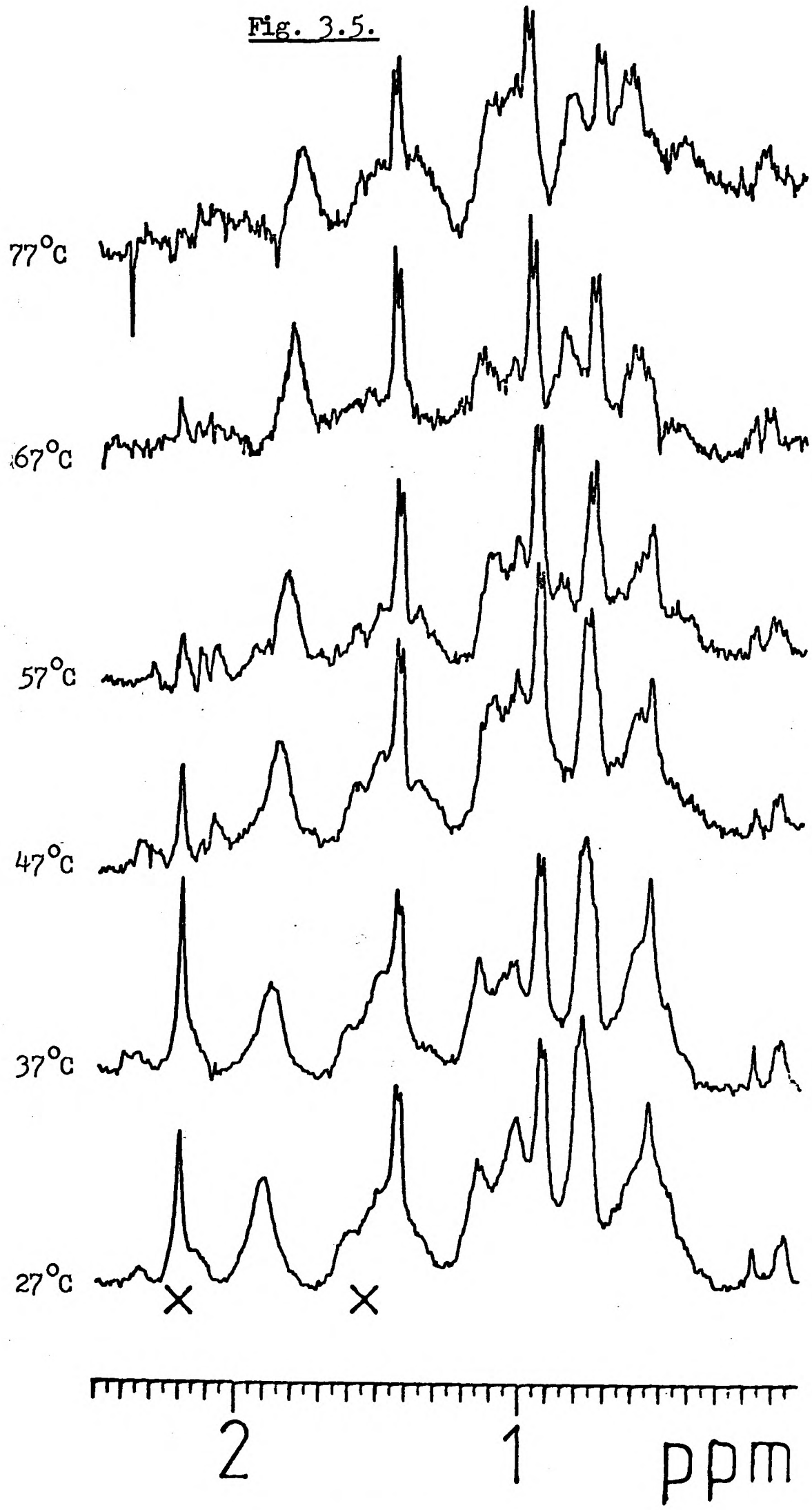
The relative orientation of Met 80, the haem group and the phenylalanine residues of horse ferrocytochrome c: on the left, ± y directions, and on the right, ± z directions. The dashed lines and circle indicate Phe 82 and Met 80 CH_3 in the -z direction. The diagram is based on the X-ray structure of tuna ferrocytochrome c (18).

Perkin's conclusion that 'arguments based on ring current effects are not strong ones for the assignment of phenylalanine and tyrosine signals (19)' This is an important conclusion for the n.m.r. study of cytochrome c and protein n.m.r. in general (29).

b) Protons close to Ile 57 and Tyr 74 on the back surface of cytochrome c

The temperature dependence of the automatic NOE difference spectra with the irradiation of the δCH_3 resonance of Ile 57 (M4) and of the meta protons resonance of Tyr 74 (A26) in ferrocytochrome c is described in Section 3.6.2. as part of a structural study of the protein structure close to these residues. In this section the combination of this NOE data and spin decoupling experiments is used to obtain resonance assignments in this region of the protein. Note, that there are few changes to the ^1H n.m.r. spectrum of ferrocytochrome c, and structure of ferrocytochrome c, between 27°C and 97°C (20). Both M4 and A26 overlap with other resonances in the spectrum of ferrocytochrome c at some ranges of temperature between 27°C and 87°C and consequently resonances can appear in the NOE difference spectra that are not due solely to the irradiation of either M4 or A26. For example, M4 shifts from -0.61 ppm (27°C) to -0.25 ppm (87°C) (see Section 3.6.1.), while the methyl resonances M2 and M3 of Leu 32 are at ca. -0.75 ppm and ca. -0.6 ppm respectively throughout the whole temperature range (10,20) and are the only resonances found in this region of the spectrum. Consequently between 27°C and 47°C, M4 and M3 partially or fully overlap and irradiation of M4 causes partial or full saturation of M3, and the NOE difference spectra for Ile 57 also contain Leu 32 NOEs; at higher temperatures only Ile 57 NOEs appear in the NOE difference spectra. In this section NOEs from Ile 57 will be considered and those from Tyr 74 will not be used, because the NOEs from Ile 57 are unambiguous at higher temperatures.

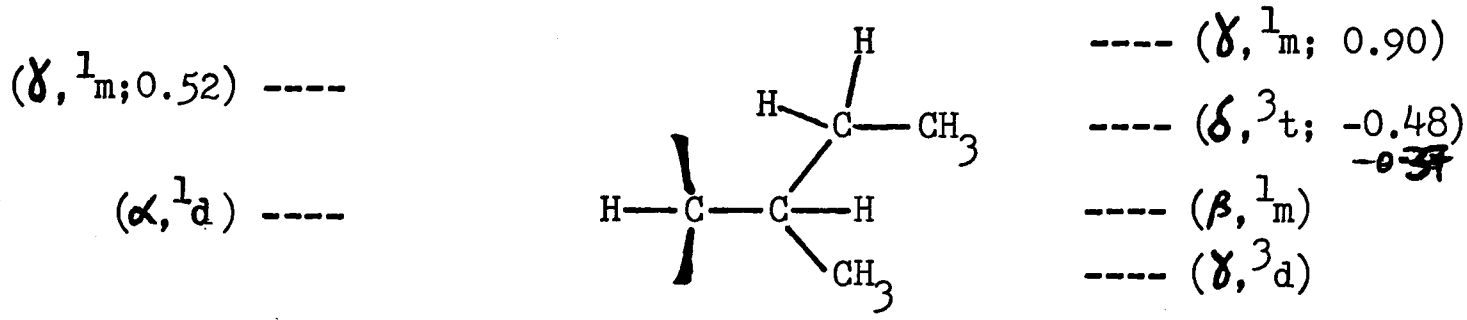
Fig. 3.5.



The selected methyl region of the NOE difference spectra from the Ile 57 δCH_3 resonance (M4) at 10°C intervals between 27°C and 77°C. The resonances marked with a cross do not appear at higher temperatures and are due to NOEs from resonance M3 (Leu 32), see text.

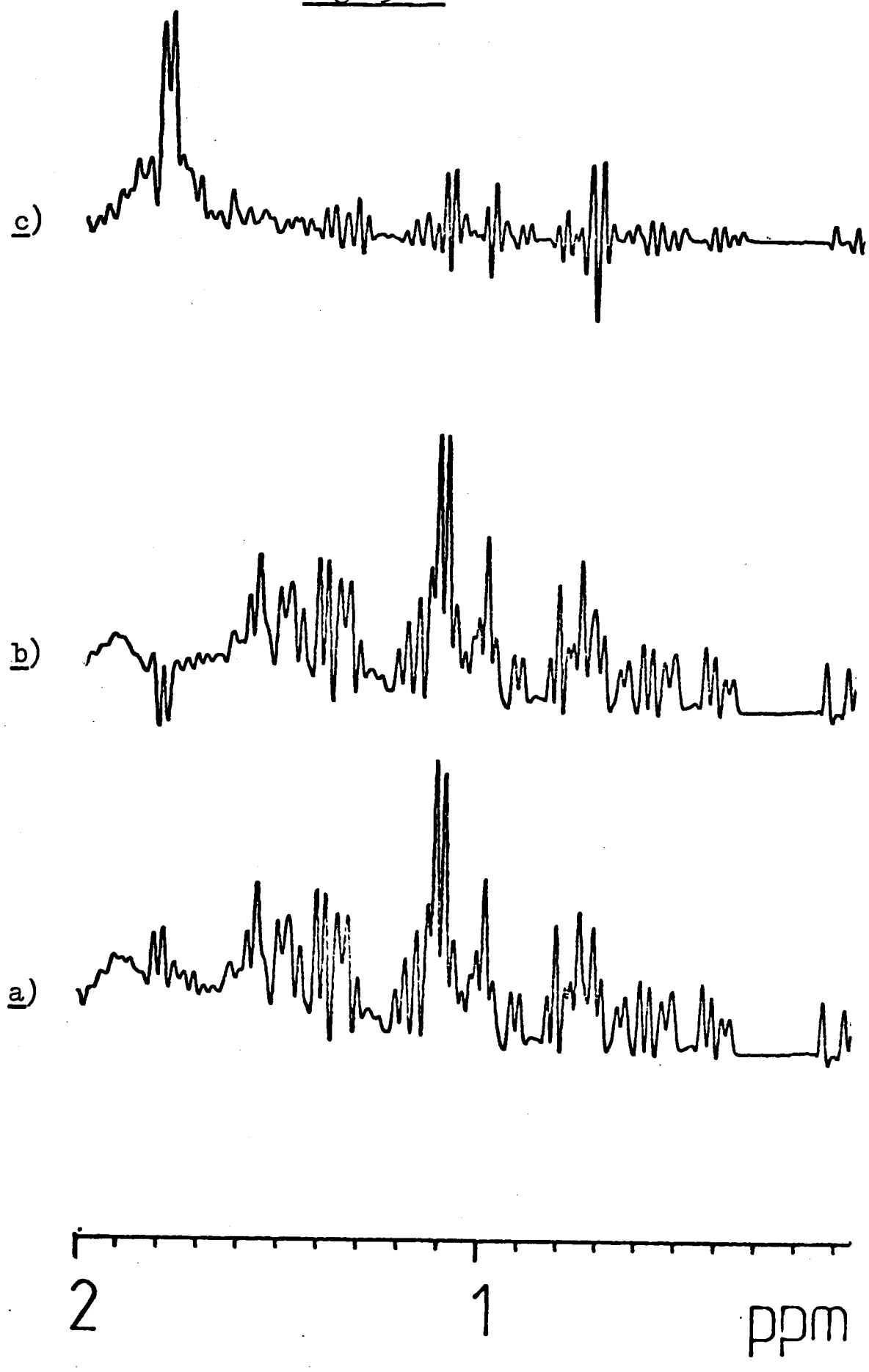
Fig. 3.5. shows the region between 2.5 ppm and 0 ppm of the NOE difference spectra from the δCH_3 resonance of Ile 57 at 10° intervals between 27°C and 77°C. In Section 3.6.2. the aromatic regions (8 ppm to 5.5 ppm) of the same spectra are shown in Fig. 3.12. In Fig. 3.5. resonances due to Leu 32 NOEs, at the lower temperatures, are marked with a cross (×). Resonances that can be seen at all temperatures and are thus due to Ile 57 NOEs are as follows; an aromatic doublet at ca. 6.65 ppm (A26, assigned to the Tyr 74 meta protons), methyl doublets at ca. 1.41 ppm, ca. 0.92 ppm and ca. 0.72 ppm, broad aliphatic multiplets at ca. 1.80 ppm, ca. 0.9 ppm and ca. 0.5 ppm. A resonance at ca. 4.5 ppm can be seen at 27°C and at 87°C, but lies too close to the ^{2}HOH resonance to be observed clearly at all temperatures. These resonances will be considered in turn, in terms of the spin coupling observed between them.

Some of these resonances will be due to other protons from the side chain of Ile 57. The figure below shows a diagram of the isoleucine side chain showing the coupling patterns and previous assignments that have been determined at 67°C (10).



The one proton multiplets of the γCH protons were assigned, because irradiating at 0.52 ppm or 0.90 ppm in ferrocytochrome c at 67°C causes M4 to decouple from a triplet to a doublet (11). Broad multiplets are seen at this positions in the NOE difference spectra (Fig. 3.5.) and are due to these protons. A further broad multiplet arises in the NOE spectra at ca. 1.80 ppm. Fig. 3.6. shows the region between 2 ppm and 0 ppm in a blank spectrum, a spectrum with time shared irradiation at 1.80 ppm

Fig. 3.6.



The spectrum of ferrocytochrome c at pH 5.3 and 57°C with a) irradiation in a blank part of the spectrum, b) irradiation at 1.80 ppm, and c) the difference spectrum a) - b). The decoupling of a doublet to a singlet at 0.72 ppm is clearly seen in c).

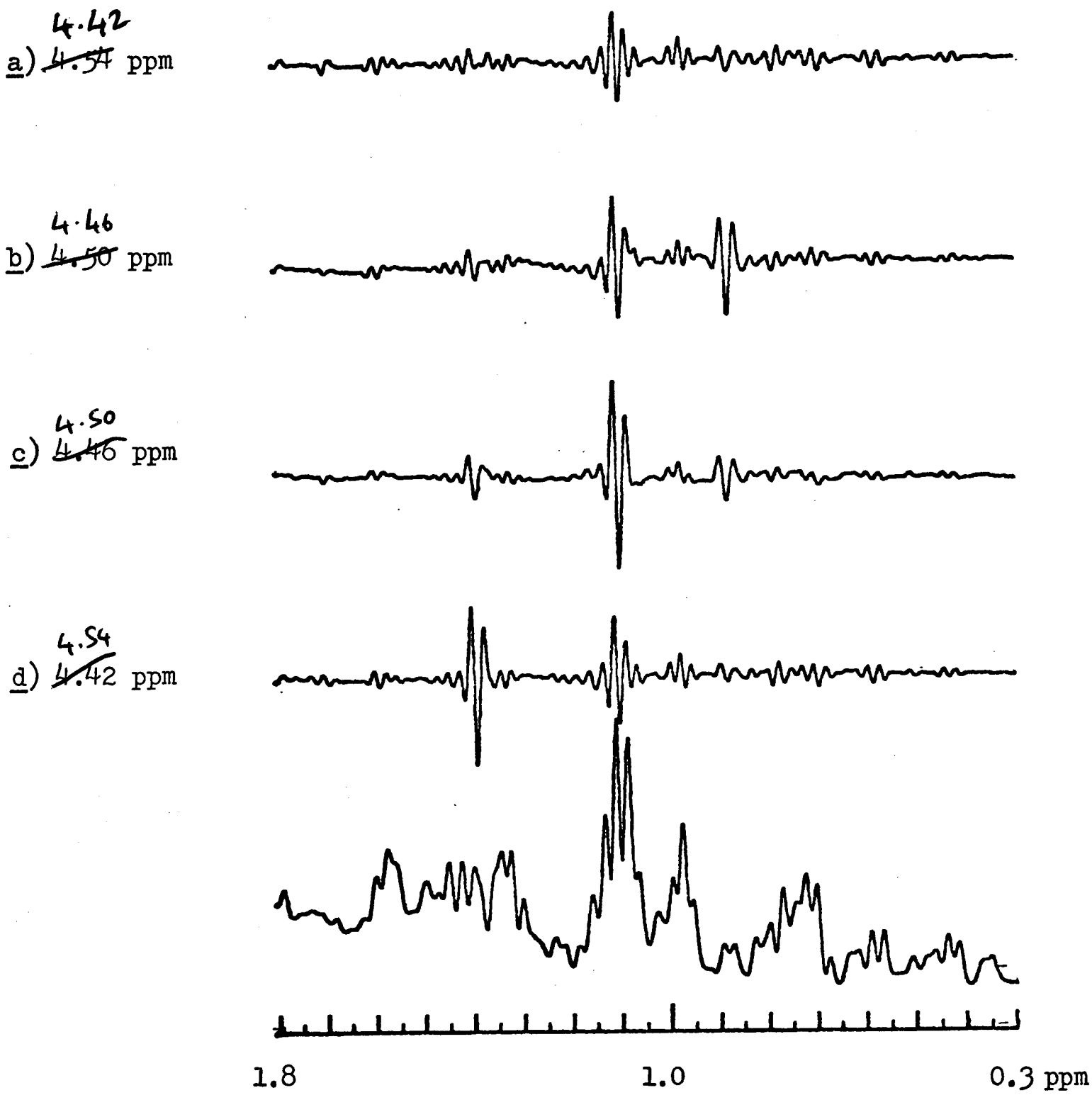
and the difference for ferrocytochrome c at 57°C. It can be seen that ι doublet at 0.72 ppm is unambiguously decoupled to a singlet. When this resonance is irradiated no effect is observed at 1.80 ppm, presumably because the proton resonance at this position is so multiply coupled and of only one proton intensity.

Methyl doublets can arise from the following amino acids; alanine, threonine, valine, leucine and isoleucine, but the nature of the decoupling at 0.72 ppm suggest that this resonance is from an isoleucine by the following reasoning and process of elimination. For an alanine or threonine we should expect to see decoupling from irradiating the doublet itself to an αCH or a βCH, respectively, in the region between 4.5 ppm and 3.5 ppm. This can be quite clearly seen in difference spectra, and an example of such decoupling is shown in Fig.3.8. in subsection c of this section. The doublet at 0.72 ppm is decoupled by irradiation at 1.80 ppm, which is too low for an αCH of alanine or a βCH of threonine, and no reverse decoupling can be demonstrated. For a valine or a leucine we should expect to see a further doublet decouple as the γ or δ methyls of these residues are both coupled to the same β or γCH proton, respectively. Only one doublet decouples in Fig. 3.6., and so the coupling observed is unique to an isoleucine. These resonances obviously come from Ile 57 and the following assignments are made at 57°C: βCH proton, 1.80 ppm; γCH_3, 0.72 ppm. Note, that no αCH decoupling was observed presumably because this resonance is close to or under the ^{2}HOH peak. It can be seen in Fig. 3.5. that the resonances at ca. 1.80 ppm and at ca. 0.72 ppm are those that suffer the greatest shifts with temperature (the resonances at ca. 0.5 ppm and at ca. 0.9 ppm can not be seen clearly enough to determine their temperature dependence), which considering the temperature dependence of the δCH_3 resonance of Ile 57 is further evidence for their assignment.

Time shared irradiation at ca. 4.5 ppm (where a resonance is seen at 27°C and 87°C in the NOE difference spectra) at 27°C and 87°C results in the decoupling of the resonances at ca. 1.40 ppm and ca. 0.90 ppm, and partial decoupling of several resonances at ca. 1.1 ppm. These effects were examined further by irradiating at 6 Hz (0.02 ppm) intervals between 4.56 ppm and 4.40 ppm at 27°C, and recording spin decoupling difference spectra from the resultant spectra and a blank spectrum. Fig. 3.7. shows the spectrum of ferrocytochrome c at 27°C between 1.80 ppm and 0.3 ppm with spin decoupling difference spectra for irradiations at 4.54 ppm, 4.50 ppm, 4.46 ppm and 4.42 ppm. This clearly shows that the resonance at ca. 1.40 ppm is coupled to a resonance at ca. 4.54 ppm, and the resonance at ca. 0.90 ppm is coupled to a resonance at ca. 4.46 ppm. Furthermore, to confirm that the resonances which are decoupled are the same as those that appear on the NOE difference spectra, the NOE difference spectra from Ile 57 were recorded with time shared irradiation at 4.54 ppm, 4.46 ppm and -5 ppm, at 27°C. The latter spectrum was identical to the conventional NOE difference spectrum, whilst the two former spectra contained singlets at 1.40 ppm and 0.90 ppm respectively. It can be seen in Fig. 3.7. that decoupling occurs ca. 1.1 ppm with irradiation throughout the range 4.56 ppm and 4.40 ppm, and this is presumably due to irradiation of Ala αCH and Thr βCH protons causing Ala βCH_3 and Thr γCH_3 proton resonances to decouple, but as there are no resonances in the NOE difference spectra at 1.1 ppm these will not be considered further. On the basis of the above results the two sets of coupled resonances are due to alanine or threonine residues and are both close to Ile 57.

NOE difference spectra from the Ile 57 δCH_3 resonance in tuna and lamprey ferrocytochromes c were recorded at 27°C. The spectra were very similar to those described above for horse ferrocytochrome c and both

Fig. 3.7.



The bottom spectrum is of ferrocytochrome c at pH 5.3 and 27°C with time shared irradiation in a blank part of the spectrum. The other spectra are spin decoupling difference spectra which result from the subtraction of spectra with time shared irradiation at a) 4.54 ppm, b) 4.50 ppm, c) 4.46 ppm, and d) 4.42 ppm from the blank spectrum.

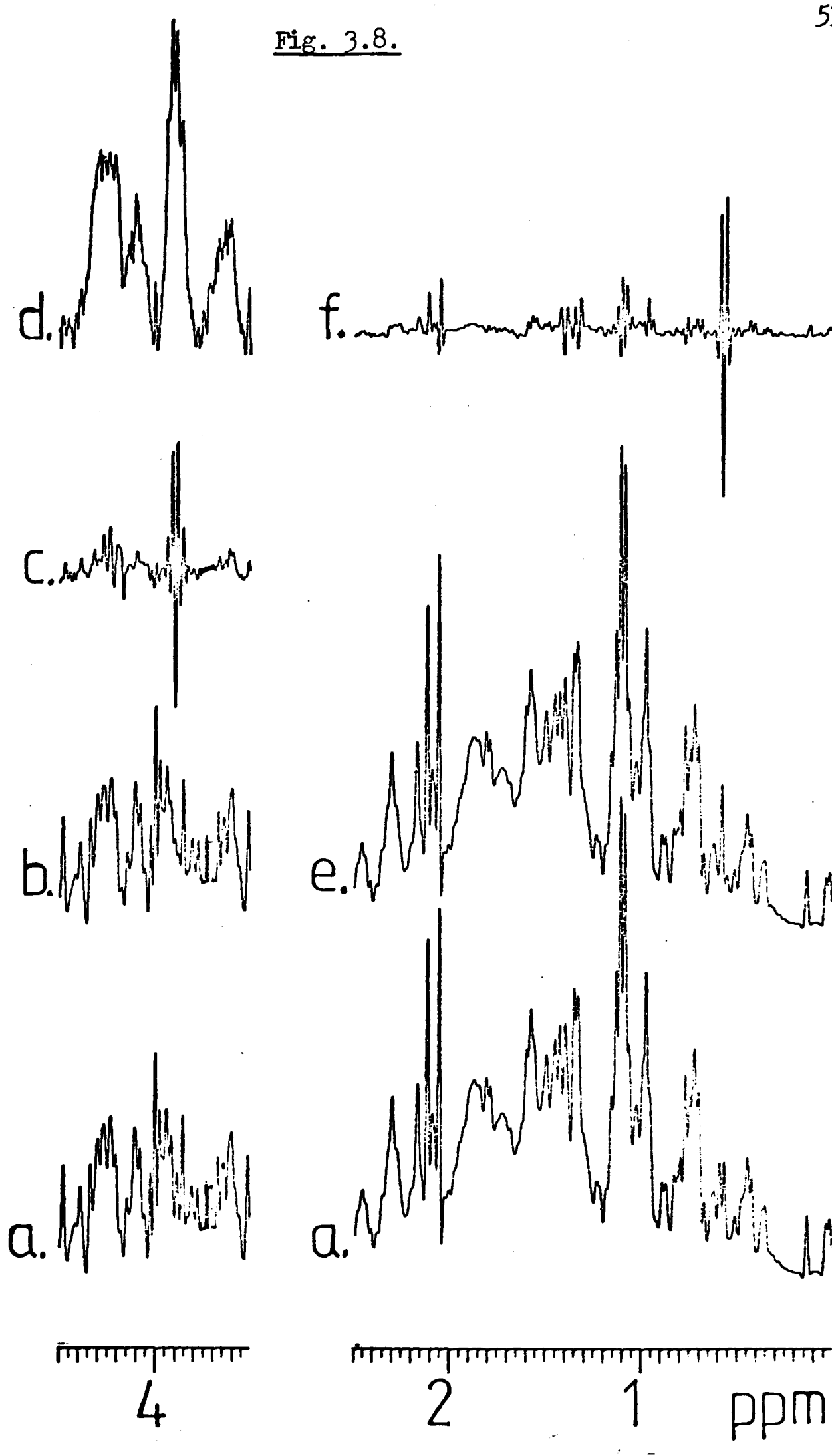
contained the methyl doublet resonances at ca. 1.40 ppm and ca. 0.90 ppm. Horse, tuna and lamprey cytochromes c contain the following common Ala and Thr residues (the sequences are given in the Appendix); Thr 19, Thr 40, Ala 43, Thr 49, Thr 63, Thr 78, Ala 83, Ala 96, Thr 102; while exam ination of the X-ray structure (18) shows that only Thr 40 (6.0 Å) and Thr 63 (6.5 Å) are close enough to Ile 57 to suffer NOE effects from it. No further distinguishing of the resonances to provide individual assignments is possible and the two sets of resonances are assigned tentatively to Thr 40 and Thr 63.

c) Protons close to Phe 82

In subsection a the resonances of Phe 82 were assigned by NOE difference spectra from Phe 82 and Met 80. In these spectra (Fig. 3.3.) it can be seen that a resonance at 0.58 ppm receives an NOE from Phe 82 and a much smaller one from Met 80. Further experiments on this proton resonance are described here.

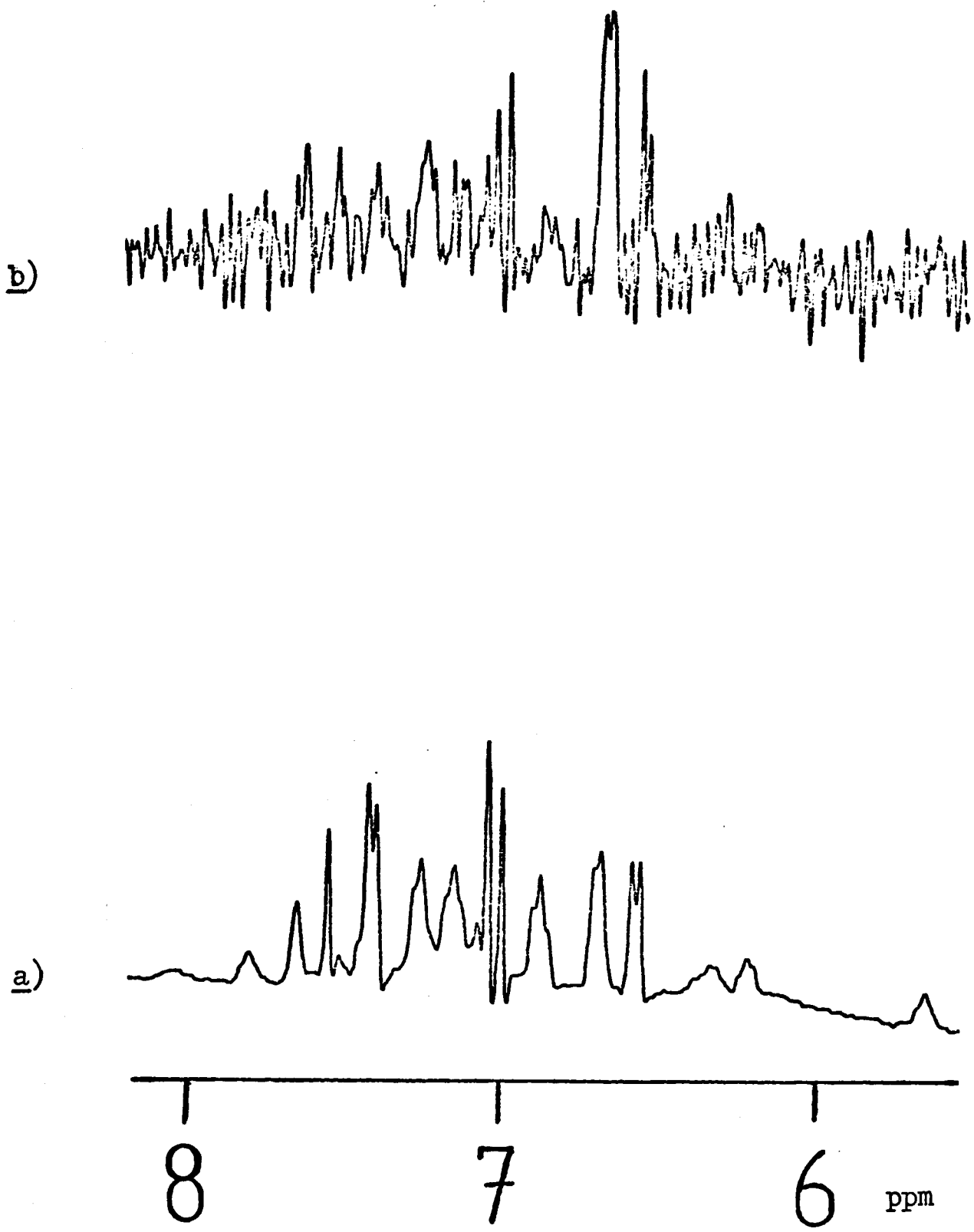
Fig. 3.8. shows the data obtained for this resonance at 27°C. a. is the blank spectrum of ferrocytochrome c, b. is the spectrum with time shared irradiation at 0.58 ppm, c. is (a. - b.) and shows the decoupling of a one proton quartet at 3.90 ppm. It can be seen by examination of a. and b. that this decoupling effect is only resolved by use of difference spectroscopy. d. is the NOE difference spectrum obtained by the irradiation of the resonance at 0.58 ppm and clearly shows the one proton quartet at 3.90 ppm. e. is the spectrum with irradiation at 3.90 ppm, and f. is (a. - e.) and shows the decoupling of the doublet at 0.58 ppm - this effect can be seen clearly from a. and e. without the use of the difference spectrum. Fig. 3.9. shows the aromatic regions of the spectra a. and d., and it can be seen that saturation of the doublet at 0.58 ppm causes an NOE effect to the ortho protons resonance (A23) of

Fig. 3.8.



a) the blank spectrum of ferrocytochrome c, b) the spectrum with time shared irradiation at 0.58 ppm, c) the spin decoupling difference spectrum, a) - b), d) the NOE difference spectrum with irradiation at 0.58 ppm, e) the spectrum with time shared irradiation at 3.90 ppm, and f) the spin decoupling difference spectrum, a) - e), all at pH 5.3 and 27°C.

Fig. 3.9.



a) The blank spectrum of ferrocytochrome c at pH 5.3 and 27°C, b) the NOE difference spectrum with irradiation at 0.58 ppm. These spectra correspond to Fig 3.8.a) and Fig. 3.8.d), respectively.

Phe 82 at 6.71 ppm.

These results show that the doublet at 0.58 ppm is coupled to a quartet at 3.90 ppm, and that these resonances originate from an alanine ot threonine close to Phe 82. The methyl group of Ala 83 is 6 Å from the ortho protons of Phe 82, and is the only alanine or threonine close to Phe 82; the resonances are assigned to Ala 83. The doublet resonance at 0.58 ppm is shown to shift in one monodeamidated ferrocytochrome c in Section 4.3., and its assignment here is important in determining the site of deamidation. Furthermore, this resonance is observed to have different chemical shifts in different species of ferrocytochromes c. For example, guanaco and dog cytochrome c differ at three amino acid positions; 44 (Val, guanaco and Pro, dog), 88 (Lys, guanaco and Thr, dog) and 103 (Asn, guanaco and Lys, dog). The Ala 83 doublet is at 0.56 ppm in guanaco and 0.58 ppm in dog ferrocytochromes c, while an extra doublet is seen in dog ferrocytochrome c at 1.27 ppm that is due to Thr 88 (23). Residue 88 is some distance from Ala 83 (>15 Å), but it is much nearer than residues 44 and 103 and it is presumably the change from positively charged Lys 88 (with its long side chain , it may be closer to Ala 83 than the distance above suggests) to the neutral Thr 88 that causes the shift to Ala 83.

Table 3.4. shows the assignments that have been obtained for horse ferrocytochrome c in Section 3.4. and it is useful to compare this table with Table 3.1., the assignments obtained before this work, and Table 3.2. the assignments obtained by the redox titration of horse cytochrome c.

Table 3.4.

^{1}H n.m.r. assignments of horse cytochrome c obtained by double resonance

The assignments are given for ferrocytochrome c at 57°C and pH 5.3

Assignment	Resonance chemical shift (and designate)	
Phe 10 o-	7.10	(A17)
Phe 10 m-	6.70	(A25)
Phe 10 p-	6.34	(A27)
Phe 82 o-	6.71	(A23)
Phe 82 m-	7.40	(A10)
Phe 82 p-	7.20	(A13)
Ile 57 βCH	1.80	
Ile 57 γCH$_3$	0.72	
Thr 40 βCH	4.54	
Thr 40 CH$_3$	1.40	
Thr 63 βCH	4.46	
Thr 63 CH$_3$	0.90	
Ala 83 αCH	3.90	
Ala 83 CH$_3$	0.58	

3.5. Studies of lamprey cytochrome c and the assignment of Phe 65

Examples of the use of eukaryotic cytochromes c from closely related species to obtain resonance assignments of single site substitutions have already been given. Apart from yielding assignments for proton resonances such studies can provide information about the effect of such amino acid sequence changes, a type of protein modification, on the tertiary structure of a protein.

Lamprey cytochrome c has 16 amino acid sequence substitutions from horse cytochrome c including phenylalanine in place of methionine at position 65 and valine instead of glutamate at position 66. A study of this protein was undertaken for the following reasons;

a) The region close to Met 65, Ile 57 and Tyr 74 on the back surface of cytochrome c is studied in many places in this thesis (e.g. Section 3.6.2.) and is well defined by ^{1}H n.m.r. (21). Met 65 and Glu 66 are conserved in most eukaryotic cytochromes c (30), and so a study of the protein from a species with changes at these positions could provide further information concerning this region of the protein. For example, the carboxyl function of Glu 66 and the hydroxyl function of Tyr 74 are very close in the structure of horse cytochrome c (18) and we may ask if a hydrogen bond forms between these two groups; and, if so, does the removal of Glu 66 in lamprey cytochrome c affect this region of the protein? Evidence is presented in Section 3.6.4. to show that the pH characteristics of the molecule are altered by this substitution. Furthermore, phenylalanine is a more hydrophobic group than methionine, and Met 65 is solvent ~~accesible~~ in horse and tuna cytochrome c (see Section 4.2.3.) and we may ask does the phenylalanine fold back into the hydrophobic core of the molecule and alter the surface structure in this region?

b) Neurosporra Crassa (a red, bread mould fungus) cytochrome c contains

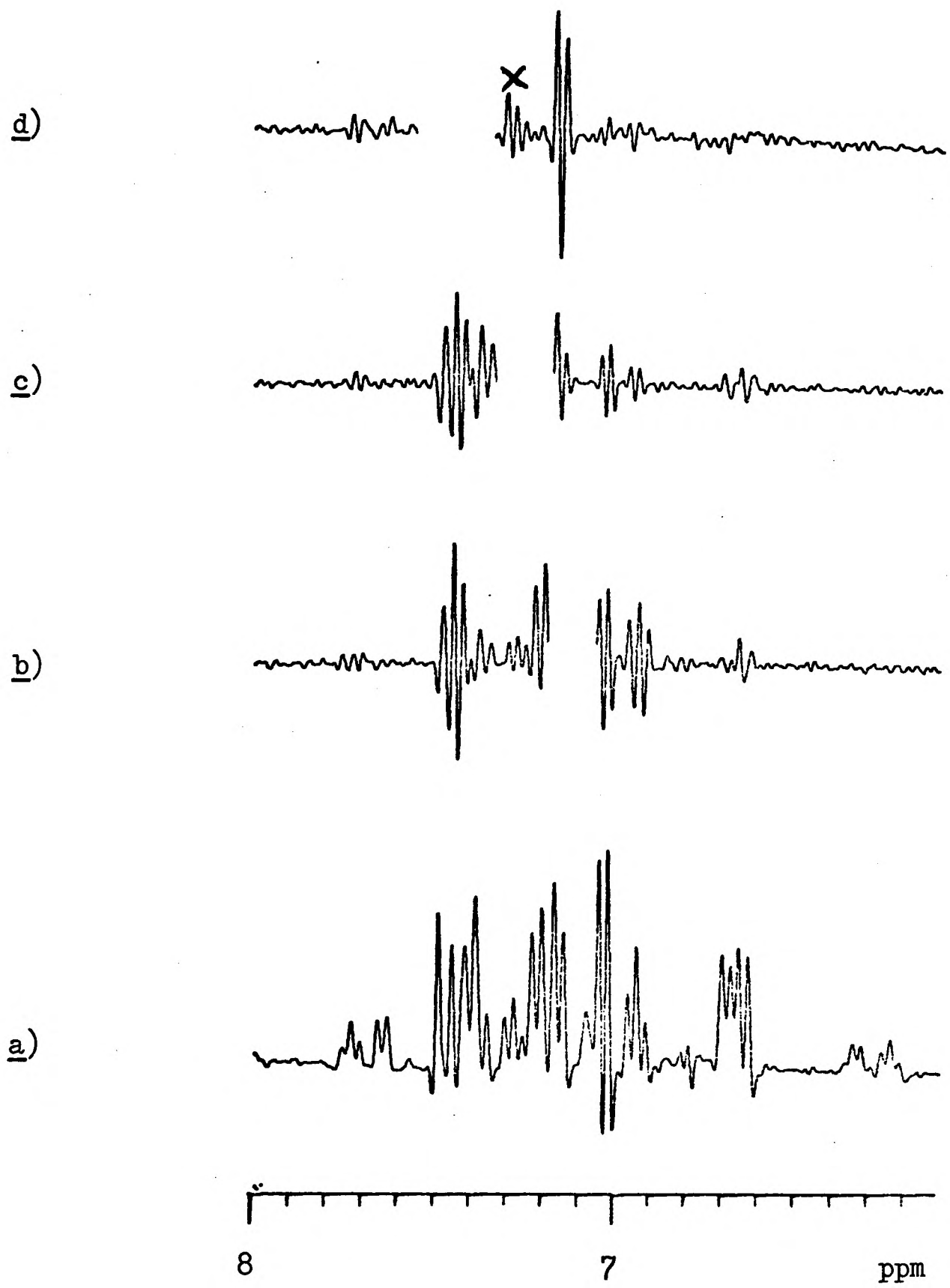
six phenylalanine residues, three of which are substitutions from horse cytochrome c; Phe -3, Phe 65 and Phe 97. The n.m.r. properties of this protein have been studied by Mr. C. Eley (23, and personal communication) and three of the phenylalanines can be assigned by comparison with horse cytochrome c (see Table 3.3.); and two first stage assignments have been made for a further two sets of phenylalanine resonances. These latter resonances are tentatively assigned to Phe -3 and Phe 65, assuming that the resonances of Phe 97 will be observed in analogous positions to those of Tyr 97 in horse cytochrome c (9,31); the validity of this logic is discussed elsewhere (23). Thus the assignment of Phe 65 in lamprey cytochrome c will confirm the assignment of one set to Phe 65 and leave the remaining set tentatively assigned to Phe -3.

Lamprey and horse cytochrome c differ in their aromatic residues at two positions; 33 (serine, lamprey and histidine, horse) and 65 (phenylalanine, lamprey and methionine, horse). Examination of the spectra of lamprey and horse ferricytochrome c at pH7 and 27°C shows the loss of a pair of singlets that are due to His 33 (9) and the gain of a two proton doublet at 6.79 ppm and a two proton triplet at 7.33 ppm in the first spectrum. Spin decoupling can be used to demonstrate that the latter resonances are coupled, and they are assigned to the ortho and meta protons of Phe 65 respectively. The para protons have not been found. Any other differences in the aromatic spectral region between horse and lamprey cytochrome c can be accounted for by small secondary shifts of the resonances by substitutions of neighbouring residues.

Assignment of Phe 65 in lamprey ferrocytochrome c is not so easy, as the spectrum in the aromatic region contains many overlapping resonances. Much has been written about resolving resonances under such circumstances (e.g. 7) and in this case only the essential data for the

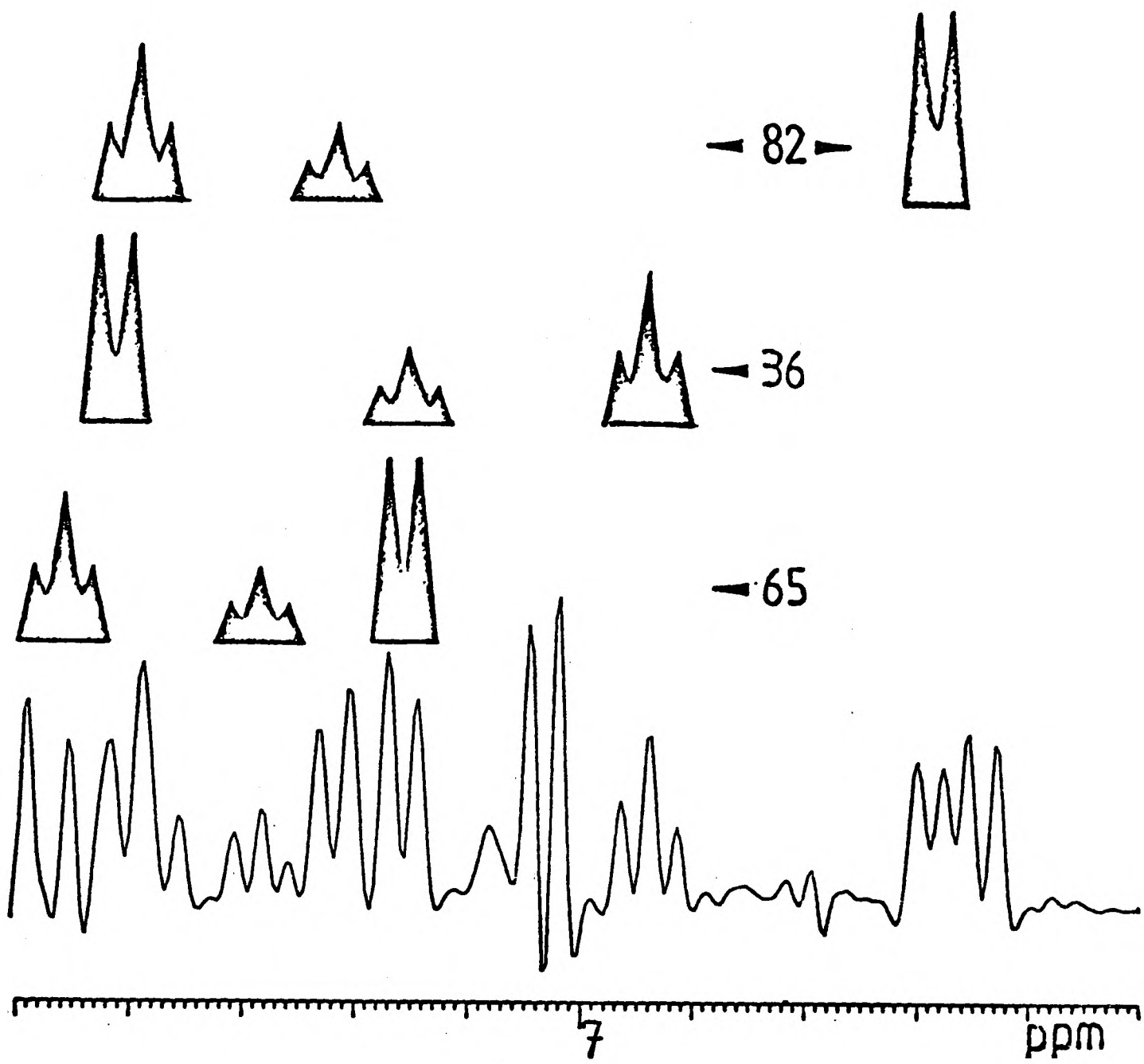
assignment of Phe 65 will be presented. Fig. 3.10. shows the aromatic regions of the blank and spin decoupling difference spectra of lamprey ferrocytochrome c at 47°C and pH 6.0 for the following conditions; a) blank b) - d) difference spectra with time shared irradiation at 7.16 ppm (b), 7.285 ppm (c) and 7.46 ppm (d). In b two two proton triplets at 7.46 ppm and 6.94 ppm can be seen to decouple. The resonance at 6.94 ppm is due to the meta protons of Phe 36 (cf. horse cytochrome c, Table 3.1.) and irradiating it decouples a doublet to a singlet at 7.415 ppm and causes a small effect at 7.16 ppm. The ortho and para proton resonances of Phe 36 are thus at 7.415 ppm and 7.16 ppm in lamprey ferrocytochrome c. c shows that irradiation at 7.285 ppm also causes the triplet at 7.46 ppm to decouple, which when irradiated itself in d causes the doublet at 7.165 ppm to decouple and the one proton triplet at 7.285 ppm to collapse to a singlet (this is not seen clearly in the difference spectrum d, because this resonance receives a large NOE from the irradiated peak - the effect in the difference spectrum is marked with a cross). These results indicate a first order assignment of the resonances at 7.46 ppm, 7.29 ppm and 7.16 ppm to the meta, para and ortho protons of a phenylalanine and as the resonances are not observed in horse ferrocytochrome c they are assigned to Phe 65. At this temperature only the para proton of Phe 10 at ca. 6.3 ppm and the para proton of Phe 46 at ca. 7.80 ppm are observed as these residues are undergoing motion that places their ortho and meta resonances in intermediate exchange; this is identical to the situation for horse ferrocytochrome c. Thus between 7.5 ppm and 6.5 ppm resonances from three phenylalanine residues (10, 65, and 82) are seen in the spectrum of lamprey ferrocytochrome c, and Fig. 3.11. shows the spectrum and the positions of these resonances (note, there are also resonances due to tyrosines, tryptophan

Fig. 3.10.



The aromatic region of the ^{1}H n.m.r. spectra of lamprey ferrocytochrome c at pH 6.0 and 47°C; a) Spectrum with time shared irradiation in a blank part of spectrum, b) spin decoupling difference spectrum with irradiation at 7.16 ppm, c) spin decoupling difference spectrum with irradiation at 7.285 ppm, d) spin decoupling difference spectrum with irradiation at 7.46 ppm.

Fig. 3.11.



The spectrum of lamprey ferrocytochrome c at pH 6.0 and 47°C. The coupling patterns of Phe 36, Phe 65 and Phe 82 are shown in the region between 7.5 ppm and 6.5 ppm. This region contains many overlapping resonances (including tyrosine, tryptophan and histidine residues), which may only be resolved in spin decoupling difference spectra (e.g. Fig. 3.10.). At this temperature only the para proton resonances of Phe 10 and Phe 46 are observed and they are outside this spectral region at 6.3 ppm and 7.80 ppm respectively.

and histidine in this region). It can be seen that few of the resonances are resolved, although the ortho proton doublets of Phe 65 and of Phe 82 and the meta triplet of Phe 36 are resolved. The region between 7.35 ppm and 7.50 ppm is particularly complicated containing the resonances of the meta protons of Phe 65 and 82, the ortho protons of Phe 36 and a singlet from His 26. The resonances here have only been identified by spin decoupling difference spectra.

Resonances are found in N. Crassa cytochrome c in very similar positions to those of Phe 65 in lamprey cytochrome c, and they are assigned to Phe 65 in this protein (23). As explained above the assignment of Phe 65 in N. Crassa cytochrome c allows the assignment of Phe -3 by a process of elimination. Table 3.5. gives the assignments of Phe -3 and Phe 65 in N. Crassa and lamprey ferricytochrome c and ferrocytochrome c with the primary chemical shift positions for a phenylalanine residue.

In horse cytochrome c the εCH_3 protons of Met 65 are assigned to resonance $M^{*}19$ at 1.94 ppm in ferricytochrome c and to resonance M11 at 2.11 ppm in ferrocytochrome c (Section 4.2.1.) and thus there is a 0.17 ppm shift upfield on this resonance upon oxidation of the protein (see Table 3.2.). For the ortho and meta resonances of Phe 65 in lamprey cytochrome c, there is a 0.19 ppm and 0.13 ppm upfield shift upon oxidation of the protein (Table 3.5.). Note also, that in lamprey ferrocytochrome c the resonances of Phe 65 all lie close (less than 0.13 ppm) to their primary chemical shift positions (those in a random coil protein or small peptide), while M11 in horse ferrocytochrome c recieves a very small 0.02 ppm upfield shift from its primary position. The chemical shift upon oxidation is due to a pseudocontact shift (7) from the paramagnetic haem centre in ferrocytochrome c, assuming that there is no conformational change between oxidation states. This is a valid assump-

Table 3.5.

Phenylalanine assignments for lamprey and Neurosporra Crassa cytochromes c

Assignment	Neurosporra Crassa+ oxidised	reduced	Primary position	lamprey oxidised	reduced
Phe 65 o-	6.99	7.20	7.29	6.97	7.16
Phe 65 m-	7.25	7.51	7.39	7.33	7.46
Phe 65 p-	----	----	7.34	----	7.29
Phe -3 o-	7.20	7.40	7.29	----	----
Phe -3 m-	7.41	7.55	7.39	----	----

+, the Neurosporra Crassa assignments are from the work of Mr. C. Eley (23).
The data is given for $^{2}H_{2}O$ solutions of the proteins at pH 7 and at 47°C.

tion for the Met/Phe 65 region of the protein (Section 2.2.1. and refs. therein). The correspondences between the shifts for primary positions in the ferrocytochromes c and the pseudocontact shifts in the ferricytochromes c for the proton resonances of Met 65 and Phe 65 in horse and lamprey cytochrome c indicate that the side chains of the two different residues lie in very similar positions with respect to the rest of the protein. No further quantification of these results is possible, but it is unlikely that the main peptide backbone in the αhelix 61 - 69 (18) is altered by the substitution at position 65; this helix is very firmly anchored in its configuration by Tyr 67 that lies right in the hydrophobic core of the protein, below the plane of the haem ring. Thus, like Met 65 in horse cytochrome c, Phe 65 in lamprey cytochrome c lies close to or on the surface of the protein; it does not fold back into the protein sufficiently to cause a conformation change to this region of the protein.

Lamprey cytochrome c is used for experiments in two further places in this thesis; a pH titration of lamprey ferrocytochrome c is described in Section 3.6.4., and the spectrum of lamprey ferricytochrome c - CN is briefly discussed in Section 6.2.

3.6.1. Structural studies close to Ile 57 in cytochrome c : Introduction

Ile 57 is a conserved residue that gives rise to a triplet methyl resonance, from its δCH_3 group, in the upfield methyl region of the spectrum of ferricytochrome c and ferrocytochrome c. The resonance is clearly resolved for horse ferrocytochrome c (M4, -0.43 ppm, 57°C) and has been cross assigned by redox titration (Section 3.3.1.) to ferricytochrome c (M*6, -0.18 ppm, 57°C). This resonance has some unusual properties, that initiated interest in the further studies that are described here. These

properties are:

a) The resonance has a strong temperature dependence in the spectra of ferrocytochrome c (unlike the majority of other resonances) in all the species (over 20 (23)) so far studied. The shift is downfield towards its primary position with increasing temperature and represents a conformation change between the δCH_3 of Ile 57 and the aromatic rings that give rise to its upfield shift that is temperature dependent. The X-ray structure (18) shows that Trp 59 and Tyr 74 are the sources of the large upfield shift on the resonance. The previous n.m.r. data shows that the position of Trp 59 with respect to the rest of the protein does not change significantly with temperature and that movements of Ile 57 and Tyr 74 can not be distinguished. This means that the conformational change results from the movement of Ile 57 or Tyr 74 or both with respect to the rest of the molecule. A temperature dependence of the NOEs in this region of the protein is discussed in Section 3.6.2., which provides further data on these movements.

b) In the spectra of ferricytochrome c, the resonance is the only resonance that does not shift towards it diamagnetic position with increasing temperature (20,21). This points to a conformational change between ferrocytochrome c and ferricytochrome c in this region of the protein. The conformation change appears to be approximately constant throughout species of cytochrome c studied by n.m.r. (23). This conformation change was originally found, ~~because~~ because M4 is one of the few resonances to suffer a significant shift between the spectra of horse ferrocytochrome c and cobalticytochrome c, which is a diamagnetic model for horse ferricytochrome c (32).

c) The resonance is pH dependent in the spectrum of ferrocytochrome c in the pH range (pH 3.9 to pH 13, 27°C) for which the protein is in its

native (state II) conformation. At high pH it shifts -0.43 ppm between pH 9.5 and pH 13 at 27°C. The only other resonances to shift in this pH range are haem meso βCH, +0.07 ppm; haem thioether bridge 4 CH, +0.04 ppm; and the Tyr 74 meta protons (A26) -0.08 ppm. Some new aromatic resonances also appear at ca. pH 12.5, and after their appearance the pH effects described become irreversible (11). Furthermore, at low pH the resonance shifts -0.06 ppm between pH 4.4 and pH 3.9 at 27°C. Later in this chapter evidence is provided as to the nature of the ionising residues that give rise to these shifts; Glu 66 at low pH and a lysine in the first 65 residues, probably Lys 39 or Lys 55, at high pH.

d) Ile 57 lies in a region of cytochrome c that is a major antigenic determinant, for the antibody response is well characterised for amino acid substitutions between species of cytochrome c in the region of residue 58 to residue 62.(33). The immune response is sensitive to very small changes of structure, and particularly to modifications to the surface regions of a protein; and the Ile 57 δCH_3 resonance is a good marker for small changes of structure induced by amino acid substitutions in the region of residue 58 to residue 62. There has been shown to be a good correlation between the chemical shift of this resonance, which varies from species to species, and the immune response generated by these species of cytochrome c in other species (22). For example the change from Lys 60 in donkey to Gly 60 in cow (these species also differ at position 89, but this is far away in the structure) is antigenic, and is reflected in the chemical shifts of M4 (-0.43 ppm, donkey and -0.49 ppm, cow), while the addition of a $-CH_2$ group at position 62 from Asp 62 in rabbit to Glu 62 in cow (these species also differ at positions 44, 89 and 92) is not immunogenic and does not affect M4 (-0.49 ppm, both). In order to further the understanding of how the immune response operates, it is necessary to

know more about the structure and dynamics of this region of the protein.

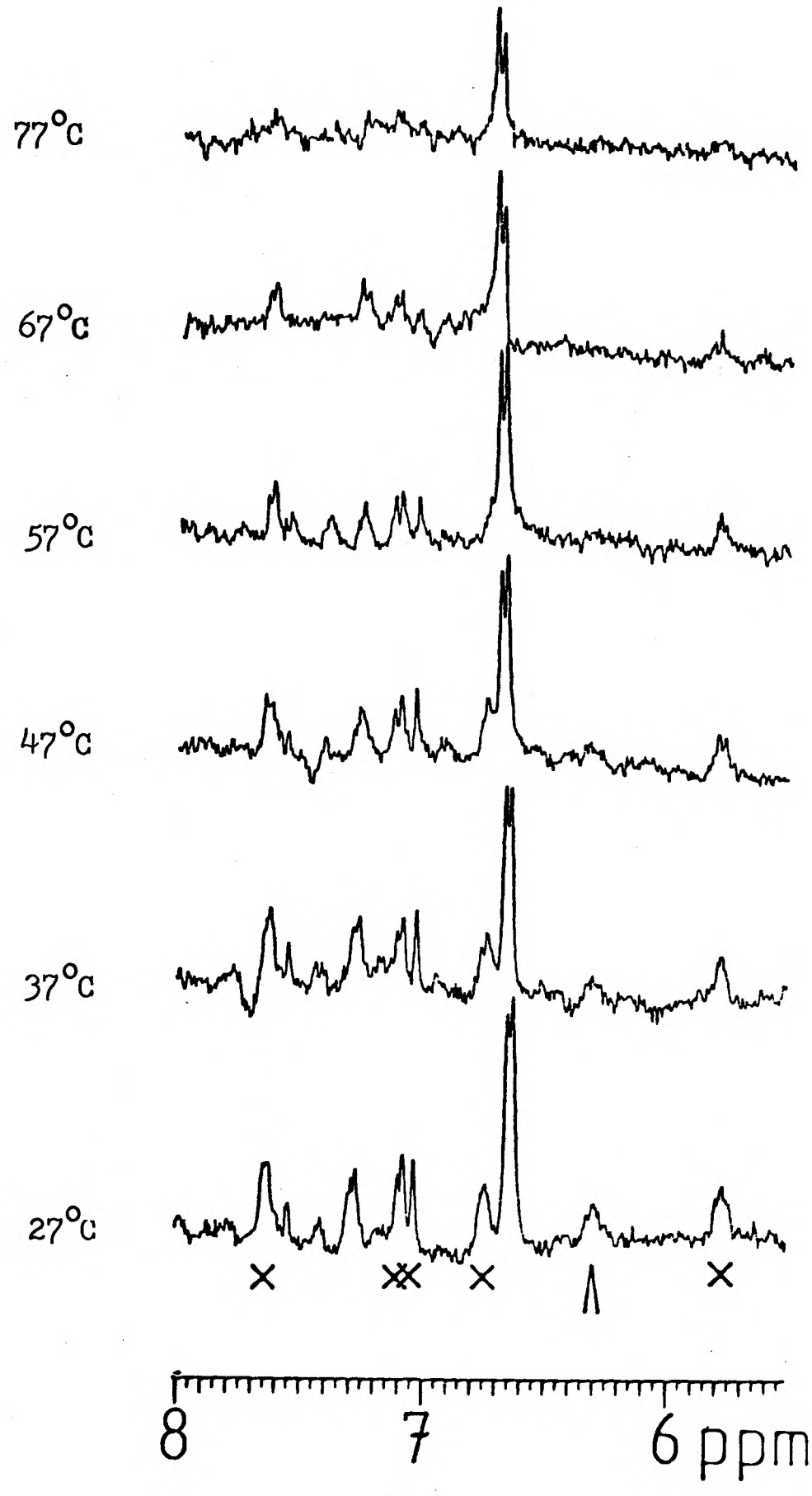
3.6.2. The temperature dependence of the NOEs between neighbouring protons close to Ile 57

In section 3.4., Fig. 3.5. shows the temperature dependence of the automatic NOE difference spectra from the δCH_3 resonance of Ile 57 in ferrocytochrome c, and these data are briefly discussed as they provides useful data for resonance assignments. Here, these data with those from the meta proton resonance of Tyr 74 are discussed and analysed semi-quantitively.

Fig. 3.12. shows the region between 8 ppm to 5.5 ppm of the NOE difference spectra from Ile 57 at 10° intervals between 27°C and 77°C, i.e. the aromatic regions of the spectra in Fig. 3.5. The five resonances from Trp 59 are marked with a cross, while the doublets at ca. 7.3 ppm and 6.65 ppm are due to the ortho and meta protons of Tyr 74 respectively (9). These spectra confirm the assignment of these latter resonances to Tyr 74 (29). The other resonances seen at 27°C, 37°C and 47°C are due to NOEs from Leu 32, whose resonances overlap with that of Ile 57 at these temperatures (a full account of this is given in Section 3.4.b)). This is confirmed by NOE experiments from the Leu 32 protons at higher temperatures. For example, the resonance at 6.3 ppm and marked with a 'Λ' is due to the para protons of Phe 10 (ca. 6 Å from Leu 32, in the X-ray model (18)) and is seen in the NOE difference spectrum from Leu 32 at 57°C.

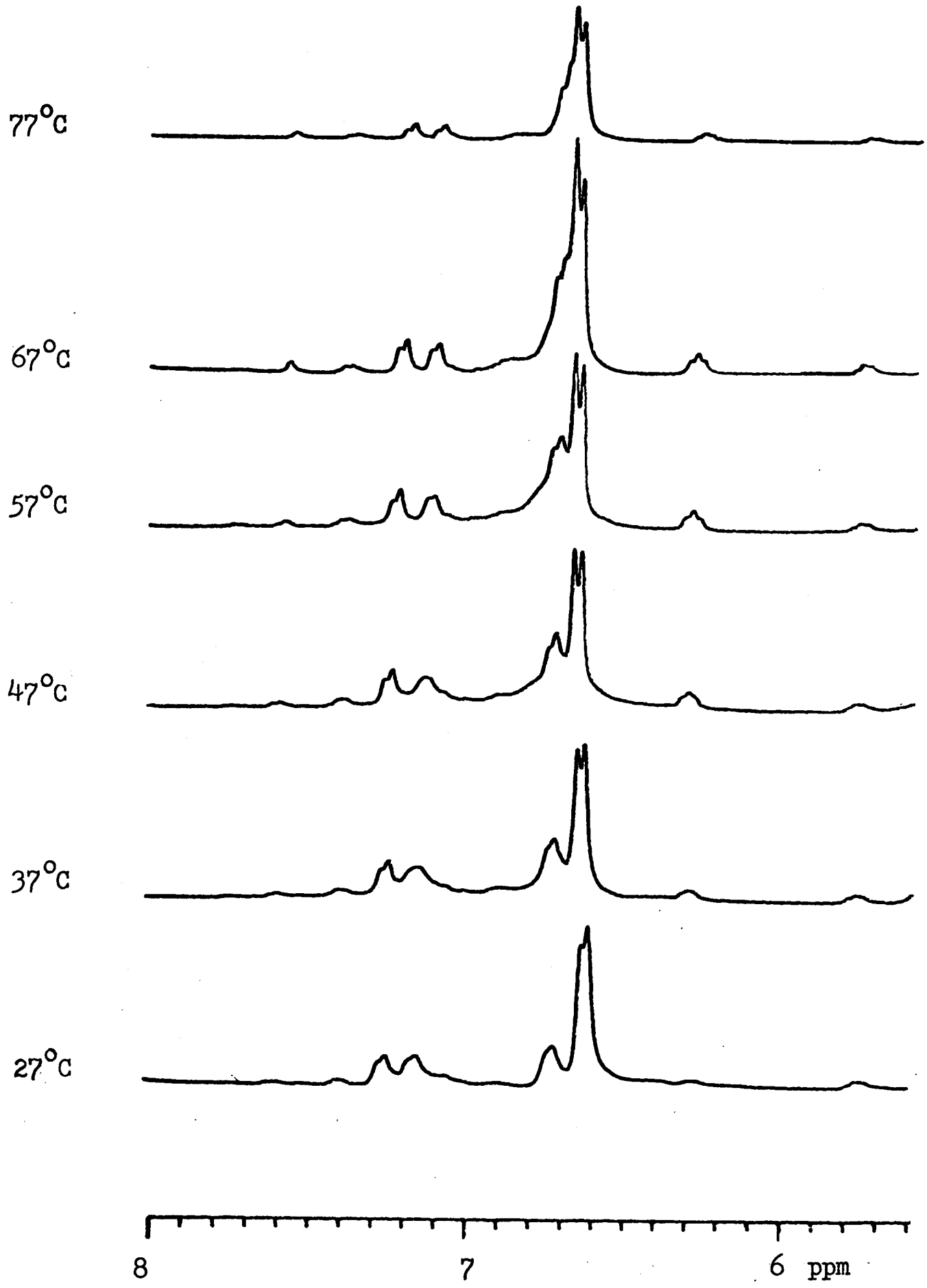
The temperature dependence of the NOE difference spectra from the Tyr 74 ortho protons resonance (A26, 6.65 ppm) has also been determined in the same temperature range. The results are not open to easy interpretation because of the number of resonances that receive the irradiation pulse at 6.65 ppm. This can be demonstrated by examination of Fig. 3.12.

Fig. 3.12.



The aromatic region of the NOE difference spectra from the Ile 57 δCH_3 resonance (M4) at 10°C intervals between 27°C and 77°C. The resonances marked with a cross are due to Trp 59, the resonance marked with a 'Λ' is from Phe 10 and recieves an NOE from Leu 32 (M3) at low temperature.

Fig. 3.13.



The aromatic region of the NOE difference spectra from the Tyr 74 meta protons resonance (6.65 ppm) at 10°C intervals between 27°C and 77°C.

(see above) and of Fig. 3.13., which shows the region between 8 ppm and 5.5 ppm for the NOE difference spectra with irradiation at the Tyr 74 meta proton resonance. The following points should be considered:

i) In Fig. 3.12. a Trp 59 resonance is seen at ca. 6.75 ppm at 27°C, which with the increase of temperature shifts towards and under the Tyr 74 meta resonance. At all temperatures irradiation of the Tyr 74 resonance causes some saturation of the Trp 59 resonance. This causes other Trp 59 resonances to appear in Fig. 3.13 at all temperatures and thus NOEs from Tyr 74 to Trp 59 resonances and NOEs from this Trp 59 resonance to other Trp 59 resonances, and particularly their respective intensities, can not be distinguished. Also this Trp 59 resonance appears in the NOE difference spectra from Ile 57, and so there will be a Trp 59 component to the NOE observed for Ile 57 in the NOE difference spectra from Tyr 74.

ii) The resonance at ca. 6.3 ppm can be seen to arise in the NOE difference spectra from Tyr 74 (Fig. 3.13) with increasing temperature; this is because the meta protons of Phe 10 are in intermediate exchange at 27°C, but appear under the Tyr 74 resonance (at 6.65 ppm) in fast exchange at higher temperatures. The resonance at 6.3 ppm is from the para protons of Phe 10, which are not affected by the ring flipping process that causes the exchange to the other Phe 10 resonances, and it receives an increasing NOE from the meta protons resonance as this resonance sharpens up under that of Tyr 74.

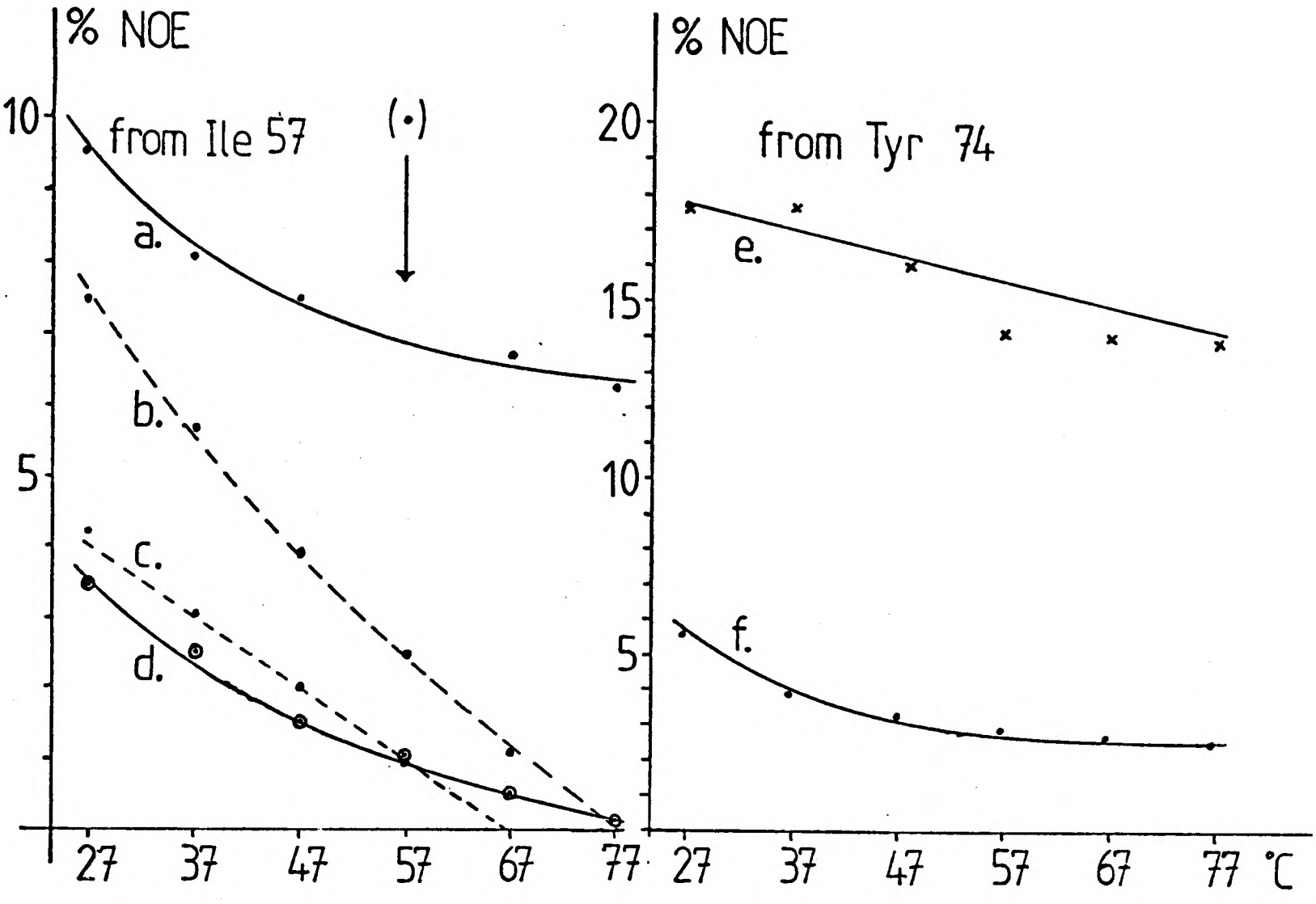
iii) A resonance at ca. 5.6 ppm is seen at 27°C in Fig. 3.13., which broadens out at higher temperatures. This effect is due to the cross saturation of the resonances of a tyrosine residue in slow exchange. The resonance at 5.6 ppm is due to Tyr 97 D and it exchanges environments with Tyr 97 C, which is at ca. 6.65 ppm at 27°C (i.e. under the resonance of Tyr 74). The recent assignments of these resonances is given in Table 3.1. (9,31).

iv) In Fig. 3.13. a further doublet resonance is seen immediately downfield from that of Tyr 74 (6.65 ppm) in the ordinary spectrum of horse ferrocytochrome c. This resonance is due to the ortho protons of Phe 82 (see Section 3.4.a)), and at all temperatures in the NOE difference spectrum from Tyr 74 the Met 80 CH_3 resonance appears due to an NOE effect from Phe 82.

Thus the NOEs observed from Tyr 74 in Fig. 3.13. have to be carefully distinguished from NOEs and other effects from Phe 10, Trp 59, Phe 82 and Tyr 97.

The intensities of the resonances of Trp 59, Tyr 74 and Ile 57 in the NOE difference spectra were calculated by integration. The results obtained are plotted against temperature in Fig. 3.14., and represent the following effects; a)the intensity of the NOE to the Tyr 74 meta protons resonance from the δCH_3 resonance of Ile 57, b) the average of the intensities of the NOEs to the C-4, C-5, C-6 and C-7 proton resonances of Trp 59 from Ile 57, c) the intensity of the NOE to the Trp 59 C-2 proton resonance from Ile 57, d) the intensity of the NOE to the ortho protons resonance of Tyr 74 from Ile 57, e) the intensity of the NOE to the ortho protons resonance of Tyr 74 from the meta protons resonance of Tyr 74, and f) the intensity of the NOE to the δCH_3 resonance of Ile 57 from the meta protons resonance of Tyr 74. Note that the NOE effect to the Trp 59 benzenoid proton resonances from Ile 57 was the same within the experimental error ($\pm$ 1% intensity units) at all temperatures, so these intensities were averaged (in the X-ray structure these protons all lie between 3 Å and 4.5 Å from the δCH_3 group of Ile 57. Plot e) is a standard to calibrate all the plots for any effect to the intensity of the NOEs upon the increase of temperature (and rotational correlation times); it can be seen that the intensity of the NOE from the meta resonance to the ortho

Fig. 3.14.



The intensities of some NOEs between protons in the Ile 57, Tyr 74 and Trp 59 region of ferrocytochrome c plotted against temperature. a) The NOE to Tyr 74 meta protons resonance from the Ile 57 δCH_3 resonance, b) the average of the NOEs to the C-4, C-5, C-6 and C-7 proton resonances from Ile 57, c) the NOE to the Trp 59 C-2 proton resonance from Ile 57, d) the NOE to the Tyr 74 ortho protons resonance from Ile 57, e) the NOE to the ortho protons resonance of Tyr 74 from the meta protons resonance of Tyr 74, and f) the NOE to the Ile 57 δCH_3 resonance fom the meta protons resonance of Tyr 74.

resonance of Tyr 74 suffers a ca. 15% decrease over the temperature range 27°C to 77°C. The data is not accurate enough to interpret this effect. The 57°C point for plot a) is an example of the problems that the resonance overlap, ~~described~~ described above in points i) to iv), can cause to the integration of the spectra. The value obtained at this point is too large, because, as inspection of Fig. 3.12. will reveal at 57°C a Trp 59 resonance lies directly under the meta proton resonance of Tyr 74. The head of an arrow from this point on Fig. 3.14. indicates an estimate of the real intensity for the tyrosine resonance alone, after an estimate if the intensity of the tryptophan resonance, based on plot b), is subtracted.

The following points about the data presented in Fig.3.14. are made:

a) The NOE from the δCH_3 resonance of Ile 57 to the meta proton resonance of Tyr 74 and vice versa both decrease with increasing temperature indicating a mutual movement from each other. (Note, there will be a very small, unquantifiable, contribution to plot f) from NOEs to Ile 57 from partial saturation of a Trp 59 resonance close to the Tyr 74 resonance (point i), above).) The NOE from Ile 57 (plot a) is about twice the magnitude of the reverse NOE (plot f)), due to the methyl group being a better 'relaxation sink'. Both NOEs have an approximately identical temperature dependence, as can be seen by the similar shapes of plots a) and f).

b) The NOE from the δCH_3 resonance of Ile 57 to the ortho proton resonance of Tyr 74 (plot d) decreases much more rapidly than that to the meta proton resonance of Tyr 74.This isclearly appreciated when the data are normalised.

c) The NOEs of the benzenoid protons (plot b)) and the C-2 proton (plot c)) resonances of Trp 59 from Ile 57 decrease more rapidly than either of the Tyr 74 NOEs.

Two further points from other studies should be considered:

d) The resonances of Ile 57 are temperature dependent in ferrocytochrome c. The δCH_3 resonance shifts from -0.61 ppm (27°C) to -0.31 ppm (77°C) (10) and the γCH_3 resonance shifts from 0.78 ppm (27°C) to 0.68 ppm (77°C) (this work, based on the assignment given in Section 3.4.b)). This indicates a temperature dependent conformation change with respect to the rings of Trp 59 and Tyr 74, which are responsible for secondary shifts on these resonances.

e) The spectrum of $(Hse)^{65}$, $(Leu)^{74}$ horse ferrocytochrome c (see Section 4.2.5.) contains a triplet from the δCH_3 group of Ile 57, which shifts from 0.12 ppm (27°C) to 0.18 ppm (77°C). In this protein Met 65 and Tyr 74 have been replaced by homoserine 65 and Leu 74, but the majority of the spectrum has a 1:1 correspondence with that of ferrocytochrome c, indicating that the modified protein has a native conformation. Note, that the Tyr 74 component to the upfield secondary shift of the Ile 57 resonance has been lost in the modified protein, but that the resonance still has a small temperature dependence, which must arise from a temperature dependence in the secondary shift from Trp 59.

Points a) to c) suggest that there is a larger temperature dependence to the secondary shift of Ile 57 from Trp 59 than from Tyr 74, as the NOEs to Trp 59 decrease more rapidly; while points d) and e) suggest the opposite conclusion as in the modified protein Ile 57 recieves only a small temperature dependence in its secondary shift from Trp 59. This apparent inconsistency is either due to the fact that the secondary shift on Ile 57, and its temperature dependence, is a function of both the distance and angle from the rings of Tyr 74 and Trp 59, while the NOEs are only dependent on the distance between the individual protons involved; or due to a change in the relative positions of Trp 59 and Ile 57 between the native

and modified proteins. It is impossible to distinguish between these two possibilities on the basis of the present data. However both sets of data indicate that the Ile 57 δCH_3 group moves relatively away from both Tyr 74 and Trp 59 as the temperature is increased. The NOE data shows that in the native protein the movement between Ile 57 and Trp 59 is greater than that between Ile 57 and Tyr 74.

A temperature dependent movement of Trp 59 relative to the rest of the protein is ruled out as its resonances are temperature independent and lie within the ring current field of the haem group and are very sensitive markers to any such movement (34). Thus the group(s) that move relative to the rest of the protein are either Ile 57 solely or both Ile 57 and Tyr 74. It is impossible to distinguish between these two possibilities. It is, however, interesting to note that the δCH_3 group of Ile 57 shifts towards its primary position with increasing temperature (point d)) while the γCH_3 resonance shifts away from its primary position; this suggests that there may be some rotation about the $\alpha-\beta$ bond of the Ile 57 residue as the temperature is increased so that the γCH_3 approachs the ring current centres and the δCH_3 moves away from them.

3.6.3. NOEs observed from Ile 57 in cytochromes c other than horse ferrocytochrome c

In the last section a semiquantitive interpretation of the temperature dependence of the NOEs observed from Ile 57 and from Tyr 74 in horse ferrocytochrome c was given. Here a brief description of preliminary NOE experiments in other cytochromes c is given.

The NOE from Ile 57 to Tyr 74 and vice versa was demonstrated for tuna and lamprey ferrocytochromes c at 27°C, and presumably could be demonstrated for all other eukaryotic ferrocytochromes c in which these residues

are conserved. It would be of interest to do the experiment for all obtainable ferrocytochromes c at a standard temperature and relate the NOE intensity to the secondary shift on the Ile 57 resonance in each protein. Robinson and Moore (personal communication) have extended this experiment to Candida krusei ferrocytochrome c which has Val 57 instead of Ile 57, and have assigned the resonances of Val 57 by the NOE from Tyr 74.

The NOEs were also demonstrated for horse ferricytochrome c at 27°C and 57°C. The NOE difference spectra were not so clear as for the ferrocytochromes c due to the irradiated resonances being regions with more overlapping resonances, but it could be seen that the intensity of NOE from Ile 57 to Tyr 74 and vice versa was smaller at the elevated temperature. This indicates that there is a similar temperature dependent conformation change in ferricytochrome c to that in ferrocytochrome c.

3.6.4. The pH dependence of Ile 57 in ferrocytochrome c at low pH; the pH titration of lamprey ferrocytochrome c

Between pH 4.4 and pH 3.5 in horse ferrocytochrome c at 27°C four resonances are observed to shift; A8 and A18 from His 26 ($pK_a \approx 3.2$ (35)), A30 from the C-6 proton of Trp 59 and M4 from the δCH_3 of Ile 57. In this range of pH glutamic and aspartic acid residues are known to accept protons and it has been proposed that the effect to M4 and possibly to A30 is caused by the ionisation of Glu 66 (11). There is most probably a hydrogen bond between Glu 66 and Tyr 74 (see Section 3.5.) and the acceptance of a proton of Glu 66 would be likely to cause a small perturbation to Tyr 74 and hence to the secondary shift due to Tyr 74 of resonance M4. Lamprey cytochrome c has a valine at position 66 and so the analogous pH titration was carried for this protein.

Spectra of lamprey ferrocytochrome c were recorded at pH 5.00, pH

4.55, pH 4.05 and pH 3.50 all at 27°C. The spectra were broad at the lower pH values, but it could be clearly seen that the corresponding resonances to A30 and M4 were not affected by the changes in pH.

These results are extremely good evidence for the ionisation of Glu 66 being responsible for the pH shifts to A30 and M4 in horse ferrocytochrome c. The effect is mediated via Tyr 74 to Ile 57, and through space to the C-6 proton of Trp 59 (Trp 59 C-6 proton is ca. 7 Å from the carboxyl group of Glu 66 (18)).

3.6.5. The pH dependence of Ile 57 in ferrocytochrome c at high pH; the pH titration of some modified cytochromes c

At 27°C and between pH 9.5 and 13.0 resonance M4 in ferrocytochrome c shifts -0.43 ppm. The only other changes to the spectrum through this pH range are shifts to haem meso βCH, +0.07 ppm; haem thioether bridge 4 CH, +0.04 ppm; and the Tyr 74 meta protons (A26), -0.08 ppm, and the appearance of some new aromatic resonances at ca. pH 12.5 (11).

In Section 5.4. the analogous pH titration for N^{ε}-acetimidylated ferrocytochrome c (fully modified with the N^{ε}-acetimidyl group at all 19 lysines) is described. In the case of this protein, the resonances A26 and M4 do not shift from their positions at pH 7 at pH values up to 12 - the protein denatures at about this pH value. New resonances are seen as in native ferrocytochrome c at pH 9.5 and above. These results are fully discussed in Section 5.4. and only one point is relevant to this section, which is as follows; the modification of all 19 lysine residues in ferrocytochrome c with a group with a raised pK_a (i.e. the N^{ε}-acetimidyl group) eliminates the high pH dependence of Ile 57 and Tyr 74. This effect is studied further in this section by the study of two suitable modifications of ferrocytochrome c.

In Section 4.1.2. the coupling of the cyanogen bromide cleaved peptide derivatives of cytochrome c, 1 to 65 (A) and 66 to 104 (BC) to form the semisynthetic $(Hse)^{65}$ cytochrome c (A.BC) is described; and in Section 4.2.1. the structure of $(Hse)^{65}$ ferrocytochrome c is shown to be very close to that of ferrocytochrome c by the comparison of their ^1H n.m.r. spectra. Two further modifications (and those which are studied here) in which either fragment A or BC has been N^{ε}-acetimidylated prior to the coupling reaction can be obtained. These are (with their nomenclatures):

aA.BC = (N^{ε}-acetimidylated lysine)$_{11}$(1 - 65);(lysine)$_8$(66 - 104); $(Hse)^{65}$ cytochrome c

A.aBC = (lysine)$_{11}$(1 - 65);(N^{ε}-acetimidylated lysine)$_8$(66 - 104); $(Hse)^{65}$ cytochrome c

These two compounds were kindly prepared by Dr. D. E. Harris and Dr. C. J. A. Wallace of the Molecular Biophysics Laboratory, Oxford. Only 1 mg. quantities were available and the compounds were used only for the study described here, as the quantities are not sufficient for further n.m.r. studies.

Spectra of the two derivatives were recorded at pH 7 and 27°C, in the reduced state, and compared to the corresponding spectra of ferrocytochrome c, $(Hse)^{65}$ ferrocytochrome c and N^{ε}-acetimidylated ferrocytochrome c (see Sections 4.2.2. and 5.2.). The following points are made:

a) The position of resonance M1, from the CH_3 group of the haem ligand Met 80, and other resolved resonances as the haem meso protons between 9.7 ppm and 9.0 ppm, and the Leu 32 CH_3 resonances at -0.60 ppm and -0.76 ppm are maintained at chemical shift positions close to that of native ferrocytochrome c in the spectra of the two compounds. This indicates that the two compounds have a native structure under the experimental

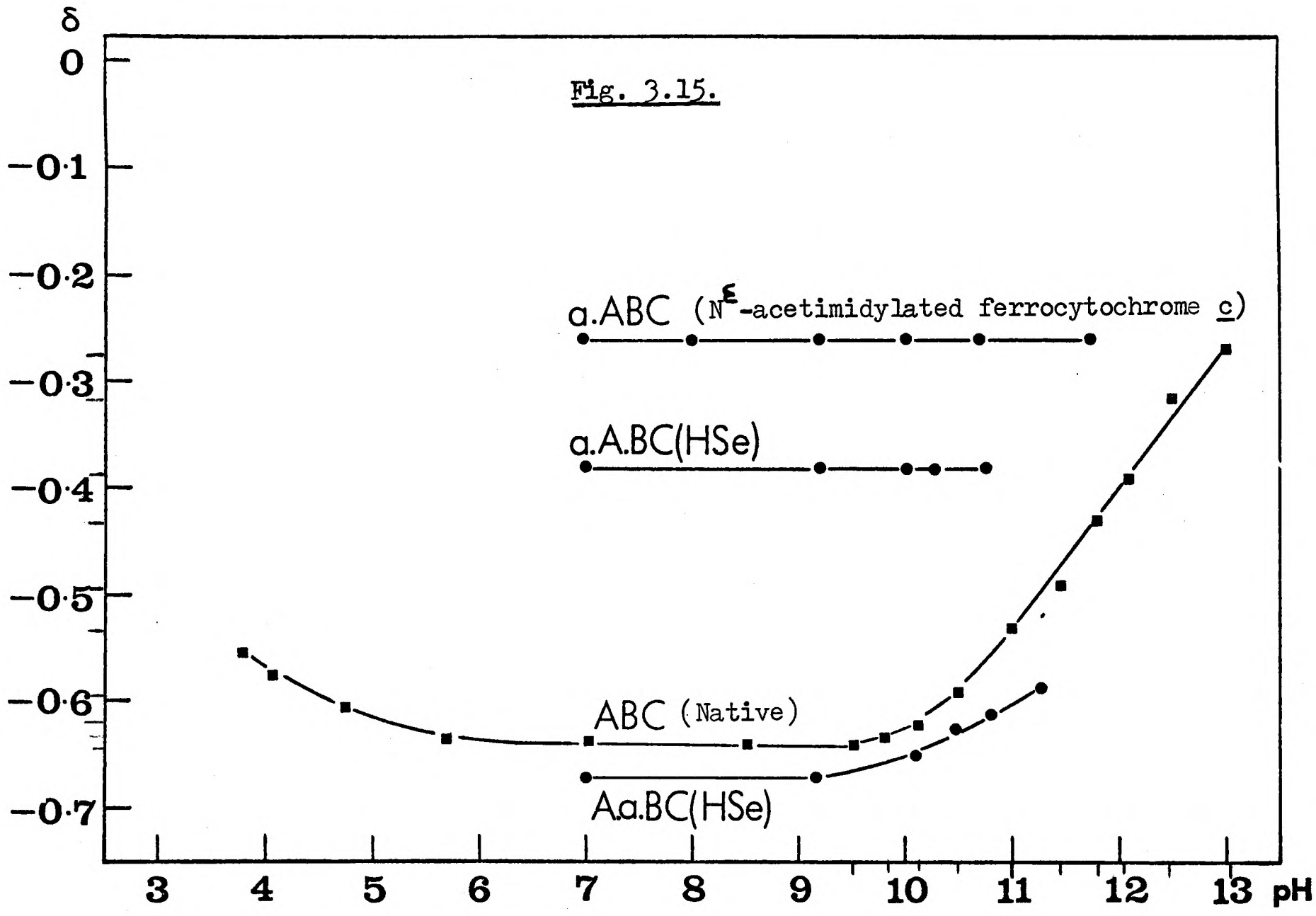
conditions, as do $(Hse)^{65}$ ferrocytochrome c and N^{ε}-acetimidylated ferrocytochrome c (Sections 4.2.2. and 5.2.).

b) The δCH_3 resonance of Ile 57, M4, is shifted from its position in ferrocytochrome c, but is clearly resolved in the upfield methyl region for all the derivatives. The origin of the shifts to the Ile 57 resonance in $(Hse)^{65}$ ferrocytochrome c and N^{ε}-acetimidylated ferrocytochrome c is discussed in Section 4.2.2 and Section 5.2., respectively. The shifts of this resonance in aA.BC and A.aBC are assumed to be due to the same origins and approximately accumulative with the modifications involved, and further discussion is not relevant here. The resolution of M4 in a non-overlapping region of the spectra for all the proteins is crucial to the main experimental data of the argument that follows, as 1 mg. quantities of aA.BC and A.aBC produce spectra in which overlapping regions may not be clearly resolved.

The pH titration in the range pH 7 to pH 11.5 at 27°C was carried out for the two compounds, and the chemical shifts of M4 against pH value in these compounds, in N^{ε}-acetimidylated ferrocytochrome c (Section 5.2.) and in ferrocytochrome c (11) is plotted in Fig. 3.15. It can be clearly seen that the modification of the first eleven lysine residues of ferrocytochrome c in aA.BC eliminates the high pH dependence of Ile 57, whereas the modification of the last eight lysine residues in A.aBC does not affect the pH characteristics of Ile 57. Before these points are discussed further the following points will be made:

i) The resonances of Tyr 74 have been firmly assigned in the ^1H n.m.r. spectrum of ferrocytochrome c (Section 3.5.2.), and although they are observed to shift slightly between pH 9.5 and pH 13.0 (see above) the tyrosine is not observed to ionise. Dr. G.Moore has carried out the

Fig. 3.15.



The plot of the chemical shift of the Ile 57 CH_3 resonance (M4) against pH for native ferrocytochrome c, and three derivatives (nomenclature explained in text), all at 27°C

^{13}C pH titration for ferrocytochrome c, and no tyrosine resonances are observed to titrate in this pH range (personal communication). Thus Tyr 74 does not ionise in this pH range (note, that the pK_a for tyrosine itself is pK_a=10.8), and can not be responsible for the high pH dependence of Ile 57.

ii) The pH dependence of Ile 57 in Neurosporra Crassa ferrocytochrome c is similar to that in horse ferrocytochrome c (23). N. Crassa cytochrome c has a non ionisable trimethyllysine group at position 72 in place of lysine 72 in horse cytochrome c.

iii) Lysine residues in horse cytochrome c that are close enough to the Ile 57 region to cause the high pH dependence are those at 72 and 73, which are close to Tyr 74, and those at 39 and 55, whose NH_3^+ group lie within 6 Å of the δCH_3 of Ile 57 according to the X-ray structure.

iv) Indirect measurements of the pH dependence of the chemical reactivities of all the lysine residues in ferrocytochrome c indicates apparent pK_a values of ca. 9.3 and ca. 10.7 for Lys 39 and Lys 55 respectively (36).

The data presented above on compounds aA.BC and A.aBC show firmly that the pH dependence of Ile 57 at high pH is due to the ionisation of a lysine residue in the first 65 residues of the protein. Points i) and ii) are consistent with this, and point iii) suggests that the lysine is either 39 or 55. On the basis of the present data it is not possible to distinguish between these two possibilities, however point iv) provides circumstantial evidence that Lys 39 may be responsible. The pH titration curve (Fig. 3.15.) for ferrocytochrome c itself is not symmetrical above pH 9 and indicates that above ca. 10.5 ionisation of further lysine, arginine and tyrosine residues occurs and causes further perturbations to Ile 57 and to the whole molecule, which finally denatures at pH 13.

3.6.6. Summary of structural studies of the region close to Ile 57

In section 3.6.1. a resumé of the unusual properties of the region of cytochrome c close to Ile 57 and Tyr 74 is given. The studies undertaken here show that;

a) An NOE can be observed between the protons of Ile 57 and Tyr 74 in eukaryotic ferrocytochrome c and ferricytochrome c.

b) The temperature dependence of the NOEs from Ile 57 and from Tyr 74 in horse ferrocytochrome c indicate that as the temperature increases the protein suffers a conformation change in this region of the tertiary structure. The δCH_3 group of Ile 57 moves away from the rings of both Tyr 74 and Trp 59 as the temperature is increased. This may be considered as a fine conformation change induced by the modification of the bulk properties of the medium.

c) The δCH_3 group of Ile 57 in horse ferrocytochrome c has a pH dependence that is attributable to the ionisation of Glu 66 at low pH (pH 3.5 to pH 4.5) and to the ionisation of either Lys 39 or Lys 55 at high pH (pH 9.5 to pH 13.0).

References: Chapter Three

1. Kalinichenko, P. (1976), Stud.Biophys., 58, 235.

2. Ernst, R.R. (1966), Adv.Mag.Res. 2, 1.

3. Bundi, A., and Wuthrich, K. (1979), 18, 285.

4. Johnson, C.E., and Bovey, F.A. (1958), J.Chem.Phys., 29, 1012.

5. Dobson, C.M., Moore, G.R., and Williams, R.J.P. (1975), FEBS letters, 51, 60.

6. Campbell, I.D., and Dobson, C.M. (1975), J.C.S.Chem.Commun., 750.

7. Levine, B.A., Moore, G.R., Ratcliffe, R.G., and Williams, R.J.P., (1979), in 'Chemistry of Macromolecules IIA', volume 24, ed. R.E.Offord, University Park Press, Baltimore.

8. Mc.Donald, C.C., and Phillips, W.D. (1973), Biochemistry, 12, 3170.

9. Moore, G.R., and Williams, R.J.P. (1980), Eur.J.Biochem. 103, 493.

10. Moore, G.R., and Williams, R.J.P. (1980), Eur.J.Biochem. 103, 503.

11. Moore, G.R. (1978), D.Phil thesis, Oxford.

12. Swanson, R., Trus, B.L., Mandel, N., Mandel, G., Kallai, O.B., and Dickerson, R.E. (1977), J.Biol.Chem., 252, 759.

13. Gupta, R.K., and Koenig, A.G. (1970), Science, 169, 1204.

14. Redfield, A.G., and Gupta, R.K. (1971), Cold Spring Harbour Symp.Quant.Biol., 36, 405.

(and) Gupta, R.K., Koenig, S.H., and Redfield, A.G. (1972), J.Mag.Res., 7, 66.

15. Stellwagen, E., and Shulman, R.G. (1973), J.Mol.Biol., 80, 559.

16. Dobson, C.M., Moore, G.R., and Williams, R.J.P. (1975), FEBS Letters, 51, 60.

17. Keller, R.M., Pettigrew, G., and Wuthrich, K. (1973), FEBS Letters, 36, 151.

18. Takano, T., and Dickerson, R.E. (1980), Proc.Natl.Acad.Sci., in press.

19. Perkins, S.J. (1980), J.Mag.Res., 38, 297.

20. Moore, G.R., and Williams, R.J.P. (1980), Eur.J.Biochem. 103, 513.

21. Moore, G.R., and Williams, R.J.P. (1980), Eur.J.Biochem. 103, 523.

22. Moore, G.R., and Williams, R.J.P. (1980), Eur.J.Biochem. 103, 543.

23. Eley, C.G.S. (1980), Part II thesis, Oxford.

24. Campbell, I.D., Dobson, C.M., Williams R.J.P., and Wright, P.E., (1975), FEBS Letters, 57, 96.

25. Abragam, A. (1961), 'Nuclear Magnetic Resonance', Oxford Univ.,Press.

26. Noggle, J.H., and Schirmer, R.E. (1971), 'The Nuclear Overhauser Effect', Academic Press, New York.

27. Kalk, A., and Berendsen, H.J.C. (1976), J.Mag.Res. 24. 343.

28. Poulsen, F.M., Hoch, J.C., and Dobson, C.M. (1980), Biochemistry, 19, 2597.

29. Boswell, A.P., Moore, G,R., Williams R.J.P., Chien, J.C.W., and Dickinson, L.C. (1980), J.Inorg.Biochem., 13, 347.

30. Dayhoff, M.O., (1968-1973), Atlas of Protein Sequence and Structure, National Biomedical Research Foundation, Maryland, U.S.A.

31. Keller, R.M., and Wuthrich, K. (1981), Biochim.Biophys.Acta. in press.

32. Moore, G.R., Williams, R.J.P., Chien, J.C.W., and Dickinson, L.C., (1980), J.Inorg.Biochem., 12, 1.

33. Urbanski, G.J., and Margoliash, E. (1977), J.Immunol., 118, 1170.

34. Cookson, D.J., Moore, G.R., Pitt, R.C., Williams, R.J.P., Campbell, I.D., Ambler, R.P., Bruschi, M., and LeGall, J. (1978), Eur.J.Biochem., 83, 261.

35. Cohen, J.S., and Hayes, M.B. (1974), J.Biol.Chem., 249, 5472.

36. Bosshard, H.R., and Zurrer, M. (1980), J.Biol.Chem., in press.

CHAPTER FOUR

Singly modified cytochromes c

4.1. Introduction

This chapter describes cytochromes c that have been modified at one (or two) site(s). In a few cases a 'modification' may be obtained by isolating cytochromes c from closely related species; for example, horse cytochrome c is an effective modification of donkey cytochrome c with an extra methyl group at position 47 (Thr, horse; Ser, donkey) and the ^{1}H n.m.r. spectra of the two proteins shows that this extra methyl group causes no changes to the time averaged three dimensional structure or dynamic structure in either oxidation state (1). This thesis describes parellel modifications, obtained by protein chemistry techniques, in other parts of the molecule. Protein chemistry has now reached a 'state of the art', which provides a large choice of methods and reagents for obtaining very specific modifications. There are two main methods, direct chemical modification and protein semisynthesis, which are now reviewed briefly and particularly with reference to cytochrome c

4.1.1. The method of chemical modification

The direct chemical modification method can produce very high yields of proteins modified at single amino acid residues, if the reagent and the type of amino acid to be modified are chosen carefully. Sometimes, only one residue of the particular amino acid type will be modified under suitable conditions, especially if that residue is activated in some way. For example, this may be the case in a globular protein if there is only one residue of a given amino acid type on the surface; though this is not a general rule, in cytochrome c the tyrosines are acetylated with inverse reactivity to that expected from the surface

structure (2). In tuna ferrocytochrome c, however, only the surface methionine at position 65 will react with iodoacetic acid at pH 5.6 to produce [Met(Cm)^{65}] tuna ferrocytochrome c (3,4), which is one of the modifications described in this chapter.

When such specificity can not be achieved the two following methods can be utilised. Firstly, biological specificity of a particular enzyme reaction can be used with the chemical reagent. An example of this is the specific iodination of Tyr 74 in cytochrome c using the lactoperoxidase-potassium iodide-hydrogen peroxide system (5); using conventional iodination techniques only Tyr 48 and Tyr 67 are iodinated (6). Secondly, an appropriate reagent is chosen and its reaction with the protein is stopped before completion so that a mixture of proteins, which have been modified singly (and more than singly) at several different residues of the same amino acid type, is obtained. The different singly modified proteins can then be separated and purified by ion exchange chromatography. Using this method nine singly modified lysine derivatives of cytochrome c were obtained with the reagents 4-chloro-3,5-dinitrobenzoate (7) and trifluoroacetic acid (8). These derivatives are of great importance to the study of the electron transfer properties of cytochrome c and, for example, were used to map out binding domains on cytochrome c for cytochrome c oxidase (9,10) and succinate cytochrome c reductase system (11). As these proteins are used in such studies of the structure to function relationship for cytochrome c it is important to ascertain to what extent such modifications affect the static and dynamic structure of the protein. The work described in this chapter was carried out in order to study this point.

4.1.2. The method of protein semisynthesis

The semisynthetic approach involves producing peptide fragments of a protein, by proteolytic cleavage or by chemical cleaving agent, that can be recoupled to recreate the original protein. Once such a method can be found for a particular protein, then one of the fragments can be replaced by a very similar peptide which is either synthesised with a slightly different sequence or obtained by a chemical modification of the original peptide. When the fragments are recoupled a protein of slightly different constitution from the original is obtained and is called a 'semisynthetic protein' (for general examples, see 12). This approach will be illustrated for cytochrome c.

If ferricytochrome c is treated with cyanogen bromide (CNBr) the protein is cleaved at its two methionine residues, 65 and 80, producing three fragments A (1 - 65), B (66 - 80) and C (81 - 104) (13). Fragment B is the shortest (15 amino acids) and thus is most easy to synthesise or modify, and fortuitively it is also a part of the peptide chain of cytochrome c where many interesting modifications are possible, as many of its residues are conserved from species to species. Thus a fragment B' can be synthesisied that contains a leucine at position 74 instead of the tyrosine that is found at this position invariantly for all eukaryotic species (14). Combined with A and C a new protein AB'C is obtained by the recoupling process. Due to the nature of the particular recoupling strategies used (15,16) the resynthesised protein has a further change at position 65; methionine is replaced by a homoserine residue, so that the final product is $(Hse)^{65}$, $(Leu)^{74}$ cytochrome c. $(Hse)^{65}$ cytochrome c itself has been produced in the following way; ferricytochrome c was treated with a threefold excess of CNBr, and the mixture of fragments passed down a Sephedex G50F column in 7% formic acid. Pure preparations

of the peptides A (1 - 65) and BC (66 - 104) were obtained (17). These fragments recouple spontaneously in a mixture of equimolar proportions that is reduced, via a non-covalent complex that provides steric enhancement to the coupling reaction (17,18,19) to produce the singly modified $(Hse)^{65}$ ferrocytochrome c. This technique has been used to create hybrids of different species of cytochrome c and series of closely related semisynthetic proteins can be produced from closely related original proteins (2). For example, horse and cow cytochrome c differ at only positions 47 (Thr, horse; Ser, cow) and 89 (Thr, horse; Gly, cow) and four possible semisynthetic proteins can be prepared, which have the following sequence differences:

A (horse).BC (horse)	Thr 47	Hse 65	Thr 89
A (horse).BC (cow)	Thr 47	Hse 65	Gly 89
A (cow).BC (hosre)	Ser 47	Hse 65	Thr 89
A (cow).BC (cow)	Ser 47	Hse 65	Gly 89

These proteins are very useful in immunological studies and have been used to define an antigenic determinant unambigously (20). Thus, the semisynthetic method can produce many derivatives of cytochrome c with very small changes in primary structure. It has the disadvantage of being time consuming and at the present 'state of the art' yielding only small quantities of product.

4.2. Modification at methionine 65 in cytochrome c: Introduction

Methionine 65 is a surface residue on the back of the cytochrome c molecule, and is close to the negatively charged groups of Asp 62 (tuna), Glu 62 (horse), Glu 66 (tuna and horse) and Glu 69 (tuna and horse) and the positively charged group of Arg 91 (tuna and horse). This surface region of the protein binds cations strongly (e.g. Gd^{3+}) and anions apparently less strongly (e.g. $Cr(CN)_6^{3-}$) (21). Met 65 serves as the

binding site for $Pt(Cl)_4^{2-}$ used in crystals of cytochrome c to produce heavy atom derivatives suitable for X-ray crystallography (22). Furthermore, Met 65 is close to Ile 57 which is a particular well defined region of the protein for ^1H n.m.r. studies (see Chapter 3). This section of the thesis concerns chemically modified $[Met(Cm)^{65}]$ tuna cytochrome c and semisynthetic $(Hse)^{65}$ horse cytochrome c. The binding of platinum complexes to Met 65 in cytochrome c and in model compounds is also investigated, and the use of tetrachloroplatinite ($Pt(Cl)_4^{2-}$) as a probe for surface methionines in globular proteins is discussed.

The horse $(Hse)^{65}$ cytochrome c was a kind gift from Dr. C. J. A Wallace of the Molecular Biophysics Laboratory, Oxford and was prepared by established proceedures (15). $[Met(Cm)^{65}]$ tuna cytochrome c was prepared by the reaction of tuna ferrocytochrome c with iodoacetic acid (see Appendix), following the proceedure of Ando et al (4). Detailed studies of the carboxymethylation of horse and beef cytochrome c (4) show that under the same reaction conditions only His 33 and Met 65 are modified. Tuna cytochrome c was used in this study to eliminate the His 33 side reaction, as it has a tryptophan at position 33 (14).

4.2.1. $(Hse)^{65}$ horse ferrocytochrome c and $[Met(Cm)^{65}]$ tuna ferrocytochrome c

The ^1H n.m.r. convolution difference spectra of horse ferrocytochrome c and $(Hse)^{65}$ ferrocytochrome c, both in ^2H_2O at pH 7.0 and 57°C are presented in Fig. 4.1. And those of tuna ferrocytochrome c and $[Met(Cm)^{65}]$ ferrocytochrome c, both in ^2H_2O at pH 5.6 and 57°C are presented in Fig 4.2. In both cases the two spectra (those of native and modified proteins) are very similar indicating that the structure of the proteins has not seriously been perturbed by the modifications. For example, the resonance of the CH_3 group of Met 80, the sixth ligand to

Figs. 4.1. & 4.2. : Figure legends

Fig. 4.1.

Resolution enhanced ^1H n.m.r. spectra of a) $(Hse)^{65}$ horse ferrocytochrome c, and b) horse ferrocytochrome c, both at pH 7 and 57°C.

Fig. 4.2.

Resolution enhanced ^1H n.m.r. spectra of a) $\left[Met(Cm)^{65}\right]$ tuna ferrocytochrome c, and b) tuna ferrocytochrome c, both at pH 5.6 and 57°C.

The resonances of Met 65 (M11, TM9), Ile 57 (M4, TM4), Tyr 74 (A11, TA9, A26, TA19) and Tyr 48 (TA22) are marked according to the resonance nomenclature scheme. Three regions of the spectra are given; the aromatic, methyl and upfield methyl regions.

Fig. 4.1.

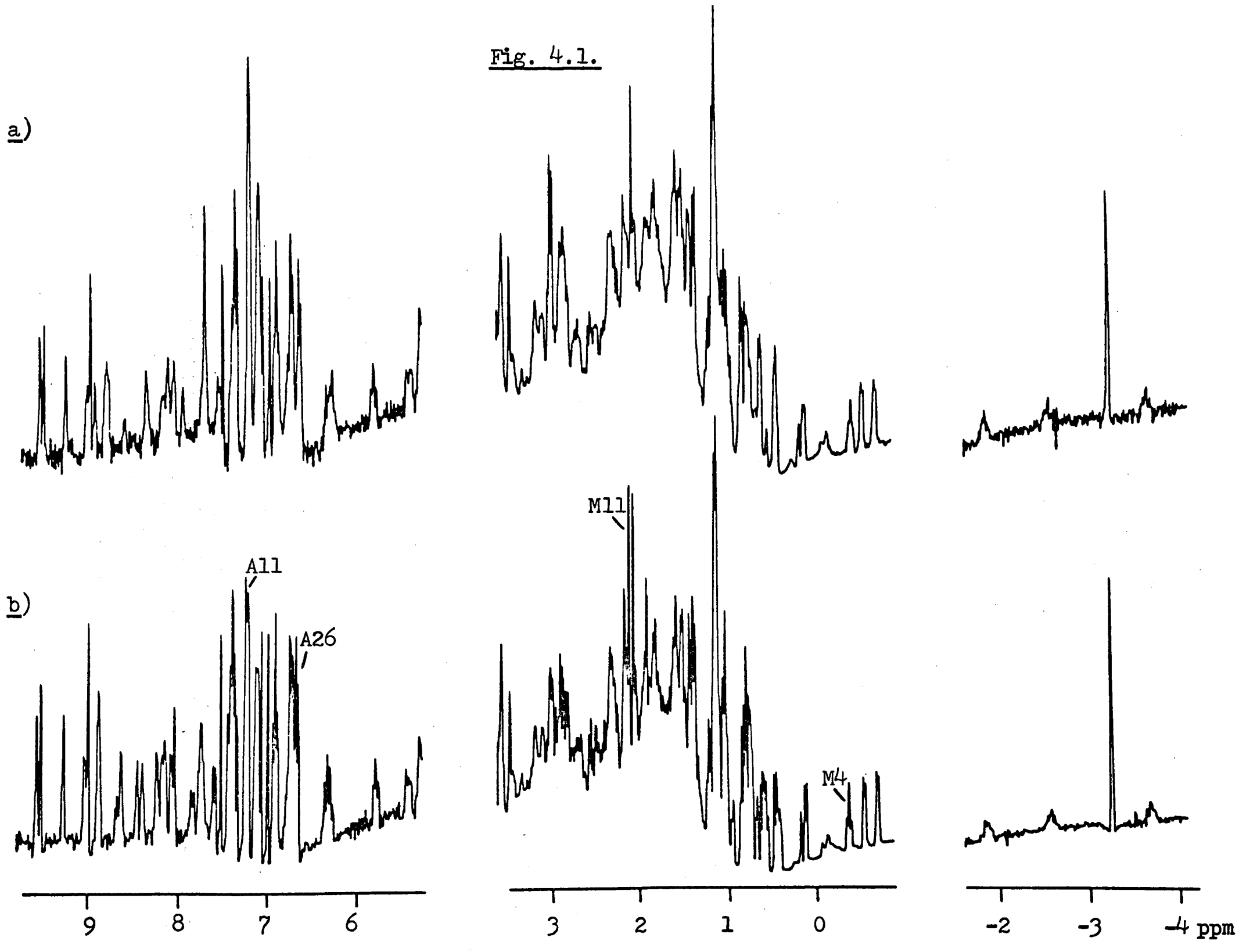
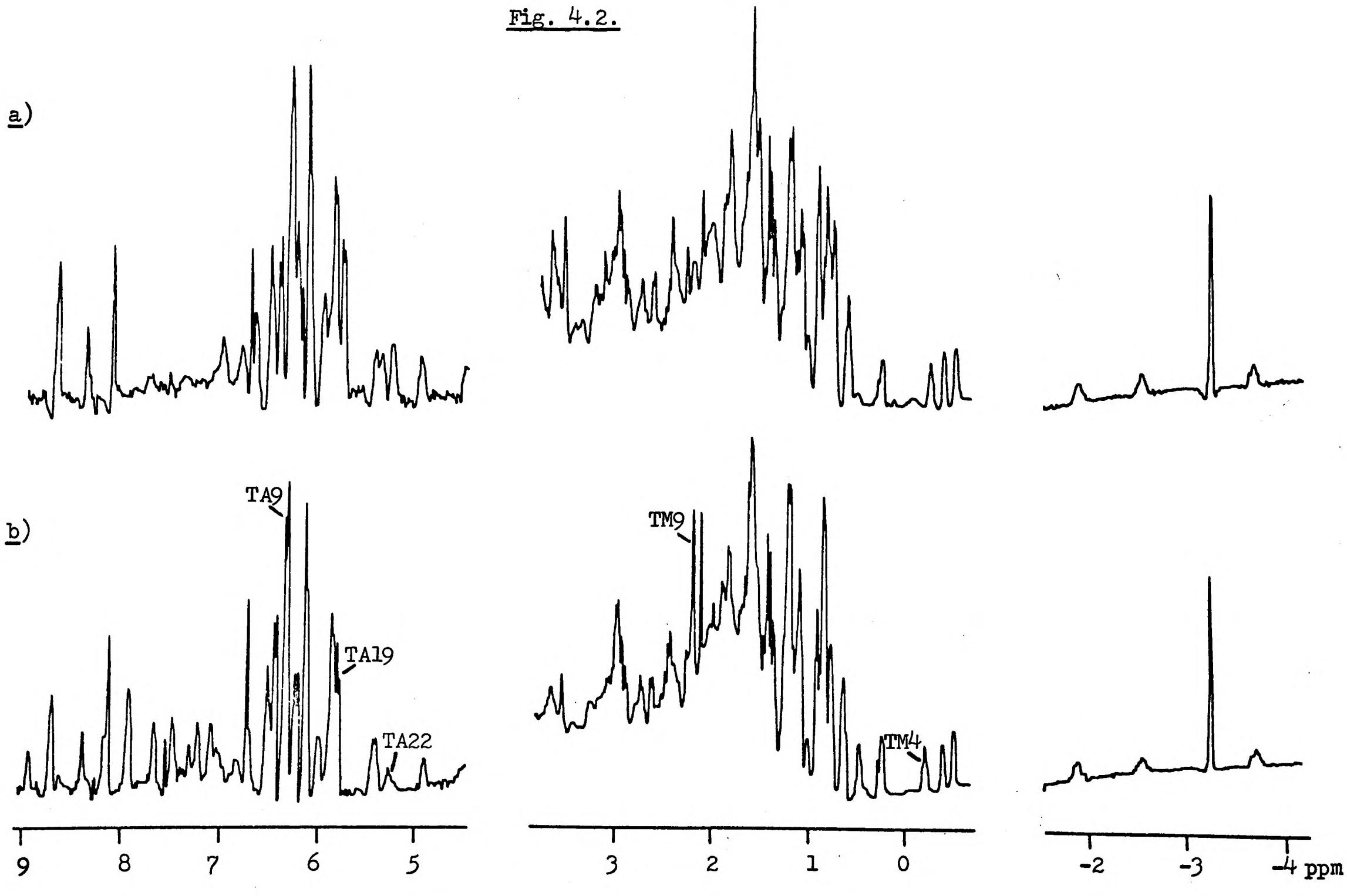


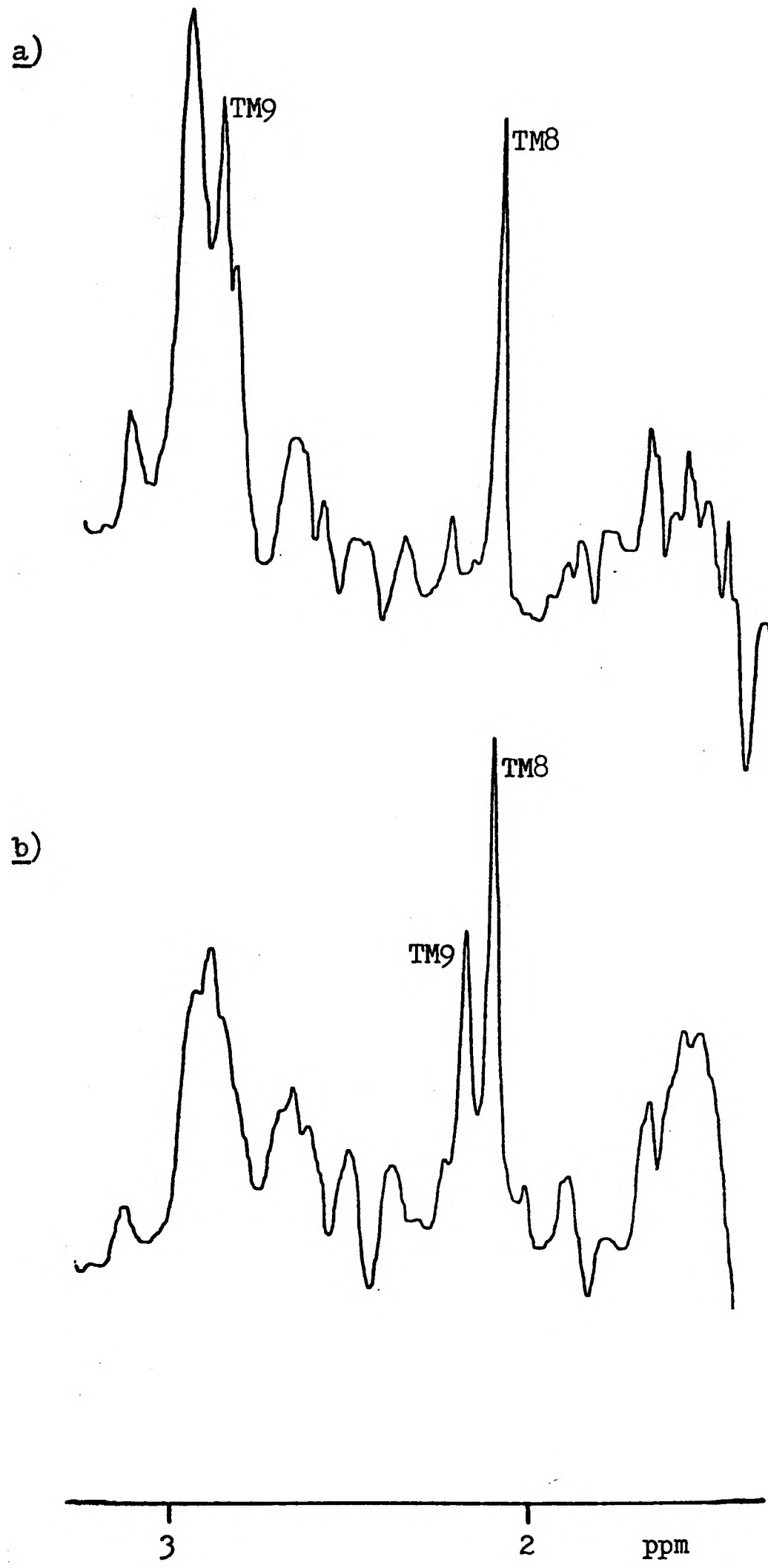
Fig. 4.2.



the haem, M1 at -3.28 ppm (horse) and TM1 at -3.25 ppm (tuna) is unaltered. This indicates that neither modification has drastically perturbed the structure of the molecule and that the haem coordination is unaffected. Both the modified ferrocytochromes c and their spectra will be discussed together.

Firstly, the singlet methyl resonances of the CH_3 group of Met 65, which are expected to be most affected by the modifications will be dealt with. There are two singlet methyl resonances at ca. 2 ppm in the spectrum of horse and tuna ferrocytochrome c, which must be due to Met 65 and the N-acetyl group; these are M11 and M10 and TM9 and TM8 respectively. In $(Hse)^{65}$ ferrocytochrome c M11 is totally removed from the spectrum and consequently is assigned to Met 65, while M10 is unaltered and is assigned to the N-acetyl group (3). In $\left[Met(Cm)^{65}\right]$ ferrocytochrome c TM8 is unaffected and is assigned to the N-acetyl group, and TM9 is shifted and located in spectra obtained using a Carr-Purcell A pulse sequence (23). The relevant region of the Carr Purcell A spectra of tuna ferrocytochrome c and $\left[Met(Cm)^{65}\right]$ ferrocytochrome c, at pH 5.6 and 57°C are shown in Fig. 4.3. TM9 was found to have moved from 2.14 ppm to 2.93 ppm, a shift of -0.79 ppm on modification. N-acetyl methionine and its carboxymethyl derivative were used to obtain primary chemical shift positions (the chemical shift value of resonances unperturbed by effects associated with the folding of a protein) of the methyl resonances, so they could be compared with the value for tuna ferrocytochrome c. ^1H n.m.r. spectra were recorded at pH 7 and at 27°C and 57°C, and the $S-CH_3$ resonances were found at 2.13 ppm in N-acetyl methionine and 2.92 ppm in N-acetyl carboxymethyl methionine, a shift of -0.79 upon modification. It can be seen that the results from the model compound agree well with those from the protein. The shift on modification is due to the sulphur bearing a

Fig. 4.3.



The aliphatic regions of the Carr-Purcell A ^{1}H n.m.r. spectra of a) tuna $\left[\text{Met}(\text{Cm})^{65}\right]$ ferrocytochrome c, and b) tuna ferrocytochrome c, at pH 5.6 and 57°C. In this type of spectrum singlets and triplets appear above the baseline, and doublets and quartets appear below the baseline.

formal positive charge in the carboxymethyl derivative. The agreement in absolute chemical shift positions between the model and the protein suggest that in the protein there is no contribution to the shifts observed on modification from the conformation of the protein before or after modification. This is in contrast to the results on the binding of small platinum complexes to Met 65, as described in Section 4.2.4. The model compound spectra also provided the chemical shift of the N-CO-CH_3 resonance in both N-acetyl methionine and N-acetyl carboxymethyl methionine; in both cases it is at 2.05 ppm. The N-acetyl group resonances in the proteins, M11 and TM9, are also at 2.05 ppm, and so suffer no secondary shifts.

Secondly, the following observations for resonances other than Met 65 are noted (this information is tabulated in Tables 4.1. and 4.2., which are based on Figs. 4.1. and 4.2. respectively):

i) Resonances M6 and TM6 that have recently been assigned to Leu 68 (24) are observed to shift by 0.04 ppm and 0.11 ppm upon modification. This residue is close to the haem group and Tyr 67, and its orientation with respect to these ring current centres is presumably altered by the modifications.

ii) The resonances of Ile 57, Trp 59 and Tyr 74 are affected by the modifications. This region of the protein has been discussed in detail in Section 3.6., where it is shown that the Ile 57 δCH_3 moves away from the rings of Trp 59 and Tyr 74 as the temperature is increased in ferrocytochrome c. In the case of the modification of Met 65 an effect is presumably transmitted through the Glu 66 - Tyr 74 hydrogen bond (this is discussed in Section 3.6.4.) causing a displacement to the ring of Tyr 74 (9.5 Å from Met 65 (25)), and so its resonances recieve a slight

Table 4.1.

Chemical shift comparison of horse ferrocytochrome c and $(Hse)^{65}$ ferrocytochrome c at pH 7 and 57°C

		Ferrocytochrome c		$(Hse)^{65}$ ferrocytochrome c
Assignment	Primary position	Resonance designate and chemical shift		Chemical shift
Met 80 CH_3	2.13	M1	-3.28	-3.28
Met 65 CH_3	2.13	M11	2.11	----
N-acetyl CH_3	2.05	M10	2.07	2.07
Ile 57 δ CH_3	0.89	M4	-0.43	-0.48
Leu 68 CH_3	0.94	M6	0.38	0.42
Trp 59 C-2	7.34	A19	6.99	6.98
Trp 59 C-4	7.65	A5	7.58	7.57
Trp 59 C-5	7.17	A24	6.68	6.70
Trp 59 C-6	7.24	A30	5.76	5.78
Trp 59 C-7	7.50	A16	7.07	7.10
Tyr 74 ortho	7.15	A11	7.22	7.21
Tyr 74 meta	6.86	A26	6,64	6.61

Table 4.2.

Chemical shift comparison of tuna ferrocytochrome c and Met(Cm)^{65} ferrocytochrome c at pH 5.6 and 57°C

		Ferrocytochrome c		Met(Cm)^{65} ferrocytochrome c
Assignment	Primary position	Resonance designate and chemical shift		Chemical shift
Met 80 CH_3	2.13	TM1	-3.25	-3.26
Met 65 CH_3	2.13	TM9	2.14	
Cm-Met 65 CH_3	2.92			2.92
Ile 57 δCH_3	0.89	TM4	-0.34	-0.39
Leu 68 CH_3	0.94	TM6	0.37	0.48
Trp 59 C-4	7.65	TA4	7.58	7.56
Trp 59 C-6	7.24	TA23	5.76	5.83
Tyr 74 ortho	7.15	TA9	7.22	7.21
Tyr 74 meta	6.86	TA19	6.67	6.65
Haem methyl 8		TH11	2.21	2.22

shift. Trp 59 is too far from Tyr 74 for its resonances to be affected by this change and so the shift caused to them is independent and reflects a change in its orientation to the haem, which is presumably transmitted via the peptide backbone from Met 65 (13 Å from Trp 59). Ile 57 lies in the ring current fields of both Trp 59 and Tyr 74 and its δCH_3 resonance is a sensitive marker of its relative orientation to these rings. The shift of this resonance upon modification reflects the changes to Trp 59 and Tyr 74; the shift is -0.05 ppm in both modifications, which is less than the difference between TM4 and M4 in the native proteins.

The shift to the resonance of Ile 57 is interesting, because structural perturbations near to Ile 57 from amino acid substitutions affects the immunochemical behaviour of cytochromes c (26) in a manner, which can be correlated to changes in the observed chemical shift position of the Ile 57 δCH_3 resonance between different species of cytochrome c (27). Horse $(Hse)^{65}$ cytochrome c and native cytochrome c have different affinities for rabbit anti-horse cytochrome c sera directed against the Ile 57 containing determinant (20), and this subtle biological change is reflected in the chemical shift change between the two molecules.

iii) There is a small shift to TH11, which is assigned to haem methyl 8 (28,29), in tuna $\left[Met(Cm)^{65}\right]$ ferrocytochrome c. This methyl group lies in the ring current field of Trp 59 (30) and the shift on modification confirms a change in the Trp 59 - haem orientation.

iv) All the above resonances, excepting Tyr 74, suffer established secondary (ring current) field effects and thus are very sensitive to changes in conformation. There are a few resonances in the region between 0.5 ppm and 1.8 ppm that suffer slight shifts, which cause the general profile of the spectra to be different between the native and modified proteins. These are shifts that are only very slightly sensi-

tive to ring currents and can not be resolved due to the overlap of resonances.

v) In Fig. 4.2. the resonances of TA15 and TA22 (marked) arise from a tyrosine residue where rotation about its C_β - C_γ bond axis is hindered (31,32). The corresponding resonances of tuna $\left[\text{Met(Cm)}^{65}\right]$ ferrocytochrome c are not shifted but are of apparent greater intensity, i.e. sharper. This implies that the rate of the rotation of the Tyr 97 ring is faster in the modified protein. It was also observed that the NH protons of the modified protein exchange for deuterons much more rapidly than those of the native protein in freshly prepared n.m.r. samples at the same temperature, pH and protein concentration. Both these observations point to tuna $\left[\text{Met(Cm)}^{65}\right]$ ferrocytochrome c as having an overall looser structure than the native protein. As shown in points i) to iv), the static structure is only affected in the direct locality of the modification; however, the flexibility of the molecule is affected a long way from the point of modification e.g. at Tyr 97 on the opposite side of the molecule. These conclusions are in accord with Stellwagen's (33) conclusion that $\left[\text{His(Cm)}^{33},\text{Met(Cm)}^{65}\right]$ horse cytochrome c has an increased susceptibility to basic and acidic denaturation compared to the native protein.

In summary, the work described in this section on two ferrocytochromes c singly modified at Met 65 shows that the protein is not grossly perturbed by the alterations and the effects are localised to the region close to Met 65 and transmitted via both a hydrogen bond and by small displacements of the peptide backbone.

4.2.2. $(\text{Hse})^{65}$ horse ferricytochrome c and $\left[\text{Met(Cm)}^{65}\right]$ tuna ferricytochrome c

These proteins were not studied to the same extent as their reduced counterparts. This was partly due to the fact that the spectra of

reduced cytochromes c, which are diamagnetic, are more easily interpreted than the spectra of oxidised cytochromes c, which are paramagnetic; and partly because the modified proteins are less stable, particularly in the oxidised state, and especially $\left[\text{Met(Cm)}^{65}\right]$ tuna ferricytochrome c, which denatures at ca. 50°C. Moore (21) has reviewed thoroughly the complicating effects of the paramagnetic ferric ion in the analysis of the spectra of oxidised cytochromes c. In the case of the oxidised modified proteins a further problem will be shown to arise, which is that the temperature dependence of the spin state of the ferric ion is different from the native proteins. This means that resonances can expect a different paramagnetic component to their chemical shift values independently of any changes in atomic structure on modification, and so a 1:1 comparison of the spectra of the native and modified proteins may not yield structural information as it did in the case of the diamagnetic reduced proteins.

Nevertheless, spectra at 27°C of the modified ferricytochromes c show that at this temperature they still are low spin and can be compared with native ferricytochrome c. Tables 4.3. and 4.4. give the chemical shift values of some resonances in $(\text{Hse})^{65}$ ferricytochrome c and horse ferricytochrome c and in $\left[\text{Met(Cm)}^{65}\right]$ ferricytochrome c and tuna ferricytochrome c, respectively, at pH 7 and 27°C. The following points are made:

i) The resonances from the two haem methyls in the downfield shifted region and the Met 80 CH_3 in the upfield hyperfine shifted region provide a good guide to the nature of the haem coordination centre. The results show that the modified and native proteins possess the same coordination, though there are some small shifts to these resonances. The most significant shift occurs to the Met 80 CH_3 and may reflect a very small displacement of this group.

Table 4.3.

Chemical shift comparison of horse ferricytochrome c and $(Hse)^{65}$ ferricytochrome c at pH 7 and 27°C

Assignment	Primary position	Ferricytochrome c: Resonance designate and chemical shift		$(Hse)^{65}$ ferricytochrome c: Chemical shift
Ring methyl 8		H^*1	35.0	35.0
Ring methyl 3		H^*2	32.4	32.3
Met 80 CH_3	2.13	M^*1	-23.5	-23.2
Met 65 CH_3	2.13	M^*19	1.94	----
N-acetyl CH_3	2.05	M^*20	1.94	1.97
Leu 68 CH_3	0.94	M^*2	-2.07	>-0.5
Ile 57 δCH_3	0.89	M^*6	-0.36	shifted

Table 4.4.

Chemical shift comparison of tuna ferricytochrome c and [Met(Cm)^{65}] ferricytochrome c at pH 7 and 27°C

Assignment	Primary position	Ferricytochrome c: Resonance designate and chemical shift		[Met(Cm)^{65}] ferricytochrome c: Chemical shift
Ring methyl 8		TH^*1	35.2	35.2
Ring methyl 3		TH^*2	32.7	32.7
Met 80 CH_3	2.13	TM^*1	-23.4	23.0
Met 65 CH_3	2.13	TM^*16	1.96	2.80
N-acetyl CH_3	2.05	TM^*17	1.96	1.96
Leu 68 CH_3	0.94	TM^*2	-2.10	>-0.5
Ile 57 δCH_3	0.89	TM^*6	-0.38	shifted

ii) $M^{*}19$ is lost from the spectrum of $(Hse)^{65}$ ferricytochrome c and $TM^{*}16$ shifts 0.84 ppm downfield in $\lceil Met(Cm)^{65} \rceil$ ferricytochrome c (0.79 ppm is the expected shift from model compounds, see Section 4.2.1.). These resonances are assigned to Met 65 and confirm the cross assignment between M11 and TM9 respectively, as determined in Section 3.3.1. The remaining singlet methyl resonances ca. 2 ppm in both modifications, $M^{*}20$ and $TM^{*}17$, are assigned to the N-acetyl group.

iii) The resonances due to Ile 57 and Leu 68 shift, though their positions in the modified proteins has not been ascertained due to resonance overlap in the spectral region they shift into.

Thus the effect of the modifications to the static structure of the protein appears to be very similar in both oxidised and reduced state - we now proceed with the temperature dependence of the spectra of the ferrocytochromes c

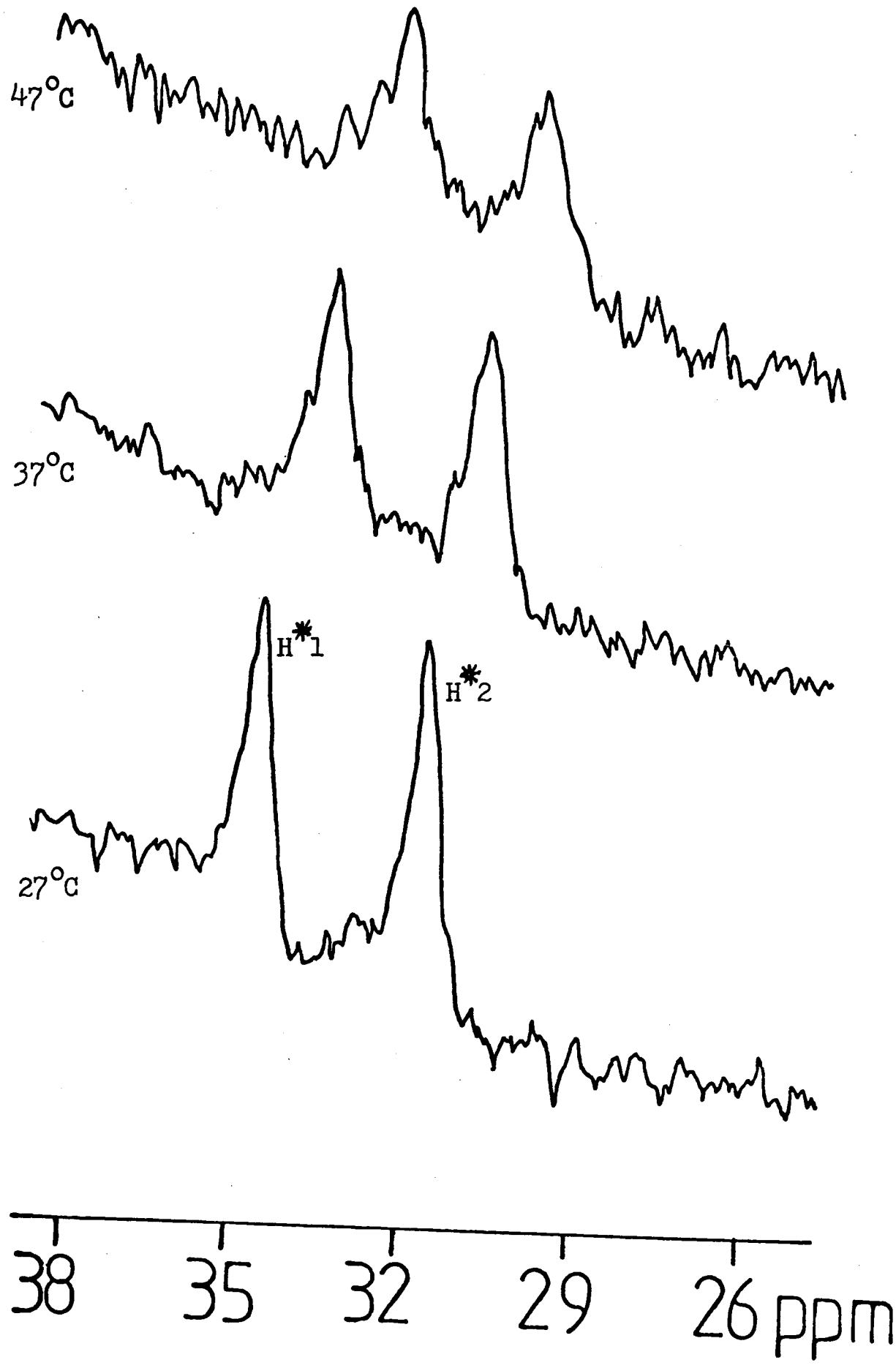
Resonances such as $H^{*}1$, $H^{*}2$ and $M^{*}1$ in native horse ferricytochrome c have virtually constant linewidths up to ca. 57°C, but above this temperature the linewidths begin to increase (21). Moore (21) interprets this as being due to the mixing in of high spin ferric states to the low spin ferric ion in native, state III horse ferricytochrome c; and notes that the 695 nm. band in the optical spectra, due to a charge transfer from sulphur (Met 80) to the ferric ion (34), is also temperature dependent with a loss of intensity starting at ca. 40°C (35) with complete disappearance at ca. 60°C. ^{1}H n.m.r. spectra show that Met 80 is still liganded to the ferric ion at 77°C, and the ^{1}H n.m.r. and optical spectral data can be rationalised in the following way. With increasing temperature the Fe — S bond becomes increasingly weaker culminating in the rupture of the bond at ca. 80°C, and the following equilibrium occurs:

$$\underset{\text{low spin}}{Fe-S} \rightleftharpoons \underset{\text{high spin}}{\begin{matrix} Fe & + & S \\ \text{or } Fe & --- & S \end{matrix}} \xrightarrow{80^{\circ}C} \text{denatured}$$

Figs 4.4. and 4.5. show the spectra of horse $(Hse)^{65}$ ferricytochrome c and tuna $[Met(Cm)^{65}]$ ferricytochrome c, respectively, at pH 7 and 27°C, 37°C and 47°C. Resonances H^*1/TH^*1, H^*2/TH^*2 from two haem methyls broaden progressively. In Fig. 4.6. the average broadening to the linewidths at half height of these resonances over that in the native proteins (constant over this temperature range, see above) is plotted for both modified proteins. These results imply that high spin 'sulphur off' ferric ion is mixed in at a much lower temperature in the modified proteins and, thus, that the modifications cause a weakening of the iron-sulphur bond (this is consistent with point i) above). This is not detected directly in the native and modified ferrocytochromes c, because the iron-sulphur bond is inherently much stronger in the reduced proteins (this is discussed in much further detail in Chapter 6), but is in accord with the faster NH-ND exchange rate noted for $[Met(Cm)^{65}]$ tuna ferrocytochrome c. The most plausible mechanism for the transmittance of an effect to the Fe — S (Met 80) bond by modification at Met 65 is based on the discussion of the effects caused to Ile 57 and Tyr 74 region in the reduced proteins (Section 4.2.1.). It is proposed that the orientation of Tyr 74 to the rest of the molecule is slightly altered in ferrocytochrome c, when Met 65 is modified. Such a change of the Tyr 74 orientation could cause a slight change to the energy of the peptide backbone between Tyr 74 and Met 80, and so cause a subtle change to the affinity of Met 80 for the haem iron. It is possible that the effect of modification to Tyr 74 and Ile 57 is greater in ferricytochromes c, as this region of the protein suffers a conformational change on change of oxidation state (Section 2.2.1. and refs. therein).

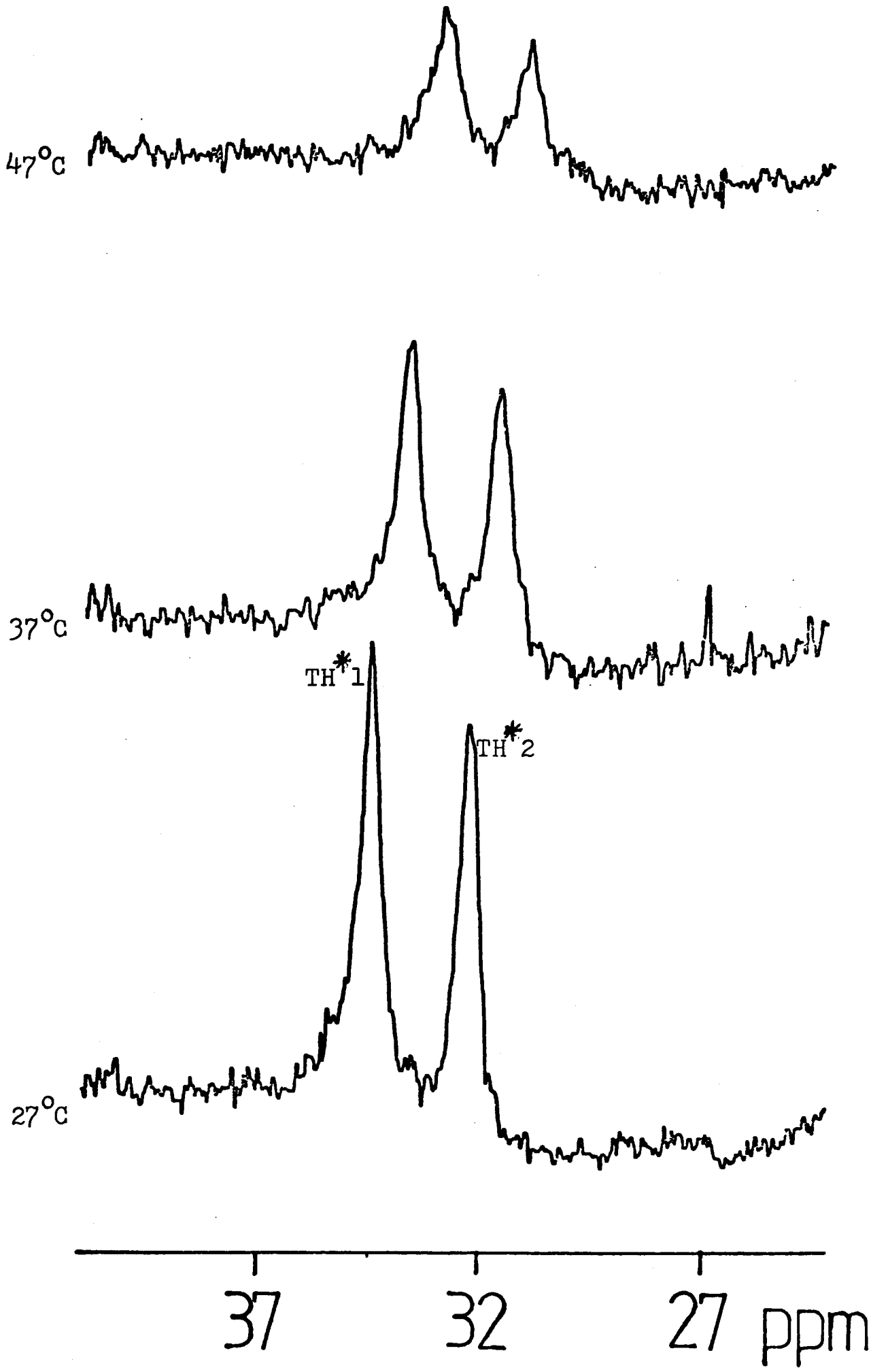
It is also of interest to note that in the ^1H n.m.r. temperature

Fig. 4.4.



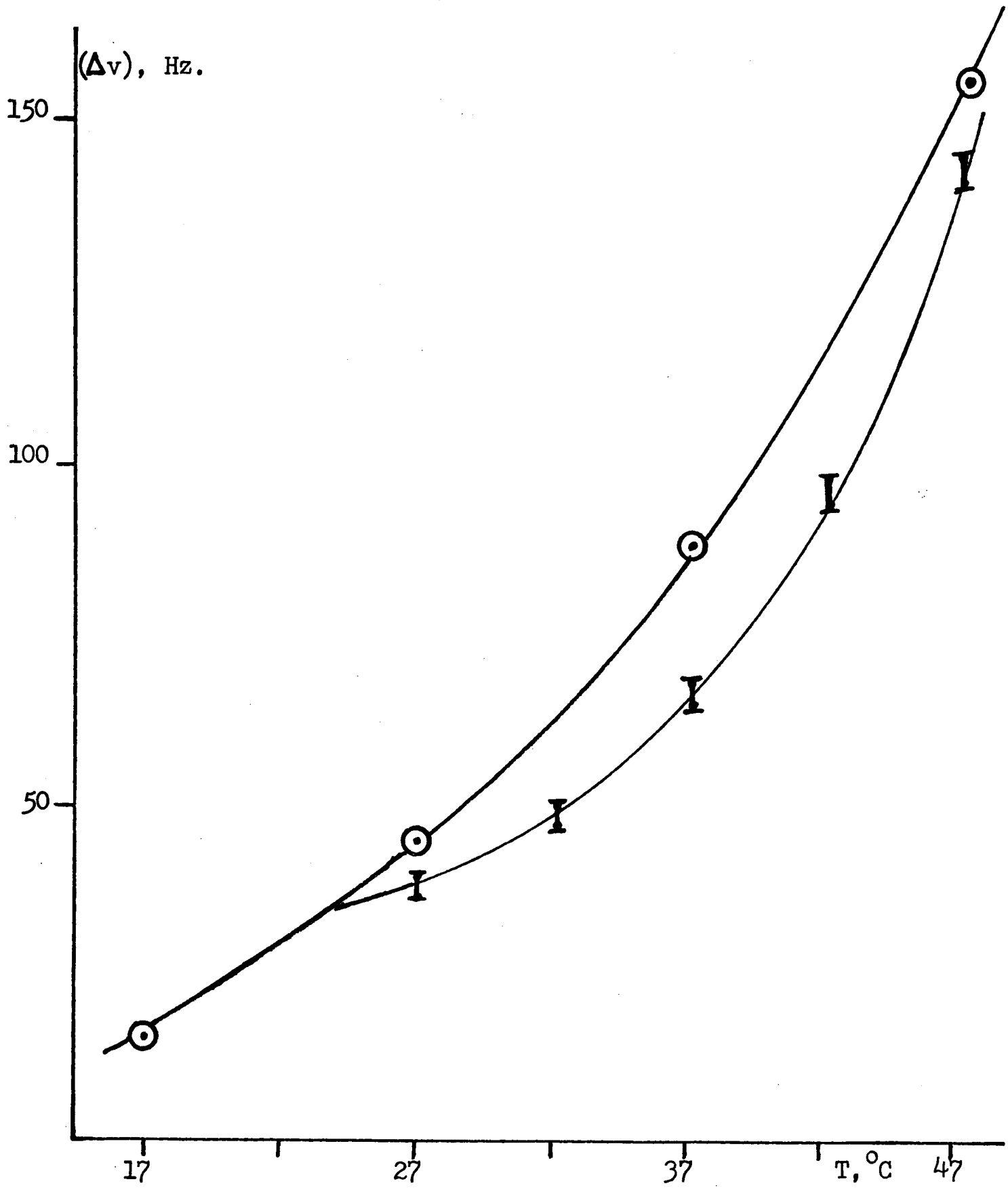
The temperature dependence of the resonances due to haem methyls 8 and 3 in horse (Hse)[65] ferricytochrome c at pH 7.

Fig. 4.5.



The temperature dependence of the resonances due to haem methyls 8 and 3 in tuna [Met(Cm)^{65}] ferricytochrome c at pH 7.

Fig. 4.6.



The average broadening (over that in the native state) to the linewidths at half height of the resonances H 1/TH 1, and H 2/TH 2 against temperature for the modified proteins, horse $(Hse)^{65}$ ferricytochrome c (⊙) and tuna $\left[Met(Cm)^{65}\right]$ ferricytochrome c (I).

denaturation studies of both the native (21) and the Met 65 modified (this work) ferricytochromes c there is no evidence for an intermediate species with an alternative sixth ligand to Met 80. This is in contrast to the chemical denaturations (reported in Chapter 7) using urea and methanol, where state IV ferricytochrome c is shown to be an intermediate. This difference in pathway must reflect a change in the relative energy levels of the native, state IV and denatured forms of ferricytochromes c that is temperature dependent. This is discussed in Section 7.3.3.

In summary, the ferricytochromes c modified at Met 65 possess a similar structure to the modified ferrocytochromes c and to native ferricytochrome c at room temperature. The modification causes a weakening of the iron-sulphur bond at the coordination centre, so that as the temperature is raised the sulphur off species dominates more than in the native protein, and a small change in structure in the locality of Met 65.

4.2.3. The binding of platinum complexes to tuna cytochrome c

In this and the next section, an ^1H n.m.r. study of the binding of two platinum complexes, $Pt(Cl)_4^{2-}$ and $Pt(NH_3)_2Cl_2$, to the sulphur atom of Met 65 in tuna cytochrome c is described. In this section the effect of such binding to the protein structure is discussed, while the next section is concerned entirely with the shift caused to the $S-CH_3$ resonance of Met 65 in the binding of the complexes. Tuna cytochrome c was chosen for this study to provide continuity with the study of carboxymethylated methionine 65 in tuna cytochrome c. This ^1H n.m.r. study of platinum complex binding to methionine in cytochrome c was undertaken with respect to the following points:

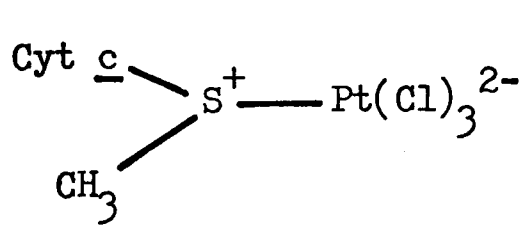
a) Tetrachloroplatinite is often used to prepare heavy atom derivatives of protein crystals, including those of tuna cytochrome c, for X-ray

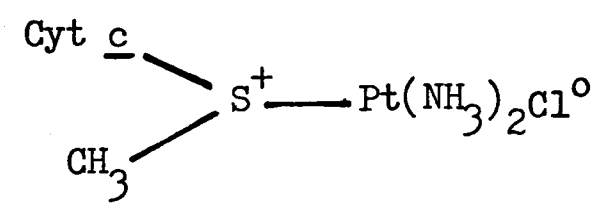
structural analysis. Thus, a knowledge of its effects on the conformation of a protein in solution in a well defined system, such as cytochrome c, is a useful test of the validity of the use of heavy atom derivatives, and the X-ray crystallographic method. The chemistry of tetrachloroplatinite with this respect has been fully discussed by Petsko et al (36).

b) The results could provide useful data as the basis for a simple, general technique that would utilise tetrachloroplatinite binding as a method for locating solvent accessible methionine residues in globular proteins, where the $S-CH_3$ singlet resonances can be clearly resolved and assigned. Indeed, with the application of the reverse logic, tetrachloroplatinite binding has previously been used by Sadler et al (37) to assign Met 29 in Ribonuclease, which is the only surface methionine in the X-ray structure.

c) It extends the data described in previous sections of this chapter concerning the effect of modification of Met 65 in cytochrome c.

The binding constants of chloride and ammonia to Pt(II) in H_2O are very high (38), and thus in the solvent used in the following experiments, unbuffered ^2H_2O, there is unlikely to be any solvent substitution of the platinite complexes before or after reaction with Met 65 of tuna cytochrome c. Assuming that the platinum complexes are bound via the $S-CH_3$ group of Met 65 and that no other groups from the protein are available for ligation (this point is discussed further in the next section), then the modifications are designated as:



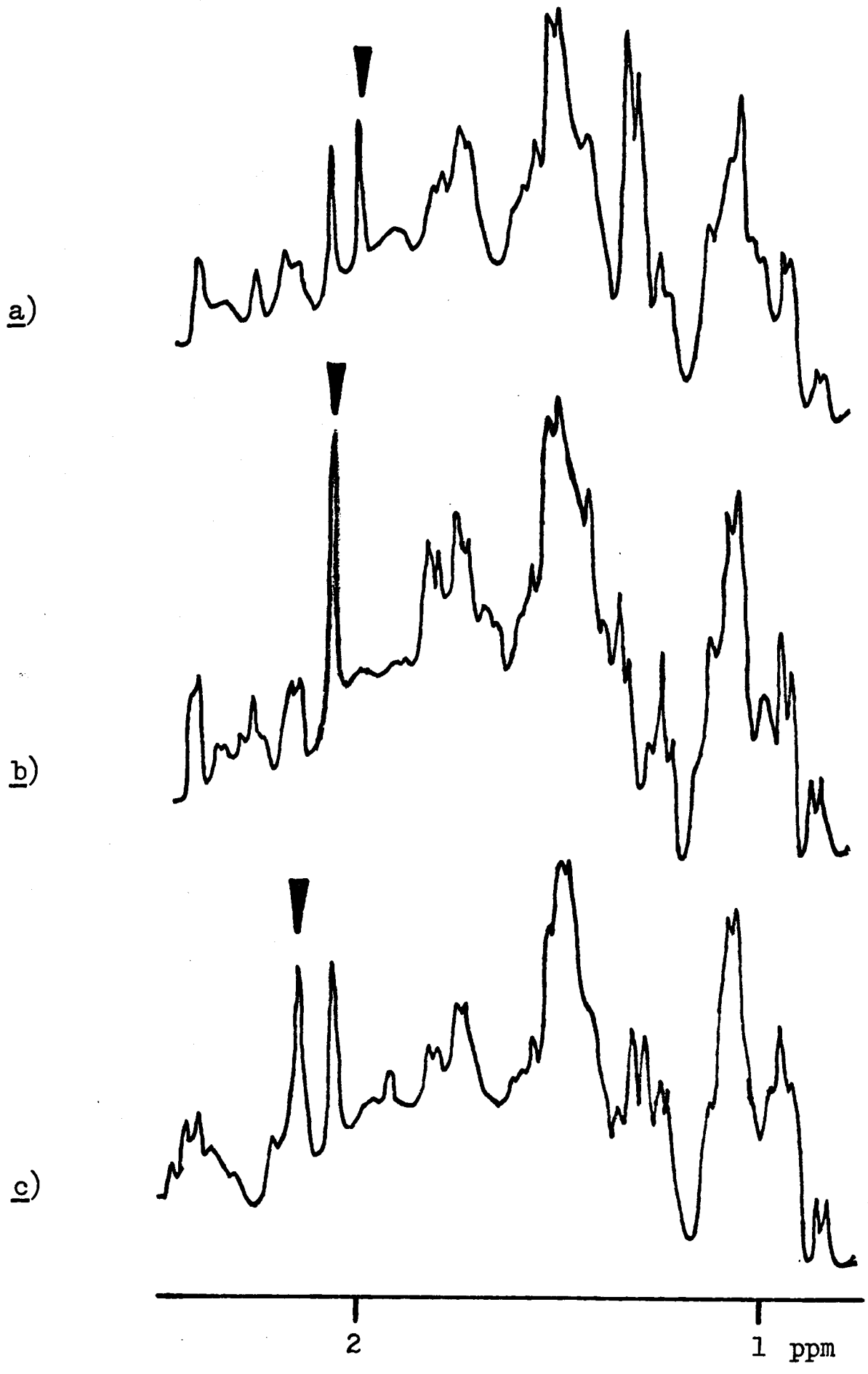


It can be seen that there is a difference in formal charge of 2 between the two species.

The ferrocytochrome c derivatives were prepared by adding a 0.1M excess of K_2PtCl_4 or $Pt(NH_3)_2Cl_2$ dissolved in ^2H_2O to a solution of tuna ferrocytochrome c at pH 5.6 in ^2H_2O in an n.m.r. tube. The reaction solution was placed under Argon and left for 24 hours before ^1H n.m.r. studies were carried out. The same proceedure was not carried out for the production of the analogous ferricytochrome c derivatives, because in this oxidation state the Fe — S bond is weaker and Met 80 is also susceptible to binding by the platinum complexes. Instead the ferrocytochrome c derivatives prepared above were retrieved after the n.m.r. experiments, oxidised with $K_3Fe(CN)_6$, passed down a G25 Sephedex column to removed the ferricyanide and freeze dried. The freeze dried powders were dissolved in ^2H_2O, adjusted to pH 5.6 and placed in n.m.r. tubes.

Spectra of tuna ferrocytochrome c and its two derivatives were recorded at pH 5.6 and 57°C. Fig 4.7. shows the region of the spectra in which the N-acetyl and Met 65 singlet resonances are found. In each of the spectra resonance TM9 which is assigned to the S-CH_3 group of Met 65 (Section 4.2.1.) is marked. It can be observed to shift upfield on modification and this will be discussed further, and with respect to model complexes, in Section 4.2.4. Table 4.5. compiles the shifts that are observed to occur to resonances in tuna ferrocytochrome c upon the binding of the platinum complexes and it is useful to compare these data with those of Table 4.2. (the analogous data for carboxymethylation). The data show that shifts occur to the same resonances in the platinite derivatives as with carboxymethylation of Met 65 in ferrocytochrome c suggesting that the three types of modification cause a similar structural change to the protein. Notably, the resonance of the Ile 57 δCH_3 group suffers a small shift, but in a different direction, with each of the platinum complexes and although no shift is observed on the

Fig. 4.7.



Part of the methyl regions of ^{1}H n.m.r. spectra of a) $PtCl_3$-tuna ferrocytochrome c, b) $Pt(NH_3)_2Cl$-tuna ferrocytochrome c, and c) tuna ferrocytochrome c at pH 5.6 and 57°C. The marked resonance in each spectrum is due to Met 65.

Table 4.5.

Chemical shift comparison of tuna ferrocytochrome c, ($PtCl_3$-Met 65) ferrocytochrome c and ($PtCl(NH_3)_2$-Met 65) ferrocytochrome c at pH 5.6 and 57°C

Resonance designate/Assignment		Primary position	Native Ferrocytochrome c Chemical shift	($PtCl_3$-Met 65) Ferrocytochrome c Chemical shift	($PtCl(NH_3)_2$-Met 65) Ferrocytochrome c Chemical shift
TM1	Met 80 CH_3	2.13	-3.25	-3.24	-3.26
TM9	Met 65 CH_3	2.13	2.11	1.96	2.05
TM8	N-acetyl CH_3	2.05	2.05	2.05	2.05
TM4	Ile 57 CH_3	0.89	-0.34	-0.36	-0.33
TA4	Trp 59 C-4	7.65	7.58	7.56	7.57
TA23	Trp 59 C-6	7.24	5.76	5.78	5.81
TA9	Tyr 74 ortho	7.15	7.22	7.22	7.22
TA19	Tyr 74 meta	6.86	6.67	6.67	6.67
TH11	Haem methyl 8		2.21	2.24	2.25

resonances of Tyr 74 (these are 'insensitive' resonances and only shift 0.02 ppm and 0.01 ppm on carboxymethylation of Met 65 (see Table 4.2.)) this result indicates that there is a small change in the relative orientation of the δCH_3 of Ile 57 to the rings of Tyr 74 and Trp 59, as previously described for $Met(Cm)^{65}$ ferrocytochrome c. In each of the three systems, the carboxymethyl derivative and the two platinum derivatives, slightly different shifts occur and hence slightly different displacements of the atomic coordinates must occur. It is not possible to draw any further information from the magnitude of the shifts, although the difference in charge between the platinite derivatives is presumably one of the factors that causes a difference in their Ile 57 shifts. Similarly the resonances of Trp 59 and haem methyl 8 (TH11, a resonance that serves as a Trp 59 - haem orientation guide (21)) are affected by the binding of platinum complexes. In summary these results indicate that the binding of $PtCl_4^{2-}$ or $Pt(NH_3)_2Cl_2$ to tuna ferrocytochrome c causes extremely small changes to the local structure of the protein near to the binding site, and that the different charges on the complexes causes very little difference to the magnitude of the effect. The effects described would not be detectable to the X-ray crystallographic method and it is very unlikely that in the X-ray structural studies (e.g. 25) the use of tetrachloroplatinite used as a heavy atom derivative caused any significant change in the conformation of tuna cytochrome c. Noting that the complexes are bulky groups; these conclusions suggest that the sulphur of Met 65 is very accessible to the solvent and that the influence of different charges on this region of the protein is virtually neglible.

Spectra of ferricytochrome c and its two platinum derivatives were recorded at various temperatures and at pH 5.6. As with the data

for [Met(Cm)^{65}] ferricytochrome c these spectra can not be easily interpreted due to the complicating effects of the spin state of the ferric ion. However the following information was obtained:

a) At 27°C the resonances from the haem centre (notably, the haem methyls and the Met 80 CH_3 group) were observed at approximately native positions and were broadened for the platinum derivatives, indicating that the modified proteins have the same coordination centre but possess more high spin ferric ion.

b) The temperature dependence of the spectra indicate that high spin ferric ion is mixed in at higher temperature than in [Met(Cm)^{65}] ferricytochrome c, and at a lower temperature than ferricytochrome c.

c) Resonance TM*16 from the S-CH_3 group of Met 65 was identified in both derivatives, and its chemical shifts are tabulated in Table 4.5.

Finally, we can use the binding of tetrachloroplatinite as a guide to solvent accessibility of methionine residues in globular proteins. Sadler et al (37) found that only one of four methionines in Ribonuclease was accessible to solvent and able to bind $PtCl_4^{2-}$ at pH 5.5, and above it is shown that only Met 65 in tuna ferricytochrome c is accessible to $PtCl_4^{2-}$ at pH 5.6. In Candida krusei cytochrome c there are three methionines 64, 80 and 98 (14) and the X-ray structure of tuna cytochrome c (25) would suggest that all of these are internal. It was decided to test this by seeing if the protein from this species would bind tetrachloroplatinite. No difference in the spectrum of Candida krusei ferrocytochrome c with and without the presence of K_2PtCl_4 could be found. This preliminary experiment suggests that residues 64, 80 and 98 are all, indeed internal.

4.2.4. The chemical shift of the S-CH_3 resonance in platinum complexes of N-acetyl methionine and methionine 65 in cytochrome c

The discussion now returns to the shifts observed to the singlet resonance of the S-CH_3 group of methionine 65 on the binding of the platinum complexes. This section describes the use of N-acetyl methionine as a model to define the shift expected to such a resonance, and discusses the differences observed between the model and protein systems. The interaction of N-acetyl methionine with tetrachloroplatinate has been studied by Dr. P. Sadler and Mr. I. Ismail. These workers have used ^{1}H, ^{13}C and ^{195}Pt n.m.r. to define the system in much more detail than the experiment described in this section; however, my results are completely compatible with their results. I am indebted to these workers for free access and discussion of their work prior to publication.

Three n.m.r. samples were made up as follows; i) 10^{-2} M N-acetyl methionine, ii) 10^{-2} M N-acetyl methionine and 10^{-2} M K_2PtCl_4 and iii) 10^{-2} M N-acetyl methionine and 10^{-2} M $Pt(NH_3)_2Cl_2$, all at pH 7 and in 0.4 ml. of $^{2}H_2O$. Spectra were recorded at 27°C and at 57°C. Table 4.6. shows the n.m.r. parameters of the S-CH_3 resonance in N-acetyl methionine, tuna ferrocytochrome c and ferricytochrome c (data extracted from Table 4.5.) free and in the presence of one equivalent of $PtCl_4^{2-}$ or $Pt(NH_3)_2Cl_2$. For N-acetyl methionine the data tabulated is for the predominant 'singlet' (singlet in terms of protons; in the presence of platinum the S-CH_3 singled is coupled due to ^{195}Pt, see below) resonance found in the spectrum. Platinum possesses an isotope, ^{195}Pt, which has a nuclear spin, $I = \frac{1}{2}$, and is found in about 30% natural abundance. The remaining ca. 70% of natural platinum is composed of an I = 0 isotope. Consequently the S-CH_3 singlet resonance becomes a ca. 1:4:1 triplet when platinum is bound to sulphur. Practically, the coupling is not observed for

Table 4.6.

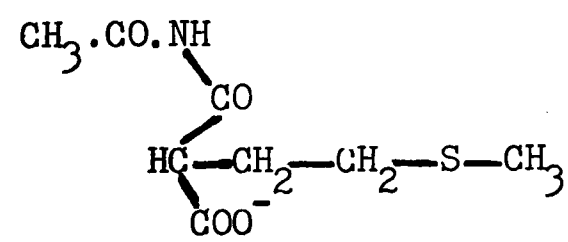
Chemical shift values for the S-CH_3 group in N-acetyl methionine and tuna cytochrome c, free and bound to platinum complexes

Species	Chemical shift values free	bound	difference
i) bound to $PtCl_4^{2-}$			
N-acetyl methionine (pH = 7)	2.04	2.34	+0.30
tuna ferrocytochrome c (pH = 5.6)	2.11	1.96	-0.15
tuna ferricytochrome c (pH = 5.6)	1.96	1.91	-0.05
ii) bound to $Pt(NH_3)_2Cl_2$			
N-acetyl methionine (pH = 7)	2.04	2.33	+0.29
tuna ferrocytochrome c (pH = 5.6)	2.11	2.05	-0.06
tuna ferricytochrome c (pH = 5.6)	1.96	1.96	0.00 +

+, this solution was reduced to check that the platinum complex was still bound.

the protein spectra because i) the ^{195}Pt nuclei has a short relaxation time which causes a substantial broadening to the sidebands; and ii) the broadened sidebands have only ca. a half proton intensity each and occur in a region of the spectrum with many overlapping resonances. In this discussion we are concerned with the shifts caused to the S-CH_3 system on the binding of platinum.

N-acetyl methionine was chosen as a model system because the only way it can ligate platinum is through its sulphur atom; the nitrogen atom is blocked, as is shown below:



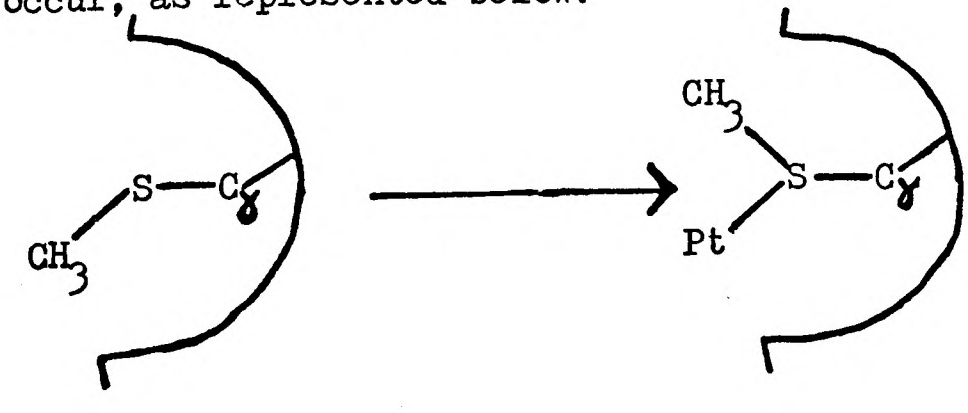
N-acetyl methionine

Thus it is assumed in 1:1 solutions of N-acetyl methionine and the platinum complex that the sulphur displaces a chloride ion and gives rise to a 1:1 complex as predominant species. Sadler and Ismail have studied the systems from ratios of 1:2 to 4:1 for N-acetyl methionine and $PtCl_4^{2-}$ and have shown that at a 1:1 ratio the above assumption is correct, although there are very complex equilibria involved in the system (39). Thus the coordination of one methionine sulphur to a platinum complex causes ca. 0.30 ppm downfield shift to the S-CH_3 resonance (Table 4.6.). This is expected from theory because when Pt binds to sulphur in the complexes, the sulphur becomes more positively charged and electrons are drawn from the methyl group. Thus the methyl group protons experience a deshielding effect and the resonance shifts downfield. Examination of Table 4.6. shows that in the protein systems the shifts are in the opposite direction. This difference between the model and protein systems suggests either that i) the mode of binding is different in the model and protein systems, or ii) a small perturbation to the Met 65 side chain occurs on the binding

of platinum to the protein that places the S-CH_3 group in a different environment with respect to the rest of the protein. These two possibilities are now discussed further.

The mode of binding in the model system has been established above, but in the protein there is the possibility of the platinum being coordinated by further ligands from the protein. Potential second ligands are other sulphur and nitrogen atoms. In cytochrome c there are three other sulphur atoms and these are all involved with binding the haem group to the peptide chain on the other side of the molecule (Met 80 and two tioether bridges), and so a further sulphur atom can be ruled out. There are many nitrogen atoms in the vicinity of Met 65; peptide backbone NHs, NH_3^+ of lysines, and the guanido group of Arg 91. At pH 5.6 none of these groups would be expected to be capable of ligation to platinum and furthermore, examination of the X-ray Labquip model shows that if platinum is bound to the surface of the molecule none of these groups would have access to it. For such access to occur a structural rearrangment would have to occur that would be expected to cause greater changes to the whole ^1H n.m.r. spectrum than are observed. Thus it seems very unlikely that there is a second ligand to the platinum from the protein.

The other possibility is for a very small perturbation to occur to the Met 65 side chain - it has to be small as no large changes are seen to the rest of the protein from the ^1H n.m.r. spectrum. For example, on the binding of a platinum complex a 180° rotation about the C_γ— S bond could occur, as represented below:



Such a rotation might facilitate the accomodation of the bulk of the platinum complex into the structure of the protein and would place the S-CH_3 group in a different environment. However, the change in environment would have to explain a secondary shift of -0.45 ppm in the case of tuna cytochrome c and $PtCl_4^{2-}$ (see Table 4.6) above the shift expected from the model complex studies. Such a secondary shift would only be expected from a ring current field or possibly due to very close proximity to a carboxyl group function. A small change of environment to the S-CH_3 group as described above does not move the group nearer to a ring current field (by examining a labquip model of the X-ray structure and calculations based on the coordinates (25)), but does move the group closer to the carboxyl function of Asp 62. The anomalous shift to the S-CH_3 group resonance in tuna cytochrome c on the binding of platinum complexes is tentatively assigned to a movement towards the carboxyl group of Asp 62, but this has to be confirmed.

In conclusion, the last two sections describe work carried out that shows:

a) Small platinum complexes, $PtCl_4^{2-}$ and $Pt(NH_3)_2Cl_2$, bind selectively at Met 65 in tuna ferrocytochrome c and cause very small changes to the structure of the molecule in both oxidation states.

b) Such platinum complexes may be used generally as a guide to the solvent accessibility of methionine residues in globular proteins.

c) there is an anomalous shift to the methionine methyl resonance of Met 65 in tuna cytochrome c on the binding of the platinum, which is not fully explicable.

4.2.5. $(Leu)^{67}$,$(Hse)^{65}$ horse cytochrome c and $(Leu)^{74}$,$(Hse)^{65}$ horse cytochrome c

In sections 4.2.1 and 4.2.2. the structure of $(Hse)^{65}$ horse cytochrome c is shown to correspond to that of native cytochrome c in both oxidation states, except in the region of the modification itself. Two of the first semisynthetic derivatives of $(Hse)^{65}$ cytochrome c to be produced were those with one of the evolutionary invariant tyrosine residues at positions 67 and 74 replaced by a leucine. A small amount of these materials were kindly loaned to Dr. G. Moore and the present author for a ^{1}H n.m.r. study by Drs. P. Boon, G. I. Tesser, and R. J. F. Nivard. This section presents some of the initial results from this study; unfortunately more materials are required to complete the study. This work extends the range of single site modifications studied to Tyr 74 on the back surface and to Tyr 67 in the haem crevice of the molecule.

Spectra were recorded of the ferrocytochromes c at pH 7 and 57°C; and Table 4.7. lists the assignments of resonances from these spectra. The following points are made:

i) The spectra bear a high resemblance to those of $(Hse)^{65}$ ferrocytochrome c (and ferrocytochrome c); this indicates that the majority of the protein fold is unaltered by the modification. The methionine 80 haem group ligand has its CH_3 resonance unaltered for $(Leu)^{74}$, $(Hse)^{65}$ ferrocytochrome c, while it is shifted 0.18 ppm downfield for $(Leu)^{67}$, $(Hse)^{65}$ ferrocytochrome c. This latter shift does not reflect a change in its bonding to the haem, but rather the removal of the Tyr 67 ring current field in this protein; Tyr 67 is packed close to the haem and Met 80 in the X-ray coordinates (25).

ii) For $(Leu)^{74}$, $(Hse)^{65}$ ferrocytochrome c the resonances of Tyr 74 are lost from the spectrum. This was originally difficult to determine as

there is a broad resonance at 6.61 ppm, the chemical shift position of Tyr 74 in $(Hse)^{65}$ ferrocytochrome c (see Fig. 4.8.). This resonance sharpens up at higher temperatures and decoupling experiments show that it is due to the meta protons of a phenylalanine residue. It is assigned to Phe 10 on the basis of a comparison with $(Hse)^{65}$ ferrocytochrome c and ferrocytochrome c. The presence of the Phe 10 meta resonance close to or under the Tyr 74 meta resonance and in intermediate exchange at 27°C in the spectrum of ferrocytochrome c is demonstrated by the temperature dependent NOEs observed by irradiating the Tyr 74 meta resonance; this is described in Section 3.6.2.ii). The Phe 10 meta resonance seems to be more clearly observable in the spectrum of $(Leu)^{74}$, $(Hse)^{65}$ ferrocytochrome c than in ferrocytochrome c. It may be that either the AA'BB'C spectrum appears at a lower temperature or that the loss of the Tyr 74 resonance provides better resolution and observation of this region of the spectrum.

iii) No Tyr 67 resonances have been found for $(Hse)^{65}$ ferrocytochrome c or ferrocytochrome c at any temperature. This is presumably because this residue's ring flipping rate is in intermediate exchange on the ^1H n.m.r. timescale at all temperatures due to constraints on its motion within the haem crevice. Consequently the removal of Tyr 67 in $(Leu)^{67}$, $(Hse)^{65}$ ferrocytochrome c does not affect its appearance in the spectrum.

iv) The resonances of Leu 32, which provide a good guide to small changes in the haem crevice as they receive a large ring current shift from the haem group, are not significantly affected in either modification. The residue lies on the other side of the haem from Tyr 67 and its resonances are not affected by the loss of this ring. Thus both modifications caused no affect to the static structural integrity of the haem crevice.

Table 4.7.

Chemical shift comparison of horse ferrocytochrome c, $(Hse)^{65}$ ferrocytochrome c, $(Leu)^{67}$, $(Hse)^{65}$ ferrocytochrome c, $(Leu)^{74}$, $(Hse)^{65}$ ferrocytochrome c

Resonance designate/	Assignment	Native Chemical shift	$(Hse)^{65}$ Chemical shift	$\{^{Leu}_{Hse}\}^{67}_{65}$, Chemical shift	$\{^{Leu}_{Hse}\}^{74}_{65}$, Chemical shift
M1	Met 80 CH_3	-3.28	-3.28	-3.10	-3.29
M4	Ile 57 CH_3	-0.43	-0.48	-0.44	0.15
	Leu 67 CH_3	----	----	-0.22	----
	Leu 67 CH_3	----	----	0.33	----
A19	Trp 59 C-2	6.99	6.98	n.d.	6.95
A5	Trp 59 C-4	7.58	7.57	n.d.	7.46
A24	Trp 59 C-5	6.68	6.70	n.d.	6.61
A30	Trp 59 C-6	5.76	5.78	n.d.	5.58
A16	Trp 59 C-7	7.07	7.10	n.d.	7.04
A11	Tyr 74 ortho	7.22	7.20	6.99	----
A26	Tyr 74 meta	6.64	6.61	6.71	----

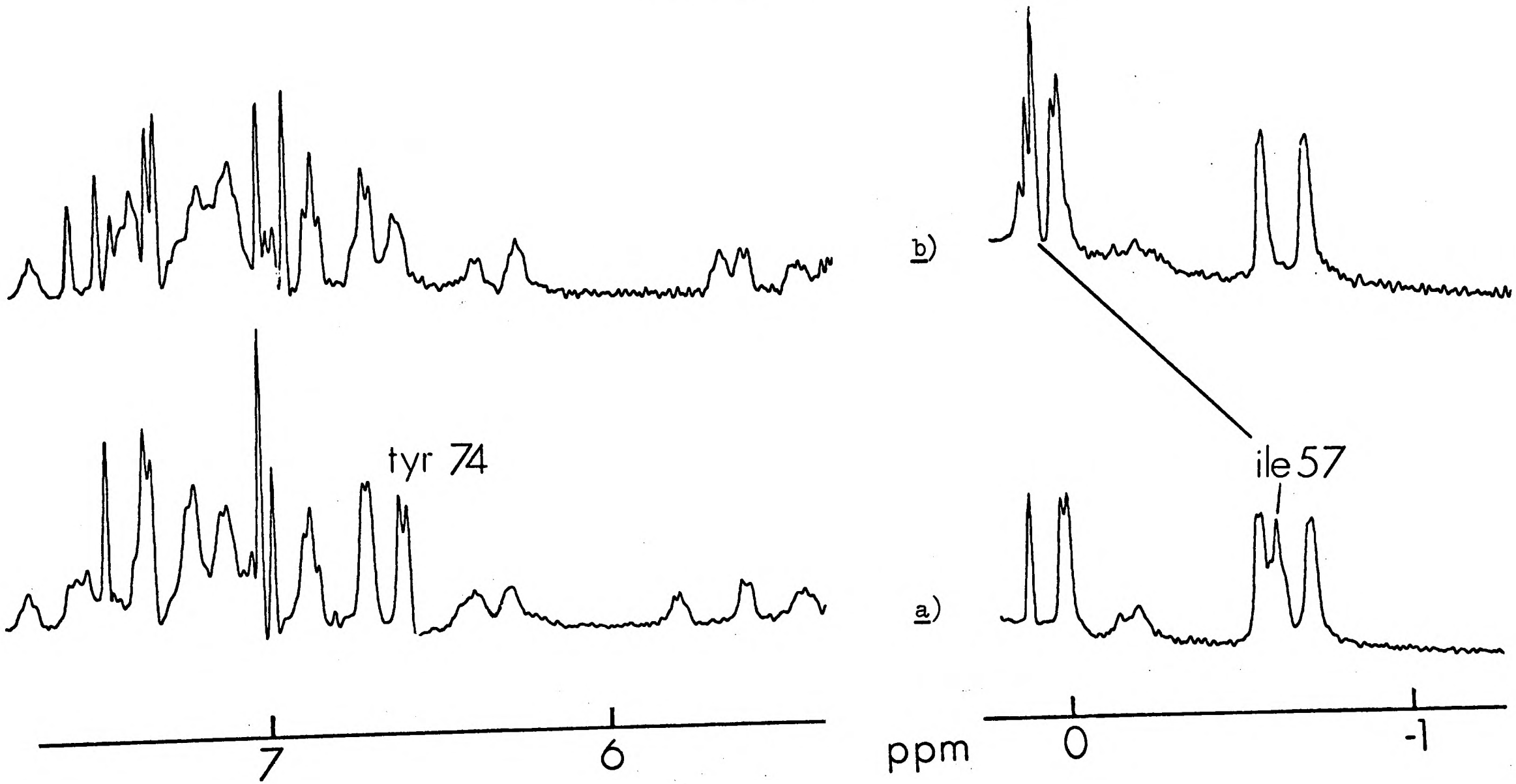
n.d., not determined.

v) Two additional methyl ~~groups~~ resonances are found in the spectrum of $(Leu)^{67}$, $(Hse)^{65}$ ferrocytochrome c at -0.22 ppm and 0.33 ppm. These are assigned to the methyl groups of Leu 67; they receive a large upfield ring current shift from the haem group. No such resonances have been found for Leu 74 in $(Leu)^{74}$, $(Hse)^{65}$ ferrocytochrome c and are presumably under the main methyl resonance area, because like the resonances of Tyr 74 in ferrocytochrome c they will not be expected to receive a large secondary shift.

vi) Fig. 4.8. shows the spectra of ferrocytochrome c and $(Leu)^{74}$, $(Hse)^{65}$ ferrocytochrome c at 27°C and pH 7. The main effect to note is the shift to the resonance of Ile 57. This results from the removal of the Tyr 74 ring current in the latter species. The temperature dependence of this resonance in the native species has been described in Section 3.6.1. In the modified protein the resonance shifts from 0.12 ppm (27°C) to 0.18 ppm (77°C). The resonance still has a large secondary shift and this must originate from the ring of Trp 59; the temperature dependence must arise from a small mutual temperature dependent displacement of the Ile 57 δCH_3 and the ring of Trp 59 in the modified protein. Note, that this resonance is not significantly affected in $(Leu)^{67}$, $(Hse)^{65}$ ferrocytochrome c (Table 4.7.).

These results indicate that the removal of either Tyr 67 or Tyr 74 from the bulk structure of $(Hse)^{65}$ ferrocytochrome c (or ferrocytochrome c) does not cause large disruptions to the protein structure. The effects observed in the ^1H n.m.r. spectra are all due to small local effects, such as the effect of removing a ring current field centre. Spectra were recorded of the ferricytochromes c that indicated that they also possessed native structures at 27°C; no further experiments were carried out.

Fig. 4.8.



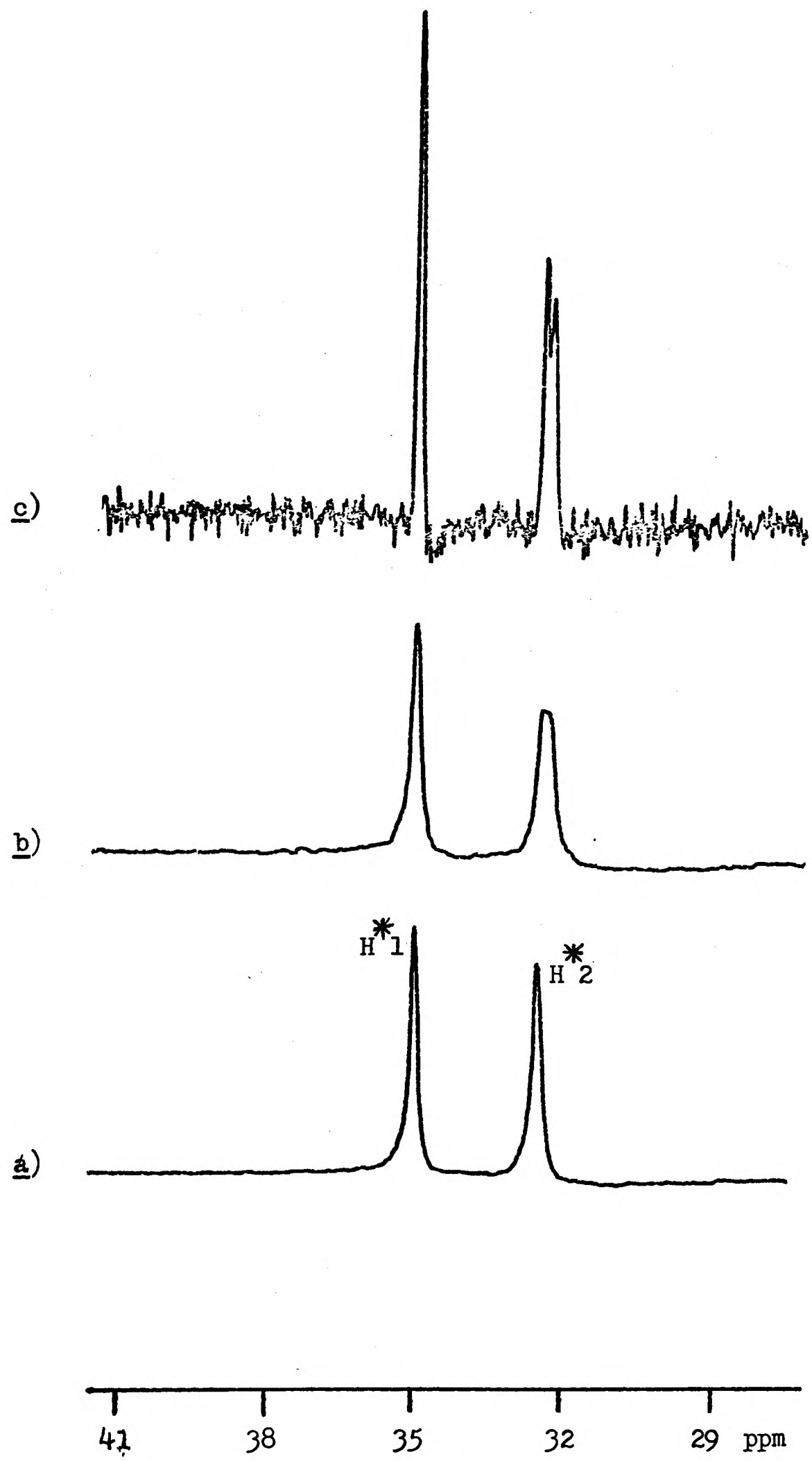
Two regions from the ^{1}H n.m.r. spectra of a) horse ferrocytochrome c, and b) horse $(Leu)^{74}$,$(Hse)^{65}$ ferrocytochrome c, at pH 7 and 27°C. The resonance(s) of Tyr 74 and Ile 57 are marked.

4.3. Monodeamidated cytochromes c

Monodeamidated cytochromes c in which one glutamine or asparagine residue of native cytochrome c has been converted to a glutamic or aspartic acid residue provide single site modifications of cytochrome c with a change of charge at the modification. Monodeamidated cytochromes c are isolated from native other artifactual forms of cytochrome c during the purification of cytochrome c and their properties have been reviewed by Margoliash (40). The normal method of isolation is by ion exchange chromatography or gel electrophoresis, as the pI of monodeamidated cytochrome c is lower than that of native cytochrome c The sample of monodeamidated cytochromes c used in this study was a kind gift from Dr. C. J. A. Wallace of the Molecular Biophysics Laboratory, Oxford, and was prepared by gel electrophoresis of Sigma Type III horse cytochrome c. The exact nature of the product obtained is not known as there are 8 possible deamidation sits in horse cytochrome c (Gln 12, Gln 16, Gln 42, and Asn 31, Asn 52, Asn 54, Asn 70 and Asn 103). However, it was thought quite likely that there might be one or two major deamidation sites, and that in this case ^{1}H n.m.r. spectroscopy might be able to yield information concerning which residues were deamidated. If this problem were to be resolved the effect of the change of charge at a specific residue could then be determined. This would compliment the work of section 4.2.which defines structural perturbations caused by the modifications at Met 65, Tyr 67 and Tyr 74.

Spectra of monodeamidated and native cytochrome c were recorded in both oxidation states, and the following points were observed:
i) Fig. 4.9. shows the hyperfine downfield shifted resonances of horse monodeamidated and native ferricytochromes c, both in $^{2}H_2O$ at pH 7.05 and 27°C. The convolution difference spectrum of the same region is

Fig. 4.9.



The far hyperfine downfield resonances (haem methyls 8 and 3) in a) the spectrum of horse ferricytochrome c, b) the spectrum of monodeamidated ferricytochrome c, and c) the resolution enhanced spectrum of monodeamidated ferricytochrome c, all at pH 7.05 and 27°C.

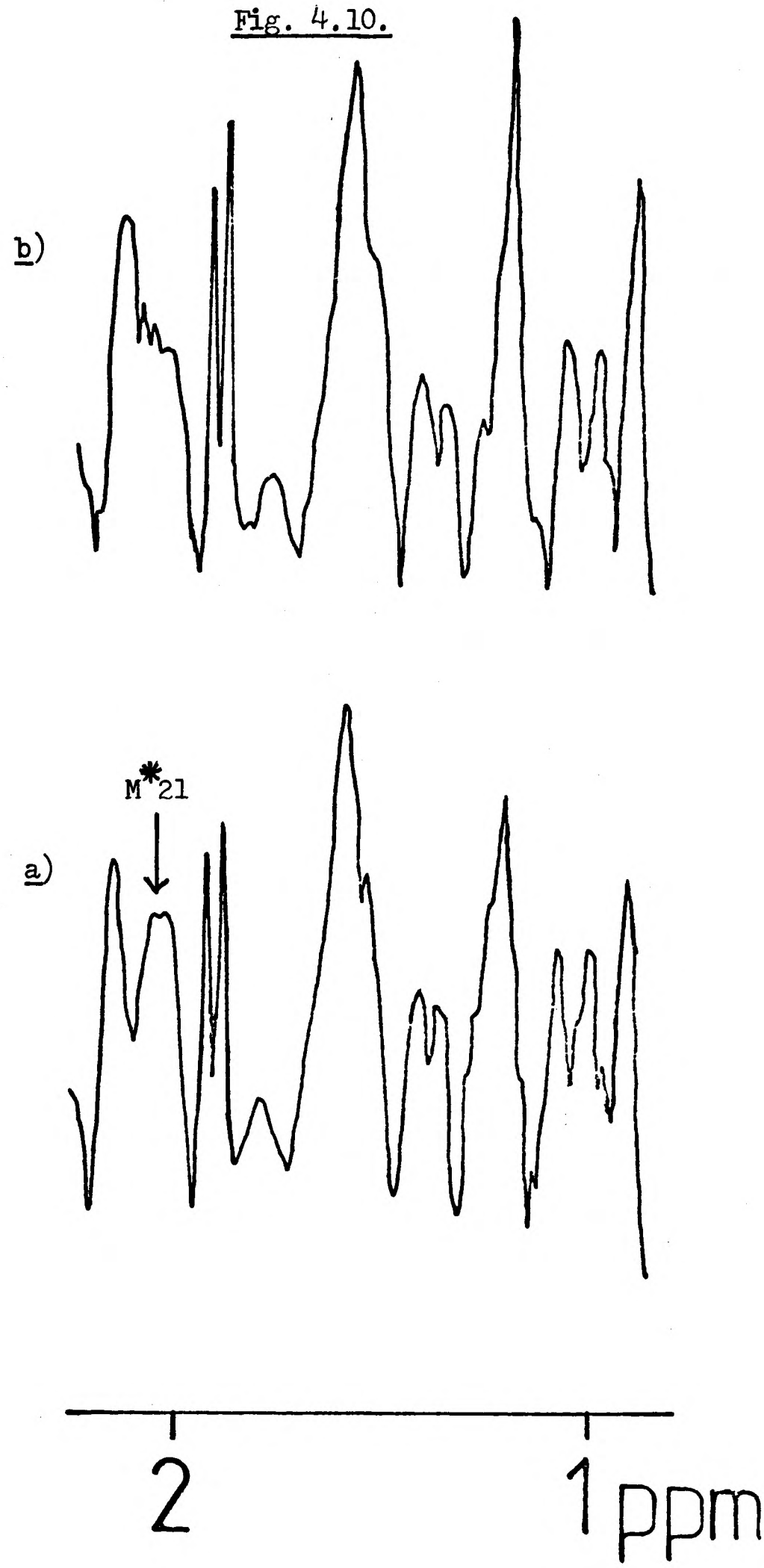
shown for the monodeamidated ferricytochrome c. In the spectrum of the modified protein mixture the resonance of haem methyl 3 is split into two components of roughly equal intensity. An examination of other regions of the spectra shows that the resonances of haem methyl 5 at 10 ppm and the two resonances of the βCH protons of haem propionate 7 at 19 ppm and 11.5 ppm are also split into two components. In each case one component is preserved at the native position and the relative intensities are approximately equal. These results point to one site of deamidation being close to the haem group, and the other main site of daemidation being far from the haem group.

ii) Fig. 4.10. shows part of the convolution difference spectra of ferricytochrome c and monodeamidated ferricytochrome c at 27°C and pH 7. Here resonance M*21, assigned to Ala 15 is split into two components. Other shifts were observed, but they could not be easily assigned due to resonance overlap.

iii) Fig. 4.11. shows the upfield shifted methyl region of the spectra of ferrocytochrome c and monodeamidated ferrocytochrome c, and the difference between them, all at 57°C and pH 7.05. The spectra show that the doublet resonances M5, M7 and λ at 0.58 ppm are split into two components of nearly equal intensity. For example, M5 appears as a triplet in the spectrum of monodeamidated ferrocytochrome c; a Carr Purcell A spectrum shows that the resonance is two overlapping doublets; and the difference spectrum (Fig. 4.11.) confirms that two overlapping doublets are present (the component above the spectrum in the difference spectrum is at the native position). The spectral profiles in other regions of the spectra from the reduced species were virtually identical.

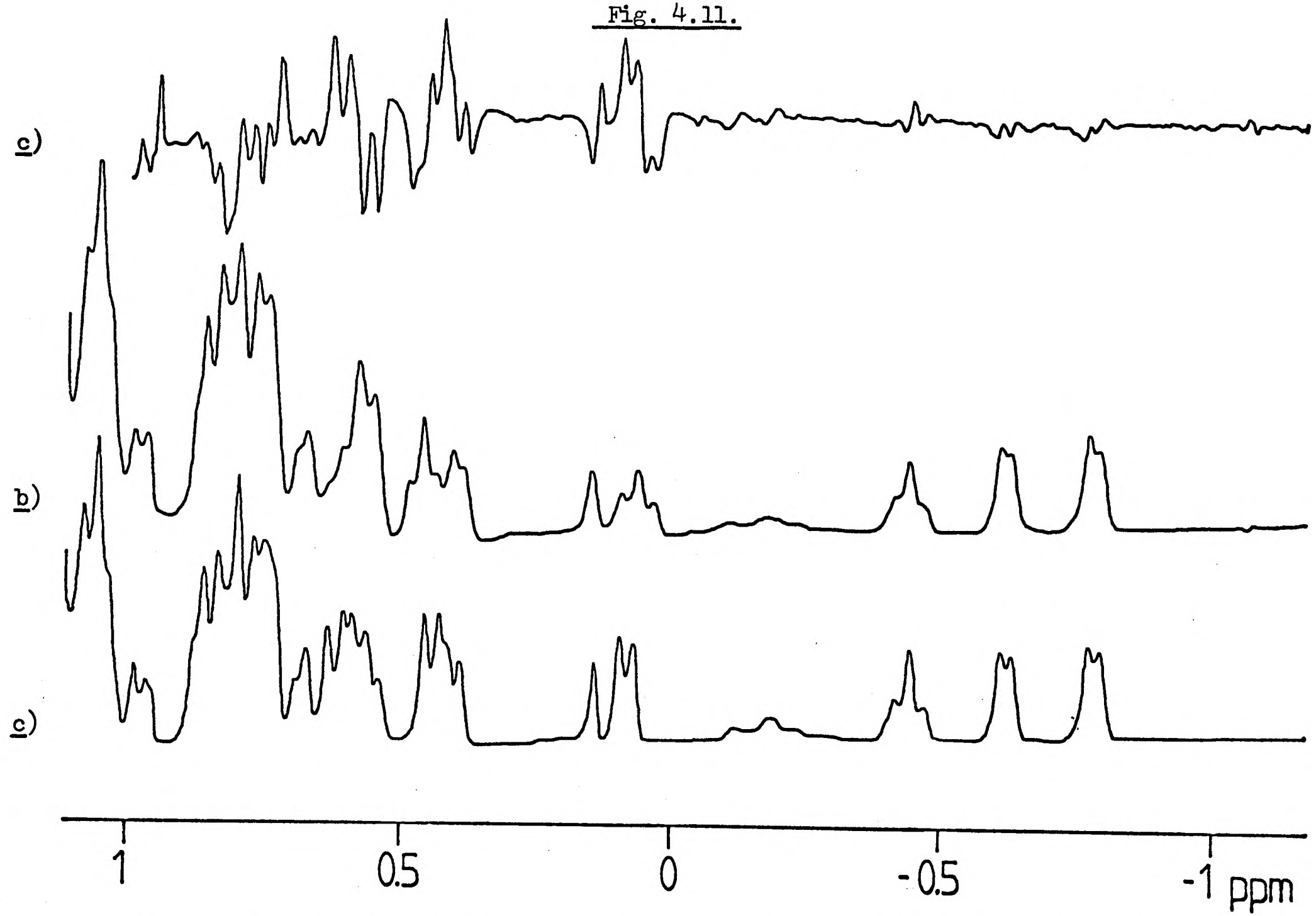
These data are summarised in Table 4.8.; two further pieces of data

Fig. 4.10.



The convolution difference spectra of a) horse ferricytochrome c, b) monodeamidated ferricytochrome c, at 27°C and pH 7. The main observation is that resonance M*21 is split into two components in the latter spectrum.

Fig. 4.11.



The upfield methyl regions of the ^{1}H n.m.r. spectra of a) horse ferrocytochrome c, b) monodeamidated ferrocytochrome c, and c) the difference between them, a) - b), all at pH 7.05 and 57°C.

Table 4.8.

Chemical shift comparison of horse cytochrome c and monodeamidated cytochrome c

Resonance designate/Assignment		Cytochrome c (native) Chemical shift	Monodeamidated cytochrome c Chemical shifts	
a) Ferricytochrome c (at pH 7.05 and 27°C)				
$H^{*}2$	Haem methyl 3	3.24	32.4	32.1
$H^{*}3$	Propionate β CH 7	19.0	19.0	18.95
$H^{*}5$	Propionate β CH 7	11.5	11.5	11.0
$H^{*}6$	Haem methyl 5	10.0	10.0	10.1
$M^{*}21$	Ala 15 CH_3	2.07	2.07	2.01
b) Ferrocytochrome c (at pH 7.05 and 57°C)				
M5	Val 20 CH_3	0.07	0.07	0.03
M7	Val 20 CH_3	0.43	0.43	0.48
+	Ala 83 CH_3	0.58	0.58	0.53

+, no designate, this resonance is assigned in Chapter 3.

from other authors' work are relevant to the interpretation of these results:

iv) Robinson et al (41) have determined the rates of deamidation in model pentapeptides of the form Gly - X - A - Y - Gly, where A is the amide residue, for all eight sequences (X - A - Y) in cytochrome c. These results are shown on Table 4.9., where the data for each amide residue is summarised.

v) Flatmark (42) has shown for beef cytochrome c that the monodeamidated cytochrome c fraction consisted almost exclusively of cytochrome c deamidated at residue 103 (the same residue observed to have the shortest $t_{\frac{1}{2}}$ for deamidation in the model pentapeptide study of Robinson et al (41)). This latter result is not consistent with the ^1H n.m.r. results, which show that there are two species of monodeamidated cytochrome c found in almost equal concentrations; but, it should be noted the methods of preparation in this study and in Flatmark's (42) were different.

No unique solution to the nature of the species present in the batch of monodeamidated cytochrome c studied in this work can be given from the data presented above, but the following is the most likely explanation. In the spectra of mondeamidated cytochromes c in both oxidation states, several resonances split into 'native' and 'shifted' resonances of about equal intensity and so the resonances that arise from one species can not be determined by a classification based on the intensities of the resonances. It is, however, most likely that the sample contains two differently deamidated cytochromes c in an ca. 1:1 mixture. The following resonances are observed to split; haem methyl 3, haem methyl 5, two CHs of propionic acid group 7, Ala 15 (ferricytochrome c) and Val 20 and Ala 83 (ferrocytochrome c) (Table 4.8.).

Table 4.9.

Summary of data concerning monodeamidated cytochromes c

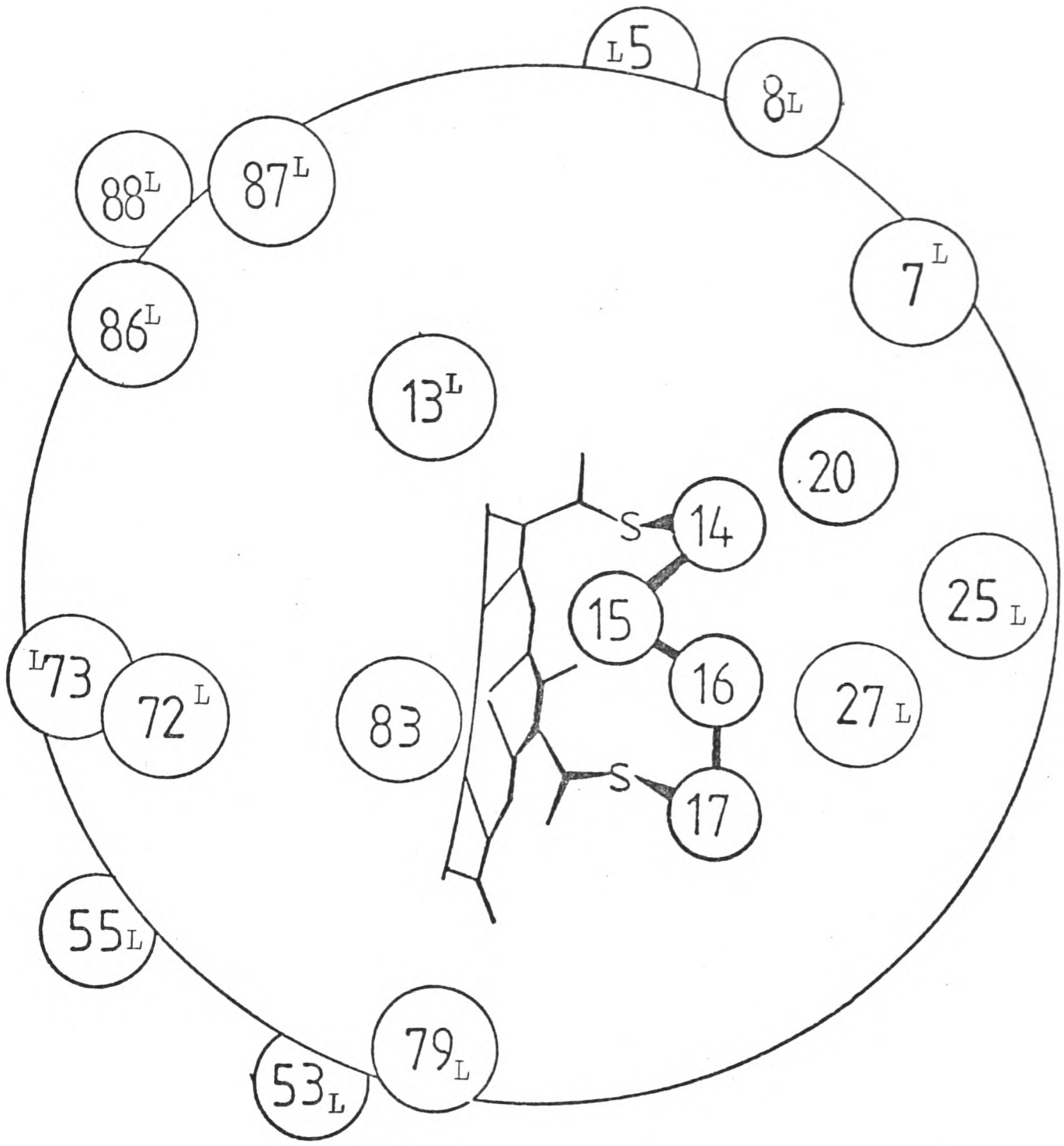
Amide residue	$t_{\frac{1}{2}}$ in model +	Flatmark's result	On surface? (X-ray)	^1H n.m.r. result
Gln 12	421 days		Surface	
Gln 16	113 days		Surface	ca. 50% of deamidated product
Gln 42	418 days		Internal	
Asn 31	227 days		Internal	
Asn 52	54 days		Close to Surface	
Asn 54	94 days		Surface	
Asn 70	80 days		Surface	
Asn 103	16 days	Deamidated in beef cytochrome c	Surface	ca. 50%

+, $t_{\frac{1}{2}}$ for deamidation of residue in model pentapeptide Gly - X - A - Y - Gly (see text) under condition given in ref. 41.

Deamidation at residue 103 is unlikely to cause any perturbation to any of these resonances; and as it lies at the extreme C terminal end of the peptide chain and is not close to any ring current centres such deamidation would be unlikely to cause any detectable changes in the spectrum. As Asn 103 is indicated by both the studies of Robinson et al (41) and Flatmark (42) all the native components of the split resonances are assigned to monodeamidated Asp 103 cytochrome c. Of the remaining amide residues Gln 16 is most likely to give rise to the shifted components of the split resonances; Ala 15, Val 20, Ala 83, and heme methyls 3 and 5, which all lie close to this residue (see Fig. 4.12.). The shifts to the other haem resonances (propionate 7) could be transmitted from Glu 16 through the electronic structure of the haem; Gln/Glu 16 is in the haem peptide.

From the studies with model pentapeptides (41) Gln 16 is the 5th most likely amide to be deamidated (Table 4.9.); however, it is possible to propose the following activation mechanism for this residue in the protein. Deamidation is catalysed by protons and hydroxide ions, but the rate of deamidation in model peptides increases with increasing pH, so the hydroxide ion is the more effective catalyst (41). In cytochrome c, Gln 16 lies on the surface of the protein and close to the cytochrome c oxidase binding surface (43), which lies at the positive end of the dipole moment of the protein (44) and contains a cluster of positively charged lysine residues (25). These features would give rise to a solvent structuring effect and would tend to attract hydroxide ions to this part of the protein surface in preference to other areas. Fig. 4.12. shows this region of the surface of the protein and shows lysine residues, haem methyls 3 and 5, residues 14 to 17, Val 20 and Ala 83 in their appropriate positions.

Fig. 4.12.



A view of the front surface of the cytochrome c molecule. This surface contains the cytochrome c oxidase binding site (43), lies at the positive end of the dipole moment of the protein (44) and contains a cluster of positively charged lysine residues. The residues which are marked are the lysine residues in this part of the molecule, and residues close to Gln 16, which is proposed as one of the two sites of monodeamidation; the lysines are identified by an 'L'.

Taking the above arguments to be correct and noting the lack of other differences between the spectra of cytochromes c and monodeamidated cytochromes c, the modifications caused at these two sites can be said to have caused very little structural perturbation to the molecule. They will, and particularly that at Gln 16, cause differences in the binding of cytochrome c oxidase and cytochrome c reductase, and would in this manner impair the biological function of the molecule.

References: Chapter Four

1. Moore, G.R., and Williams, R.J.P. (1980), Eur.J.Biochem. 103, 503.

2. Prof. H.A. Harbury, personal communication.

3. Boswell, A.P., Moore, G.R., Williams, R.J.P., Wallace, C.J.A., Boon, P.J., Nivard, R.J.F., and Tesser, G.I. (1981), Biochem.J., 193, 493.

4. Ando, K., Matsubara, H., and Okunuki, K. (1966), Biochem.Biophys.Acta., 118, 240.

5. Osheroff, N., Feinberg, B.A., Margoliash, E., and Morrison, M., (1977), J.Biol.Chem., 252, 7743.

6. Pal, P.K., Verma, B., and Myer, Y.P. (1975), Biochemistry, 14, 4325.

7. Brautigan, D.L., Ferguson-Miller, S., and Margoliash, E. (1978), J.Biol.Chem., 253, 130.

8. Ahmed, A.J., Smith, H.T., Smith, M.B., and Millett, F.S. (1978), Biochemistry, 17, 2479.

9. Ferguson-Milller, S., Brautigan, D.L., and Margoliash, E. (1978), J.Biol.Chem., 253, 149.

10. Smith, H.T., Staudenmayer, N., and Millett, F. (1977), Biochemistry, 16, 4971.

11. Staudenmayer, N., Ng, S., Smith, M.B., amd Millett, F. (1977), Biochemistry, 16, 600.

12. 'Semisynthetic Peptides and Proteins', Academic Press, London and New York, Offord, R.E., and DiBello, C. (eds).

13. Margoliash, E., Smith, E.L., Kreil, G., and Tuppy, H. (1961), Nature, 192, 1125.

14. Dayhoff, M.O. (1968 - 1973), Atlas of Protein Sequence and Structure, National Biochemical Research Foundation, Silver Springs, Maryland, U.S.A.

15. Boon, P.J., Tesser, G.I., and Nivard, R.J.F. (1979), Proc.Natl.Acad.Sci. U.S.A., 76, 61.

16. Wallace, C.J.A., and Offord, R.E. (1979), Biochem.J., 179, 169.

17. Corradin, G., and Harbury, H.A. (1974), Biochem.Biophys.Res.Comm., 61, 1400.

18. Barstow, L.E., Young, R.S., Yakali, E., Sharp, J.J., O'Brien, J.C., Berman, P.W., and Harbury, H.A. (1977), Proc.Natl.Acad.Sci. U.S.A., 74, 4248.

19. Boon, P.J., Tesser, G.I., and Nivard R.J.F. (1978), in 'Semisynthetic Peptides and Proteins' (Offord, R.E. and Di Bello, C., eds.), pp.115 - 126, Academic Press, London and New York.

20. Berman, P.W., and Harbury, H.A. (1980), J.Biol.Chem., 255, 6133.

21. Moore, G.R. (1978), D.Phil thesis, Oxford.

22. Swanson, R., Trus, B.L., Mandel, N., Mandel, G., Kallai, O.B., and Dickerson, R.E. (1977), J.Biol.Chem., 252, 759.

23. Campbell, I.D., Dobson, C.M., Williams, R.J.P., and Wright, P.E. (1975), FEBS Letters, 70, 96.

24. Moore, G.R., and Robinson, M., personal communication.

25. Takano, T., and Dickerson, R.E. (1980), Proc.Natl.Acad.Sci,

26. Urbanski, G.J., and Margoliash, E. (1977), J.Immunol., 108, 1170.

27. Moore, G.R., and Williams, R.J.P. (1980), Eur.J.Biochem., 103, 543.

28. Keller, R.M., and Wuthrich, K. (1978), Biochim.Biophys.Acta., 533, 195.

29. Redfield, A .G., and Gupta, R.K. (1971), Cold Spring Harbour Symp.Quant.Biol., 36, 405.

30. Moore, G.R., and Williams, R.J.P. (1980), Eur.J.Biochem., 103, 493.

31. Campbell, I.D., Dobson, C.M., Moore, G.R., Perkins, S.J., and Williams, R.J.P. (1976), FEBS Letters, 70, 96.

32. Keller, R.M., and Wuthrich, K. (1981), Biochim.Biophys.Acta., in press.

33. Stellwagen, E. (1968), Biochemistry, 7, 2496.

34. Schecter, E., and Saludjian, P. (1967), Biopolymers, 5, 788.

35. Schejter, A., and George, P. (1964), Biochemistry, 3, 1045.

36. Petsko, G.A., Phillips, D.C., Williams, R.J.P., and Wilson, I.A. (1978), J.Mol.Biol., 120, 345.

37. Sadler, P.J., Benz, F.W., and Roberts, G.C.K. (1974), Biochim.Biophys.Acta., 359, 13.

38. 'Advanced Inorganic Chemistry', Cotton, A., and Wilkinson, G. ((1975), Wiley, New York.

39. Sadler, P.J., and Ismail, I., personal communication.

40. Ferguson-Miller, S., Brautigan, D.L., and Margoliash, E. (1977), in 'The Porphyrins' (D.Dolphin, ed.), Academic Press, New York.

41. Robinson, A.B., McKerrow, J.H., and Legaz, M. (1974), Int. J.Peptide Res., 6, 31.

42. Flatmark, T. (1966), Acta.Chem.Scand., 20, 1487.

43. Brautigan, D.L., Ferguson-Miller, S., Tarr, G.E., and Margoliash, E. (1978), J.Biol.Chem., 253, 140.

44. Koppenol, W.H., Vroonland, Ch., Braams, R., Ferguson-Miller, S., and Brautigan, D.L. (1977), Abstracts, 175th Natl. Meeting of the American Chemical Society, Chicago, Ill., BIOL 85.

CHAPTER FIVE

Studies of cytochromes c fully modified at positively charged lysine residues

5.1. Introduction

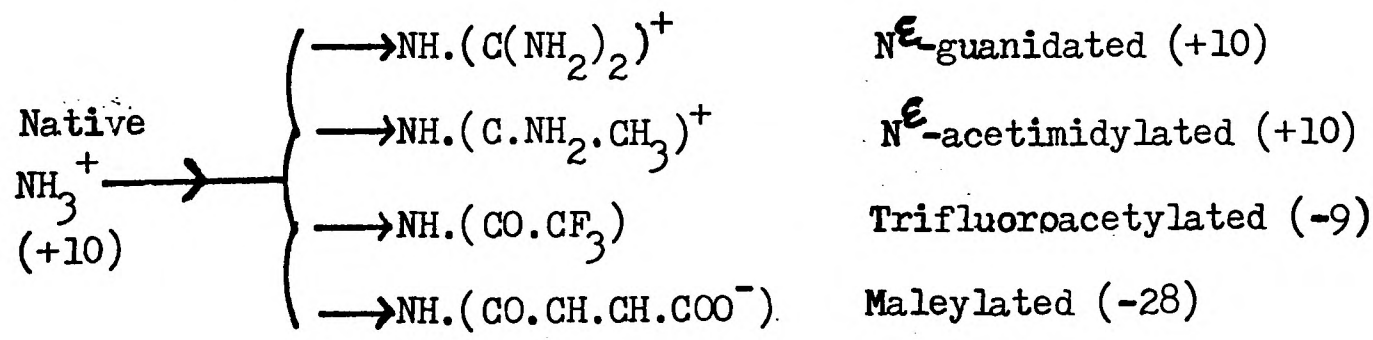
The amino acid sequence of horse cytochrome c contains 19 lysine residues, many of which are conserved throughout the whole range of eukaryotic species (1). Some of these residues are believed to play important roles in binding the physiological partners of cytochrome c (1,2,3) and in stabilising the native conformation of the protein (4). The distribution of charged groups in a protein contributes to its electric potential (5) and this in turn could modify the electronic structure (or more precisely, the distribution of the unpaired electron) in ferricytochrome c (see later). This study of fully modified lysine derivatives of cytochrome c was carried out for the following reasons:

a) Modified cytochromes c are used by protein chemists to investigate the role of different residues in physiological reactions. An implicit assumption in such work is that the modification causes little or no change to the structure of the protein, and usually the presence of the 'native' 695 nm. band in the absorption spectrum of the modified protein is taken as evidence for a native type conformation (see for example, 6,7,8,9). A study by ^{1}H n.m.r. would point to any small changes of conformation that do occur on modification.

b) Some n.m.r. studies of the protein interactions between cytochrome c and cytochrome c peroxidase have been reported (10) in which resonances of the haem group in ferricytochrome c were used to monitor complex formation. It would be of interest to know whether the haem group shifts indicate a specific or non-specific binding. The fully modified lysine

derivatives can be used to determine the effect of changing the surface charge on cytochrome c (as would occur in the formation of a complex with cytochrome c peroxidase) on the unpaired electron of the haem group that gives rise to contact and pseudocontact shifts on these resonances.
c) Lysine ionisation in proteins can lead to pH dependent conformational changes within other parts of the molecule. The study of proteins with blocked lysine residues (either non ionisable groups or ionisable groups with a different pK_a to lysine itself) can then yield information on the role of lysine ionisation in proteins. An example of this is the pH dependence of the Ile 57 residue in ferrocytochrome c that is discussed in Section 3.6.4., and for which the initial evidence of its connection to a lysine residue is presented in Section 5.4. of this chapter.
d) The changing of the surface charge of a protein can affect its structure, and for a globular protein can cause it to unfold. Such is the case for maleyl cytochrome c, which can be unfolded from a globular structure close to that of native cytochrome c at room temperature to a 'random coil' protein by the increase of temperature - an n.m.r. study of this is described in Section 5.3. of this chapter.

This work describes the ^1H n.m.r. spectra and solution structures of four fully modified lysine derivatives of cytochrome c, so chosen that the charge is either maintained, neutralised or reversed. The modifications described are as follows (the bracketed figures being the approximate charge at pH 7 for each of the modified proteins):



They were kindly prepared by Dr. D. E. Harris of the Molecular Biophysics Laboratory, Oxford (the methods of preparation are given in the Appendix). The following abbreviation are used in this chapter; N^{ε}-acet., N^{ε}-acetimidylated cytochrome c; N-guanid., N-guanidated cytochrome c; TFA, Trifluoroacetylated cytochrome c; and Maleyl, Maleylated cytochrome c.

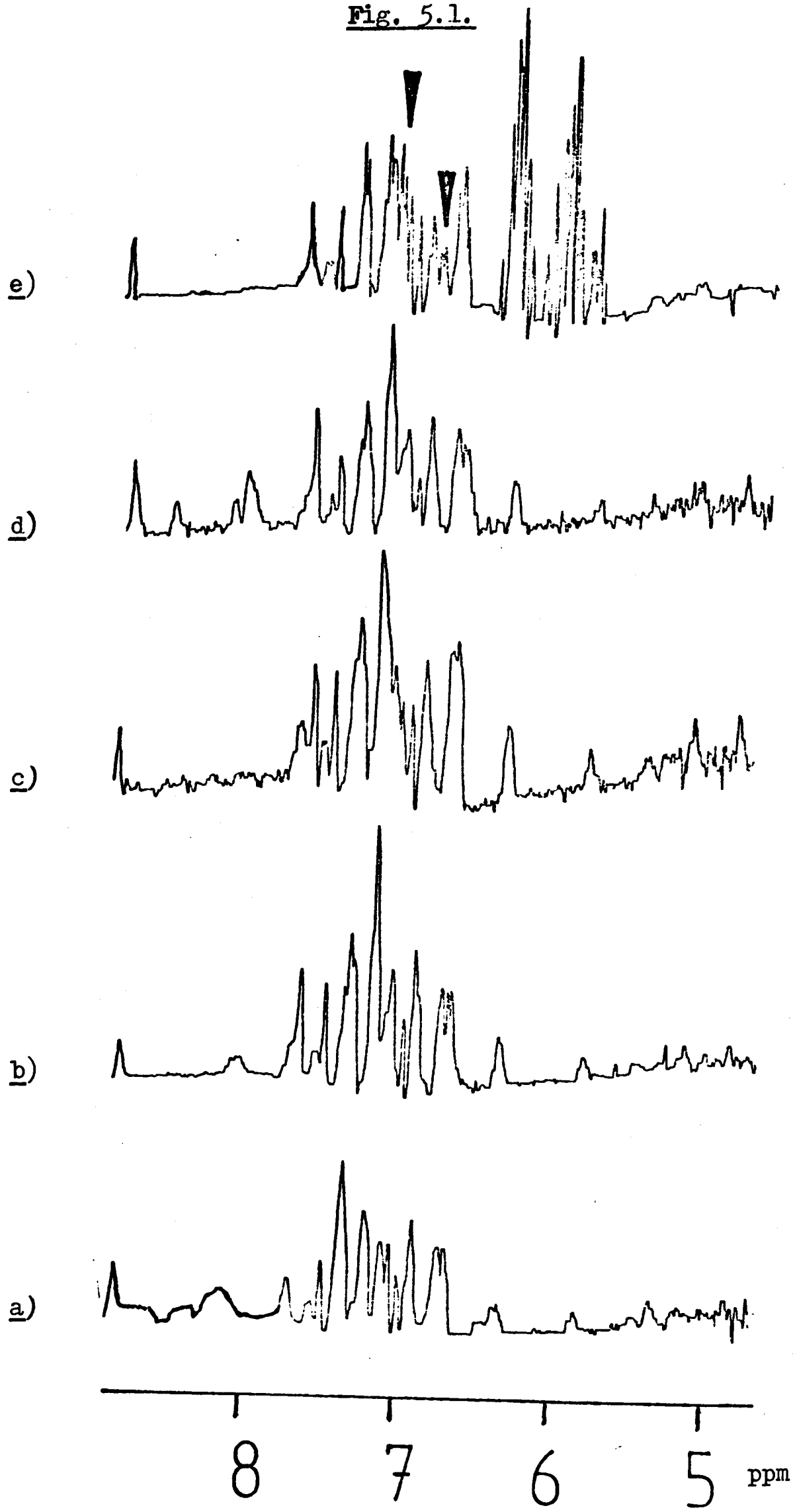
5.2. The solution structures of cytochrome c and four fully lysine modified derivatives

Three regions of the spectra of horse ferrocytochrome c and four fully modified lysine derivatives of it at pH 7 and 57°C are shown in Figs. 5.1. to 5.3. The resemblances between the spectra are striking, and the differences come from the following two sources:

a) In two of the modifications extra non-exchangeable protons have been added to the protein on modification. These are the 19 N^{ε}-acetimidyl methyl groups and 19 maleyl groups, which give rise to 19 singlet resonances between 2.0 ppm and 2.3 ppm (Fig 5.2.) in N^{ε}-acetimidylated ferrocytochrome c and 38 one proton doublets between 5.5 and 6.5 ppm (Fig. 5.1.) in maleyl cytochrome c, respectively. In both cases all the protons can be accounted for indicating that the modifications have gone to completion. By spectral simulation the individual chemical shifts of the 19 (or $2 \times 19 = 38$) resonances can be determined, and in each case there is a main band of resonances centred on the average chemical shift position comprising of ca. 13 resonances and there are ca. 3 resonances both upfield and downfield of the central band. This indicates that most of the modified side chains are in closely the same chemical environment, but that ca. 6 of the modified side chains are in different from average chemical environments.

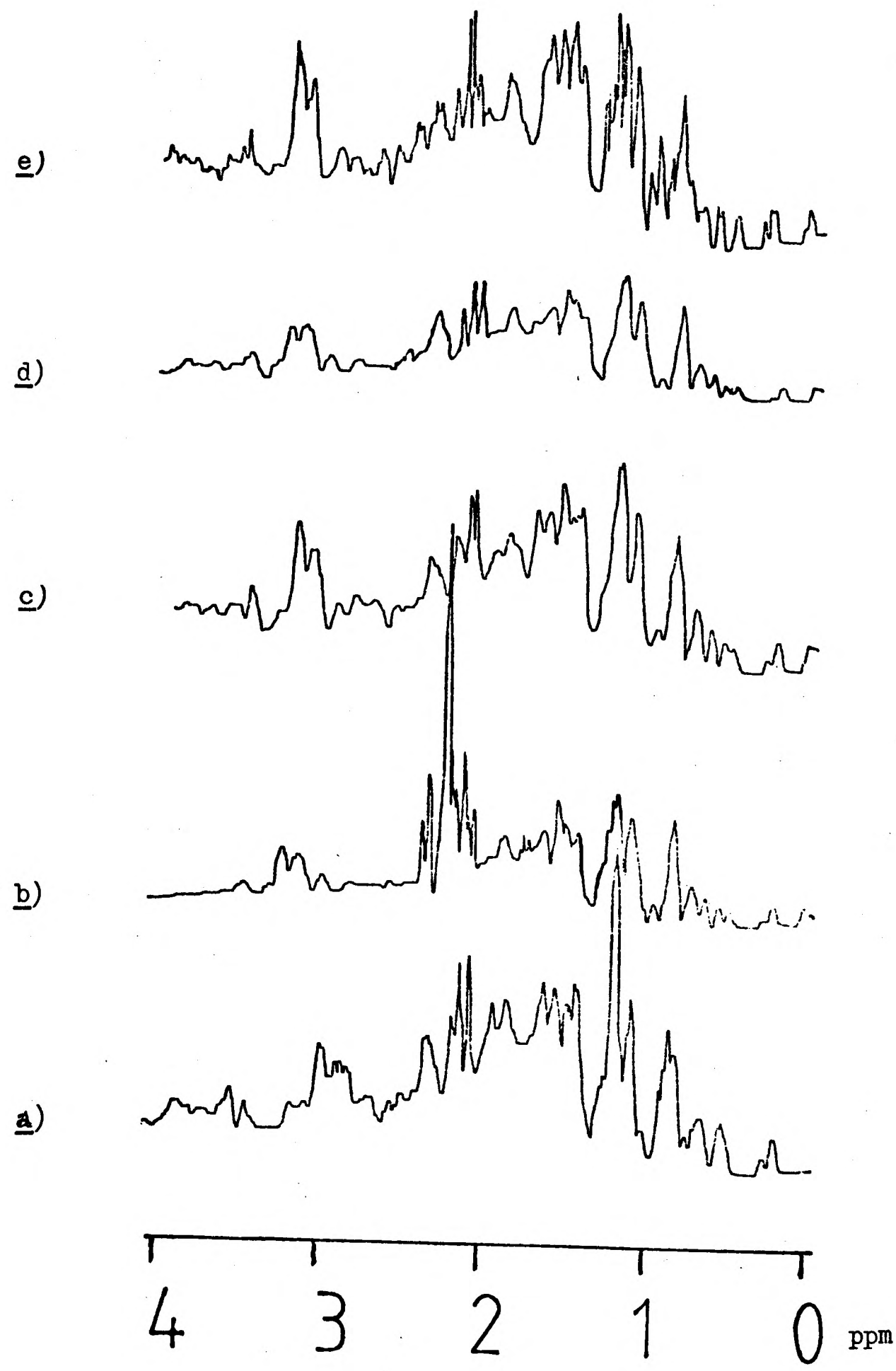
The 19 ε-CH_2 groups of the lysine residues are found a a broad hump

Fig. 5.1.



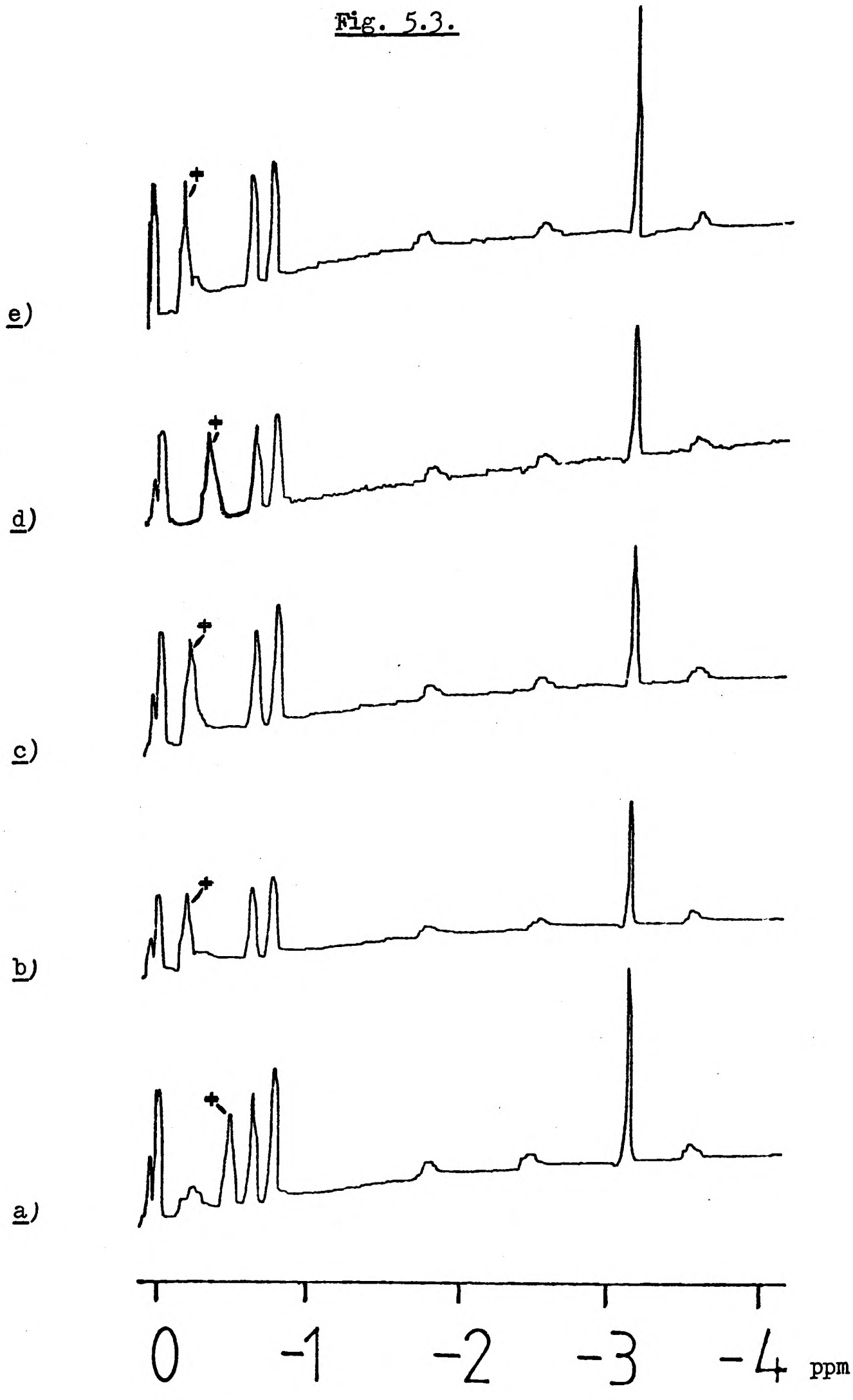
The aromatic regions of the ^{1}H n.m.r. spectra of a) native, b) N^{ε}-acet., c) N-guanid., d) TFA., and e) maleyl ferrocytochromes c, at pH 7 and 57°C. An extra pair of doublets observed for maleyl ferrocytochrome c are indicated (see text).

Fig. 5.2.



The methyl regions of the ^{1}H n.m.r. spectra of a) native, b) N--acet., c) N-guanid, d) TFA., and e) maleyl ferrocytochromes c, at pH 7 and 57°C.

Fig. 5.3.



The upfield methyl regions of the ^1H n.m.r. spectra of a) native, b) N^{ε}-acet., c) N-guanid., d) TFA., and e) maleyl ferrocytochrome c, at pH 7 and 57°C. The resonance of the Ile 57 δCH_3 group is marked for each protein (+)

Table 5.1.

Chemical shift comparison of native and fully modified horse ferrocytochromes c at pH 7 and 57°C

Resonance/Assignment		Native	N^{ε}-acet+	N-guanid+	TFA+	Maleylated+
M1	Met 80 CH_3	-3.28	-3.28	-3.28	-3.28	-3.28
M2	Leu 32 CH_3	-0.76	-0.76	-0.76	-0.76	-0.76
M3	Leu 32 CH_3	-0.60	-0.60	-0.60	-0.60	-0.60
M4	Ile 57 CH_3	-0.43	-0.13	-0.13	-0.23	-0.08
M6	Leu 68 CH_3	0.38	0.34	0.34	0.33	0.30
M10	N-acetyl CH_3	2.07	+	2.07	2.08	2.08
M11	Met 65 CH_3	2.11	+	2.09	2.09	2.09
Many resonances (from Thr residues)		1.00-1.50	There is a change of lineshape in this region of the spectrum (see text)			
A26	Tyr 74 m-	6.64	6.65	6.65	6.64	6.67
A30	Trp 59 C-6	5.74	5.76	5.76	5.76	++
	Tyr 48 m-	Not seen for these proteins at this temperature due to exchange broadening (see text)				6.85
	Tyr 48 o-					7.15

+, under resonances from N^{ε}-acetimidyl groups; ++, under resonances from maleyl groups.

+abbreviations, see text.

of overlapping triplets in the spectrum of ferrocytochrome c at ca. 3 ppm. On modification this broad hump shifts downfield in each case and the order of the magnitude of the shifts is as follows:

N-guanid (0.2 ppm) < N^{ε}-acet (0.3 ppm) < TFA ≈ Maleylated (0.35 ppm)

These shifts to the average positions of the ε-CH_2 resonances reflects a change in the chemical environment of the protons caused by the modification and provides no useful structural information.

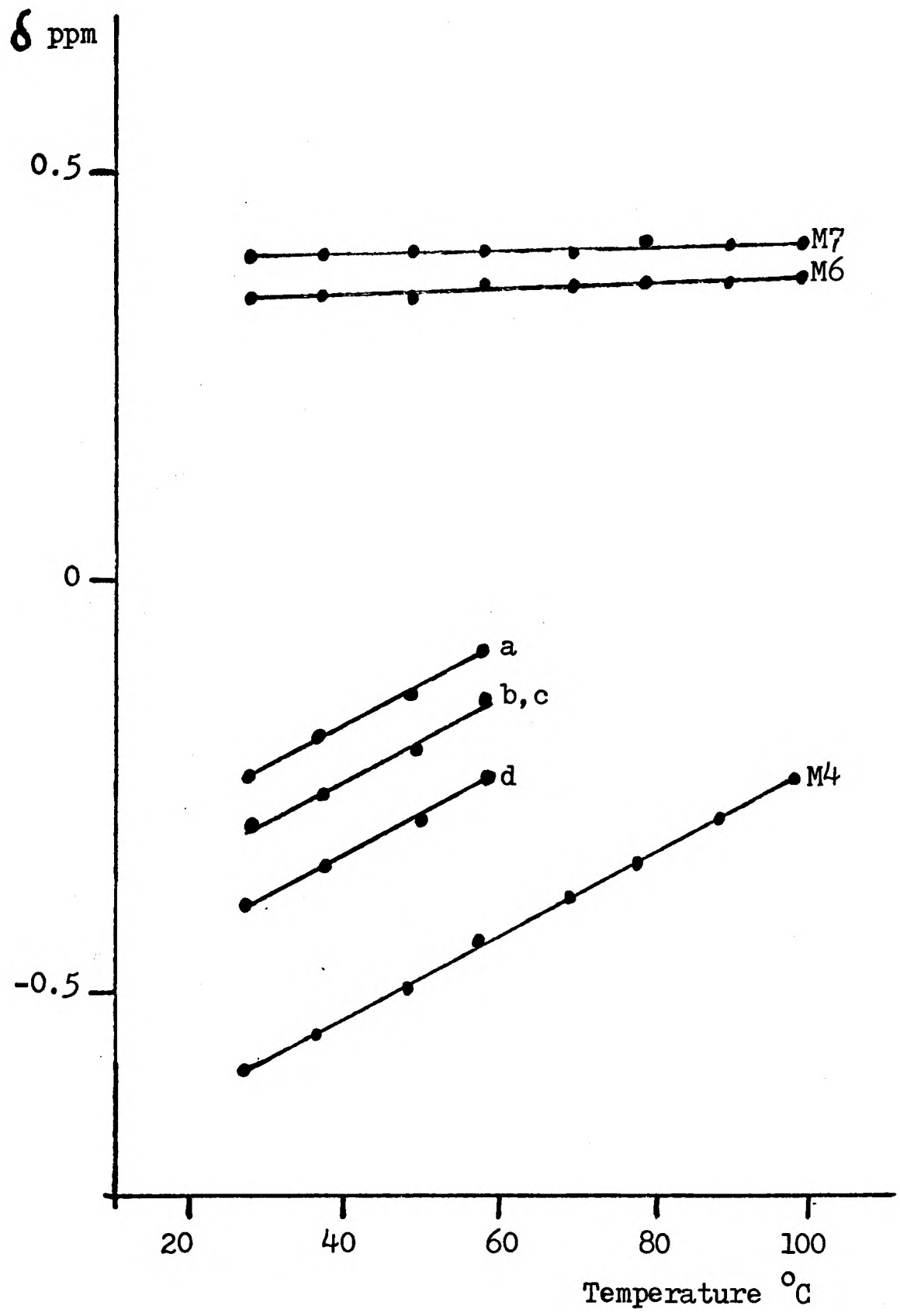
b) The chemical shift positions of a few resonances of the native protein are altered in the modified proteins. Table 5.1. lists the chemical shifts of these resonances in the native and four modified ferrocytochromes c. The following points are made:

i) The resonances of Met 80 and Leu 32, which are sensitive markers to the internal structure of the molecule, remain unaltered. The resonance of Leu 68 another internal residue suffers a small shift.

ii) Several resonances suffer small shifts, and in particular the region between 1.0 ppm and 1.5 ppm in the methyl region of the spectrum has a different overall profile for native and each of the modified ferrocytochromes c (Fig. 5.2.). The resonances from the 10 threonine residues of cytochrome c are found here; 9 of which are surface residues (11) and would expect to recieve shifts on their resonances from the surface perturbations caused by the lysine modifications.

iii) Very small shifts occur to the resonances of Trp 59 and Tyr 74, and a large shift occurs to the δCH_3 resonance of Ile 57. Despite the large shift suffered by this latter resonance upon modification it maintains a similar temperature dependence to that in the spectrum of the native protein; this is shown in Fig. 5.4. In chapter 3 this temperature dependence was discussed and its maintenance in the modified proteins suggest that no significant conformation change has occured between

Fig. 5.4.



The temperature dependences of resonance M4 (due to Ile 57) in native ferrocytochrome c (M4), and a) maleyl, b) N -acet., c) N-guanid., and d) TFA ferrocytochromes c. In each case this is the only resonance to suffer large temperature dependent shifts in the ferrocytochromes c. For comparison the temperature dependences of two other resonances (M6. M7) in native ferrocytochrome c are given.

Ile 57 and the rings of Trp 59 and Tyr 74, as it is the relative movement between these residues with the increase of temperature that gives rise to the effect. The chemical shift position of the Ile 57 resonance is presumably altered due to a small displacement of one, two or all of the residues (Ile 57, Trp 59 and Tyr 74) with respect to the rest of the protein on modification. There are also very small shifts to resonances from Trp 59 and Tyr 74, which suggest that these residues are slightly perturbed to the rest of the protein structure. The effects to the individual residues could be expected to arise from the modification of the following lysines; Lys 39 and Lys 55 (Ile 57), Lys 55 and Lys 60 (Trp 59) and Lys 72 and Lys 73 (Tyr 74). Note that the titration of Lys 39 or Lys 55 causes a large shift to the resonance of Ile 57 (Section 3.6.4.) and that the substitution of Lys 60 in donkey cytochrome c for glycine in cow cytochrome c (the only other change is at position 89; Thr, donkey; Gly, cow) causes the resonance to shift 0.06 ppm upfield.

iv) A 1:1 correlation is found for most resonances between the spectra of the native and fully modified ferrocytochromes c, which indicates that at 57°C and pH 7 the static structures of the proteins are virtually identical.

v) In the case of maleyl ferrocytochrome c an extra pair of coupled doublets are observed at ca. 6.85 ppm and ca. 7.15 ppm (marked with a '▼' in Fig. 5.1.). Further discussion of these is deferred until Section 5.3.

The ferricytochromes c were studied, but not to the same extent; shifts due to modification were observed to the hyperfine shifted resonances that suffer contact and pseudocontact shifts from the paramagnetic haem group. Chemical shift values for these and some other resonances are given in Table 5.2. for the native and fully modified ferricytochromes

Table 5.2.

Chemical shift comparison of native and fully modified horse ferricytochrome c at pH 7 and 27°C

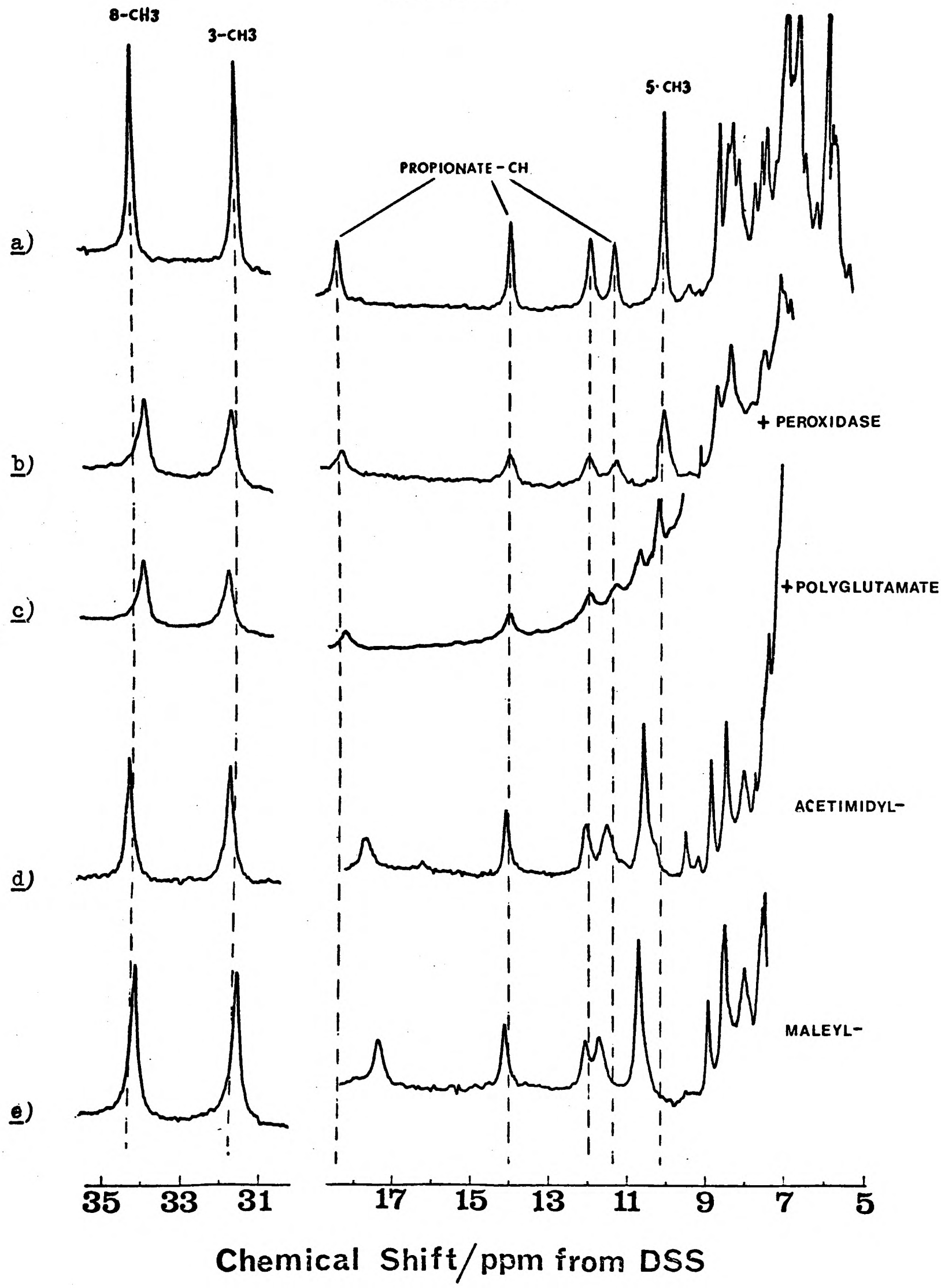
Resonance/Assignment		Native	N -acet+	N-guanid+	TFA+	Maleylated+
H*1	Ringmethyl 8	35.0	35.0	35.0	34.9	34.6
H*2	Ringmethyl 3	32.4	32.5	32.5	32.5	32.0
H*3	Propionate CH 7	19.0	18.0	18.0	17.9	17.5
H*4	Propionate CH 6	14.3	14.3	14.3	14.3	14.3
H*5	Propionate CH 7	11.5	11.9	11.9	11.9	11.9
H*6	Ringmethyl 5	10.0	10.7	10.8	10.8	10.85
M*1	Met 80 CH_3	-23.5	-23.3	-23.3	-23.3	-23.4
M*6	Ile 57 CH_3	-0.37	$\geq$ 0.0	$\geq$ 0.0	$\geq$ 0.0	$\geq$ 0.0 (+)
M*2	Leu 68 CH_3	-2.07	-1.92	-1.92	-1.90	-1.87

+, M*6 can not be found in the modified proteins, but it does lie in the region upfield of 0.0 ppm in any of the proteins.
+, abbreviations, see text.

c at 27°C and pH 7. In general the spectra of the native and fully modified proteins show many resemblances and few differences, as is the case for the reduced proteins, and so the structure of the modified and native ferricytochromes are closely related.

The effects to the resonances of the haem group are important as the behaviour of these resonances upon the modification of the surface of cytochrome c is relevant to the interpretation of n.m.r. studies of the interaction of cytochrome c with cytochrome c peroxidase (henceforth, CCP). Fig. 5.5. shows the downfield hyperfine shifted region of the spectra of ferricytochrome c, ferricytochrome c in the presence of CCP and in the presence of polyglutamate (Sigma Type 1, Mol.Wt. = 15000), and N^{ε}-acet. ferricytochrome c and maleylated ferricytochrome c (The present author is indebted to Dr. G. J. Mc.Clune for the spectra with CCP and polyglutamate). Note, firstly, that the apparent binding of CCP to cytochrome c can be mimicked by the interaction of cytochrome c with polyglutamate. The interaction of CCP to cytochrome c is specific and involves a 1:1 complex (10,12), while the interaction with polyglutamate is assumed to be non specific and to involve all regions of positive charge on the surface of the protein. Thus, the ^{1}H n.m.r. results shown in Fig. 5.5. do not distinguish any specificity of reaction for CCP and cytochrome c, and the shifts induced by CCP to the cytochrome c haem resonances can not be interpreted in terms of a specific geometry for the CCP - cytochrome c complex (13). An indication of the source of these shifts comes from the observation of similar shifts to the haem resonances on the modification of the lysine residues of ferricytochrome c (Table 5.2. and Fig. 5.5.). The conclusion from these data is that the chemical shifts of the haem resonances of ferricytochrome c are

Fig. 5.5.



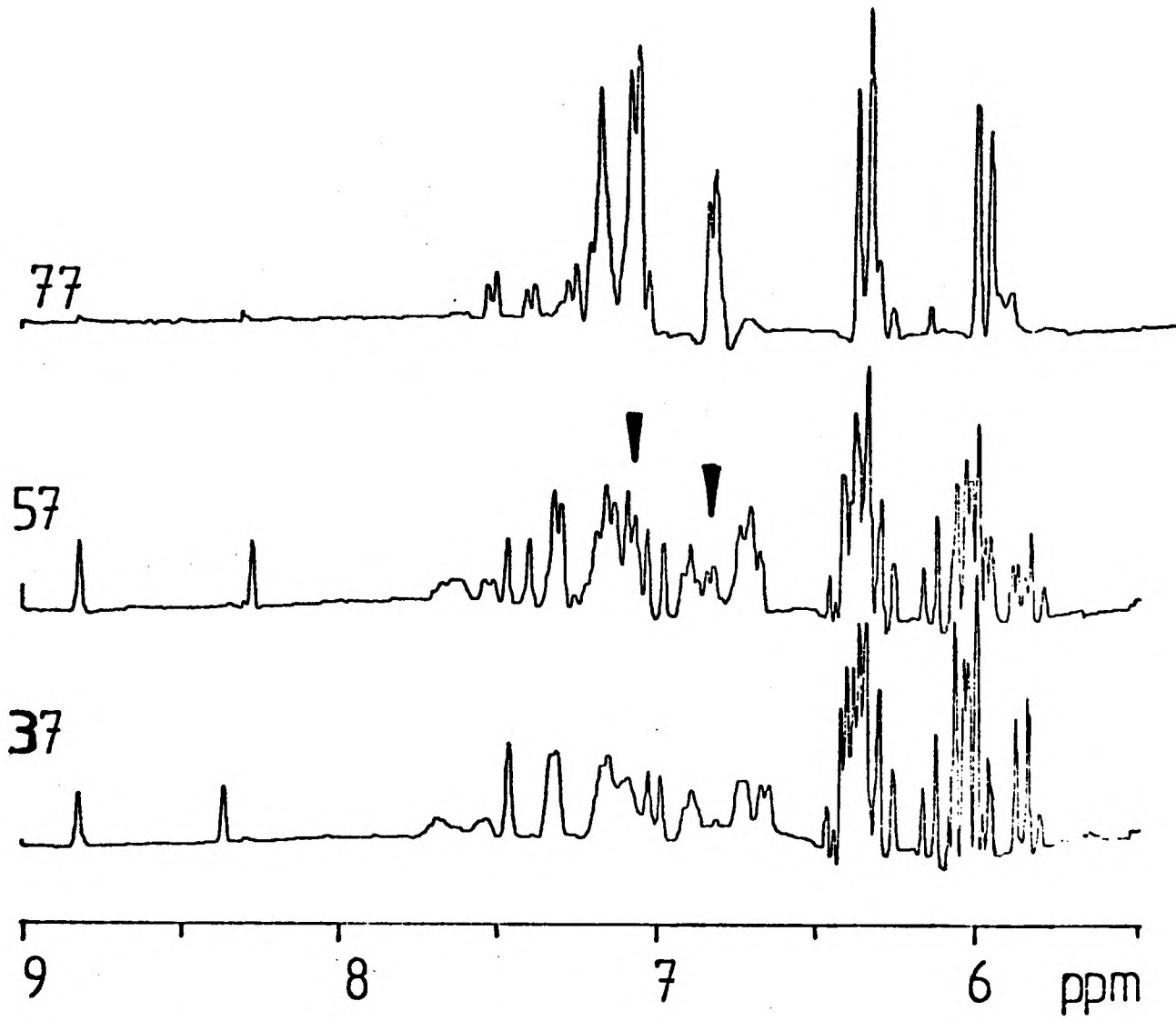
The downfield hyperfine shifted region of the ^{1}H n.m.r. spectra of a) horse ferricytochrome c, b) ferricytochrome c in the presence of cytochrome c peroxidase, c) ferricytochrome c in the presence of polyglutamate (Sigma Type 1), d) N^{ε}-acet. ferricytochrome c, and e) maleylated ferricytochrome c, all at pH 7 and 27°C.

sensitive to the distribution of the unpaired electron, which gives rise to paramagnetic contact and pseudocontact shifts of these resonances, being altered by the nature of the charged groups on the surface of the protein. It is not clear from the present data whether this part of the alteration is due to minor conformation perturbations at the haem centre or whether it results solely from an electrostatic effect. It is worth noting here that Koppenol (14) has calculated that modification of single lysine residues can change the direction of the electric dipole potential of ferricytochrome c by angles of up to 45° from that in the native protein. The binding of the negatively charged surface of CCP or of polyglutamate or the modification of all the lysines in cytochrome c would also be expected to alter the electric dipole, which presumably interacts with the one unpaired electron of ferricytochrome c. The observations described above are an indication of the contribution which n.m.r. can make to the study of protein - protein interactions, particularly those involving cytochrome c.

5.3. Maleyl cytochrome c

In Section 5.2. it is stated that an extra pair of doublets, which are spin coupled, are found in the spectrum of maleyl ferrocytochrome c compared to that of ferrocytochrome c, at pH 7 and 57°C. Fig. 5.6. shows the aromatic regions of the spectrum of maleyl ferrocytochrome c at 37°C, 57°C, and 77°C at pH 6.6. At 37°C the spectrum is identical to that of ferrocytochrome c (except for a few small shifts, Section 5.2.), while at 57°C the coupled doublets have appeared and at 77°C the spectrum is that of a random coil protein. At 77°C the 38 one proton doublets of the maleyl group have collapsed to 2 nineteen proton doublets, and the rest of the aromatic spectral region resembles the superimposed spectra of four

Fig. 5.6.



The aromatic regions of the ^{1}H n.m.r. spectra of maleyl ferrocytochrome c at pH 7 and at 37°C, 57°C and 77°C
A pair of coupled doublets which are not seen in the spectrum of ferrocytochrome c under the same conditions are marked in the spectrum at 57°C.

tyrosines, four phenylalanines, one tryptophan and two histidine as free amino acids. When maleyl ferrocytochrome c is studied at intermediate temperatures the ^1H n.m.r. spectra show that the pair of doublets increase in intensity from zero at 37°C to two proton intensity each at 67°C, while the rest of the spectrum still resembles that of ferrocytochrome c. The doublets are at the random coil positions(or primary positions) of the resonances of a tyrosine residue and exhibit spin decoupling typical of a tyrosine. The midpoint of the transition from the structured to random coil spectrum occurs at ca. 70°C, and the resonances of the two forms of the protein are in slow exchange. No cross saturation effects between resonances in the folded and unfolded states could be detected indicating that even at the elevated temperature the rate of unfolding is slower than the time of the n.m.r. experiment (ca. 0.3 seconds).

The very sharp transition indicates that the transition between folded and unfolded forms of maleyl ferrocytochrome c is cooperative. When the temperature of the sample is reduced to 57°C, after being at 77°C for half an hour, the spectrum obtained is a superposition of the native and random coil spectra at about 50% intensity each. When the sample is cooled and a spectrum is recorded three days later at 57°C the spectrum is completely native and is identical to the spectrum obtained before the sample was heated to 77°C (Fig. 5.6.). Thus there is a hysteresis for the folding/unfolding process of maleyl ferrocytochrome with the increase/decrease of temperature.

The assignments of resonances due to Tyr 74 and Tyr 97+ in ferrocytochrome c has already been briefly decribed in Chapter 3 (Table 3.1.). The resonances of Tyr 97+ are only observed at 27°C; at 37°C they are in intermediate exchange, and thus at temperatures between 37°C and 67°C

+, the resonances of Tyr 48 are now also assigned.

only resonances from Tyr 74 are seen in the spectrum of ferrocytochrome c. On the basis of this the extra pair of tyrosine doublets in the spectrum of maleyl ferrocytochrome c must arise from either Tyr 48, Tyr 67 or Tyr 97, the remaining tyrosine residues. The resonances are unlikely to arise from Tyr 67, because there is a no weakening of the iron sulphur bond observed by following the Met 80 resonance in the spectra until the sharp transition at 70°C and hence the haem crevice containing Tyr 67 remains intact until this temperature. Even if some unspecified loosening of the haem crevice structure occurs that is not seen in the spectra but were to cause the Tyr 67 resonances to appear in the spectrum they would be expected to recieve a large shift from the ring current field of the haem group (of a similar magnitude to the resonances of Leu 67 in $(Leu)^{67}$, $(Hse)^{65}$ ferrocytochrome c, see Section 4.2.5.), whereas the observed resonances are at the random coil positions of tyrosine resonances. Thus, these resonances are assigned to either Tyr 48 or Tyr 97, and their appearance just prior to the temperature denaturation step indicate that the structure of either the hydrophobic domain containing Tyr 48 or that containing Tyr 97 is perturbed by a general loosening of the structure of the protein. This effect is not observed for the denaturation of ferrocytochrome c by chemical agents, as described in Chapter 7.

There are two main theories to explain the hysteresis in the unfolding and folding of proteins, and these are reviewed in Chapter 7. The experimental evidence suggests the proline isomerisation model (15). In the unfolding of maleyl ferrocytochrome c there is a sharp transition at 70°C involving the loss of Met 80 as sixth ligand to the iron and the gain of solvent access to the haem group, which exhibits hysteresis. In Chapters 6 and 7 much evidence from this work and from other authors

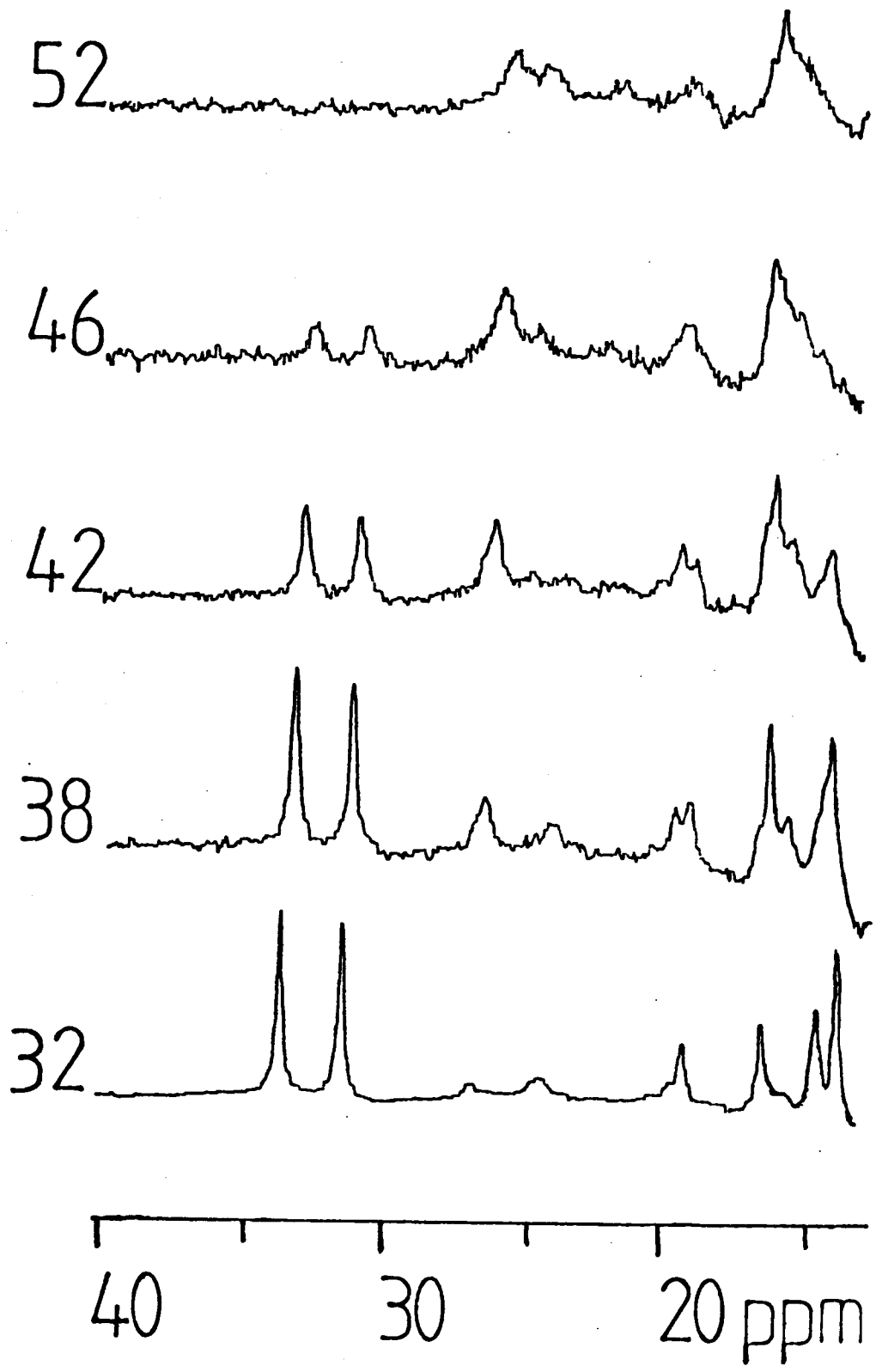
is presented, which indicates that the religation to Met 80 is the last step in the refolding of cytochrome c. In horse maleyl cytochrome c there are prolines at positions 30, 44, 71 and 76. Pro 71 and 76 are conserved in all eukaryotic cytochromes c that have been sequenced (16), and lie in an αhelix 71 - 76 that is close to the haem group in the native structure (17) and is close to Met 80 in sequence. The configurations of these two prolines are critical to the folding of this αhelix and hence to it folding into the haem crevice correctly to enable Met 80 to coordinate the iron atom succesfully. Thus, it is proposed that the hysteresis observed for the thermal unfolding/refolding of maleyl ferrocytochrome c results from a slow folding phase that depends upon the isomerisation of Pro 71 or Pro 76 or both to the correct (native) configuration as its rate determining step. This agrees with Stellwagen's (23) work that shows that the isomerisation of Pro 44 has no effect on the kinetics of folding of cytochrome c. This is summarised in the following equilibrium:

$$\text{Native} \underset{\text{Met 80 on}}{\overset{\text{Met 80 off}}{\rightleftharpoons}} U_f \underset{\text{Pro 71/76 isomerisation}}{\rightleftharpoons} U_s$$

where U_f and U_s are fast and slow refolding forms of maleyl ferrocytochrome c, respectively.

The thermal denaturation was also studied for maleyl ferricytochrome c. Fig. 5.7. shows the downfield hyperfine shifted region of the spectrum of maleyl ferricytochrome c at temperatures between 32°C and 58°C, at pH 7 and unbuffered in ^2H_2O. It can be seen that the native haem methyls at 34.4 ppm and 32.1 ppm reduce in intensity and broaden, and haem methyl resonances from an intermediate form at 26.6 ppm and 19.5 ppm arise in the spectrum up to 52°C and these then loose intensity

Fig. 5.7.



The downfield hyperfine shifted region of the spectra of maleyl ferricytochrome c at pH 7 and temperatures between 32°C and 58°C, unbuffered in $^{2}H_{2}O$.

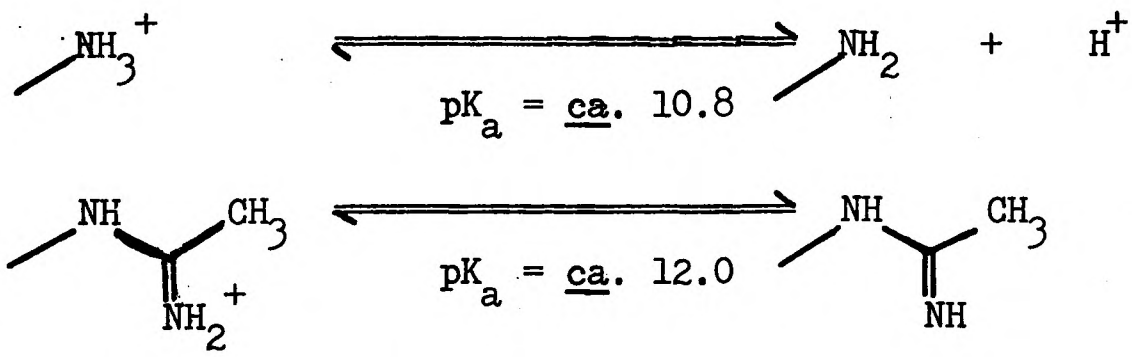
giving rise to a final high spin species. The native and intermediate resonances are of equal intensity at 42°C, and this is the denaturation temperature under these conditions; note, that the transition is much less cooperative than in the reduced protein. The intermediate is low spin, but its haem methyl resonances do not possess the same chemical shifts (23.8 ppm and 21.3 ppm) as those of state IV ferricytochrome c which is observed as an intermediate in the chemical denaturation of ferricytochrome c (Chapter 7). However Table 5.2. shows that the 'native haem methyl' resonances in maleylated ferricytochrome c can be shifted up to 1.5 ppm, and it is likely that similar shifts could occur to the haem methyls in any other state (e.g. in state IV). It is not, therefore, possible to characterise the nature of the low spin intermediate state in the unfolding of maleylated ferricytochrome c from this data.

Maleyl ferricytochrome c has also been studied by Schejter who showed that at room temperature in a 2×10^{-6}M solution, the protein was denatured and lacked a 695 nm. band (9). The addition of salt or lowering of the temperature restored the sample to a native configuration that exhibited the 695 nm. band. The efficacy of salts in restoring the 695 nm. band was found to be : $NaCl < Na_2SO_4 < MgCl_2$; which indicated that there is a dependence not only on the ionic strength, but also on the concentration and charge of the cation. Despite this complication the results of Schejter can be shown to be compatible with those reported above in the following way. Assuming that at pH 7 maleyl ferricytochrome c has a net charge of -28 units, a simple calculation shows that for 2×10^{-6}M maleyl ferrocytochrome c at 20°C the maximum ionic strength (from both the protein and from added salt) required to restore 90% of the

695 nm. band is 10^{-3}M (9). In the ^{1}H n.m.r. experiments unbuffered solutions of 5×10^{-3}M maleyl ferricytochrome c were used, and the ionic strength of these solutions from the protein is ca. 2M. Thus the high ionic strength in the n.m.r. sample gives rise to the elevated denaturation temperature as compared with the absorption spectra samples (9). The denaturation temperature is 42°C for the n.m.r. sample and at 20°C the protein in this sample is in the fully native state.

5.4. The pH titrations of N^{ε}-acetimidylated cytochrome c

The acetimidylation of lysine residues increases their average primary pK_a value (that in the isolated amino acid, or in a random coil protein) from ca. 10.8 to ca. 12.0 (18):



The relevant ionisations are shown above. Thus it is expected that any conformational changes due to the ionisation of lysine residues in cytochrome c will be expected at higher pH values in N -acetimidylated cytochrome c. For this reason a pH titration was carried out on both the oxidised and reduced proteins between pH 7 and pH 12.

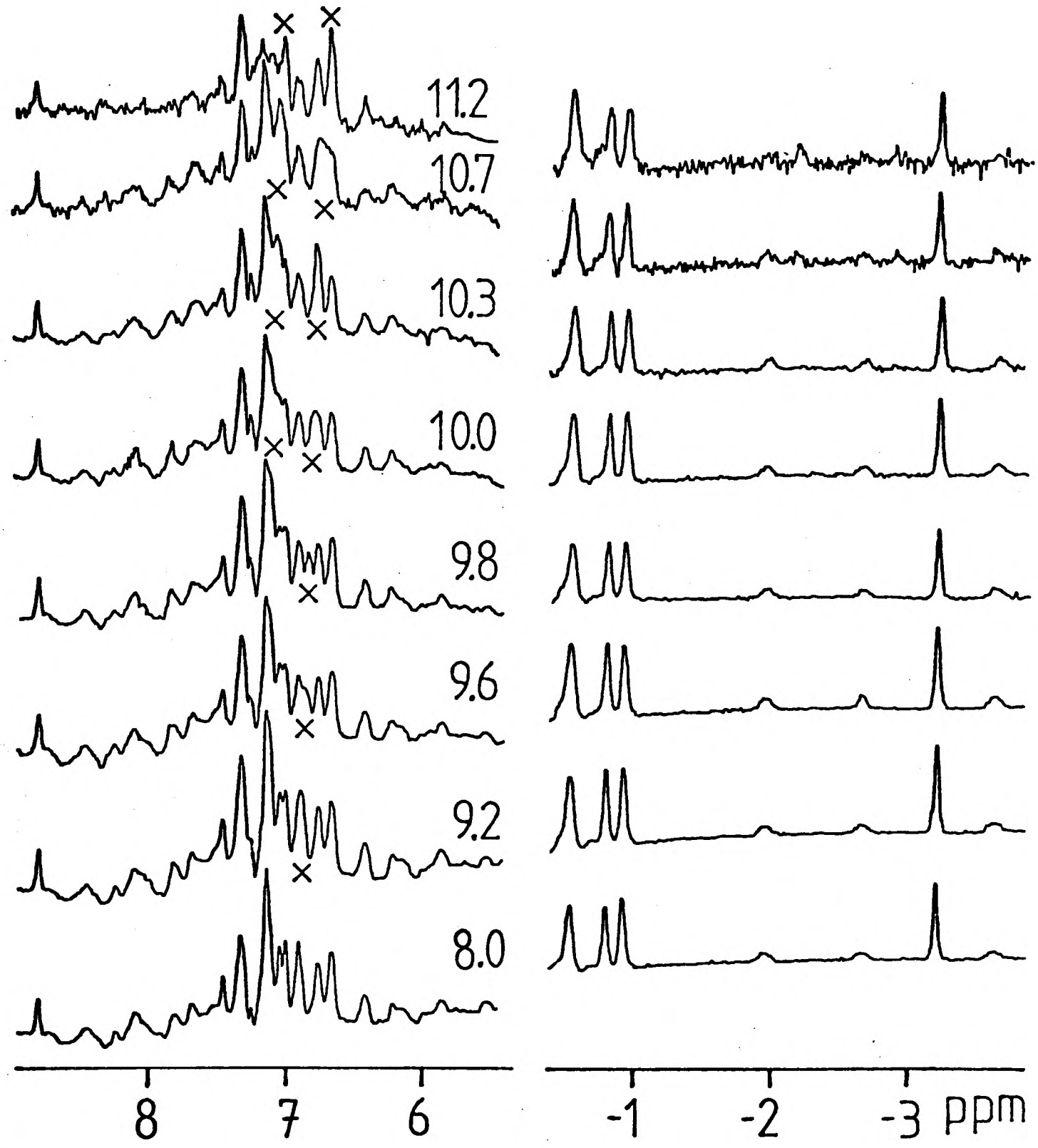
The pH titration of N^{ε}-acetimidylated ferricytochrome c yielded very little useful data and will only be discussed briefly. Ferricytochrome c undergoes an alkaline pK at 9.2 from its native low spin state III to another low spin species, state IV (Section 2.3.), and the transition is characterised by the loss of the native 695 nm. band from

the absorption spectrum. N^{ε}-acetimidylated ferricytochrome c undergoes a similar transition to a low spin species with a pK_a of 9.5 (18). There is much discussion of the nature of the sixth ligand in the high pH form (state IV) of ferricytochrome c (Section 2.3.) and some authors propose a lysine residue and probably Lys 79. If this is so, and N - acetimidylated ferricytochrome c follows a similar mechanism (i.e. an N^{ε}-acetimidylated lysine and probably that at position 79 is the sixth ligand in the high pH form) then we might expect to observe an extra methyl resonance at high pH for N^{ε}-acetimidylated ferricytochrome c from the methyl of a N^{ε}-acetimidylated lysine ligand recieving a large pseudocontact and contact shift (i.e. an analogous resonance to that from the methyl of Met 80 ($M^{*}1$) in native ferricytochrome c). The pH titration by ^{1}H n.m.r. for native and N^{ε}-acetimidylated ferricytochrome c was carried out at 27°C, and the pK_a values described above for the loss of the 695 band were confirmed by those values for the loss of the Met 80 methyl resonance. However, no extra methyl resonance was found in the case of the latter protein at pH values above the pK_a. This does not provide evidence either for or against the ligation of a N^{ε}-acetimidylated lysine, as the change of state at the pK may drastically alter contact and pseudocontact shifts expected for a group ligated to the paramagnetic low spin haem and the resonance may be obscured in the main region of resonances between 10 ppm and 0 ppm.

Fig. 5.8. shows the aromatic region and upfield methyl region of the spectra of N^{ε}-acetimidylated ferrocytochrome c at pH values between 8 and 11.25, and at 27°C. The following points are to be noted:

a) The methyl resonance of Met 80, the haem axial ligand, M1 at -3.28 ppm is unaffected between pH 8 and pH 11.2; thus the overall structure

Fig. 5.8.



The aromatic (left) and upfield methyl (right) regions of the ^1H n.m.r. spectra of N -acetimidylated ferrocytochrome c at 27°C and various pH values between 8.0 and 11.25. The pH values of the individual spectra are marked on the diagram. Two resonances are marked with a cross in the aromatic region which are observed to shift with increasing pH (see text).

of the protein is unaffected during this pH range.

b) The resonance at -0.26 ppm, M4, is due to the δCH_3 of Ile 57, and does not shift between pH 8 and pH 11.2. The corresponding resonance in native ferrocytochrome c has a pH dependent shift during this range of pH (20, and Section 3.6.1.). This result shows that the modification of all 19 lysine residues in ferrocytochrome c eliminates the pH dependence in alkaline solutions of Ile 57; and that this dependence is, thus, due to a lysine residue ionising, presumably in the proximity of Ile 57. Further experiments are described in Section 3.6.4., that limit the lysine residue responsible down to one of two out of the total of nineteen in the protein. This point is not discussed further here.

c) At elevated pH new resonances appear in the aromatic part of the spectrum. These are marked with a cross in Fig. 5.8. It can be seen that both the newly observed resonances shift upfield with increasing pH. The resonance that starts under a resonance at ca. 6.9 ppm at pH 8 and titrates to under a resonance at ca. 6.6 ppm has a pK_a of ca. 10.2. Note that the meta protons of a random coil tyrosine residue titrate from ca. 6.8 ppm to ca. 6.5 ppm with increasing pH with a pK_a of ca. 10.8 (20). The protein is not stable enough at these pHs for spin decoupling experiments on this resonance, but the only other resonance that suffers a large shift in this pH range is that which is at ca. 7.0 ppm at pH 11.2. These are the two resonances marked with a cross, and it is assumed that they arise from the same tyrosine residue.

Thus between pH 8.0 and pH 11.2 N^{ε}-acetimidylated ferrocytochrome c remains in its native conformation and the only significant new effects over native ferrocytochrome c are the loss of the Ile 57 pH dependence and the titration of one tyrosine residue. Moore (21) notes that some

new aromatic resonances arise in the spectrum of native ferrocytochrome c at ca. pH 12.8 and 27°C. It is not certain that it is the same resonances appearing in both native and modified ferrocytochromes c, and note that they appear at different pH values, 12.8 and 9.2 respectively. Firstly, we will assume that they appear from the same residue and make the following tentative explanation and assignment. The resonances of Tyr 74 and Tyr 97 are assigned at this temperature for ferrocytochrome c (21,22) and corresponding resonances are found for N^{ε}-acetimidylated ferrocytochrome c at this temperature, while no resonances have been found for either Tyr 48 and Tyr 67 at any temperature for both species. ≠ The absence of these latter resonances is presumably due to the residues being in some hindered motion in their hydrophobic domains causing the resonances to be in intermediate exchange on the ^1H n.m.r. timescale. Tyr 67 is in the hydrophobic core of the molecules and if it ionises during the pH range of the titrations we would expect very large changes to occur to the spectrum. Thus the new resonances are assigned to Tyr 48, which is in a hydrophobic domain close to the surface of the proteins. The resonances appear at pH 9.2 for N^{ε}-acetimidylated ferrocytochrome c and at pH 12.8 for ferrocytochrome c; and this is most likely caused by an alteration to the surface structure and packing around Tyr 48 on modification, so that the motion of the residue is no longer hindered above pH 9 in its ionised state, while in the native protein the motion is hindered until ca. pH 12.8 although the tyrosine has ionised at a lower pH (the resonances that appear in ferrocytochrome c are seen at the chemical shift positions of the resonances that have ionised in N^{ε}-acetimidylated ferrocytochrome c).

Secondly, an alternative explanation that does not assume that the

resonances are from the same residue in ferrocytochrome c and in N^{ε}-acetimidylated ferrocytochrome c is as follows; the resonances that appear in N^{ε}-acetimidylated ferrocytochrome c are from Tyr 48 in its ionised state for the reasons given above, and they can only arise from the 'native' state II form as they are seen to arise in the spectrum while the rest of the spectrum is unaltered. However, the resonances that appear in ferrocytochrome c at pH 12.8 could be due to any tyrosine residues in state III ferrocytochrome c at their random coil positions, as the protein denatures at just above pH 12.8 and some state III form may be in the spectrum while state II predominates. It is not possible to distinguish between these two possibilities at present, but in both cases the resonances in N^{ε}-acetimidylated ferrocytochrome c are due to Tyr 48 and indicate that the region close to this residue is perturbed by the modification, as for both explanations Tyr 48 has an enhanced mobility in the modified ferrocytochrome c. Lys 39, Lys 53 and Lys 55 are close enough in the secondary structure to cause such a perturbation. Note, that the perturbation is not detected in the spectra at pH 7 (Section 5.2.) and is only apparent at the elevated pH values.

References: Chapter Five

1. Dickerson, R.E., and Timkovitch, R. (1975), In 'The Enzymes' (P.D.Boyer, editor), Third Edition, Vol XI, pp. 397 - 547.

2. Brautigan, D.L., Ferguson_Miller, S., Tarr, G.E., and Margoliash, E. (1978), J.Biol.Chem., 253, 140.

3. Ferguson-Miller, S., Brautigan, D.L., and Margoliash, E. (1978), J.Biol.Chem., 253, 149.

4. Osheroff, N., Borden, D., Koppenol, W.H., and Margoliash, E. (1980), J.Biol.Chem., 255, 1689.

5. Koppenol, W.H., Vroonland, C.A.J., and Braams, R. (1978), Biochim.Biophys.Acta., 503, 499.

6. Hettinger, T.P., and Harbury, H.A. (1964), Proc.Natl.Acad.Sci.,52, 1469.

7. Fanger, M.W., and Harbury, H.A. (1965), Biochemistry 4, 2541.

8. Offord, R.E., and Harris, D.E. (1977), Biochem.J., 161, 21.

9. Schejter, A., Zuckerman, M., and Aviram, I. (1979), J.Biol.Chem., 254, 7042.

10. Gupta, R.K., and Yonetani, T. (1973), Biochim.Biophys.Acta., 292, 502.

11. Taborsky, G., and McCollum,K. (1979), J.Biol.Chem., 254, 7069.

12. Pettigrew, G. (1978), FEBS Lett., 86, 14.

13. Boswell, A.P., McClune, G.J., Moore, G.R., Williams, R.J.P., Pettigrew, G.W., Inubishi,T., Yonetani, T., and Harris, D.E. (1980), Biochem.Soc.Trans., 8, 637.

14. Dr. W.H.Koppenol, personal communication.

15. Schmid, F.X., and Baldwin, R.L. (1979), J,Mol.Biol., 133, 285.

16. Dayhoff, M.O. (1968 - 1973), Atlas of Protein Sequence and Structure, National Biochemical Research Foundation, Silver Springs, Maryland, U.S.A.

17. Takano, T., and Dickerson, R.E. (1980), Proc.Natl.Acad.Sci.,

18. Harris, D.E. (1977), D.Phil. thesis, Oxford.

19. Moore, G.R., and Williams, R.J.P. (1980), Eur. J.Biochem., 103, 513.

20. Bundi, A., and Wuthrich, K. (1979), Biopolymers, 18, 285.

21. Moore, G.R. (1978), D.Phil. thesis, Oxford.

22. Wuthrich, K., and Keller, R.M. (1981), Biochim.Biophys.Acta., accepted for publication.

CHAPTER SIX

^{1}H n.m.r. studies of derivatives of cytochrome c with Met 80 replaced as sixth ligand to the haem group

6.1. Introduction

In Section 2.4. the structures and stabilities of state III ferricytochrome c and state II ferrocytochrome c, the native states of cytochrome c at neutral pH, are discussed. Both these states have an imidazyl (His 18) fifth ligand and a sulphuryl (Met 80) sixth ligand coordinating the haem group, around which the protein folds in a well defined conformation that differs only slightly between the two oxidation states (1,2,3). The work described in this chapter concerns states of cytochrome c in which the methionyl sixth ligand is replaced at neutral pH through a combination of the ~~midification~~ modification of Met 80 itself and/or the addition of the exodogeneous ligand CN^-. The main aim of the work is to investigate the role of the iron - sulphur (haem to methionyl ligand) bond in native cytochrome c and to characterise the effect of its loss on the rest of the protein structure.

The following points from the work of previous authors concerning the role of the iron - sulphur bond should be noted:

a) Ferrocytochrome c is more stable than ferricytochrome c to extremes of pH and temperature and to chemical denaturation (see, for example, 2). There are two main reasons for this; i) the iron - sulphur bond is much stronger in ferrocytochrome c than in ferricytochrome c, and ii) the most recent X-ray structure of ferricytochrome c and of ferrocytochrome c show that there is an increased polarity in the haem crevice of ferricytochrome c (3) (it is difficult to separate this factor from factor i), as it almost certainly contributes to the weakening of the

iron - sulphur bond in ferricytochrome c) and that the peptide chain is slightly different between the two molecules for residues 44 to 58.

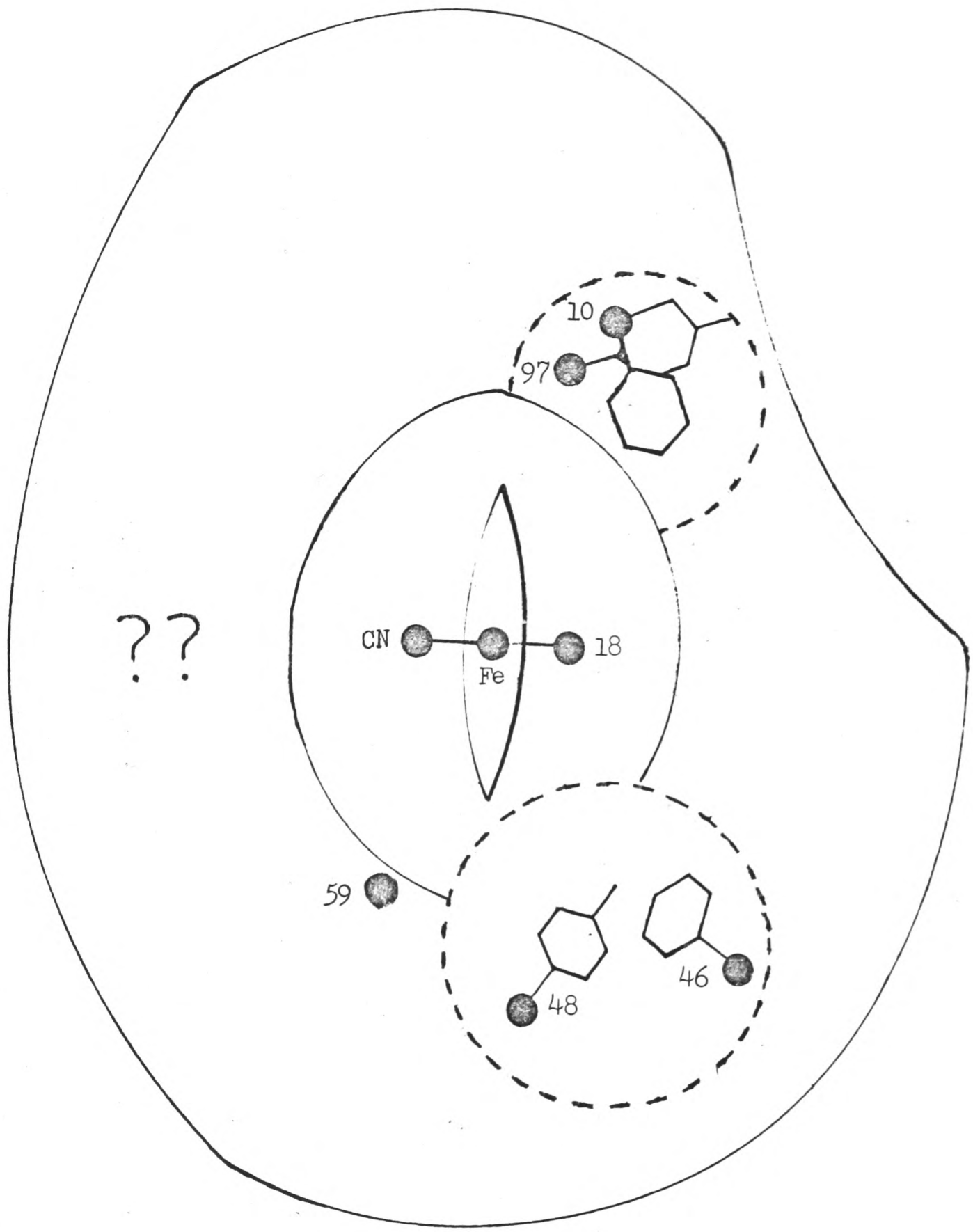
b) Tanuichi et al have shown that; i) porphyrin cytochrome c, in which the iron atom has been removed (i.e. the iron - methionyl and iron - histidyl cross linking bonds are missing in this protein), is folded into a compact and structured configuration around the empty porphyrin ring (4); while apo-cytochrome c, in which the porphyrin ring is also missing, is random coil (5). Thus the porphyrin ring itself is an important hydrophobic template for the folding of the protein.

ii) Fragments of cytochrome c obtained by a 'clean' proteolytic cleavage combined to form bimolecular non-covalent complexes with a native type structure (6) by an initial bimolecular process involving packing around the haem and the creation of the hydrophobic core of the complex (characterised by the quenching of the tryptophan flourescence of Trp 59) followed by a unimolecular process of reorganisation of the polypeptide chain and the coordination of Met 80 to the haem iron (characterised by the appearance of the 695 nm. band) (7).

The results show that the the formation of the iron - sulphur bond is the last step in the refolding processes of ferricytochrome c and ferrocytochrome c; and that in the case of ferricytochrome c the coordination of the sulphur atom causes little more than the final fine structure adjustment to the stability and strucure of the protein.

Fig. 6.1. shows a schematic diagram of a proposed penultimate state in the refolding of cytochrome c, and is on the same scale and from the same angle as Figs. 2.1. and 2.2., for the purposes of comparison. Met 80 is not coordinated, but the haem crevice on this side of the protein is maintained as a hydrophobic domain although the precise structure here is not known (as indicated by the question mark). Trp 59 is close to

Fig. 6.1.



A schematic diagram of a proposed penultimate state in the refolding of cytochrome c, based on the results of previous authors (see text), on the same scale and from the same angle as Figs. ?.1. and 2.2. The haem crevice is maintained without Met 80, and Trp 59 is close to/in the haem the haem crevice; two hydrophobic domains centred on Phe 10 and Phe 46 are a long way from Met 80 and are most likely preserved in their native configuration. The structure of the left hand side of the molecule is perturbed in an uncharacterised manner.

its native position, adjacent to the haem; and the haem structure and the peptide backbone are not drastically perturbed on the His 18 side of the molecule. The aim of the work of this chapter is to try and define this structure more thoroughly by studying the ^{1}H n.m.r. spectra of compounds, which may be used as models for it.

In this chapter the basic starting materials are horse cytochrome c and carboxymethylated horse cytochrome c, prepared by the method of Schejter (8). As discussed in Section 6.2. in further detail, the product of carboxymethylation by this method is monomeric, but consists of six electrophoretically distinct species (9), each of which contains carboxymethylated methionines at positions 65 and 80. The six distinct species presumably possess other modifications by side reactions at His 33 and at lysine residues. The product (henceforth, Cm-cytochrome c) was not further purified for this work as the heterogeneity of the samples did not appear to have caused measurable shifts on the ^{1}H n.m.r. spectra (see later). In this chapter the ^{1}H n.m.r. spectra of the following species are compared: Cm-ferricytochrome c-CN, ferricytochrome c-CN and ferricytochrome c; and Cm-ferrocytochrome c-CN, ferrocytochrome c-CN (an unstable species, half life $t_{\frac{1}{2}}$=2 minutes), and ferrocytochrome c.

6.2.1. The ^{1}H n.m.r. spectra of ferricytochrome c-CN and Cm-ferricytochrome c-CN : Introduction

The main aim of the work of this section will be to compare the spectra of ferricytochrome c-CN and Cm-ferricytochrome c-CN to that of ferricytochrome c, and so determine the effects caused by the loss of Met 80 as sixth ligand to the structure of the latter species. In Section 6.3. the corresponding comparison for the reduced species will

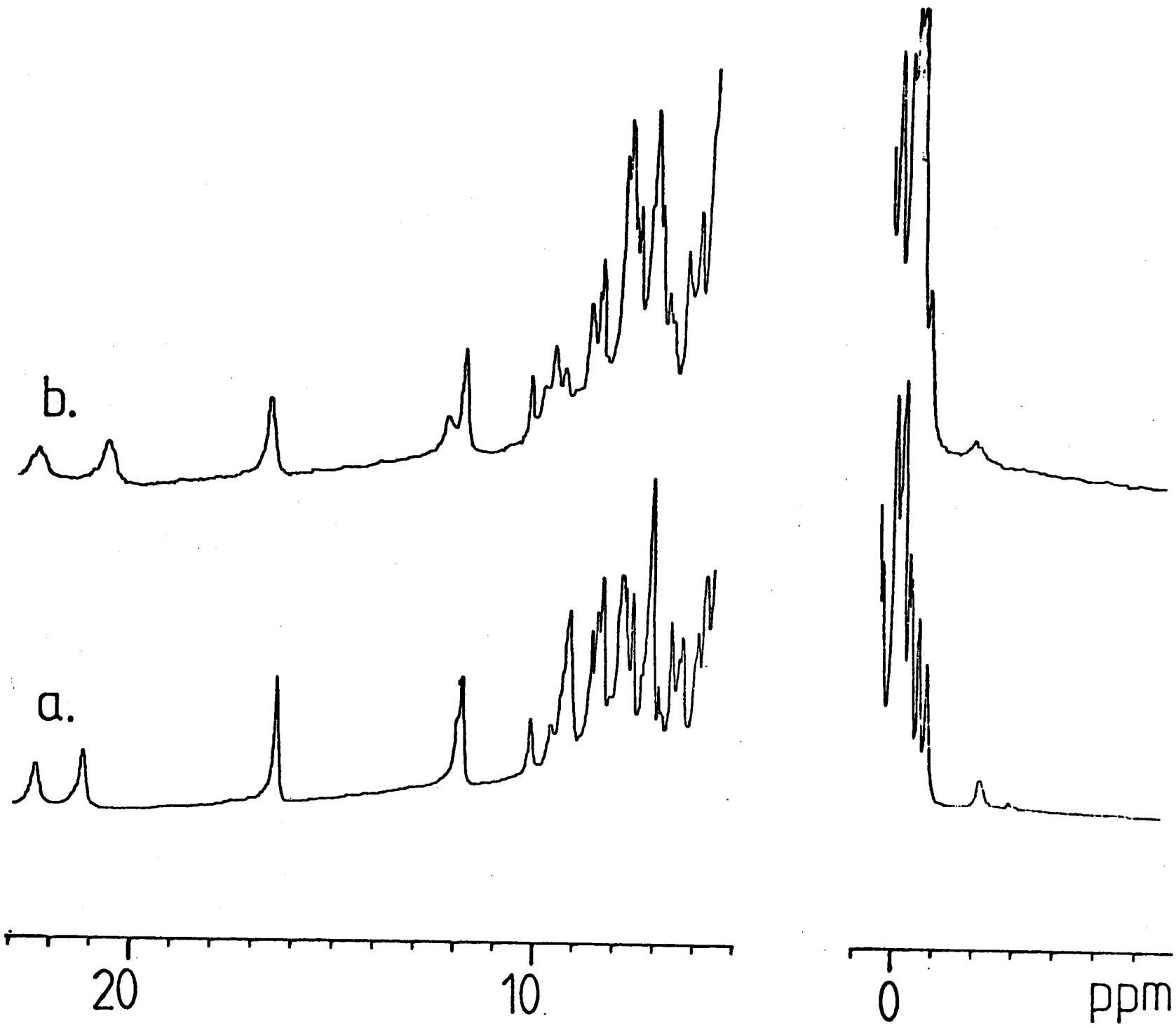
be given. An essential prerequisite of this work is the determination of the extent of the change induced to the structure of ferricytochrome c-CN by carboxymethylation of Met 65 and Met 80 (and other groups by minor side reactions), and it is to this that I first turn.

6.2.2. A brief comparison of the spectra of ferricytochrome c-CN and Cm-ferricytochrome c-CN

Fig. 6.2. shows the wide sweep width spectra of ferricytochrome c--CN and Cm-ferricytochrome c-CN at pH 7 and 27°C. It can be seen that the spectra are very similar although there are small chemical shift differences and the spectrum of the latter species is broader, though note that the broadening is selective and not uniform to the whole spectrum.

Cyanide possess a very strong ligand field effect and presumably binds to the haem of both species equally strongly giving rise to a low spin ferric ion ($S=\frac{1}{2}$) for both. Thus the differences in linewidth are not expected to be from variations in paramagnetic relaxation caused to the resonances by differences in the spin state of the ferric ions of the two species. This is borne out by the very similar contact and pseudocontact shifts that the haem resonances of the two species (resonances in the downfield parts of the spectra in Fig. 6.2.) suffer. The broadening is not due to polymerisation of the Cm-ferricytochrome c-CN sample, as it is not selective. Brunori et al (9) found that the product of carboxymethylation is monomeric, but consists of a number (5 or 6) of electrophoetically distinct species, that can be separated by isoelectric focusing. Furthermore, each of these species was found to be identical in the following experiments; a spectrophotometric pH titration between pH 6 and pH 9 in the Soret region of Cm-ferrocytochrome c (also followed by temperature jump experiments at 412 nm.

Fig. 6.2.



The wide sweep width ^{1}H n.m.r. spectra of a) ferricytochrome c-CN, and b) Cm-ferricytochrome c-CN, both at pH 7 and 27°C.

Table 6.1.

Chemical shift comparison of ferricytochrome c, ferricytochrome c-CN and Cm-ferricytochrome c-CN at pH 7 and 27°C

Ferricytochrome c			Ferricyto-chrome c-CN	Cm-ferricyto-chrome c-CN
Resonance designate and chemical shift			Chemical shift	Chemical shift
H*1	Haem methyl 8	35.0	22.7	22.7
H*2	Haem methyl 3	32.4	21.4	20.9
H*6	Haem methyl 5	10.0	16.4	16.6
H*8	Haem methyl 1	7.3	11.55	11.60
One proton peak		---	11.65	12.00
M*1	Met 80 CH_3	-24.8	2.1+	2.9 (Carboxymethyl Met 80)
M*19	Met 65 CH_3	1.94	2.1+	2.9 (Carboxymethyl Met 65)
M*20	N-acetyl	1.97	2.1+	2.1

+, see Section 6.2.3. for discussion and tentative ~~assignemnts~~ assignments of these resonances.

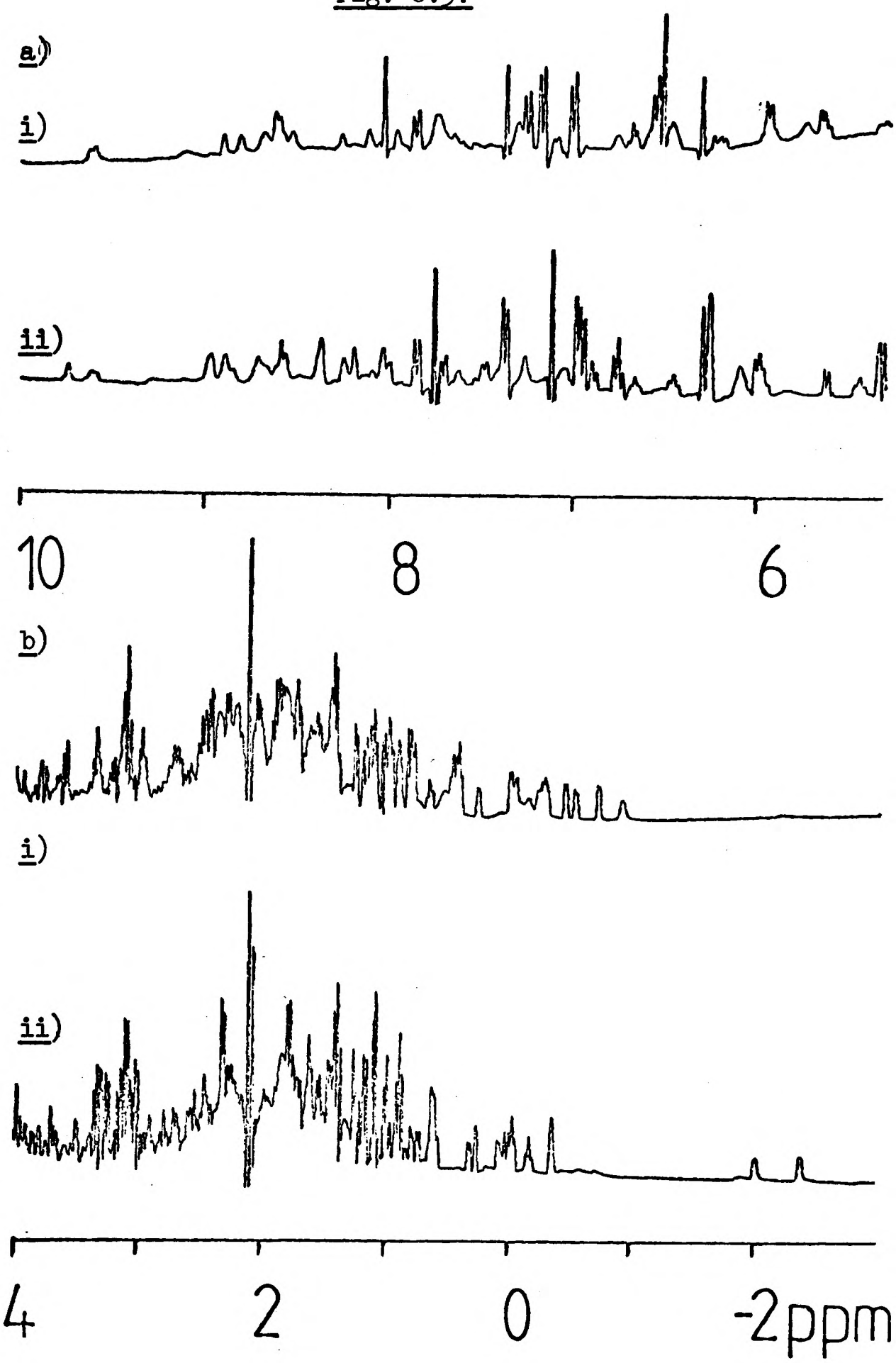
through the same pH range), the ligand binding (CN, CO, O_2, ethyl isocyanide) to Cm-ferrocytochrome c at neutral pH, and the kinetics of CO binding to the same as a function of pH (10). Thus the six species must be virtually identical and presumably differ only by the modification, by side reactions, of His 33 and single lysine residues. In Section 5.2. the effect to the haem resonances of the modification of lysine residues in ferricytochrome c is discussed and it is shown that small shifts can occur to the resonances probably due to a small alteration of the electronic structure of the haem on modification. The broad linewidths in the spectrum of Cm-ferricytochrome c-CN are almost certainly due to the superposition of the resonances of the six species contributing to the spectrum, which will not be at identical chemical shift positions for the six different species.

Table 6.1. shows a comparison of the chemical shifts of the haem methyl resonances and some other resonances for ferricytochrome c, ferricytochrome c-CN, and Cm-ferricytochrome c-CN (the main component of the resonances is given for this species). The results in the table (and from comparison of the spectra it is based upon, Fig. 6.2.) show that the spectra of ferricytochrome c-CN and Cm-ferricytochrome c-CN are very similar. The resonances of Met 80 in these compounds will be discussed in the next section. No further comparisons will be made and I will now concentrate on the spectrum of ferricytochrome c-CN, as it is better resolved due to sharper linewidths.

6.2.3. The spectrum of ferricytochrome c-CN

Fig. 6.3. shows the spectra of a) the aromatic region and b) the methyl region of ferricytochrome c and ferricytochrome c-CN at pH 7 and 37°C, for the purpose of comparison. Decoupling experiments were carried out according to the usual proceedures to provide first stage

Fig. 6.3.



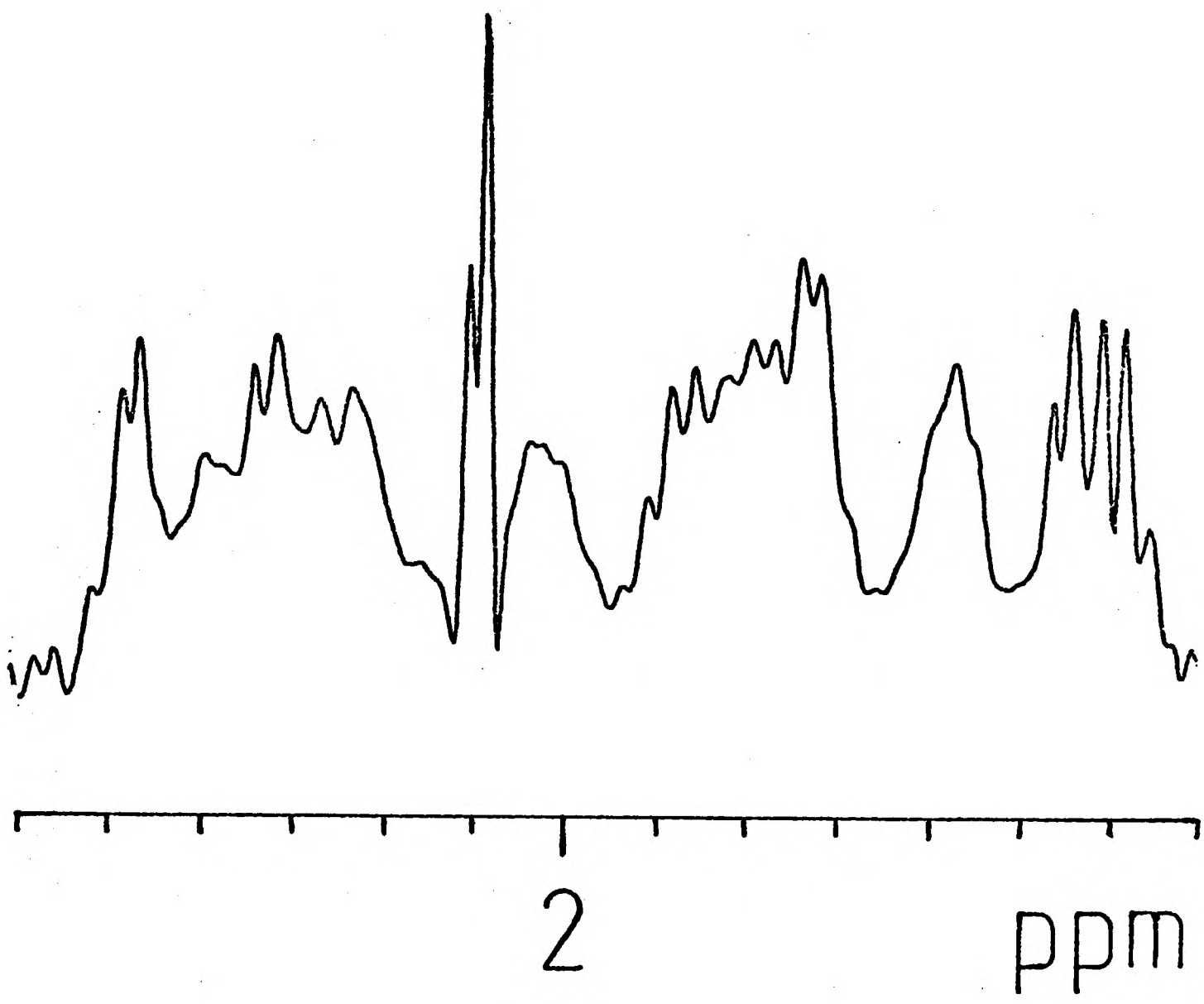
a) The aromatic region, and b) the aliphatic region of the ^1H n.m.r. spectra of i) ferricytochrome c-CN and ii) ferricytochrome c, both at pH 7 and 37°C.

assignments at 37°C and 47°C in the spectrum of ferricytochrome c-CN. Second stage assignments were made to sets of coupled resonances, where possible, and the results are tabulated, with the previous second stage assignments for ferricytochrome c, in Table 6.2. The following points are noted for these assignments:

i) The assignment to Met 80 is tentative and is based on the following arguments. Fig. 6.4. shows the region between 2.6 ppm and 1.3 ppm in the spectrum of ferricytochrome c-CN at pH 7 and 27°C. There are two sharp peaks at ca. 2.1 ppm which overlap and have an apparent intensity of ca. 1:2, and are due to a total of two or three methyl singlets. At higher temperatures these singlets overlap completely in one peak. An attempt was made to integrate the spectrum (with no resolution enhancement or line broadening functions applied to it) in Fig. 6.4. using the integration routine of the Aspect 2000 computer; however due to the mass of overlapping resonances in this region no unambiguous result could be obtained. The initial conclusion is that there may well be three methyl singlets at ca. 2.1 ppm, which would be from the N-acetyl group, Met 65 and Met 80. A Carr-Purcell A spectrum was recorded for ferricytochrome c-CN and no other singlet methyls were found, but this does not prove that the resonance of Met 80 is not to be found elsewhere in the spectrum if it recieves paramagnetic relaxation from the haem centre (if it were broadened it would not be expected to appear in the Carr-Purcell spectrum). However, it is of interest to note that while no Met 80 ^{13}C methyl resonance is found for ferricytochrome c (due to broadening by paramagnetic relaxation from the haem centre) the resonance is found in the ^{13}C n.m.r. spectrum of ferricytochrome c-CN (11).

Furthermore, a spectrum of lamprey ferricytochrome c-CN was recorded under the same conditions, as this protein contains Phe 65 instead of Met 65. Only one peak is seen at ca. 2.1 ppm between 27°C

Fig. 6.4.



The region between 2.6 ppm and 1.3 ppm in the ^{1}H n.m.r. spectrum of ferricytochrome c-CN at pH 7 and at 27°C. The two sharp peaks at ca. 2.1 ppm, which overlap and have an apparent intensity of 1:2, are due to a total of two or three methyl singlets; these resonances are discussed fully in the text.

and 47°C for this protein (which could not be integrated satisfactorily, re. the horse protein) and also no other singlet methyl resonances were found in a Carr-Purcell A spectrum. Thus the peak at ca. 2.1 ppm may well contain resonances from Met 80 and the N-acetyl group. On the basis of the above, the Met 80 methyl resonance is assigned tentatively to a third singlet at ca. 2.1 ppm in horse ferricytochrome c-CN and to a second singlet at ca. 2.1 ppm in lamprey ferricytochrome c-CN.

In horse Cm-ferricytochrome c-CN there is only one peak at ca. 2.1 ppm, due presumably to the N-acetyl group, but a further peak of singlet(s) is found at ca. 2.9 ppm and is not seen for ferricytochrome c-CN. This latter peak is assigned to carboxymethyl methionine 65 (and possibly, 80), see Table 6.1., and the shift due to carboxymethylation is in agreement to that obtained from model compound studies (0.79 ppm downfield) with N-acetyl methionine (see Section 4.2.1.). Clearly more work is required to confirm the assignments of Met 80 and carboxymethyl Met 80 in these compounds.

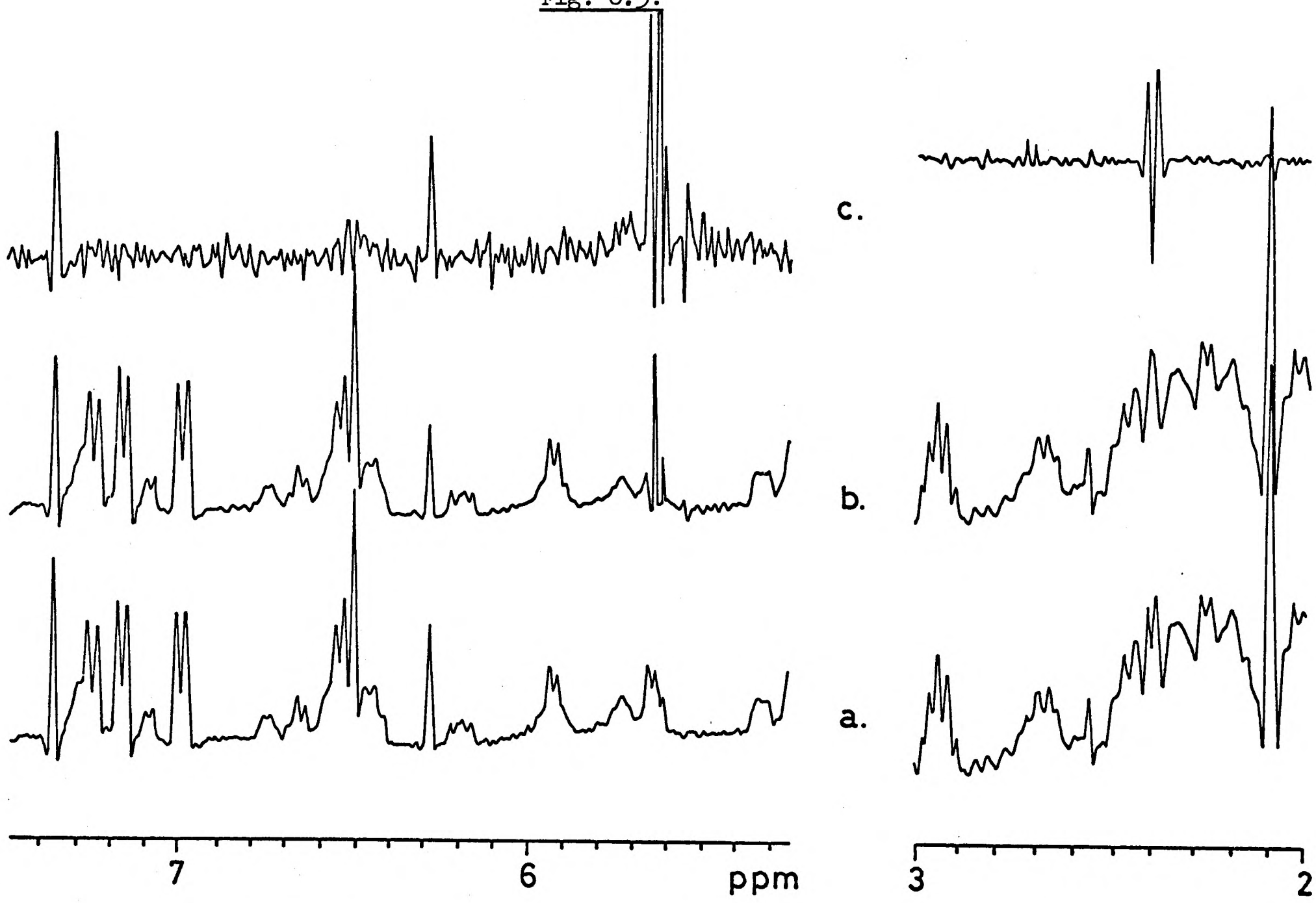
If the Met 80 resonance is found for ferricytochrome c-CN at ca. 2.1 ppm, as proposed above, then it is near to its random coil position and thus recieves little secondary shift from the paramagnetic haem group. This would mean that the coordination of CN^- instead of Met 80 causes a considerable displacement of Met 80 from the haem.

ii) A quartet resonance at 5.92 ppm is coupled to a doublet at 2.39 ppm; and in the spectrum of ferricytochrome c-CN recorded in 90% H_2O : 10% ^2H_2O the quartet exhibits further coupling, which suggests that it is due to the αCH of an alanine (the further coupling being due to the peptide NH protons). These resonances are not seen in a spectrum of pigeon ferricytochrome c-CN, which has Ser 15 instead of Ala 15 (horse), and are thus assigned to Ala 15. This is exactly analagous to the assignment in the native protein (12). There is a good correlation

between the secondary shifts to the resonances of Ala 15 in ferricytochrome c and ferricytochrome c-CN (see Table 6.3.). The spatial distribution of Ala 15 with respect to the haem group is fixed by the requirements of the peptide chain between the thioether - haem linkages at Cys 14 and Cys 17 to fit between pyrrole rings 1 and 2; consequently the displacement of Met 80 can not cause a conformational change to this part of the protein. Thus the difference in secondary shifts must solely (there are no other secondary shift centres near to Ala 15) be due to changes in the pseudocontact shift magnitude between the two molecules caused by changes at the coordination centre. The correlation shows that we may use the differences in pseudocontact shifts (based on secondary shifts) for other resonances as a guide to any conformational changes on the binding of CN^- to ferricytochrome c.

iii) Fig. 6.5. shows the spectra of ferricytochrome c-CN with time shared irradiation a) at 10 ppm, b) at 5.64 ppm and c) the difference between them. A decoupling effect to a methyl at 2.39 ppm from a one proton quartet at 5.64 ppm is demonstrated; but there are also further effects, the decrease of intensity of two sharp singlets at 7.36 ppm (due to His 26) and 6.29 ppm. In 90% H_2O : 10% ^2H_2O it is not clear whether there is further coupling to the quartet; this is analogous to the situation for C^*3 in ferricytochrome c. The assignment of these resonances by comparison with ferricytochrome c (12) is either to a threonine, most likely Thr 19 (αCH, 6.29 ppm; βCH, 5.64 ppm; γCH_3, 2.39 ppm) or to an alanine, most likely Ala 43 (αCH, 5.64 ppm; βCH_3, 2.39 ppm) and to a threonine, most likely Thr 19 (αCH, 6.29 ppm), see Table 6.2. ; in the former case the NOE from the quartet is observed in the same residue and in the latter case it is observed between two close residues, in

Fig. 6.5.



The spectra of ferricytochrome c-CN with time shared irradiation at a) 10 ppm, b) 5.64 ppm and c) the difference between them. See text for further details.

Table 6.3.

Secondary shift comparison for some resonances of ferricytochrome c and ferricytochrome c-CN at pH 7 and 37°C

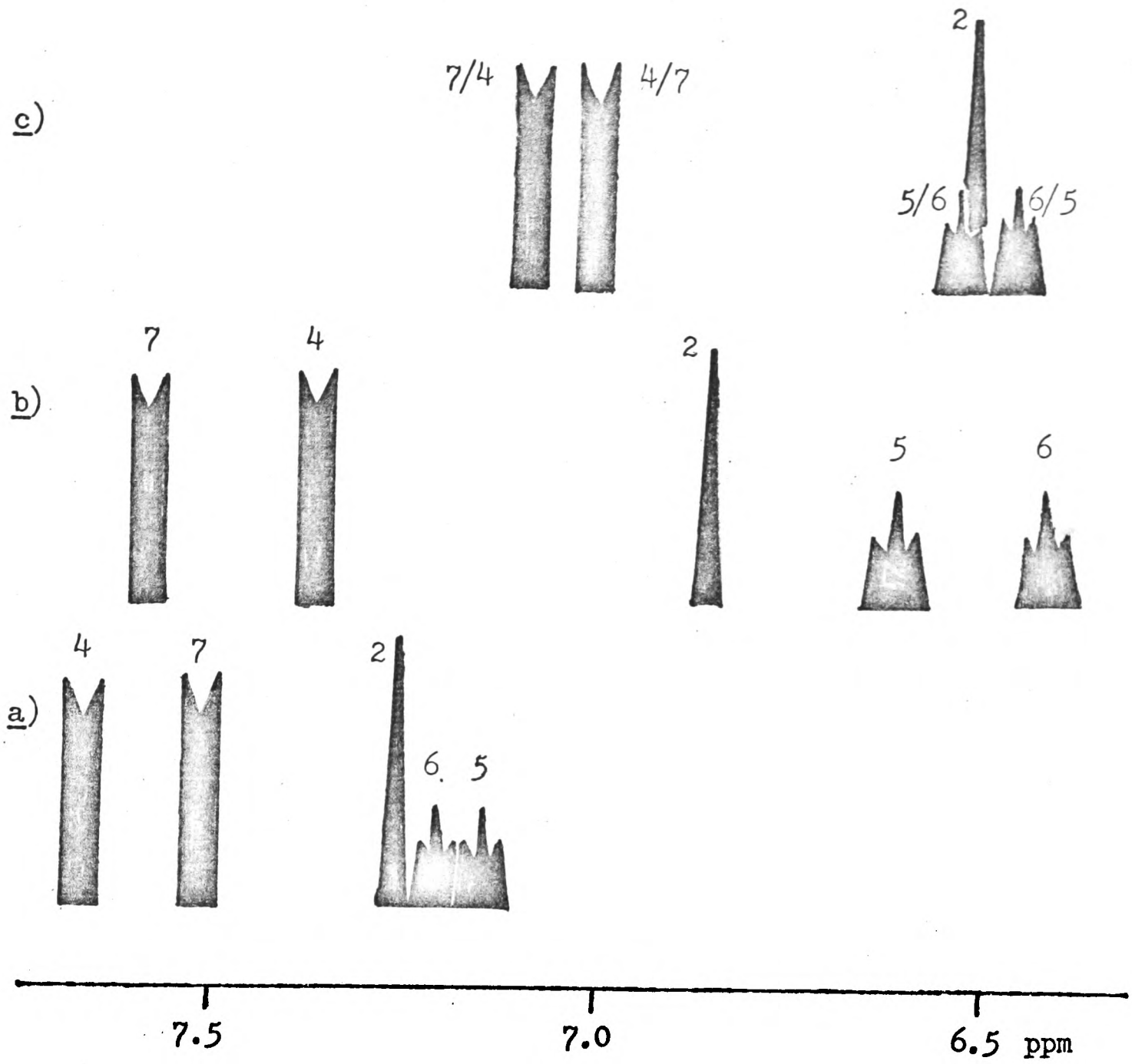
Assignment	Ferricytochrome c		Ferricytochrome c-CN	
	Chemical shift	Secondary shift	Chemical shift	Secondary shift
Ala 15 αCH	5.85	+1.50	5.92	+1.57
Ala 15 βCH_3	2.10	+0.70	2.25	+0.85
His 26 C-2	7.63	-0.49	7.36	-0.76
His 26 C-4	6.99	-0.15	6.50	-0.64
Thr 19 αCH(?)	6.15	+1.80	6.29	+1.94
Thr 19 βCH(?) Ala 43 αCH(?)	5.47	+1.25 +1.12	5.64	+1.42 +1.29
Thr 19 γCH_3(?) Ala 43 βCH_3(?)	2.07	+0.84 +0.67	2.39	+1.16 +0.99

both cases an NOE from the quartet to the close residue of His 26. Table 6.3. gives the secondary shift comparison for these resonances; it can be seen that there is only a small difference for the resonances of Thr 19 or of Ala 43 and Thr 19. The size of the difference of the shifts to His 26 indicate that residue undergoes a small conformational change upon the displacement of Met 80 by CN^-..

iv) Fig. 6.6. shows the resonance patterns of L-Trp, Trp 59 in ferricytochrome c and Trp 59 in ferricytochrome c-CN at 37°C. It can be seen that, although the shifts are different for the two proteins, the general upfield shifts for Trp 59 in ferricytochrome c are enhanced in ferricytochrome c-CN. The residue must lie in similar environments next to the haem group for both proteins. A similar result has been found by other authors; Myer et al (13) have found that the tryptophan floures-cence is more quenched in ferricytochrome c-CN than in ferricytochrome c; and Allerhand et al (14) have assigned the ^{13}C resonances of Trp 59 in ferricytochrome c and ferricytochrome c-CN by the partially relaxed Fourier Transform (PRFT) method and shown that the residue is in similar environments in both proteins. The result is important as it shows that Trp 59 is folded into the haem crevice despite Met 80 being displaced in ferricytochrome c; this complements the work of Tanuichi, outlined above (6,7). Presumably Trp 59 is folded into the haem crevice in the penultimate state in the folding of cytochrome c, and its hydrophobic interaction with the haem group is an important stabilising structural element for the haem crevice.

v) Resonances due to Phe 82 in ferricytochrome c are lost in the spectrum of ferricytochrome c-CN. No other resonances from phenylalanine residues in ferricytochrome c-CN, apart from Phe 36, are found. Phe 82 would be expected to be displaced by the displacement of Met 80, but the

Fig. 6.6.



The resonance patterns of a) L-Trp, and Trp 59 in b) ferricytochrome c, and c) ferricytochrome c-CN in the ^{1}H n.m.r. spectra at pH 7 and 37°C. The resonances of the benzenoid protons in ferricytochrome c-CN have not been uniquely assigned.

fact that no other resonances are found suggests that it is involved in some motion and is in intermediate exchange; it may well be folded back into the haem crevice. Similarly Phe 10 and Phe 46 presumambly maintain the motion that causes them to be in intermediate exchange in ferricytochrome c, and thus the alteration of the haem coordination (on the Met 80 side) has not disrupted the hydrophobic domains in which these residues lie on the His 18 side of the molecule (Fig. 2.1.).

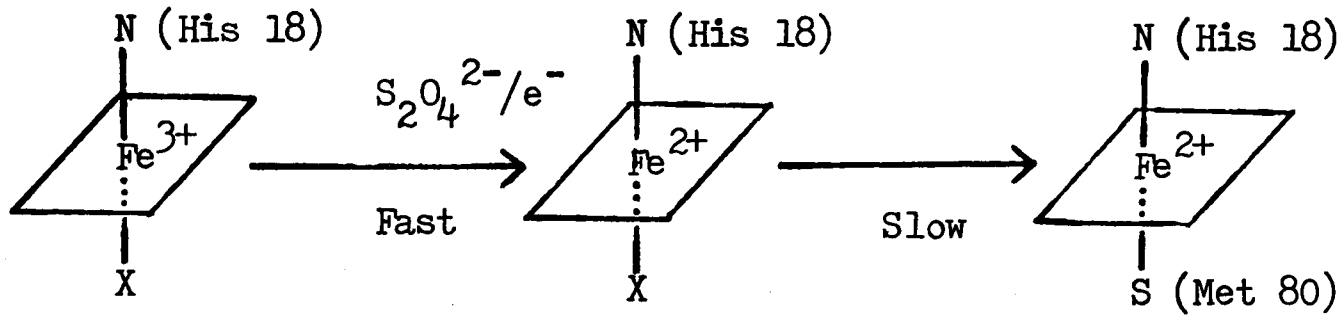
vi) Two sets of tyrosine resonances are found, one of which is tentatively assigned to Tyr 74, the only tyrosine for which resonances are seen in the native protein. There is a large shift to these resonances on the displacement of Met 80 by CN^-, which would be expected as Tyr 74 is close to Met 80. The further set could arise from Tyr 48, 67 or 97, but the regions of the protein close to Tyr 48 (Phe 46) and Tyr 97 (Phe 10) have already (in v) above) been shown to be unaltered by the modification and so they are unlikely to arise from these groups. The new resonances most likely arise from Tyr 67, which is very close to Met 80 in ferricytochrome c; note that the resonance of Leu 68 suffers a large shift, see vii) below, and is also close to Met 80 and Tyr 67.

vii) Examination of Fig. 6.3. shows that the upfield methyl region beyond 0 ppm is very different between the two proteins. Notably the resonances of the thioether methyl of Cys 14 and the methyl from Leu 68 have been drastically shifted at least 1.5 ppm downfield. As Leu 68 suffers a significant displacement from the haem with the coordination of CN^-, we would expect that much of the back of the molecule is altered; for example, the αhelix 61 to 69 is anchored to the haem crevive by Leu 68. The resonance of Met 65 does indeed suffer a shift between ferricytochrome c and ferricytochrome c-CN (see Table 6.2.), which might be expected if the helix is significantly perturbed.

The results show that there are a number of differences between the structures of ferricytochrome c and ferricytochrome c-CN (and Cm-ferricytochrome c-CN), which are not just in the region close to Met 80. On the whole the structure of the molecule is unaltered on the His 18 side of the molecule, though a change is found in the vicinity of His 26, and the haem crevice is maintained as hydrophobic as possible on both sides of the haem (e.g. Trp 59 is maintained in a native type position in the haem crevice, and the evidence suggests that Phe 82 may well fold back into the haem crevice) despite the disruption of the iron-methionyl bond.

6.3.1. The study of the unstable species ferrocytochrome c-CN : Introduction

Like native cytochrome c, cytochrome c-CN can exist in two oxidation states. By contrast, the redox potentials of the two species are different such that ferricytochrome c-CN is not reducible with ascorbate (15) and ferrocytochrome c-CN is a metastable species which spontaneously rearranges to native ferrocytochrome c with a half life at room temperature of ca. 2 minutes. Early ^{1}H n.m.r. work of Wuthrich (16) showed that when ferricytochrome c was reduced with dithionite the initial step is fast reduction followed by a slow step involving the dissociation of CN^- and its replacement by Met 80 as sixth ligand giving rise to the final product of native ferrocytochrome c. A similar pathway is observed for the reduction and rearrangement of ferricytochrome c-N_3, (ferricytochrome c-CN) and ferricytochrome c at pH 10 (state IV) when the reducing agent is a hydrated electron in pulse radiolysis experiments (17), and can be represented generally in the following scheme:



Cm-ferrocytochrome c will bind CN^- at pH 7 and form a stable complex (9), and as ferricytochrome c-CN is quite stable the rearrangement of ferrocytochrome c-CN must reflect the greater strength of the iron methionyl bond (relative to the iron - CN bond) in ferrocytochrome c over that in ferricytochrome c. In this and the next sections experiments are described that define the structure of ferrocytochrome c-CN and Cm-ferrocytochrome c-CN in order to try and establish what structural changes occur between these species and native ferrocytochrome c. The next section describes the recording of a high resolution ^1H n.m.r. spectrum of ferrocytochrome c-CN.

6.3.2. The recording of a spectrum of ferrocytochrome c-CN

A spectrum was obtained on the Oxford Enzyme Group Bruker WH270 spectrometer in the following manner: A 10^{-2}M sample of horse ferricytochrome c-CN was prepared in ^2H_2O at pH 7. The sample volume was 0.5 ml. and the remainder of the n.m.r. tube was flushed out under a stream of Argon and sealed under a 'subaseal". The sample was placed in the spectrometer magnet at 27°C, 'locked on' and 'shimmed up' and a spectrum was recorded to check the resolution. The sample was then removed from the magnet, and reduced with 0.05 ml. of a freshly prepared solution of 5×10^{-2}M sodium dithionite in ^2H_2O at pH 7 delivered to the sealed n.m.r. tube by a Hamilton microsyringe. The sample was immediately replaced in the magnet and allowed to equilibrate for thirty seconds from the moment of reduction, during which time the spectrometer was 'locked on'

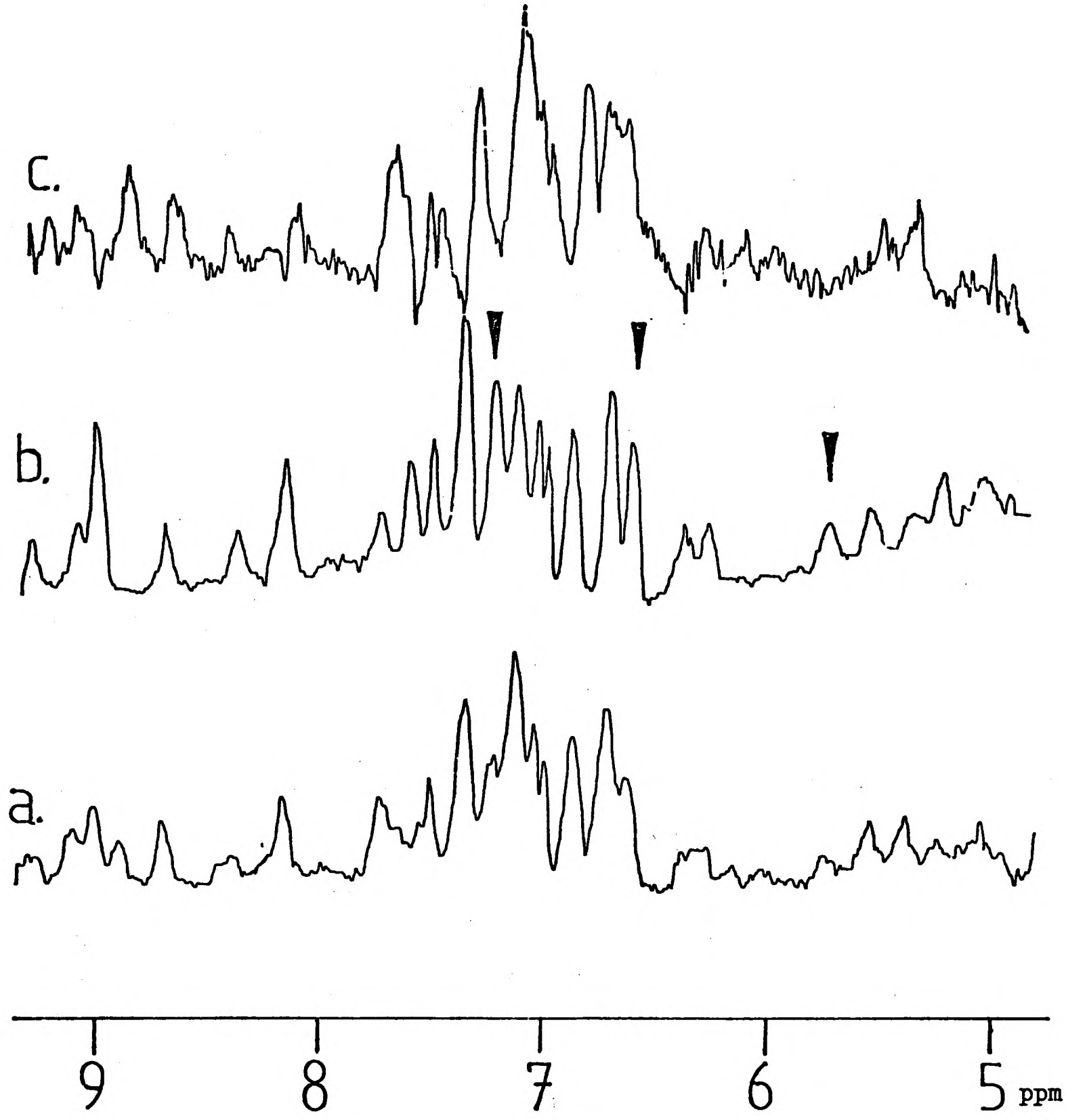
to the deuterium signal. It was found by practice attempts that no further 'shimming up' was required at this stage.

Immediately, after the equilibration period a series of ten spectra, each of 400 scans and requiring three minutes accumulation time, were recorded and stored on disc. The spectra collected were, as time proceeded, the superimposed spectra of increasing concentrations of ferrocytochrome c and decreasing concentrations of ferricytochrome c-CN. The ninth and tenth spectra in the series were of pure ferrocytochrome c, and the intensity of the Met 80 CH_3 resonance at -3.28 ppm in these spectra was used to calculate the relative concentrations of ferrocytochrome c and ferrocytochrome c-CN in the previous spectra. Such an analysis revealed that the first spectrum contained ca. 55% ferrocytochrome c and the second spectrum contained ca. 90% ferrocytochrome c. 55% of spectrum 10 (pure ferrocytochrome c) was subtracted from spectrum 1 (55% ferrocytochrome c, 45% ferrocytochrome c-CN) to yield a spectrum corresponding to 400 scans of 4.5×10^{-3}M ferrocytichrome c-CN at pH 7 and 27°C. The results of this proceedure are shown in Figs. 6.7., 6.8. and 6.9., which show respectively the aromatic, aliphatic and upfield methyl regions of a) spectrum 1, b) spectrum 10, and c) spectrum 1 - 0.55 spectrum 10, all at pH 7 and 27°C. These will be discussed in the following section. The experimental conditions outlined above were found to be optimum for the recording of a high resolution ^1H n.m.r. spectrum of ferricytochrome c-CN, though a variety of conditions were tried; at lower temperatures (e.g. 17°C and 7°C) the complex has a longer halflife, but the spectra are much broader.

6.3.3. The spectrum of ferrocytochrome c-CN

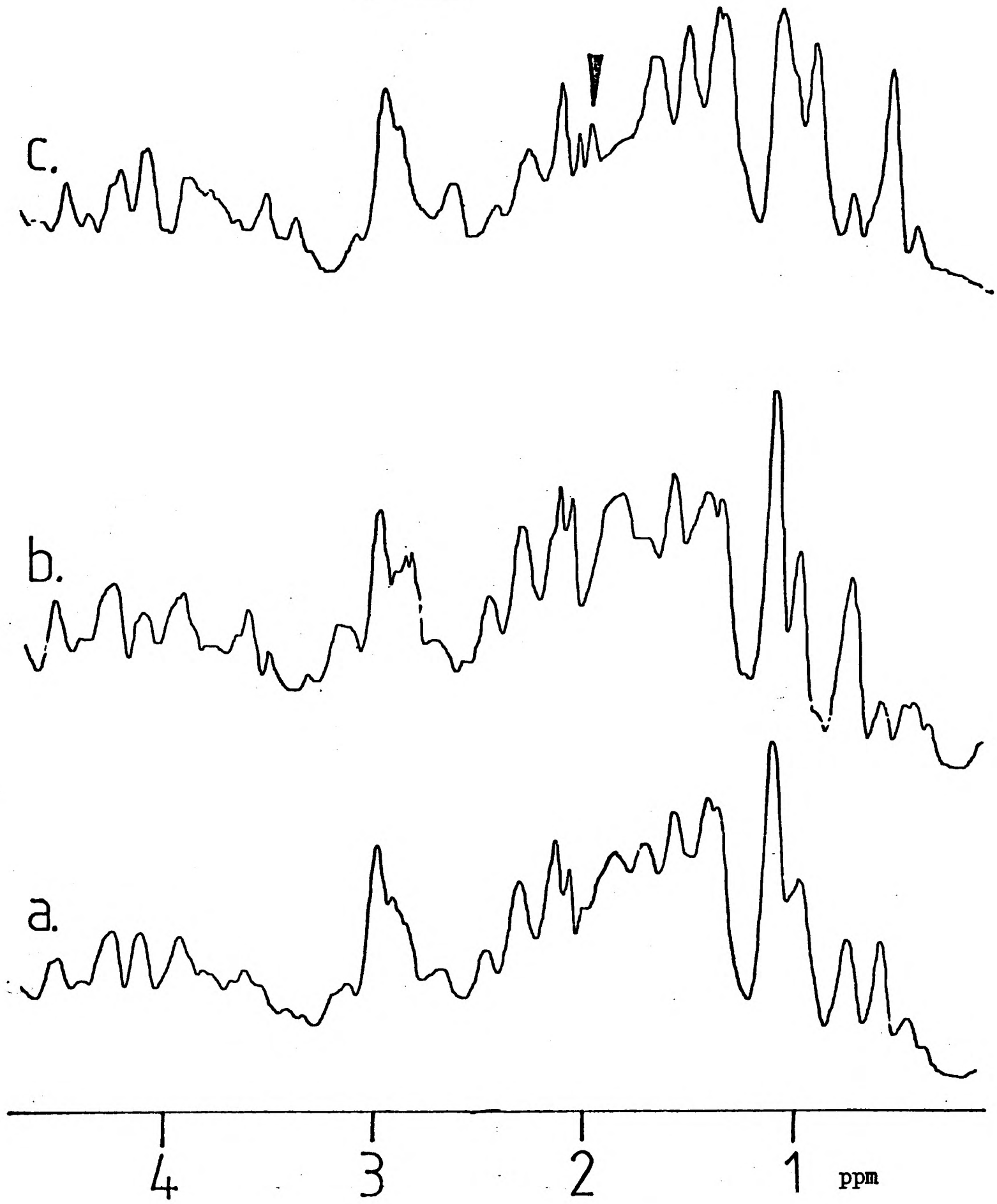
The spectra of ferrocytochrome c and ferrocytochrome c-CN may be readily compared as the proton resonances in each protein only suffer

Fig. 6.7.



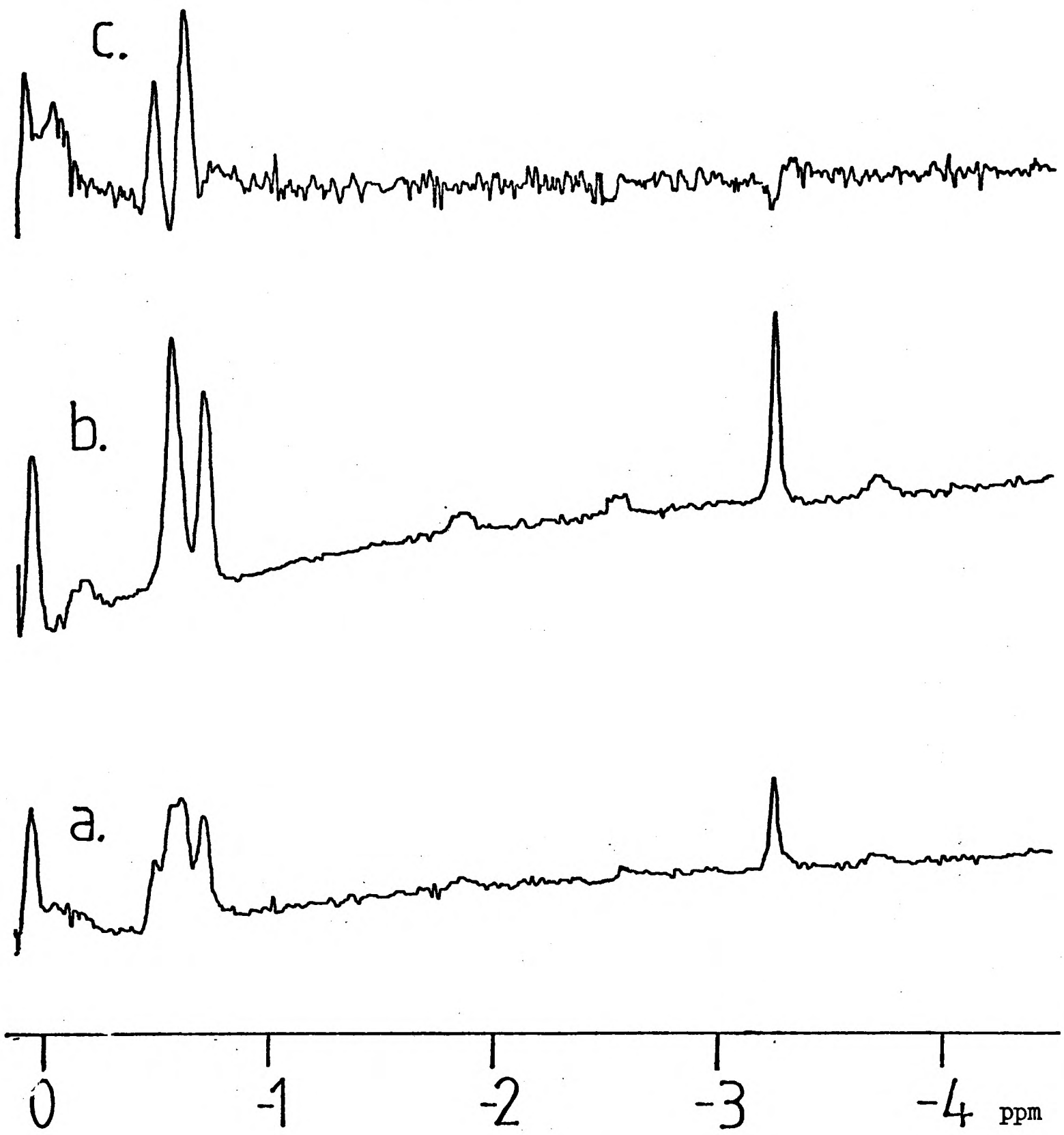
The aromatic regions of the ^{1}H n.m.r. spectra of a) 55% ferrocytochrome c, 45% ferrocytochrome c-CN, b) ferrocytochrome c, and c) ferrocytochrome c-CN (a) - 0.55 b)), all at pH 7 and 27°C. See text of Section 6.3.2. for details.

Fig. 6.8.



The aliphatic regions of the ^{1}H n.m.r. spectra of a) 55% ferrocytochrome c, 45% ferrocytochrome c-CN, b) ferrocytochrome c, and c) ferrocytochrome c-CN (a) - 0.55 b)), all at pH 7 and 27°C. See text of Section 6.3.2. for details.

Fig 6.9.



The upfield methyl regions of the ^{1}H n.m.r. spectra of a) 55% ferrocytochrome c, 45% ferrocytochrome c-CN, b) ferrocytochrome c , and c) ferrocytochrome c-CN (a) - 0.55 b)), all at pH 7 and 27°C. See text of Section 6.3.2. for details.

diamagnetic secondary shifts, though note that the ring current field of the haem group may be slightly different in each protein, as the different ligation will presumably perturb the electron density in the porphyrin ring. This is in contrast to the case of ferricytochrome c and ferricytochrome c-CN, where, as discussed in Section 6.2., there is also a paramagnetic secondary shift. An initial comparison of the spectra shows that they share a very similar general shape. The following specific points are made:

a) The CH_3 resonance of Met 80 is found at -3.28 ppm in ferrocytochrome c (see Fig. 6.9.b)), due to recieving a large upfield shift from the ring current of the haem group. No corresponding resonance is found in this region for ferrocytochrome c-CN. This result is expected as Met 80 is displaced by CN^- in this protein. However an extra singlet methyl resonance is found at 2.00 ppm (marked with a in Fig. 6.8.c)), and this resonance is not present in the spectrum of Cm-ferrocytochrome c-CN (Section 6.4.); it is assigned to Met 80. Note, that the resonance is shifted upfield from its primary position (2.13 ppm (18)) by 0.13 ppm, which indicates that the methionine methyl still lies in the upfield ring current shift cone of the haem ring although it is substantially displaced by CN^-.

b) Singlet methyl resonances from the N-acetyl group and Met 65 are found in the spectrum of ferrocytochrome c at 2.07 ppm and 2.11 ppm, respectively (see Section 4.2.1.). The spectrum of ferrocytochrome c--CN contains such resonances at 2.08 ppm and 2.12 ppm (Fig. 6.8.c)), the latter not being present in the spectrum of Cm-ferrocytochrome c -CN. Thus the resonances are assigned to the N-acetyl group and Met 65 respectively. These are virtually identical positions to those found for the resonances in the native protein and are close to their primary positions.

c) Between -0.4 ppm and -0.8 pppm in ferrocytochrome c (Fig. 6.9.b)) there are three methyl resonances assigned to the following groups; Ile 57 δCH_3 (-0.61 ppm), Leu 32 CH_3 (-0.60 ppm) and Leu 32 CH_3 (-0.76 ppm) (12). In the corresponding region of ferrocytochrome c-CN there are only two methyl resonances at -0.53 ppm and -0.67 ppm (Fig. 6.9.c)), and these are tentatively assigned to Leu 32, by comparison. Only small changes in secondary shift are observed to the resonances of Leu 32, which have large upfield ring current shifts from the haem group, when CN^- displaces Met 80; this either reflects a small conformational change to the methyl groups with respect to the haem or a small change in the ring current field of the haem or both. Thus the Leu 32 resonances provide an n.m.r. marker to the structural integrity of the His 18 side of the haem crevice, and show that little change has been caused by the change in cooordination.

d) The loss of the Ile 57 δCH_3 resonance from the spectral region discussed above reflects changes in the conformation of the group with respect to its sources of upfield ring current shifts, namely Tyr 74 and Trp 59. The major change is most likely a displacement of Tyr 74 which is close to sequence to Met 80 and would be expected to suffer an effect with the change of coordination. Note, that in native cytochrome c the change in oxidation state and hence the strength of the iron - sulphur bond is transmitted to the surface of the protein at Ile 57 and Tyr 74 and detected by the sensitive characteristics of the Ile 57 resonance.

e) There are several differences between the aromatic spectral regions of the proteins (Fig. 6.7.). These will be examined further by spin decoupling experiments on Cm-ferrocytochrome c-CN in Section 6.4. Briefly, the resonances of Tyr 74 at 7.21 ppm and 6.61 ppm of ferro-

cytochrome c (marked with a ▼ in Fig 6.7.b)) are not seen in the spectrum of ferrocytochrome c-CN; this is consistent with paragraph d) above. One of the benzenoid proton resonances of Trp 59 that can be clearly resolved for ferrocytochrome c is that of the C-6 proton at 5.76 ppm (marked with a ▼ in Fig. 6.7.b)); the corresponding resonance can not be seen for ferrocytochrome c-CN (Fig. 6.7.c)).

f) The four meso protons of the haem group of ferrocytochrome c are assigned to individual resonances at 9.62 ppm, 9.62 ppm, 9.32 ppm and 9.03 ppm at 27°C (19); it is difficult to find the corresponding resonances for ferrocytochrome c-CN, but there are two sets of two nearly overlapping resonances at ca. 8.9 ppm and ca. 8.75 ppm (Fig 6.7.c)), which are likely candidates. This is confirmed by the assignments of resonances at 8.93 (2), 8.75 and 8.73 to the haem meso in Cm-ferrocytochrome c-CN (see Section 6.4.2.). These large differences in chemical shift may be due to conformational changes near to the haem group (e.g. the removal of the ring current centre of Tyr 67, on coordination of CN^-) and/or change to the ring current field and electron density distribution in the haem group. This will be discussed further with respect to the results for Cm-ferrocytochrome c-CN, see Section 6.4.2.

In summary, a sample of the metastable ferricytochrome c-CN and the product of its dissociation, native ferrocytochrome c, could be obtained by the reduction of a pure solution of ferricytochrome c-CN by dithionite. A spectrum was recorded from which the ferrocytochrome c component could be subtracted to yield a difference spectrum corresponding to ferrocytochrome c-CN. Comparison with that of ferrocytochrome c showed that ; i) groups on the Met 80 side of the haem group were altered (e.g. Tyr 74), and ii) some groups on the His 18 side of the haem group were largely unaltered (e.g. Leu 32).

Furthermore, ferrocytochrome c-CN is a good model for the penultimate state in the refolding pathway of ferrocytochrome c, which exists immediately prior to the coordination of Met 80. The results show that much of the hydrophobic core of the protein, particularly on the His 18 side of the molecule, is already in a 'native' type structure at this stage of the refolding pathway.

6.4.1. Studies of Cm-ferrocytochrome c-CN : Comparison with ferrocytochrome c-CN

Although a spectrum of ferrocytochrome c-CN can be obtained, no detailed experiments such as spin decoupling may be carried out because of the compounds short lifetime. However Cm-ferrocytochrome c-CN is stable and can be used for such experiments, if it may be shown to possess a similar tertiary structure to ferrocytochrome c-CN.

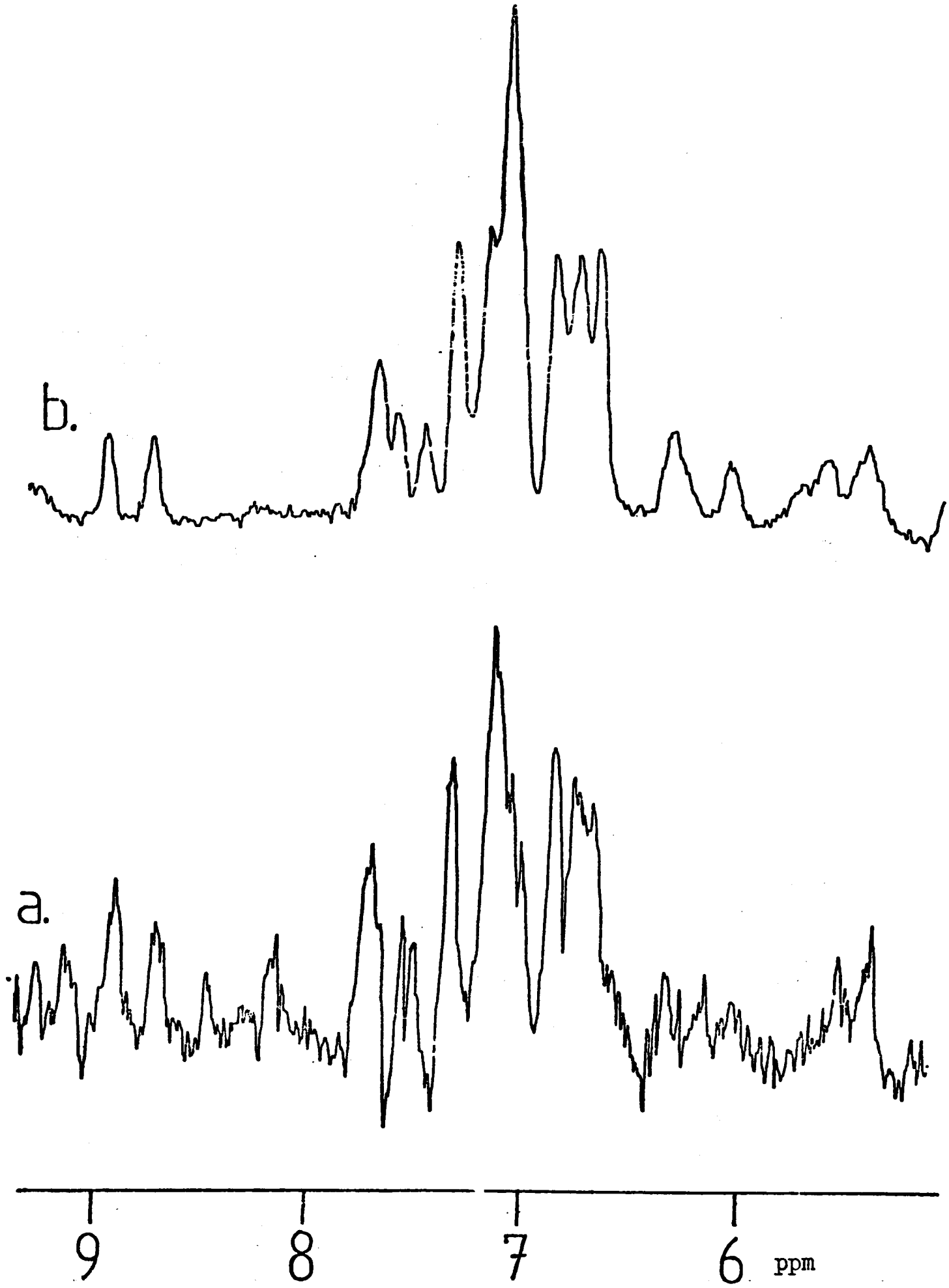
Figs.6.10 and 6.11 show two regions of a) the spectrum of ferrocytochrome c-CN and b) the spectrum of Cm-ferrocytochrome c-CN, both at pH 7 and 27°C. The spectra can be seen to be extremely similar and to possess the same general lineshape; the following points are noted:

a) Resonances at 2.00 ppm and 2.11 ppm in the spectrum of ferrocytochrome c-CN, that were assigned to Met 80 and Met 65 are not found in the spectrum of Cm-ferrocytochrome c-CN. These groups have been carboxymethylated in the latter protein.

b) The upfield methyl region (chemical shift 0.5 ppm), which provides a fine structure fingerprint of the tertiary structure of the protein due to the resolution of ring current shifted resonances is virtually identical for the two proteins. Note the resonances of Leu 32 are in identical positions at -0.53 ppm and -0.67 ppm for both species.

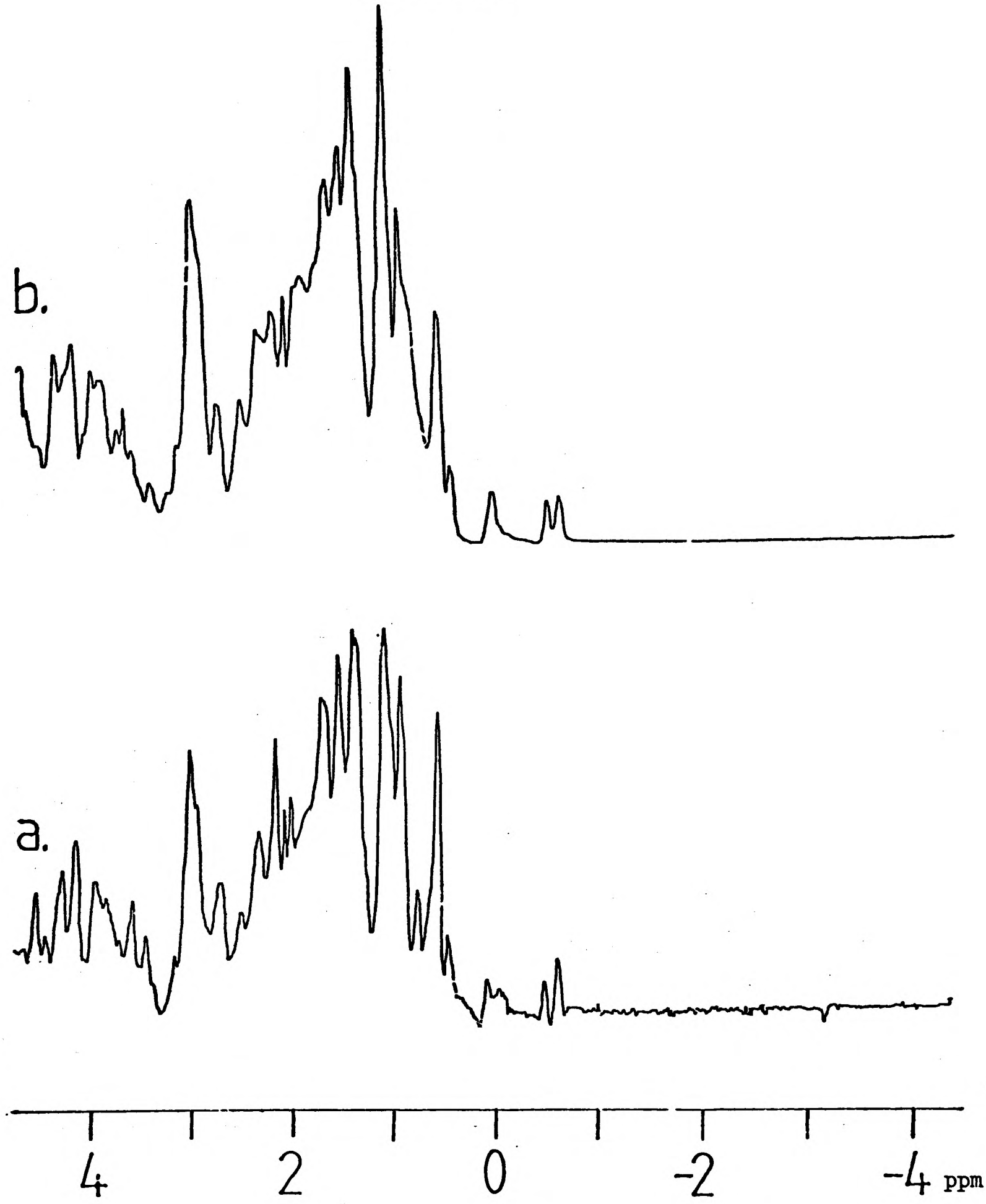
c) The aromatic regions (Fig. 6.10) are similar, but not identical.

Fig 6.10.



The aromatic regions of the ^{1}H n.m.r. spectra of a) ferrocytochrome c-CN and b) Cm-ferrocytochrome c-CN, both at pH 7 and 27°C.

Fig. 6.11.



The aliphatic and upfield methyl regions of the ^{1}H n.m.r. spectra of a) ferrocytochrome c-CN and b) Cm-ferrocytochrome c-CN, both at pH 7 and 27°C.

No resonances corresponding to those of Tyr 74 in ferrocytochrome c are found in the spectrum of either protein.

d) Resonances from the haem meso protons are found at the same positions as in ferrocytochrome c-CN.

e) The spectrum of Cm-ferrocytochrome c-CN is broader. This is due to Cm-cytochrome c being composed of six species.

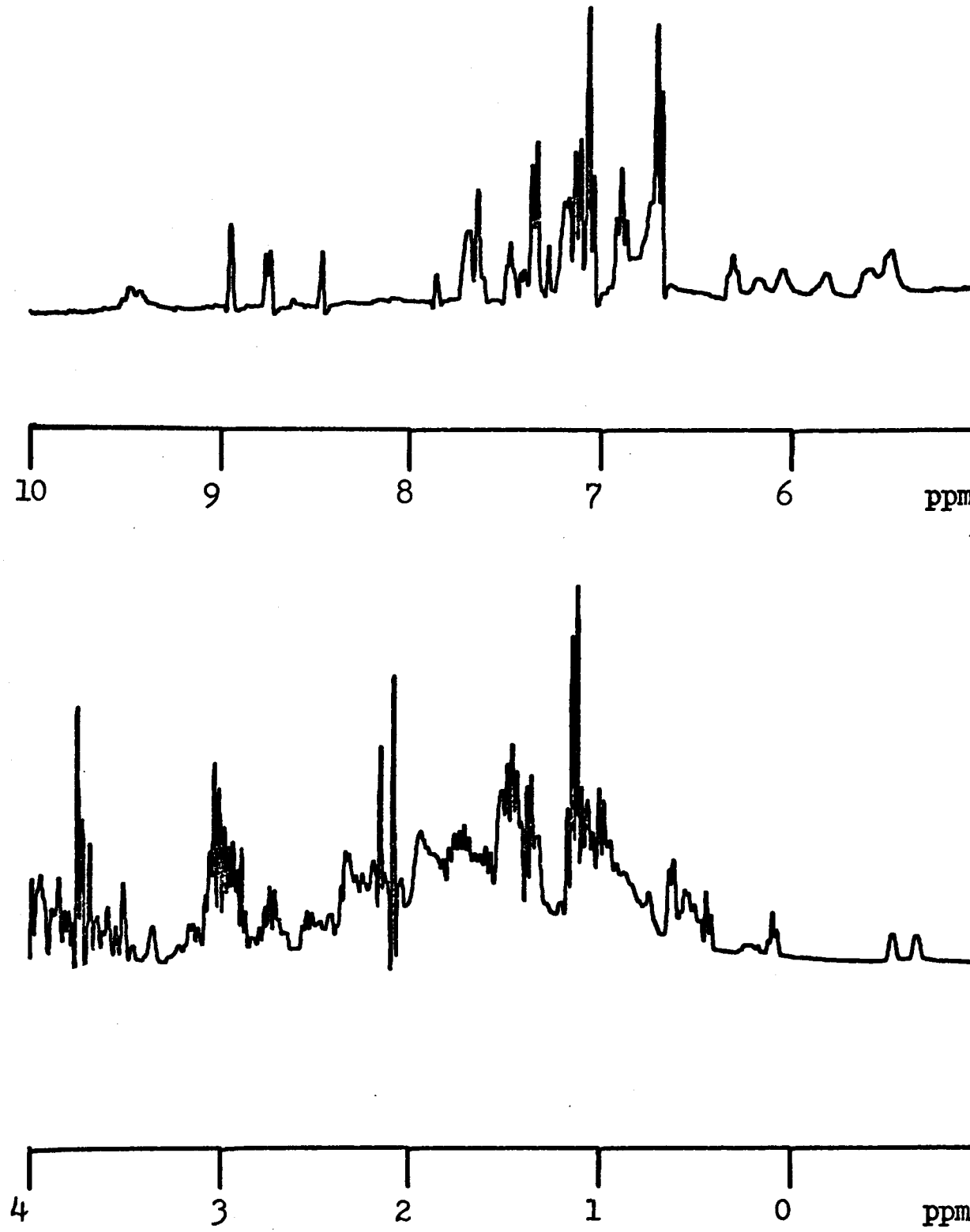
In summary the spectra of ferrocytochrome c-CN and Cm-ferrocytochrome c-CN are similar enough for the latter to be used as an n.m.r model of the former.

6.4.2. The spectrum of Cm-ferrocytochrome c-CN

Fig. 6.12. shows the spectrum of Cm-ferrocytochrome c-CN at pH 6.5 and 57°C. A number of assignments can be made by comparison to the spectra of ferrocytochrome c and ferrocytochrome c-CN; further assignments are now described based on NOE experiments and spin decoupling experiments.

NOE difference spectra were recorded with irradiation at the proton singlets at 8.93 ppm (2), 8.75 ppm, 8.73 ppm and 7.87 ppm. The first four singlets come from haem meso protons and a total of four methyl singlets were found in the three NOE difference spectra. These are assigned to the four haem methyl resonances for Cm-ferrocytochrome c-CN. No individual assignments may be made for the haem meso and methyl resonances without further NOE experiments. The methyl resonances are at positions that correspond fairly closely to their positions in the native protein, but as pointed out in Section 6.3.3. the meso proton resonances suffer fairly large shifts. This suggests that there may be a σ-bonding effect to the meso protons, which is insignificant to the methyl protons. The binding of CN^- would be expected to withdraw

Fig. 6.12.



The spectrum of Cm-ferrocytochrome c-CN at pH 6.5 and 57°C.

electron density from the ring and consequently electron density may be withdrawn from the meso protons by a σ bond effect. Such deshielding would be expected to lead to upfield shifts to the haem meso proton resonances as is observed. Irradiation of the latter singlet at 7.87 ppm gave rise to a ca. 20% NOE effect to a singlet at 7.60 ppm, and caused a much smaller NOE to the resonance at -0.53 ppm, which is due to Leu 32. The two singlets at 7.87 ppm and 7.60 ppm are assigned to a histidine on the basis of their large mutual NOE and to His 33 on the basis of the NOE to Leu 32. There are then three remaining singlets in the aromatic region at 7.58 ppm and 7.06 ppm (2) and these are assigned to His 26 and Trp 59. These and later results are tabulated in Table 6.4. The chemical shift positions of His 26 and His 33 can be seen to correlate well between the spectra of Cm-ferrocytochrome c-CN and ferrocytochrome c.

The spectrum is much altered from that of ferrocytochrome c in the region upfield of 0.5 ppm. As already described the resonances of Leu 32 (M2/M3) are maintained with only a small shift (see Section 6.3.3.c)); however the resonances from Met 80 (M1), Ile 57 (M4), His 18 (A32/A33), Leu 68 (M6) and the resonances M5 and M7 are all drastically perturbed. The shifts to Met 80 and His 18 are due to the change in coordination at the haem; the shift to His 18 must be downfield into the main methyl region and corresponds to a combination of any change in ring current field felt by His 18 and a bond effect through the ferrous ion from the CN^-. The change in the ring current shift of His 18 results either from a change in the ring current field of the haem itself (note, the shifts already observed to the haem mesos and methyls) and/or a lengthening of the ferrous - nitrogen (His 18) bond.

In order to try and locate the position of the resonance of the Ile 57 δCH_3 group, a Carr-Purcell A spectrum was recorded, in which

Table 64.

Chemical shift comparison of ferrocytochrome c and Cm-ferrocytochrome c-CN at pH 6.5 and 57°C

Assignment	Ferrocytochrome c Resonance designate	Ferrocytochrome c and chemical shift	Cm-ferrocytochrome c-CN Chemical shift	
Haem meso αCH	H3	9.32	8.93	no individual assignments see text
Haem meso βCH	H2	9.59	8.93	
Haem meso γCH	H1	9.62	8.75	
Haem meso δCH	H4	9.04	8.73	
Ringmethyl 1	H9	3.49	3.35	no individual assignments see text
Ringmethyl 3	H7	3.88	3.70	
Ringmethyl 5	H8	3.60	3.51	
Ringmethyl 8	H11	2.21	2.31	
His 18 C-2	A32	0.50	>0.5	
His 18 C-4	A33	0.13	>0.5	
His 26 C-2	A8	7.52	7.58	
His 26 C-4	A18	7.06	7.06	
His 33 C-2	A1	7.85	7.87	
His 33 C-4	A6	7.30	7.60	
Trp 59 C-2	A19	6.99	7.06	
Trp 59 C-4	A5	7.60		These results have to be confirmed see text
Trp 59 C-5	A24	6.70	6.70	
Trp 59 C-6	A30	5.74	5.46	
Trp 59 C-7	A16	7.09	7.06	
Phe 10 p-	A27	6.34	6.23	

Table 6.4.(cont.)

Phe 36 o-	A9	7.40	7.35
Phe 36 m-	A20	6.89	6.87
Phe 36 p-	A15	7.10	7.05
Tyr 74 o-	A11	7.22	7.02
Tyr 74 m-	A26	6.64	6.62
Tyr ??			7.06
Tyr ??			6.65
Tyr ???			7.18
Tyr ???			7.76
Leu 32 CH_3	M2	-0.76	-0.67
Leu 32 CH_3	M3	-0.60	-0.53
Ile 57 δ CH_3	M4	-0.43	> 0.5
Leu 68 CH_3	M6	0.38	> 0.5

There are no individual assignments for tyrosines see text

this resonance, a triplet, would be above the baseline, whereas the majority of other resonances in this region are doublets and appear below the baseline. No such triplet resonance could be found in the region upfield of 0.5 ppm, and the resonance at 0.5 ppm which appears to be a triplet is infact two overlapping doublets. This indicates that the region of the protein close to Ile 57 is disrupted in the modification. If the disruption concerned solely the loss of Tyr 74 as a ring current centre then the resonance of Ile 57 might be expected to be in the 0.2ppm to 0.0 ppm region of the spectrum as it is in $(Leu)^{74}$, $(Hse)^{65}$ ferrocytochrome c (Section 4.2.5.). As the resonance must be in the region downfield of 0.5 ppm, the results show that the orientation between Trp 59 and Ile 57 is altered as well as the displacement of Tyr 74 with the displacement of Met 80.

Decoupling experiments were performed in the aromatic region of the protein; and is some cases spin echo double resonance (SEDR) experiments were required to clearly ascertain the nature of the decoupling. However, the decoupling experiments are still incomplete, because the spectrum of Cm-ferrocytochrome c-CN is broader than that of ferrocytochrome c (due to six contributing species in the former) and some decoupling effects could not be clearly resolved. The results are tabulated in Table 6.4. and the following points are made:

a) A one proton triplet is found at 6.23 ppm and irradiation of it causes no other effects to the spectrum, so it is assigned to a phenylalanine residue in intermediate exchange. A similar resonance is found at 6.34 ppm in the native protein and is assigned to Phe 10 (Setion 3.4.). The resonance is assigned to Phe 10 by comparison; and the similar exchange properties and secondary shift between native and modified proteins for this resonance indicate that the static and dynamic region of this

region of the protein (on the His 18 side of the molecule) are not affected by the deligation of Met 80.

b) Resonances from a phenylalanine residue in fast exchange are found at 7.35 ppm, 6.87 ppm and 7.05 ppm; these resonances are assigned to Phe 36, by comparison to the native protein.

c) No other resonances due to phenylalanine residues have been found, although at least the para proton resonances (one proton triplets can be difficult to observe) of two other phenylanine residues must appear in the spectrum. Note, Phe 82 is affected considerably by the modification as resonances are not found for it; as in the case of ferricytochrome c -CN (Secton 6.2.3.) its resonances may be in intermediate exchange, which may result from the resonance being folded back into the haem crevice after the displacement of Met 80.

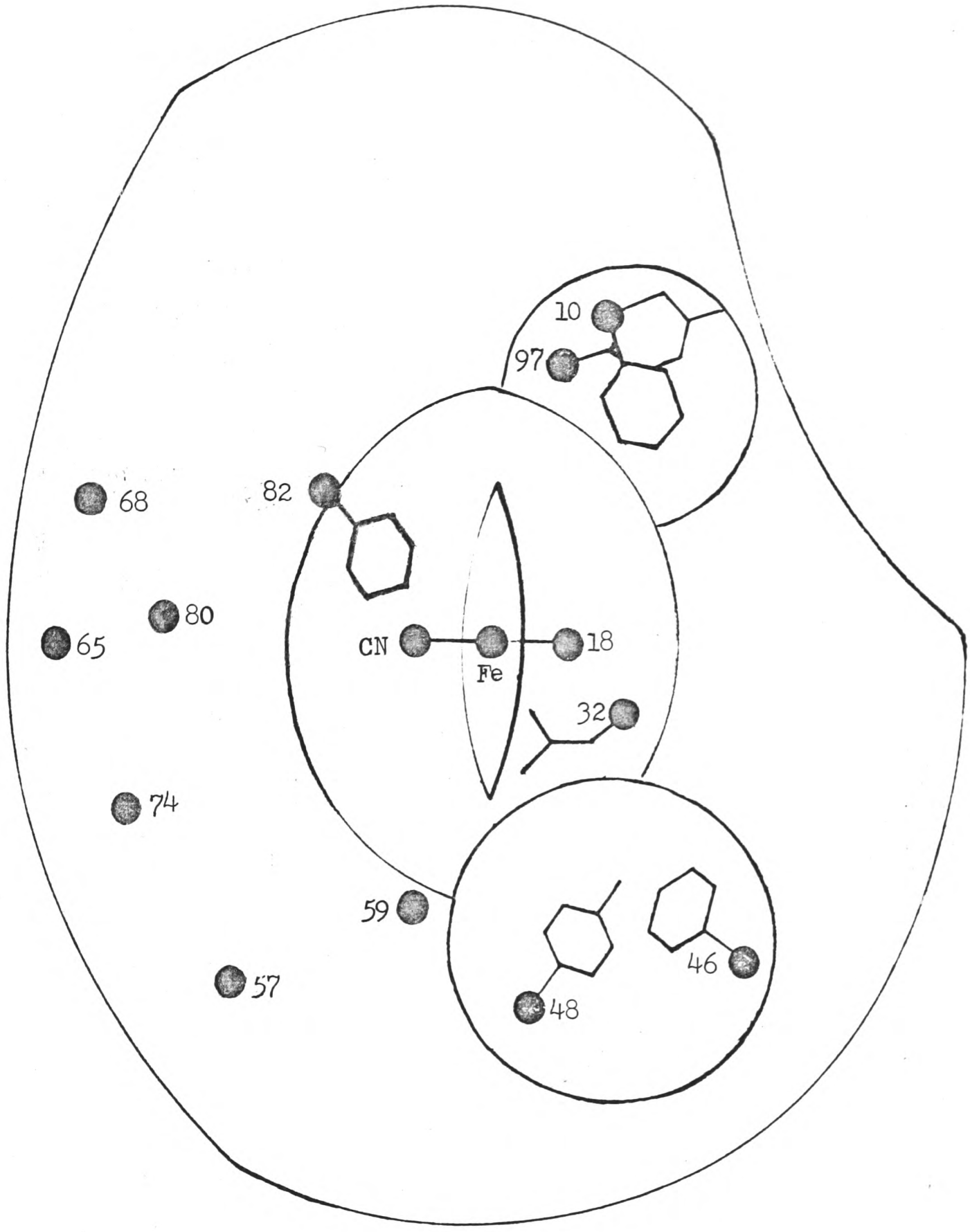
d) Three sets of coupled doublets are found, of which the set at 7.76 ppm and 7.18 ppm can be clearly seen by ordinary decoupling. Irradiation between 7.10 ppm and 7.00 ppm at 0.01 ppm intervals reveals that two independent doublets exist at 6.65 ppm and 6.62 ppm, which are decoupled by irradiation at 7.06 ppm and 7.02 ppm. Decoupling with SEDR shows that both the 7.06 ppm and 7.02 ppm resonances are doublets; this could not be observed by ordinary decoupling at 6.65 ppm and 6,62 ppm. Second stage assignments for these sets of resonances can not be easily made, but one set will almost certainly arise from Tyr 74 and further set will most likely arise from Tyr 67 displaced from the haem crevice.

e) No resonances have been unambiguously assigned to Trp 59 in Cm-ferro-cytochrome c-CN, except the C-2 proton resonance. A one proton triplet at 5.46 ppm is a likely candidate for the C-6 proton and irradiation at this resonace by SEDR causes effects at 6.70 ppm, consistent with a triplet decoupling to a doublet, and at 7.06 ppm, consistent with a

doublet decoupling to a singlet. However further effects such as back decoupling from 7.06 ppm or 6.70 ppm has not yet been detected. These resonances are tentatively assigned to Trp 59 (Table 6.4.), but further experiments are required to confirm this. If the assignment is correct these resonances are in similar positions to that in the native protein which suggests that Trp 59 is not drastically altered by the displacement of Met 80; this is the case found for ferricytochrome c-CN (Section 6.3.3).

In conclusion, the spectra of ferrocytochrome c-CN and Cm-ferrocytochrome c-CN bear resemblances to that of ferrocytochrome c, and parts of the structures of the molecules are not drastically altered. For example, the resonances of Leu 32, which provide a guide to the integrity of the His 18 side of the molecule suffer only insignificant shifts, and the region close to Phe 10 maintains its features of hindered dynamic motion. There are also large differences between the spectra particularly to resonances that are due to residues on the Met 80 side of the protein, for example, Ile 57, Leu 68, Tyr 74 and Phe 82. Phe 82 appears to fold back into the haem crevice as its resonances are not found, and are most likely in intermediate exchange. These conclusions are the same as those which were given from the comparison of the spectra of ferricytochrome c and ferricytochrome c-CN. Both ferricytochrome c-CN and Cm-ferrocytochrome c-CN may be considered as models for the penultimate state in the folding pathway of cytochrome c. Fig. 6.13. is a diagram based on Fig. 6.1. which illustrates the results of this chapter. These results provide a basis for further experiments into the penultimate state in the unfolding pathway of cytochrome c. If the major components of Cm-cytochrome c were to be isolated and characterised in n.m.r sized quantities further useful ^{1}H n.m.r. work could be carried out with pure Cm-ferrocytochromec c-CN

Fig. 6.13.



A schematic diagram of a proposed penultimate state in the refolding of cytochrome c, based on the results of this chapter, on the same scale and from same angle as Figs 2.1., 2.2., and 6.1. The haem crevice is maintained without Met 80, Trp 59 is close to/in the haem crevice, Leu 32 is virtually in an identical configuration with respect to the haem as in the native protein, and Phe 82 is folded back into the haem crevice. Approximate positions of several residues in the left hand side of the molecule, which suffer displacements from the native protein, are given.

References: Chapter Six

1. Bosshard, H.R., and Zurrer, M. (1980), J.Biol.Chem., in press.

2. Moore, G.R., and Williams, R.J.P. (1980), Eur.J.Biochem., 103, 523.

3. Takano, T., and Dickerson, R.E. (1980), Proc.Natl.Acad.Sci., in press.

4. Fisher, W.R., Taniuchi, H., and Anfinsen, C.B. (1973), J.Biol.Chem., 248, 3188.

5. Stellwagen, E., Rysavy, R., and Babul, G. (1972), J.Biol.Chem., 247, 8074.

6. Parr, G.R., and Taniuchi, H. (1978), J.Biol. Chem., 253, 5381.

7. Parr, G.R., and Taniuchi, H. (1979), J.Biol.Chem., 254, 4836.

8. Schejter, A., and George, P. (1965), Nature, 206, 1150.

9. Brunori, M., Wilson, M.T., and Antonini, E. (1972), J.Biol.Chem..247, 6076.

10. Wilson, M.T., Brunori, M., Rotilio, G.C., and Antonini, E. (1973), J.Biol.Chem., 248, 8162.

11. Schejter, A., Lanir, A., Vig, I., and Cohen, J.S. (1978), J.Biol.Chem., 253, 3768.

12. Moore, G.R., and Williams, R.J.P (1980), Eur.J.Biochem., 103, 503.

13. Tsong, T.W. (1976), Biochemistry, 15, 5467.

14. Oldfield, E., Norton, R.S., and Allerhand, A. (1975), J.Biol.Chem., 250, 6381.

15. George, P., and Schejter, A. (1964),
J.Biol.Chem., 239, 1504.

16. Wuthrich, K. (1969), Proc.Natl.Acad.Sci., 63, 1071.

17. Blumenfeld, L.A., and Davidov, R.M. (1979),
Biochim.Biophys.Acta., 549, 255.

18. Bundi, A., and Wuthrich, K. (1979), Biopolymers, 18, 285.

19. Keller, R.M., and Wuthrich, K. (1978), Biochim.Biophys.Acta.,
533, 195.

CHAPTER SEVEN

Studies of the unfolding of cytochromes c

7.1. Introduction

Recently much emphasis has been placed on the dynamic nature of the structure of proteins and its relevance to the structure - function relationship. For example, Williams (1) states that the best description of a globular protein is expressed in terms of the following generalised equilibrium:

Ground ensemble of states pH = 7, T = 25°C ⟷ Excited ensemble(s) of states at another pH, Temperature, ion binding or denaturing condition

For a protein with a well defined backbone fold, such as cytochrome c (2), the 'ground ensemble of states' contains all the individual rotational and vibrational states and their occupancies in one potential energy minimum (i.e. there is only one native conformation, which is much more stable than the next lowest energy (denatured) state). The denaturation of a protein causes a major shift in the above equilibrium from left to right, and may be considered a type of modification (as defined for this thesis in Chapter 1). Denaturation studies are useful to the study of globular protein structure for the following reasons:

a) They probe the stability of the ground state under a variety of conditions.

b) The unfolding pathway (and, if the principal of microscopic reversibility holds, the folding pathway), and the intermediates on it, may be determined for a particular denaturant.

c) Similarities between pathways for different denaturant agents may point to a general pathway, and may define the importance of certain structural elements in stabilising the protein in its native conformation.

d) Some dynamic degrees of freedom in the native state of a protein may correspond to in extreme to configurations that approximate to one of the denatured states, for example translational movements between hydrophobic domains (see next section). Thus denaturation studies can provide information concerning the dynamics of the ground state.

This chapter consists, firstly of a brief review on the theories of protein folding (both the proposed folding mechanisms and the general structures of folded globular proteins), and secondly of experimental results and discussion on the unfolding of cytochrome c by the denaturants urea and methanol. The unfolding pathways with these reagents is shown to differ between the two oxidation states of cytochrome c.

7.2. A brief resume of the theories of protein folding and the dynamic structure of proteins

Anfinsen (3) has shown that all the information necessary for a protein to acquire a unique and complex structure is stored in its linear amino acid sequence. Much progress has been recently made in the prediction of protein structure from amino acid sequence (see, for example 4,5), but we are interested here in the nature of the folding pathway.

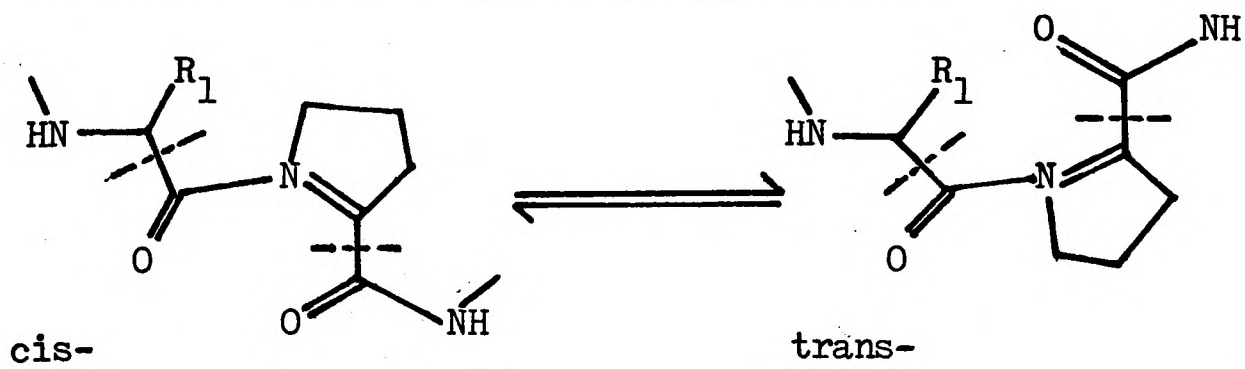
The central observation is that for many proteins the folding process occurs in two distinct kinetic phases (6), namely, the slow phase and the fast phase, and this can be summarised for the well characterised protein, Ribonuclease A (much research has been carried out on the folding of this protein (6,7)) in the following equilibrium:

$$U_s \underset{\text{slow}}{\rightleftharpoons} U_f \xrightarrow{\text{fast}} \text{Native}$$

where U_s, U_f and Native are the slow refolding denatured form, the fast refolding denatured form and the native form of the protein, respectively.

We will consider the slow refolding phase first.

Two theories are proposed to account for the biphasic kinetics, described above; the cluster model (8) and the proline isomerisation model (6). In the cluster model the protein is said to exist in equilibrium between native and denatured macrostates, which are both distributions of microstates (or microscopic configurations). The unfolding and folding of the protein are then described by the time evolution of the distribution function of the states, which mathematically can be approximated into two well separated kinetic phases; the slow process corresponds to conversion between the native and denatured microstates and the fast process to the redistribution within the macrostates. The proline isomerisation model is based on the fact that the slow refolding phase occurs at a rate that is very similar to that at which proline peptide bonds interconvert between cis and trans isomers (6 and refs. therein), as shown by the equilibrium:



cis- trans-

In a denatured protein the prolines will soon reach their equilibrium distribution of cis and trans isomers in the absence of contraints of the native conformation, where with rare exceptions the prolines are found in either one or other configuration (more often trans, which is also slightly more stable). The slow refolding phase corresponds to molecules in which the prolines have to reisomerise before complete folding can take place and the fast phase to molecules with their prolines already in their correct configuration. The experimental evidence supports the proline isomerisation model (9).

The fast folding phase is typically of the order of 1 - 100 microseconds, and this rapidity suggests that the protein molecule is directed along a (set of) particular pathway(s) to its ground state configuration, as it could not possibly explore even a few of its possible conformations in this time. It is believed that in aqueous media one or more nucleation centres form, which are composed of hydrophobic residues that aggregate in a cluster and that once formed these determine a limited set of pathways to the ground state. Scheraga (7) has found such a centre for Ribonuclease, which can be shown to never be totally unfolded by thermal denaturation in aqueous media (10). As the protein folds, further hydrophobic domains fold and the rest of the protein finds its most stable state, which is most likely determined by short range effects (11). The effects of covalent cross linking structures, such as disulphide bridges between cysteines, have been shown to be small for the folding processes of both Bovine Pancreatic Trypsin Inhibitor (12) and Ribonuclease (13). This parellels the minor role of the iron - methionyl bond in the folding of cytochrome c as compared to cytochrome c - CN, as described in Chapter 6. In order to evaluate the stability of a protein in its ground ensemble of states the dynamic motions of the hydrophobic domains in this state must be considered. To this end a brief description of the two current theories of globular protein structure is now given.

The first evidence for the internal flexibility of globular proteins in their native states came from the observation that interior amide protons exchange with protons of the solvent (14). General exchange rates for amide exchange were obtained by methods as infra red spectroscopy and tritium tracer experiments (15), and were analysed in terms of a two state 'breathing' motion for the protein (16,17,18).

More recently, high resolution ^1H n.m.r. studies have shown that aromatic groups in a protein are able to undergoe rotational 'flipping' motions about the C_β-C_γ bond (19,20), and have provided individual rates of amide proton exchange (21 and refs. therein,22,23).

A particularly well characterized protein with these respects is the Bovine Pancreatic Trypsin Inhibitor (henceforth, BPTI), and three main properties were initially found by Wuthrich and Wagner for this system (21). These are:

a) The amide proton exchange rate is slow (ca. 10^4 to 10^8 times slower than in unfolded peptides) and there are different rates for different types of amide protons within the interior (for example, in general the protons of βsheets exchange slower than those ofαhelixes).

b) The amide proton exchange rate could be correlated with the thermal stability of the globular conformation (this will be shown to be only true under limiting conditions, as those of the experiments (21), later).

c) The rotational 'flipping' motions of aromatic rings are not correlated with either thermal stability or amide exchange rate (24).

Wuthrich and Wagner suggested a 'dynamic multistate model' for globular protein structure, which is consistent with the above properties (21) and has the following basic characteristics:

i) Globular proteins are densely packed structures containing interior hydrophobic clusters, which are connected by interacting polar groups (mediated via hydrogen bonds) and are shielded from the solvent.

ii) Differences in the weak bonding interactions between the clusters and at the interfaces cause different time dependent fluctuations (intermolecular translational and rotational motions) of the hydrophobic clusters relative to each other. Such fluctuations open up the hydrogen bonded secondary structures and allow solvent access and amide exchange.

The different rates for individual amide protons arise from the different time dependent fluctuations in different regions of the tertiary structure; i.e. amide exchange results from the same motions that give rise to thermal denaturation (see iii) below). This process of amide exchange will be referred to as process a later.

iii) Thermal denaturation arises when the thermal forces involved in the fluctuations equilise the forces of binding between the different domains.

iv) The mobility of aromatic rings is correlated to the internal structural flexibility of the individual hydrophobic clusters, and hence, is not related to the thermal stability or amide exchange rate. Note, that recent experiments of Wagner (25), in which the flipping rate of aromatic rings in BPTI was determined at a variety of pressures up to 1200 atmospheres have led to the determination of the activation volumes, ΔV^{+}, of the individual ring flipping processes.

However, Woodward and Hilton (26) have obtained the exchange rates for the amide protons in BPTI over the temperature range 22°C to 77°C and the pH range 1 to 12, a much wider range of conditions; and have shown that the kinetics are more complex than previously thought (21) and that they are not consistent with the mechanism (process a) given above. These authors propose a model with an additional exchange mechanism, process b, where minor motions (small amplitude atomic fluctuations - with a characteristic low activation energy (ca. 20 - 30 kcal mol^{-1})) within very localised segments of the protein permit gradual penetration of solvent molecules into the protein core; this mechanism is not affected by or connected with thermal unfolding. Process a has by contrast a higher activation energy (ca. 65 kcal mol^{-1}). According to this model process a is expected to dominate in the limiting conditions of Wuthrich and

Wagner's (21) experiments; process b becomes more important at high pH values and low temperatures. Tests to distinguish between the two models are not yet available and the literature debate continues (27).

Process b involves small fluctuations that are important dynamic features of the ground state ensemble of a folded globular protein, while process a involves a larger scale disruption of structure that utilises states from the excited ensemble (i.e. states approximating to unfolded states). Process a is of particular interest to the studies of the folding/unfolding of globular proteins as it suggests that hydrophobic clusters are 'stable pockets' within structures that act as archetectural pillars; a view, which is entirely consistent with the role of hydrophobic clusters in initiating protein folding pathways (7), as outlined above. Thus the role of hydrophobic clusters is important in both the folding pathway of a globular protein and the maintenance of rotational and translational degrees of freedom within its structure. In cytochrome c the main hydrophobic domain is the haem crevice (the haem group and the surrounding hydrophobic sidechains), while there are two smaller pockets that contain Tyr 48 and Tyr 97 that have hindered motion (19,28) (see Fig. 2.2.).

7.3. ^{1}H n.m.r. studies of the denaturation of cytochrome c

Many studies have been undertaken on the denaturation of cytochrome c by simple denaturants as alcohols, urea and guanidine hydrogen chloride (see for examples 29-34). In this study horse cytochrome c was titrated with d^{4}-methanol, d^{4}-urea and d^{5}-guanidine hydrochloride and the denaturation was studied by ^{1}H n.m.r. Typically, samples of freeze dried protein were made up in either oxidised or reduced state in 0.5 ml. $^{2}H_{2}O$ and placed in n.m.r. tubes (under Argon for the reduced samples) under suba seals. Denaturants as guanidine hydrochloride and urea were made

up at the required pH and concentration in ^2H_2O, and d^4-methanol was used in its pure form. Titrations were carried out by adding the denaturant solution to the sample in the n.m.r. tube via a 0.1 ml. Hamilton syringe.

Solutions containing urea, and particularly guanidine hydrochloride, are very viscous and the rotation of protein molecules dissolved in such solutions is hindered. This effects the rotational correlation time, τ_R (35), and causes broadening to the resonances in the ^1H n.m.r. spectrum of the protein. The spectra for the guanidine hydrochloride titrations were so broadened that the data were uninterpretable and consequently will not be considered further. The titration with methanol caused little broadening to the spectra and these results are the most facile to interpret; they will be described and discussed first.

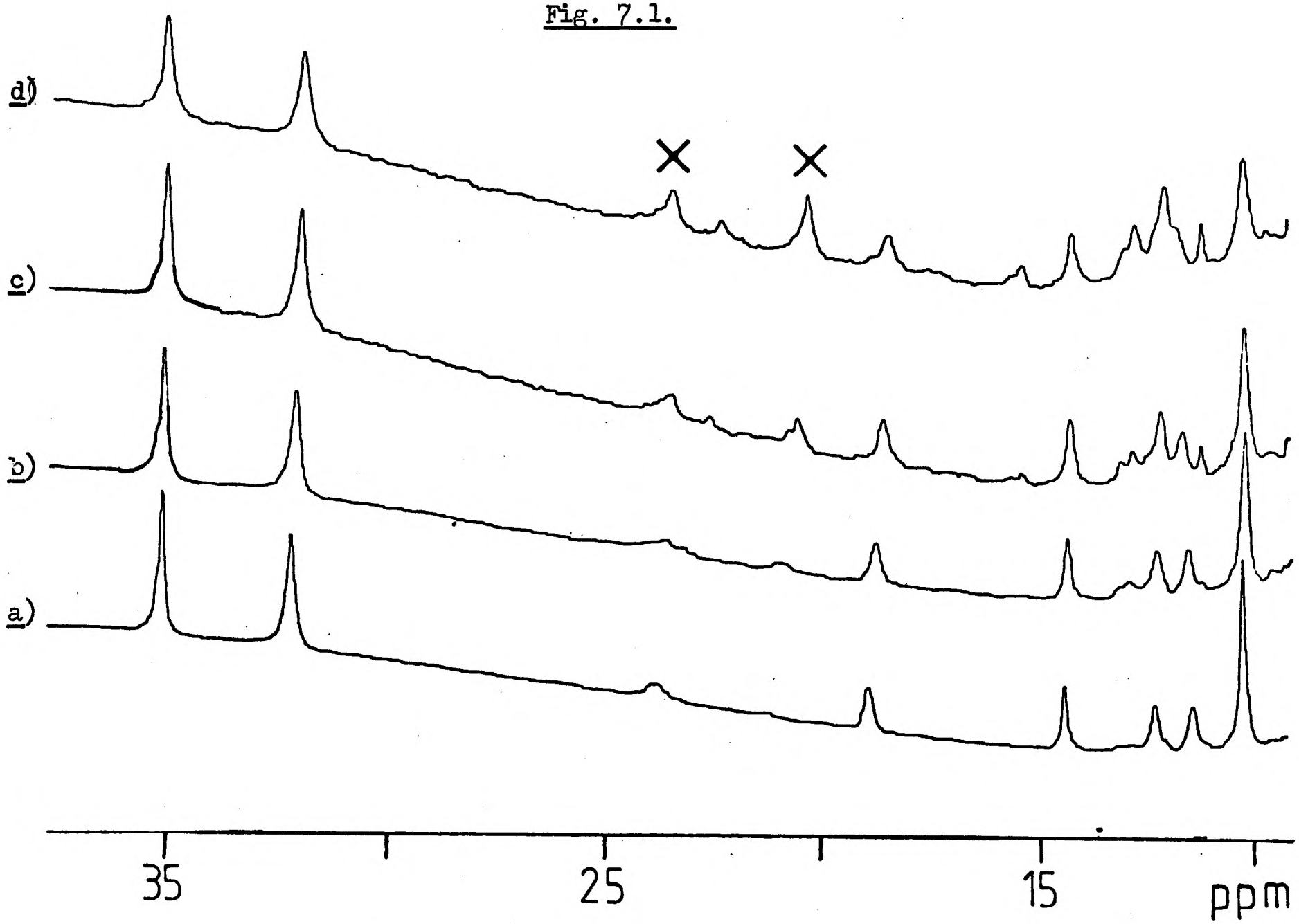
7.31. The methanol titration of ferricytochrome c

Fig. 7.1. shows the downfield regions of the large sweep width spectra of ferricytochrome c at pH 7 and 27°C in a) 100% ^2H_2O , b) 85% ^2H_2O : 15% d^4-MeOD , c) 70% ^2H_2O : 30% d^4-MeOD and d) 60% ^2H_2O : 40% d^4-MeOD. The following points are significant:

a) The native haem methyls resonances at 35.0 ppm and 32.4 ppm (36) broaden, shift towards their diamagnetic positions and loose intensity. Two new resonances arise concurrently in the spectrum at 23.8 ppm and 21.3 ppm (marked with a X), which correspond to the haem methyl resonances in the high pH, state IV, form of ferricytochrome c (37). These resonances reach maximal intensity at ca. 40% methanol and are lost from the spectrum at 55% methanol leaving a spectrum that is typical of a 'random coil' protein.

When the titration is carried out at pH 5.3 at 27°C the methanol does not affect the haem methyl resonances significantly until ca. 40% methanol, where upon they loose intensity rapidly on further methanol additions. At this point the haem methyl resonances due to state IV

Fig. 7.1.



The downfield hyperfine shifted regions of the ^{1}H n.m.r. spectra of ferricytochrome c at pH 7 and 27°C in a) 100% $^{2}H_2O$, b) 85% $^{2}H_2O$: 15% d^4-MeOD, c) 70% $^{2}H_2O$: 30% d^4-MeOD, and d) 60% $^{2}H_2O$: 40% d^4-MeOD.

ferricytochrome c can be observed at very low intensity (less than 10%).
b) The haem methyl resonance at ca. 10.0 ppm, the Met 80 CH_3 resonance at ca. -23.8 ppm and the thioether methyl resonance of the haem linked residue Cys 17 at ca. -2.2 ppm are also observed to broaden and shift towards their diamagnetic positions and loose intensity with increasing methanol concentrations.
c) Some resonances due to peptide residues can be observed to shift, particularly at concentrations of less than 20% methanol. Notably, the δ-CH_3 resonance of Ile 57 at -0.18 ppm and the CH_3 resonance of Leu 68 at -2.4 ppm (38) shift as methanol is added.

These results indicate that ferricytochrome c unfolds in the presence of methanol via at least one intermediate form. The intermediate, that is detected is the high pH, state IV, form of ferricytochrome c, in which Met 80 is displaced from the haem and a new sixth ligand coordinates which causes the iron to remain low spin and is probably a lysine residue (Section 2.3.2.). Preliminary results have shown that Cm-ferricytochrome c at pH 7 is a good model for state IV ferricytochrome c and that as the acidity is increased this compound looses the state IV ligand with a pK of ca. 5.8. This is consistent with the variation in the methanol titrations at pH 5.3 and at pH 7; in the former case only a small amount of state IV ferricytochrome c is detected, while in the latter case a significant amount of state IV ferricytochrome c is detected during the titration (see point a) above). Thus this species does not provide an intermediate in the unfolding pathway at pH 5.3 and so the spectra remain largely unaltered until 40% methanol, and the transition from native to denatured becomes more cooperative than at pH 7. Dickerson and Drew (33) also note that there is an increasing cooperativity for the native to denatured transition for the methanol denaturation of ferricytochrome c,

as monitored by the 695 nm. band in optical spectra, as the pH is decreased. There is much discussion of the nature of the sixth ligand in state IV ferricytochrome c (Section 2.3.2.), but as the species can be detected at low intensity even at pH 5.3 the suggestion that the ligand is hydroxide is unlikely to be correct (39).

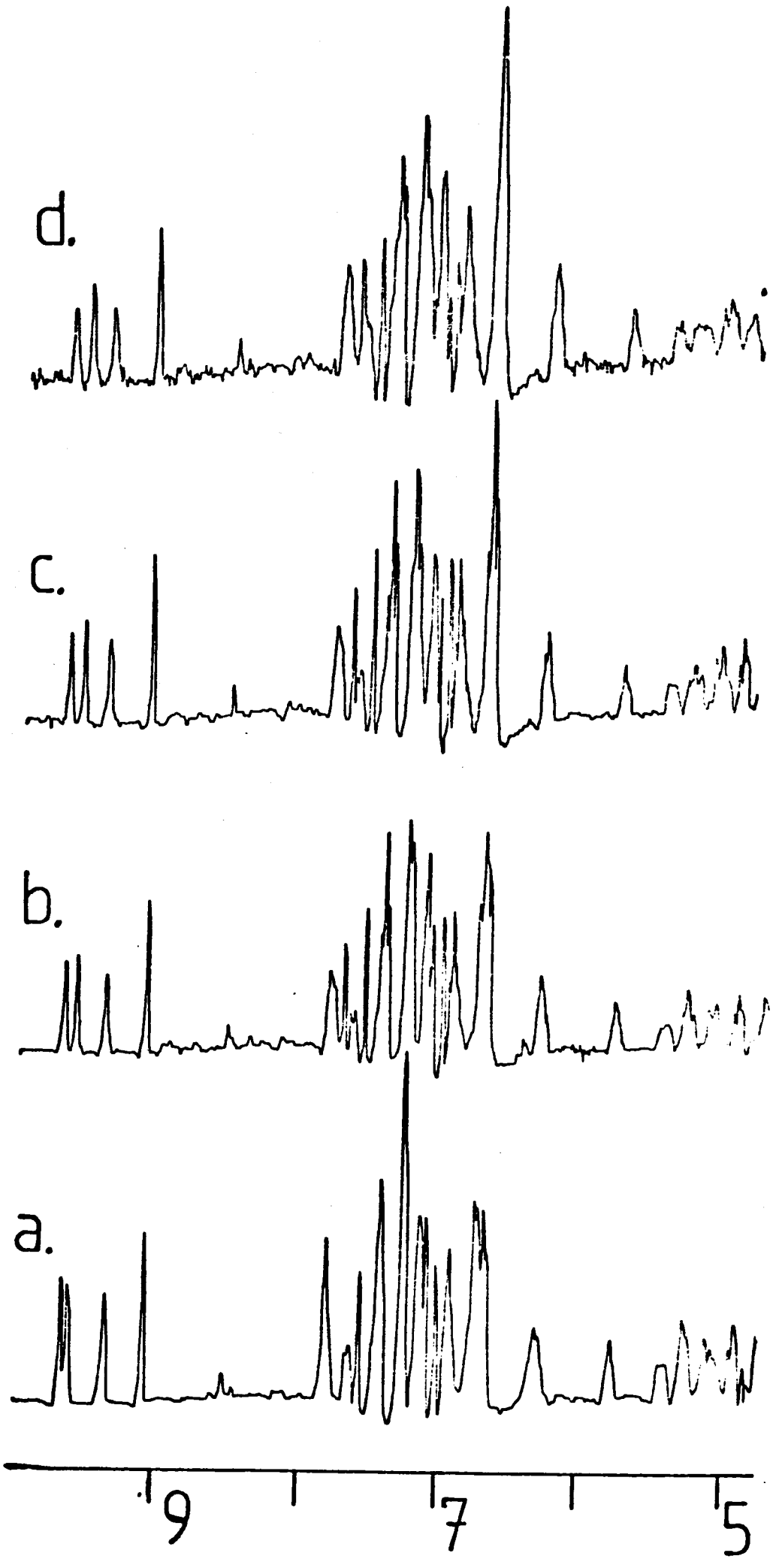
Broadening and shifting is observed to all the resonances close to the haem group and this probably results from an exchange phenomenum between state III and state IV ferricytochrome c as increasing concentrations of state IV are produced with increasing methanol concentrations.

7.3.2. The methanol titration of ferrocytochrome c

Titrations were carried out at pH 7 and at 27°C and 57°C; the results at the two different temperatures were not significantly different. At the higher temperature the spectra obtained were better resolved and these results will be discussed here; Fig. 7.2 shows the spectra of ferrocytochrome c at pH 7 and at 57°C in a) 100% ^2H_2O , b) 80% ^2H_2O : 20% d^4-MeOD , c) 70% ^2H_2O : 30% d^4-MeOD , and d) 60% ^2H_2O : 40% d^4-MeOD. It can be seen that the spectra are largely unaltered by the additions of methanol; they remain so until ca. 50% methanol at which point there is a sharp transition to a random coil spectrum. There are shifts to several resonances that increase as a linear function of the concentration of methanol and these are tabulated in Table 7.1. along with some resonances that do not shift for comparison. Note that the resonance at 3.65 ppm is due to CD_2HOD and increases with increasing methanol. The following points are made:

a) The protein undergoes a sharp transition to a random coil protein at ca. 50% methanol; no intermediates are detected. This is in contrast to the case for ferricytochrome c, described above. There are two main reasons for this; i) the iron sulphur bond is stronger in ferrocytochrome

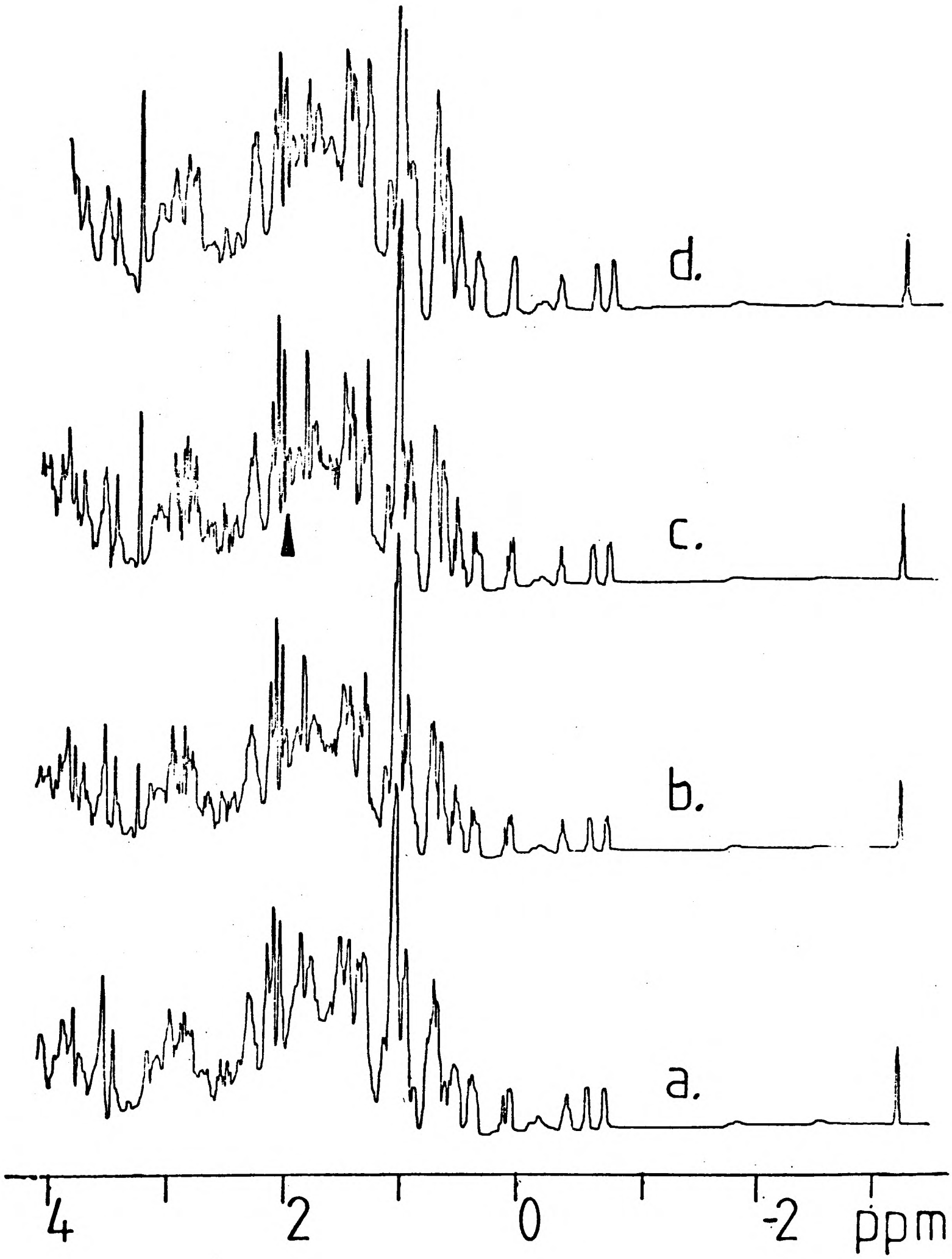
Fig. 7.2.i.



The aromatic regions of the ^{1}H n.m.r. spectra of ferrocytochrome **c** at pH 7 and 57°C in a) 100% $^{2}H_2O$, b) 80% $^{2}H_2O$: 20% d^4-MeOD c) 70% $^{2}H_2O$: 30% d^4-MeOD, and d) 60% $^{2}H_2O$: 40% d^4-MeOD.

Part ii) of this diagram is given on the following page.

Fig. 7.2.ii.



The aliphatic regions of the ^{1}H n.m.r. spectra of ferrocytochrome c at pH 7 and 57°C under the same solvent conditions that are given for part i) of this diagram on the previous page.

Table 7.1.

Methanol induced chemical shifts to resonances in ferrocytochrome c

Resonance designate and Assignment		Chemical shift at pH 7, 57°C in 100% ^2H_2O	Induced shift at pH 7, 57°C in 60% ^2H_2O:40% MeOD
H1	Haem meso γCH	9.62	0.00
H2	Haem meso βCH	9.58	-0.07
H3	Haem meso αCH	9.32	+0.04
H4	Haem meso δCH	9.04	0.00
M4	Ile 57 δCH_3	-0.43	+0.12
M10	N-acetyl CH_3	2.07	+0.02
M11	Met 65 CH_3	2.11	+0.01
Unkown doublet ?		2.05	-0.03
Unknown broad ?		ca. 1.93	+0.03
---	Thr 40/63 CH_3 +	1.40	+0.02
Ala/Thr doublets (inc. Thr 89 CH_3)		1.36	+0.02
Unknown doublet ?		0.91	>+0.03
A11	Tyr 74 o-	7.22	-0.02
A26	Tyr 74 m-	6.64	+0.02
A23	Phe 82 o-	6.71	-0.02
A30	Trp 59 C-6	5.74	0.00

+, see Section 3.4. for assignment of this resonance.

N.B. The point of interest is the relative shift, as gives a guide to relative denaturation (i.e. induced shift / secondary shift)

c than in ferricytochrome c. At pH 7 50% methanol is required to break it and force the haem cleft open, and at this concentration of methanol the rest of the protein structure can no longer be stabilised. ii) Wilson's (40) work shows that Cm-ferricytochrome c at pH 7 is a good model for state IV ferricytcchrome c (a low spin species, S = $\frac{1}{2}$), the intermediate in the methanol denaturation of ferricytochrome c, but that Cm-ferrocytochrome c at pH 7 is a high spin species with a high field ligand. Hence ferrocytochrome c has no equivalent intermediate to State IV ferricytochrome c at pH 7.

Therefore the backbone tertiary structure of ferrocytochrome c at pH 7 and at 57°C remains unaltered up to ca. 50% methanol. Any changes seen in the ^{1}H n.m.r. spectrum at concentrations of methanol below this level will reflect the binding of methanol to the surface of the protein.

b) Of the four meso proton resonances from the haem group which are individually assigned (41) the two buried ones (γ and δ, Table 7.1.) are not affected by the methanol, while the two other protons (α and β) that are close to the surface are shifted. These latter two resonances are also shifted by pH variations in the solvent (42).

c) Several methyl group resonances shift, but due to the overlap in this region of the spectrum the shifts are difficult to assign. The firmly assigned resonances from the surface residues of Met 65 and the N-acetyl group suffer small shifts. An unassigned doublet found under the N-acetyl resonance at 2.05 ppm in 100% ^2H_2O is shifted to a position where it is clearly resolved in the spectrum in 30% MeOD (see Fig.7.2.c), the resonance is indicated).

Many shifts occur between 1.30 ppm and 1.60 ppm , where most of the alanine and threonine methyl resonances are found (a total of 16 are expected from horse cytochrome c). Many shifts might be expected for the

resonances from threonines, as Taborsky and McCollum (43) have shown that all 10 threonines, except Thr 49, in horse cytochrome c are solvent accessible. Of note (Table 7.1.) a methyl resonance at 1.40 ppm from Thr 40 or Thr 63 (Section 3.4.) and methyl resonances at 1.36 ppm, under which Thr 89 is found (44), are observed to shift.

Similarly the lineshape between 0.85 ppm and 0.65 ppm is observed to change. The resonances here are mainly methyl resonances from valines, leucines and isoleucines that do not recieve large secondary shifts. It is of note that the methyl resonances of Leu 32 at -0.60 ppm and at -0.76 ppm which recieve very large secondary shifts and which are markers to the internal hydrophobic core of the molecule are unshifted (Fig. 7.2.)

d) The resonances of Ile 57 and Tyr 74 are observed to shift (Table 7.1.). This reflects a small change in structure in this region and in their mutual juxtaposition in the structure. A similar change is induced to this flexible region if the molecule by the increase of temperature (see Section 3.6.1.). The residues are close to the surface of the molecule.

e) The resolved resonances of Trp 59 (e.g. the C-6 proton at 5.74 ppm) are not observed to shift. This residue lies in the hydrophobic core of the molecule and is not expected to be affected by solvent perturbation; the C-6 resonance is affected at low pH, but the reason is not understood (see Section 3.6.4.).

The above points show that ferrocytochrome c undergoes a sharp cooperative transition to a random coil protein at 50% methanol. Before this transition no intermediates are detected, but many resonances from surface residues are perturbed. The perturbations caused to the surface of the protein may be considered as a type of modification caused by change in the bulk medium - this modification is very small as the protein remains intact up to the denaturation transition.

7.3.3. The comparison of the methanol denaturations of ferricytochrome c and of ferrocytochrome c

Taken together the results from the last two sections indicate that ferricytochrome c and ferrocytochrome c possess different unfolding pathways at pH 7. The main reason for this is the stronger iron - methionyl cross linking bond in the latter species. The first major step in each case is the breaking of this bond and it is presumably triggered by the perturbation of the protein surface by the solvent, which becomes increasingly less polar as the methanol titration proceeds. For the oxidised protein a small quantity of 'sulphur off' species is produced by low concentrations of methanol, which are not great enough to affect the majority of the groups in the hydrophobic core of the molecule (particularly that on the His 18 side of the haem). A new species is stabilised as an intermediate, state IV ferricytochrome c, with another cross linking bond between the iron and a strong field ligand from the protein chain giving rise to a low spin ferric ion. This new bond is broken at higher concentrations of methanol (ca. 45%), which provide a hydrophobic enough solvent medium to cause disruption of the hydrophobic core of the molecule; a random coil protein is produced. The reduced protein does not produce any significant amount of 'sulphur off' species until ca. 50% methanol, at which point a random coil protein is formed without any detectable intermediate.

These points may be summarised in two phase diagrams of the free energy of the protein configurations against hydrophobicity of solvent (or % methanol for the two proteins). These are shown in Fig. 7.3., and clearly show the difference in the unfolding pathways. In Section 4.2.2. it is pointed out that during the temperature denaturation of ferricytochrome c (and $\left[\text{Met(Cm)}^{65}\right]$ ferricytochrome c and $(\text{Hse})^{65}$ ferricytochrome c)

that no intermediate is detected. Thus there is a difference in the thermal denaturation and the chemical denaturation pathways. Fig. 7.4. shows a phase diagram of the free energy of ferricytochrome c states against temperature and comparison with Fig. 7.3. clearly indicates the difference in pathways.

The conclusions outlined above differ from those of Dickerson and Drew (33) , who studied the methanol denaturation of cytochrome c, in two respects:

a) These authors suggest the following scheme for the denaturation of ferricytochrome c,

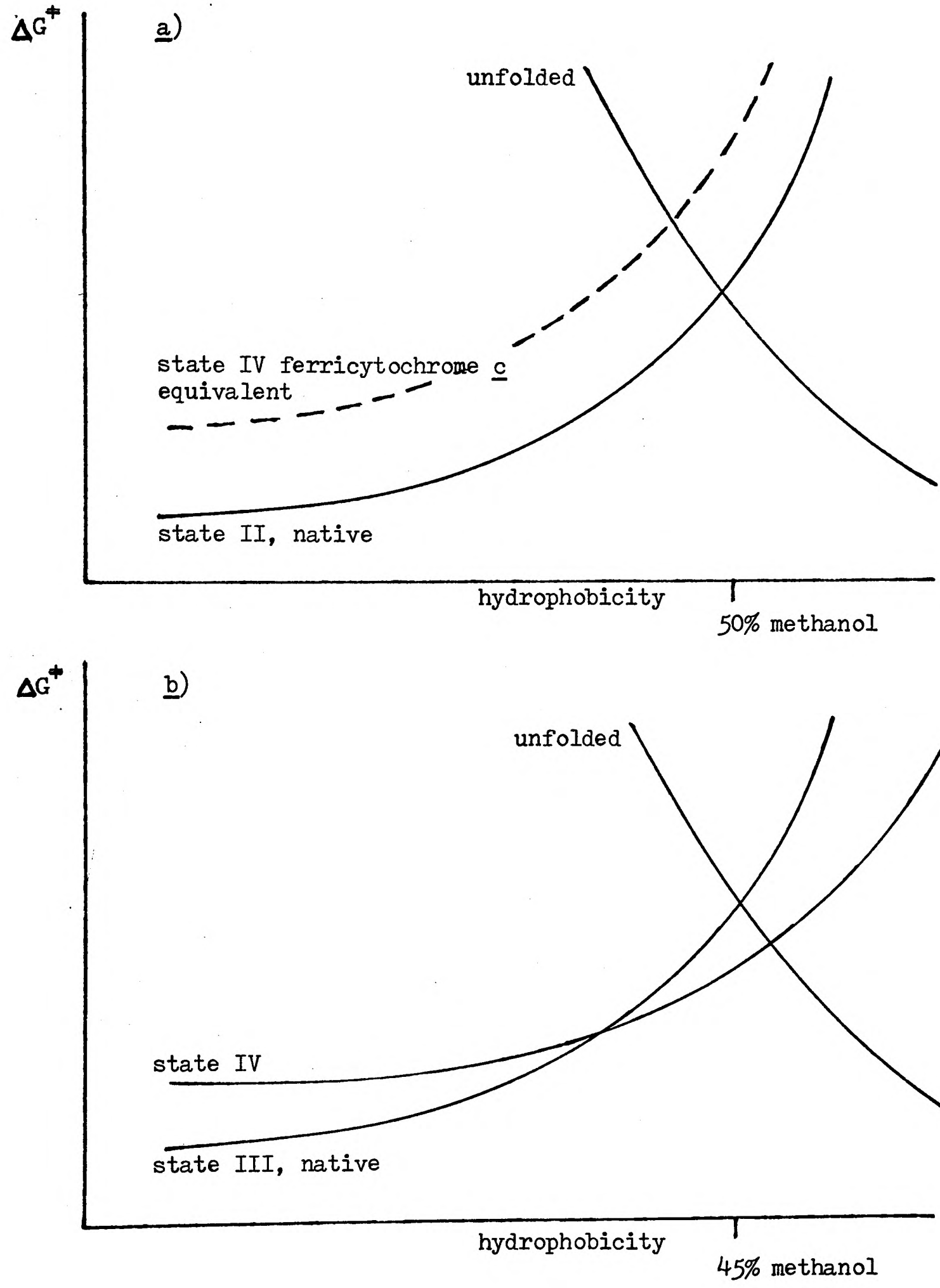
$$\text{Native} \underset{1\ \text{MeOH}}{\rightleftharpoons} \text{I} \underset{10\ \text{MeOH}}{\rightleftharpoons} \text{II} \rightleftharpoons \text{III} ,$$

where states II and III correspond to 'state IV' and random coil proteins in the scheme of this work. State I is said to be a slightly perturbed configuration of the native state characterised by a slight enhancement in the absorbance of the 695 nm. band. Kinetic and equilibrium data show that state I results from the binding of one methanol molecule to a single site on the molecule. No such state can be detected in the denaturation by methanol as studied by ^{1}H n.m.r. However a similar state is found in the urea denaturation as studied by ^{1}H n.m.r. (see next section).

b) The above authors propose the same scheme for ferrocytochrome c, although their evidence is at best circumstantial. A central conclusion from my work is that this is not the case, and that ferricytochrome c and ferrocytochrome c do not possess the same unfolding pathway with increasing concentrations of methanol.

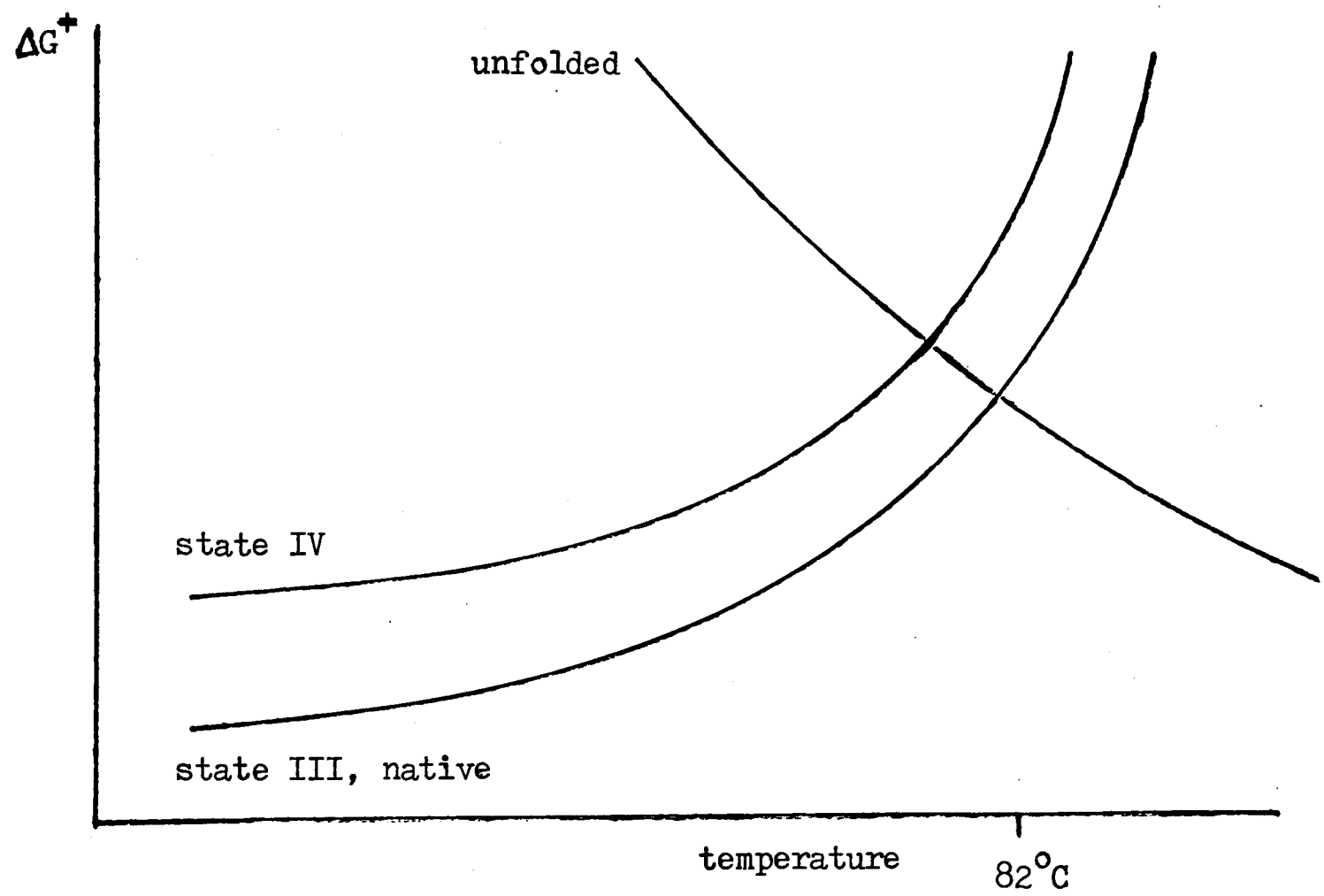
This work shows that ferricytochrome c is an exception to the two state unfolding principle, which is that typically protein denaturation is a two state (native and denatured) phenomenon (45).

Fig. 7.3.



Suggested diagrams of the relative free energies for states of a) ferrocytochrome c, and b) ferricytochrome c against hydrophobicity (or % methanol) of solvent. The diagrams indicate the difference in the unfolding pathways of ferricytochrome c and ferrocytochrome c.

Fig. 7.4.



Suggested diagram of the relative free energies for states of ferricytochrome c against temperature; this diagram can be compared to Fig. 7.3.b., which illustrates the difference in the unfolding pathways of ferricytochrome c with chemical denaturants and temperature.

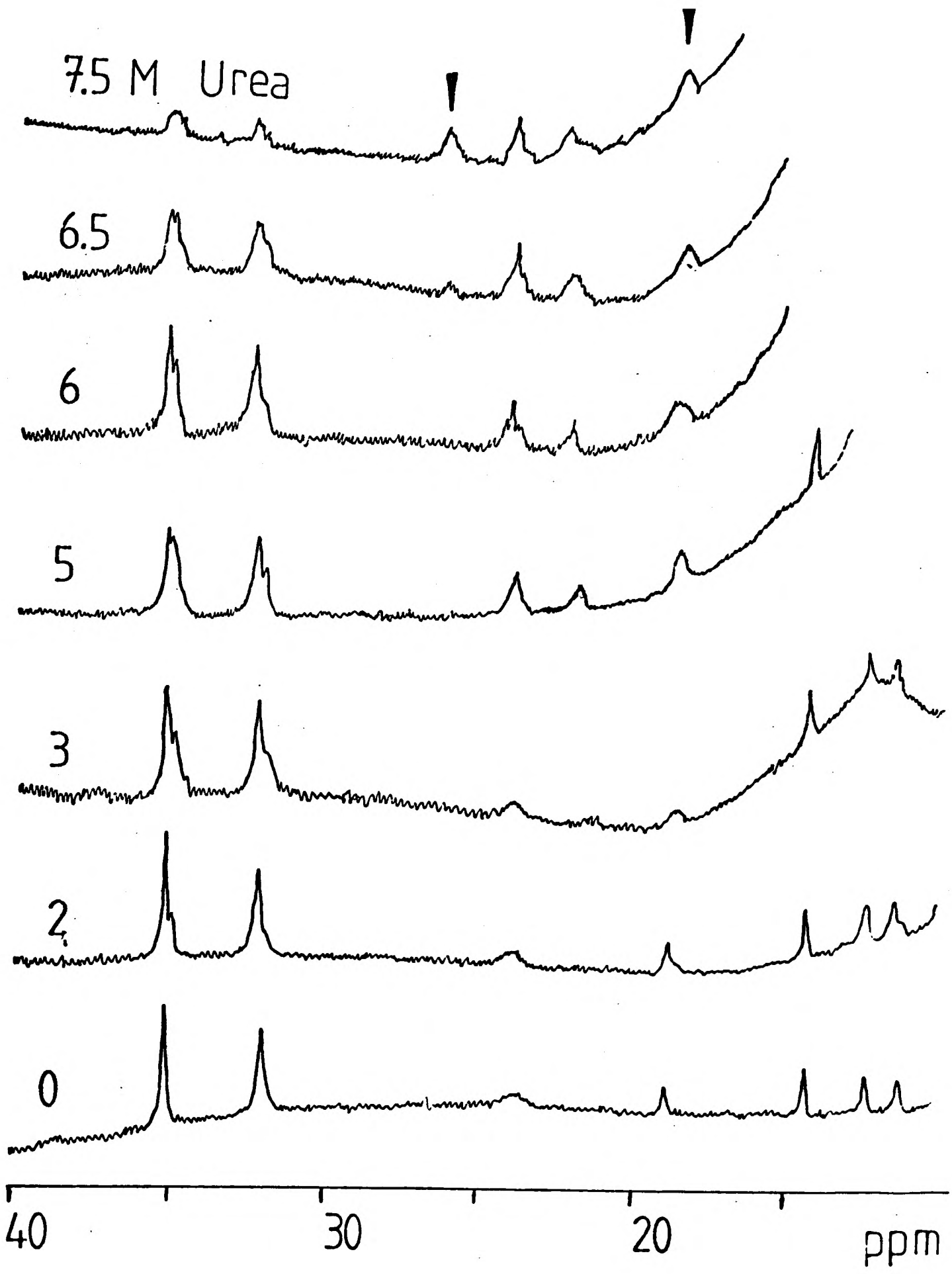
7.3.4. The urea titrations of ferricytochrome c

Several titrations were carried out because it was discovered that there is an effect from the concentration of the ferricytochrome c itself. Initially 10 samples of 8×10^{-4}M ferricytochrome c at pH 7 were made up in ^2H_2O solutions that contained concentrations of d^4-urea from 0 to 9M. This experiment was designed to be an ^1H n.m.r. experimental equivalent to the urea titration, as described by Myer et al (34), that followed the absorption of the 695 nm. band and the fluorescence efficiency of Trp 59. Interpretable ^1H n.m.r. spectra were obtained, but the signal to noise was low due to the low concentration (in n.m.r. terms) of the protein. A second experiment was therefore carried out, in which the samples were 3.8×10^{-3}M in protein, but identical in other respects. The results were similar to those of the first experiment, but not identical so a third titration was carried out to ascertain the effect of dilution (in terms of the protein) in a 5M urea sample; a 3.8×10^{-3}M solution of ferricytochrome c in 5M urea was diluted in steps with 5M urea, all in ^2H_2O at pH 7. This latter titration indeed indicated a dilution effect in the ferricytochrome c itself, but the results did not totally agree with those of the previous titration. In all cases the above experiments were carried out in unbuffered solutions with no added salt concentration except from the protein itself. Thus the experiments so far carried out must be said to be incomplete, but they do provide a basis for further experiments. They are now discussed in turn.

Fig. 7.5. shows the downfield hyperfine shifted resonances for 8×10^{-4}M ferricytochrome c in ^2H_2O solution at pH 7 and 27°C containing various concentrations of d^4-urea. The following points are noted:

a) Between 0 and 5M urea the two haem methyl resonances of native

Fig. 7.5.



The downfield hyperfine shifted region of the ^{1}H n.m.r. spectra of ferricytochrome c at pH 7 and 27°C in various concentrations of solutions containing urea. The protein concentration is 8×10^{-4} M. The marked resonances are referred to in the text.

ferricytochrome c diminish, while two resonances arise slightly upfield of each (by ca. 0.1 ppm) in the spectrum. These latter two resonances are assigned to the same haem methyls in a slightly non-native state.
b) At concentrations of urea of 5M and above both the above sets (the native and their slightly upfield counterparts) of haem methyl resonances diminish in intensity and the corresponding resonances of haem methyls in state IV ferricytochrome c arise at 23.8 ppm and 21.3 ppm (37).
c) In 9M urea two resonances at 26.5 ppm and 19.0 ppm (these appear at low intensity in the spectrum at 7.5M urea, Fig. 7.5., and are marked) are the only resonances found in this region of the spectrum. They are assigned to two haem methyl groups in a low spin fully denatured state. This state is not the same as state IV ferricytochrome c though it could possess the same sixth ligand and differ from state IV ferricytochrome c in only a conformation change of the peptide. To test the possibility that urea itself might be the ligand in this state a spectrum of the ferric haem undecapeptide (kindly prepared by Dr. K. Kimura) was recorded in 9M urea - no resonances similar to those found for the native protein (haem methyls in a low spin state) were found and the sample gave a typical high spin spectrum. The ligands for the protein in 9M urea must, therefore, originate from the protein.

These results indicate that the denaturation of ferricytochrome c by urea at pH 7 involves at least two intermediates. The first intermediate can be observed at concentrations of urea as low as 2M and is characterised by two haem methyl resonances slightly upfield from their positions in the native protein. The second intermediate observed by ^{1}H n.m.r. is state IV ferricytochrome c, as observed in the methanol denaturation. The final state is another low spin species. These results are compatible with the most recent study of the urea denaturation of

ferricytochrome c (at similar concentrations of the protein) by other techniques, as following the absolute extinction coefficient of the 695 nm. band (determined by the method of Kaminsky et al (46)) and the fluorescence efficiency of Trp 59 (34). Briefly, these authors' results show that the following scheme may be drawn up:

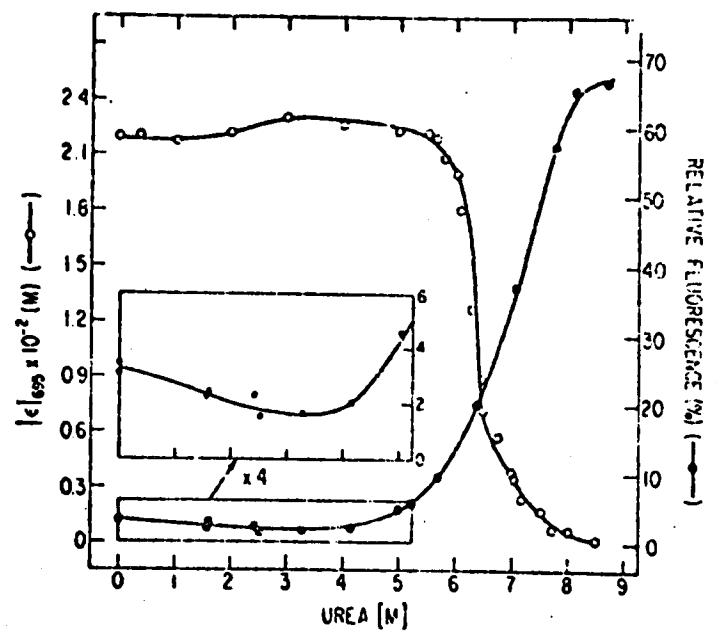
$$\underset{A_{695}=219}{\text{Native}} \overset{2.5M}{\rightleftharpoons} \underset{A_{695}=229}{X_1} \overset{6.2M}{\rightleftharpoons} \underset{A_{695}=82}{X_2} \overset{7.0M}{\rightleftharpoons} \underset{\substack{A_{695}=0 \\ \text{Low spin}}}{\text{Denatured}} \quad [\text{Urea}]$$

The first transition is characterised by a slight enhancement of the absorption at 695 nm. and a slight lowering of the Trp 59 fluorescence efficiency (i.e. it is quenched by the haem group more). The second and third transitions can only be resolved by curve fitting to the 695 nm. absorption profile between 5M and 7.5M urea, while in this region the tryptophan fluorescence gives a symmetrical profile; the two relevant diagrams from the paper of Myer et al (34) are shown in Fig. 7.6. No evidence of further changes of state are observed above 7.5M urea by these techniques.

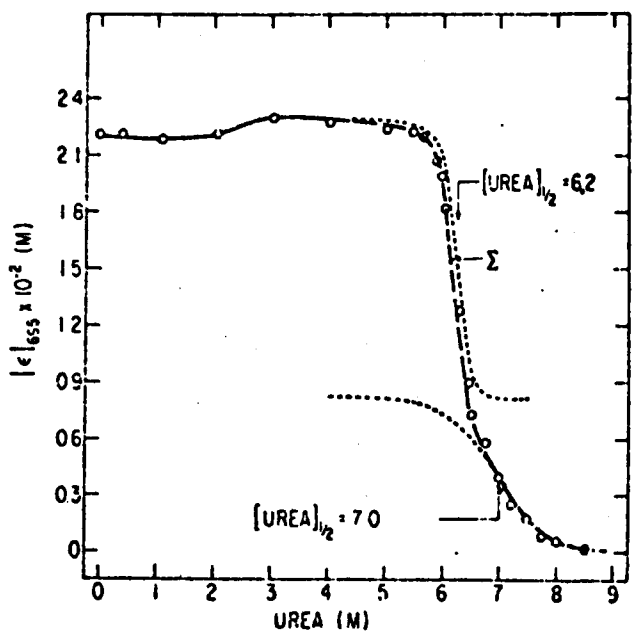
States N, X_1, and X_2 in this scheme all possess a Met 80 - haem bond, as indicated by the presence of the 695 nm. band, and it is of interest to note that the absolute extinction of the 695 nm. band in X_2 is calculated from the curve fitting (see Fig. 7.6.) to be $82M^{-1}$, which is very close to that obtained for the methionine complex of the haem undecapeptide (a model for the iron sulphur bond, without any influences from the peptide part of the protein) (47). This suggests that in X_2 the Met 80 - haem bond is preserved but there is a large disruption to the peptide backbone, and that in N and X_1 the protein conformation (native and nearly native, respectively) contributes to the absorptivity of the

Fig. 7.6.

i)



ii)



These two figures are taken from the paper of Myer et al (34).

i) Denaturation profiles of horse heart ferricytochrome c with urea from changes of the 695 nm. band (o) and the fluorescence efficiency (●) of Trp 59; ii) Resolution of the high molar urea 695 nm. absorption - urea denaturation transition. (...) Resolved components; (---) summation of two resolved components; (——) unresolved denaturation profile. The absorption profile is from i).

Conditions: 0.05M phosphate, 0.2M KCl, varying concentrations urea, pH 7, 22°C; protein concentration, 2 10^{-6}M.

695 nm. band. No evidence for X_2 is seen in the n.m.r. experiments, presumably due to its small concentration during the denaturation even between urea concentrations of 6M and 7M. Clearly, X_1 corresponds to the first n.m.r. intermediate and the slight enhancement of the 695 nm. band absorption is related to the same structural change that causes the upfield shifts on the haem methyl resonances. Combining all the evidence outlined above the following scheme for the urea denaturation of ferricytochrome c may be written:

	Native	2.5M ⇌	X_1	6.2M ⇌	X_2	7.0M ⇌	X_3	8.0M ⇌	Denatured	[Urea]
A_{695}	219		229		82		0		0	
Trp 59 fl. eff.	3%		1.9%		n.d.		70%		70%	
^1H n.m.r. work	upfield shifts on two haem methyls		n.d.		State IV ferri c		low spin, same ligand as X_3 ?			

State X_1 is this scheme corresponds to state I of Dickerson and Drew's (33) scheme for the methanol denaturation of ferricytochrome c (see Section 7.3.3.).

Thus there is a good correlation between the physicochemical data for the urea denaturation of ferricytochrome c in solutions below 10^{-3}M in the protein. However this concentration is at the limit at which good ^1H n.m.r. spectra may be obtained for proteins. Two further experiments at higher concentrations of the protein are now described.

Firstly, the previous experiment was carried out with the concentration of the protein at 3.8×10^{-3}M. In this case the following points were observed from the spectra obtained:

i) Up to 5M urea the spectra remain completely native, though they were slightly broadened as the solutions became more viscous with the increase

of the urea concentration. No resonances are seen slightly upfield and adjacent to the native haem methyl resonances (i.e. state X_1 is not observed) during this range of urea concentration or at higher concentrations.

ii) Above 6M urea resonances from the haem methyls of state IV ferricytochrome c are observed in the spectra.

iii) At pH 8.5 urea the ratio of the intensities of the haem methyl resonances from the native and state IV ferricytochromes c is ca. 1:1. At higher concentrations of urea, at this protein concentration, the solutions become too viscous to afford ^{1}H n.m.r. spectra.

Thus the increase in the concentration of the protein itself appears to suppress the denaturation reaction and the existence of state X_1 as an equilibrium intermediate in the denaturation process. As a further test of this effect a sample of 3.8×10^{-3}M ferricytochrome c in 5M urea was diluted with 5M urea solution and ^{1}H n.m.r. spectra were recorded. The spectra are shown in Fig. 7.7. (the protein concentrations in 10^{-3}M (mM) are marked on the figure). There are significant changes to these spectra:

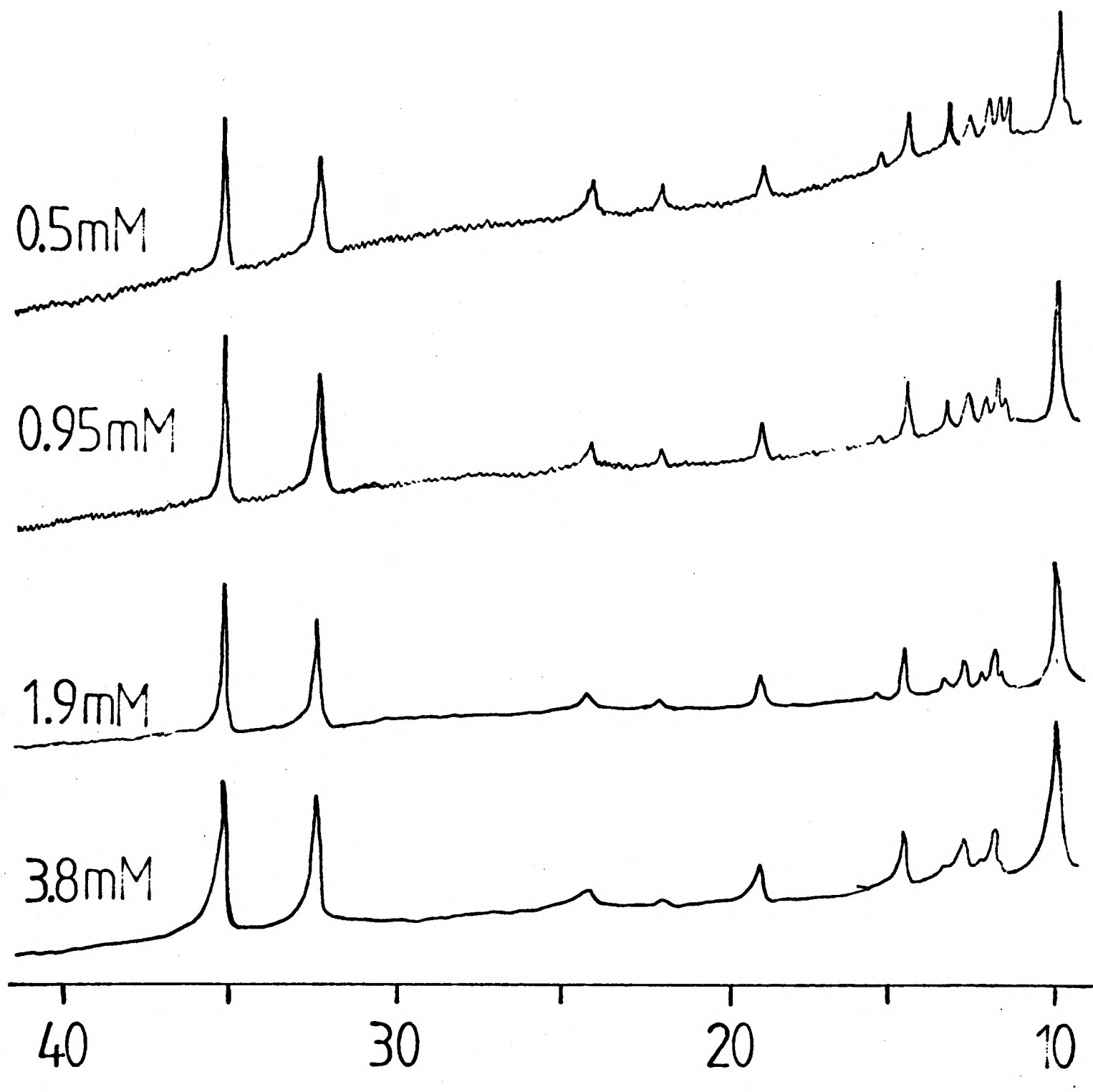
i) The spectra sharpen up as the sample is diluted. This is because the viscosity of the samples is determined by both the concentration of the protein and of the urea. In this case the urea concentration is constant at 5M, and the change in the protein concentration significantly alters the viscosity of the solution.

ii) As the concentration of ~~urea~~ protein is decreased resonances from haem methyls in state IV ferricytochrome c at 23.8 ppm and at 21.3 ppm increase in intensity.

iii) There are changes in the spectrum between 11 ppm and 14 ppm (see Fig. 7.7.).

The latter two experiments show discrepancies with the first

Fig. 7.7.



The downfield hyperfine regions of the spectra of various concentrations of ferricytochrome c (marked on the individual spectra) in 5M urea $^{2}H_{2}O$ solution. The spectra were recorded on the same sample of ferricytochrome c, which was progressively diluted with 5M urea solution.

experiment, which is discussed more fully as it is at a similar protein concentration to other physicochemical studies of the urea denaturation of ferricytochrome c. The dilution of the 3.8×10^{-3}M sample changed the spectrum, but did not give a spectrum that was identical to that originaly recorded at a lower concentration of protein. Thus, the main conclusion from the above experiments is as follows; at a protein concentration of 8×10^{-4}M the ^{1}H n.m.r. data for the urea denaturation of ferricytochrome c agrees very well with data from other physicochemical techniques (34). As the protein concentration is increased the denaturation is suppressed, but the nature of the concentration effect has yet to be determined.

7.3.5. The urea denaturation of ferrocytochrome c

Ferrocytochrome c was titrated with d^4-urea at pH 7 and at 82°C (this temperature afforded sharper resonances than the standard of 57°C) and ^{1}H n.m.r. spectra were recorded. The results are similar to those reported for the methanol titration of Section 7.3.2.; the protein undergoes a sharp transition to a random coil protein at ca. 6M urea and before this no intermediates are detected. Before the transition some effects to ^{1}H n.m.r. spectrum are observed:

a) The resonances from the internal meso protons are unaffected, while those from the surface meso protons are observed to shift.

b) The lineshapes of the spectra in the region of resonances from threonine methyl groups changes slightly as the concentration of urea is increased. The resonances of Leu 32 do not shift.

c) The resonances of Ile 57 and Tyr 74 are observed to shift.

d) No resonances of Trp 59 are observed to shift.

Thus ferrocytochrome c is denatured by methanol and by urea in very similar ways. First, the surface of the protein is perturbed (or modified) as the solvent hydrophobicity increases, and secondly the protein undergoes a cooperative two state denaturation (Section 7.3.2.).

7.3.6. The denaturation pathways of cytochrome c : Summary

The previous parts of this chapter show that cytochrome c, in either of its oxidation states, undergoes the same denaturation transitions with either methanol or urea; the processes do differ between the the oxidation states. Ferrocytochrome c undergoes one two state transition between native and fully denatured states. Ferricytochrome c has at least one main intermediate, state IV ferricytochrome c, in its denaturation profiles with either denaturant. The difference in pathways between oxidation states results from two facts; i) ferricytochrome c is less stable than ferrocytochrome c, mainly due to a weaker iron - sulphur cross linking bond, and ii) ferrocytochrome c can not form a stable, equivalent intermediate to state IV ferricytochrome c. Free energy diagrams of each protein against hydrophobicity of solvent are postulated (Fig. 7.3.) that account for the difference in denaturation profiles. The main stability of cytochrome c in both oxidation states arises from the wrapping of hydrophobic residues around a hydrophobic template, the haem group, and the positioning of hydrophilic residues on the surface with easy access to the aqueous solvent. The four cross linking bonds in the protein act to enhance this stability, and one of these the iron sulphur bond is much stronger in the reduced protein.

References: Chapter Seven

1. Williams, R.J.P. (1979), Biol.Rev. 54, 389.

2. Takano, T., and Dickerson, R.E. (1980), Proc.Natl. Acad.Sci.

3. Anfinsen, C.B. (1973), Science, 181, 223.

4. 'Protein Folding', (1980), ed. Rainer Jaenicke, Elsevier/North Holland, New York.

5. Sternberg, M.J.E., and Thornton, J.M. (1978), Nature, 271, 15.

6. Brandts, J.F., Halvorson, H.R., and Brennan, M. (1975), Biochemistry, 14, 4953.

7. Burgess, A.W., and Scheraga, H.A. (1975), J.Theor.Biol., 53, 403.

8. Kanehisa, M.I., and Tsong, T.Y. (1978), J.Mol.Biol., 124, 177.

9. Schmid, F.X., and Baldwin, R.L. (1979), J.Mol.Biol., 133, 285.

10. Matheson, R.R., and Scheraga, H.A. (1979), Biochemistry, 18, 2437.

11. Lewis, P.N., Momany, F.A., and Scheraga, H.A. (1971), Proc.Natl.Acad.Sci., 68, 2203.

12. Creighton, T.E. (1977), J.Mol.Biol., 113, 313.

13. Creighton, T.E. (1979), J.Mol.Biol., 129, 411.

14. Linderstrom-Lang, K. (1955), Chem.Soc.Spec.Publ., 2, 1.

15. Englander, S.W., Downer, N.W., and Teitelbaum, H. (1972), Ann.Rev.Biochem., 41, 903.

16. Hvidt, A., and Nelson, S.O. (1966), Advan. Prot.Chem., 21, 287.

17. Tanford, C. (1970), Advan.Prot.Chem., 24, 1.

18. Richarz, R., Sehr, P., Wagner, G., and Wuthrich, K. (1979), J.Mol.Biol., 130, 19.

19. Campbell, I.D., Dobson, C.M., Moore, G.R., Perkins, S.J., and Williams, R.J.P. (1976), FEBS letters, 70, 96.

20. Wuthrich, K., and Wagner, G. (1975), FEBS letters, 50, 265.

21. Wagner, G., and Wuthrich, K. (1979), J.Mol.Biol., 134, 75.

22. Patel, D.J., and Canuel, L.L. (1976), Proc.Natl.Acad.Sci., 73, 1398.

23. Moore, G.R., and Robinson, M. (1981), manuscript in preparation.

24. Wagner, G., and Wuthrich, K. (1978), Nature, 275, 247.

25. Wagner, G. (1980), FEBS letters,

26. Woodward, C.K., and Hilton, B.D. (1979), Ann.Rev.Biophys.Bioeng., 8, 99.

27. Wuthrich, K, and Wagner, G. (1980), J.Mol.Biol.,

28. Keller, R., and Wüthrich, K. (1981), Biochim.Biophys.Acta., in press.

29. Knapp, J.A., and Pace, C.N. (1971), Biochemistry, 13, 1289.

30. Henkins, R.W., and Turner, S.R. (1979), J.Biol.Chem., 17, 8110.

31. Land, E.J., and Swallow, A.J. (1976), Biochem.J., 157, 781.

32. Creighton, T.E. (1979), J.Mol.Biol., 129, 235.

33. Drew, H.R., and Dickerson, R.E. (1978), J.Biol.Chem., 253, 8420.

34. Myer, Y.P., MacDonald, L.H., Verma, B.C., and Pande, A. (1980), Biochemistry, 19, 199.

35. Levine, B.A., Moore, G.R., Ratcliffe, R.G., and Williams, R.J.P. (1979), Chemistry of Macromolecules IIA, Vol. 24, pp. 77-140, ed. R.E.Offord, University Park Press, Baltimore.

36. Redfield, A.G., and Gupta, R.K. (1971), Cold Spring Harbour Symp.Quant.Biol., 36, 405.

37. Gupta, R.K., and Koenig, S.H. (1971), Biochem.Biophys. Res.Comm., 45, 1134.

38. Moore, G.R., Eley, C.G.S., Clayden, N.J.(1981), unpublished results.

39. Pettigrew, G.W., Aviram, I., and Schejetr, A. (1976), Biochim.Biophys.Res.Comm., 68, 807.

40. Wilson, M.T., Brunori, M., Rotillo, G.C., and Antonini, E. (1973), J.Biol.Chem., 248, 8162.

41. Keller, R.M., and Wuthrich, K. (1978), Biochim.Biophys.Acta., 533 195.

42. Moore, G.R., and Williams, R.J.P. (1980), Eur.J.Biochem, 103, 513.

43. Taborsky, G., and McCollum, K. (1979), J.Biol.Chem., 254, 7069.

44. Moore, G.R., and Williams, R.J.P. (1980), Eur.J.Biochem, 103, 503.

45. Baldwin, R.L. (1975), Annu.Rev.Biochem., 44, 453.

46. Kaminsky, L.S., Miller, V.J., and Davison, A.J. (1973), Biochemistry, 12, 2215.

47. Schecter, E., and Saludjian, P. (1967), Biopolymers, 5, 788.

CHAPTER EIGHT

Summary and Conclusion: Overview of chemically modified cytochromes c described in this thesis

The first task of this thesis was to confirm previous assignments and obtain further assignments of resonances in the ^{1}H n.m.r. spectra of eukaryotic cytochromes c. This was achieved by the cross assignment of resonances in ferricytochrome c and ferrocytochrome c by a redox titration between the two species (Section 3.3.1.), by spin decoupling and NOE experiments (Section 3.4.), by sequence comparisons (Section 3.5.) and by chemical modifications (e.g. Met 65, Section 4.2.1.). Often the previous assignments were tentatively based upon indirect and sometimes circumstantial evidence (2,3); in the work of this thesis many of these assignments have been confirmed by direct and independent methods, which have not only yielded the assignment data, but has turned the original data (such as, pH titrations and titrations with resonance broadening reagents) into useful structural information. Further experiments are now being carried out on the basis of these data. Many examples of the confirmation of earlier tentative assignments are given in Chapter 3, where the assignment of twenty four new resonances in eukaryotic cytochromes c is described. Table 8.1. gives the assignments resulting from this work.

The central theme of this thesis is the study of the structural changes accompanying the perturbation, chemical modification or denaturation of eukaryotic cytochrome c. Within this framework a wide range of differing degrees of perturbation have been described, from that of

Table 8.1.

Assignments obtained for eukaryotic cytochrome c

Resonance designate and Assignment		Chemical shift	Species	Method
M11	Met 65 CH_3	2.11	ferrocytochrome c (horse and tuna)	chemical modification and redox titration
M10	N-acetyl CH_3	2.07	ferrocytochrome c (horse and tuna)	ditto
M*19	Met 65 CH_3	1.94	ferricytochrome c (horse and tuna)	ditto
M*20	N-acetyl CH_3	1.94	ferricytochrome c (horse and tuna)	ditto
M*6	Ile 57 δCH_3	-0.18	ferricytochrome c	redox titration
	Ile 57 βCH	1.80	ferrocytochrome c	spin decoupling and NOEs
	Ile 57 γCH_3	0.72	ferrocytochrome c	ditto
	Ala 83 αCH	3.90	ferrocytochrome c	ditto
	Ala 83 βCH_3	0.58	ferrocytochrome c	ditto
	Thr 40 βCH	4.54	ferrocytochrome c	ditto
	Thr 40 γCH_3	1.40	ferrocytochrome c	ditto
	Thr 63 βCH	4.46	ferrocytochrome c	ditto
	Thr 63 γCH_3	0.90	ferrocytochrome c	ditto
	Leu 67 CH_3	-0.22	$(Leu)^{67}$,$(Hse)^{65}$ ferrocytochrome c	
	Leu 67 CH_3	0.33	ditto	
A17	Phe 10 o-	7.10	ferrocytochrome c	spin decoupling and NOEs
A25	Phe 10 m-	6.70	ferrocytochrome c	ditto
A27	Phe 10 p-	6.34	ferrocytochrome c	ditto
	Phe 65 o-	7.16	lamprey ferrocytochrome c, spin decoupling	($47^{o}C$)
	Phe 65 m-	7.46	ditto	
	Phe 65 p-	7.29	ditto	

Table 8.1. (cont.)

	Phe 65 o-	6.97	lamprey ferricytochrome c, spin decoupling (47°C)
	Phe 65 m-	7.33	ditto
A*5	Tyr 74 o-	7.62	ferricytochrome c redox titration
A*12	Tyr 74 m-	6.82	ferricytochrome c ditto
A23	Phe 82 o-	6.71	ferrocytochrome c NOEs
A10	Phe 82 m-	7.40	ferrocytochrome c ditto
A13	Phe 82 p-	7.20	ferrocytochrome c ditto
A*18	Phe 82 o-	6.20+	ferricytochrome c redox titration
A*18	Phe 82 m-	6.20+	ferricytochrome c ditto

+, the o- and p- resonances of Phe 82 in ferricytochrome c overlap.

All assignments are given for the proteins at pH 7 and at 57°C, unless otherwise stated.

the increase in the temperature of the bulk medium to that of the complete denaturation by chemical reagents. Each of these facets of the properties of eukaryotic cytochrome c have important implications to the structure (including dynamic features) of the molecule. These will now be discussed in turn.

a) In Section 3.6.1. the reasons are outlined for the interest that has been shown , recently (4), in the region of the molecule close to Ile 57. In brief, it is one site of a conformational difference between ferricytochrome c and ferrocytochrome c, it is in the region of one of the antigenic determinants of the molecule, its resonance in ferrocytochrome c is the only one to show a large temperature dependence and it has unusual pH characteristics. The latter effect has been shown to be attributable to the ionisation of Glu 66 at lower pH and the ionisation of either Lys 39 or Lys 55 at higher pH. Studies of the temperature dependence of the NOEs to and from the δCH_3 resonance of Ile 57 in ferrocytochrome c show that this group moves away from the rings of Tyr 74 and Trp 59 with the increase of temperature, though the actual displacement of atomic coordinates is very small and probably reflects a rotation of the Ile 57 side chain about its C_α-C_β bond. It has previously been shown that the increase of temperature causes no 'swelling' of the molecule (5), as is observed for some other globular proteins (6) and that there are two regions of the protein which possess hindered motion (7,8). Thus the structure of the native protein is a compact and solid configuration; the perturbations near Ile 57 with pH and temperature change are on the surface and do not affect the interior of the molecule.

b) Several differing single site modifications at Met 65, Tyr 67, Tyr 74 and at Met 80, and four modifications of all the lysine residues in the molecule have been studied. Modification of Met 65, a surface

residue, and of all the lysine residues of the molecule, also surface residues, cause only very small perturbations to the molecule, the most detectable of these being a small displacement close to Ile 57. In only one of the lysine modifications, maleyl cytochrome c, are the properties of the molecule radically altered; in this molecule the complete reversal of charges on the surface of the molecule cause it to unfold reversibly with increasing temperature. In both the Met 65 (to $(Hse)^{65}$ or $\left[Met(Cm)^{65}\right]$) and the lysine modifications the charges on the molecule are altered and in the latter case surface bonds and salt bonds are broken (9). The fact that despite this the molecules are still in a native structure except for minor surface perturbations shows that the surface structure is not a major factor in the determination of the structure of the molecule.

Tyr 74 may be considered as a residue on the boundary of the surface and the interior of the molecule and Tyr 67 as a residue in the haem crevice of the molecule. Two modifications have been described where these residues are replaced by a less bulky leucine residue. In both cases this causes no major disruption of the molecule, presumably because it does not perturb the hydrophobic core drastically. It is worth contrasting these results with that of the replacement of Tyr 67 with a more bulky nitrotyrosine residue; Myer et al (10) have shown this to cause large disturbances to the structure and properties of the molecule, and illustrates the extremely fine criteria that exist for the molecular packing of the haem crevice.

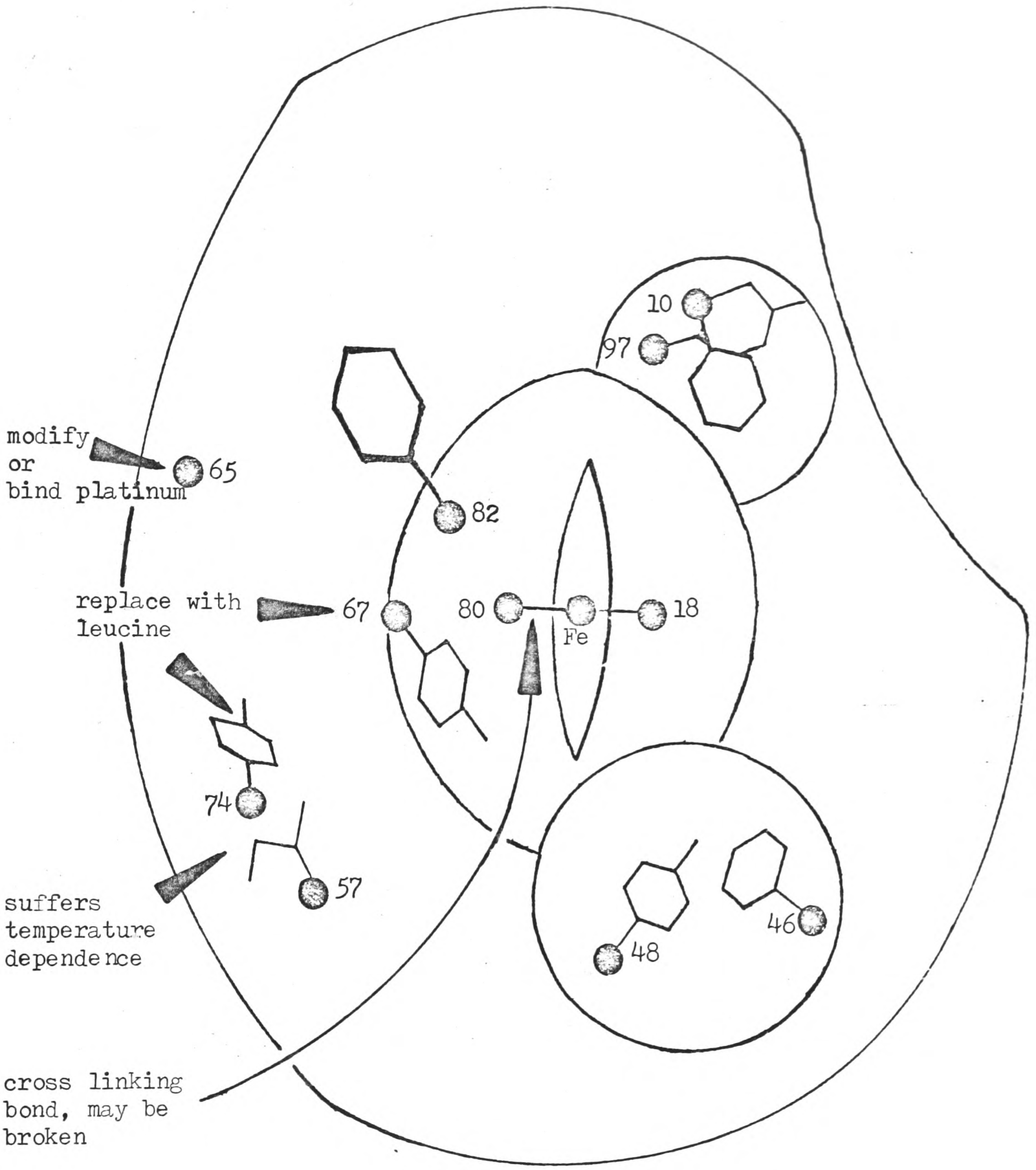
c) So far the studies have shown that the cytochrome c molecule may be modified on its surface without drastic effect and that the important factor in the stability of the molecule is the packing in the hydrophobic core of the molecule. One way to cause a large perturbation of the molecule is, therefore, to disrupt the haem crevice. In this

thesis, experiments are described where this is achieved in two differing ways; either the cross linking iron - sulphur bond is modified or the protein is denatured with chemical denaturant. In the latter case, as the hydrophobicity of the solvent is increased the molecule becomes less stable due to the charged groups on its surface and finally it rearranges to a more stable structure. One important result from the study is that the pathways of denaturation are different for ferricytochrome c and ferrocytochrome c. This difference largely results from the haem crevice being extra stabilised in ferrocytochrome c by the stronger iron-sulphur bond. The haem crevice for ferricytochrome c is disrupted more easily and an intermediate, state IV ferricytochrome c, is produced; in this species many elements of the haem crevice are preserved and further hydrophobicity of the solvent is required to produce an unwound protein.

Cytochrome c-CN is a good model for the penultimate species in the refolding pathway of cytochrome c. The studies, here, show that this species has a haem crevice with many similar properties to that of the native protein, and that much of the structure on the His 18 side of the protein is preserved. This, again, illustrates the importance of the packing of the core of the molecule; as the protein refolds a 'cytochrome c like' molecule is produced where much of the molecular packing is already complete; the final step is the coordination of Met 80 as sixth ligand and a rearrangement to enable this.

Fig. 8.1. shows the cytochrome c molecule with the sites of modification indicated, and Table 8.2. gives a summary of the chemical modifications studied. The overall view of the cytochrome c molecule, described here, is compatible with that required for the biological function of cytochrome c. The molecule is stable with a well defined peptide backbone, the iron atom that carries the extra electron is well insulated from the external medium by the extremely well packed

Fig. 8.1.



A schematic diagram (based upon Fig. 2.2.) of eukaryotic cytochrome c showing some of the sites of modifications, which are described in this thesis. The sites of monodeamidation and the locations of lysine residues (Section 4.3. and Chapter 5) may be best appreciated from Fig. 4.12.

Table 8.2.

Modifications and Perturbations of eukaryotic cytochrome c

Method	Summary of effect	Section of thesis
Temperature increase	Perturbation to area of surface, close to Ile 57, Trp 59, and Tyr 74. Studied by NOEs between proton resonances of these groups.	3.6.1.,3.6.2.,3.6.3.
pH variation between 3.5 and 12 in ferrocytochrome c	Small perturbations to some surface residues. Large effect to resonance of Ile 57 due to ionisation of Glu 66 at low pH and of Lys 39 or Lys 55 at high pH.	3.6.4.,3.6.5.,5.4.
Substitution of Met 65 by Phe 65.	Phe 65, a hydrophobic residue, in Lamprey cytochrome c appears to occupy a similar position to that of Met 65 in other cytochromes c, i.e. on the surface. The helix 61-69 is not altered.	3.5.
Modification of Met 65 by protein semisynthesis, (Hse), and by carboxymethylation)	Resonances due to Ile 57, Leu 68, Trp 59, and Tyr 74 are affected. All lie close to Met 65, and the effect may be transmitted through the Glu 66 - Tyr 74 hydrogen bond. The iron - sulphur bond is substantially weakened in the oxidised species.	4.2.1.,4.2.2.
Binding of platinum complexes to Met 65	Small local effect similar to the above. Anomalous shift to the resonance of Met 65.	4.2.3,4.2.4.
Semisynthetic replacement of Tyr 67 or Tyr 74.	These residues may be replaced by less bulky Leu groups. The modifications cause only small perturbations to the structure.	4.2.5.
Monodeamidated cytochromes c	Changing the charge of two surface residues (Asn 103 and Gln 16 ??) causes only very small local effects.	4.3.
Modification of lysine residues	Full modification of the 19 Lys residues in horse cytochrome c causes only a small effect to the structure of the molecule; the region close to Ile 57 is affected.	Chapter 5

Table 8.2 (cont.)

Maleyl cytochrome c	Maleylation of all the lysine residues in cytochrome c gives rise to a species that may be reversibly unfolded with the increase of temperature. This result is also reported by Schejter et al (1).	5.3.
Modification of the iron-sulphur bond at the coordination centre.	The breaking of this bond gives a species that is reorganised on the Met 80 side of the molecule; however the haem crevice and rest of the molecule is preserved on the His 18 side of the molecule. The modified molecule approximates to the penultimate state in the refolding of cytochrome c.	Chapter 6
Chemical denaturation of cytochrome c.	Urea and methanol unfold the protein with very similar pathways. The pathway is different, however, for the oxidised and reduced proteins. Oxidised cytochrome c unfolds via an intermediate which is State IV ferricytochrome c; no equilibrium intermediates are detected for ferrocytochrome c.	Chapter 7

hydrophobic core of the protein. The electron can only be accepted from or donated to a biological partner in a reaction of well defined specificity.

References: Chapter Eight

1. Schejter, A., Zuckerman, M., and Aviram, I. (1979), J.Biol.Chem., 254, 7042.

2. Moore, G.R., and Williams, R.J.P. (1980), Eur.J.Biochem, 103, 493.

3. Moore, G.R., and Williams, R.J.P. (1980), Eur.J.Biochem, 103, 503.

4. Moore, G.R., and Williams, R.J.P. (1980), Eur.J.Biochem, 103, 523.

5. Moore, G.R., and Williams, R.J.P. (1980), Eur.J.Biochem, 103, 533.

6. Williams, R.J.P. (1979), Biol.Rev., 54, 389.

7. Campbell, I.D., Dobson, C.M., Moore, G.R., Perkins, S.J., and Williams, R.J.P. (1976), FEBS Letters, 70, 96.

8. Keller, R.M., and Wuthrich, K. (1981), accepted for publication.

9. Osheroff, N., Borden, D., Koppenol, W.H., and Margoliash, E. (1980), J.Biol.Chem., 255, 1689.

10. Pal, P.K., Verma, B., and Myer, Y.P. (1975), Biochemistry, 14, 4325.

A P P E N D I X A

Basic theory and techniques of nuclear magnetic resonance spectroscopy

i) The proton magnetic resonance effect

All electrons and some nuclei possess a property called 'spin' (1). The spin of a nucleus is usually given by the symbol, I, called the spin quantum number (1). This thesis is concerned with the hydrogen nucleus or proton, which has a spin of $I=\frac{1}{2}$. Quantum mechanics shows that the angular momentum of a nucleus is given by

$$\hat{I} = \sqrt{I.(I+1)} \; . \; (h/2\pi) \qquad (1.)$$

and is a vector quantity. The magnetic moment or dipole of the nucleus is then given by

$$\hat{\mu} = q.h.\hat{I} \; / \; 4\pi.m \qquad (2.)$$

and measured in units of $A.m^2$ or $J.T^{-1}$ (Joules per Tesla). Because the nuclei can not be considered a point charge equation 2 has to be modified to

$$\hat{\mu} = g.q.h.\hat{I} \; / \; 4\pi.m \quad , \qquad (3.)$$

which is normally simplified to

$$\hat{\mu} = g.\beta_N.\hat{I} \qquad (4.)$$

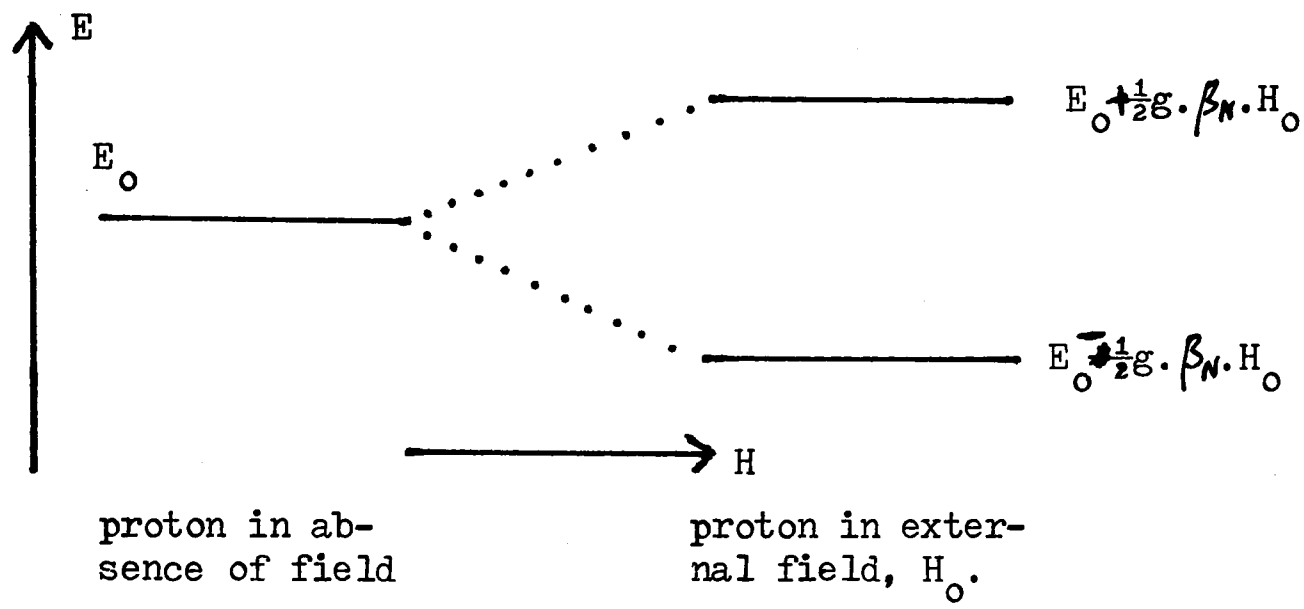
where β_N is the 'nuclear magneton', and the value for the proton given by equations 3 and 4 is

$$\beta_N = e.h \; / \; 4.m_p.\pi \; = 5.050 \times 10^{-27} \; J.T^{-1}$$

and g is the 'nuclear g factor' and is experimentally determined for the proton as g = 5.585.

When the nucleus is placed in a magnetic field of strength, H_o, there is an interaction between the field and the magnetic dipole of magnitude $\mu_z.H_o$, where μ_z is the component of the dipole along the reference direction (that, along which the magnetic field is applied). For a

nucleus of spin $I=\frac{1}{2}$, $\hat{I}_z$ is equal to $\frac{1}{2}$ or $-\frac{1}{2}$ and hence in the presence of an external magnetic field there are two energies allowed to the dipole. The concept is shown figuratively in the diagram, below.



Theoretically there is a transition possible between the two energy levels of the nuclear magnetic dipole, when it is in the magnetic field. The energy of this transition is given by

$$\Delta E = (E_o+\tfrac{1}{2}g.\beta_N H_o) - (E_o-\tfrac{1}{2}g.\beta_N H_o)$$

and has a characteristic frequency

$$\nu_o = \Delta E/h = g.\beta_N H_o / h \qquad (5.)$$

The detection of an absorption of energy at this characteristic frequency is the basis of nuclear magnetic resonance spectroscopy. The effect was predicted by Pauli (2) and was first detected by Bloch in 1948 (3).

At this point we may calculate the frequency of a resonance of an isolated proton using the values of g and β_N already quoted and in a magnetic field of 6.34 Tesla (the magnetic field strength of the superconducting magnet used for some of the experiments in this thesis) using equation 5.

$$\nu_o = g.\beta_N H_o / h = \frac{5.585\times5.05\times10^{-27}\times6.34}{6.63\times10^{-34}} = 270\times10^{6} \text{ Hz}$$

Thus the appropriate frequency for studying protons with such a magnetic field is 270 MHz and falls in the short wave radio frequency region.

ii) The parameters obtained in proton nuclear magnetic resonance spectroscopy

If a molecular substance, such as a protein, containing protons is placed in a magnet of fixed field strength and a broad band of radio-frequency radiation of suitable energy is applied to the sample it should be possible to detect absorptions of the radiation at certain frequencies by a suitable experimental apparatus and record a proton magnetic resonance spectrum. Such a spectrum would contain a number of 'resonances' corresponding to protons absorbing at different frequencies and each resonance would be defined by a number of parameters (4). These parameters are the chemical shift, δ; the intensity, I; the spin-spin coupling constant, J; and two relaxation times, T_1 and T_2; and these will now be considered in turn.

a) The chemical shift, δ: The absorption of energy of an isolated proton is modified when the proton is found in a molecule, because the electronic structure around the proton causes magnetic shielding of the nuclear spin by nature of its own interaction with the external magnetic field. Equation 5 becomes modified to

$$\nu_o = g.\beta_N.H_{eff} / h \text{ , where } H_{eff} = H_o (1 - \sigma). \quad (6.)$$

H_o is the applied magnetic field and H_{eff} is the effective shielding at the nucleus, and σ is the shielding constant. Absorptions over a range of frequencies are possible for protons found in different chemical environments, and a scale of frequencies can be calibrated with reference to a standard. This scale is independent of the magnetic field strength. This is the chemical shift, δ, scale and measured in parts per million (ppm), so that

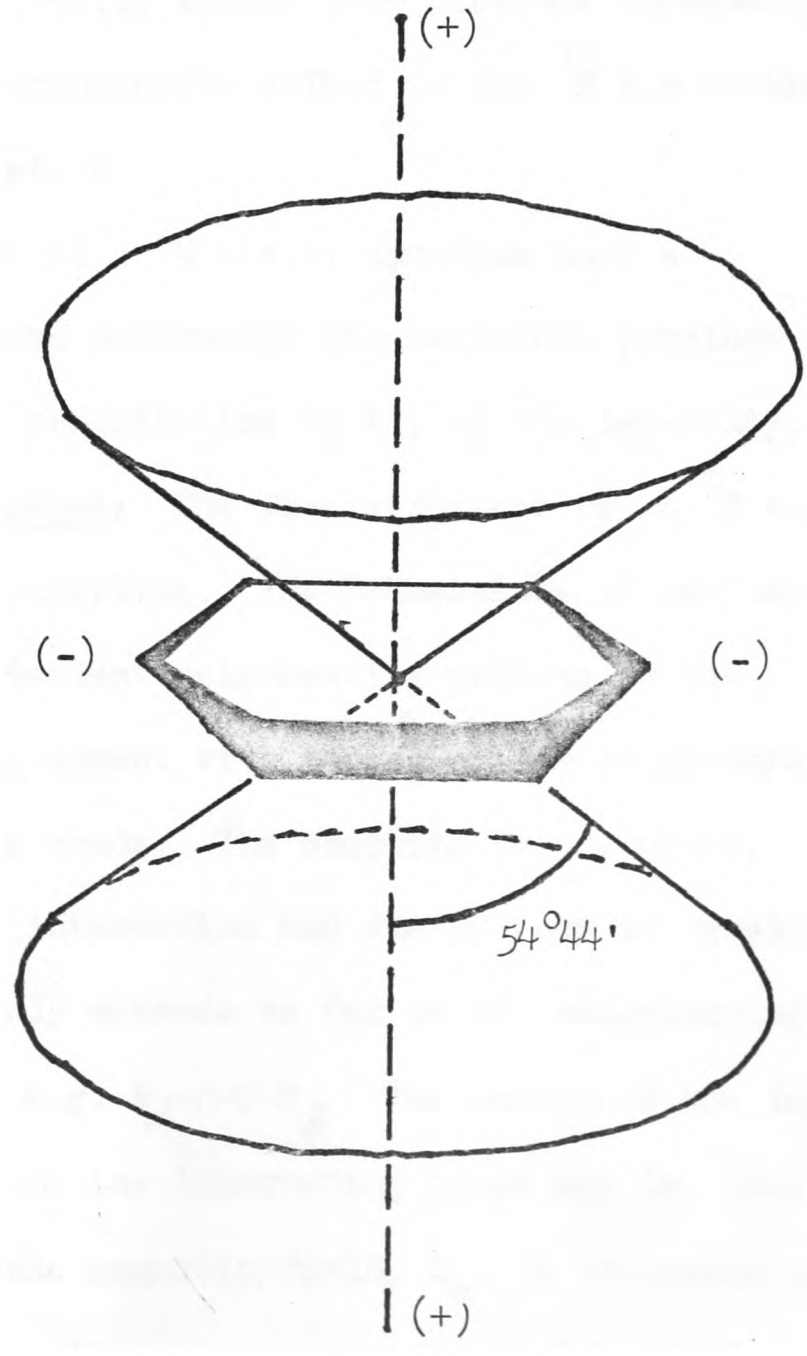
$$\delta(\text{ppm}) = \frac{H_{eff}(\text{ref}) - H_{eff}(\text{sample proton})}{H_o}$$

which can be rearranged to

$$\delta(\text{ppm}) = \frac{\nu(\text{ref}) - \nu(\text{sample proton})}{\nu_o} \quad (7.)$$

where v_o is the spectrometer frequency as determined in equation 5. All chemical shifts in this thesis are quoted in ppm downfield from a Tetramethylsilane (TMS) reference. For diamagnetic molecules almost all the resonances for protons lie in the range 0 - 10 ppm.

There are two types of perturbations to the chemical shift of a resonance which are relevant to discussions of the ^1H n.m.r. spectra of cytochrome c; these are the diamagnetic ring current effect and the paramagnetic effect of the ferric ion in ferricytochrome c. The delocalised electrons of the aromatic rings of phenylalanine, tryptophan, tyrosine, histidine residues or the haem group generate an anisotropic local magnetic field, which is illustrated by the ring current field for a benzene ring, which is shown below.



Ring - current field for a benzene ring. The current produces a local magnetic field that opposes the externally applied field above and below the plane of the ring, but that reinforces it elsewhere. The cone separates the shielding (+) and deshielding (-) regions. The additional chemical shift given to a resonance of a proton found close to the ring is given by

$$\delta_{rc} \propto (3.\cos^2\theta - 1) / r^3$$

The effects of ring current fields lead to secondary shifts (δ_{rc}) for resonances in the ^1H n.m.r. spectrum of cytochrome c, which may provide useful structural information. The paramagnetic effects to proton resonances in a protein containing paramagnetic centres (such as metal ions) are two fold; these are the two mechanisms, the contact shift, and the pseudocontact shift. The contact shift is a 'through bonding effect', while the pseudocontact shift is the result of a dipolar interaction through space. The magnitude of the pseudocontact shift for a proton resonance in the field of a rhombic metal ion, as in ferricytochrome c, is given below:

$$\delta_{pc} = D\,(3.\cos^2\theta - 1) / r^3 + D'\,(\sin^2\theta.\cos 2\omega / r^3)$$

where D and D' are constants. For axial symmetry D' reduces to zero and pseudocontact shifts may be easily turned into distance information (5). For full account of the paramagnetic effect to the ^1H n.m.r. spectrum of ferricytochrome c see ref. 6.

b) The intensity, I: Resonances in a ^1H n.m.r. spectrum have a Lorentzian lineshape, and the area underneath the resonance provides a measure of the number of nuclei contributing to it, or the intensity.

c) The spin - spin coupling constant: The fine structure of an ^1H n.m.r. spectrum arises from spin - spin coupling. The transitions of one proton are coupled to those of non equivalent neighbouring protons by the coupling of its nuclear magnetic moment with those of its neighbours via electrons in the intervening bonds. The coupling is therefore, through bond', and is a scalar interaction and for protons is rarely larger than 10 Hz; it usually only extends as far as the neighbouring protons linked through 3 bonds, e.g. H_α-C-C-H_β. The energy of the interaction depends upon the nature of the intervening bonds and is, thus, independent of the strength of the magnetic field, H_o. A resonance where such coupling is present has more than one component and is called a 'multiplet'. The multiplicity of a resonance is dependent upon the

neighbouring protons and their spins; for a proton with n neighbours in equivalent environments the multiplicity is (n+1) and the intensity of the individual components is given by the binomial coefficients for the number n. The interaction is removed, when one (or more) of the nuclei is caused to undergo very rapid transitions between its own energy levels. This may be achieved by the irradiation at the frequency of the neighbouring proton(s), and is the basis of the spin decoupling technique, which is a very powerful method for relating resonances from the same amino acid residue in protein ^1H n.m.r. spectra.

d) The two relaxation times, T_1 and T_2: Under equilibrium conditions for a sample of nuclei in a constant magnetic field there will be a small excess of nuclei in the lower energy level (as determined by the Boltzmann distribution, typically ca. 1 proton for every 10^5 protons distributed between the two states). The n.m.r. method is insensitive compared to other spectroscopic methods due to the small number of transitions that may occur, and for 'saturation' of the energy levels not to occur it relies upon excited nuclei returning to the lower energy level sufficiently rapidly under the experimental conditions. Spontaneous reemission is slow at radiofrequencies, but there are two relaxation mechanisms open to the excited nuclei; these are spin-lattice relaxation and spin-spin relaxation. Each resonance in an n.m.r. spectrum is characterised by a spin-lattice relaxation time (T_1) and a spin-spin relaxation time (T_2). Spin-lattice relaxation of a nucleus is induced by the rapidly fluctuating magnetic fields of neighbouring nuclei that are undergoing rapid thermal motion, and T_1 describes the average lifetime of the excited nucleus before it dissipates its energy as thermal energy to the other nuclei in the sample. This acts directly to maintain an excess of nuclei in the lower energy level. Spin-spin relaxation limits the lifetime of an excited nucleus by mutual exchange of the orientation of its nuclear

magnetic dipole with a neighbouring nucleus of the same kind; this is a process of energy redistribution within the spin system, but does not help maintain an excess of nuclei in the lower energy level.

All the processes which cause spin lattice relaxation also cause spin spin relaxation and there are usually further mechanisms of spin spin relaxation that do not affect the populations in the energy levels and so $T_2 \leqslant T_1$. The resonance linewidth is related to these relaxation times since, on average, a nucleus remains in the excited state for no longer than the time T_1. Thus it follows from the Heisenberg Uncertainty principle that

$$\Delta\nu_{\frac{1}{2}} \geqslant \frac{1}{\pi T_2} \geqslant \frac{1}{\pi T_1}$$

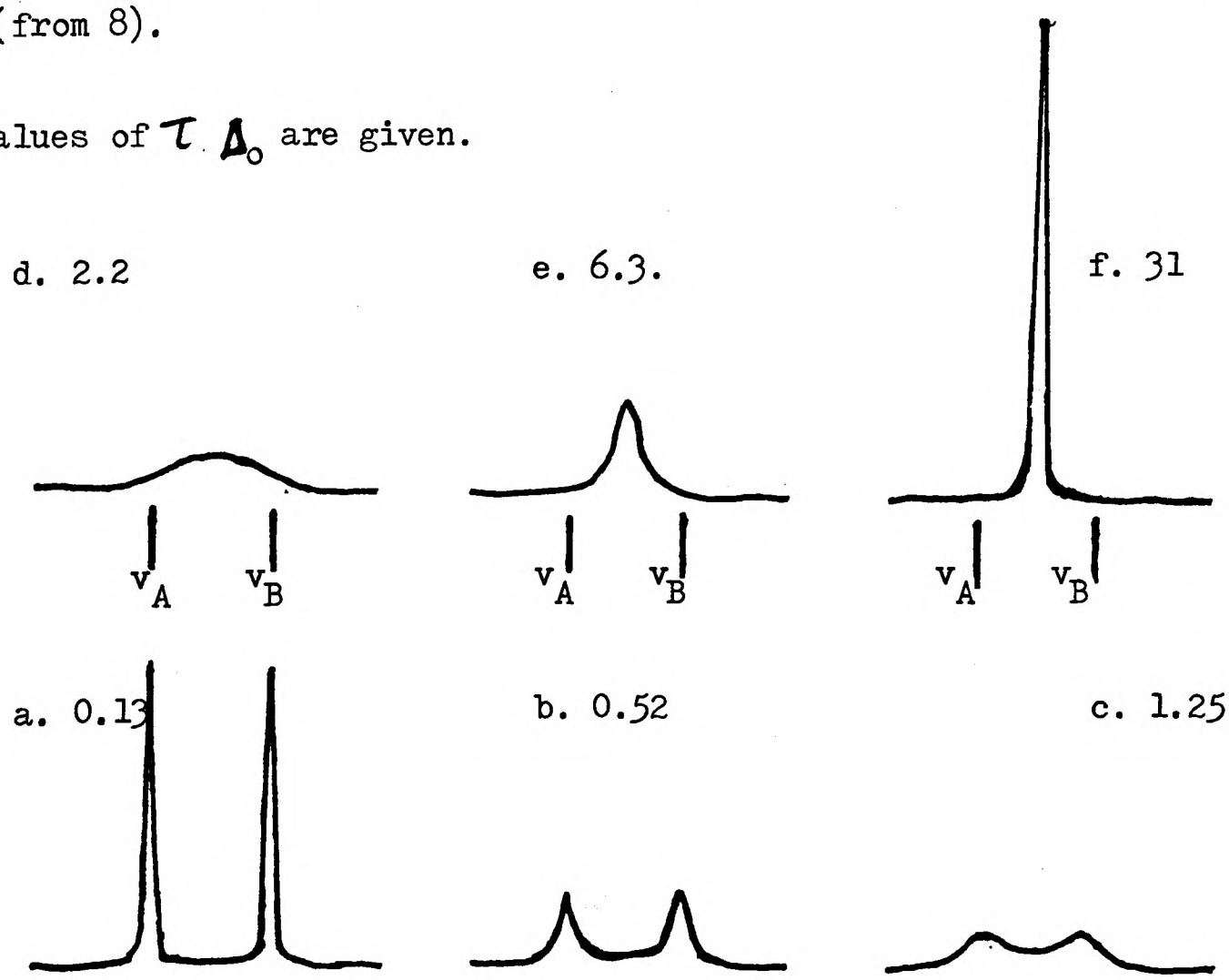
where $\Delta\nu_{\frac{1}{2}}$ is the resonance linewidth in Hz at half the peak height. Both relaxation mechanisms are directly influenced by molecular motion; rapid thermal motion in liquids leads to long spin lattice relaxation times (T_1) and to a lessening of the time during which two nuclei remain in phase (the correlation time, τ_c) such that they may exchange their spin orientation (spin spin relaxation) giving rise to large T_2 values. In an ideal situation $\Delta\nu_{\frac{1}{2}} = \frac{1}{\pi T_2}$ but other factors contribute to resonance linewidths, such as exchange of nuclei between non-equivalent environments.

When the spin-lattice relaxation of one nucleus is dipolar coupled to that of a second nucleus, the saturation of the energy levels of nucleus A will perturb the equilibrium distribution of nucleus B, leading to a change in the intensity of its resonance. This is the Nuclear Overhauser effect, and since it arises from a dipolar mechanism its magnitude is dependent upon the nuclear separation ($I\,(NOE) \propto r^{-6}$) and so it is a technique of potential use for providing data on internuclear distances (7).

iii) Exchange processes

If a given nucleus can exchange between two magnetically inequivalent sites, A and B, in which it has resonance frequencies, ν_A and ν_B, and lifetimes at each site, τ_A and τ_B, respectively, then the n.m.r. spectrum of the nucleus may be drastically altered from what is found for the nucleus at one site. We will assume that the nucleus has an equal probability of being in each site and so has an identical lifetime in each site, i.e. $\tau = \tau_A = \tau_B$, and describe the types of exchange that may occur under these conditions; these are illustrate in the diagram below, which shows the lineshapes for various values of exchange lifetime relative to the difference in frequency between the two equally populated site (from 8).

The values of $\tau\,\Delta_o$ are given.



In very slow exchange the lifetime at each site is so long that if a given nucleus is at site A it will absorb radiofrequency and relax back to its ground state many times before it leaves site A and enter site B (e.g. a.). In fast exchange the nucleus exchanges between site A

and site B with a rate that is fast to $(\nu_A - \nu_B)$, or Δ_o, measured in Hz, and under these conditions it experiences the average of the two environments and a single resonance is observed at resonance frequency (e,g, f.) $\nu_e = \frac{1}{2}(\nu_a + \nu_B)$. Between these limiting cases a range of exchange rates lead to differing varieties of intermediate types of spectra (e.g. b.,c., d., and e.) The approximate definitions and conditions of these types of exchange are:

a) Slow exchange $\tau.\Delta_o \gg 1$

b) Intermediate exchange $\tau.\Delta_o \approx 1$

c) Fast exchange $\tau.\Delta_o \ll 1$

When there is non-equal occupation of the two sites (i.e. the nucleus spends f_A and f_B amounts of its times at sites A and B respectively) under the fast exchange condition then the resonance is observed at the weighted average of its frequencies at the two sites, i.e. $\nu_e = f_A\nu_A + f_B\nu_B$. An example of this type of fast exchange exchange situation is the redox titration of cytochrome c described in Chapter 3; as the titration proceeds f_A increases from 0 to 1, and f_B vice versa.

The phenomenum of exchange leads to the broadening of the resonances between the two limits of very slow exhange and very fast exchange. For slow exchange the linewidth is given by

$$\left(\frac{1}{T_2}\right) = \left(\frac{1}{T_{2A}}\right) + \left(\frac{1}{\tau_A}\right)$$

where $(1/T_2)$ is the observed linewidth, and $(1/T_{2A})$ is the linewidth at site A with no exchange. For the fast exchange suituation the linewidth is

$$\left(\frac{1}{T_2}\right) = \left(\frac{f_A}{T_{2A}}\right) + \left(\frac{f_B}{T_{2B}}\right) + \frac{4\pi^2.f_A.f_B.(\Delta_o)^2}{\left(\frac{1}{\tau_A}\right) + \left(\frac{1}{\tau_B}\right)}.$$

iv) The Fourier transform method

An n.m.r. spectrum of a sample may be recorded in several ways, the two best known ways being the frequency continuous wave technique, and the Fourier transform method. In the first method the spectrometer sweeps through the appropriate range of frequencies and an absorption spectrum is recorded. In the Fourier transform method, a powerful pulse of radio frequency radiation is applied to the sample for a short time (ca. 5 S), which excites the nuclei to a non-equilibrium state. After the pulse the collection of nuclei relax towards their equilibrium state and a 'free induction decay' may be recorded; in practice many free induction decays are recorded and summed up to average out the noise. The free induction decay is of the form, $f = \left[\sin (\nu_i - \nu_o).t\right] . \exp(-t/T_{2i})$, where ν_i is the frequency of the i^{th} nucleus, ν_o is the frequency of the applied pulse, and T_{2i} is the T_2 of the i^{th} nucleus. Thus this free induction decay contains the same information as the conventional continuous wave spectrum, the resonance frequencies (ν_i) and the linewidths ($\propto 1/T_{2i}$) for all the nuclei in the sample. The data may be turned into a conventional spectrum by the process of Fourier transformation performed by a computer, usually built into the spectrometer.

This technique has several advantages. A signal to noise improvement of a factor of 10 for a given spectral collection time is easily achieved, which corresponds to a saving in time of a factor of 100. This has extended greatly the range of concentrations which it is feasible to study (e.g. down to 10^{-3}M with protons), and it also makes it possible to study nuclei as ^{13}C in natural abundance. Information is obtained from the whole spectral region simultaneously, whereas in continuous wave spectroscopy only a small part of the spectrum is inspected at one time. This has led to a large number of sophicated techniques involving pulse sequences for Fourier transform spectroscopy, which are not available

in continuous wave spectroscopy. Lastly, mathematical functions may be applied to the collected free induction decay, to increase the signal to noise level, or to increase the resolution (e.g. multiplication with a Gaussian function (9); these functions are readily available in the computer software of modern spectrometers.

v) Double resonance and other techniques

Any number of pulses may be applied to a sample in a Fourier transform spectrometer, before or during the collection of the free induction decay, in practice the only restrictions imposed are due to the limitations of the spectrometer. In double resonance experiments two radiofrequency pulses are applied to the system. Three main types of double resonance experiment are used in the work of this thesis.

An important technique is the presaturation of the solvent resonance (usually ^{2}HOH in this study); usually the solvent resonance is irradiated selectively for a long period (0.5 seconds or more) before the observation pulse is applied to all the protons in the system and the free induction decay is collected. By adjusting the power of the pulse on the solvent the optimum spectrum may be achieved, where the solvent resonance is nearly removed (so that it is less intense than the largest other resonance in the spectrum) and the rest of the spectrum suffers the least perturbation possible. The saturation of the solvent resonance can lead to an enhancement of the signal to noise as the removal of a large resonance from the signal means that the sensitivity of the instrument may be turned up without overloading the ~~eletronics~~ electronics or the computer memory.

The Nuclear Overhauser effect has previously been mentioned in this appendix; it is the change in intensity of a resonance caused by the saturation of another resonance, when the protons giving rise to

the resonances are dipolar coupled. It is usually observed by applying a gated irradiation pulse to a resonance prior to accumulating the free induction decay. Under conditions where spin diffusion (10) does not occur it may be expected to be observed between spin coupled resonances and resonances of adjacent groups.

Spin decoupling has also been mentioned earlier in this appendix; if a multiplet resonance is irradiated with a time-shared pulse, resonances previously coupled to it behave as if they are not longer coupled. Time-shared irradiation is the application of a selective irradiation pulse during the collection of the free induction decay.

As stated earlier many pulse sequences are now available in Fourier transform spectroscopy. One very useful application of these pulse sequences is that of the simplication of spectra on the basis of the multiplet structure of the resonances. Such a technique used in this thesis is the recording of spin echo spectra, or Carr-Purcell A spectra. In a Carr-Purcell A spectrum singlets and triplets appear above the baseline and doublets and quartets below the baseline; decoupling may also be performed in the Carr-Purcell A sequence and this is called spin echo double resonance (SEDR). For example, if a doublet is decoupled to a singlet, the resonance appears below the baseline in a Carr-Purcell A spectrum with irradiation off the resonance it is coupled to and above the baseline with irradiation on the correct resonance.

For nuclear Overhauser, spin decoupling and SEDR experiments it is often useful to record difference spectra and sometimes automatic difference spectra. Both may be easily carried out on modern spectrometers and ease the interpretation of data acquired in regions of spectra when there is resonance overlap. Many examples of difference spectra are given in this thesis.

References: Appendix A

1. Pauling, L., and Wilson, E.B. (1935), 'Introduction to Quantum Mechanics', McGraw-Hill Book Co., New York.

2. Pople, J.A., Schneider, W.G., and Bernstein, H.J. (1959), 'High Resolution Nuclear Magnetic Resonance', Mc-Graw-Hill Book Co., New York.

3. Bloch, F. (1946), Phys. Rev., 70, 460.

4. Carrington, A., and McLachlan, A.D. (1967), 'Introduction to Magnetic Resonance', Harper and Row, New York.

5. Barry, C.D., Martin, D.R., Williams. R.J.P., and Xavier, A.V. (1974), J.Mol.Biol., 84, 491.

6. Moore, G.R., D.Phil thesis (1978), Oxford.

7. Noggle, J.H., and Schirmer, R.E. (1971), 'The Nuclear Overhauser effect', Academic Press, New York.

8. Becker, E.D. (1969), 'High Resolution NMR', Academic Press, New York.

9. Ernst, R.R. (1966), Adv.Mag.Res., 2, 1.

10. Kalk, A., and Berendsen, H.J.C. (1976), J.Mag.Res., 24, 343.

A P P E N D I X B

Materials and Methods

i) General

Cytochromes c from horse (Sigma, type VI), tuna (Sigma, type XI), pigeon (Sigma, type XIV) and Candida krusei (Sigma, type VII) were obtained from the Sigma Chemical Co., Lamprey cytochrome c was a gift from Dr. R. Wever, University of Amsterdam, Holland.
The modified cytochromes c were obtained and prepared as described below.

All the protein samples were obtained in a semi pure form as lyophilised powders, predominantly in the oxidised forms, unless otherwise stated. In order to obtain the proteins in a pure form and to remove small molecules and ions, such as sodium chloride, acetate and ferricyanide, the lyophilised proteins were dissolved in double distilled H_2O (typically 0.1 g. of protein in 0.5 ml. of H_2O and adjusted to pH 7) and oxidised with $K_3Fe(CN)_6$ before being run down a Pharmacia K9/30 column (bed dimension 0.9 ✖ 30 cms., volume ca. 20mls.) packed with Sephedex G25 at 4°C. The flow rate down the column was adjusted so the cytochrome c was eluted ca. 15 minutes after the application. The eluate was lyophilised and the powder stored at 4°C.

Solutions of these powdered proteins were prepared for ^1H n.m.r. studies by dissolution of weighed quantities of the lyophilised solids in the required amount of 99.8% ^2H_2O (typically ca. 0.4 ml). These solutions were adjusted to the required pH (unadjusted for isotope effects (1)) with additions of ^2HCl and NaO^2H solutions in a range of concentrations (10^{-3}M, 10^{-2}M, 10^{-1}M and 1M). These solutions were placed in n.m.r. tubes. To produce n.m.r. samples of the reduced proteins the solutions were reduced in the n.m.r. tubes with sodium ascorbate or sodium dithionite (typically a 0.1M solution at the same pH as the protein

and prepared immediately prior to use) and the n.m.r. tubes were flushed under a stream of Argon for ca. 20 minutes and sealed under a suba seal.

ii) Preparation of the modified cytochromes c

Tuna $\left[\text{Met(Cm)}^{65}\right]$ ferrocytochrome c (50 mgs.) was prepared from tuna ferrocytochrome c by an adaption of the method of Ando et al (2,3). for bovine cytochrome c. To ensure that Met 80 remained unmodified, the reaction with iodoacetic acid was performed at pH 5.6 (2,3). The modified protein was purified at 4°C by passage down a column of Sephedex G25 equilibrated with 0.1M NaCl followed by dialysis against three changes (ca. 1000 ml) of double distilled water. The resulting solution was freeze dried and stored at 4°C.

Horse $(\text{Hse})^{65}$ cytochrome c was prepared in accordance with published proceedures (4,5). Ferricytochrome c (approx. 200 mgs.) was treated with a three-fold excess of CNBr in accordance with the method of Corradin and Harbury (6), and the mixture of peptide fragments was filtered on a column (2 × 135 cm) of Sephedex G50 (fine grade) in 7% formic acid. The pure preparations of fragments 1-65 and 66-104 were freeze dried, and the fragments were redissolved in 0.1 M potassium phosphate buffer at pH 7.0, such that the resulting solution was 0.1M in each. The mixture was reduced and maintained in the reduced state for 24 hours as described by Wallace and Offord (1979), and the solution was then filtered on a Sephedex G-50 (fine grade) column. The first peak to emerge was resynthesised $(\text{Hse})^{65}$ ferrocytochrome c. After being freeze dried the protein was redissolved in 0.1M potassium phosphate buffer, pH 7.0, made in 8M urea, in accordance with the proceedure of Boon et al (4), and filtered through a Sephedex G25 column (1.5 × 30 cm.) in 0.1M phosphate buffer at pH 7.0. The haem containing peak was loaded on to a CM-cellulose 23 column (1.5 × 30 cm.) and eluted as a single peak with a 0.1-0.2M potassium phosphate gradient at pH 7.0. The resulting solution contained pure $(\text{Hse})^{65}$ cytochrome c

(final estimated purity greater than 95%). $K_3Fe(CN)_6$ was added to ensure complete oxidation, or ascorbic acid was added to ensure complete reduction. Samples for n.m.r. were prepared by using an Amicon ultrafiltration cell fitted with a UM10 membrane. Four cycles of concentration and dilution with ^2H_2O at pH 7.0 were sufficient to eliminate 99.9% of H_2O and avoided further freeze drying of the sample. The final volume of the sample was 0.4 ml. and the protein concentration was 5×10^{-3}M. This sample was then placed in an n.m.r. tube and stored at 4°C. All the initial steps in preparing the $(Hse)^{65}$ cytochrome c were carried out by Dr. C.J.A. Wallace, Molecular Biophysics, Oxford.

Samples of $(Leu)^{67}$, $(Hse)^{65}$ cytochrome c and $(Leu)^{74}$, $(Hse)^{65}$ cytochrome c were loaned, prepared and ready for use in n.m.r. tubes, to Dr. G. Moore and myself by Drs. P. Boon, G.I. Tesser, and R.J.F. Nivard. Further details of their preparation are given in ref. 4.

The sample of monodeamidated cytochromes c was a kind gift from Dr. C.J.A. Wallace. The sample was prepared by the gel electrophoresis of horse cytochrome c (Type III, Sigma) in a semi pure form, which contains ca. 10% monodeamidated forms.

The four fully modified lysine derivatives were prepared by Dr. D.E. Harris, Molecular Biophysics, Oxford, according to the following proceedures. Fully guanidated cytochrome c was prepared by the reaction of O-methylisourea hydrogen sulphate with cytochrome c according to the method of Hettinger and Harbury (7); the reaction was carried out for 400 minutes at room temperature (8). The solution of modified protein was desalted by gel filtration of a Sephedex G50 (fine grade) column equilibrated in 4×10^{-2}M potassium phosphate at pH 7.2. The product was finally purified by passage down a cation exchange column (SP-Sephadex C-25) and freeze dried. An amino acid analysis showed that the extent of guanidation was greater than 98%. Fully N^{ε}-acetimidylated cytochrome c was prepared

by the reaction of methyl acetimidate.HCl with cytochrome c according to the method outlined by Wallace and Offord (5). The same purification proceedures were applied as for guanidated cytochrome c, and an amino acid analysis showed that acetimidylation was greater than 98%. Trifluoroacetylated cytochrome c was prepared by the method of Fanger and Harbury (9), in which cytochrome c is reacted with S-ethylthioltrifluoroacetate. After purification an amino acid analysis showed that trifluoroacetylation was greater than 95%. Maleyl cytochrome c was prepared by the reaction of maleic anhydride with cytochrome c under the conditions used by Pettigrew (10). The protein was purified by binding to an anion exchange column and an amino acid analysis showed that maleylation was greater than 99%.

Carboxymethyl- (Cm-) cytochrome c was prepared by a method based upon the original preparation by Schejter and George (11,12). A solution of 10^{-1}M NaCN, 2×10^{-1}M iodoacetic acid and 10^{-1}M potassium phosphate was made up at pH 7.0. 50 mgs of horse ferricytochrome c (Sigma, type VI) was dissolved in 5 ml. of this solution. The protein exists as ferricytochrome c-CN in this solution, so that both Met 65 and Met 80 are modified. The solution was left for 24 hours at room temperature, by which time the reaction is complete (11). The solution was then dialysed against 2×10^{-2}M potassium phosphate (3 changes, at pH 7), and then passed down a Sephedex G25 column (0.9×30 cms.) to remove the phosphate buffer. The solution was freeze dried. From this powdered solid n.m.r. samples of Cm-ferricytochrome c-CN and Cm-ferrocytochrome c-CN were prepared. For the former the required amount of weighed solid was dissolved in ^2H_2O at pH 7 and a 1 eq. excess of 1M NaCN in ^2H_2O at pH 7 was added; the resultant solution was placed in an n.m.r. tube. Because of the susceptibility of Cm-ferrocytochrome c-CN to autooxidation n.m.r samples of this species were made by flushing out an n.m.r. tube of

the oxidised species with Argon and sealing it under a subaseal. Then a 2 eq. excess of sodium dithionite(2×10^{-1}M, made immediately prior to use) at pH 7 was added to the sample in the n.m.r. tube via a Hamilton syringe.

iii) Other materials

^2H_2O (99.8%) was obtained from Merck, Sharp and Dohme. NaO^2H (40% in ^2H_2O, isotopic purity 99%) and ^2HCl (~ 5% in ^2H_2O, isotopic purity 99.6%) were obtained from CIBA. The sodium salt of 2,2-dimethyl-2-silapentane-5-sulphonate (DSS) was obtained from Merck, Sharp and Dohme. Oxygen free Argon was obtained from B.O.C. and from Air Products. Standard buffer solutions for standardising pH meters were supplied by B.D.H.

$K_3Fe(CN)_6$, DL-N-Acetylmethionine, iodoacetic acid (biochemical grade), ascorbic acid, sodium dithionite, methyl acetimidate.HCl, urea and maleic anhydride were all supplied by B.D.H. O-methylisourea hydrogen sulphate was supplied by the Aldrich Chemical Co.. S-ethylthioltrifluoro-acetate was supplied by the Eastman Chemical Co. $Pt(NH_3)_2Cl_2$ and K_2PtCl_4 were kindly loaned by Dr. M.J. Cleary of Johnson Matthey. Deuterated urea and methanol (d^4) was obtained from Merck, Sharp and Dohme.

N.m.r. tubes were obtained from the Wilmad Glass Co., and were cleaned prior to use with chromic acid, washed with a solution of ethylenediaminetetraacetic acid, rinsed several times with doubly distilled water and dried in an oven at 120°C.

iv) Collection of n.m.r. data

All the n.m.r. spectra presented in this thesis are Fourier Transform spectra (Appendix A). Spectra were recorded at 270 MHz, using a Bruker GFX 270 spectrometer fitted with an Oxford instrument Co. superconducting Magnet, and at 300 MHz, using a Bruker WH-300 spectrometer fitted with an Oxford Instrument Co. superconducting magnet. The 270 MHz spectrometer was used for the bulk of the experiments. Both spectrometers are equipped with computers for collecting and manipulating the data, disc storage systems (used in conjunction with Nashua 4415 or DD15-8 cartridges) and deuterium field frequency locks. The spectrometers require samples of volumes of about 0.35 ml.

Normally spectra of a frequency width (sweep width) of 5000 Hz were collected. For the paramagnetic ferricytochrome c molecule and its derivatives spectra were sometimes collected over a 20000 Hz sweep width in order to observe resonances suffering contact and pseudocontact shifts. Data was collected over 4096 or 8192 data acquisition points and the spectra were usually Fourier transformed over 8192 data points.

All chemical shifts values are quoted downfield of DSS, though dioxan was more often used as the internal standard; it gives a resonance at 3.741 ppm downfield of DSS.

References: Appendix B

1. Kalinichenko, P. (1976), Stud. Biophys., 58, 235.

2. Ando, K., Matsubara, H., and Okunuki, K. (1966), Biochim.Biophys.Acta., 118, 240.

3. Ando, K., Matsubara, H., and Okunuki, K. (1966), Biochim.Biophys.Acta., 118, 256.

4. Boon, P.J., van Raay, A.J.M., Tesser, G.I., and Nivard, R.J.F. (1979), FEBS Letters, 108, 131.

5. Wallace, C.J.A., and Offord, R.E. (1979), Biochem.J., 179, 169.

6. Corradin, G., and Harbury, H.A. (1974), Biochem.Biophys. Res.Comm., 61, 1400.

7. Hettinger, T.P., and Harbury, H.A. (1964), Proc.Natl.Acad.Sci.USA., 52, 1469.

8. Stellwagen, E., Smith, L.M., Cass, R., Ledger, R., and Wigus, H. (1977), Biochemistry, 16, 3672.

9. Fanger, M.W., and Harbury, H.A. (1965), Biochemistry, 4, 2541.

10. Pettigrew, G.W., Aviram, I., and Schejter, A. (1976), Biochem.Biophys.Res.Comm., 68, 807.

11. Schejter, A., and George, P. (1965), Nature, 206, 1150.

12. Brunori, M., Wilson, M.T., and Antonini, E. (1972), J.Biol.Chem., 247, 6076.

APPENDIX C

Aminoacid sequences of cytochromes c

	10	20	25
Horse	@.G.D.V.E.K.G.K.K.I.F.	V.Q.K.C.A.Q.C.H.T.V.	E.K.G.G.K.
Tuna	@.G.D.V.a.K.G.K.K.t.F.	V.Q.K.C.A.Q.C.H.T.V.	E.n.G.G.K.
Pigeon	@.G.D.i.E.K.G.K.K.I.F.	V.Q.K.C.s.Q.C.H.T.V.	E.K.G.G.K.
Lamprey	@.G.D.V.E.K.G.K.K.v.F.	V.Q.K.C.s.Q.C.H.T.V.	E.K.a.G.K.

	35	45	53
H	H.K.T.G.P.N.L.H.G.L.	F.G.R.K.T.G.Q.A.P.G.	F.T.Y.T.D.A.N.K.
T	H.K.v.G.P.N.L.w.G.L.	F.G.R.K.T.G.Q.A.e.G.	y.s.Y.T.D.A.N.K.
P	H.K.T.G.P.N.L.H.G.L.	F.G.R.K.T.G.Q.A.e.G.	F.s.Y.T.D.A.N.K.
L	H.K.T.G.P.N.L.s.G.L.	F.G.R.K.T.G.Q.A.P.G.	F.s.Y.T.D.A.N.K.

	63	73	81
H	N.K.G.I.T.W.K.E.E.T.	L.M.E.Y.L.E.N.P.K.K.	Y.I.P.G.T.K.M.I.
T	s.K.G.I.v.W.n.n.d.T.	L.M.E.Y.L.E.N.P.K.K.	Y.I.P.G.T.K.M.I.
P	N.K.G.I.T.W.g.E.d.T.	L.M.E.Y.L.E.N.P.K.K.	Y.I.P.G.T.K.M.I.
L	s.K.G.I.v.W.n.q.E.T.	L.f.v.Y.L.E.N.P.K.K.	Y.t.P.G.T.K.M.I.

	91	101	104
H	F.A.G.I.K.K.K.T.E.R.	E.D.L.I.A.Y.L.K.K.A.	T.N.E.
T	F.A.G.I.K.K.K.g.E.R.	q.D.L.v.A.Y.L.K.s.A.	T.s.
P	F.A.G.I.K.K.K.a.E.R.	a.D.L.I.A.Y.L.K.q.A.	T.a.k.
L	F.A.G.I.K.K.e.g.E.R.	k.D.L.I.A.Y.L.K.K.s.	T.s.E.

The sequence data were taken from 'Atlas of Protein Sequence and Structure' (National Biomedical Research Foundation, Silver Spring, Maryland, U.S.A.) compiled by M.O.Dayhoff (1968) and its later supplements (1973, 1976).

@, acetylated amino terminus.

A D D E N D A

D.Phil thesis (1981) A.P.Boswell

Page	Line	Correction
Abstract	9	temperature
12	9	pKs
17	19	N^{ε}-acetimidylated
27	bottom	is→are
28	8	assigned→assign
29	20	documented (15))
30	24	$A^{*}11$
34	13	$M^{*}19$, $M^{*}20$
47	19	(δ,^{3}t; -0.37)
47	22	γCH
51	diagram	a) 4.42 ppm, b) 4.46 ppm, c) 4.50 ppm and d) 4.54 ppm
51	4	irradiation at a) 4.42 ppm, b) 4.46 ppm, c) 4.50 ppm and d) 4.54 ppm from the blank spectrum.
55	4	6 Å
57	24	accesible→accessible
59	16	delete;'the effect in the difference spectrum is marked with a cross'
66	3	haem meso βCH
70	7	ortho→meta
73	4	described
77	13	haem meso βCH
77	16, 17, 24	N^{ε}-acetimidylated
78	7,10,12,21	ditto
79	1, 6,17	ditto
80	3	add ' , all at 27°C.'

88	21	was→is
102	Leu 68	>-0.5 for both Tables 4.3. and 4.4.
114	12	TM*16
115	8	insert: methionine' with tetrachloroplatinite' has
117	17	upfield→downfield
119	21	b) The binding of such platinum ...
122	19	temperatures
123	1	groups→resonances
127	5	βCH
127	19	insert M7 'and' at
130	Table	Propionate βCH 7
135	4	will→may
135	6	would→could
155	bottom	Tur→Tyr
156	17	hydrophobic
156	26	solvent
157	9	This→Thus
157	13	insert: Stellwagen's '(23)' work
167	add 23.	Stellwagen, E. (1979), J.Mol.Biol., 135, 217.
160	17	N^{ε}-acetimidylated
161	12	M*1
165	1	N^{ε}-ace-
174	bottom	assignments
181	6	displacements
194	14	marked with a '[illegible]' in Fig. 6.8.c)
196	1	marked with a '[illegible]' in Fig. 6.7.b)
196	5	marked with a '[illegible]' in Fig. 6.7.b)

225	19	Many→Several
231	3	insert: the 'concentration of the' ferricytochrome c
231	12, 16	3.8×10^{-3}M
239	3,7	3.8×10^{-3}M , 8×10^{-3}M
266	24	electronics

The mistake $CH_3 \rightarrow \delta CH_3$ should be corrected on the following pages and lines: 7,2; 17,15; 34,19; 45,4; 96,22; 102,Table; 110,27; 113,4; 123,19; 195,3; 195,16.

256	equation 3	$\hat{\mu} = g \cdot q \cdot h \cdot \hat{I} / 4\pi M$
256	4	$\hat{\mu} = g \cdot \beta_N \cdot \hat{I}$.
256	16	$\beta_N = e.h./4.m_p.\pi = 5.050 \times 10^{-27}$ J T^{-1}
259	1	in equation 5 .
260	19	arises
260	21	those
264	1	Δ_o
264	bottom equation	$\left(\frac{1}{T_2}\right) = \left(\frac{f_A}{T_{2A}}\right) + \left(\frac{f_B}{T_{2B}}\right) + \frac{4\pi^2 f_A \cdot f_B \cdot (\Delta_o)^2}{\left(\frac{1}{\tau_A}\right) + \left(\frac{1}{\tau_B}\right)}$.

204	all three sets of tyrosine resonances should be bracketed