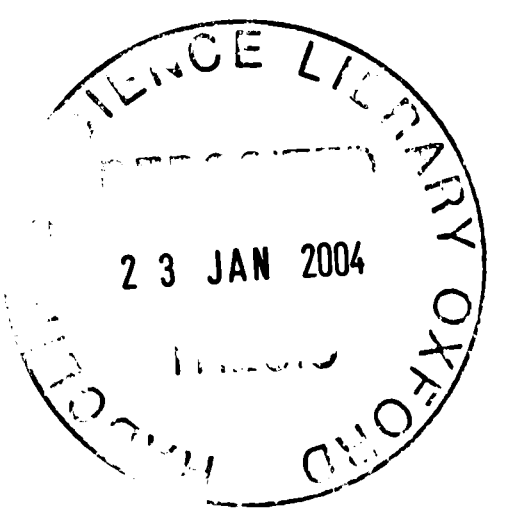
In vitro Studies on Cytochrome *c* Biogenesis

Oliver Daltrop

St. Edmund Hall

August 2003

A thesis submitted to the board of the Faculty of Biological Sciences of the University of Oxford in partial fulfillment of the requirements for the degree of Doctor of Philosophy



In vitro Studies on Cytochrome *c* Biogenesis

Oliver Daltrop, St. Edmund Hall, D.Phil., Trinity Term 2003.

C-type cytochromes are essential for almost all organisms and are mainly involved in electron transport; they are characterised by the covalent attachment of heme to protein through two thioether bonds to a CXXCH peptide motif.

This thesis describes the development of *in vitro* systems to establish chemical aspects of the process of cytochrome *c* maturation. Initially, the uncatalysed reaction of heme and apocytochrome *c* from *Hydrogenobacter thermophilus* cytochrome c_{552} was studied *in vitro*, yielding the desired thioether bonds under mild conditions and in the absence of any biosynthesis apparatus. The reaction proceeded via a *b*-type cytochrome intermediate, showing the ability of the apoprotein to bind heme non-covalently prior to thioether bond formation. It was also determined that the two cysteine residues of the CXXCH motif can form a disulfide. Optimal reaction conditions for thioether bond formation required both the heme and the cysteine residues to be reduced. Mechanistic insights were gained by showing that the thioether bonds can form independently from one another. Furthermore, it was shown that, in the case of *H. thermophilus* apocytochrome, thioether bonds can form stereoselectively with respect to the α, γ mesoaxis of heme.
These findings were extended to a broader range of apocytochromes. It was discovered that mitochondrial apocytochromes *c* from horse heart and yeast also formed thioether bonds with heme, as well as the bacterial *Paracoccus denitrificans* apocytochrome c_{550}. It was established that apocytochromes show a trend to bind hydrophobic ligands or heme to yield *b*-type cytochromes. Thioether bonds can form *in vitro* in these heme-protein complexes.

In the second part of this thesis the heme chaperone CcmE, which is part of the cytochrome *c* biogenesis apparatus in many Gram-negative bacteria, was studied as an extension to the establishment of *in vitro* systems. It was discovered that CcmE can bind heme initially non-covalently and then covalently upon reduction of the heme. However, CcmE seems to have a preference for ferric rather than ferrous heme. The involvement of the vinyl groups of heme is suggested. Mutation of the heme-binding histidine residue of CcmE established the involvement of this residue in the covalent binding of heme for the *in vitro* reaction; mechanistic insights were gained from the observation that a histidine to cysteine mutant could still bind heme covalently *in vivo* and *in vitro*. Effects of the presence of a His tag on CcmE were shown and are discussed. Furthermore, *in vitro* heme transfer from CcmE to certain apocytochromes *c* was achieved.
All these *in vitro* results mimic, and thus have implications for, the molecular pathway of heme transfer during the complex process of *c*-type cytochrome maturation *in vivo*.

Some of the work in this thesis has been published:

In vitro formation of a *c*-type cytochrome.
Daltrop, O., Allen, J. W. A., Willis, A. C. & Ferguson, S. J. (2002) *Proc Natl Acad Sci U S A* **99**, 7872-6.

The CcmE protein of the *c*-type cytochrome biogenesis system: unusual *in vitro* heme incorporation into apo-CcmE and transfer from holo-CcmE to apocytochrome.
Daltrop, O., Stevens, J. M., Higham, C. W. & Ferguson, S. J. (2002) *Proc Natl Acad Sci U S A* **99**, 9703-8.

C-type cytochromes: diverse structures and biogenesis systems pose evolutionary problems.
Allen, J. W. A., Daltrop, O., Stevens, J. M. & Ferguson, S. J. (2003) *Philos Trans R Soc Lond B Biol Sci* **358**, 255-66.

Cytochrome *c* maturation: The *in vitro* reactions of horse heart apocytochrome *c* and *Paracoccus dentrificans* apocytochrome c_{550} with heme.
Daltrop, O. & Ferguson, S. J. (2003) *J Biol Chem* **278**, 4404-9.

Interaction of heme with variants of the heme chaperone CcmE carrying active site mutations and a cleavable N-terminal His tag.
Stevens, J. M., Daltrop, O., Higham, C. W. & Ferguson, S. J. (2003) *J Biol Chem* **278**, 20500-6.

Stereoselective *in vitro* formation of *c*-type cytochrome variants from *Hydrogenobacter thermophilus* containing only a single thioether bond.
Daltrop, O., Smith, K. M. & Ferguson, S. J. (2003) *J Biol Chem* **278**, 24308-13.

Acknowledgements

Firstly, I would like to thank Professor Stuart Ferguson for all his support, encouragement and continuous supervision. I am very grateful for all the shared scientific ideas and wisdom.

During this project I was in receipt of a studentship from the University of Oxford in association with St. Edmund Hall.

The second part of this project is collaborative in nature and I am particularly grateful to Dr. Julie Stevens for all the help especially with experimental methodologies. I would also like to thank Drs. James Allen and Chris Higham for their contributions, especially the extensive proof-reading. I gratefully acknowledge Lin Hong for the production of C11A and C14A variants of *Hydrogenobacter thermophilus* cytochrome c_{552} and Professor Kevin M. Smith for the supply of heme derivatives.

The presence of many individuals in the Ferguson lab made the time particularly enjoyable. I am most grateful to Rich Zajicek, Dr. Will Fosdike, the three post-docs mentioned above, and all the past and present members of the 5th floor people. Practical advice was appreciated as was the time spent during lunches, coffee breaks, in pubs and on park benches. Inspirational and taralysing moments were shared.

Personally, I would like to thank anyone who interacted with me over the last three years, bought me pints, coffees or whatever. I hope I will resist the corporate life style, though it is entertaining to hear about it. Football played successfully by Bayern München and Deutschland would also be pleasurable.

I am grateful to the family and friends I have.

And finally, I would like to dedicate this thesis to my family.

Abbreviations

AMS	4-acetamido-4'-maleimidyl-stilbene-2,2'-disulfonate
ANS	8-anilino-1-naphthalenesulfonate
APS	ammonium persulfate
ca.	*circa*
Ccm	cytochrome *c* maturation
$CcmE^{sol}$-C-His_6	soluble version of CcmE with a C-terminal His tag
CD	circular dichroism
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
EPR	electron paramagnetic resonance
ES	electrospray
FPLC	fast protein liquid chromatography
g	gram
h	hour
HPLC	high pressure liquid chromatography
IPTG	isopropyl-β-D-thiogalactopyranoside
k	kilo
K_d	(equilibrium) dissociation constant
k_{obs}	(observed) rate constant
l	liter
LB	Luria-Bertani
μ	micro
m	milli; meter
M	molar
MALDI	matrix-assisted laser desorption ionisation
MCD	magnetic circular dichroism
min	minute
MS	mass spectrometry
n	nano

N-His_6-$CcmE^{sol}$	soluble version of CcmE with a N-terminal His tag;
NMR	nuclear magnetic resonance
OD	optical density
p	piko
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
s	second
SAM	self-assembled monolayer
SDS	sodium dodcylsulfate
SDM	site-directed mutagenesis
Tat	twin arginine translocation
TEMED	*N,N,N',N'* –tetramethylethylenediamine
TFA	trifluoroacetic acid
Tris	tris-(hydroxymethyl)aminomethane
UV	ultra violet
V	Volts

Contents

CHAPTER 1

INTRODUCTION

C-TYPE CYTOCHROMES: DIVERSE STRUCTURES AND BIOGENESIS SYSTEMS AND THE COVALENT ATTACHMENT OF HEME TO PROTEIN VIA THIOETHER BONDS

1.1. CYTOCHROMES

The term cytochrome originates from the Greek meaning cellular pigment (Greek words *cytos*, meaning cell, hollow vessel and *chroma*, meaning colour). Cytochromes belong to the group of heme-containing proteins, which are ubiquitous in nature and have a variety of functions ranging from oxygen transport and storage (*e.g.* hemoglobin and myoglobin), to catalysis (*e.g.* cytochrome P450) and electron transfer. These proteins can be classified according to the type of heme present and the nature of its attachment to the polypeptide. The structure of heme and three of its derivatives are shown in Figure 1.1 (Falk & Smith 1975).

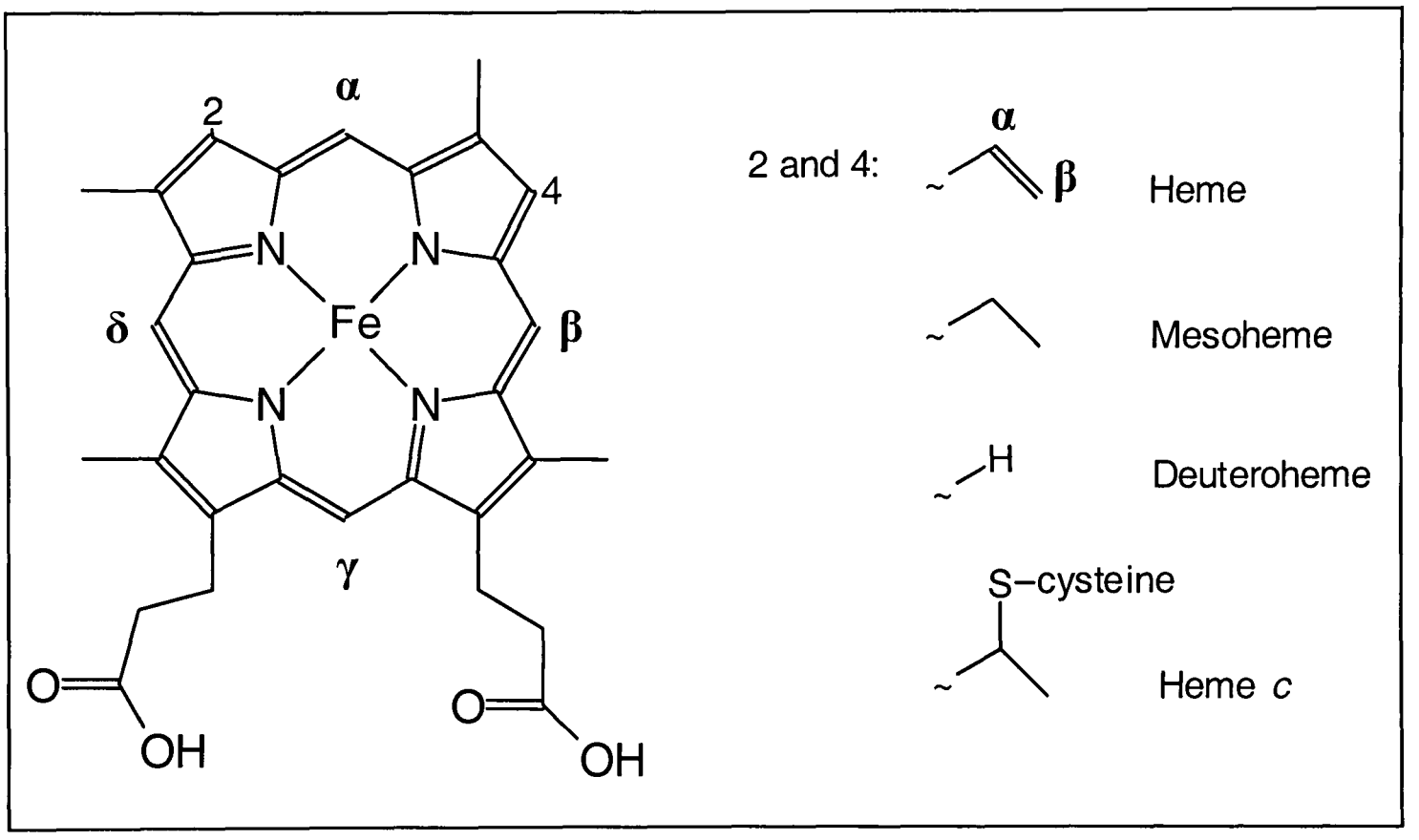


Figure 1.1. Structure of heme (Fe-protoporphyrin IX) and three of its derivatives, mesoheme, deuteroheme and "heme *c*" (Kojo & Sano 1981). The 2- and 4-positions are labelled using the "Fischer System of Nomenclature". The labelling of the methylene carbons of the porphyrin moiety is shown to allow discussion of the symmetry aspects of the molecule. The classification of the vinyl carbons of heme itself is also given at the top. ~ abbreviates the Fe-porphyrin and represents the connectivity at the pyrrole carbons 2 and 4. Protoporphyrin IX is the organic component of heme, where the iron is replaced by two protons.

1.2. *c*-TYPE CYTOCHROMES

c-type cytochromes, a widespread class of proteins essential for the life of almost all organisms, pose both a structural and an evolutionary puzzle. Cytochromes *c* are characterized by the covalent attachment of heme (iron-protoporphyrin IX) to a polypeptide chain via two (or rarely one) thioether, *i.e.*, carbon-sulfur, bonds which are generated as a result of the reaction of the thiol groups of cysteine residues with the α-carbon on the vinyl groups of heme (Barker & Ferguson 1999; Moore & Pettigrew 1990; Pettigrew & Moore 1987; Scott & Mauk 1995). An example of such a thioether bond is shown in Figure 1.1 in the heme derivative heme *c* (Kojo & Sano). The two cysteine residues almost always occur in the amino acid sequence CXXCH where the histidine is an axial ligand to the heme iron. A schematic representation of the heme binding and coordination of the CXXCH motif including the stereochemistry of the thioether bonds (see Section 1.6.2.1.) is shown in Figure 1.2.

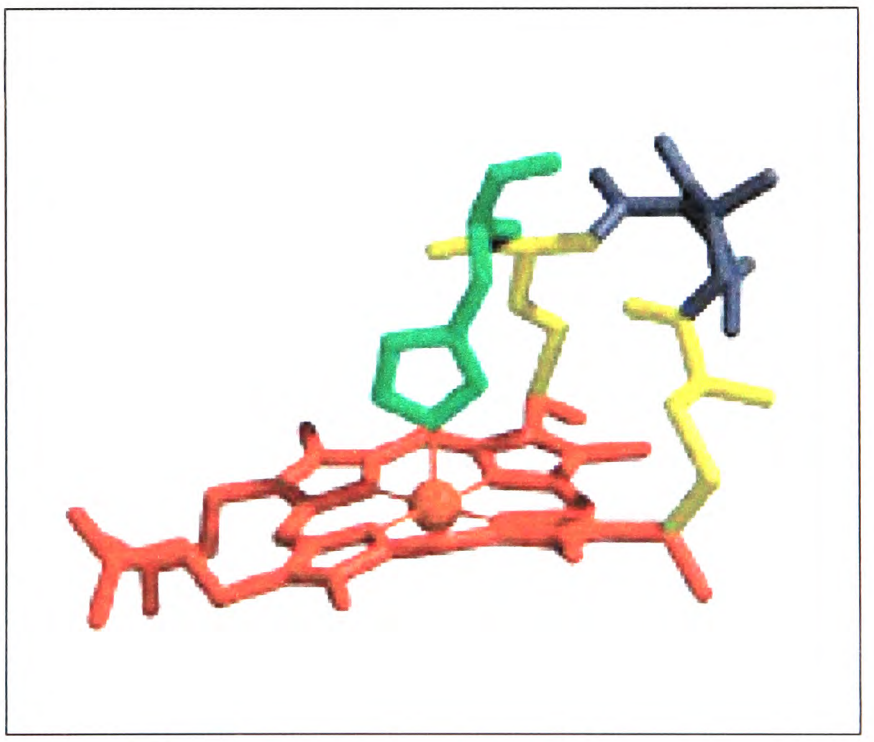


Figure 1.2. Schematic representation (reproduced from Barker & Ferguson 1999) of the attachment of heme (red) to the CXXCH motif of the protein moiety in a cytochrome *c*. The cysteine side chains are shown in yellow and form stereospecific thioether bonds. The histidine side chain is shown in green, coordinated as the axial ligand to the heme-iron.

Almost any residues (except cysteine) may be found in the XX positions; very rarely there are three or four residues between the two cysteines (Barker & Ferguson 1999). An example of a cytochrome with two out of four of its hemes attached via CXXXXCH heme-binding motifs is *Desulfovibrio vulgaris* Hildenborough holocytochrome c_3 (Herbaud et al. 2000). Recently, it has been demonstrated that an additional residue can be inserted into the CXXCH motif of *Rhodobacter sphaeroides* cytochrome c_2 to yield a CXAXCH peptide to which the heme became bound covalently in a functional cytochrome *c* (Rios-Velazquez et al. 2001).

The cytochrome *c* from mammalian and fungal mitochondria are by far the best known *c*-type cytochromes, but there is a very great range of other such proteins, often with several hemes attached per polypeptide chain, which are not related to these mitochondrial cytochromes *c*. In the fungal, vertebrate and invertebrate mitochondrial respiratory chain, two types of *c*-type cytochrome are found (Wilkins & Wilkins 1997). One is designated cytochrome c_1 and is part of the cytochrome bc_1 complex (ubiquinol:cytochrome *c* oxidoreductase; Complex III). The heme of this protein is the electron acceptor from the Fe_2S_2 iron-sulfur centre (the cofactor of the Rieske protein). The implications for electron and proton transfer from the elucidation of the structure of this complex have been reviewed (Hunte 2001; Munro et al. 2000). The electron acceptor from the c_1 heme of the bc_1 complex is the heme-iron of cytochrome *c*, which shuttles electrons between cytochrome bc_1 and cytochrome *c* oxidase by diffusing along the surface of the inner mitochondrial membrane. The structure of the yeast cytochrome c_1-domain of the bc_1 complex is shown in Figure 1.3. Cytochrome *c* is water-soluble and was one of the first proteins to have its structure solved (Dickerson et al. 1971). A space filling model of mitochondrial cytochrome *c* is shown in Figure 1.4 (Banci et al. 1997), illustrating that an edge of the buried heme group is exposed close to the surface of the protein to facilitate electron transfer.

Figure 1.3. The crystal structure of *Saccharomyces cerevisiae* cytochrome c_1 (Lange et al. 2001); viewed using RasMol and the data from the PDB submission 1KB9. The c_1 domain is shown from residue Met-62 to Ala-259 in yellow. The porphyrin moiety is shown in red with the heme iron in blue. The cysteine residues Cys-101 and Cys-104 forming the thioether bonds to heme are shown in green. The axial ligands to the heme iron His-105 and Met-225 are shown in black.

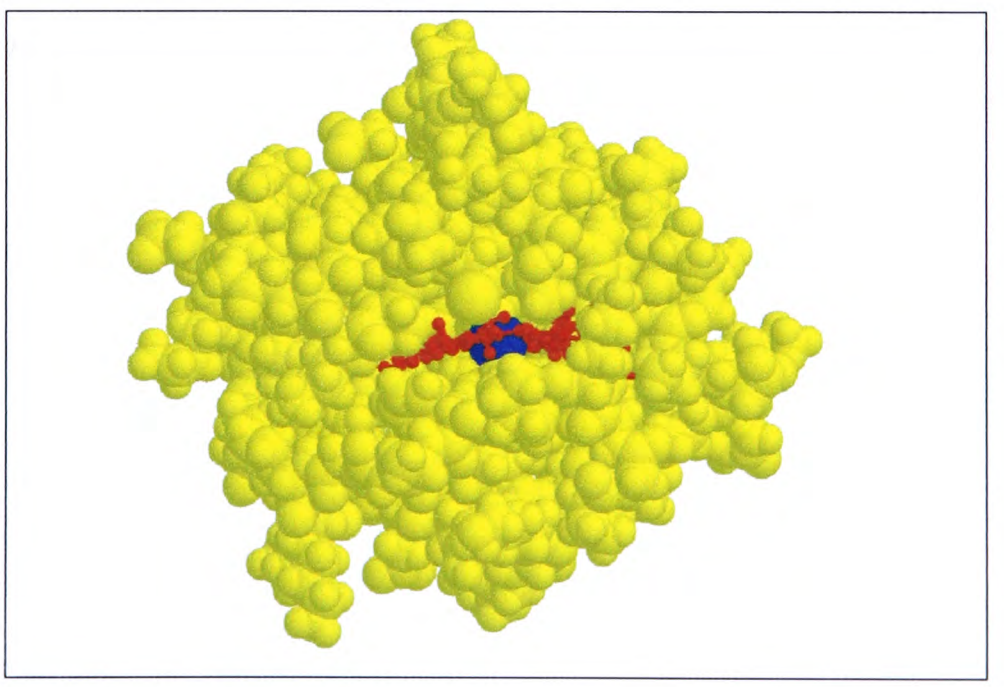


Figure 1.4. The solution structure of oxidised horse heart cytochrome *c* (Banci et al. 1997); viewed using RasMol and the data from PDB entry 1AKK. The protein residues are shown as space filling model. The porphyrin is shown in ball and stick representation in red and the heme iron in blue.

Cytochrome *c* oxidase is a terminal oxidase, which couples the reduction of molecular oxygen to water to the translocation of protons from the matrix (inside of the mitochondrion) across the inner membrane into the inter-membrane space, which is between the inner and outer mitochondrial membrane. This respiratory complex (Complex IV) is also known as heme-copper oxidase as it contains two copper and two heme redox centres between which the electrons are transferred and channelled to the oxygen binding site (Abramson et al. 2001). The inter-membrane space which is the location of cytochrome *c*, is referred to as the P-(positive) side of the inner membrane because protons are moved in this direction from the matrix by the proton translocating components of the respiratory chain. The matrix is correspondingly called the N-(negative) side. It is notable that in eukaryotic cells cytochrome *c* plays a recently discovered and surprising role in apoptosis (Martinou et al. 2000).

In bacteria, cytochromes *c* are involved in many more electron transfer reactions. In Gram-negative bacteria, cytochrome *c* is located in the periplasm, the compartment between the cytoplasmic membrane and the cell wall (Ferguson 1991). It is, therefore, on the P-side of the cytoplasmic membrane. Cytochromes *c* are essential in respiratory chains, for example donating electrons to cytochrome cd_1 nitrite reductase in denitrification (Berks et al. 1995); further examples are described elsewhere (Ferguson 2001). The structure of *Hydrogenobacter thermophilus* cytochrome c_{552}, a bacterial cytochrome *c*, is shown in Figure 1.5. *c*-type cytochrome centres are also found in the active sites of many enzymes, for example cytochrome *c* nitrite reductase from *Sulfurospirillum deleyianum* (Einsle et al. 1999) and hydroxylamine oxidoreductase from *Nitrosomonas europaea* (Igarashi et al. 1997).

In the remainder of this introduction the advantages that might accrue from covalent attachment of a heme group to protein are described and the biosynthesis of heme is considered. This leads on to discussion of the evolution of both cytochromes *c* and their biogenesis systems, which interestingly differs between different cell types. Finally, the challenging chemistry involved in thioether bond formation is discussed.

1.3. Possible Advantages of Forming Thioether Bonds

Barker and Ferguson (1999) sought to identify reasons that would explain the advantage(s) conferred by the thioether bonds in cytochromes *c*. One suggestion is that from Wood (1983), who noted that because in Gram-negative bacteria these proteins are located in the periplasm, covalent attachment of the heme would guard against a finite rate of dissociation of heme from a non-covalent complex with protein (i.e. a *b*-type cytochrome). Any such dissociation could be followed by passage of heme through the cell wall and hence loss to the medium external to the cell. It is impossible to disprove this hypothesis absolutely, but a number of considerations argue against it being the main reason for occurrence of covalent heme attachment in *c*-type cytochromes. These are: (i) Although in principle heme might pass readily through a cell wall, its hydrophobic nature does mean that it would adsorb onto the wall and hence would not necessarily be readily lost to the cell (i.e. it could remain available for incorporation into heme binding proteins); (ii) there are now several known extracellular proteins that have non-covalently bound heme, in effect *b*-type cytochromes (Barker & Ferguson 1999). One example is an enzyme involved in cellobiose degradation (Hallberg et al. 2000); (iii) there are several periplasmic *b*-type cytochromes now recognized. Of the latter, the enigmatic (its function is uncertain and it is rare in other species) cytochrome b_{562} in *Escherichia coli* (Arnesano et al.

1999, and references therein) is arguably the best known. More recently discovered examples include a group of related *b*-type cytochromes that are involved in the oxidation of ethylbenzene or dimethyl sulfide and in reduction of selenate (Hanlon et al. 1996; Kniemeyer & Heider 2001; Schroder et al. 1997). Furthermore, it has proved possible to direct the formation, in substantial amounts, of stable holocytochromes b_5 and P450, and of hemoglobin, to the periplasm of *E. coli* (Goldman et al. 1996; Kaderbhai et al. 2000; Karim et al. 1993); severe problems with loss of heme from the periplasm might have been expected to pose an impediment to such synthesis. A final consideration is that the specialized d_1 heme of the periplasmic respiratory enzyme cytochrome cd_1 nitrite reductase is bound non-covalently (Fulop et al. 1995; Hill & Wharton 1978), despite the significant biosynthetic expense of this heme (Zumft 1997).

1.3.1. Comparison of the physico-chemical properties of related *b*- and *c*-type cytochromes

If the presence of thioether bonds in cytochromes *c* cannot be explained in terms of a heme retention mechanism in the periplasm, other possibilities must be considered. One explanation may be that the thioether bonds confer advantageous features in terms of reduction potentials or stability on the proteins in which they occur. Tests of such proposals could include the comparative study of a *c*-type cytochrome from which the thioether bonds have been removed, or of a *b*-type cytochrome to which they have been added. The first of these strategies has been inaccessible until recently because there had been no report of the production of a properly folded variant of a *c*-type cytochrome that lacked the thioether bonds.

Cytochrome *c* maturation normally requires specialised biogenesis systems. However, exceptionally, heme may be covalently attached to the cytochromes c_{552} from *Hydrogenobacter thermophilus* and *Thermus thermophilus* in the cytoplasm of *E. coli*, apparently without the action of any biosynthesis proteins (Keightley et al. 1998; Sambongi & Ferguson 1994b; Sinha & Ferguson 1998); the usual location of *c*-type cytochrome formation in bacteria is the periplasm. The cytoplasmically produced *H. thermophilus* protein, which has a typical cytochrome *c* fold (Hasegawa et al. 1998), folds correctly and its physical properties are in many respects indistinguishable from those of protein matured by a dedicated biogenesis apparatus in *H. thermophilus* itself (Karan et al. 2002). The structure of this cytochrome *c* is shown in Figure 1.5.

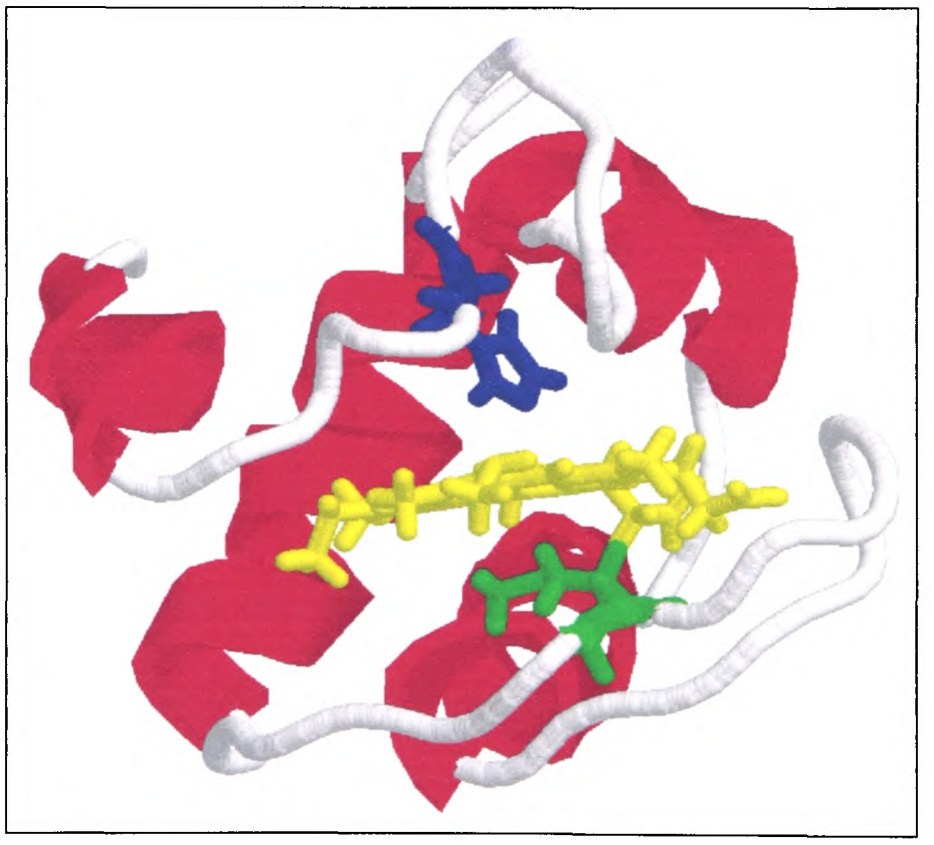


Figure 1.5. The solution structure of *Hydrogenobacter thermophilus* cytochrome c_{552} (Hasegawa et al. 1998); viewed using RasMol and the data from PDB entry 1AYG. The four α-helices are shown in red and the heme cofactor in yellow. The heme-iron is axially ligated by histidine 14 (blue) and methionine 59 (green).

It has been proposed that cytoplasmic assembly occurs because the apoproteins of *H. thermophilus* and *T. thermophilus* cytochrome, normally found in thermophilic organisms (optimum growth at 70+ °C), adopt a pre-folded conformation that can bind heme when produced in the mesophile *E. coli* (Sambongi & Ferguson 1994b; Sinha & Ferguson 1998). This idea is consistent with the observation that replacement of both of the *H. thermophilus* cytochrome c_{552} heme-binding cysteine residues by alanines (in a C11A/C14A variant) results in cytoplasmic formation of a *b*-type cytochrome whose heme is not covalently bound to the polypeptide and can be removed reversibly *in vitro* (Tomlinson & Ferguson 2000a). More significantly, the ability to create such a protein has allowed a direct comparison between a *c*-type cytochrome and a *b*-type cytochrome that, in principle, differ only in the fact that the heme is covalently attached to the polypeptide in the former but not in the latter (Tomlinson & Ferguson 2000a).

Biophysical studies of wild type *H. thermophilus* cytochrome c_{552} and of the C11A/C14A variant showed that the gross secondary structures of the two proteins were the same, as judged by their virtually identical far-UV circular dichroism spectra; NMR spectroscopy and sequence analysis showed that the *b*-type variant retained the His/Met heme iron coordination of the *c*-type (Tomlinson & Ferguson 2000a). However, the *b*-type (C11A/C14A) cytochrome was structurally less stable, with the guanidine hydrochloride unfolding midpoint occurring at a concentration 2 M lower than for the wild-type protein. The reduction potential was only 75 mV lower than that of the *c*-type cytochrome (Tomlinson & Ferguson 2000a). This difference is extremely small given that variations of several hundreds of millivolts can arise in the reduction potential of *c*-type cytochromes due to differences such as the extent of solvent exposure of the heme (Tezcan et al.

1998) and indicates that modulation of reduction potentials is probably not a key reason for the covalent attachment of heme to polypeptide. The *b*-type variant protein was also shown to be stable with respect to loss of heme in dialysis experiments over a period of days (Tomlinson & Ferguson 2000a), further arguing against heme retention as the basis of covalent attachment.

Additional exploitation of the cytoplasmic assembly in *E. coli* of *H. thermophilus* cytochrome c_{552} and variants allowed a more thorough investigation of the significance of two thioether bonds in cytochromes *c*. Variants of the protein in which each of the cysteines that form the covalent linkages to heme had been mutated to alanines (*i.e.*, with AXXCH or CXXAH heme-binding motifs) could be isolated and their physico-chemical properties investigated (Tomlinson & Ferguson 2000b). For each of these proteins, the heme is covalently attached to the polypeptide through a single thioether bond. Remarkably, the single thioether bond-containing proteins have similar properties, including thermal stability and reduction potential, to the wild type CXXCH protein (Tomlinson & Ferguson 2000b). In combination with the work showing that the AXXAH variant of cytochrome c_{552} is much less stable than the CXXCH form (Tomlinson & Ferguson 2000a), it can be concluded that covalent attachment of heme via either of the thioether bonds is sufficient to confer considerable stability and that these bonds *per se* contribute little to the setting of the reduction potential. The reverse approach has also been attempted, *i.e.*, selective introduction of one or two cysteine residues into *E. coli* cytochrome b_{562} or bovine liver cytochrome b_5, positioned such that they could form covalent bonds with the vinyl groups of heme (Arnesano et al. 2000; Barker et al. 1993; Barker et al. 1995). An NMR characterisation of one such variant (b_{562} R98C; CXXXH heme-binding motif)

demonstrated formation of the thioether bond and also that the overall secondary and tertiary structures of the protein were very similar to those of the wild-type cytochrome b_{562}. The single thioether bonded *c*-type variant of cytochrome b_{562} was shown to have a significantly increased stability, compared to that of wild type b_{562}, toward thermal and chemical denaturation (Arnesano et al. 2000). Thus, conversion of a *c*-type cytochrome to a *b*-type and conversion of a *c*-type to a *b*-type indicates, in both cases, that formation of the thioether bonds confers considerably greater stability on the product cytochrome; this holds true even with only a single thioether bond. The absence of naturally occurring ZXXCH or CXXZH heme-binding motifs from bacterial cytochromes *c* (in proteins discovered to date) may relate to the coexistence of the assembly pathway with that for formation of disulfide bonds in the bacterial periplasm (Section 1.5.2.2).

1.3.2. Packing of hemes

In the bacterial world, especially as part of the biological nitrogen and sulfur cycles, there are many *c*-type cytochromes with multiple hemes per polypeptide chain. Examples include the octaheme hydroxylamine oxidoreductase (Igarashi et al. 1997), the tetraheme NapC/NirT/TorC family (Cartron et al. 2002; Roldan et al. 1998), the 65 kDa sixteen heme containing protein Hmc (high molecular mass cytochrome *c*) from sulfate reducing bacteria (Czjzek et al. 2002; Florens & Bruschi 1994; Matias et al. 2002), and cytochrome c_3 which is, remarkably, a tetraheme protein of only *ca.* 16 kDa molecular weight (Higuchi et al. 1984). Such proteins bear no resemblance to the two vertebrate/invertebrate/fungal *c*-type cytochromes (*c* and c_1), but form structurally related families (in which the positions of the hemes can often be overlayed) even when there is little sequence conservation between members of the family (e.g. pentaheme nitrite reductase,

hydroxylamine oxidoreductase and flavocytochrome fumarate reductase) (Barker & Ferguson 1999). It is striking that in these proteins the hemes are clustered together. Barker and Ferguson (1999) have argued that only by fixing the hemes spatially via their thioether bonds can such clustering be achieved. Certainly it is the case that in general the amino acid to heme ratio in this type of protein is much lower than it is in heme proteins with non-covalently bound heme. It is probably also significant that dense packing of hemes allows extremely rapid electron transfer between the heme centres (Page et al. 1999), an essential part of the function of these cytochromes, which are often involved in multi-electron oxidoreductase reactions. This property is ideal for enzymes such as the pentaheme nitrite reductase which catalyses the six-electron reduction of nitrite to ammonia, or hydroxylamine oxidoreductase, which catalyses the four-electron oxidation of hydroxylamine to nitrite. However, it should be noted that there are many other examples of proteins with a dense packing of non-covalently bound cofactors. One example is the photosystem 1 of thylakoids. In this case the chlorophylls (closely related to hemes) are either positioned between transmembrane helices or in loops between those helices. It has been pointed out previously (Ferguson 2001) that *c*-type cytochromes with the heme attached in a transmembrane part of the protein have not been observed; at least one outer membrane *c*-type cytochrome is now thought to have a globular conformation on the external surface of the cell (Richardson 2000). Thus at present multi-heme *c*-type cytochrome formation is tentatively correlated with a requirement to have a cluster of hemes in a globular protein. It should also be reiterated that many cytochromes *c* (notably vertebrate/invertebrate/fungal cytochrome *c* and the bacterial respiratory electron transport cytochromes c_2) carry only a single heme. Therefore, the ability to pack many hemes into economical polypeptides with conserved relative orientations

cannot be the only reason for covalent heme attachment in the cytochrome *c* fashion, but it may be an evolutionary consequence of the development of systems for such attachment (or, indeed, vice versa).

The structures of *c*-type cytochromes fall into several groups that have no obvious relation to one another (Barker and Ferguson, 1999). However, the coordination of the heme iron, with a few exceptions, is either His/His or His/Met. The former ligation occurs frequently in obligate anaerobes such as sulfate reducing bacteria; it results in a reduction potential typically 200 mV (or more) lower than that with the latter coordination (Moore & Pettigrew 1990; Pettigrew & Moore 1987) and is therefore more consistent with electron transfer in the reduced (pre-atmospheric oxygen) biosphere associated with the early stages of life on Earth. Thus if His/His coordinated *c*-type cytochromes evolved first, then we can envisage the His/Met coordination that is found in the cytochromes *c* from many aerobic bacteria and vertebrates, invertebrates and fungi as a later adaptation. Perhaps the most puzzling *c*-type cytochrome of all is cytochrome *f*, which has no known relatives outside the plastids (Martinez et al. 1994). This protein has, despite its misleading name, heme covalently attached to polypeptide through two thioether bonds. Its axial iron ligands are histidine and the N-terminal amino group of the polypeptide. Other unusual cases include the active site *c*-type cytochrome centre of the pentaheme nitrite reductase, which is pentacoordinate with a proximal lysine ligand (from a CXXCK heme-binding motif) (Einsle et al. 1999), and the cytochrome *c´* family, which also have pentacoordinate iron but with the typical cytochrome *c* proximal histidine ligand (Lawson et al. 2000).

Finally, in considering the evolutionary origin of *c*-type cytochromes, one might note that the CXXC peptide motif is found in other proteins including the thioredoxin class which, see section 1.5.2, is intimately involved in at least one cytochrome *c* biogenesis pathway. The CXXC motif is also found in many iron-sulfur proteins (Johnson 1994), which are generally regarded as having occurred before cytochromes during evolution.

1.4. HEME BIOSYNTHESIS

Before discussing the different cytochrome *c* biogenesis systems, the current understanding of the pathways for heme biosynthesis are mentioned to highlight the importance of heme chemistry for cytochrome *c* synthesis. The first step in the synthesis of heme is the generation of δ-aminolevulinic acid (ALA). Two different biogenesis routes for ALA are found in nature. In archaea, bacteria (except for α-proteobacteria), plants and algae, glutamate is condensed onto $tRNA^{Glu}$ carrying the UUC anticodon, which may also be present from the glutamyl-$tRNA^{Glu}$ pool for protein synthesis (O'Brian & Thöny-Meyer 2002). NADPH dependent reduction followed by a transamination reaction yields ALA (Figure 1.6(a)). Structural and functional details of the two enzymes (glutamyl-tRNA reductase and GSA aminotransferase, the latter also called glutamate-1-semialdehyde 2,1-aminomutase) involved in these steps are reviewed elsewhere (Moser et al. 2002).

In non-photosynthetic eukaryotes, where the first reaction in heme biosynthesis takes place in the mitochondrion, and α-proteobacteria, glycine and succinylCoA are condensed by the pyridoxal phosphate-containing enzyme, δ-aminolevulinic acid synthase (ALA synthase). The reaction is summarised in Figure 1.6.(b) (O'Brian & Thöny-Meyer 2002).

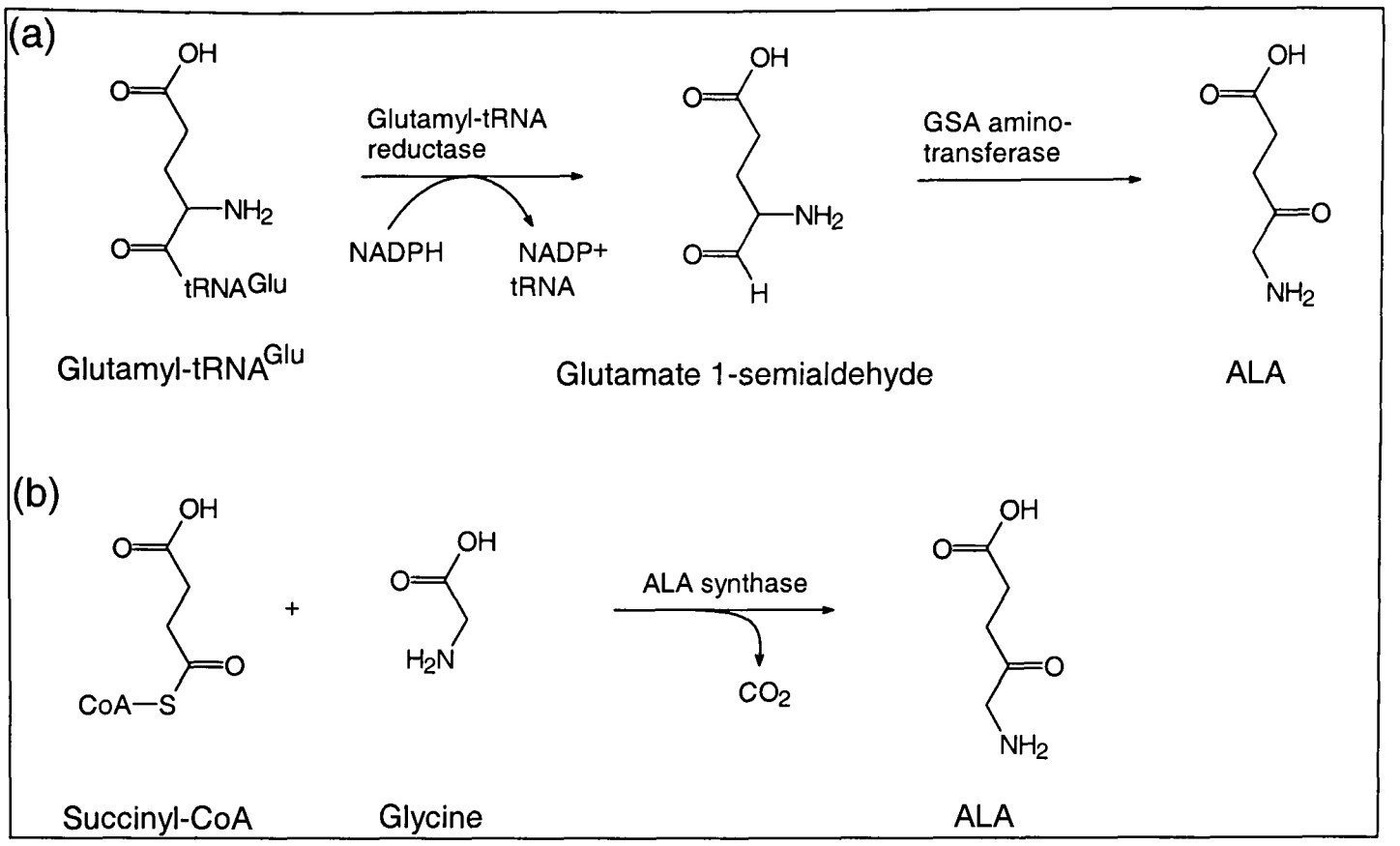


Figure 1.6. The two synthetic routes leading to δ-aminolevulinic acid (ALA) production (O'Brian & Thöny-Meyer 2002).

After synthesis of ALA, the chemical steps of the heme biosynthesis pathway are essentially identical in prokaryotes and eukaryotes and involve seven enzyme-mediated reactions (Figure 1.7). Condensation of two molecules of ALA to produce the pyrrole ring compound porphobilinogen in a Knorr-type reaction is catalysed by ALA dehydratase (Warren et al. 1998). Two active site lysine residues are proposed to function as Schiff bases (Shoolingin-Jordan et al. 2002). For non-photosynthetic eukaryotes, mitochondrial δ-aminolevulinic acid (ALA) has to be transported to the cytosol, where ALA dehydratase (also called porphobilinogen synthase or hydroxymethylbilane synthase) functions.

Head-to-tail condensation of four molecules of porphobilinogen produces the linear tetrapyrrole intermediate, hydroxymethylbilane (PBG), a reaction which is catalysed by porphobilinogen deaminase (PBG deaminase; also called uroporphyrinogen I synthase). Hydroxymethylbilane is cyclised to the asymmetric

tetrapyrrole ring, uroporphyrinogen III. This step is mediated by uroporphyrinogen III synthase (Schubert et al. 2002).

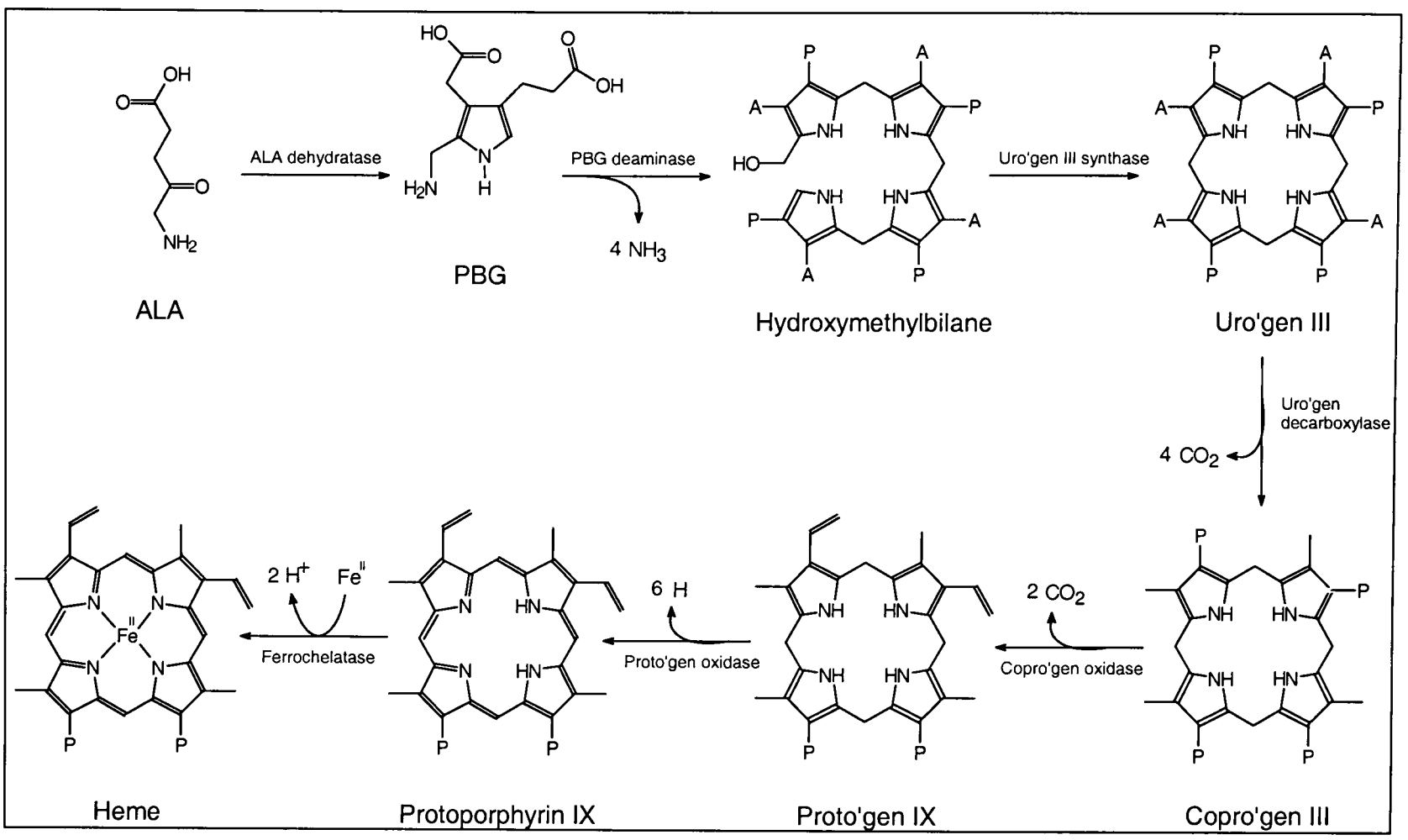


Figure 1.7. The biosynthesis of heme from ALA. The abbreviations used are PBG: porphobilinogen; Uro'gen: uroporphrinogen; Copro'gen: coproporphyrinogen; Proto'gen: protoporphyrinogen; A: acetate side chain; P: propionate side chain. Reproduced from Warren et al. (1998).

The acetate substituents of uroporphyrinogen III are all sequentially decarboxylated by the enzyme uroporphyrinogen III decarboxylase. The resultant products have methyl groups in place of acetate yielding coproporphyrinogen III (Warren et al. 1998). The propionate residues of this intermediate are decarboxylated, yielding vinyl substituents on the two pyrrole rings producing the colourless protoporphyrinogen IX. This process is catalysed by either oxygen-dependent or oxygen-independent coproporphyrinogen III oxidase in aerobic or anaerobic and facultative anaerobic organisms, respectively (Dailey 2002). In non-

photosynthetic eukaryotes, coproporphyrinogen III is transported to the interior of the mitochondrion after the decarboxylation of the propionates (Voet & Voet 1995).

Protoporphyrinogen IX is converted to protoporphyrin IX by protoporphyrinogen IX oxidase. The oxidase reaction requires molecular oxygen in most cases and results in the loss of six protons and six electrons, yielding a completely conjugated ring system, giving rise to a red coloured product. In some prokaryotes an oxygen independent coproporphyrinogen oxidase can be expressed, facilitating respiratory metabolism during hypoxia or anaerobiosis (Dailey 2002). In the final reaction of heme biosynthesis, Fe^{2+} is inserted into the ring system generating heme. The enzyme catalysing this reaction is known as ferrochelatase. Interestingly, it has been shown that the inserted iron has to be in its (reduced) Fe^{2+} state (Dailey 2002). It is unclear, how, and in which oxidation state, heme is then transported to the location of cytochrome *c* maturation, *i.e.*, the periplasm for Gram-negative bacteria and the intermembrane space of the mitochondrion for eukaryotes (see Section 1.6.2.2).

1.5. CYTOCHROME *C* BIOGENESIS STRATEGIES

Remarkably, three very different cytochrome *c* biogenesis systems have been identified to date. This in itself suggests that there must be one or more strong reasons for the adoption of thioether bonds for anchoring heme to protein in cytochromes *c*. The simplest system is apparently unrelated to the other two and operates, for example, in the mitochondria of fungal, invertebrate and vertebrate cells.

1.5.1. Mitochondrial cytochrome *c* formation

Studies on yeast led to the discovery of the enzyme heme lyase which catalyses the attachment of the heme group to the apocytochrome *c* (Basile et al. 1980; Veloso et al. 1981). At least in the case of fungi, these lyases are each specific for a particular cytochrome (i.e. one for cytochrome *c*, one for cytochrome c_1) (Steiner et al. 1996). However, only a single enzyme homologous to the yeast lyases is found in the genomes of human and mouse (that enzyme having a high degree of sequence similarity to both the yeast cytochrome *c* lyase and the cytochrome c_1 lyase). Remarkably, plant mitochondria do not contain heme lyase but use instead at least part of a bacterial cytochrome *c* biogenesis system for assembly of cytochromes *c* and c_1 (Section 1.5.3). Heme lyases do not act on bacterial apocytochromes *c* (Sanders & Lill 2000). There is no obvious heme lyase 'ancestor' in an archaeal or bacterial genome and thus the origin of this protein is puzzling. Its absence from plants may also have implications for understanding the evolution of the eukaryotic cell. It is intriguing that heme lyase is found in *Dictyostelium discoideum* and *Plasmodium falciparum* organisms that are distinct from, and may pre-date, yeast or *Neurospora* in evolutionary terms.

The ability to express yeast mitochondrial cytochrome *c* in the cytoplasm of *E. coli* if the heme lyase is co-expressed provides strong evidence that only one ancillary protein is required to promote thioether bond formation in this case (Pollock et al. 1998). It has also been shown that the yeast lyase can promote attachment of heme, in low yield, to a variant of human mitochondrial cytochrome *c* carrying an AXXCH heme-binding sequence (Tanaka et al. 1990). Additionally, a C14S variant of yeast *iso*-1-cytochrome *c* (i.e. with a SXXCH motif) has been isolated from the cytoplasm of *E. coli* following its co-expression with the yeast

cytochrome *c* heme lyase (Rosell & Mauk 2002). Thus it appears that the heme lyases, sometimes referred to as cytochrome *c* maturation System III, do not have a strict requirement for the CXXCH motif, which implies in turn that unlike for bacterial cytochrome *c* assembly (Section 1.5.2) a disulfide bond is not formed within this motif during the heme lyase type of cytochrome *c* biogenesis. This idea is consistent with the successful expression of yeast cytochrome *c* (CXXCH motif) in the *E. coli* cytoplasm provided the heme lyase is co-expressed (Pollock et al. 1998), since disulfides would not be expected to form in the reducing environment of this compartment of the cell. The mode of action of the heme lyases is not established; while it may be catalysis of formation of the thioether bonds between heme and cysteine, other possibilities must be considered (Section 1.6.2). A conserved amino acid CPV motif has been proposed to bind heme (Steiner et al. 1996). Ferrous heme has been shown to be required for efficient heme incorporation into apocytochromes by heme lyase (Nicholson & Neupert 1989; Tong & Margoliash 1998).

1.5.2. The cytochrome *c* maturation (Ccm) system

Many Gram-negative bacteria (the α– and γ-proteobacteria) use proteins known as the cytochrome *c* maturation (Ccm) system (named for the genes *ccmABCDEFGH* found in *Escherichia coli* but also sometimes called System I) for cytochrome *c* biogenesis. Analogues of at least some of these proteins are also found in plant and protozoal mitochondria. Note that various different names are given to the equivalent genes from organisms other than *E. coli*, but here the Ccm nomenclature proposed by Page et al. (1998) is used; the cytochrome *c* biogenesis nomenclature is surveyed elsewhere (Thöny-Meyer (2000)). Furthermore, at least four additional gene products are essential for cytochrome *c* maturation in *E. coli*.

These are DsbA and DsbB (Metheringham et al. 1995; Metheringham et al. 1996), which are disulfide bond forming proteins in the periplasm, and DipZ (DsbD) and TrxA (thioredoxin) (Crooke & Cole 1995; Reid et al. 1998), which provide reductant to the periplasm via a disulfide chain. Very recently it has been shown, however, that DsbA and DsbB are not required for cytochrome *c* maturation in *Rhodobacter capsulatus* (Deshmukh et al. 2003). The bacterial Ccm system is complex and generally quite poorly understood; nevertheless, it is extremely flexible with regard to substrate and can catalyse the formation of cytochromes *c* of diverse prokaryotic and eukaryotic origin (Sambongi et al. 1996; Sanders & Lill 2000; Schlarb et al. 1999).

In Gram-negative bacteria, *c*-type cytochromes are found in the periplasm, or anchored to the cytoplasmic membrane with the covalently attached heme groups on the periplasmic side (Ferguson 1991; and references therein). Heme synthesis occurs in the cytoplasm (Section 1.4.), whereas heme attachment to apocytochromes occurs in the oxidising environment of the periplasm. The apocytochromes are transported to the periplasm via the general type II secretion (Sec) pathway (Manting & Driessen 2000; Thöny-Meyer & Kunzler 1997). The Ccm proteins (Figure 1.9) are all located in the periplasm and/or cytoplasmic membrane and the Ccm system functions periplasmically.

A number of studies on ABC transporters have suggested that the proteins CcmA and CcmB form a transporter for some component required by the Ccm system (Section 1.5.2.1). CcmC appears to be essential for the delivery of heme to the membrane-anchored protein CcmE and is sufficient for this function under some conditions (Ren & Thöny-Meyer 2001). CcmC interacts directly with CcmE via a

tryptophan-rich motif forming the first part of the putative periplasmic heme delivery system (Ren & Thöny-Meyer 2001). An additional role for CcmC has been proposed because the absence of CcmC affects the conversion of ferribactin to pyoverdine, probably by affecting oxidative steps that take place in the periplasm (Baysse et al. 2002).

Among the better-understood Ccm proteins is CcmE, which has been purified, partially characterized and identified as a protein that covalently binds heme *en route* to a *c*-type cytochrome (Reid et al. 1998; Schulz et al. 1998). This protein, which is referred to as a heme chaperone, forms a biologically novel type of covalent bond between a conserved histidine residue (H130 in *E. coli*) and heme, although the nature of this bond has not yet been established. CcmE has been shown to transfer its covalently bound heme to apocytochrome *c in vivo* (Schulz et al. 1998; Schulz et al. 1999). However, the mechanism of heme attachment to CcmE and subsequent release are unknown. Recently, structures of the CcmE apoproteins, *i.e.*, without the heme bound, from two different bacterial species have been reported (Arnesano et al. 2002; Enggist et al. 2002); these show that the heme-binding histidine is exposed on the protein surface. A structure of the *E. coli* heme chaperone CcmE is shown in Figure 1.8 (Enggist et al. 2002); it shows the β-barrel and the exposed, proposed heme-binding histidine residue.

Further studies have highlighted the essential nature of the histidine residue in terms of binding and releasing heme for cytochrome *c* maturation (Schulz et al. 1998). A hydrophobic patch is proposed to be formed by amino acids aiding heme-binding by the heme chaperone (Enggist et al. 2002). CcmE has been identified in many species of bacteria, as well as in *Arabidopsis thaliana* (Spielewoy et al.

2001). The protein could have several possible roles, such as heme storage and/or control of regio- and stereochemical attachment of heme to apocytochrome *c*, as well as catalysis of covalent heme attachment to these proteins.

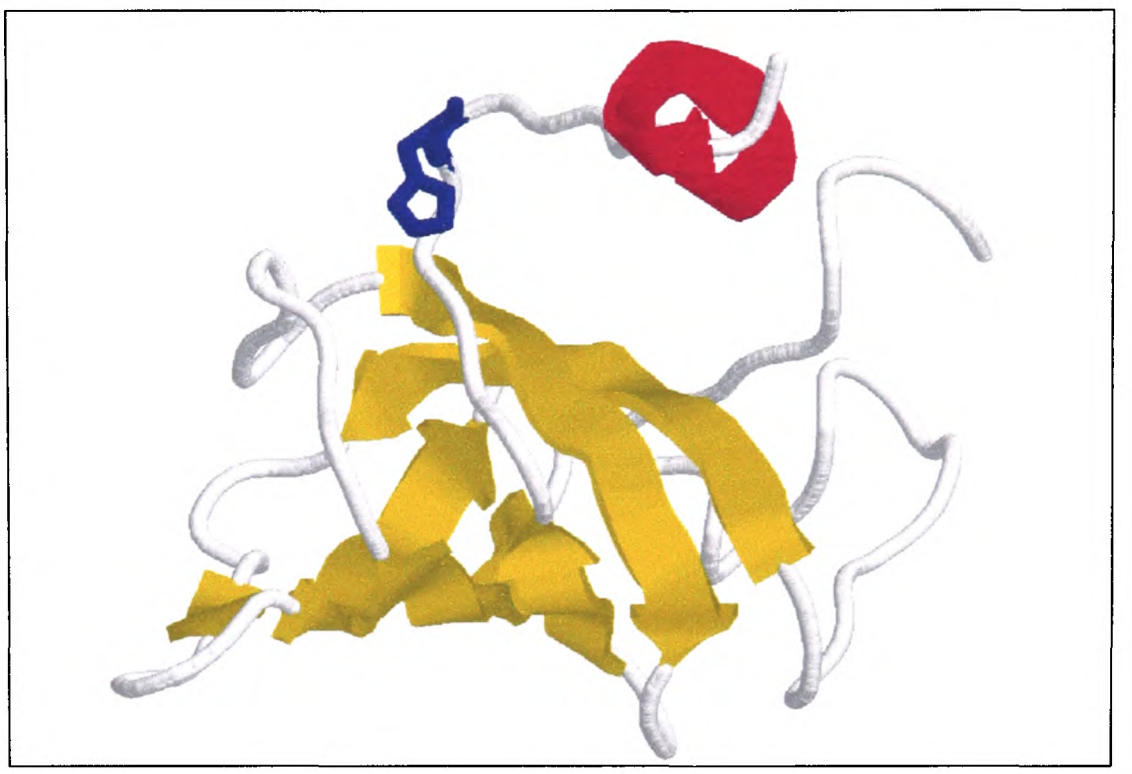


Figure 1.8. The solution structure of *E. coli* apo-CcmE (Enggist et al. 2002); viewed using RasMol and the data from PDB entry 1LIZ. The six-stranded β-sheet wrapped to a closed β-barrel is shown in yellow, the exposed histidine residue 130, which is proposed to bind heme covalently (Schulz et a. 1998), is highlighted in blue and the C-terminal α–helix is shown in red. Interestingly, most of the C-terminal region of the protein is disordered.

CcmD is a small integral membrane protein that appears to stabilise CcmE in the membrane (Schulz et al. 2000). CcmF and H have been proposed to function as a heme lyase (Ren et al. 2002), catalysing the covalent attachment of heme to apocytochrome *c*. CcmG and CcmH are thioredoxin-like proteins; CcmG is postulated to reduce disulfide bonds in apocytochrome *c* (Reid et al. 2001). It is notable that the absence *in vivo* of DsbD (Sambongi & Ferguson 1994a) and CcmH (Fabianek et al. 1999) can be overcome by addition of a low molecular weight thiol-containing compound such as Coenzyme M (2-mercaptoethane-

sulphonic acid). Thiol-redox pathways in Gram-negative bacteria are reviewed extensively elsewhere (Fabianek et al. 2000; Ritz & Beckwith 2001).

A schematic representation of the cytochrome *c* maturation (ccm) pathway is shown in Figure 1.9.

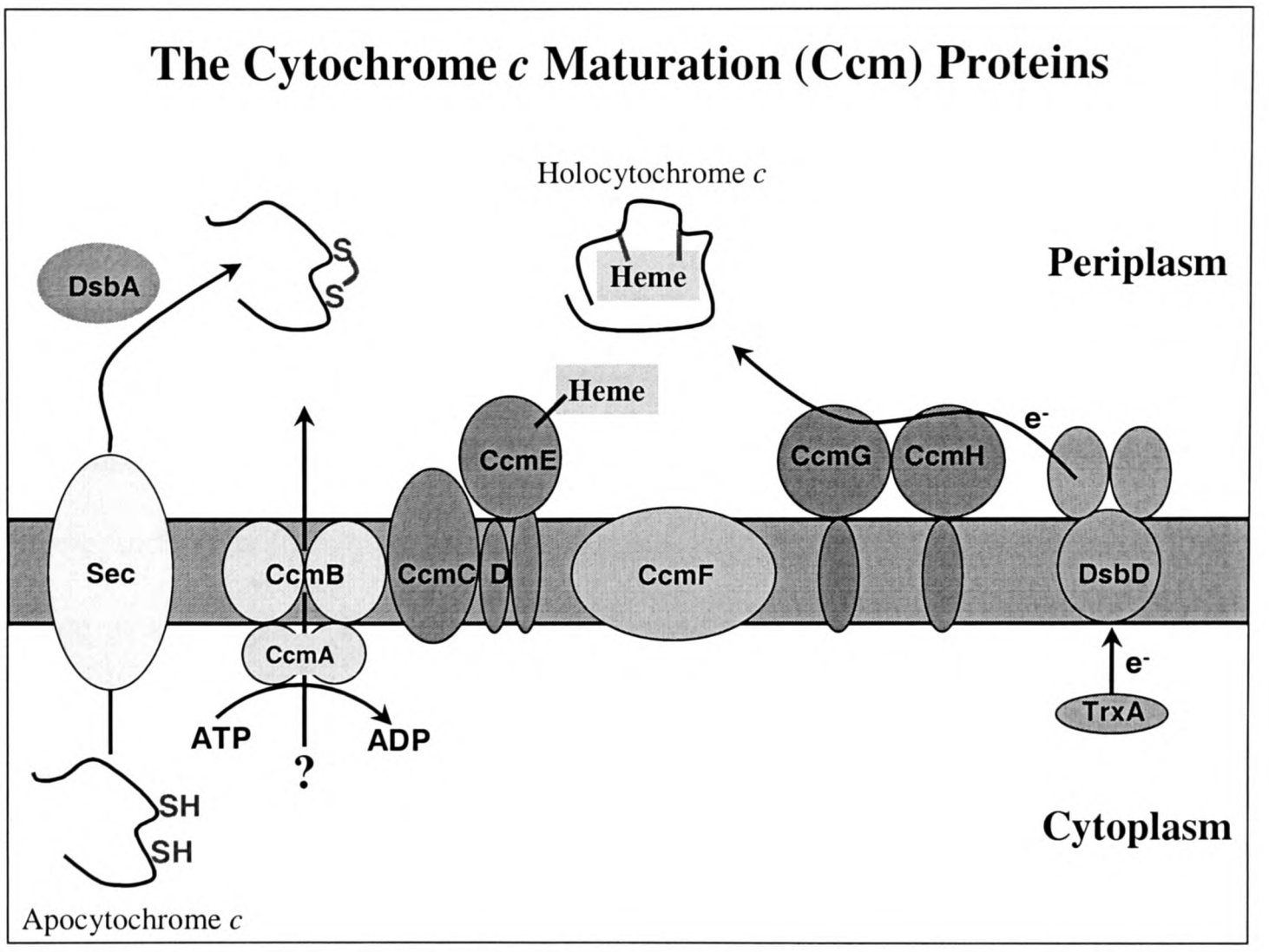


Figure 1.9. Schematic representation of the proteins involved during cytochrome c maturation by the Ccm system adapted from Page et al. (1998). Note that DsbA does not seem to be required for all organisms producing cytochrome *c* using this biogenesis system (Deshmukh et al. 2003).

1.5.2.1.Transporters associated with the Ccm system

CcmA and CcmB form elements of an ATP-dependent ABC transporter whose substrate is not known. Several components may need to be transported from the bacterial cytoplasm for covalent attachment of heme to protein to occur in the

periplasm. Some evidence from protein stability and co-immunoprecipitation experiments suggests that a complex of CcmABCD exports heme from its site of biosynthesis in the cytoplasm (Goldman & Kranz 2001 review the evidence in some detail). However, alternative evidence is accumulating that CcmAB is not a straightforward heme transporter from cytoplasm to periplasm. Disruption of *ccmA* did not inhibit accumulation of holocytochrome b_{562} in the periplasm of *E. coli* (Goldman et al. 1996; Throne-Holst et al. 1997). The route whereby heme reaches this protein is unknown; however, the accumulation of the apoprotein in the periplasm is dependent on the Sec pathway, *i.e.*, it is unlikely that the heme binds to cytochrome b_{562} in the cytoplasm before the protein is translocated to the periplasm. Supplementation of the growth media with heme did not stimulate *c*-type cytochrome formation in *ccmA* or *ccmB* mutants of *Paracoccus denitrificans* (Page et al. 1997a). Furthermore, *in vitro* heme uptake into everted membrane vesicles was neither ATP-dependent, nor was it different in a *ccmA* mutant when compared with the wild-type (Cook & Poole 2000). So if CcmAB does not transport heme, another substrate has to be considered. Mutants deficient in *ccmA* or *ccmB* could not form holo-CcmE, although very high level overexpression of CcmC complemented for this deficiency (Schulz et al. 1999). However, when the Δ*ccmA* mutant was complemented by overexpression of CcmC, holo-CcmE accumulated, but holocytochrome *c* did not (Schulz et al. 1999). These observations suggest that the substrate for CcmAB probably functions in the cytochrome *c* biogenesis process after heme attachment to CcmE, but might, depending on the precise role of CcmC, also function in that heme attachment. Such a factor may, for instance, be involved in accelerating heme-transfer from holo-CcmE to apocytochrome *c* or, specifically, in heme ligation to the apocytochrome *c*. Since correct attachment of heme to apocytochrome *c* requires

that the heme iron be reduced (ferrous) (Barker et al. 1993), another candidate substrate for CcmAB is a specific (ideally one-electron) ferric heme reductant.

CcmF has a predicted topology of 11 transmembrane helices (Goldman et al. 1998; Rios-Velazquez et al. 2003) and thus, speculatively, seems a plausible candidate for a second transporter in the Ccm system (Pearce et al. 1998). CcmF is essential for this mode of cytochrome *c* maturation, functions after covalent attachment of heme to CcmE (Reid et al. 1998; Schulz et al. 1998), and has been shown to interact with CcmE and CcmH (Ren et al. 2002), so it seems most unlikely to be transporting heme or a catalytic agent for holo-CcmE formation. However, possible transport substrates for CcmF are reductants for heme or a catalytic agent for heme transfer from CcmE to apocytochrome. Others have proposed that CcmF is a component of a bacterial heme lyase system (Ren et al. 2002) or, since most of the highly conserved residues of CcmF are periplasmic, a heme transport system along the outer surface of the periplasmic membrane (Goldman & Kranz 2001).

In *E. coli*, an additional ATP-dependent ABC transporter, CydDC, is also related to cytochrome *c* biogenesis. Both *cydD* and *cydC* mutants were shown to be deficient in cytochromes *c* (Goldman et al. 1996; Poole et al. 1994). This transporter is associated with maintaining the redox balance of the periplasm, but seems very unlikely to transport heme (Cook & Poole 2000; Goldman et al. 1996). Thus the substrate of CydDC may be required for use by the Ccm system (as discussed above for CcmAB), or its absence may deleteriously affect the functions of one, or a combination of, the thioredoxin-like Dsb proteins, CcmG and CcmH (Section 1.5.2.2). Recently, it has been shown that CydDC can export cysteine into the periplasm, and it is proposed that this thiol-containing compound is the

physiological substrate of the ATP-binding cassette-type transporter CydDC (Pittman et al. 2002).

1.5.2.2. The Ccm system and disulfide bonding in the apocytochrome CXXCH heme-binding motif

The Ccm system functions in the periplasm alongside the Dsb system which generates disulfide bonds. The CXXC motif in some proteins, including members of the Dsb family, can be converted into an intramolecular disulfide. Generation of a disulfide in a *c*-type cytochrome polypeptide before attachment of the heme would seem at first sight to be an undesirable reaction; it would be necessary for any intramolecular disulfide in apocytochrome *c* to be reduced before the thiols can react with the vinyl groups of heme. However, mutants of *E. coli* deficient in DsbA, DsbB (the oxidant for DsbA) or DsbD (which transfers electrons from the cytoplasm to the periplasm) were all unable to synthesize *c*-type cytochromes (Metheringham et al. 1996; Reid et al. 1998; Sambongi & Ferguson 1994a). This suggests that the Ccm system has evolved to function in series with the Dsb system such that as the apocytochrome polypeptide emerges from the Sec transporter proteins it is first oxidized by DsbA. CcmG and CcmH both have the thioredoxin motif (CXXC), and it has been proposed that at least one of these serves to reduce apocytochrome *c* before heme attachment, ultimately by transferring electrons from DsbD (Fabianek et al. 2000; Reid et al. 2001).

Such proposed interactions of the Ccm and Dsb systems require substantiation in several ways. First, there has been no example of an apocytochrome *c* with a disulfide bond between the apocytochrome cysteines. Second, the requirement for the Dsb system could reflect the need for a disulfide bond in one or more of the

Ccm proteins; it is not clear if the phenotypes of the various Dsb mutants arose because of the inability of these mutants to process a disulfide involving apocytochrome *c*, one or more of the Ccm proteins, or indeed a combination of these proteins, *i.e.*, an intermolecular disulfide bond between apocytochrome *c* and a Ccm protein. In this regard it is notable that a Gram-negative bacterial protein secretion system has recently been shown to require a disulfide in the secretion apparatus, rather than, as previously thought, in the secreted substrate itself (Pugsley et al. 2001). Fabianek *et al.* (Fabianek et al. 2000) have proposed that formation of a disulfide in the apocytochrome *c* pre-folds the protein to facilitate heme attachment.

A recent investigation into cytochrome *c* biogenesis by the Ccm system has highlighted required roles for both of the cysteine residues in the apocytochrome heme-binding motif (Allen et al. 2002). As described above, *Hydrogenobacter thermophilus* cytochrome c_{552}, which has a typical CXXCH heme-binding motif, and mutants with AXXCH and CXXAH motifs, can, exceptionally, be expressed as stable holocytochromes in the cytoplasm of *E. coli* (section 1.3.1 and Tomlinson & Ferguson 2000b). By targeting these proteins to the periplasm of *E. coli* using the signal peptide of a bacterial cytochrome *c*, the ability of the Ccm system to covalently attach heme to proteins with one or two cysteine residues in the heme-binding motif was assessed. Expression of the endogenous *E. coli* Ccm proteins was repressed by growing cells in aerobic conditions, but the Ccm proteins could be provided by their expression from a plasmid as required. Only the wild-type protein, with two cysteines, was effectively processed by the Ccm system and thus accumulated in the periplasm as a holocytochrome. With the signal sequence and the *ccm* plasmid present, essentially 100% of the wild type cytochrome was

periplasmic. In contrast, the mutant cytochromes were found in the periplasm only in small amounts even when the Ccm proteins were co-expressed (in each case less than 5% of the total cytochrome after subtractions for cytoplasmic contamination and endogenous cytochrome production by *E. coli*). Assessment of these data and the relative yields of the cytochromes allowed estimation of the upper limit of the activity of the Ccm proteins towards the single cysteine substrate apocytochromes as 2% of that towards the wild-type (CXXCH) apocytochrome *c*. These data (Allen et al. 2002), reporting at the level of the apocytochrome *c* rather than of the biogenesis proteins, are further strong evidence that an intramolecular disulfide bond involving the two cysteine residues of apocytochrome *c* is an intermediate in the Ccm type of cytochrome *c* biogenesis, and/or that the ultimate functional recognition determinant of the Ccm system requires the two cysteines in the heme-binding motif. An additional possibility is that both cysteine residues in the CXXCH motif play specific and required roles in the maturation pathway. It may be, for example, that the Ccm system is unable to process single cysteine apocytochromes *c* because two cysteine residues are required to release heme from holo-CcmE. Note, however, that any rationalisation of these observations (Allen et al. 2002) must allow for the fact that the Ccm proteins are active with substrate apocytochromes that either have naturally, or have been mutated to have, CXXXXCH or CXXXCH binding-motifs (Herbaud et al. 2000; Rios-Velazquez et al. 2001), and thus it is the two cysteines that are important, rather than their precise linear sequential arrangement. Also, the periplasmic protein DsbC from *E. coli* (and some other bacteria) has a CXXCH motif, but is not a *c*-type cytochrome (McCarthy et al. 2000); thus, the *c*-type cytochrome heme-binding motif on its own is not sufficient for covalent attachment of heme by the Ccm system. No (naturally occurring) cytochromes with heme attached through a single thioether

bond have been observed to date in bacteria, which is consistent with an inability of the Ccm system to process them.

If the Ccm system is organized to handle a disulfide-bonded apocytochrome then an interesting point arises with respect to plant and some protozoal mitochondria. Unlike fungal, invertebrate and vertebrate cell mitochondria, these seem to use some components of the Ccm system (at least CcmB, CcmC, CcmE and CcmF (Spielewoy et al. 2001)). If the analogy is drawn with the operation of the Ccm system in bacteria, then one might expect that there would be an intramolecular disulfide in an apocytochrome *c* in the intermembrane space of plant mitochondria. There does not appear to be any evidence for or against this compartment being sufficiently oxidizing for this to occur. Note, however, that analogues of CcmG or CcmH, the potential disulfide reductases of the Ccm system, have not been identified to date in plant mitochondria (Spielewoy et al. 2001). The CcmG and CcmH proteins from the bacterium *Rhodobacter capsulatus* have been estimated to have E°′ values of -300 and -210 mV, respectively, for reduction of their disulfide bonds; a peptide designed as a model for an apocytochrome *c* had an E°′ value of -170 mV (Setterdahl et al. 2000). In plant mitochondria it may be that the E°′ is such that CcmG and CcmH are not needed. Cytochromes *c* in which heme is attached to a single cysteine residue ((F/A)XXCH motif) have been isolated from some protozoan cells (Pettigrew et al. 1975; Priest & Hajduk 1992), but it is not yet known which cytochrome *c* biogenesis system is used by these organisms. If any eukaryote were found to have both the Ccm system and single cysteine attachment of heme in a cytochrome *c* then, by implication, the Ccm system can be modified when it operates outside bacteria to cope with a single cysteine heme-binding motif.

1.5.3. System II

In addition to the heme lyases and the Ccm proteins, there is a third system for the biogenesis of *c*-type cytochromes, known as System II. The latter is found in some Gram-negative bacteria (β– and ε–proteobacteria), Gram-positive bacteria, cyanobacteria and plant and algal chloroplasts (Kranz et al. 2002). This system uses at least four proteins for cytochrome *c* biogenesis (Beckett et al. 2000). CcsA (also called ResC) and CcsB (ResB) are proposed to form the heme transport and heme lyase system (Goldman & Kranz 2001), but this is far from certain. As discussed above, it is clear that in many Gram-negative bacteria the formation of *c*-type cytochromes occurs alongside disulfide bond formation in the periplasm. Page et al. (Page et al. 1997b) suggested that one reason for the complexity of the cytochrome *c* maturation apparatus in such organisms might be this co-existence. The absence of the Dsb system from the Gram-negative organism *Helicobacter pylori* led to the suggestion that the absence of the Ccm system in this organism might be related to an absence of a disulfide bond forming apparatus. *H. pylori* has the System II type of cytochrome *c* biogenesis apparatus, as does *Bacillus subtilis*, a Gram-positive organism from which the Dsb system was also apparently absent. However, this plausible correlation was undermined by the subsequent finding that System II was present in a Gram-positive organism, *Bordetella pertussis* (Beckett et al. 2000; Kranz et al. 2002), which also contained the Dsb system. In this organism, both DsbD and CcsX (a protein containing the CXXC thioredoxin motif and anchored on the outer face of the membrane) were shown to be essential for biosynthesis of all endogenous cytochromes *c* (Beckett et al. 2000).

More recently Erlendsson and Hederstedt (Erlendsson & Hederstedt 2002) have argued that *B. subtilis* has counterparts of DsbA and DsbB, called BdbD and BdbC

respectively. It is suggested that in combination the latter proteins generate a disulfide in the CXXCH motif of a *c*-type cytochrome. Electrons would be provided subsequently by CcdA (a clear relative of DsbD) and probably a protein called ResA, which is a homologue of CcsX; the latter has been shown to be involved in *c*-type cytochrome synthesis in *B. pertussis* (above). ResA has also been shown in *Bacillus subtilis* to be a thiol-disulfide oxidoreductase. It is proposed to be required for the reduction of the cysteinyls in the heme-binding site of apocytochromes *c* (Erlendsson et al. 2003). Thus it appears that some bacterial *c*-type cytochrome syntheses involve formation of a disulfide within the CXXCH motif. However, this may not be obligatory in the case of the *Bacillus* system (System II); Erlendsson and Hederstedt have shown that *c*-type cytochrome synthesis can occur in the absence of both CcdA and the Bdb system. In contrast, it should be noted that the absence of both DsbA and DsbD from *E. coli* did not permit cytochrome *c* synthesis by the Ccm proteins (System I) (Metheringham et al. 1996). Furthermore, Simon et al. (2002) have proposed that the *Wolinella succinogenes* System II biogenesis apparatus can covalently attach four hemes to a variant of NrfH, a tetraheme subunit of nitrite reductase, even when one of the CXXCH heme-binding motifs has been mutated to SXXCH. An overview of the current understanding of System II for cytochrome *c* biogenesis is summarised in Figure 1.10.

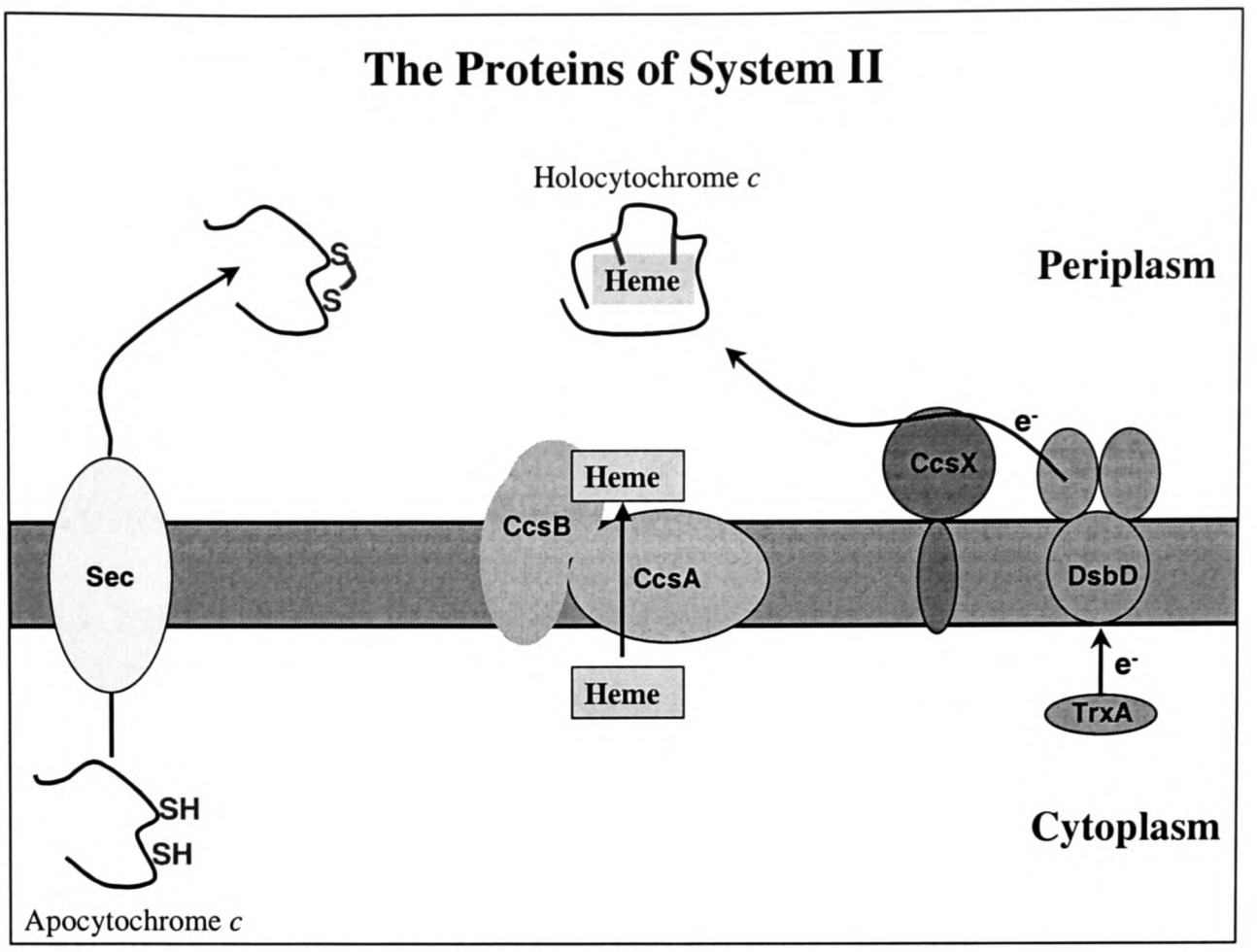


Figure 1.10. Schematic representation of the proteins involved in System II for cytochrome *c* biogenesis as exemplified by *Bordetella pertussis*. Reproduced from Kranz et al. (2002).

1.5.4. Unusual cytochrome *c* biogenesis

It is worth making a few further remarks concerning the biogenesis of an unusual cytochrome *c*. NrfA is a periplasmic enzyme that reduces nitrite by six-electrons to ammonia. It contains five covalently bound hemes; four of these are bound through the typical CXXCH cytochrome *c* motif, but the active site heme is bound through a CW(S/T/N)CK motif. In *E. coli*, which uses the System I (Ccm) biogenesis apparatus, three additional biogenesis genes (*nrfEFG*) are dedicated to attaching the heme to this CXXCK motif (Eaves et al. 1998; Thöny-Meyer 2000). NrfE is an analogue of CcmF, NrfG of the C-terminal region of CcmH and NrfF of the N-terminal region of CcmH. (Note that in some bacteria, the analogues of these two parts of CcmH are transcribed from separate genes (Thöny-Meyer 1997)). Interestingly, in *W. succinogenes*, which uses the System II cytochrome *c*

biogenesis apparatus, it is proposed that heme attachment to NrfA requires the additional dedicated System II gene *nrfI*; this gene has no obvious homology to *nrfEFG* (Kranz et al. 2002; Pisa et al. 2002; Simon et al. 2000).

1.6. CHEMICAL ASPECTS OF THIOETHER BOND FORMATION

1.6.1. Synthetic aspects of thioether bond formation

The biogenesis of *c*-type cytochromes involves the formation of thioether bonds, *i.e.*, carbon-sulfur single covalent bonds. Various synthetic studies on this type of bond have been performed, giving insight into the chemical aspects of this bond formation. Thioethers (sulfides) are synthetically prepared by alkylation of thiols in the presence of base. The base generates the alkanethiolate, which reacts, for example, with haloalkanes by an S_N2 process (Vollhardt & Schore 1994; Whitham 1995).

Syntheses involving the addition of thiol moieties to vinyl groups are known, best exemplified by the addition of cysteine to 5-vinyluracil under aqueous acidic conditions (Jones et al. 1987), which is shown below (Figure 1.11).

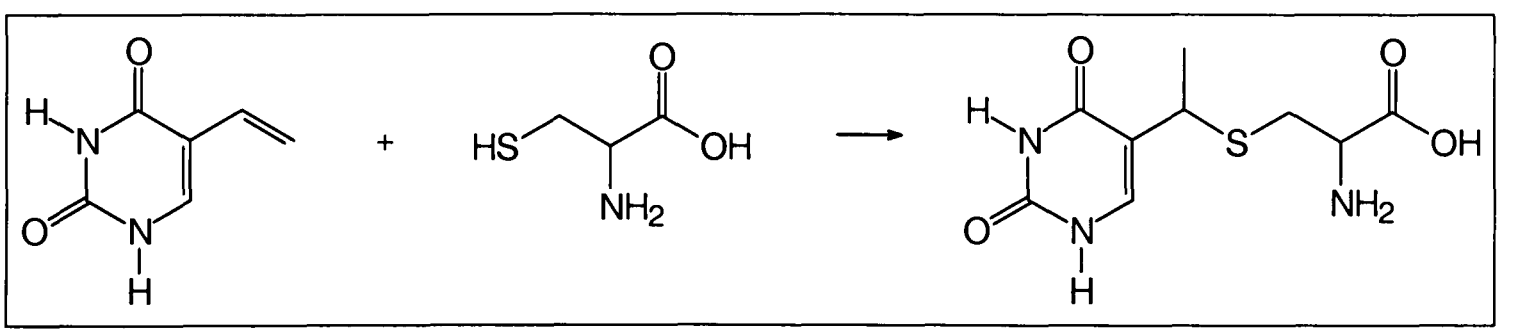


Figure 1.11. Scheme showing the reaction of cysteine with 5-vinyluracil, which yields 5-(1-cysteinylethyl) uracil. Reproduced from Jones et al. (1987).

Preparation of the 2,4-bis-cysteinyl derivative of protoporphyrin IX (described as porphyrin *c*) was first carried out under acidic conditions more than 60 years ago (Neilands & Tuppy 1960; Theorell 1939). Modified approaches have involved the regioselective attachment of cysteines via the dibromide adduct formed from the reaction of hematoporphyrin, a protoporphyrin IX derivative which has α-hydroxyethyl substituents in the position of the vinyl groups (cf. Figure 1.1), and hydrogen bromide (Karagianis et al. 1993; Scourides et al. 1986). Iron can be inserted into the porphyrin by standard procedures for metallation of tetrapyrroles (Falk & Smith 1975; Shedbalkar et al. 1988).

A direct approach to obtaining Fe-porphyrin *c* (heme *c*) in solution has also been reported and involves a radical mechanism (Kojo & Sano 1981). Thioether bonds have been reported to form on the surface of a gold electrode coated with mercaptoalkanes. Self-assembled monolayers (SAMs) containing the thiol functionality formed thioether bonds with heme which was physi-sorbed onto the SAM coated electrode surface (Pilloud et al. 2000; Pilloud et al. 1998).

The implications from these *in vitro* studies for the processes that must occur *in vivo* are unclear, but these synthetic studies, often under conditions that are harsh compared with biological environments highlight the chemical problems, which need to be overcome *in vivo* under physiological conditions.

1.6.2. Mechanistic insights into cytochrome *c* maturation

Mechanistically, formation of the thioether bonds in cytochromes *c* is very interesting. It presumably requires protonation of the β-carbon of the heme vinyl group and thioether bond formation between the cysteine sulfur from the

polypeptide and the heme vinyl α-carbon. An important feature of the *c*-type cytochrome thioether bonds is the regio- and stereospecificity of the heme-protein attachment (Section 1.6.2.1); the mechanism of how biological systems create the universally conserved stereospecificity is unresolved but intriguing. In the case of the fungal, invertebrate and vertebrate mitochondrial system, the proposed mechanism of heme-attachment involves binding of the heme by the heme lyase ((Steiner et al. 1996) and section 1.5.1).

The catalytic mechanism of the bacterial Ccm system is particularly complex; the mechanism of the heme transfer to apocytochrome *c* is poorly understood. One of the key aspects of the Ccm system is the attachment of heme to the heme chaperone CcmE, which involves the formation of a novel histidine-heme bond (Schulz et al. 1998). Very recently it has been reported that a covalent bond can form spontaneously in reducing conditions between a histidine residue and the α-carbon of the 2-vinyl group of heme in hemoglobin from the cyanobacterium *Synechocystis* sp. PCC 6803 (Vu et al. 2002). The reaction did not occur when redox inert zinc protoporphyrin IX was used, implicating the iron atom as crucial for such chemistry.

1.6.2.1. Control of covalent heme attachment by cytochrome *c* biogenesis systems

In all naturally produced cytochromes *c* for which high-resolution structures have been obtained, the heme is always attached covalently to the two cysteines of the heme-binding motif with the same stereochemistry (represented in Figure 1.2, Section 1.2). The importance of this stereochemistry with respect to the physiochemical properties of the cytochrome *c* is unclear. However, as discussed

by Barker and Ferguson (1999), strictly controlled reaction conditions are needed to obtain a regio- and stereospecific attachment of heme to an apocytochrome *c*. This is illustrated in the work of Barker et al. (1993) who reported that oxidizing conditions may produce a mixture of product cytochromes *c* because the ferric heme reacted incorrectly. The need for such controlled reaction conditions is also apparent from the observations of Keightley et al. (Keightley et al. 1998) on the cytoplasmic assembly in *E. coli* of a structurally different cytochrome c_{552} from *Thermus thermophilus.* In the latter case, *in vivo* addition of heme in the cytoplasm to apoprotein lacking a periplasmic targeting sequence, which therefore prevented heme attachment by the periplasmically functioning Ccm apparatus of *E. coli*, generated a mixture of three major products. In one of these, the heme was rotated 180° around its α,γ mesoaxis and a single thioether bond formed between residue cysteine 14 and the 2-vinyl group of heme, whilst cysteine 11 formed an intermolecular disulfide bond (McRee et al. 2001). The latter cysteine normally bonds to the 2-vinyl group. Clearly all cytochrome *c* biogenesis systems have to ensure that such misattachment does not occur. Note that when *T. thermophilus* cytochrome c_{552} was co-expressed in the periplasm of *E. coli* with the Ccm proteins, the product was essentially indistinguishable from that made by *T. thermophilus* itself (Fee et al. 2000).

1.6.2.2. Implications of the heme binding for the folding of apocytochrome *c*

In work on horse heart cytochrome *c* two interesting observations have been made. Upon addition of heme to apocytochrome, a *b*-type cytochrome complex formed in which the heme was non-covalently bound in a heme pocket with the iron axially coordinated by amino acid(s) (Dumont et al. 1994). This *b*-type cytochrome complex had substantial structure as shown by CD spectroscopy (Dumont et al.

1994) and studies with antibodies against holocytochrome *c* (Goldberg et al. 1999). The antibodies recognised the *c*- and *b*-type heme-protein complexes, but not the apocytochrome.

It has also been shown that the cysteine thiols of horse heart apocytochrome *c* can undergo a nucleophilic reaction onto brominated electrophilic carbons of hydrophobic molecules (Kang & Carey 1999), indicating a potential reaction mode of the cysteine thiols. The covalently bound hydrophobic molecule also induced substantial ordering of the protein structure as judged by CD spectroscopy. These observations are in agreement with the substantial folding of the apocytochrome of the C11A/C14A mutant of *H. thermophilus* cytochrome c_{552} (Wain et al. 2001) and the *b*-type cytochrome (Tomlinson & Ferguson 2000a). The investigation and implications of the folding of cytochrome *c* are reviewed extensively elsewhere (Englander et al. 1998; Yeh et al. 1998).

1.6.2.3. Periplasmic heme delivery in Gram-negative bacteria

Delivery of heme to the periplasm is an issue both for cytochrome *c* and periplasmic *b*-type cytochrome formation; the means of delivery could be different, and specific, for each of these classes of protein. How the cytoplasmically made heme is transported to the periplasm for bacterial cytochrome *c* biosynthesis is unknown, but, as described above (section 1.5.2.1), there is scarce evidence it has anything to do with the ATP-dependent transporter of the Ccm system (where that system is used) and some to the contrary. It is not known how heme crosses any biological membrane, and it is noteworthy that cytochrome *c* biogenesis always occurs on the opposite side of a membrane from that of the final step of heme synthesis. It is probable that another ATP-dependent

transporter, CydDC, does not, despite earlier proposals, transport heme across the cytoplasmic membrane of *E. coli* and cysteine has now been shown to be a substrate for this transporter (Cook & Poole 2000; Pittman et al. 2002). Cook and Poole (2000) discuss extensively the conflicting evidence about heme transport across the cytoplasmic membrane by such mechanisms as energy dependent transport, specific and non-specific diffusion processes and processes where periplasmic apocytochromes "pull" heme out of the lipid bilayer. For System II cytochrome *c* biogenesis, it has been proposed that the non-ATP dependent protein CcsA, possibly in complex with CcsB, transports the heme across the membrane for attachment to apocytochrome *c* (Kranz et al. 2000). *b*-type cytochromes are rare in the bacterial periplasm; an inability of the cytochrome *c* biogenesis systems to process (or perhaps not to interfere with synthesis of) proteins with non-covalently bound heme could, in part, account for this rarity. However, cytochrome b_{562} can form in large quantities in the periplasm of *E. coli* irrespective of the extent of expression, or disruption, of the *ccm* genes (Allen et al. 2002; Goldman et al. 1996; Throne-Holst et al. 1997). Naturally occurring periplasmic *b*-type cytochromes (e.g. b_{562}, dimethyl sulfide oxidase) have Sec signal sequences, so the heme does not become bound to the protein in the cytoplasm, as must also be the case with experiments where exogenous cytochromes were periplasmically targeted (Kaderbhai et al. 2000; Karim et al. 1993). Since they are rare, it is possible that the specific nature of the periplasmic apocytochrome *b* may be important for the process of heme acquisition. The non-covalently bound d_1 heme of the cytochrome cd_1 type nitrite reductases is transported to the periplasm by a specific transporter that uses the Tat system (Heikkila et al. 2001), but it should be noted that this heme is significantly more biosynthetically demanding than Fe-protoporphyrin IX and is only found in cytochromes cd_1.

1.6.3. Rationale for periplasmic cytochrome *c* formation

Why are *c*-type cytochromes not naturally made in the cytoplasm in a catalysed reaction? Folded holocytochromes *c* could be exported to the periplasm by the twin arginine translocation (Tat) system (Robinson & Bolhuis 2001; Sargent et al. 2002). Perhaps this would work for mono-heme cytochromes *c*, but the multi-heme cytochromes *c*, with their clustered arrays of *c*-type centres, might need to be assembled via correctly formed intermediate disulfides. Otherwise, thioether bonds might form between a wrong pair of cysteines and the heme moiety. In general, disulfide bonds cannot form in the cytoplasm as it is a reducing environment. Therefore, if it is advantageous to assemble *c*-type cytochromes via disulfide bonds, this would provide a rationale for a periplasmic route (Ferguson 2001). To date, multiheme cytochromes *c* have only been found in organisms that use the Ccm and System II biogenesis systems (which as described above (sections 1.5.2 and 1.5.3) can interact with disulfide bond forming apparatus). In contrast, multiheme cytochromes *c* have not been identified in organisms that use the heme lyase biosynthesis system, for which the balance of evidence presently suggests that a disulfide bond does not normally form in the CXXCH heme-binding motif (section 1.5.1).

1.7. Aims of this Project

One of the main questions for cytochrome *c* maturation is how thioether bonds form between the cysteine residues of apocytochrome *c* and the heme-vinyl groups. Therefore, a key aspect to be studied was the interaction of heme with apocytochrome *c*. A promising candidate for investigating an apocytochrome *c* was *Hydrogenobacter thermophilus* cytochrome c_{552}, both for the reasons described in section 1.3.1 and as a consequence of the observation that other

cytochromes might not be as stable, especially with regard to heme extraction methodologies. *In vitro* studies with apocytochrome *c* and heme were expected to lead to further insights into the basic requirements and conditions needed for cytochrome *c* maturation. Following from this, a fresh look at the interaction between other apocytochromes *c* and heme could be undertaken.

A key protein of the Ccm system is CcmE, which forms a covalently heme-bound intermediate in cytochrome *c* maturation. It was therefore desirable to study interactions of the bacterial apo-CcmE with heme *in vitro*, so as to identify the conditions under which both covalent attachment to CcmE and the transfer of heme from holo-CcmE to apocytochromes might occur. Mutagenesis experiments of the heme-binding histidine residue to amino acids with reactive or unreactive side chains were anticipated to be a further focus of attention.

CHAPTER 2

MATERIALS AND METHODS

2.1. BACTERIAL STRAINS AND PLASMIDS

The bacterial strains used in this study are listed in Table 2.1. The plasmids used in this work are given in Table 2.2. Plasmids produced during this work using site directed mutagenesis are given in Table 2.3.

Table 2.1. Bacterial strains used in these studies.

Strain	Used for	Source/Reference
E. coli DH5α	Plasmid DNA production	Lab stock/Hanahan 1983
E. coli XL2-blue	Plasmid DNA production	Stratagene/Bullock et al. 1987
E. coli JCB7123	Protein expression	Lab stock/Iobbi-Nivol et al. 1994
E. coli JM109 (DE3)	Protein expression	Lab stock/Yanisch-Perron et al. 1985
E. coli XL1-blue	Protein expression	Stratagene/Bullock et al. 1987

Table 2.2. Plasmids used for this work.

Plasmid	Expressed protein(s)	Source/Reference
pKHC12	*H. thermophilus* cytochrome c_{552}	Lab stock/Sanbongi et al. 1991
pKPD1	*P. denitrificans* cytochrome c_{550}	Lab stock/Sambongi & Ferguson 1994b
pEC86	CcmABCDEFGH	Prof. Thöny-Meyer/Arslan et al. 1998
pCcmE'	$CcmE^{sol}$-C-His_6	Dr. Stevens/Daltrop et al. 2002b
pE151	N-His_6-$CcmE^{sol}$	Dr. Stevens/Stevens et al. 2003

2.2. OLIGONUCLEOTIDE PRIMERS

Oligonucleotides (Table 2.3) were synthesised by Sigma Genosys and 5'phosphorylated during synthesis, followed by desalting for operational purity.

Table 2.3. Oligonucleotides used for mutagenesis and resulting plasmids constructed.

Primer	**Sequence (5'-3')**	**Plasmid made**
*pET22b*H130AF	GCCGATGAAAACTATACGCCG	**pE222**
*pET22b*H130AR	TTTCGCCAGCACTTCTTTCGC	
*pET22b*H130CF	TGCGATGAAAACTATACGCCG	**pE223**
*pET22b*H130CR	TTTCGCCAGCACTTCTTTCGC	
*pET15b*H130AF	GTGCTGGCGAAAGCCGATGAAAACTATACGCCG	**pE152**
*pET15b*H130AR	CGGCGTATAGTTTTCATCGGCTTTCGCCAGCAC	
*pET15b*H130CF	GTGCTGGCGAAATGCGATGAAAACTATACGCCG	**pE153**
*pET15b*H130CR	CGGCGTATAGTTTTCATCGCATTTCGCCAGCAC	
*pET15b*HMAAF	GGTGCCGCGCGGCAGCGCTGCGCTCGAGTCGAATATCG	**pE154**
*pET15b*HMAAR	CGATATTCGACTCGAGCGCAGCGCTGCCGCGCGGCACC	
C552AXXHHF	CAAGCAAAAGGGCGCCATGGCTCATCACGATCTGAAAGC	**pOli1**
C552AXXHHR	GCTTTCAGATCGTGATGAGCCATGGCGCCCTTTTGCTTG	
C552HXXAHF	CAAGCAAAAGGGCCATATGGCTGCCCACGATCTGAAAGC	**pOli2**
C552HXXAHR	GCTTTCAGATCGTGGGCAGCCATATGGCCCTTTTGCTTG	

2.3. BACTERIAL GROWTH CONDITIONS AND MEDIA

2.3.1. Liquid medium

The composition of the media used is listed in the tables below.

Table 2.4. LB Medium

Ingredient	**Amount (g l^{-1})**
Tryptone	10
Yeast Extract	5
NaCl	10

Table 2.5. 2 x YT Medium

Ingredient	**Amount (g l^{-1})**
Tryptone	16
Yeast Extract	10
NaCl	5

2.3.2. Growth of bacteria on solid medium

Bacteria were grown routinely on agar plates with media as described above containing 1.5 % agar (Melford labs).

2.4. GENETIC TECHNIQUES

2.4.1. Purification of plasmid DNA

Plasmid preparation was performed using the Qiagen Plasmid Mini or Midi (for large scale cloning) Prep systems (Qiagen), using the kit as per the manufacturer's instructions. Plasmid DNA was stored at –20 °C.

2.4.2. Restriction digestion of DNA

Restriction digestion was carried out for analysis of plasmid DNA. Restriction enzymes (Roche, New England Biolabs) were incubated with DNA according to the manufacturer's instructions, using the buffer systems supplied, for 1.5 h at 37 °C. Digested DNA was subjected to agarose gel electrophoresis.

2.4.3. Agarose gel electrophoresis

DNA samples were analysed for their gel mobility by agarose gel electrophoresis. 6 x gel loading dye (10 mM EDTA, 50 % v/v glycerol, 0.5 % bromophenol blue) was added to the DNA samples. These were routinely loaded on agarose gel slabs (40 mM Tris-acetate, pH 8.0, 1 mM EDTA, 1 % agarose, ethidium bromide 0.2 μg ml^{-1}), which were run in TAE buffer (40 mM Tris-acetate, pH 8.0, 1 mM EDTA) at a constant current of 60 mA. Ethidium bromide facilitated DNA visualisation using ultraviolet radiation.

2.4.4. Polymerase chain reaction (PCR) methods

PCR amplification was carried out in 0.5 ml thin-walled Eppendorf tubes using a Techne Genius thermocycler. Either of two DNA polymerases were used routinely, PLATINUM Pfx (Invitrogen) or Pfu (Stratagene). Both enzymes were used according to the manufacturers' instructions. The PCR conditions were

chosen according to the site directed mutagenesis methodology (section 2.4.6) used. Standard conditions were denaturation for 2 min at 95 °C, followed by 18 cycles of denaturation (95 °C, 1 min), primer annealing (50 °C, 1 min) and extension (72 °C, 1 min/kb DNA for Pfx or 2 min/kb for Pfu). Finally a single extension step (72 °C, 10 min) was added when Pfx was used.

2.4.5. DNA sequencing

Plasmid DNA was sequenced by the in house DNA sequencing facility using v.3 Big Dye terminators on an Applied Biosystems I377 sequencer.

2.4.6. Site directed mutatgenesis (SDM)

The expression vector for $CcmE^{sol}$-C-His_6 (pCcmE'), *i.e.*, the soluble construct of CcmE with a C-terminal hexahistidine tag, was constructed by Dr. Julie Stevens as described (Daltrop et al. 2002) and produces the soluble periplasmic region of the protein from residue Ser32 with a cleavable *pelB* signal sequence for periplasmic targeting of the protein. Amino acid substitutions in this expression vector were performed using the ExSite PCR-based SDM method (Stratagene) using the primers pET22bH130AF and -R for the H130A mutant, and pET22bH130CF and -R for the H130C mutant, respectively (as listed in Table 2.3). PCR conditions were chosen as per manufacturers' instructions.

The vector for the cytoplasmic expression of the thrombin-cleavable N-terminal hexahistidine-tagged version of CcmE (N-His_6-$CcmE^{sol}$) was constructed by Dr. Julie Stevens as described (Stevens et al. 2003) yielding the plasmid pE151. The nucleotide sequence for this construct of CcmE is given in the APPENDIX. Mutations were made in this plasmid using the QuikChange method (Stratagene).

The mutations made were H130A using the primers pET15bH130AF and –R, H130C using the primers pET15bH130CF and –R, as well as mutation of the His and Met (both to Ala) that remain on the protein following thrombin cleavage, using the primers pET15bHMAAF and –R (Table 2.3). PCR conditions were chosen as per manufacturer's instructions for the QuikChange method. All SDM procedures on plasmids containing CcmE variants were carried out in collaboration with Dr. Julie Stevens.

Two variants of cytoplasmically expressed *H. thermophilus* cytochrome c_{552} were made using pKHC12 as the template. Mutations were made using the QuikChange kit (Stratagene) and primers C552AXXHHF and -R and C552HXXAHF and -R for the AXXHH and HXXAH heme-binding motif variants, respectively. The manufacturer's protocol was followed. The gene encoding *H. thermophilus* cytochrome c_{552} is given in the APPENDIX. All of the resulting plasmids were sequenced to confirm that only the desired mutations had been incorporated.

2.4.7. Ligation of DNA fragments to plasmid vectors

DNA fragments obtained from PCR (section 2.4.5) using the Excite SDM methodology were ligated with T4 DNA ligase (Roche). Ligation reactions were incubated overnight at 16 °C in the buffer supplied by the manufacturer following the manufacturer's recommendations for optimal DNA ligation conditions.

2.4.8. Transformation of competent *E. coli* with plasmid DNA

Strains obtained from Stratagene (Table 2.1) were transformed as in the manufacturer's instruction manual.

For strains from lab stocks, typically 200 µl of competent cells were thawed on ice and incubated with plasmid DNA on ice for 30 min. The cells were heat shocked at 42 °C in a water bath for 1.5 min followed by immediate placement on ice for 10 min. When pEC86 was co-transformed, LB medium (1 ml, filter-sterilised, Whatman, 0.45 µm) was added and cells were left to recover at 37 °C for 1 h. Cells were centrifuged, resuspended in 50 µl and plated on agar containing the appropriate antibiotics as required.

2.5. PROTEIN BIOCHEMISTRY TECHNIQUES

2.5.1. Sodium dodecyl sulphate polyacrylamide electrophoresis (SDS-PAGE)

The following buffers were used for SDS-PAGE, as described in the protocol for the discontinuous buffer system (Laemmli 1970):

4 x separating buffer: 18.7 g Tris Base, 0.4 g SDS, HCl to pH 8.8, water to 100 mls;

4 x stacking buffer: 6.06 g Tris Base, 0.4 g SDS, HCl to pH 6.8, water to 100 mls;

5 x running buffer: 15 g Tris Base, 5 g SDS, 72 g glycine, water to 100 mls;

3.3 x loading buffer: 125 mM Tris-Cl, pH 6.75, 20% (v/v) glycerol, 4% SDS, 0.02% bromophenol blue. For reductive conditions DTT (50 mM) was added.

Samples were prepared by adding 20 µl protein solution to 5 µl 3.3 x loading buffer. The protein sample was denatured by heating in boiling water for 10 min. Prestained protein markers (New England Biolabs, Beverly, USA) were used when staining for covalently bound heme was performed. Ultra low range molecular weight markers (Sigma; 10µl) were diluted with water (130µl) and 3.3 x loading buffer (60µl). Protein preparations were analysed using a 4% stacking gel and an appropriate acrylamide concentration in the separating gel (Table 2.6).

Table 2.6. Composition of the SDS polyacrylamide gels.

Component	4 % stacking	12 %	15 %	17.5 %
30 % acrylamide/ml	0.4	2.0	2.5	2.91
4 x separating buffer/ml	-	1.25	1.25	1.25
4 x stacking buffer/ml	0.75	-	-	-
Water/ml	1.85	1.75	1.25	0.83
10% APS/µl	30	50	50	50
TEMED/µl	3	2	2	2

PAGE was carried out on a Mini Protean III system (BioRad) at constant voltage (80 V/gel). If single gels were run, a buffer dam was used in the gel system.

2.5.1.1. Coomassie Blue staining

Gels were stained for 10 min in Coomassie staining solution (2.5 g/l Coomassie Blue, 45 % methanol, 45 % Glacial acetic acid; filtered). The stained gels were destained by boiling in water for 10 min. For improved destaining, gels were incubated in an aqueous solution containing 10 % glacial acetic acid and 30 % methanol until the non-specifically adsorbed Coomassie Blue was desorbed sufficiently to minimise the background colour.

2.5.1.2. Staining for covalently bound heme

Heme staining was performed as described in Goodhew et al. (1986). A gel was placed in sodium acetate (pH 5.0, 250 mM, 70 ml) for 30 min. 3,3',5,5'-Tetramethylbenzidine (33 mg) was added in a methanol solution (30 ml) and the gel was left to equilibrate for ten minutes on a rocking platform. Hydrogen

peroxide (30 % solution, 0.3 ml) was added and staining was complete in 5-10 min.

2.5.2. High Pressure Liquid Chromatography (HPLC)

High pressure liquid chromatography was performed on a Beckman System Gold apparatus. A C_{18} reverse-phase column (Vydac: 4.6 mm i.d., pore size 300Å, length 25 cm, 5 μm particle size) was routinely used. Trifluoroacetic acid (TFA) (0.1%) was added to all solvents used as the mobile phase. All solvents were thoroughly degassed under vacuum before use. In a standard method, 90 μl of solution was injected and a 20-80% acetonitrile gradient was run for 30 min, after equilibrating the column with 20/80 acetonitrile/water (0.1% TFA). Quantitative analysis of the proportion of CcmE containing heme was performed using this method with detection of absorbance at 280 and 408 nm.

2.5.3. Fast Protein Liquid Chromatography (FPLC)

Fast Protein Liquid Chromatography was performed with the following Pharmacia system: LCC-500/501 controller, two P-500 pumps, a solenoid valve, a 0.6 ml fluoroplastic mixer, an MV-7 control valve, a uv-1 monitor and a FRAC-100 fraction collector. Solutions were filtered through a 0.45μm cellulose nitrate membrane (Whatman). The column was equilibrated in appropriate buffer (see section 2.6) and the protein sample was applied via a 50 ml loop. The flow rate was 20.0 ml/min.

2.5.4. Mass spectrometry techniques

2.5.4.1. Electrospray mass spectrometry (ESMS)

Electrospray ionisation mass spectrometry (ESMS) was performed on a Micromass Bio-Q II-ZS triple quadrupole atmospheric pressure mass spectrometer equipped

with an electrosray interface by Drs. Robin Aplin and Neil Oldham or using an autosampler. Standard conditions used were, cone voltage 30 V; HV lens voltage 3.5 V; capillary voltage 0.45 V; using 50 % (v/v) acetonitrile in water as the mobile phase with 0.1 % (v/v) formic acid as the proton source. Samples (10 μL) were introduced into the electrospray source via a loop injector as a solution (20 pmol μl^{-1} in 1:1 water:acetonitrile, 1% formic acid) at a flow rate of 10 μl min^{-1}.

2.5.4.2. Matrix-assisted laser desorption ionisation mass spectrometry (MALDI-MS)

MALDI-MS was performed on a Micromass MALDI ToF Mass Spectrometer in combination with a Time-of-Flight detector for analysis by Dr. Robin Aplin and Mr. Colin Sparrow. Protein samples were used at 5 pmol/μl in a α-cyano-4-hydroxycinnamic acid matrix, 10 mg/ml in 70 % water/ 30 % acetonitrile (0.1 % TFA). Ionisation was achieved with a Nitrogen laser pulse (4 ns pulse, 150-250 μm beam, 337 nm, 180 microjoules) at the sample spot. Below 10,000 Daltons the instrument was operated in Reflectron mode.

2.5.5. UV/visible absorption spectroscopy

UV/visible absorption spectra were acquired with a Lambda 2 spectrophotometer (Perkin-Elmer) in quartz cuvettes (Helma) with a pathlength of 0.2 or 1 cm or in disposable UV cuvettes (Merck) with a pathlength of 1 cm.

2.5.5.1. Determination of protein concentrations

The concentrations of heme-containing proteins were determined using extinction coefficients for dithionite-reduced protein samples as previously reported (Table

2.7). Concentrations of apoproteins were calculated using the extinction coefficients at 280 nm determined using the method of Gill and von Hippel (1989), whereby the cysteine residues are assumed to be reduced. These extinction coefficients are given in Table 2.7. The amino acid sequences of the proteins are given in the APPENDIX. In the case of highly concentrated solutions of protein, aliquots were diluted with buffer to give a final maximum absorbance of less than 1.

Table 2.7. Extinction coefficients of proteins used in this work.

Protein	Extinction coefficient for (reduced) hemoprotein (mM^{-1} cm^{-1}) ; wavelength (nm)	Extinction coefficient for apoprotein (mM^{-1} cm^{-1}) at 280 nm	Reference
Yeast iso-1-cytochrome *c*	27.7; 550	12.1	(1)
H. thermophilus cytochrome c_{552}			
wild-type (CXXCH)	27.7; 552	15.2	(2)
C11A variant	27.7; 557	15.2	(2)
C14A variant	29.8; 556	15.2	(2)
P. denitrificans cytochrome c_{550}	30.0; 550	15.3	(3)
Horse heart cytochrome *c*	29.5; 550	10.9	(4)
Spinach cytochrome *f*	36.0; 554	-	(5)
P. laminosum cytochrome *f*	31.5; 556	-	(6)
CcmE (either construct)	-	7.68	

References:

(1) (Kluck et al. 2000)

(2) (Allen et al. 2002)

(3) (Koppenhöfer 1998)

(4) (Hulse et al. 1988)

(5) (Metzger et al. 1997)

(6) (Schlarb et al. 1999)

2.5.5.2. Pyridine hemochrome spectra

The absorption spectra of reduced hemes in the presence of hydroxide and pyridine are characteristic of the type of Fe-porphyrin present, as well as of any modifications to it (*e.g.* covalent attachment to the polypeptide in a *c*-type cytochrome). Such pyridine hemochrome spectra were obtained according to the method of Bartsch (Bartsch 1971). Reduced heme-protein solutions (400 µl) were thoroughly mixed with a 40 % pyridine (v/v) - sodium hydroxide (0.3 M) solution (400 µl). Spectral analysis was carried out after an incubation time of 5 min. The extinction coefficients for pyridine hemochrome spectra were taken from Falk (1964); for free heme an extinction coefficient of 34.4 mM^{-1} cm^{-1} at 556 nm and for *c*-type cytochromes $\varepsilon = 29.1$ mM^{-1} cm^{-1} at 550 nm was used.

2.5.6. Fluorescence spectroscopy

Fluorescence measurements were made using a Perkin Elmer LS 50B fluorimeter in a quartz cuvette (Helma) with a pathlength of 1 cm.

2.5.6.1. Determination of heme dissociation constants

The dissociation constant for heme was determined by Dr. Julie Stevens. The quenching of the intrinsic protein fluorescence was measured with excitation at 280 nm and emission from 285-350 nm (slit widths 5 nm) with increasing heme concentrations (100 μM and 1 mM stocks in DMSO); K_d values were determined by standard double-reciprocal plot analysis.

2.5.6.2. Determination of ANS dissociation constants and ANS displacement

ANS (8-anilino-1-naphthalenesulfonate (Sigma), 1 mM stock in 50 mM sodium phosphate buffer, pH 7.0) binding was examined by protein fluorescence quenching as described for heme, as well as by the enhancement of ANS fluorescence (Stryer 1965). This was measured by excitation at 380 nm and emission from 420-540 nm. The displacement of ANS by heme was measured by the decrease in ANS fluorescence at 480 nm upon addition of aliquots of heme (100 μM and 1 mM in DMSO) to a mixture of protein and ANS (25 μM and 750 μM, respectively). Fluorescence intensities were corrected for the values of free ANS and heme. Data was analysed using standard double-reciprocal plots and Rosenthal plot analysis.

2.5.7. Circular dichroism spectroscopy

Far UV circular dichroism (CD) spectra were recorded on a Jasco J720 spectropolarimeter with a quartz cuvette (Helma) with a pathlength of 0.1 cm.

2.5.8. Analytical ultracentrifugation

Equilibrium analytical ultracentrifugation was carried out in collaboration with Drs. Julie Stevens and Russell Wallis. The protocol for the equipment (using a Beckman Optima XL-A instrument) used is published by Wallis and Drickamer (1997). This technique was used to establish the self-association state of an apo-CcmE protein variant in the concentration range of 16-65 μM.

2.5.9. Ellman's test

Ellman's reagent was used according to the protocol outlined by Riddles et al. (1983).

2.5.10. AMS labelling

Thiol modification with 4-acetamido-4'-maleimidyl-stilbene-2,2'-disulfonate (AMS; Molecular Probes Inc., Leiden, Netherlands) (Vestweber & Schatz 1988) was carried out by incubating either reduced or oxidized apoprotein (20 μM) in freshly prepared AMS solution (15 mM AMS, 100 mM Tris-HCl (pH 7.5), 1 mM EDTA, 100 mM NaCl, 1 % SDS) for 45 min at 37 °C with occasional agitation. Buffer exchange to sodium phosphate buffer (50 mM, pH 7.0) was achieved with Centricon Centrifugal filter units (YM-10; Millipore, Bedford, USA) before analysis of the proteins.

2.5.11. Thrombin cleavage

Thrombin cleavage of N-His_6-$CcmE^{sol}$ and mutants thereof was performed using a Thrombin CleanCleave Kit (Sigma) according to the manufacturer's instructions. Uncleaved protein was removed by re-applying the reaction mixture to a Ni^{2+}-Sepharose column.

2.5.12. General laboratory techniques

2.5.12.1. pH measurements

pH was determined at room temperature using a KCl electrode and a pH meter (Hanna Instruments). Calibration was performed with standard buffers at pH 7.0 and pH 4.0 or pH 10.0. The pH of solutions was adjusted by addition of HCl (2 M) or NaOH (2 M).

2.5.12.2. Bradford assays

The concentrations of protein solutions were be measured using this method in some cases. A calibration sample was prepared by adding 1 ml of Bradford reagent (100 mg Coomassie blue G, 50 ml 95% ethanol, 100ml 85% phosphoric acid, 850 ml water; filtered; BioRad) to 100µl of water. 1 ml of Bradford reagent was added to the protein solution (10 µl) diluted with water (90 µl). The absorption of the sample was measured relative to the calibration sample at λ=595nm (Pharmacia spectrophotometer). The concentration of the protein sample was obtained by comparing the absorption value to a standard calibration curve measured with bovine serum albumin for the Bradford reagent.

2.5.12.3. Dialysis

Dialysis tubing (Snakeskin^{TM}, Pierce; $MW_{cut\ off}$: 3,500 Da) was routinely used for all proteins and all buffer systems. The tubing containing the protein solution was placed in the appropriate buffer exchange system, placed in the cold room and stirred continuously for several hours. The buffer was replaced at least three times after several hours of dialysis to ensure maximum dialysis efficiency.

2.6. PROTEIN PRODUCTION AND PURIFICATION PROTOCOLS

Media and glassware were sterilised in an autoclave (Astell) at 121°C, 15 psi, for a minimum of 20 minutes.

2.6.1. *Hydrogenobacter thermophilus* cytochrome c_{552}

Recombinant *Hydrogenobacter thermophilus* cytochrome c_{552} was purified from the cytoplasm of *E. coli* XL1-Blue, which had been transformed according to standard procedures (described in section 2.4.8) with the plasmid pKHC12 (Sanbongi et al. 1991), using the method described by Tomlinson and Ferguson (2000a) with small modifications.

Growth and harvesting of cells

Colonies of transformants grown on LB agar plates (Table 2.4), containing 100 μg ml^{-1} ampicillin for selection purposes, were used to inoculate 5 ml 2 YT medium (Table 2.5) in Falcon tubes. These precultures were grown under aerobic conditions at 37 °C overnight with shaking at 200 rpm for 14 h. The starter cultures (aliquots of 2.5 ml) were used to inoculate 2 l flasks containing 2 YT medium (each 750 ml; 100 μg ml^{-1} ampicillin). Incubation under the same conditions as the starter culture was performed until an OD_{595} of 0.9 was reached. Transcription of the *H. thermophilus* cytochrome c_{552} gene was induced with isopropyl-β-D-thiogalactopyranoside (IPTG; final concentration of 1 mM) and cells were incubated for approximately 6 h at 37 °C and 200 rpm. Cells were recovered by centrifugation (Beckman JA-10 rotor; 4,000 x g, 15 min, 4 °C), resuspended in 50 mM Tris-HCl pH 8.0 (20 ml), containing 10% (v/v) glycerol, and stored at –20°C. After thawing, the cells were lysed by sonication (MSE Soniprep 150, Fisons); 4 x

30 seconds with 2 min intervals for cooling on ice). The cell debris was removed by centrifugation (Beckman JA-20; 12,000 x g, 15 min, 4 °C).

Ion-exchange chromatography

The supernatant containing the soluble extract was applied to a FPLC-column containing 500 ml CM52-Sepharose fast-flow resin. The column had been pre-equilibrated with 50 mM Tris-HCl, pH 8.0, and was developed over 600 ml with a linear gradient of 0-300 mM NaCl in Tris-HCl, pH 8.0. Fractions of 20 ml were collected. All fractions that contained considerable amounts of red protein were pooled and retained for the next step of the purification. Protein eluted at a concentration of approximately 125 mM NaCl. Pooled fractions were concentrated to a volume of 1-2 ml in a Vivaspin centrifugal concentrator with a 5 kDa cut-off membrane.

Gel-filtration chromatography

As the final chromatographic step 1-2 ml of the concentrated protein solution was applied to a low pressure gel-filtration column (C 26/100) containing 500 ml G50 Sephadex resin (Sigma), which had been equilibrated with 50 mM potassium phosphate buffer, pH 7.0. The column was eluted using the same buffer at a flow rate of 15 ml h^{-1}. 2 ml fractions were collected, and those containing pure *H. thermophilus* cytochrome c_{552}, as judged by SDS-PAGE analysis, were pooled, concentrated and stored at –80 °C.

The C11A and C14A mutants of *H. thermophilus* cytochrome c_{552} (Tomlinson & Ferguson 2000b) were produced and purified using the same protocol by Mrs Lin Hong.

2.6.2. *Paracoccus denitrificans* cytochrome c_{550}

Paracoccus denitrificans cytochrome c_{550} was overexpressed in *E. coli* using the method described by Richter et al. (2002). This procedure was carried out in collaboration with Carsten Richter and Dr. Christopher Higham. The production and purification protocol is reproduced from Richter (2002). Competent cells of *E. coli* strain 7123 were co-transformed with the plasmids pKPD1 (complete gene for the cytochrome c_{550}) (Table 2.2) and pEC86 (cytochrome *c* maturation system) (Table 2.2) according to standard procedures (section 2.4.8) and plated on LB agar plates containing 35 μg ml^{-1} chloramphenicol and 100 μg ml^{-1} ampicillin to select for cells containing both plasmids. The recombinant *Paracoccus denitrificans* cytochrome c_{550} was purified as outlined below.

Growth and Harvesting of the cells

Colonies of transformants from agar plates were used to directly inoculate 2 l flasks containing 750 ml of LB medium. To prevent growth of cells devoid of one or both plasmids 100 μg ml^{-1} ampicillin and 35 μg ml^{-1} chloramphenicol were added to the medium. Cells were grown under aerobic conditions at 37 °C with vigorous shaking. A second pulse of ampicillin (100 μg ml^{-1}) was added after 6 h. 1 ml aliquots from the flasks were spun down in a micro centrifuge after 24 h. If cell pellets were pink, the contents of the flasks were harvested, if not, a third pulse of ampicillin (100 μg ml^{-1}) was added, and cells were allowed to grow for another three hours. Harvesting was achieved by centrifugation (JA-10 rotor; 4,000 x g, 15 min, 4 °C).

Preparation of the periplasmic extract

Pink cells were resuspended in polymyxin buffer (100 mM Tris-HCl, 100 mM NaCl, pH 8). One tablet of protease inhibitor cocktail complete^{TM} (Boehringer Mannheim) and 1 mg ml^{-1} polymyxin B sulfate were added. The solution was incubated for 15 minutes at 37 °C. Cell debris was spun down by centrifugation (Beckman JA-20 rotor; 17,500 x g, 20 min, 4 °C). The supernatant was retained and showed a typical *c*-type cytochrome visible absorption spectrum, with the cytochrome being approximately 50 % reduced.

Q-Sepharose column

The periplasmic extract was diluted 1:1 with 50 mM Tris-HCl, pH 8, to decrease the concentration of sodium chloride. It was then applied to a Q-Sepharose column (FPLC, Pharmacia) equilibrated with 50 mM Tris-HCl, pH 8. The column was developed with a linear gradient of 0-500 mM NaCl in 50 mM Tris-HCl, pH 8, and fractions of 20 ml were collected. The cytochrome c_{550} eluted at approximately 180 mM NaCl. Red fractions were pooled and concentrated to a volume of 2 ml using Vivaspin centrifugal concentrators (5 kDa cut-off membrane).

Size-exclusion chromatography

The concentrated cytochrome c_{550} solution was then subjected to size-exclusion chromatography. A G50 column (Sigma) was equilibrated with 50 mM potassium phosphate buffer, pH 7. The protein was applied to the column in batches of approximately 80 mg in a volume of *ca.* 2 ml. At a flow rate of 14 ml h^{-1} the cytochrome c_{550} typically eluted from the column after 4-5 h. It was collected in fractions of 5 ml. The purity was assessed by SDS-PAGE. Fractions with

$A_{410,ox}/A_{280,ox}$ > 3.5 were retained and concentrated to *ca.* 2 mM in a Vivaspin centrifugal concentrator. The purified protein was stored at –80 °C until needed.

2.6.3. Horse heart cytochrome *c*, yeast iso-1-cytochrome *c* and spinach cytochrome *f*

Horse heart cytochrome *c*, yeast iso-1-cytochrome *c* and spinach cytochrome *f* were obtained from Sigma and used without further purification.

2.6.4. *Phormidium laminosum* soluble fraction of cytochrome *f*

The soluble fraction of *P. laminosum* cytochrome *f* was kindly supplied by Drs. William Turner, Beatrice Schlarb-Ridley and Prof. Chris Howe, who used the published protocol for the purification of the protein (Schlarb et al. 1999). The cytochrome was used as supplied.

2.6.5. Variants of *Escherichia coli* CcmE

Studies on this protein were carried out in collaboration with Drs. Julie Stevens and Christopher Higham. The protocol for the production and purification of soluble CcmE variants is given below.

For expression of holo-CcmE, competent cells of *E. coli* JM109 (DE3) were co-transformed with the various plasmids for CcmE variants (Table 2.2 and 2.3) and pEC86 (Table 2.2) according to standard procedures (section 2.4.8) and plated on LB agar plates containing 35 μg ml^{-1} chloramphenicol and 100 μg ml^{-1} ampicillin to select for cells containing both plasmids. The co-expression of these proteins was found to be essential for production of holo-CcmE. High levels of apo-CcmE were expressed in the periplasm in the absence of pEC86 and/or without addition of 35 μg ml^{-1} chloramphenicol to the medium.

Growth and Harvesting of cells

Colonies of transformants grown on LB agar plates (Table 2.4), were used to inoculate 5 ml LB medium in Falcon tubes. These precultures were grown under aerobic conditions at 37 °C overnight with shaking at 200 rpm for 14 h. The starter cultures (aliquots of 2.5 ml) were used to inoculate 2 l flasks containing LB medium (each 500 ml; 100 μg ml^{-1} ampicillin and 35 μg ml^{-1} chloramphenicol for holo-forms of CcmE). *E. coli* cultures were grown aerobically in LB medium to mid-exponential phase and induced with 1 mM IPTG for 5 hours at 37 °C. Cells were harvested by centrifugation (Beckman JA-10 rotor; 4,000 x g, 15 min, 4 °C) and resuspended in 50 mM Tris-HCl, pH 7.4, 250 mM NaCl. The periplasmic fraction was prepared by adding polymyxin B sulfate to the cell suspension to a final concentration of 1 mg/ml, followed by incubation at 37 °C for 30 min and centrifugation (Beckman JA-20 rotor; 17,500 x g, 20 min, 4 °C).

Ni^{2+}- affinity chromatography

The periplasmic extract was applied to a Ni^{2+}-chelating Sepharose column equilibrated with 50 mM Tris-HCl, pH 7.4, to purify proteins with a hexahistidine tag. The column was washed with 10 column volumes of the same buffer and the protein was eluted from the column with 100 mM imidazole, 50 mM Tris-HCl, pH 7.4.

Hydrophobic-interaction chromatography

Separation of holo- from apo-CcmE was performed by Dr. Chris Higham using a Source Phenyl hydrophobic-interaction FPLC column equilibrated in 50 mM Tris-HCl, pH 8, 30% ammonium sulfate. Elution was achieved with a gradient of 30-0% ammonium sulfate.

Preparations of cytoplasmically produced protein

The cytoplasmically-expressed variants of CcmE (from the plasmids pE151, pE152, pE153 and pE154; Table 2.3) were purified in the same way, except that the cells were sonicated on ice three times for 30 sec to prepare the cell extracts, and the buffer contained 300 mM NaCl throughout. The cell debris was removed by centrifugation (Beckman JA-20 rotor; 11,500 x g, 15 min, 4 °C).

2.7. PRODUCTION OF APOCYTOCHROMES

2.7.1. Apocytochromes *c*

Several procedures for the production of apocytochrome *c* were attempted. The various available procedures are listed in Fontana et al. (1973). The method, which proved applicable to the cytochromes *c* in this study, was the treatment of the heme-protein with silver sulfate to break the thioether bonds, a process initially reported by Paul (1950; 1951) and modified by Fisher et al. (1973). Silver sulfate (40-50 mg) was incubated for 30 min in degassed acetic acid solution (0.1 M; 20 ml) in the dark at 37 °C. Cytochrome *c* (0.5 μmol) was added and the solution was incubated for 1.5 to 3 h. The solution was thoroughly dialysed either against sodium acetate (50 mM, pH 5.0) or sodium phosphate buffer (50 mM, pH 7.0). The protein was incubated with DTT (0.2 M final concentration) for 2 h. The protein was concentrated (Vivaspin centrifugal concentrators with 5 kDa cut-off membrane) was applied to a G50 column (Sigma), which was equilibrated with 50 mM potassium phosphate buffer, pH 7. This chromatographic step was used to obtain apocytochrome *c* and separate it from holocytochrome. Fractions were checked by SDS-PAGE analysis followed by heme-staining (section 2.5.1). It was observed that after the treatment with DTT solutions had a single absorption maximum at 389 nm, presumably arising from the cleaved heme-derivative Fe-

hematoporphyrin, and did not contain holocytochrome *c*. The chromatographic separation step could be omitted in these cases.

2.7.2. C11A/C14A variant of *H. thermophilus* apocytochrome

Apocytochrome of the C11A/C14A mutant of *H. thermophilus* was prepared by Drs. Esther Tomlinson and James Allen according to published procedures (Tomlinson & Ferguson 2000a). The kindly supplied protein was used without further purification.

2.8. HEME ADDITION EXPERIMENTS

2.8.1. Heme addition to apocytochromes *c*

Hemin (Sigma) or mesoheme (Frontier Science) was used from a stock solution (1 mM) in dimethylsulfoxide (DMSO). Reconstitution of cytochromes was achieved by the addition of apoprotein to (typically 2-5 μM) heme (or mesoheme) in sodium phosphate buffer (pH 7.0 unless otherwise stated, 50 mM) at 25 °C. Fe-porphyrin solutions were reduced with disodium dithionite. Apoprotein was kept reduced by the addition of 5 mM DTT. The presence of oxygen was avoided by thoroughly sparging all solutions with humidified argon. Reactions were carried out in the dark. Free heme and aggregated protein was removed by passing the solution through PD-10 desalting columns (Amersham Biosciences). The kinetics of cytochrome c_{552} formation were monitored at wavelengths characteristic of the *b*-type cytochrome intermediate leading to the formation of cytochrome c_{552}, and of cytochrome c_{552} itself. Kinetic data were analysed using TableCurve (Jandel Scientific, California).

2.8.2. Heme addition to apo-CcmE variants

Heme or mesoheme was added to an apo-CcmE' solution in 50 mM sodium phosphate buffer, pH 7.0. A range of heme concentrations was used, but concentrations exceeding 20 μM sometimes failed to stay in homogeneous solution in some cases as seen by formation of precipitate, especially in the absence of His-tags. To achieve quantitative (1:1) loading of CcmE' with heme, 1 equivalent of protein was incubated with 1.1 equivalents of ferric heme at room temperature. Excess heme was removed by passing the heme-protein containing solution through a PD-10 desalting column. Heme was reduced with disodium dithionite (Sigma). Potassium cyanide was added when required to a final concentration of 2 mM from a concentrated stock solution in 200 mM phosphate buffer, pH 7.0. Potassium ferricyanide was added to oxidise reduced samples. The half-life of the *b*-type heme- or mesoheme-CcmE' complex upon reduction was measured by the decrease in the absorption spectrum Soret bands at 425 nm and 407.5 nm for heme and mesoheme, respectively, over a time period of five minutes.

2.8.3. Studies on the covalent attachment of heme to CcmE variants

Low molecular weight thiol-containing compounds were added to a 20 μM solution of the quantitatively formed *b*-type ferric heme-CcmE' complex. Dithiothreitol (DTT, Avocado), propane-1,3-dithiol (Avocado), cysteine (Sigma) or 2-mercaptoethanesulfonic acid (Sigma) were added from 100 mM stock solutions to give final concentrations of 5 mM in the protein solution. Non-covalently bound heme was removed by treating the heme-protein solution with 1 M imidazole (in 50 mM sodium phosphate buffer, pH 7.0) overnight at 4 °C in the dark, followed by extensive dialysis. Solutions were deoxygenated by thoroughly sparging with humidified argon. Reactions were carried out in the dark.

2.9. HEME TRANSFER STUDIES FROM A HOLO-CCME VARIANT TO APOCYTOCHROME *C*

H. thermophilus apocytochrome c_{552} (10 μM), or the apo C11A/C14A variant (Tomlinson & Ferguson 2000a), and an equimolar amount of *E. coli* holo-CcmE' were incubated in 50 mM sodium phosphate buffer, pH 7.0 at 25 °C for 18 hours. *H. thermophilus* apocytochrome c_{552} C11A and C14A variants (15 μM), horse heart apocytochrome *c* (15 μM) and yeast apo iso-1-cytochrome *c* (15 μM) were incubated with *E. coli* CcmE (35 μM) in 50 mM sodium phosphate buffer, pH 7.0 at 25 °C for 10 hours. Samples were reduced by the addition of disodium dithionite to a final concentration of 5 mM. Alternatively, samples were reduced with disodium dithionite and DTT, both to a final concentration of 2 mM. Solutions were thoroughly sparged with humidified argon and reactions were carried out in the dark.

CHAPTER 3

IN VITRO STUDIES ON THE INTERACTION OF HEME WITH *HYDROGENOBACTER THERMOPHILUS* APOCYTOCHROME C_{552}

SUMMARY

Apocytochrome *c* from *Hydrogenobacter thermophilus* was produced and characterised. It was shown that the Cys-Met-Ala-Cys-His motif in this peptide can form an intramolecular disulfide bond. Binding studies with 8-anilino-1-naphthalenesulfonate provide evidence for the presence of a hydrophobic pocket in the apocytochrome. The reaction of apocytochrome *c* and heme was studied. When the disulfide in the apoprotein was reduced, a *b*-type cytochrome, with non-covalently bound heme, could form. Data are presented to demonstrate the formation of thioether bonds between the heme vinyl-substituents and the cysteine residues of the apocytochrome *c*. Moreover, contrary to opinion of thirty years standing, a *c*-type cytochrome formed correctly from ferrous heme and reduced apoprotein *in vitro*, under mild conditions and in the absence of any biosynthesis apparatus. Mechanistic studies were performed including pH profiles and time course experiments. There are implications for understanding *in vivo* cytochrome *c* assembly.

3.1. INTRODUCTION

A key aspect of cytochrome *c* maturation is the interaction of heme with apocytochrome *c*. However, formation of the *c*-type cytochrome thioether bonds between cysteine residues and the vinyl substituents of heme is not chemically facile and is poorly understood (Barker & Ferguson 1999; CHAPTER 1). Side products have been considered to be readily formed (Barker & Ferguson 1999; Barker et al. 1993). Sano and co-workers demonstrated the covalent addition of protoporhyrinogen to bovine apocytochrome *c* (Sano & Granick 1961; Sano & Tanaka 1964). However, their reaction was conducted under harshly acidic conditions (pH 3.5 or below), the porphyrin they used was reduced by six electrons compared with the physiologically relevant protoporphyrin IX, and it did not contain the iron atom. It is now accepted that cytochrome *c* biosynthesis requires the covalent attachment of heme to the polypeptide, rather than the attachment of porphyrin with subsequent incorporation of iron (Colleran & Jones 1973; Garrard 1972; Hennig & Neupert 1981; Pettigrew & Moore 1987). Since the seminal observations of Anfinsen and coworkers on the properties of apocytochrome *c* thirty years ago (Fisher et al. 1973), it has been widely believed that uncatalysed covalent attachment of heme to apoprotein cannot occur *in vitro*.

The unusual cytoplasmic formation of *H. thermophilus* cytochrome c_{552} in *E. coli* (see CHAPTER 1) and the properties of the double alanine C11A/C14A mutant (Tomlinson & Ferguson 2000a) suggested that investigation of the reaction between heme and the apo wild type form of the protein may prove instructive. In this chapter, the results of a study of the reaction between *H. thermophilus* apocytochrome c_{552} and heme are reported.

3.2. RESULTS

3.2.1. Apocytochrome production

The covalently attached heme was removed from *H. thermophilus* cytochrome c_{552} by treatment of the protein with silver sulfate (Fisher et al. 1973; see CHAPTER 2). Formation of the apoprotein (and removal of the silver) was demonstrated by the absence of a band on heme stained SDS-PAGE gels (Figure 3.1), Electrospray mass spectrometry (ES-MS) (calculated mass of apoprotein 8,700 Da, observed mass 8,699 Da), MALDI-TOF MS (8,697 Da), by the disappearance of bands characteristic of heme-containing cytochrome c_{552} from the absorption spectrum (data not shown) and the disappearance of the holocytochrome *c* fold as judged from CD spectra (Figure 3.2).

3.2.2. Disulfide bond formation within the apocytochrome

An intramolecular disulfide bond formed in the apoprotein between the cysteine residues of the CXXCH heme-binding motif (these are the only cysteines in the protein (see APPENDIX for the sequence)), as demonstrated with Ellman's Reagent (Riddles et al. 1983), which indicated two free thiols per mole of reduced protein and zero per mole of oxidised protein. This was confirmed by treatment of the apoprotein prepared in oxidising conditions with cyanogen bromide (CNBr). This experiment was carried out by Anthony C. Willis (University of Oxford). CNBr cleaves a peptide specifically after methionine residues (Means & Feeney 1971). In *H. thermophilus* cytochrome c_{552}, the amino acid sequence of the heme-binding motif is CMACH (Hasegawa et al. 1998). Following digestion of the apoprotein with CNBr, one of the fragments isolated by HPLC had two N-termini present in equimolar amounts; these contained the sequences NEQLAKQ (the N-terminus of the polypeptide) and ACHDLK, showing that the protein had cleaved after the

methionine between the two cysteine residues. On reduction of this fragment with dithiothreitol, two separate peptides, one with each of these same two N-terminal sequences, were resolved by HPLC. This demonstrates that the oxidised protein contained a disulfide bond, which was broken on reduction. MALDI-TOF and ES-MS spectra gave masses of 8,697 and 8,699 Da, respectively, for the undigested oxidised apoprotein, indicating that it was monomeric, *i.e.*, that the disulfide bond between the two cysteines of the CMACH sequence was intramolecular. Air oxidation was sufficient to form the disulfide, which is the first to be observed between the heme-binding cysteines of a *c*-type cytochrome. Circular dichroism (CD) spectra indicated that formation of this disulfide bond induced secondary structure in the protein relative to the reduced (free thiol) state (Figure 3.2). The CD spectrum of reduced apocytochrome c_{552} is very similar to that reported for the apo C11A/C14A (*b*-type cytochrome) variant (Tomlinson & Ferguson 2000a).

3.2.3. Formation of a *b*-type cytochrome intermediate

Mixing reduced *H. thermophilus* apocytochrome c_{552} (no disulfide bond) with ferrous (Fe(II)) heme in the presence of dithiothreitol (DTT) at pH 7.0 resulted in an increase in absorption around 423 nm relative to that of heme alone; the same trend was observed around 528 and 560 nm, bands characteristic of the presence of a reduced cytochrome (Figure 3.3.A). The pyridine hemochrome spectrum of this mixture had its α-band at 556 nm (Figure 3.3.B), indicating that the heme contained two unreacted vinyl groups (Bartsch 1971; Falk 1964). These results show that the apoprotein and heme initially form a *b*-type cytochrome, in which the heme is not covalently attached to the peptide but in which its iron atom is coordinated by two amino acid side chains from the protein. With a protein concentration of 10 μM and a stoichiometric quantity of heme in the presence of 5

mM DTT, formation of this cytochrome *b* was complete in *ca.* 20 min at 25 °C. Very similar kinetics were observed on mixing the apo C11A/C14A mutant of *H. thermophilus* cytochrome c_{552} with heme under otherwise identical conditions. Formation of the *b*-type cytochrome from apocytochrome c_{552} and heme was observed to be pH dependent, its rate increasing from pH 8 to pH 6. This acceleration may correlate with the protonation state of the histidine residue of the CXXCH motif, although there is no direct evidence that this is a ligand to the heme iron in the *b*-type cytochrome. The main factor that influenced the rate of *b*-type cytochrome formation was the concentration of DTT present in the reaction mixture. DTT competes for axial ligation sites of the iron in heme. The replacement of DTT as a ligand during heme uptake by the apoprotein is interpreted as slowing the rate of *b*-type cytochrome formation. In the absence of DTT, formation of the *b*-type intermediate occurred within the mixing time under otherwise identical conditions (Tomlinson & Ferguson 2000a).

The presence of a hydrophobic pocket in apocytochrome c_{552}, which, presumably, facilitates *b*-type cytochrome formation, was shown by protein fluorescence quenching experiments with ANS. The dissociation constant for ANS of apocytochrome c_{552} was *ca.* 10^{-5} M. Interestingly, the affinity for ANS was not affected by the presence of the disulfide bond within the CXXCH motif within the protein. These data are in good agreement with previously published results on the apoprotein of the C11A/C14A variant of *H. thermophilus* cytochrome c_{552} (Wain et al. 2001).

3.2.4. Formation of thioether bonds to yield a *c*-type cytochrome

Following formation of the *b*-type cytochrome from the mixture of wild type *H. thermophilus* apocytochrome c_{552} and heme in reducing conditions, the maximum

at 560 nm in the protein absorption spectrum shifted slowly to 552 nm (Figure 3.3.A). After 18 hours, the pyridine hemochrome spectrum of the purified reduced protein had a resolved band at 549.7 nm (Figure 3.3.B), characteristic of saturation of both vinyl groups of the heme (Bartsch 1971; Falk 1964). These observations decisively indicate the formation of a *c*-type cytochrome, an interpretation which was substantiated further by demonstration of the absence of free thiol groups with Ellman's reagent, heme staining of the proteins on SDS-PAGE gels (Figure 3.1), treatment of the heme-containing protein with acidified-acetone and ES-MS analysis in denaturing conditions; in the latter three experiments, non-covalently bound heme dissociates from protein but covalently bound heme does not. The visible absorption spectra of both the ferric (Fe(III)) and ferrous forms of the *in vitro*-synthesised cytochrome *c*, together with its reduced pyridine hemochrome spectrum, were indistinguishable from those of holoprotein prepared from *E. coli* (Figures 3.3.A,B; oxidised spectra not shown); the CD spectra of the oxidised cytochrome *c* made *in vivo* or *in vitro* demonstrated that each must have a very similar, and probably, same protein fold (Figure 3.2). The data show that these two forms of cytochrome c_{552} are identical with respect to both the environment of the heme and overall conformation. The ES-MS spectrum of the *in vitro* product cytochrome *c* had a peak at the correct mass for *H. thermophilus* holocytochrome c_{552}.

An analogue of the *b*-type cytochrome formed on addition of reduced Fe-mesoporphyrin to reduced apoprotein. The product had visible absorption maxima at 550, 519 and 415 nm and did not heme stain on an SDS-PAGE gel (Figure 3.1). Mesoporphyrin has ethyl groups in the positions of the vinyl groups of protopophyrin (Figure 1.1; Falk 1964) and therefore cannot form thioether bonds

with the polypeptide. There was no evidence for any other type of covalent attachment of mesoporphyrin to apoprotein. SDS-PAGE analysis including heme-staining methodology (Figure 3.1, lane 4) showed that the holoform of C11A/C14A mutant of *H. thermophilus* cytochrome c_{552} (Tomlinson & Ferguson 2000a) did not form any covalent bonds to the heme moiety under otherwise identical conditions.

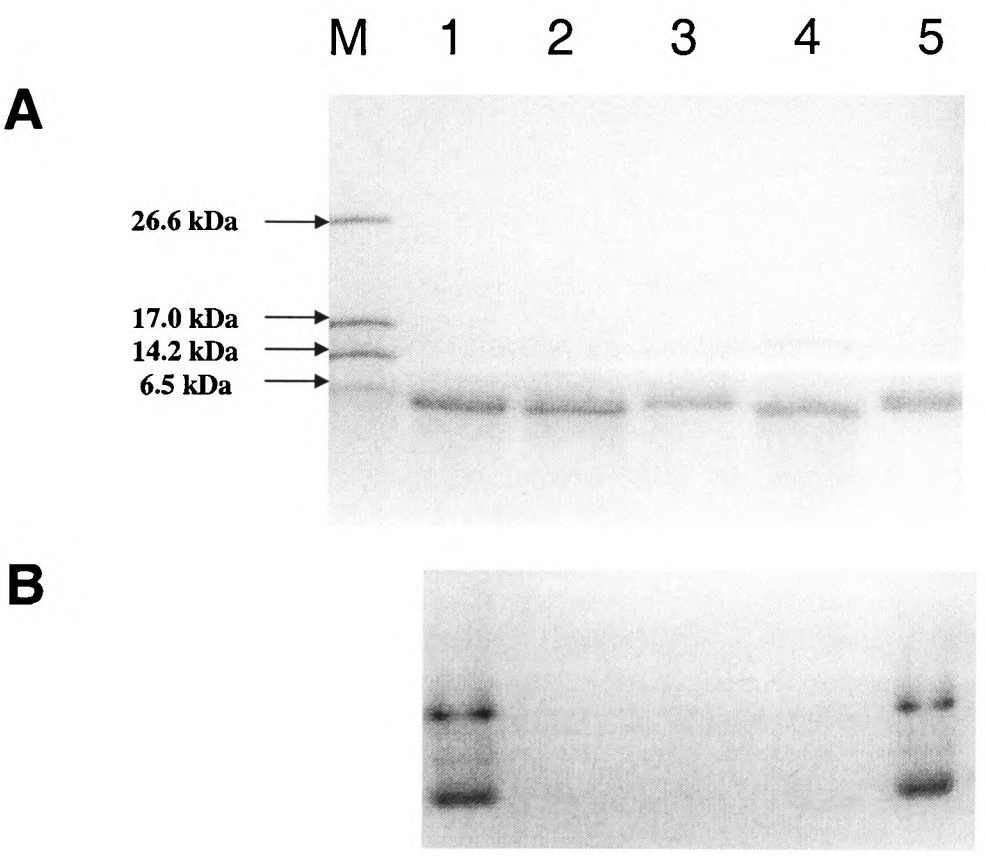


Figure 3.1. SDS/17.5% PAGE analysis stained with **A.** Coomassie Blue or **B.** activity stained for covalently bound heme. Lane M shows molecular weight markers. (1) Wild-type *Hydrogenobacter thermophilus* cytochrome c_{552} as purified from the cytoplasm of *E. coli*. (2) Apocytochrome c_{552} produced from the holoprotein. (3) The *b*-type cytochrome analogue formed from apoprotein and Fe-mesoporphyrin. (4) The C11A/C14A mutant of *H. thermophilus* cytochrome c_{552}, which is a *b*-type cytochrome (Tomlinson & Ferguson 2000a). (5) Reconstituted cytochrome c_{552} produced by the reaction of wild type apocytochrome and heme. The higher molecular weight bands in Lanes 1 and 5 of the heme stained gel are due to polymerised protein; they are not visible using the less sensitive Coomassie stain. For Coomassie staining, 200 pmoles of protein were applied to each lane of the gel, for heme staining, 20 pmoles were applied.

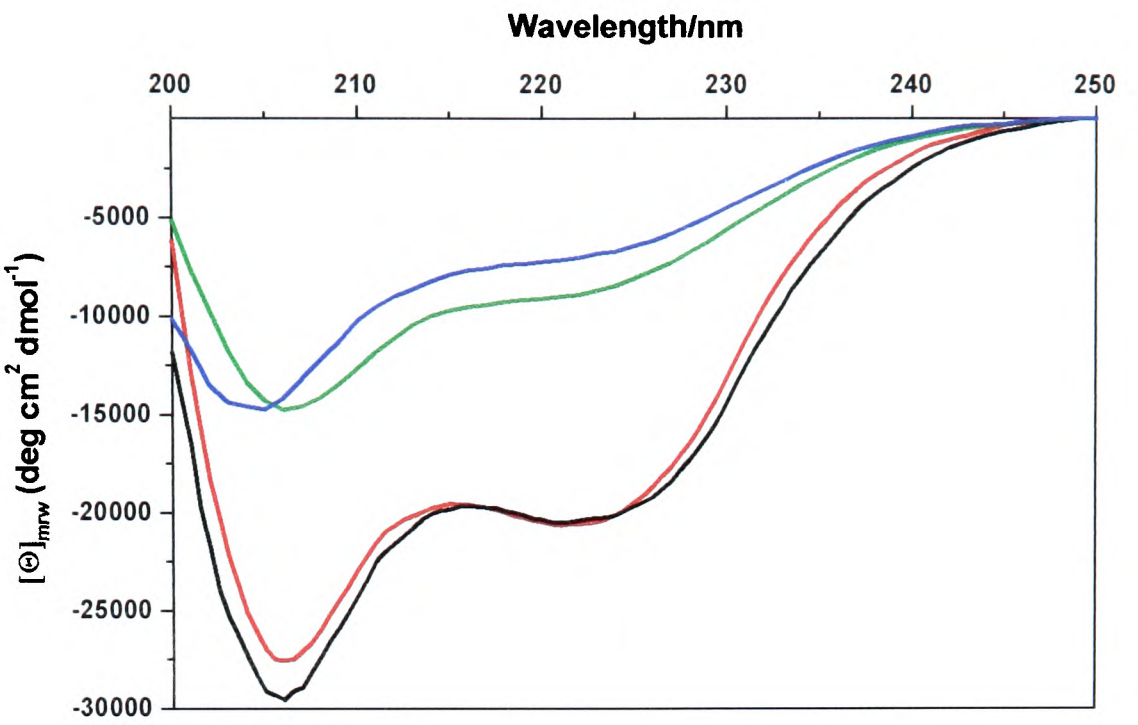
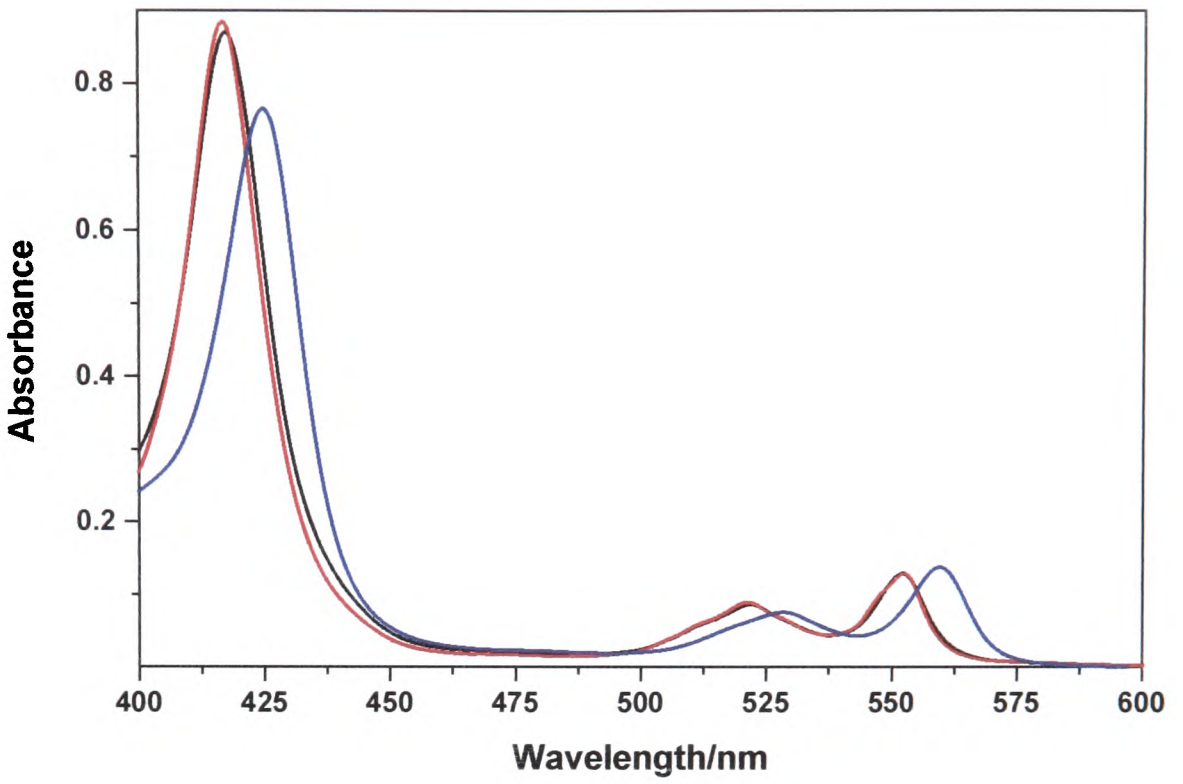


Figure 3.2. Circular dichroism spectra of *H. thermophilus* apocytochrome c_{552} reduced with dithiothreitol (blue), apocytochrome c_{552} air oxidised (green), *H. thermophilus* holocytochrome c_{552} as purified from *E. coli* (red) and reconstituted cytochrome c_{552} formed by adding heme to apoprotein (black). $[\Theta]_{mrw}$ is mean molar ellipticity per residue. Spectra were recorded using 25 μM protein in 20 mM sodium phosphate buffer, pH 7.0.

A



B

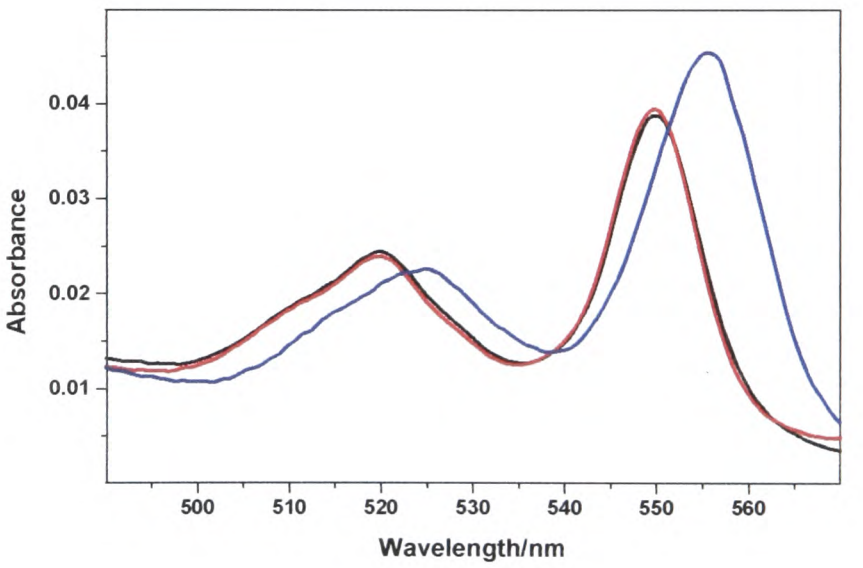


Figure 3.3. **A**. Absorption spectra and **B**. pyridine hemochrome spectra of wild-type *Hydrogenobacter thermophilus* cytochrome c_{552} as purified from *E. coli* (red, largely obscured by the black line), reconstituted cytochrome c_{552} (black) recorded 18 hours after adding heme to apoprotein and the *b*-type cytochrome intermediate (blue) formed 30 minutes after adding heme to apoprotein. Absorption spectra were recorded using 5 μM protein in 50 mM sodium phosphate buffer, pH 7.0; pyridine hemochrome spectra were obtained using 1.25 μM protein in 19% (v/v) pyridine and 0.15 M NaOH. All samples were reduced using disodium dithionite.

3.2.5. Mechanistic insights into *c*-type cytochrome formation

Formation at pH 7.0 of the covalent bond(s) in the product *c*-type cytochrome occurred with biphasic kinetics. The first phase, accounting for *ca.* 70% of the total change, had an observed first order rate constant of 0.4 h^{-1}; this value was independent of the initial heme and apoprotein concentrations, implying that the reaction was intramolecular. The second phase (*ca.* 30% of the amplitude) had a rate constant of *ca.* 0.15 h^{-1}. By analogy with many hemoproteins that contain non-covalently bound heme, heme may incorporate into the *b*-type cytochrome intermediate leading to cytochrome c_{552} as a mixture of isomers in which the heme is rotated by 180° about the α,γ-meso axis (Banci et al. 2000; La Mar et al. 1984; McLachlan et al. 1986; Wu et al. 1991; Yamamoto & La Mar 1986). For other heme proteins (Banci et al. 2000; La Mar et al. 1984; McLachlan et al. 1986; Wu et al. 1991; Yamamoto & La Mar 1986), the initial proportions of the two isomers varied and equilibration was observed following such heme binding, ultimately resulting in > 85% of the non-covalently bound heme having a single orientation. At pH 7.0, this reorientation typically occurred on a timescale of hours (La Mar et al. 1984; McLachlan et al. 1986; Yamamoto & La Mar 1986), comparable to the rate for the second phase of *in vitro* holocytochrome c_{552} formation. Therefore, these observations are consistent with a mechanism in which heme can covalently bind the cysteine thiols of apocytochrome c_{552} in only one orientation. It follows that heme in the incorrect orientation in the *b*-type intermediate must reorient before covalent attachment can occur. Hence the portion of the heme that initially binds the apocytochrome *c* in the correct orientation can relatively rapidly form holocytochrome *c* (the first observed phase), while covalent attachment of the remainder of the heme is rate limited by its reorientation (the second phase). Support for this idea is found in the structure of *H. thermophilus* cytochrome c_{552}

produced in the cytoplasm of *E. coli*; the heme orientation and the stereochemistry of each of the thioether bonds are homogeneous in such protein (Paul D. Barker, University of Cambridge, and Stuart J. Ferguson, unpublished observations) and the same as for all cytochromes *c* made by biogenesis proteins (Barker & Ferguson 1999).

After 2, 3.25 and 5 h of reaction between apocytochrome c_{552} and heme, *i.e.*, during the first phase of cytochrome c_{552} formation, protein was treated with acidified-acetone or guanidine hydrochloride to remove any non-covalently bound heme (Tomlinson & Ferguson 2000a; Tomlinson & Ferguson 2000b). At each of these time points, in the absorption spectrum of the resolubilised protein the α-band maximum was at 552.5 nm, and in the pyridine hemochrome spectrum the corresponding band was at 550 nm, observations indicative of cytochrome *c* with the heme covalently bound to the polypeptide through two thioether bonds. The equivalent wavelengths for cytochrome c_{552} with a CXXAH or AXXCH heme-binding motif are 553 nm for both pyridine hemochrome spectra and 556 and 558 nm for the absorption spectra, respectively (Tomlinson & Ferguson 2000b). Thus, the reaction kinetics for the *in vitro* cytochrome *c* formation described in the present work suggest that the faster first phase involves reaction of one of the cysteine thiols with heme as the rate determining step, with subsequent faster formation of the second thioether bond; an alternative is that both thioether bonds form in a concerted fashion. The second thioether bond might form faster because of the covalent fixation of the heme due to the first thioether bond, which may position the reactive cysteine residue in close proximity to the vinyl group of heme with a lower extent of freedom of movement compared to the non-covalently bound heme-protein complex. Additionally, (stereo)electronic effects may

influence the second thioether bond formation when one of the heme vinyl groups is changed to a saturated group containing a bond between the α-carbon and a sulfur of the protein backbone. In principle, the covalent heme attachment could be monitored by NMR. However, the apocytochrome c_{552} is not stable at the millimolar concentrations this technique would require (note that to follow the reaction kinetics early in the heme attachment process, it would be necessary to identify resonances from each of the thioether bonds, some of which are intrinsically broad, in a small percentage of the total protein).

In vitro cytochrome *c* formation from heme and apo C11A and C14A variants of *H. thermophilus* cytochrome c_{552}, both of which form holocytochromes with a single thioether bond in the cytoplasm of *E. coli* (Tomlinson & Ferguson 2000b), proceeded similarly to the wild type (double cysteine) protein. Both mutants showed biphasic kinetics with the amplitudes and rate constants of both phases at pH 7.0 being approximately equal to those for wild type cytochrome c_{552} (more detailed analysis is shown in CHAPTER 4). Thus, there is no evidence that the two thioether bonds can form at substantially different rates, while the observation that the slower phase of the reaction is the same for both single and double cysteine proteins supports the idea that heme reorientation is rate limiting.

The chemistry of thiol attachment to the vinyl groups of heme is poorly understood and therefore studies were also undertaken of the kinetics of this *in vitro* cytochrome *c* reconstitution as a function of pH. The rate of first phase of the reaction increased as the pH was lowered (k_{obs} = 0.9, 0.4 and 0.3 h^{-1} at pH 6.0, 7.0 and 8.0, respectively). Thus, protonation of a titratable group near the vinyl group(s) of the heme that could act as a proton donor, or perhaps protonation of the

β-carbon of the vinyl group itself, is presumably mechanistically more important in the physiological pH region than deprotonation of the cysteine thiols. Addition of cysteine to the vinyl group of 5-vinyluracil in acidic conditions has been shown to proceed *via* a carbonium ion intermediate (Jones et al. 1987); the particular local environment of the vinyl groups within a *c*-type cytochrome might facilitate a similar reaction mechanism. The second phase of the covalent heme attachment reaction showed no significant pH dependence in this range.

3.2.6. Effect of the oxidation states of the apocytochrome and heme on *c*-type cytochrome formation

It is commonly believed that reducing conditions are required for the *in vivo* synthesis of *c*-type cytochromes (Barker & Ferguson 1999; Nicholson & Neupert 1989; Tong & Margoliash 1998); reductant may be necessary to ensure that the cysteine residues have free thiol groups and/or to provide ferrous heme. On mixing oxidised (disulfide bonded) *H. thermophilus* apocytochrome c_{552} and ferric (Fe(III)) heme, no reaction was observed. The visible spectrum of the oxidised heme broadened slightly upon addition of apoprotein. These data are interpreted as the weak association of heme with apoprotein, which is substantiated by the observation from the ANS fluorescence data that heme can displace ANS from the protein. However, in the presence of the disulfide bond the ligation of the heme did not change markedly upon addition of apoprotein, as judged from the absorption spectrum. It may be that in the oxidised apoprotein the normal heme ligand is distorted such that coordination to the heme iron is sterically not feasible.

When ferric heme was reacted with reduced apoprotein, products different from *H. thermophilus* cytochrome c_{552} were obtained, as shown by ES-MS analysis,

absorption and pyridine hemochrome spectra. A comparison of the absorption spectra of wild-type cytochrome c_{552} (absorption maxima at 417, 521 and 552 nm) and the product of this reaction (absorption maxima at 416, 520 and 550 nm) is shown in Figure 3.4. The pyridine hemochrome spectrum of the cytochrome product of ferric heme and reduced apoprotein had an α-band at 546 nm compared with 549.5 nm in the wild-type cytochrome. The reaction kinetics were much faster and no additional product formation was observed after *ca.* 45 minutes. Despite the fact that ferric heme had been added, features of a reduced cytochrome were observed in the visible spectrum.

These data can be interpreted as resulting from reduction of the heme iron by the free thiols of the apocytochrome cysteine residues and creation of highly reactive thiyl radicals (Barker et al. 1993). The ES-MS analysis showed a high tendency for incorporation of atomic oxygen (protein masses increased in increments of 16 Da). This concurs with previous proposals (Barker & Ferguson 1999; Barker et al. 1993) that interaction of reduced apocytochromes *c* with ferric heme *in vivo* is far from optimal for generating the correct covalent attachment.

When reduced heme was mixed with oxidised apoprotein in the absence of excess reductant, a small maximum around 557 nm was observed in the α-band region of the absorption spectrum. This observation is interpreted as a fraction of the disulfide being reduced by the ferrous heme, resulting in the reaction of reduced apoprotein with either ferric or ferrous heme. In these reaction conditions a mixture of products was obtained. Overall, the data concerning the various oxidation states of apoprotein and heme iron show that to form effectively the

correct product *c*-type cytochrome, both the heme iron and disulfide bond of the apocytochrome must be reduced.

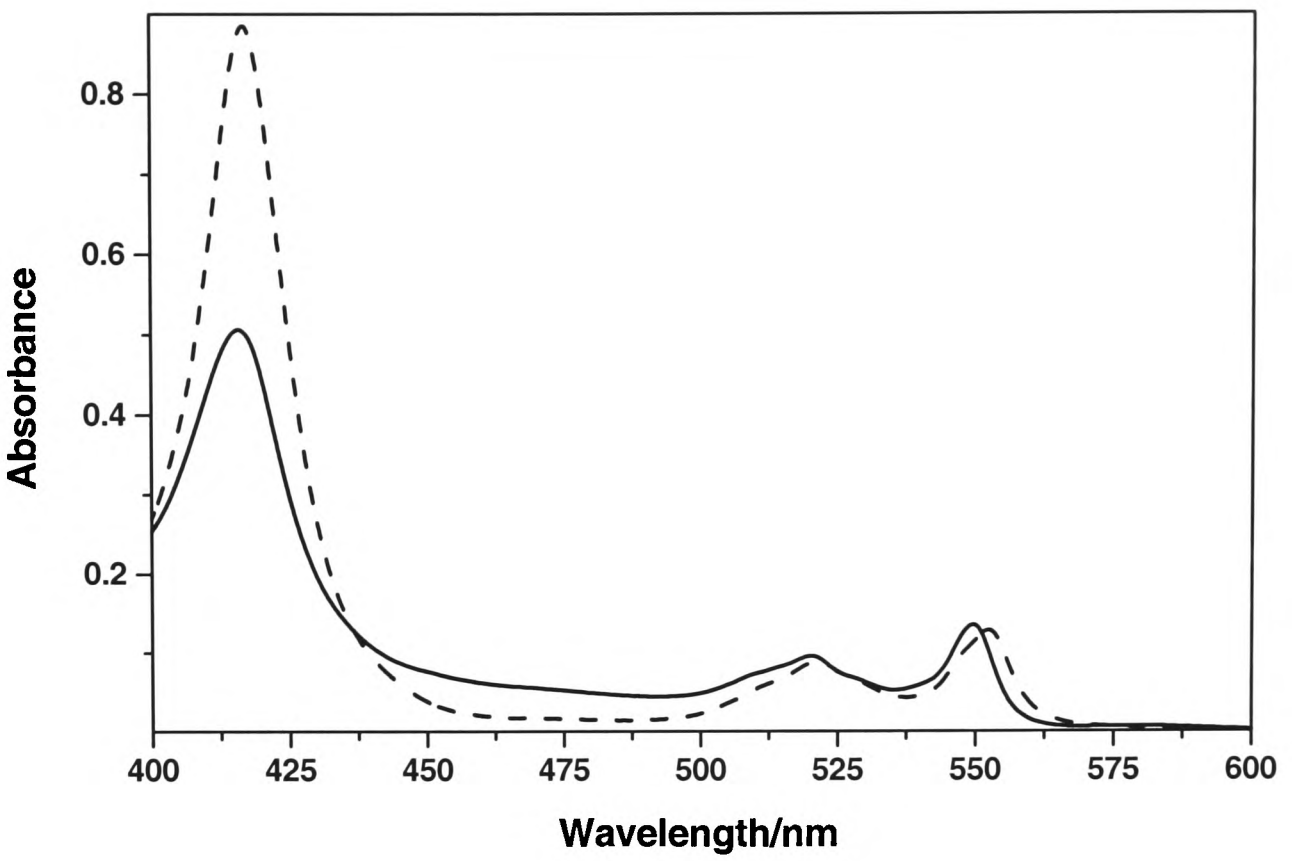


Figure 3.4. Absorption spectra of wild-type *Hydrogenobacter thermophilus* cytochrome c_{552} as purified from *E. coli* (— — —) and reconstituted cytochrome *c* (———) recorded 45 minutes after adding ferric heme to reduced apoprotein. Absorption spectra were recorded using 5 μM protein in 50 mM sodium phosphate buffer, pH 7.0. Both samples were reduced using disodium dithionite.

3.3. DISCUSSION

The data presented in this chapter show that a *c*-type cytochrome, containing heme attached to a polypeptide through two thioether bonds, can form *in vitro* without the action of any biosynthesis proteins. Initially, the apocytochrome c_{552} from *H. thermophilus* bound heme non-covalently, involving coordination of amino acid side chains to the heme-iron. It is extremely likely that the highly conserved histidine residue of the CXXCH motif, which binds the heme in the holo *c*-type

cytochrome, provided one of these axial iron ligands. Unfortunately, no ferricytochrome spectrum could be obtained for the *b*-type cytochrome complex of reduced apocytochrome and ferric heme, which may have allowed the examination of the spectrum around 695 nm. This spectral region is indicative of methionine coordination to a ferric heme iron (Scott & Mauk 1995).

Subsequent to *b*-type cytochrome formation from apocytochrome c_{552} and heme, thioether bonds formed, owing, presumably, to the proximity of the vinyl groups of the heme and the cysteine residues of the protein. The presence of reduced heme (and reduced apoprotein) avoided formation of side-products observed when ferric heme was used. It is probable that heme must be reduced when any *c*-type cytochrome is made *in vivo*, as has already been deduced for mitochondrial cytochrome *c* (Nicholson & Neupert 1989; Tong & Margoliash 1998). A requirement for reduced apoprotein is consistent with the roles in bacteria of some of the cytochrome *c* maturation proteins, namely CcmG and H (Page et al. 1998; Thony-Meyer 1997) as disulfide reductases in the periplasm (Fabianek et al. 2000; Page et al. 1998; Ritz & Beckwith 2001); Ccm proteins may also keep heme reduced. The present work shows that a disulfide bond can form in the CXXCH heme binding motif of an apocytochrome. The bacterial periplasm has a very active system for catalysing the formation of disulfide bonds (Dsb) (Fabianek et al. 2000; Page et al. 1998; Ritz & Beckwith 2001); the reversal or avoidance of the propensity of the CXXCH motif of an apocytochrome to form a disulfide in the cell in any organism is clearly an important factor in the biogenesis of this class of protein.

Very many studies (Englander et al. 1998; Scott & Mauk 1995; Yeh et al. 1998) have been made of the refolding of mitochondrial cytochrome *c* (closely related in

structure to the cytochrome c_{552} studied here) following denaturation *in vitro*, which leaves the thioether bonds to the heme intact. The relevance of such observations to *in vivo* formation of mitochondrial cytochrome *c* is unclear. It is possible that the mitochondrial heme lyase enzymes (Page et al. 1998; Steiner et al. 1996) catalyse heme attachment to the unfolded protein, which would then fold as in the *in vitro* studies. However, the present data suggest that consideration should be given to the notion that the apoprotein adopts a folded conformation with a nascent heme binding site, as might also be deduced from the observations of Dumont et al. (1994). Therefore, different roles of the heme lyase have to be considered. These will be discussed later (CHAPTER 5). In any case, it is undoubtedly shown in the present work that the vinyl groups of heme are more reactive towards cysteine thiols than has been previously thought.

An issue, which is discussed, but not addressed experimentally, is the stereochemistry at the α-carbon of the vinyl-substituents in heme. Experiments to try to elucidate stereochemical aspects of thioether bond formation during cytochrome *c* formation are described in CHAPTER 4.

CHAPTER 4

STEREOSELECTIVE *IN VITRO* FORMATION OF *C*-TYPE CYTOCHROME VARIANTS OF *HYDROGENOBACTER THERMOPHILUS* CYTOCHROME C_{552} CONTAINING ONLY A SINGLE THIOETHER BOND

SUMMARY

In the previous chapter, *in vitro* formation of *Hydrogenobacter thermophilus* cytochrome c_{552} was demonstrated. Here it is shown that variants of *H. thermophilus* c_{552} with just one cysteine in the heme-binding motif, which could covalently bind heme only via a single thioether bond, can also form *in vitro*. Furthermore, the apocytochromes with either AXXCH or CXXAH in the binding motif reacted with 2-vinyldeuteroheme (2-VDH) and 4-vinyldeuteroheme (4-VDH) resulting, predominantly, in covalent attachment between Cys-11 and the 2-vinyl moiety and Cys-14 and the 4-vinyl functionality. These observations showed that the covalent attachment of heme to apocytochrome was stereoselective, indicating that the initial non-covalent complexes between apoprotein and heme have to be in the correct stereochemical orientation for preferential promotion of thioether bond formation. The heme derivatives 2-VDH and 4-VDH were also reacted with wild-type *H. thermophilus* c_{552} to yield other forms of cytochrome *c* containing only one thioether bond. These results showed that the formation of the two thioether bonds in typical *c*-type cytochromes can occur independently from one another.

4.1. INTRODUCTION

In the previous chapter, *in vitro* formation of a *c*-type cytochrome containing two thioether bonds was reported. Naturally occurring cytochromes containing only one thioether bond are rare (Barker & Ferguson 1999; also CHAPTER 1). However, variants engineered in the CXXCH binding motif, where one cysteine was replaced with an alanine residue by site-directed mutagenesis, for a bacterial cytochrome *c* (Tomlinson & Ferguson 2000b) and for mitochondrial yeast iso-1-cytochrome *c* (Rosell & Mauk 2002) have aided the understanding of this class of proteins and the requirement for covalent attachment of the heme group to the polypeptide.

In this chapter the *in vitro* reaction between AXXCH and CXXAH variants of apocytochrome *H. thermophilus* c_{552} and heme will be reported. Furthermore, the reaction of the apocytochromes with the heme derivatives 2-vinyl deuteroheme (2-VDH) and 4-vinyl deuteroheme (4-VDH) is investigated. The latter two molecules (structures shown in Figure 4.1) have only one vinyl group and are thus hybrids between heme and deuteroheme (Figure 1.1).

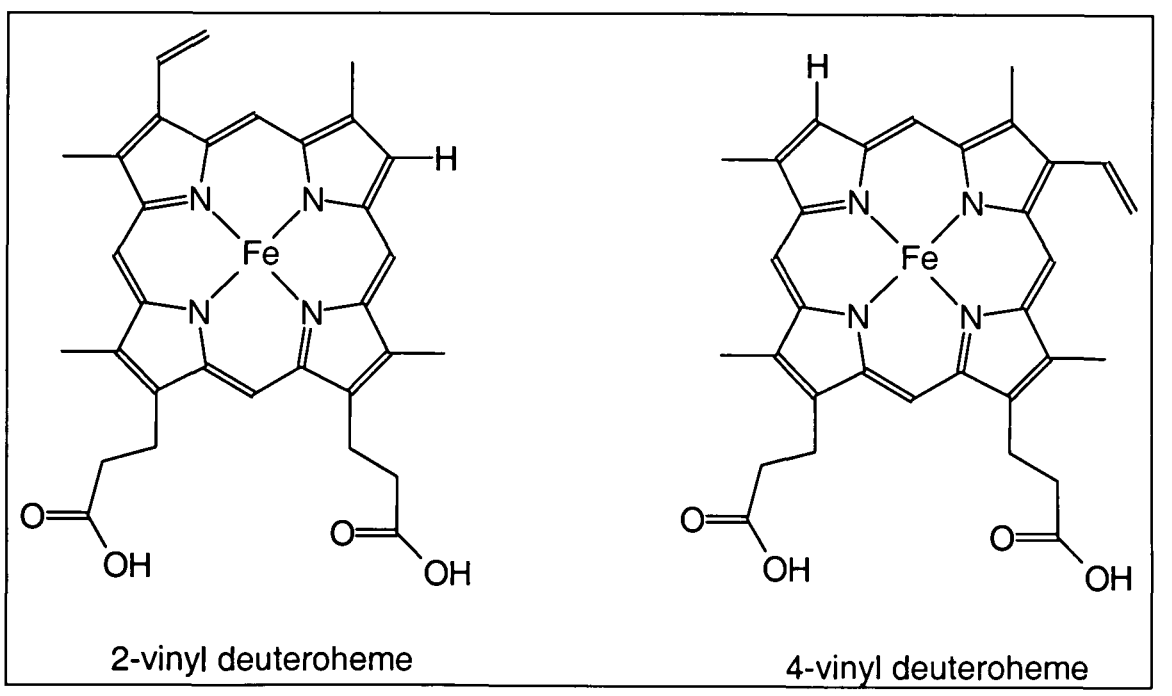


Figure 4.1. Structures of 2–vinyl deuteroheme (2-VDH) and 4-vinyl deuteroheme (4-VDH) is studied.

4.2. RESULTS

4.2.1. Apocytochrome production and characterisation

C11A and C14A variants of *H. thermophilus* cytochrome c_{552} were shown to be pure by SDS-PAGE analysis (Figure 4.2, lanes a, respectively). The apocytochromes were shown to be devoid of heme as judged by heme-stained SDS-PAGE analysis (Figure 4.2, lanes b, respectively). The presence of protein was shown by staining the same gels with Coomassie Blue after the heme staining had been carried out. The absence of characteristics of cytochromes in the visible spectra, and the disappearance of the cytochrome *c* fold as shown by CD analysis (Figure 4.3) were also indicative of the removal of the covalently bound heme from the proteins. It was observed that in the absence of DTT bands appeared in the SDS-PAGE corresponding to a dimer. This data is interpreted as the formation of an intermolecular disulfide bond between the cysteine residues of the apocytochromes *c*.

4.2.2. *In vitro* thioether bond formation with heme

Mixing either C11A or C14A variants of *H. thermophilus* apocytochrome c_{552} with ferrous heme in the presence of dithionite and DTT at pH 7.0 resulted in the formation of a non-covalent heme-protein complex as judged by absorption spectroscopy. These *b*-type cytochrome complexes had bands in the visible spectra around 424, 528 and 559 nm (Figures 4.4a and 4.3b for the C11A and C14A variants, respectively; Table 4.1). The pyridine hemochrome spectra of these mixtures had their α-bands at 556 nm, indicating that in each case the heme contained two intact vinyl groups. The intermediate *b*-type cytochrome formed quantitatively to yield a 1:1 heme-protein complex for the C11A protein. In the case of the C14A protein, a fraction of the apoprotein (up to 40 %) was unable to

bind heme for unknown reasons. These results are consistent with previous observations on wild-type cytochrome c_{552} and the AXXAH mutant, which were both shown to initially form a *b*-type cytochrome on mixing apocytochrome *c* and heme (CHAPTER 3; Tomlinson & Ferguson 2000a).

Following formation of the *b*-type cytochrome from the mixture of C11A or C14A variants of *H. thermophilus* cytochrome c_{552} and heme in reducing conditions, the maxima at 560 nm in the protein absorption spectra progressively shifted to either 556 (C11A) or 557 nm (C14A) (Figure 4.4a and 4.4b). After 15 hours, the pyridine hemochrome spectra of the purified reduced proteins had resolved bands at 553 nm, characteristic of saturation of one vinyl group of the heme (Moore & Pettigrew 1990; Tomlinson & Ferguson 2000b). Heme staining of the proteins on SDS-PAGE gels (Figure 4.2, lanes d), after treating the heme-containing protein with acidified-acetone; were further indication of the formation of a thioether bond between heme and polypeptide. The visible absorption spectra of the ferrous forms of the *in vitro* synthesised cytochromes *c*, together with their reduced pyridine hemochrome spectra (Table 4.1), were almost identical to those of the holoproteins produced in the cytoplasm of *E. coli* (Table 4.1 and Figures 4.4a and 4.4b for the C11A and C14A variants, respectively). These data show that the *in vivo* and *in vitro* produced forms of the cytochromes *c* are almost identical with respect to both the environment and the type of chemical modification of the heme.

For both the C11A and C14A variants, the initial rates of thioether bond formation were very similar to that observed for the reaction of heme with wild-type *H. thermophilus* cytochrome c_{552} containing a CXXCH motif (CHAPTER 3). However, owing to the smaller differences between the visible spectra of the non-covalently

and covalently bound heme-protein complexes, it was not possible to elucidate unambiguously whether biphasic kinetic behaviour was apparent for the single cysteine variants.

An analogue of the *b*-type cytochrome intermediate formed on addition of reduced Fe-mesoporphyrin (Figure 1.1) to both reduced apocytochrome variants (C11A and C14A). The product had visible absorption maxima around 549, 519 and 416 nm (Table 4.1) and did not heme stain on an SDS-PAGE gel (Figure 4.2, lanes c for the C11A and C14A variant, respectively). Therefore, the vinyl groups of heme are required for the covalent attachment of the metal-containing prosthetic group to the protein.

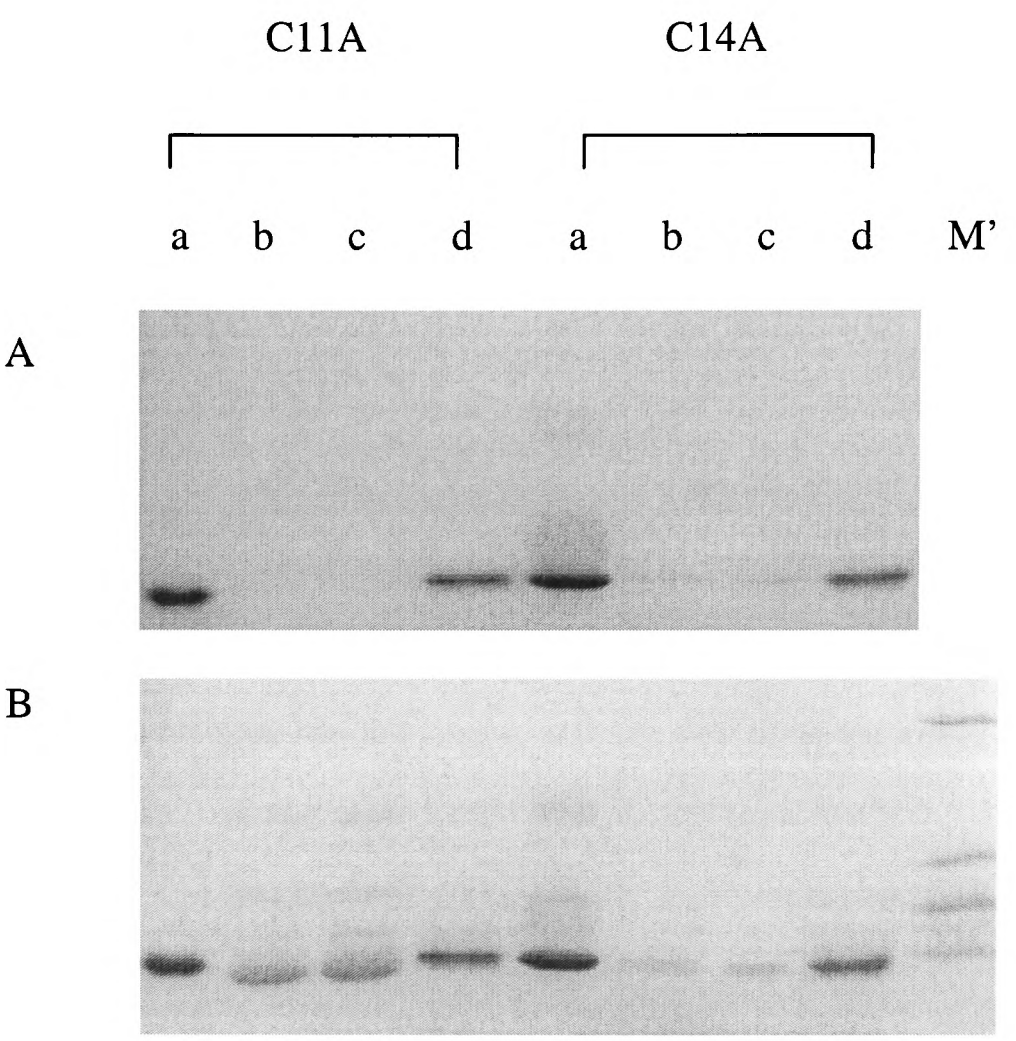


Figure 4.2. SDS/15% PAGE of C11A and C14A variants of *H. thermophilus* cytochrome c_{552}. **A** activity stained for covalently bound heme followed by **B** Coomassie Blue staining. Analysis of the C11A variant of *H. thermophilus* cytochrome c_{552} is shown on the left and of the C14A variant on the right. (a) *In vivo* produced holocytochrome, (b) apocytochrome, (c) mesoheme-containing cytochrome and (d) *in vitro* produced holocytochrome. M' is ultra-low range protein marker (Sigma) corresponding to 6.5, 14.2, 17.0 and 26.6 kDa from bottom to top, respectively. 200-300 pmoles of protein were applied to each lane of the gel. Note that the procedure of sequentially staining the gels for heme and then protein means that proteins with heme covalently attached tend to stain disproportionately strongly for protein owing to carry over of stain.

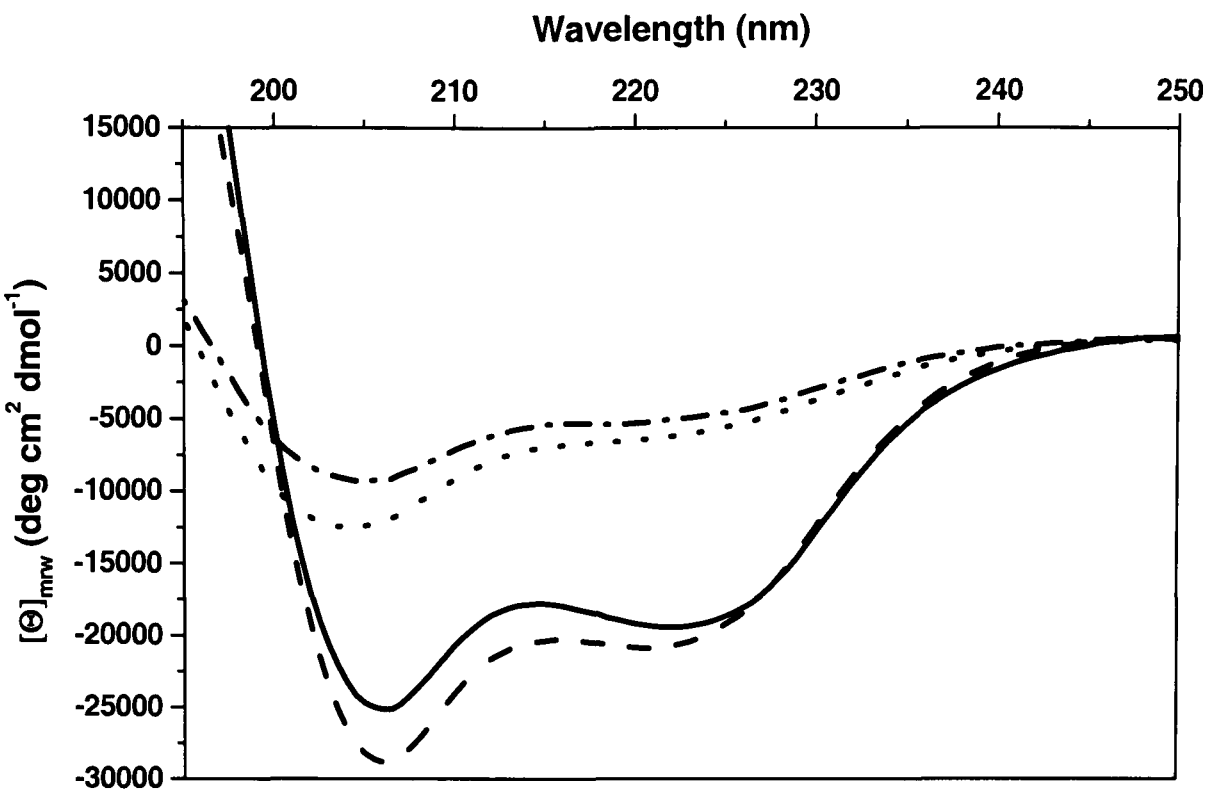


Figure 4.3. Circular dichroism spectra of the apo (*in vitro* produced) and holo (*in vivo* produced) forms of the C11A and C14A variants of *H. thermophilus* cytochrome c_{552}. The oxidised holoform (———) and apoform (- - - -) of the C11A variant and the holoform (— — —) and apoform (·— ·— ·) of the C14A variant. $[\Theta]_{mrw}$ is mean molar ellipticity per residue. All proteins were at a concentration of 20 μM in 20 mM potassium phosphate buffer, pH 7.0.

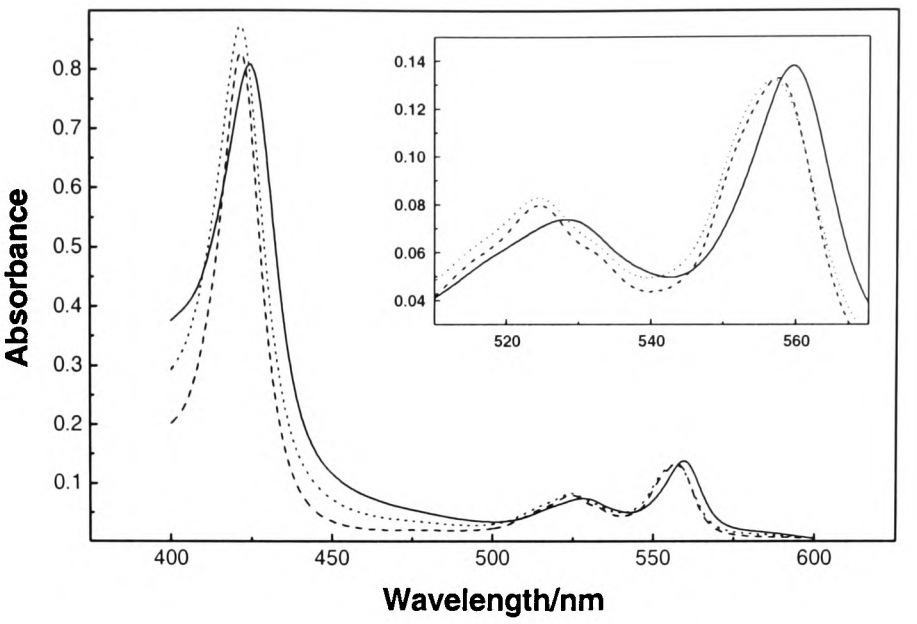


Figure 4.4a. Comparison of the visible spectra of various forms of the C11A mutant of *H. thermophilus* cytochrome c_{552} (~5 μM). The spectra of the *b*-type cytochrome complex of apocytochrome *c* and heme obtained after 10 minutes of incubation under reductive conditions (———); the reaction product of heme and apocytochrome after 15 hours (- - - -); the *in vivo* produced protein (— — —). Absorption spectra were recorded using 5 μM protein in 50 mM sodium phosphate buffer, pH 7.0.

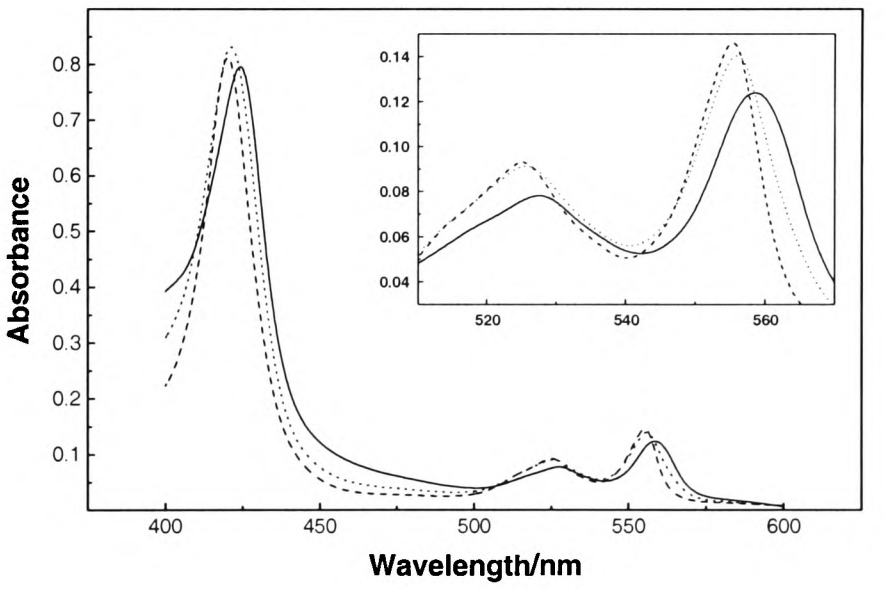


Figure 4.4b. Comparison of the visible spectra of various forms of the C14A mutant of *H. thermophilus* cytochrome c_{552} (~5 μM). The spectra of the *b*-type cytochrome complex of apocytochrome *c* and heme obtained after 10 minutes of incubation under reductive conditions (———); the reaction product of heme and apocytochrome after 15 hours (- - -); the *in vivo* produced protein (— — —). Absorption spectra were recorded using 5 μM protein in 50 mM sodium phosphate buffer, pH 7.0.

Table 4.1. Absorption maxima of various species derived from *Hydrogenobacter thermophilus* cytochrome c_{552}.

Protein species		Wavelength/nm			
		Reduced protein			Pyridine hemochrome (α band)
AXXCH mutant	Heme *b*-type complex	424.5	528.5	559.5	556
	Mesoheme complex	415	520	549	546
	Holo produced *in vitro*	421.5	524.5	557	553
	Holo produced *in vivo*[¶]	421.5	525	557.5	553
	2-VDH *b*-type complex	419	522	554	550
	2-VDH *c*-type complex	-	-	-	549.5
	4-VDH *b*-type complex	417	522	552.5	550
	4-VDH *c*-type complex	414	519.5	549	547.5
CXXAH mutant	Heme *b*-type complex	424	528	559	556
	Mesoheme complex	415	519.5	549.5	546
	Holo produced *in vitro*	421	525.5	556	553
	Holo produced *in vivo*[¶]	420	525	555.5	553
	2-VDH *b*-type complex	418.5	523	554	550
	2-VDH *c*-type complex	416	519.5	552.5	548
	4-VDH *b*-type complex	416.5	522	552.5	550
	4-VDH *c*-type complex	-	-	-	550
Wild-type CXXCH	2-VDH *b*-type complex	418.5	523	554	550
	2-VDH *c*-type complex	415	519	552	547.5
	4-VDH *b*-type complex	418	522	552.5	550
	4-VDH *c*-type complex	414	519.5	548.5	547

Spectra were recorded in 50 mM potassium phosphate buffer, pH 7.0; heme-proteins were reduced with disodium dithionite.

[¶] The values obtained in the present work differ slightly from those stated in Tomlinson, E. J. & Ferguson, S. J. (2000b).

4.2.3. *In vitro* thioether bond formation with 2-VDH and 4-VDH

In order to elucidate whether the reaction of the vinyl-groups of heme with the cysteine residues of the apoproteins was selective with respect to the α,γ mesoaxis of heme, thioether bond formation was studied with mono-vinyl heme derivatives in combination with single-cysteine mutants of *H. thermophilus* cytochrome c_{552}.

When either heme derivative (2-VDH and 4-VDH, donated by Professor Kevin M. Smith, Louisiana State University) was added to the C11A mutant of *H. thermophilus* apocytochrome c_{552} in the presence of DTT and dithionite, a *b*-type cytochrome intermediate was observed (Table 4.1 and Figures 4.5a and b for addition of 2-VDH and 4-VDH, respectively). Very similar spectra were obtained for synthetic cytochromes obtained following addition of mono-vinyl heme derivatives to peptides known to form cytochrome *b* maquettes (Kalsbeck et al. 1996). Following formation of the *b*-type cytochrome complex on addition of the C11A mutant of *H. thermophilus* cytochrome c_{552} to 2-VDH, the visible spectrum obtained remained unchanged for 15 hours, as did the pyridine hemochrome spectrum, which had an α-band maximum at 550 nm. This value is the average of the pyridine hemochrome maxima of the α-bands of heme (containing two vinyl groups in the 2- and 4-position) and deuteroheme (containing two hydrogens at the 2- and 4-position (Falk 1964)). These observations suggested that no reaction had occurred between Cys-14 and the 2-vinyl moiety of the heme derivative. SDS-PAGE analysis, including heme staining to test for covalently bound heme, suggested that very little covalent attachment of 2-VDH to the apoprotein had occurred (Figure 4.6, lane 4). In contrast, the spectrum of the mixture of the C11A mutant of *H. thermophilus* apocytochrome c_{552} and 4-VDH had changed after 15 hours (Table 4.1 and Figure 4.5b). The pyridine hemochrome spectrum had an α-

band maximum around 547.5 nm. These data suggested that a reaction between the 4-vinyl group of 4-VDH and the Cys-14 of the apocytochrome had taken place, which was confirmed by heme staining in conjunction with SDS-PAGE analysis (Figure 4.6, lane 5) showing that heme was covalently attached to the protein. Approximately 80 % of *b*-type cytochrome intermediate reacted to form covalently attached heme-protein as judged by pyridine hemochrome analysis. It is unclear why the reaction was not stoichiometric as it was with heme itself. Possible explanations include precipitation of the incorrect rotational isomer *b*-type cytochrome, which then did not equilibrate with its reactive rotational isomer, or the yield being underestimated due to the problem of estimating the extinction coefficients for the novel cytochromes with single thioether bonds.

When the C14A mutant of *H. thermophilus* apocytochrome c_{552} was reacted with the heme derivatives 2-VDH and 4-VDH, the reactivity was reversed relative to the C11A variant. Again, a *b*-type cytochrome intermediate was observed following addition of either 2- or 4-VDH to C14A apocytochrome (Table 4.1 and Figures 4.7 a and b for addition of 2-VDH and 4-VDH, respectively) as was a pyridine hemochrome spectrum with an α-band of 550 nm. With 4-VDH, no further spectral change was observed over 15 hours, nor was covalent heme attachment detected by SDS-PAGE (Figure 4.6, lane 7). However, for the 2-VDH derivative, a spectral change did occur over 15 hours (Table 4.1 and Figure 4.7a). The resultant pyridine hemochrome spectrum had an α-band maximum at 548 nm. SDS-PAGE analysis confirmed that a reaction had occurred, yielding a protein with covalently bound heme (Figure 4.6, lane 6). As described earlier, not all of the C14A apoprotein bound heme to form the non-covalent complex. The same was observed for binding of the mono-vinyl hemes, but in the case of 2-VDH it

was estimated, in common with C11A protein and 4-VDH, that around 80% of the bound VDH became covalently attached.

Overall the data in this chapter show that the reaction of the cysteine residues of *H. thermophilus* cytochrome c_{552} with heme vinyl-groups is selective with respect to the vinyl group and cysteine residue. In the C11A and C14A variants of *H. thermophilus* cytochrome c_{552}, Cys-11 reacts with the 2-vinyl group of heme and Cys-14 with the 4-vinyl moiety. However, a minor side reaction (up to 10 %) leading to non-selective thioether bond formation cannot be excluded as shown by the slight heme staining of the product of the reaction of the C11A mutant with 2-VDH (Figure 4.6, lane 4).

The kinetics of the reactions of the single cysteine variants of *H. thermophilus* cytochrome c_{552} with mono-vinyl heme derivatives were difficult to determine due to the broad absorption bands of the intermediate *b*-type cytochrome (Figures 4.5b and 4.6a). The broadness of the spectral features of these intermediates might reflect the less rigid heme-binding pocket. The lack of the steric hindrance induced by an additional vinyl-group of heme might lead to lower constraints on the heme orientations within the protein.

Finally, it was shown that wild type *H. thermophilus* apocytochrome c_{552} (Figure 4.6, lane 1) was able to bind both 2-VDH and 4-VDH covalently (Figure 4.6, lane 2 and 3, respectively), reacting via a *b*-type cytochrome intermediate (Table 4.1 and Figures 4.8 a and b for reaction of wild-type apoprotein with 2-VDH and 4-VDH, respectively). Thus it behaves similarly to the reaction with heme (CHAPTER 3). The kinetics of the reaction of the mono-vinyl heme derivatives with wild-type protein containing two cysteine residues were, in so far as could be determined, similar to those for the reaction of heme with apoprotein (CHAPTER 3). However, it again remained unclear whether a biphasic kinetic behaviour was observed.

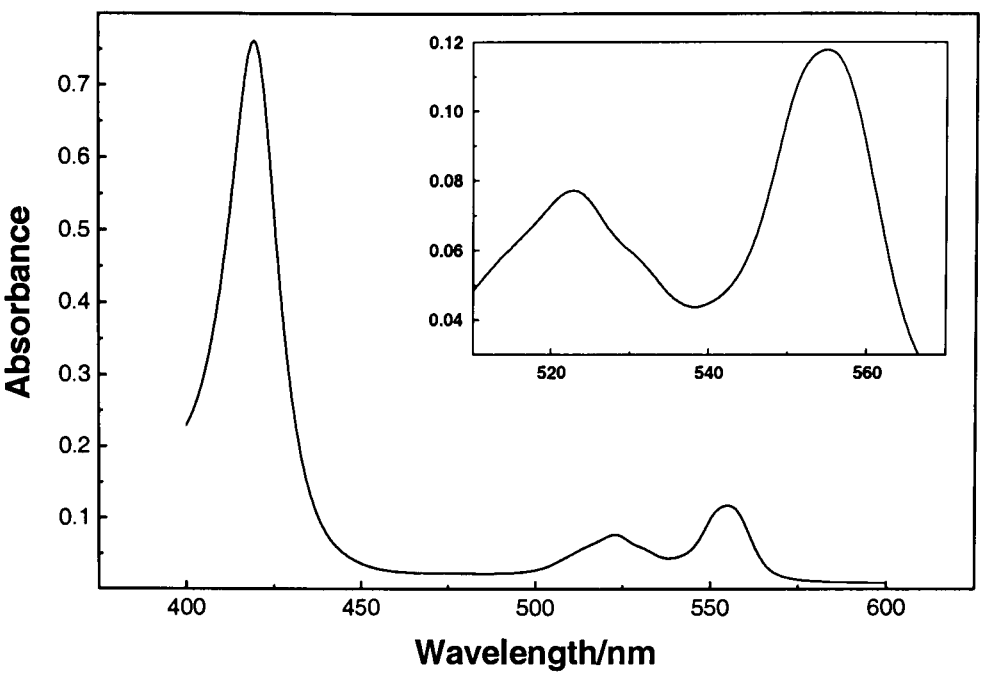


Figure 4.5a. Visible spectrum of the C11A mutant of *H. thermophilus* cytochrome c_{552} incubated with 2-VDH (~5 μM). The absorption spectrum was recorded in 50 mM sodium phosphate buffer, pH 7.0, after ten minutes and was unchanged after 15 h.

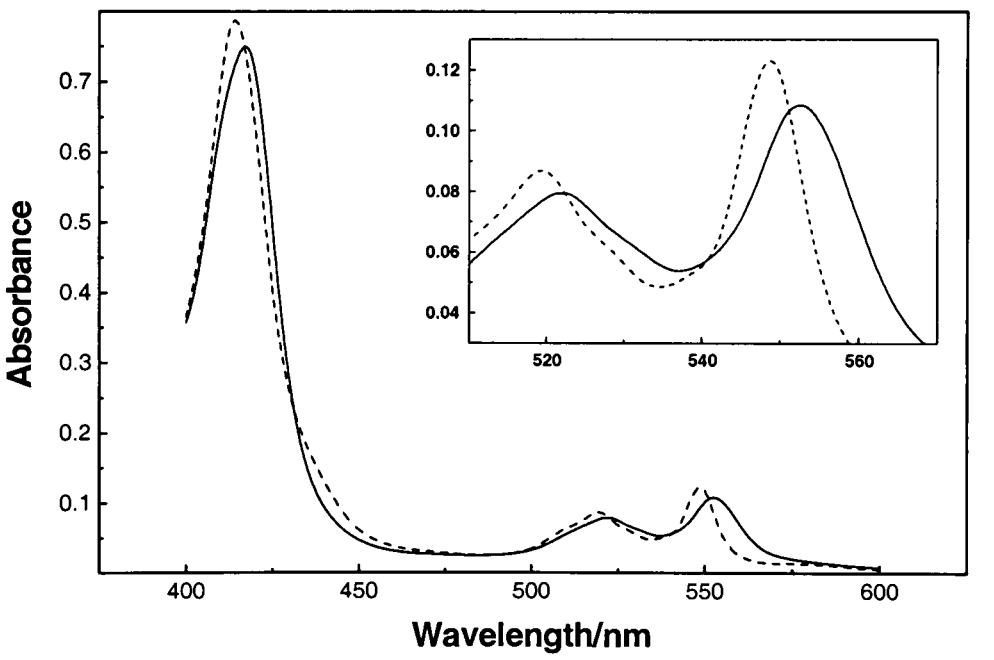


Figure 4.5b. Comparison of visible spectra of non-covalent and covalent complexes of the C11A mutant of *H. thermophilus* cytochrome c_{552} with 4-VDH (~5 μM). The spectrum of the *b*-type cytochrome complex between apocytochrome *c* and 4-VDH obtained after 10 minutes of incubation under reductive conditions (———) and of the reaction product formed from heme and apocytochrome after 15 hours (- - - -). Absorption spectra were recorded in 50 mM sodium phosphate buffer, pH 7.0.

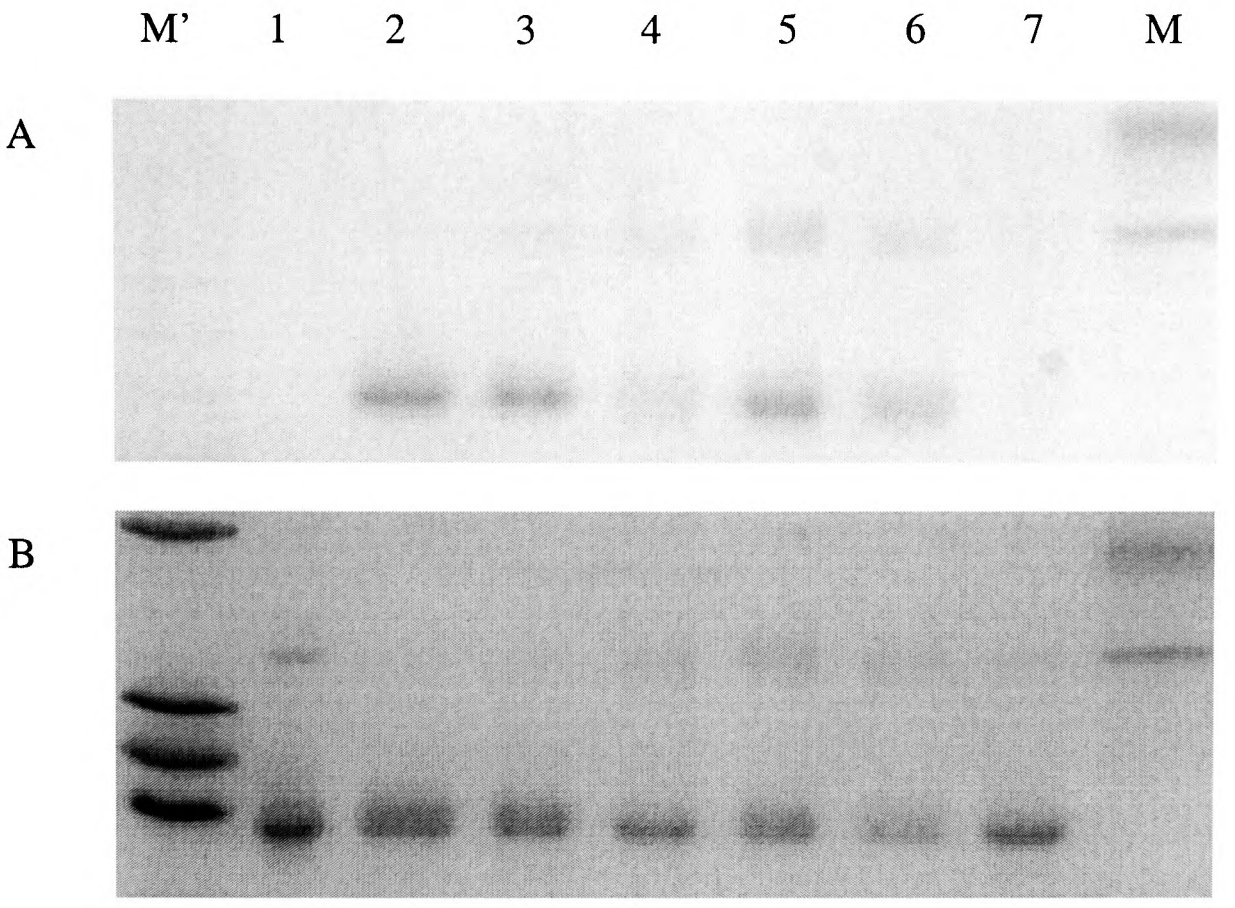


Figure 4.6. SDS/15% PAGE of *in vitro* formed cytochrome variants of *H. thermophilus* c_{552} following incubation with mono-vinyl heme derivatives. **A** activity stained for covalently bound heme followed by **B** Coomassie Blue staining. Lane 1: Apoform of wild-type (CXXCH) *H. thermophilus* cytochrome c_{552}. Lane 2: Wild-type apoprotein reacted with 2-VDH. Lane 3: Wild-type apoprotein reacted with 4-VDH. Lane 4: The C11A mutant of *H. thermophilus* cytochrome c_{552} reacted with 2-VDH. Lane 5: The C11A mutant of *H. thermophilus* cytochrome c_{552} reacted with 4-VDH. Lane 6: The C14A mutant of *H. thermophilus* cytochrome c_{552} reacted with 2-VDH. Lane 7: The C14A mutant of *H. thermophilus* cytochrome c_{552} reacted with 4-VDH. All reactions were carried out with 5 μM protein and an equimolar amount of heme or heme derivative and the reaction time was 15 hours. M' is ultra-low range protein marker (Sigma) corresponding to 6.5, 14.2, 17.0 and 26.6 kDa from bottom to top, respectively. M is prestained molecular protein marker and the bands correspond nominally to 25 and 16.5 kDa. 200-400 pmoles of protein were applied to each lane of the gel. As noted in the legend to Figure 4.2, the relative staining by Coomassie Blue in different lanes is affected by stain retained from the heme staining.

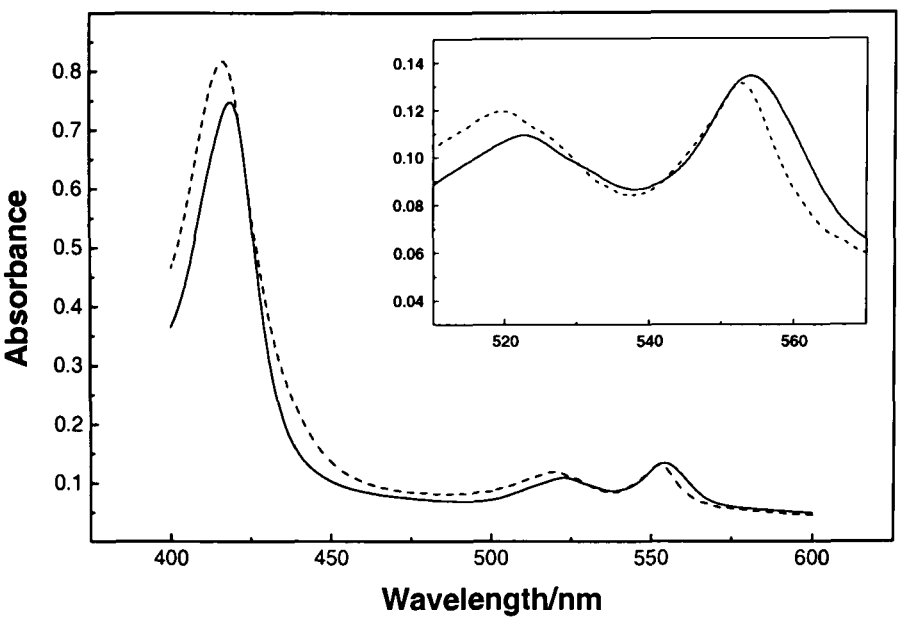


Figure 4.7a. Comparison of visible spectra of non-covalent and covalent complexes of the C14A mutant of *H. thermophilus* cytochrome c_{552} and 2-VDH (~5 μM). The spectrum of the *b*-type cytochrome complex of apocytochrome *c* and 2-VDH obtained after 10 minutes of incubation under reductive conditions (———) and the reaction product formed from heme and apocytochrome after 15 hours (- - - -). Absorption spectra were recorded in 50 mM sodium phosphate buffer, pH 7.0.

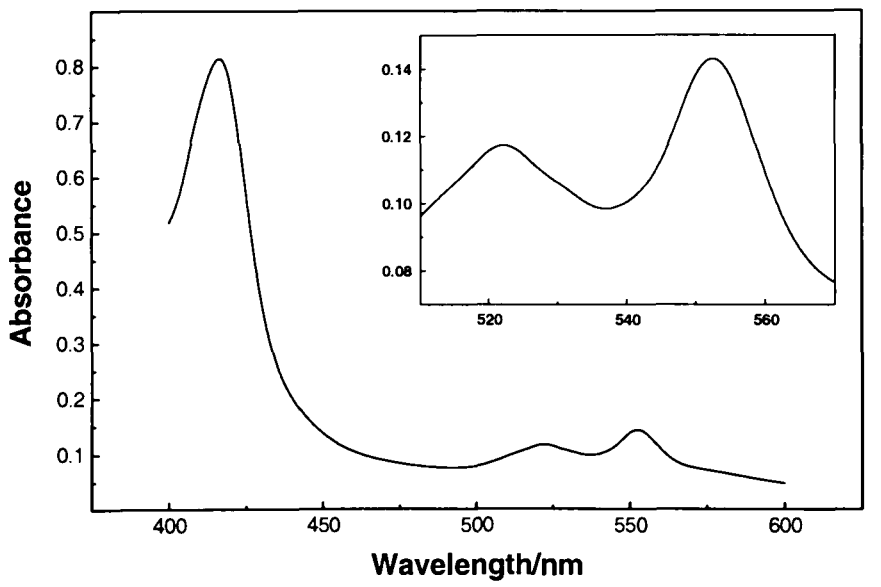


Figure 4.7b. Visible spectrum of the C14A mutant of *H. thermophilus* cytochrome c_{552} incubated with 2-VDH (~5 μM). The absorption spectrum was recorded in 50 mM sodium phosphate buffer, pH 7.0, after ten minutes and was unchanged after 15 h.

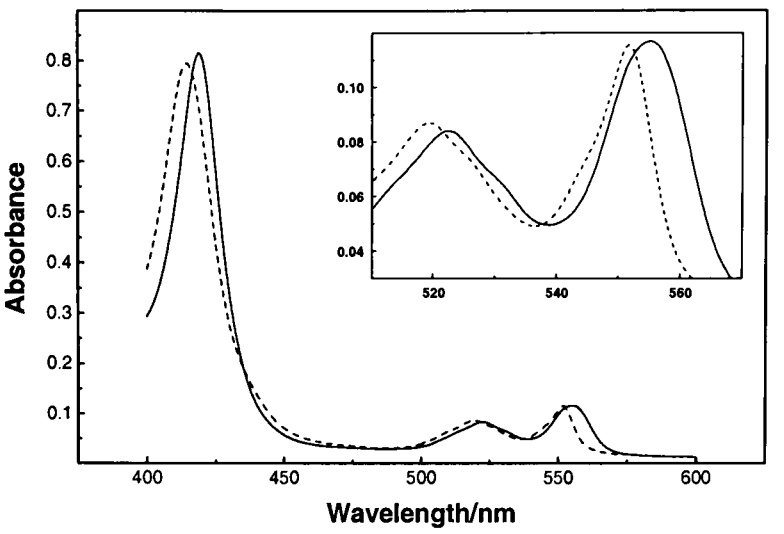


Figure 4.8a. Comparison of the visible spectra of non-covalent and covalent complexes of wild-type *H. thermophilus* cytochrome c_{552} with 2-VDH (both ~5 μM). The spectrum of the *b*-type cytochrome complex between apocytochrome *c* and 2-VDH obtained after 10 minutes of incubation under reductive conditions (———) and the reaction product of heme and apocytochrome after 15 hours (- - - -). Absorption spectra were recorded in 50 mM sodium phosphate buffer, pH 7.0.

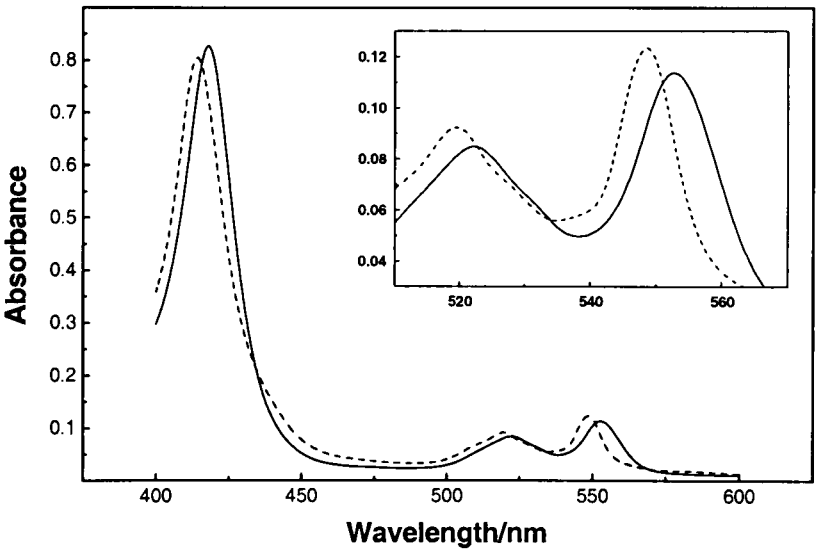


Figure 4.8b. Comparison of the visible spectra of non-covalent and covalent complexes of the wild-type *H. thermophilus* cytochrome c_{552} with 4-VDH (both ~5 μM). The spectrum of the *b*-type cytochrome complex between apocytochrome *c* and 4-VDH obtained after 10 minutes of incubation under reductive conditions (———) and the reaction product of heme and apocytochrome after 15 hours (- - - -). Absorption spectra were recorded in 50 mM sodium phosphate buffer, pH 7.0.

4.3. DISCUSSION

C11A and C14A variants of *H. thermophilus* apocytochrome c_{552}, each having one cysteine in the heme-binding motif, have been shown to attach heme covalently *in vitro* via thioether bonds. This observation is evidence that the unusual *in vivo* formation of these cytochrome variants in the cytoplasm of *E. coli* (Tomlinson & Ferguson 2000b) arises from spontaneous thioether bond formation between heme and apocytochrome.

Apoforms of *H. thermophilus* cytochrome c_{552} have been shown previously to be relatively compact, to be able to recognise heme rapidly and adopt the characteristics of a *b*-type cytochrome (CHAPTER 3; Tomlinson & Ferguson 2000a; Wain et al. 2001). It was suggested for wild-type *H. thermophilus* cytochrome c_{552}, but not proved, that the heme may initially bind in two orientations (CHAPTER 3), related by a 180° rotation around the α,γ mesoaxis of heme (see below). Wild type *H. thermophilus* cytochrome c_{552}, carrying the CXXCH motif, goes on to form a *c*-type cytochrome *in vitro* under reducing condition (CHAPTER 3). It was argued that this product had the heme covalently attached in only one orientation. The data presented in this chapter provide strong confirmation of this proposal since each of the two mono-vinyl derivatives of heme is selective for the single cysteine variant of the cytochrome *c*; the cysteine in each case is the one that forms the thioether bond with the particular vinyl group in the wild-type holocytochrome *c*. Hence stereoselective thioether bond formation is observed with respect to the heme rotational isomers of the intermediate *b*-type cytochrome complexes relative to the α,γ mesoaxis of heme. It is noteworthy that in all naturally formed *c*-type cytochromes (*i.e.*, those made by biogenesis systems) the 2-vinyl group of heme becomes attached to the N-terminal cysteine of the CXXCH motif and the 4-vinyl

moiety reacts with the cysteine adjacent to the histidine coordinating to the heme iron (Barker & Ferguson 1999; also section 1.6.2.1).

In general, non-covalently bound heme is found predominantly in one orientation in proteins such as globins and *b*-type cytochromes. However, mixtures of heme orientations that are related by a 180° rotation around the α,γ mesoaxis of heme can be seen following addition of heme to an apoprotein *in vitro* (La Mar et al. 1984; McLachlan et al. 1986; Yamamoto & La Mar 1986). In many cases the apoproteins are thought to present a nascent binding site that can be initially occupied by heme in either orientation. Such binding sites have asymmetric features, which result in one heme orientation being thermodynamically favoured. In the case of *c*-type cytochromes it has not been thought that there is a nascent heme pocket in the apoproteins, but recent work on both *H. thermophilus* cytochrome c_{552} (CHAPTER 3; Wain et al. 2001) and mitochondrial cytochrome *c* (section 1.6.2.2 and CHAPTER 5) challenges this view. The structural features of this putative nascent site are not known and therefore it may, in principle, accommodate heme in either of the two rotational isomeric orientations. On the other hand, the heme binding pocket of the apoprotein may have sufficient of the asymmetric features seen in the holoprotein (Hasegawa et al. 1998) to ensure preferential binding of heme in one rotational isomeric position. In the former of the two alternatives the results presented imply that the stereochemical features of the site in *H. thermophilus* cytochrome c_{552} are such that only a 2- or 4-vinyl group in the same location as in the holoprotein can approach a cysteine thiol sufficiently closely to overcome kinetic constraints on *in vitro* thioether bond formation. The second alternative implies that the nascent heme-binding site in the apoprotein is sufficiently structured so as to exclude its occupancy by heme to yield the "wrong"

rotational isomer. In this case bond formation between a mono-vinyl heme and a "wrong" cysteine can be readily envisaged as not feasible. These considerations also imply that the single thioether bond variants of *H. thermophilus* cytochromes *c* formed in the cytoplasm of *E. coli* (Tomlinson & Ferguson 2000b) have predominantly heme attached in one rotational orientation around the α,γ mesoaxis of heme.

The strict stereospecific requirement for covalent attachment of heme to *H. thermophilus* apocytochrome c_{552} contrasts with the situation seen for uncatalysed *Thermus thermophilus* cytochrome *c* synthesis in the cytoplasm of *Escherichia coli*. In that case an inversion of heme relative to its α,γ mesoaxis can be related to misattachment of heme in one of the major products identified (McRee et al. 2001). This contrast makes the acquisition of structural information about the *H. thermophilus* apoprotein (Wain et al. 2001) an intriguing prospect.

To the best of my knowledge, it is not known what advantage(s) arise from incorporating heme to yield only one rotational isomer *in vivo* in either *c*-type cytochromes, as is found in all known examples, or into other heme containing proteins such as globins or *b*-type cytochromes in a non-covalent fashion. Evolutionary points have been discussed for *c*-type cytochromes (Kojo et al. 1989). Rotational isomerism may arise *in vivo* either due to stereoselective heme release after its biosynthesis or because heme-containing proteins have evolved to form the heme-pocket such that only one rotational heme-protein isomer yields optimal heme-protein interactions required for efficient functioning of the metalloprotein.

Little is known about the chemistry that underpins thioether bond formation from cysteine thiols and vinyl-groups of heme. The present observations show, at least for the *in vitro* studies, that the reaction of either vinyl group of heme to form the first thioether bond is independent of the presence of the second vinyl group. However, where both the second vinyl group and two cysteine residues are present, the formation of the second thioether bond is faster than the first, at least as evidenced by the failure to detect a significant concentration of single-thioether bond product during reaction of heme with the CXXCH protein (CHAPTER 3). The observation that heme lyase can attach heme covalently to apocytochromes with only one cysteine residue in the CXXCH motif (Rosell & Mauk 2002; Tanaka et al. 1990) suggests that heme lyase might accelerate the process studied here *in vitro*. Mechanistic implications for the catalytic functions of the heme lyase will be discussed in the following chapter (CHAPTER 5).

CHAPTER 5

THE *IN VITRO* REACTIONS OF HORSE HEART APOCYTOCHROME *C*, YEAST ISO-1-APOCYTOCHROME *C* AND *PARACOCCUS DENITRIFICANS* APOCYTOCHROME C_{550} WITH HEME

SUMMARY

The *in vitro* formation of *Hydrogenobacter thermophilus* cytochrome c_{552} was reported in CHAPTER 3. In this CHAPTER it is reported that thioether bonds can form spontaneously *in vitro* between heme and the apocytochromes *c* from horse heart, yeast and *Paracoccus denitrificans* following the formation of *b*-type cytochrome intermediates. The apocytochromes from horse heart and *P. denitrificans*, but not their holo forms, bind 8-anilino-1-naphthalenesulfonate, indicating that these apoproteins each have an affinity for a hydrophobic ligand. Furthermore, for these two apocytochromes *c*, an intramolecular disulfide bond can form between the cysteines of the CXXCH heme-binding motif of the *c*-type cytochromes. The *in vitro* reaction of apocytochromes *c* with heme to yield holocytochromes *c*, and their tendency to form a disulfide, have implications for the different systems in various organisms responsible for cytochrome *c* maturation *in vivo*.

5.1. INTRODUCTION

Following the report of the *in vitro* formation of holocytochrome c_{552} from *H. thermophilus* (CHAPTER 3), an important point to be established was whether the spontaneous attachment of heme to apocytochrome *c* is a reaction also applicable to other cytochromes *c*. Since holocytochrome c_{552} from *H. thermophilus* forms, exceptionally, in the cytoplasm of *E. coli* (Sambongi & Ferguson 1994b; Sinha & Ferguson 1998), its spontaneous *in vitro* formation might not necessarily be anticipated to occur for all *c*-type cytochromes. The thioether bond formation described for *H. thermophilus* cytochrome c_{552} in CHAPTER 3 involved a *b*-type cytochrome (*i.e.*, non-covalent) intermediate. Prompted by the previously reported similar ability of the horse heart apocytochrome to bind heme to yield a species characteristic of a *b*-type cytochrome (Dumont et al. 1994), the reaction of horse heart apocytochrome *c* with heme was recognised to require reinvestigation.

The formation of *c*-type cytochromes is especially interesting in light of the different biosynthetic pathways found in various organisms (reviewed in CHAPTER 1 and elsewhere (Kranz et al. 1998; Page et al. 1998; Thöny-Meyer 2002)), by which heme is attached to the apoproteins *in vivo*. In some eukaryotes, a single enzyme, designated heme lyase, facilitates the attachment of the heme moiety to the apocytochrome *c* (Steiner et al. 1996). This system is exemplified by horse (*Equus caballus*) heart and yeast (*Saccharomyces cerevisiae*) mitochondria. Therefore, the reaction between yeast iso-1-apocytochrome *c* and heme, which is assembled very similarly to horse heart cytochrome *c in vivo*, was also identified for study *in vitro*.

Many Gram-negative bacteria use a system that involves more than ten gene products to ensure correct cytochrome *c* maturation (ccm) in the periplasm (CHAPTER 1; Page et al. 1998; Thöny-Meyer 2002; Thöny-Meyer 1997). An example of a cytochrome *c* that is matured *in vivo* using this biosynthesis apparatus is cytochrome c_{550} from the mesophilic bacterium *Paracoccus denitrificans* (Sambongi & Ferguson 1994b); this protein was therefore also obtained for the study of the reaction of heme with the apocytochrome.

A very interesting candidate for studying the potential *in vitro* formation of thioether bonds is cytochrome *f*, which is matured using cytochrome *c* biogenesis system II (Section 1.5.3). Two sources of this type of protein were available; cytochrome *f* from spinach (*Spinacea oleracea*), and the soluble fraction of cytochrome *f* from the cyanobacterium *Phormidium laminosum*.

Thus, the cytochromes *c* discussed in this CHAPTER are assembled by different cytochrome *c* maturation systems *in vivo*. A comparison of the reactions of heme and cytochromes *c in vitro* with respect to the heme attachment mechanism may be anticipated to have implications for the molecular basis of heme attachment by the different biogenesis systems *in vivo*.

5.2. RESULTS

5.2.1. Apocytochrome production and characterisation

5.2.1.1. Horse heart cytochrome *c*

Horse heart cytochrome *c* was shown to be pure by SDS-PAGE analysis (Figure 5.1, lane a) and had a mass of 12,359 Da (calculated: 12,360 Da) as shown by ES-MS analysis. The *in vitro* produced apocytochrome was shown to be devoid of

heme as judged by SDS-PAGE analysis followed by staining for covalently bound heme (Figure 5.1, lane b), the absence of cytochrome characteristics in the visible absorption spectra, the disappearance of the cytochrome *c* fold as shown by CD analysis (Figure 5.2) and ES-MS analysis, which resulted in a mass of 11,746 Da (calculated masses 11,742 Da and 11,744 Da for the oxidised and reduced protein, respectively). It is noteworthy that addition of one oxygen atom to the apoprotein occurred, as judged by the appearance of peak at 11,760 Da in the ES-MS, if the reaction time for the removal of heme was not kept to a maximum of two hours.

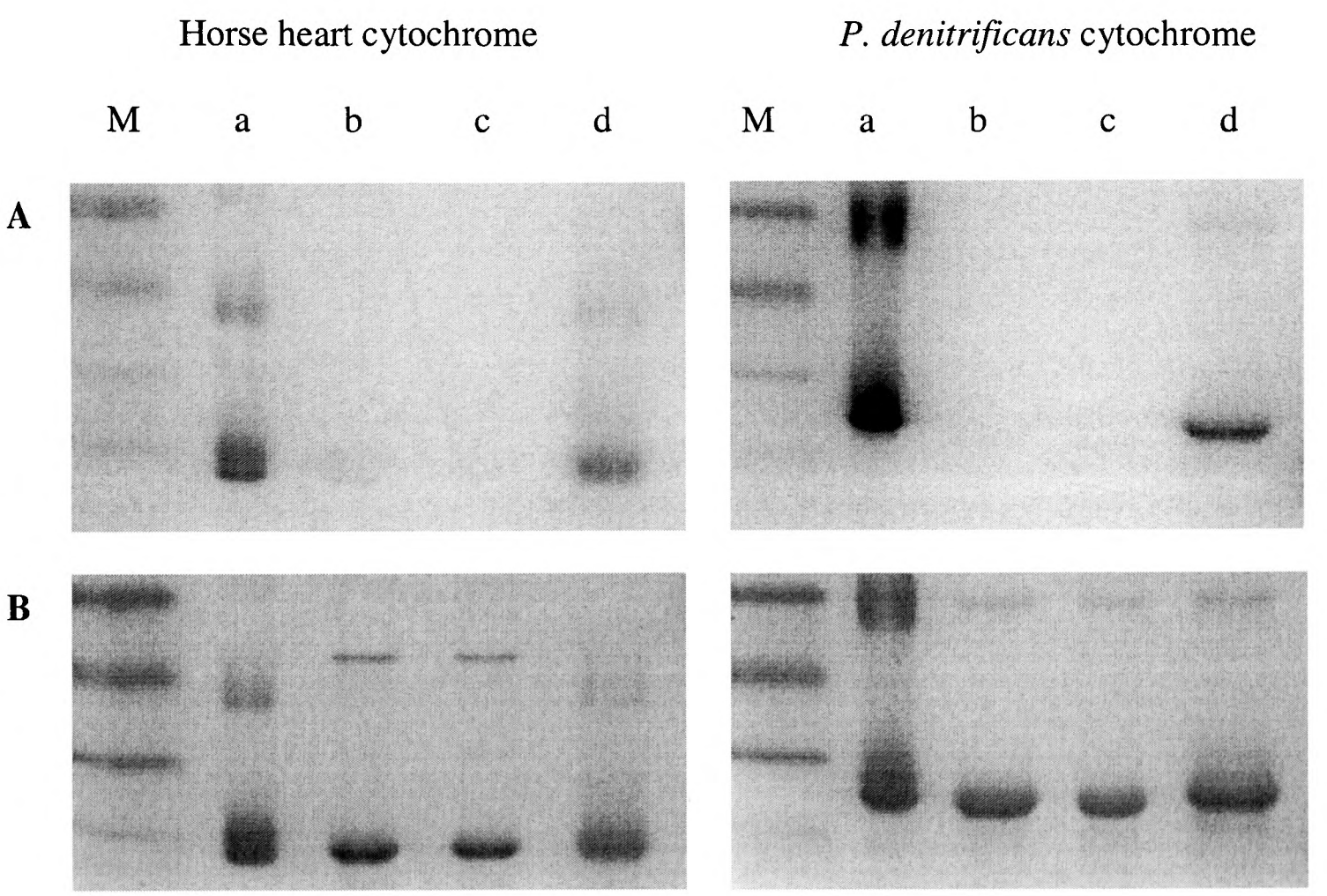
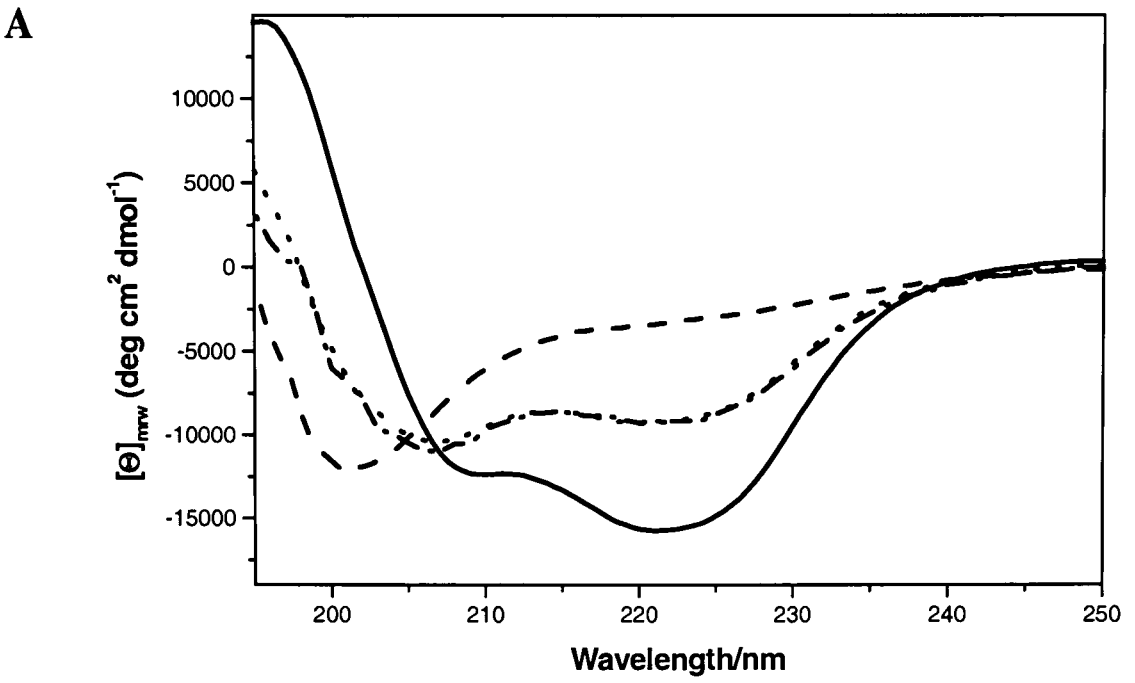


Figure 5.1. SDS/15% PAGE of various forms of horse heart cytochrome *c* and *Paracoccus denitrificans* cytochrome c_{550}. **A** activity stained for covalently bound heme followed by **B** Coomassie Blue staining. (a) *In vivo*-produced holocytochrome, (b) apocytochrome, (c) mesoheme-containing cytochrome and (d) *in vitro*-produced holocytochrome. M is prestained protein marker corresponding nominally to 16.5, 25, 32.5 and 47.5 kDa from bottom to top, respectively. 200-400 pmoles of protein were applied to each lane of the gel.



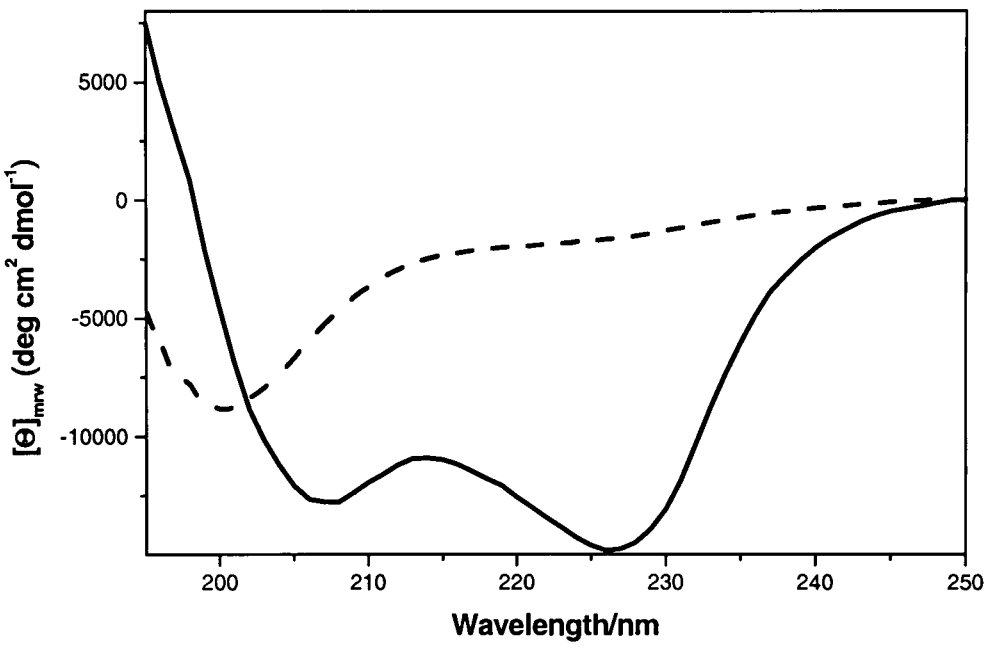


Figure 5.2. Circular dichroism spectra of various forms of (**A**) horse heart cytochrome *c* and (**B**) *Paracoccus denitrificans* c_{550}. Oxidised holocytochrome *c* (———) and apocytochrome *c* (— — —) are presented for the proteins from both sources. For horse heart cytochrome *c*, the reaction product of apocytochrome and heme is shown (·— ·— ·) and compared with the simulated CD spectrum obtained by adding the spectra of holoprotein and apocytochrome in the ratio 2:3 (- - - -), which overlaps with the former spectrum for most of the spectral range. $[\Theta]_{mrw}$ is mean molar ellipticity per residue. Spectra were recorded using 20 μM protein in 20 mM sodium phosphate buffer, pH 7.0.

When the apoprotein was dialysed extensively overnight, the cysteines within the CXXCH heme-binding motif (CAQCH for equine cytochrome *c*) formed a disulfide bond as shown by Ellman's reagent (Riddles et al. 1983), which accounted for 0.1 equivalents of thiol per mole of protein instead of the 2 equivalents that were detected in the starting material. This disulfide was shown to be intramolecular because ES-MS analysis gave the monomeric protein mass, and the polypeptide migrated as a monomer in SDS-PAGE analysis under non-reducing conditions (Figure 5.1, lane b). Further evidence to show the presence of a disulfide was obtained from thiol-labelling studies of reduced and oxidised apocytochrome with AMS. Oxidised apoprotein was incapable of reacting with the alkylating agent, whereas the reduced apoprotein was labelled as shown by SDS-PAGE (Figure 5.3A); there was a heavier protein band for modified polypeptide (lane b, Figure 5.3A). After incubation with AMS, oxidised apoprotein (lane a, Figure 5.3A) migrated at a weight corresponding to non-reacted oxidised and reduced apocytochrome (lane c and d, respectively, Figure 5.3A). In contrast to *H. thermophilus* apocytochrome c_{552} (CHAPTER 3), no difference was observed between the CD spectra of oxidised and reduced apocytochrome.

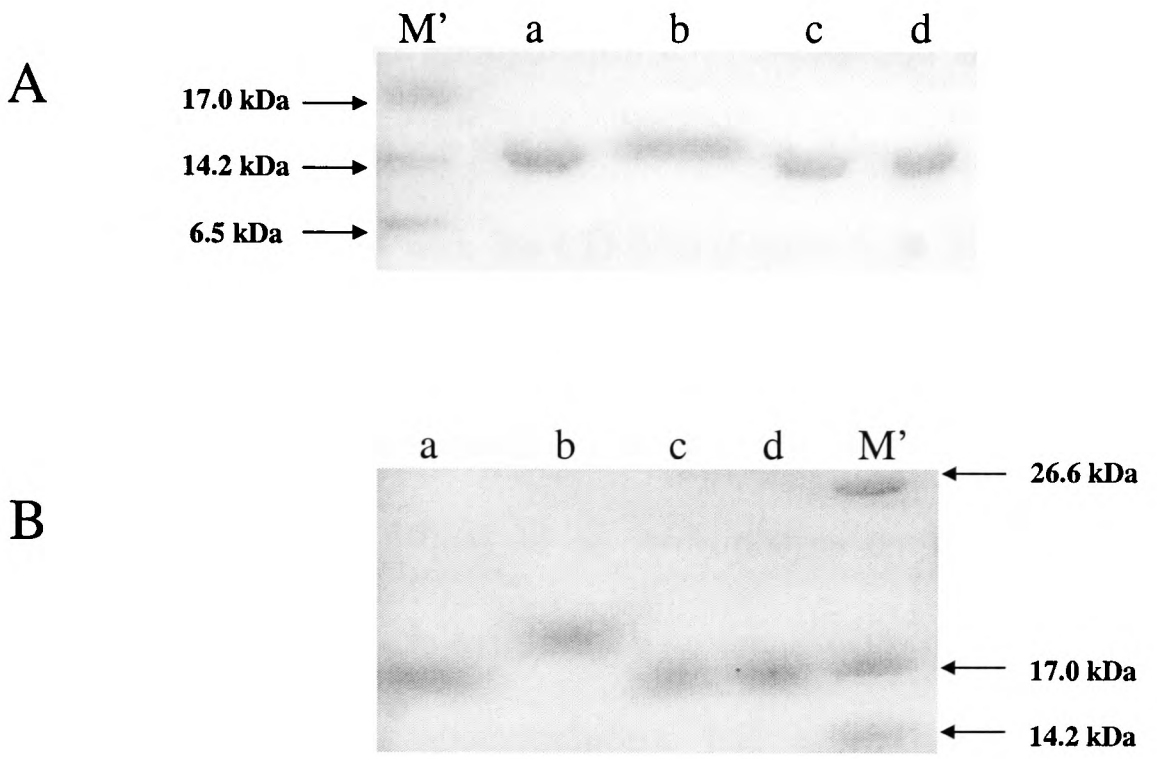


Figure 5.3. A and B: SDS/17.5%-PAGE analysis of susceptibility of various forms of apocytochromes *c* to modification of their cysteine thiols by AMS. **(A)** corresponds to horse heart apocytochrome *c* and **(B)** shows analysis of *P. denitrificans* apocytochrome c_{550}. M' is ultra low range molecular weight marker (Sigma) with the bands corresponding to the masses indicated in the figure. Lane (a) oxidised apocytochrome incubated with AMS labelling solution; (b) reduced apoprotein treated with AMS under identical conditions to the polypeptide shown in lane 1; (c) oxidised apoprotein; (d) reduced apocytochrome. The gels were stained with Coomassie Blue and 200-300 pmoles of protein were loaded to each lane.

The affinity of horse heart apocytochrome *c* for the hydrophobic ligand ANS was measured as described in CHAPTER 2. The binding isotherms showed one transition corresponding to a dissociation constant of 60 (±20) μM for a single binding site for the oxidised (disulfide-containing) protein (Figure 5.4). The absence of the disulfide did not alter the ANS affinity substantially (K_d : 70 (±20) μM for reduced protein in the presence of 2 mM DTT). Interestingly, the protein fluorescence emission maximum was around 355 nm, indicating that the fluorescing tryptophan

(Trp-59 in horse heart apocytochrome *c*) is exposed to an aqueous environment. This observation suggests that the apoprotein is largely unfolded (Ladokhin 2000), which is in agreement with the CD data (Figure 5.2). The original holocytochrome neither bound ANS nor gave rise to fluorescence features of a tryptophan residue exposed to a polar environment. The structure of the protein, which shows that the tryptophan (residue 59) is in an hydrophobic environment close to the heme (Bushnell et al. 1990), is consistent with this observation. The ANS fluorescence in the presence of the apocytochrome had a maximum around 475 nm, with excitation at 380 nm, indicating that the ANS moiety was in a relatively hydrophobic environment (Stryer 1965). Addition of ANS to either holoprotein or buffer gave rise to an ANS fluorescence maximum around 515 nm, which is in agreement with ANS being exposed to an aqueous environment and not bound to a hydrophobic site on the holoprotein (Stryer 1965).

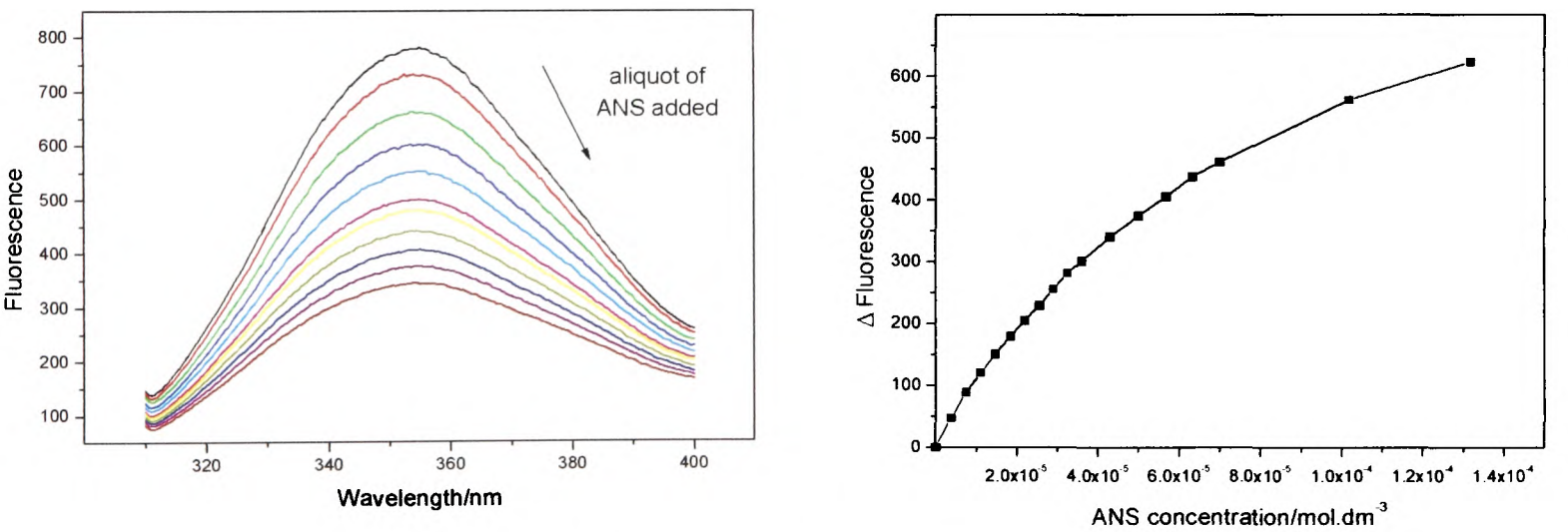


Figure 5.4. A Protein fluorescence quenching upon addition of ANS aliquots to horse heart apocytochrome *c*. Typical fluorescence spectra are shown on the left, the saturation curve obtained is given on the right. Spectra were recorded using 5 μM protein in 20 mM sodium phosphate buffer, pH 7.0.

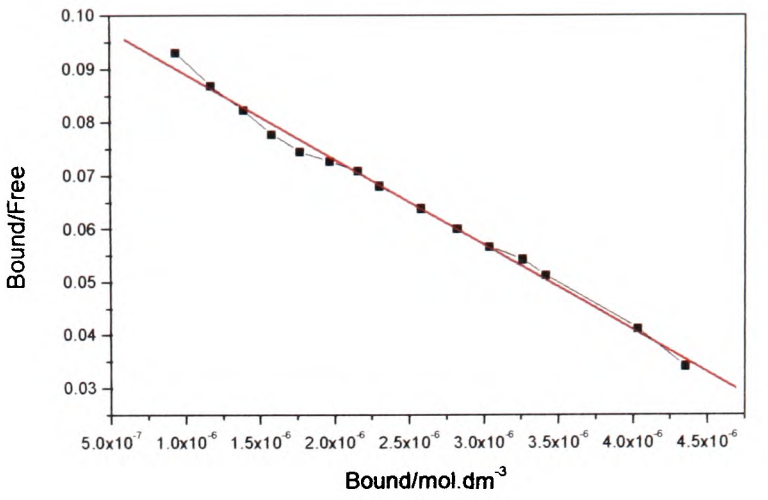


Figure 5.4. B: The Rosenthal plot obtained from the data shown in panel A. The slope shown in red corresponds to the ANS binding affinity and the intercept at the x-axis compared with the concentration of protein used allows calculation of the number of binding sites of ANS per molecule of protein.

It was shown that ANS, which is known to bind to the heme pocket in apomyoglobin (Stryer 1965), was displaced from horse heart apocytochrome *c* protein upon addition of just over one equivalent of heme per equivalent of protein, suggesting that the hydrophobic ligand ANS binds to the same site as heme (Figure 5.5).

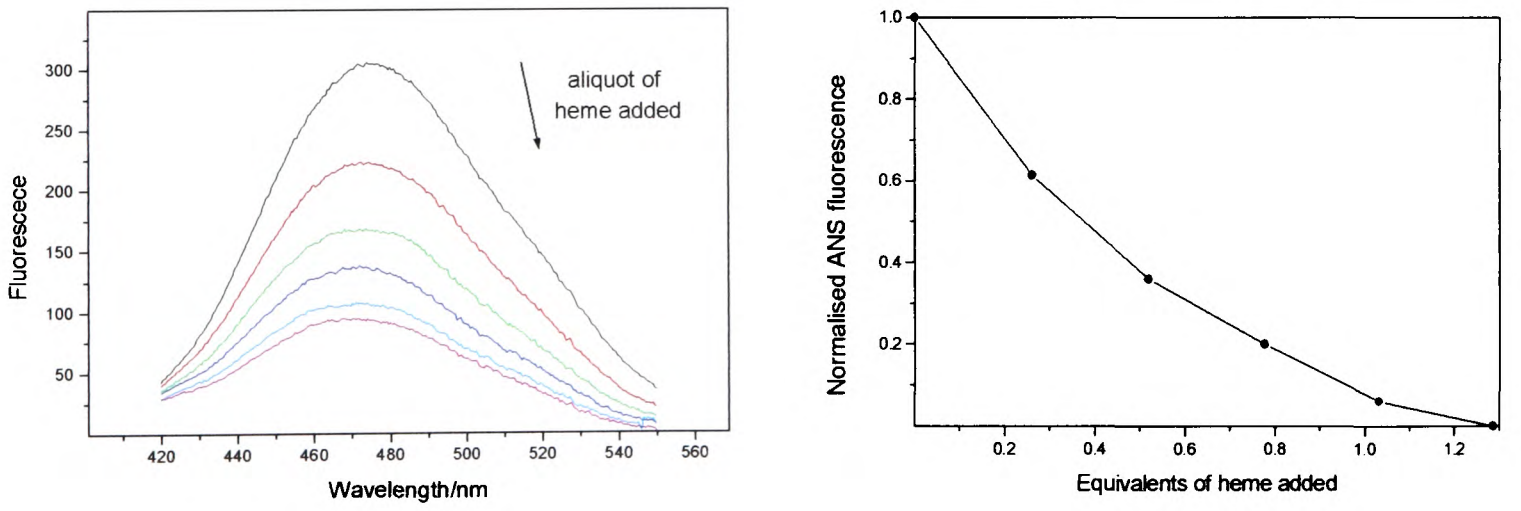


Figure 5.5. ANS fluorescence quenching upon addition of aliquots of heme. The difference spectra of the ANS fluorescence minus the free ANS fluorescence are shown on the left, the normalised ANS fluorescence is plotted against equivalents of heme added relative to protein on the right.

5.2.1.2. *Paracoccus denitrificans* cytochrome c_{550}

P. denitrificans cytochrome c_{550} was shown to be pure by SDS-PAGE analysis (Figure 5.1, lane a) and ES-MS analysis showed a mass of 15,028 Da (calculated: 15,028 Da). The apocytochrome was shown to be devoid of heme, as determined using the same techniques described above for horse heart apocytochrome *c*. SDS-PAGE analysis (Figure 5.1, lane b) and the CD spectrum (Figure 5.2) of the apocytochrome are shown. The ES-MS data showed a mass of 14,409 Da (calculated: 14,410 Da and 14,412 Da for oxidised and reduced cysteines, respectively).

Overnight dialysis of the apoprotein resulted in the formation of a disulfide between the cysteines of the CXXCH motif (CKACH for *P. denitrificans* cytochrome c_{550}; cf. APPENDIX) as shown by Ellman's reagent (Riddles et al. 1983), which accounted for zero equivalents of thiol per mole of protein compared to two equivalents in the initially produced apoprotein. The monomeric state of the protein shown by ES-MS and SDS-PAGE analyses under non-reducing conditions (Figure 5.1, lane b) demonstrates that this disulfide bond is intramolecular. AMS labelling was also performed with reduced and oxidised protein. Apoprotein containing a disulfide was unable to bind any label, whereas reduced apocytochrome reacted with two AMS moieties as shown by ES-MS and SDS-PAGE analyses (Figure 5.3B). The oxidised protein after incubation in AMS solution had a mass of 14,410 Da, indicating that no alkylation had taken place. Reduced apocytochrome had a mass peak at 15,420, which is interpreted as the covalent binding of two AMS molecules (0.5 kDa each) and one sodium adduct. The increased mass was also apparent by SDS-PAGE analysis (lane b, Figure 5.3B), relative to unreactive (lane a, Figure 5.3B) and non-reacted protein (lanes c and d, Figure 5.3B).

An ANS-protein dissociation constant of 55 (±15) μM for oxidised apocytochrome c_{550} was obtained by fluorescence experiments analogous to those described for horse heart apocytochrome *c*, demonstrating that the apoprotein has a pronounced affinity for hydrophobic ligands. The absence of the disulfide bond (*i.e.*, in the presence of 2 mM DTT) did not affect the ANS affinity (K_d : 55 (±20) μM). The solvent exposure of the tryptophan residues and the overall fold of the apocytochrome c_{550}, as judged by CD spectroscopy (Figure 5.2), are analogous to those for horse heart apocytochrome *c*; they suggest a largely unfolded structure. Holocytochrome c_{550} was incapable of binding ANS, but heme could displace ANS from the apocytochrome, indicating both hydrophobic molecules competed, most probably directly, for the same binding site.

5.2.1.3. Yeast iso-1-cytochrome *c*

Yeast iso-1-cytochrome *c* (Sigma) ran as a single band in SDS-PAGE and was used without further purification. The mass of the holoprotein was 12,704 Da (calculated 12,704 Da) as shown by ES-MS. The preparation of apocytochrome did not stain for covalently bound heme on SDS-PAGE analysis (Figure 5.6), nor give rise to cytochrome characteristics in visible absorption or CD-spectra (Figure 5.7). The mass of the apocytochrome was 12,087 Da (calculated 12,088 Da). The oxidation state of the thiol moieties within this apocytochrome was difficult to determine as an additional cysteine residue (cys-107) is present (cf. APPENDIX). After prolonged dialysis, the thiol content decreased from three moles per protein to 0.4 per protein as judged by analysis with Ellman's reagent. A mixture of disulfides between the cysteines, including intermolecular disulfide bonds, is assumed to have formed. The formation of intermolecular disulfide bonds between

cys-107 in this protein is known to occur in the holocytochrome (Cutler et al. 1987).

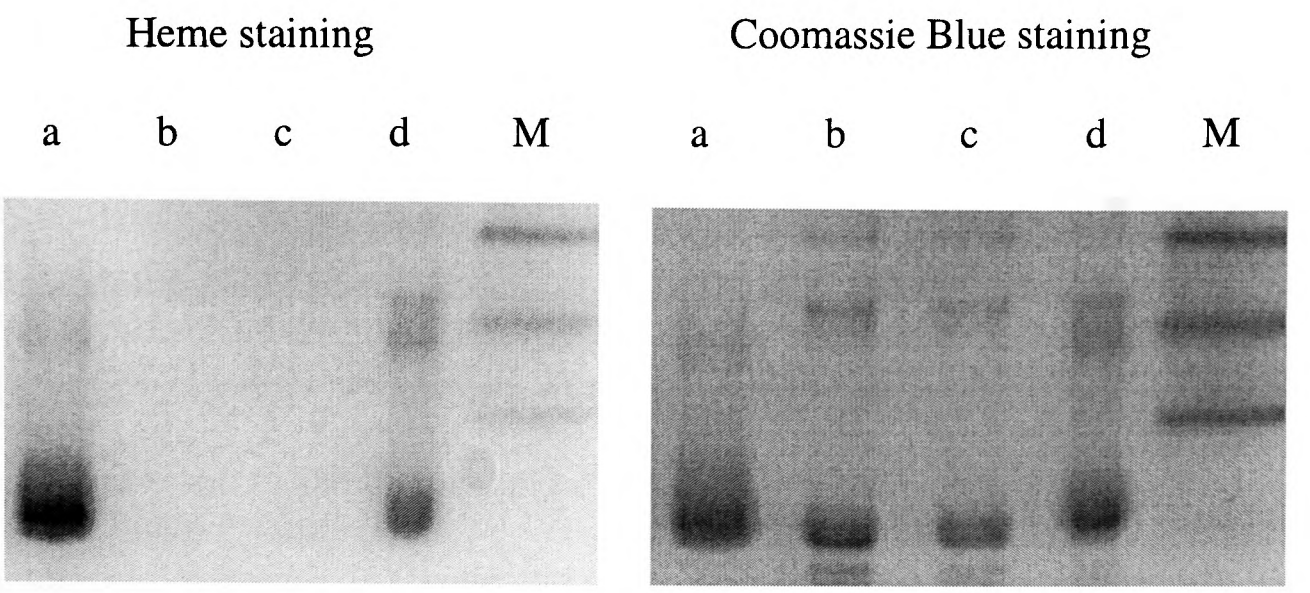


Figure 5.6. SDS/15% PAGE of various forms of yeast iso-1-cytochrome *c* subjected to heme staining (left) followed by Commassie Blue staining (right). (a) *In vivo*-produced holocytochrome, (b) reduced apocytochrome, (c) mesoheme-containing cytochrome and (d) *in vitro*-produced holocytochrome. M is prestained protein marker corresponding nominally to 47.5, 32.5, 25 (and a faint band for 16.5) kDa from top to bottom, respectively. 200-400 pmoles of protein were applied to each lane of the gel.

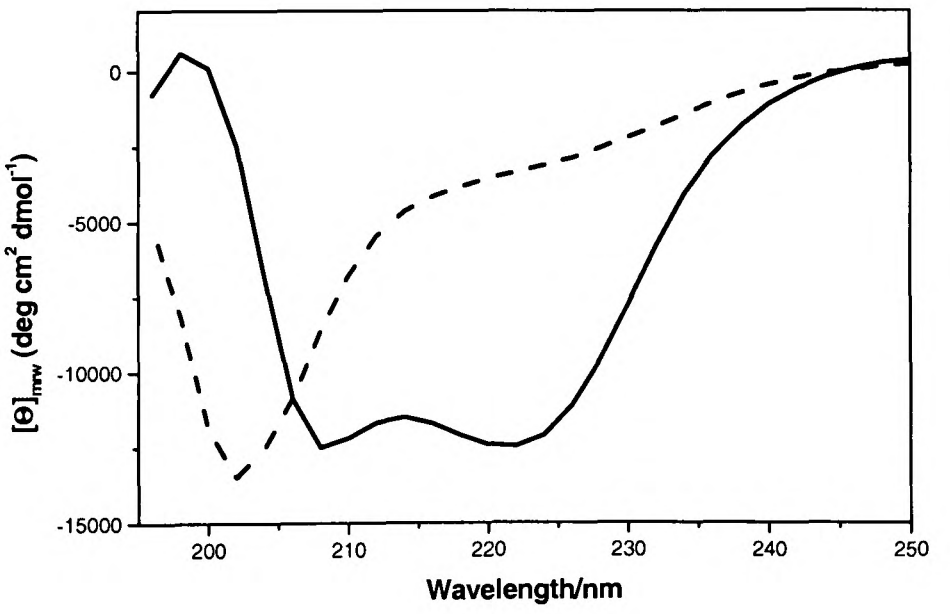


Figure 5.7. Circular dichroism spectra of yeast iso-1-cytochrome *c*. Oxidised holocytochrome *c* (———) and apocytochrome *c* (— — —) are shown. $[\Theta]_{mrw}$ is mean molar ellipticity per residue. Spectra were recorded using 20 μM protein in 20 mM sodium phosphate buffer, pH 7.0.

5.2.1.4. Spinach and *Phormidium laminosum* cytochrome *f*

Both cytochromes were of high purity as shown by SDS-PAGE analysis (Figure 5.8, lane a and d, for spinach and *P. laminosum* cytochrome *f*, respectively; the latter protein was kindly supplied by Drs. William Turner, Beatrice Schlarb-Ridley and Prof. Chris Howe, University of Cambridge). However, the heme extraction methodology led to precipitation and aggregation of protein, such that insufficient quantities of either apoprotein were obtained for experimental purposes (Figure 5.8, lane b and e, for spinach and *P. laminosum* cytochrome *f*, respectively). Even a ten-fold decrease in acetic acid concentration did not improve the yield of spinach apocytochrome *f* (Figure 5.8, lane c). Therefore, no further studies could be undertaken for this type of *c*-type cytochrome.

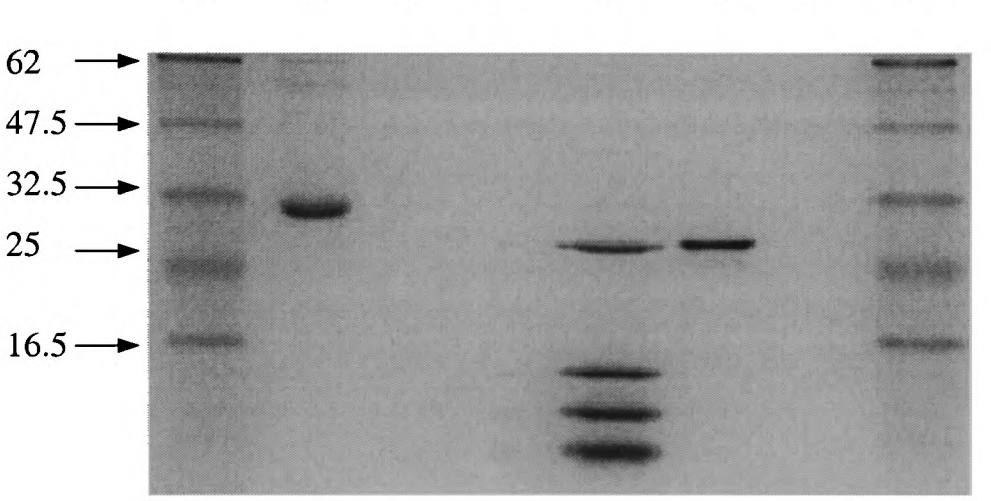


Figure 5.8. SDS/12% PAGE of spinach and *Phormidium laminosum* cytochrome *f* stained with Coomassie Blue. (a) Spinach holocytochrome *f*. (b) Supernatant after incubating cytochrome *f* in heme extraction solution for 2 hours. (c) Supernatant of cytochrome *f* reacted with silver sulfate in 0.01 M acetic acid. (d) *P. laminosum* holocytochrome *f*. (e) Supernatant after incubating protein from lane d under heme extraction conditions for 2 hours. M is prestained protein marker corresponding nominally to masses as indicated. M' is molecular weight marker corresponding to 6.5, 14.2, 17.0 and 26.6 kDa from bottom to top, respectively. 200-300 pmoles of protein were applied to lanes a and d of the gel.

5.2.2. Reaction of apocytochromes with heme

5.2.2.1. Horse heart cytochrome *c*

Addition of ferrous heme to reduced horse heart apocytochrome *c* (no disulfide bond) in the presence of 5 mM DTT at pH 7.0 resulted in the appearance in the visible spectrum of characteristics of a reduced *b*-type cytochrome (Figure 5.9a and Table 5.1) with absorption maxima at 424 nm, 528 and 559 nm. The pyridine hemochrome spectrum of this mixture had its α-band at 556 nm (Table 5.1), indicating that the heme was not modified and contained two unreacted vinyl groups (Bartsch 1971). These results, showing the formation of a non-covalent complex between heme and horse heart apocytochrome *c*, are consistent with previous studies on this protein (Dumont et al. 1994).

Following nearly quantitative formation of the *b*-type cytochrome from the mixture of horse heart apocytochrome *c* and heme under reducing conditions, the maximum at 559 nm in the absorption spectrum shifted to 550 nm over time (Table 5.1). After 48 hours, the visible spectrum of protein, following gel-filtration chromatography to remove any unbound heme and protein aggregates, showed features both of a *b*- and *c*-type cytochrome in a ratio of approximately 2:3 (Figure 5.9a). After 72 hours, the visible spectrum contained broad peaks, but had absorption maxima very similar to those of the original holoprotein (Figure 5.9b and Table 5.1) and the pyridine hemochrome spectrum of the reduced protein after gel-filtration chromatography had a resolved band at 549.5 nm (Table 5.1), characteristic of saturation of both vinyl groups of the heme (Bartsch 1971). CD spectra of the desalted, oxidized cytochrome *c* produced *in vitro* after 72 hours of incubation showed a mixture of spectral features of holoprotein and apoprotein (Figure 5.2). It was deduced from the ratio of the Soret band relative to the

absorption at 280 nm in the absorption spectrum that 40 % of the apoprotein had formed thioether bonds to heme and 60 % of the protein was present in the apoform. This was calculated knowing that in the original (oxidised) holoprotein the ratio of A_{280}:A_{410} is 1:4. Heme is assumed to have dissociated from 60 % of the initial *b*-type cytochrome complex and unbound heme to have been removed upon gel-filtration chromatography. The reconstituted holocytochrome *c* prepared in the present work had a CD spectrum that was almost identical to a spectrum that could be simulated by adding the spectra of the apocytochrome and the original (purchased) holocytochrome in a ratio of 3:2 (Figure 5.2). It was determined experimentally that CD spectra of these two components are additive, *i.e.*, the presence of either form of the protein did not affect the fold or, therefore, the CD spectrum of the other.

Consequently, it was concluded that 60 % of the *b*-type cytochrome intermediate had not formed a *c*-type cytochrome after 72 h. Why this is case is unclear. Possible reasons for this difference in reactivity may arise from the orientation of the heme relative to its rotation around the α,γ mesoaxis, or an unreactive fold of the apocytochrome around the prosthetic group.

A noteworthy point is that the CD spectrum of the horse heart cytochrome *c* generated *in vitro* had a great similarity not with only that of the previously reported *b*-type cytochrome complex (Dumont et al. 1994), but also with an apocytochrome derivative where the cysteines had been reacted with a hydrophobic molecule (Kang & Carey 1999). Hence a definite conclusion about whether the full holocytochrome *c* fold is gained upon reaction of apocytochrome with heme cannot be reached. Separation of holo- and apocytochromes by addition

of ammonium sulfate (Goldberg et al. 1999) was not achieved to a satisfactory extent with our putative mixture of reconstituted cytochrome *c* and unreacted apoprotein.

Table 5.1. Absorption maxima of various forms of cytochrome species.

Protein species		Wavelength/nm			
		Reduced protein			Pyridine hemochrome (α-band)
Horse heart cytochrome *c*	Holo produced *in vivo*	415	520	550	549.5
	Holo produced *in vitro*	416	520	550	549.5
	b-type complex	424	528	559	556
	Mesoheme complex	416	519	549	546
P. denitrificans cytochrome c_{550}	Holo produced *in vivo*	415	521.5	550	549.5
	Holo produced *in vitro*	417	523	552	552.5
	b-type complex	423	528.5	558.5	556
	Mesoheme complex	414	519.5	548.5	546
Yeast iso-1-cytochrome *c*	Holo produced *in vivo*	415	521	549.5	549.5
	Holo produced *in vitro*	417	521	551	549.5
	b-type complex	423	528	559	556
	Mesoheme complex	415	519	549	546

Spectra were recorded with protein in 50 mM potassium phosphate buffer, pH 7.0, and reduced by disodium dithionite.

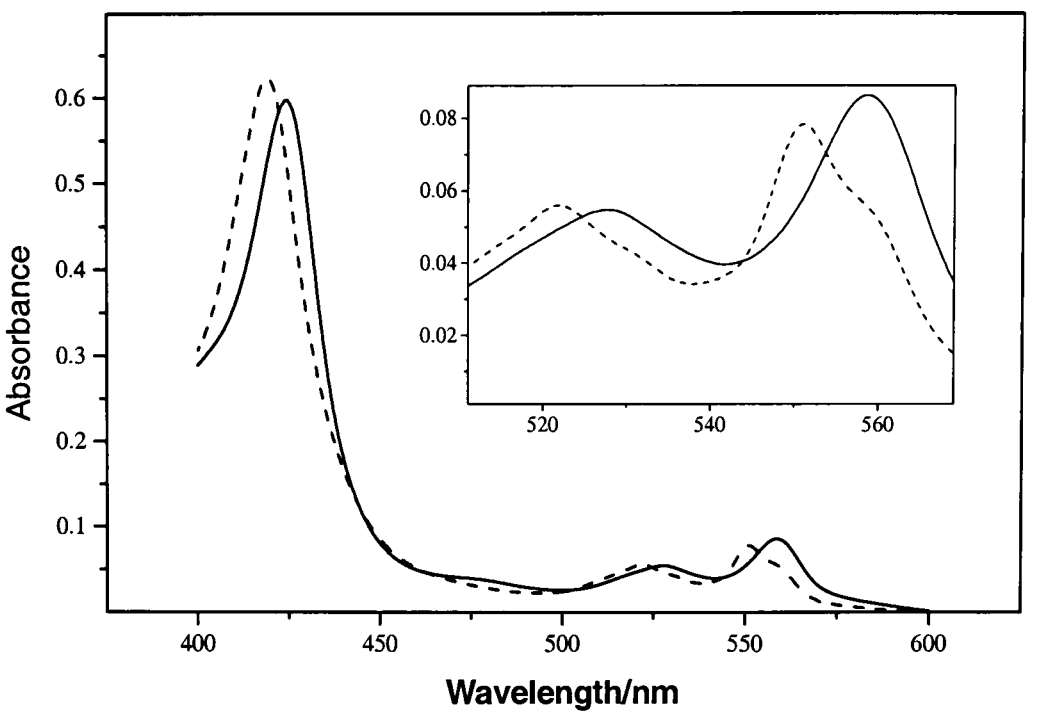


Figure 5.9a. Visible absorption spectra of horse heart apocytochrome *c* (5 μM) following mixing with an equimolar amount of heme under reductive conditions. The solid line shows a spectrum, corresponding to that of a *b*-type cytochrome, obtained after 10 minutes after mixing. The dashed line was obtained after 48 hours of incubation. Spectra were recorded using in 20 mM sodium phosphate buffer, pH 7.0.

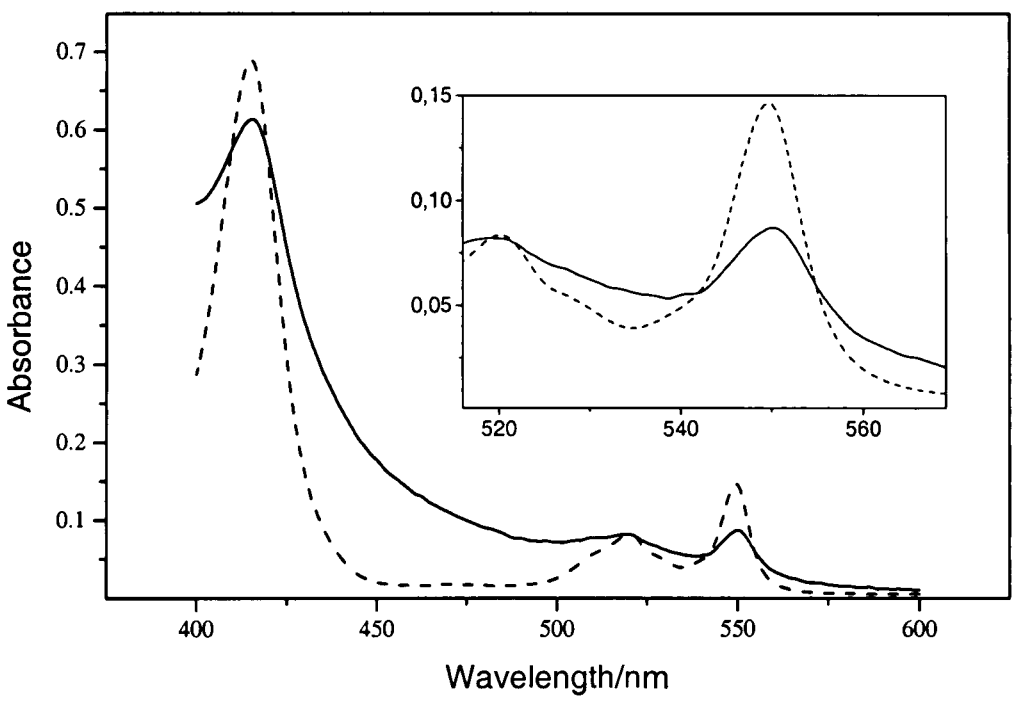


Figure 5.9b. Visible spectra of horse heart cytochrome *c* (5 μM) produced either *in vivo* (— — —) or *in vitro* after 72 hours incubation under reductive conditions and subsequent purification (———). Spectra were recorded in 20 mM sodium phosphate buffer, pH 7.0.

The observations made following mixing of horse heart apocytochrome *c* and heme with incubation for 72 h clearly indicate the formation of a *c*-type cytochrome, an interpretation which was substantiated further by heme staining of the proteins on SDS-PAGE gels (Figure 5.1, lane d), including treatment of the heme-containing protein with acidified-acetone (Goodhew 1986); in these experiments, non-covalently bound heme dissociates from protein but covalently bound heme does not. The data show *in vitro* formation of horse heart cytochrome *c*; the latter has not been previously reported to form from the *b*-type cytochrome intermediate (Dumont et al. 1994; Goldberg et al. 1999).

An analogue of the *b*-type cytochrome intermediate formed upon addition of reduced Fe-mesoporphyrin to reduced apocytochrome *c*. The product had visible absorption maxima at 549, 519 and 416 nm and did not heme stain on an SDS-PAGE gel (Figure 5.1, lane c).

The requirement for both the heme and the apoprotein to be reduced for *in vitro* thioether bond formation (CHAPTER 3 and 4) was substantiated. Oxidised apoprotein (containing a disulfide), when reacted with ferric heme, did not give rise to the characteristic visible spectrum of a cytochrome.

5.2.2.2. Yeast iso-1-cytochrome *c*

Mixing ferrous heme and reduced yeast iso-1-apocytochrome *c* in the presence of DTT led to the formation of a *b*-type cytochrome, as judged by visible absorption spectroscopy (Table 5.1). The spectral features shifted within 32 hours to give the spectrum of holocytochrome *c* (Table 5.1). In order to show that covalent attachment of heme to the protein had taken place, pyridine hemochrome spectra

(Table 5.1) and SDS-PAGE analysis (Figure 5.6, lane 4) were obtained. Both results are consistent with the interpretation that thioether bonds had formed between heme and protein. Addition of ferrous mesoheme to reduced apoprotein gave rise to a mesoheme-protein complex as judged by visible absorption spectroscopy (Table 5.1). This visible spectrum did not change over time and had a constant pyridine hemochrome α-band maximum at 546 nm. Together with the data from SDS-PAGE which suggested that no covalent bond had formed between mesoheme and protein (Figure 5.6, lane 3), these data substantiate the interpretation that thioether bonds had formed when heme was used. Recall that the heme-vinyl groups are required for covalent attachment of heme to protein in cytochromes *c*.

From these data, it can be concluded that yeast iso-1-cytochrome *c* has very similar properties to horse heart cytochrome *c* with respect to the interaction of the apoprotein with heme. These data are consistent with the observation that heme lyase from yeast can be used to mature horse heart cytochrome *c* in the cytoplasm of *Escherichia coli* (Patel et al. 2001). The sequence identity of both apocytochromes *c* is 59 % (using CLUSTAL W). The slightly faster rate of *in vitro* cytochrome *c* formation for yeast iso-1-cytochrome *c* compared with horse heart cytochrome *c* and the higher yield of yeast holocytochrome *c* (*ca.* 70 %) may suggest that the yeast apocytochrome *c* maintained a more compact structure after the heme extraction procedure, allowing it to bind heme covalently more effectively than horse heart apocytochrome *c*.

5.2.2.3. *Paracoccus denitrificans* cytochrome c_{550}

Similar to the observations with the mitochondrial cytochromes *c*, when reduced *Paracoccus denitrificans* apocytochrome c_{550} was reacted with ferrous heme, spectral features of a *b*-type cytochrome intermediate were observed after a few minutes (Figure 5.10 and Table 5.1). Over a time period of several hours, spectral bands appeared at 552, 523 and 417 nm. A spectrum of the heme-protein solution after 5 hours of incubation under reductive conditions is shown (Figure 5.10). SDS-PAGE analysis of the reaction product showed that heme was covalently attached to the protein (Figure 5.1, lane d). Incubation of ferrous mesoheme with reduced apocytochrome did not result in covalent attachment (Figure 5.1, lane c), suggesting that in the reaction with heme thioether bonds had formed between the protein and the heme vinyl groups. This interpretation was substantiated by the pyridine hemochrome spectrum of the reaction product; this had an α-band at 552.5 nm, which in combination with the visible absorption spectrum, shows that more than half of the vinyl groups had been saturated. These data are interpreted as reflecting a mixture of *b*- and *c*-type cytochrome present in the reaction mixture. The pyridine hemochrome spectrum of the reaction of mesoheme and apoprotein, which also yields a *b*-type cytochrome (Table 5.1), remained unchanged from the pyridine hemochrome of mesoheme itself. Again, formation of cytochrome *c* from the *b*-type cytochrome intermediate was not quantitative. A similar ratio as in the horse heart cytochrome *c* experiments described above remained as *b*-type cytochrome. However, the fraction of apocytochrome *c* that could form the *b*-type cytochrome in the case of *P. denitrificans* cytochrome c_{550} was very low (5 % of the starting material overall), suggesting that the heme extraction procedure had adversely affected the ability of the majority of the apoprotein to form a hydrophobic pocket. A similar effect has been seen for the ANS binding data for

this apoprotein. However, it is clear from the data that reaction of heme and *Paracoccus denitrificans* apocytochrome c_{550} can lead, to some extent, to formation of holoprotein with covalently attached heme *in vitro*.

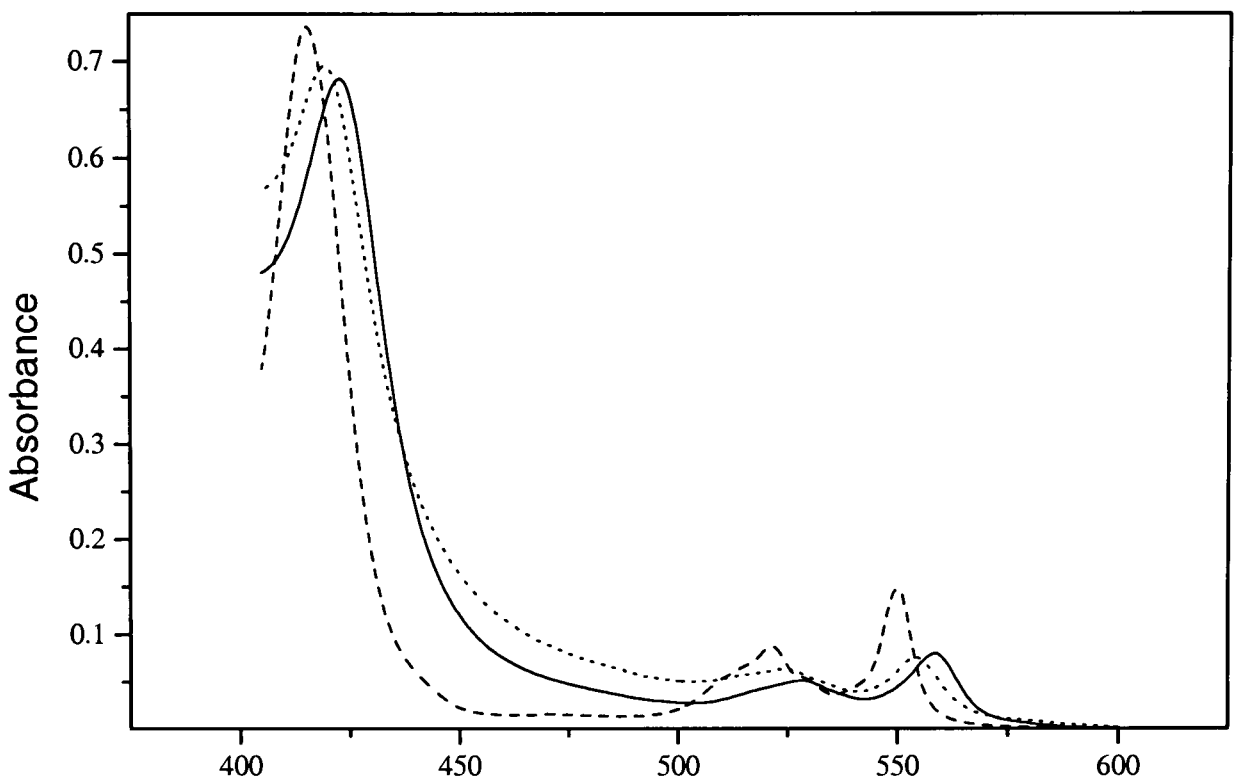


Figure 5.10. Comparison of the visible spectra of various forms of *P. denitrificans* cytochrome c_{550} (5 μM). The spectrum of the *b*-type cytochrome complex of apocytochrome *c* and heme obtained after 10 minutes of incubation under reductive conditions (———); the reaction product of heme and apocytochrome after 5 hours (- - - -); the *in vivo* produced protein (— — —). Spectra were recorded in 20 mM sodium phosphate buffer, pH 7.0.

5.3. DISCUSSION

In this chapter, data are presented on the production of apocytochromes *c* from a bacterial and two eukaryotic organisms, and the reaction of the cysteine thiols of these apocytochromes with the vinyl moieties of heme is characterised. All these proteins have very similar properties, with regard to their apo forms and their

reactions with heme. It is shown that the cysteines of the conserved CXXCH motif can form an intramolecular disulfide in horse heart and *P. denitrificans* apocytochromes *c*. This observation raises the question as to whether this oxidation occurs *in vivo* for the respective proteins in either organism. For the bacterial (Ccm) system (section 1.5.2) it has been suggested that such a disulfide bond is an important intermediate during cytochrome *c* maturation due to the presence of the disulfide bond forming (Dsb) proteins in the periplasm (Ferguson 2001). Furthermore, it has been proposed that proteins (CcmG/H and DsbD) required for cytochrome *c* biogenesis are involved in the reduction of this disulfide bond in the apocytochrome (Allen et al. 2002; Thöny-Meyer 2002). Why this *in situ* reduction of an apocytochrome has evolved is unclear, but an internal protection mechanism against metallation of the coordination site of the reduced apocytochrome might offer one explanation.

Heme attachment to mitochondrial apocytochrome *c* occurs in the intermembrane space (Diekert et al. 1999). The *in vivo* oxidation state of the cysteines in mitochondrial apocytochromes *c* has not been investigated. Whether, as it is shown here *in vitro*, a disulfide bond forms during the *in vivo* protein maturation process, will depend on both the reduction potential of its locus and kinetic factors. If formation of a disulfide must be avoided, a thioredoxin-like protein could be required in the intermembrane space (Laloi et al. 2001). A thioredoxin (Trx3) and its reductase (Trr2) are known to be present in yeast mitochondria, but submitochondrial localisation to the matrix has not been definitely established (Pedrajas et al. 1999). These proteins can in any case be deduced to be dispensable for cytochrome *c* biogenesis in yeast, as mutants carrying specific disruptions in the corresponding genes were able to grow normally under aerobic, respiratory

conditions, which require participation of cytochrome *c* in the mitochondrial respiratory chains. However, in the case of *Arabidopsis thaliana*, it has been recognised, on the basis of the genome sequence that there may be a yet to be characterised thioredoxin that is targeted to the intermembrane space (Laloi et al. 2001). Alternatively, the formation of holocytochrome *c* may happen sufficiently fast that the two thiols of apocytochrome *c* cannot form a disulfide during its lifetime in the intermembrane space following delivery from the reducing environment of the cytoplasm. Such a kinetic constraint would suffice if the rate of disulfide bond formation in the intermembrane space were to occur on a similar time scale to that *in vitro* (hours as observed in the present work). The presence of the CcmABCEF components of one cytochrome *c* biogenesis pathway, but not of the thioredoxin-like CcmG component, in plant mitochondria (Spielewoy et al. 2001) might support the latter view.

The binding of heme to the apoform of mitochondrial cytochrome *c* clearly involves some ordering of the polypeptide chain. This is evident from several biophysical studies (Dumont et al. 1994; Goldberg et al. 1999). A non-covalent complex between heme and the apoprotein of *H. thermophilus cytochrome* c_{552} generates a protein structure that is very similar to that of the native protein with covalently bound heme (Tomlinson & Ferguson 2000a). Whilst it appears that the structure of the non-covalent complex between heme and mitochondrial apocytochrome *c* does not fully resemble the native, holo structure (Dumont et al. 1994; Goldberg et al. 1999), studies with two monoclonal antibodies that scarcely recognise the apoprotein, but bind with comparable affinities to both the non-covalent heme-apoprotein complex and the holoprotein, indicate that the former must be similar to the latter in respect of quite specific structural features at the

epitope regions (Goldberg et al. 1999). The present work shows that such similarities are evidently sufficient to permit the vinyl groups of heme to be oriented appropriately, in at least a fraction of the molecules, for reaction with the thiol groups which have previously been shown to exhibit nucleophilic reactivity (Kang & Carey 1999). The effect of covalently bound hydrophobic moieties on the folding of cytochrome *c* has also been discussed (Kang & Carey 1999).

The affinity of the horse heart and *P. denitrificans* apoproteins toward hydrophobic ligands and the ability of the apoproteins to bind heme in a *b*-type cytochrome complex has potentially important implications for the catalytic strategy of the enzymes involved in cytochrome *c* maturation. It is unclear from previous studies how the heme lyase functions in mitochondria, except for the proposal that it binds heme via a conserved CPX motif (Steiner et al. 1996). For the bacterial Ccm system, two proteins have been proposed to function as a heme lyase (Ren et al. 2002), but the heme attachment is more complex due to the presence of a heme chaperone, CcmE (Schulz et al. 1998; CHAPTERS 6-8). However, in view of the data in this work (CHAPTERS 3-5) showing that the *b*-type cytochrome can spontaneously react to form thioether bonds yielding holocytochrome *c*, two key conditions have to be achieved by the catalytic systems. Firstly, the heme group has to be kept reduced to give optimal reactivity for cysteine-thiol and heme-vinyl group reaction partners, including avoidance of side products. This is in agreement with previous studies (Nicholson & Neupert 1989; Tong & Margoliash 1998). Secondly, the orientation of the heme relative to its rotation around the α,γ mesoaxis has to be correct to yield the required stereochemistry at the prochiral α-carbon of the vinyl substituents of the heme moiety (CHAPTER 4; Barker & Ferguson 1999). McRee *et al.* showed that a recombinant cytochrome *c* from

Thermus thermophilus could be improperly matured *in vivo* without any catalytic assistance in the cytoplasm of *E. coli*; problems arose due to both heme inversion and intermolecular disulfide bond formation between the cysteine residues that are usually involved in heme binding (McRee et al. 2001). The latter behaviour was clearly reflected in the visible absorption spectrum, which was different from the reported, properly matured holocytochrome *c*, and the pyridine hemochrome spectrum, which was not indicative of two thioether bonds to the heme-vinyl groups (McRee et al. 2001). In the present work, for the mitochondrial cytochromes *c*, two thioether bonds have formed between heme and protein. In the case of *in vitro* reaction of heme with *P. denitrificans* apocytochrome c_{550} it is not clear whether the reaction yielded a mixture of *b*- and *c*-type cytochromes or whether it also contained species with only one thioether bond. Owing to the presence of DTT during the course of the reaction, no reaction product containing an intermolecular disulfide bond was obtained *in vitro*.

In the *in vitro* reactions between heme and apocytochromes *c*, the oxidation state of the heme can be controlled, but the heme delivery is non-specific with respect to the stereochemical aspects of the heme orientation. The stereochemistry of the thioether bond in the *in vitro* produced cytochromes *c* has not been determined. However, the fact that a fraction of the mitochondrial *b*-type cytochrome intermediates remained unreacted, might hint that incorrect orientation of heme does not lead to thioether bond formation in those cases, suggesting that the *in vitro* cytochrome *c* formation is stereoselective. In the case of the *in vitro* formation of *Hydrogenobacter thermophilus* c_{552} there is very strong evidence that the reaction is stereoselective (CHAPTER 4).

An interesting point arises from the observation that neither *P. denitrificans* cytochrome c_{550} (Sambongi & Ferguson 1994b) nor mitochondrial cytochrome *c*, in the absence of a heme lyase (Pollock et al. 1998), can form in the cytoplasm of *Escherichia coli*, in contrast to *H. thermophilus* c_{552} (Sinha & Ferguson 1998). These observations might reflect the stability and the folding state of the apoproteins, but not the intrinsic difference in their reactivity towards heme, which is shown from previous (CHAPTER 3) and present work (this chapter) to be similar for the these apocytochromes *c*. The higher stability and partial folding (Wain et al. 2001) of *H. thermophilus* c_{552} apocytochrome may lead to a slower rate of degradation in the cytoplasm of *E. coli* and a higher heme affinity compared with the proteins studied in this work. Therefore, in addition to the aspects mentioned above, another function of heme lyase may be to increase the heme affinity of the apocytochrome *c*.

In summary, it is shown that three *c*-type cytochromes, from a mesophilic bacterium and two eukaryotic organisms, can form *in vitro* from the reaction of apocytochrome and heme. The reaction generally seems to proceed from a *b*-type cytochrome intermediate, provided the potential disulfide between the conserved cysteine residues is avoided and the heme-iron is reduced.

CHAPTER 6

STUDIES ON HEME BINDING TO VARIANTS OF *ESCHERICHIA COLI* CCME

SUMMARY

The uptake of heme by a soluble form of the heme chaperone CcmE containing a C-terminal or N-terminal His-tag, and the covalent attachment of heme to CcmE, were achieved *in vitro*. *E. coli* apo-CcmE^{sol}-C-His$_6$ preferentially bound to ferric, with high affinity (K_d: 200 nM), rather than ferrous heme. The preference for ferric heme was confirmed by competition with 8-anilino-1-naphthalenesulfonate, which bound to a hydrophobic pocket in apo-CcmE. Reduction under certain conditions of the ferric heme-CcmE complex, which has characteristics of a *b*-type cytochrome, resulted in covalent attachment of heme to protein. The resulting *in vitro*-produced holo-CcmE was found to be identical to the *in vivo*-produced holo-CcmE, demonstrating that unmodified Fe-protoporphyrin IX is incorporated into CcmE. Only non-covalent binding of mesoheme to CcmE was observed, thus implicating at least one vinyl group in covalent binding of heme to CcmE. Control experiments showed that CcmE^{sol} lacking a His tag binds 8-anilino-1-naphthalenesulphonate and heme, the latter both non-covalently and via a covalent bond from the histidine side chain, similarly to the tagged proteins, thus countering an argument that the His tag may be entirely responsible for heme binding. However, the His tag did appear to enhance the rate of *in vitro* covalent heme binding and to affect the heme ligation in the ferric *b*-type cytochrome form.

6.1. INTRODUCTION

Having studied the interaction of heme with apocytochromes *c* (CHAPTER 3-5), it was considered of interest to investigate the effect of a heme chaperone on this process, thus possibly providing insight into the *in vivo* *c*-type cytochrome biogenesis system used by many Gram-negative bacteria and also in plant mitochondria.

CcmE has been identified as the heme chaperone in this cytochrome *c* maturation pathway; it binds heme covalently via a conserved histidine residue (H130 in *E. coli*) before transferring the heme to apocytochromes (Schulz et al. 1998; also see CHAPTER 1). Recently, structures of the CcmE apoproteins from two different bacterial species have been reported (Arnesano et al. 2002; Enggist et al. 2002), which show that the heme-binding histidine is exposed on the protein surface, but provide no clue as to the unusual properties of this residue.

To investigate the interactions between heme and apo-CcmE and the nature of the covalent bond between protein and heme, studies were undertaken with a soluble domain of CcmE and heme *in vitro*. The protein initially used in these *in vitro* studies had a His tag at the C terminus. Control experiments involved studies on a CcmE variant containing a cleavable N-terminal His tag; the latter approach addressed the possibility that a His tag causes non-physiological heme binding.

6.2. RESULTS

6.2.1. Characterisation of CcmE^{sol}-C-His$_6$

In vivo-produced apo-CcmE^{sol}-C-His$_6$, overproduced in *E. coli*, was shown to be highly pure by SDS-PAGE (Figure 6.1, lane 2) and complete cleavage of the periplasmic targeting sequence was confirmed by determining the mass of the purified protein by electrospray mass spectrometry (Molecular mass: calculated

15,516 Da, observed 15,517 Da). Similarly, *in vivo*-produced holo-CcmE^{sol}-C-His$_6$ was of high purity (Figure 6.1, lane 1); however, HPLC analysis showed that no more than one fifth of the protein contained covalently bound heme. Hydrophobic interaction FPLC was used to separate the apo- from the holo-form, enabling the production of pure holo-CcmE^{sol}-C-His$_6$. The molecular mass of holo-CcmE^{sol}-C-His$_6$ was shown by mass spectrometry to be 16,132 Da (calculated mass for apoprotein (15,516 Da) plus heme (616 Da) = 16,132 Da) ruling out the possibility that a modified form of heme was incorporated into the heme chaperone. The visible absorption spectrum of the reduced holo-CcmE^{sol}-C-His$_6$ and its pyridine hemochrome spectrum (Table 6.1) were essentially identical to those reported previously (Schulz et al. 1998). The data from an analytical ultracentrifugation experiment indicated that apo-CcmE^{sol}-C-His$_6$ was present as a monomer in solution (data not shown).

6.2.2. Non-covalent binding of heme to CcmE

The addition of oxidized heme to apo-CcmE^{sol}-C-His$_6$ resulted in a change in the visible spectrum, compared with that of ferric heme, within the mixing time. The species formed was stable at room temperature for several hours. The remarkable stability of this non-covalent complex was apparent from HPLC analysis. Under the HPLC conditions described (CHAPTER 2), apo-CcmE^{sol}-C-His$_6$ eluted at 50 % acetonitrile/water, whereas the heme-CcmE^{sol}-C-His$_6$ complex eluted at 55 % acetonitrile/water, without separation of the heme from the protein. The spectrum of the non-covalent heme-CcmE complex in aqueous buffer is shown (Figure 6.2; Table 6.1). Addition of cyanide to this species led to a shift of the Soret band from 413 to 421 nm, and an appearance of spectral characteristics of a low spin 6-coordinate heme iron centre (Figure 6.2; Table 6.1), which is interpreted as binding

of cyanide to the ferric iron (Abraham et al. 2001) and a change from a high to a low spin state of the iron. Upon reduction of the oxidised heme-CcmE^{sol}-C-His$_6$ complex with disodium dithionite in the absence of cyanide, the visible spectrum instantaneously showed characteristics of a *b*-type cytochrome with 6-coordinate low spin heme iron (Figure 6.2; Table 6.1). The pyridine hemochrome spectrum at this stage had an α-band at 556 nm, which is characteristic of heme with two free vinyl groups and shows that the heme was not modified (*i.e.*, bound covalently) immediately following its addition to apo-CcmE^{sol}-C-His$_6$.

After reduction of the quantitatively loaded heme-CcmE^{sol}-C-His$_6$ complex with disodium dithionite, the initial absorption peaks of the reduced *b*-type cytochrome subsequently decreased in intensity and peaks appeared which are characteristic of free ferrous heme (data not shown). These data reflect the dissociation of the ferrous heme-CcmE^{sol}-C-His$_6$ complex, which was confirmed by separation of protein and heme by size-exclusion chromatography. The half-life of this reduced *b*-type cytochrome species was approximately 35 seconds. Reoxidation of the ferrous heme and CcmE^{sol}-C-His$_6$ mixture, by the addition of potassium ferricyanide, resulted in the quantitative reformation of the ferric *b*-type complex, showing the reversibility of the process. This observation implies that apo-CcmE, unusually, favours the coordination of ferric heme.

The affinity of apo-CcmE^{sol}-C-His$_6$ for heme was measured by Dr. Julie Stevens by protein fluorescence quenching; the binding isotherms appeared to show two transitions, suggesting that there are two binding sites with different affinities for heme (data not shown). Dissociation constants of 200 nM and 10 μM were determined from double-reciprocal plots, corresponding to a high-affinity and a

low-affinity binding site respectively. Binding of the hydrophobic ligand ANS was examined and it also appeared to bind to two sites on the protein as observed for heme, though with substantially lower affinities (K_d: 2 μM and 100 μM). To confirm that the ANS, which is known to bind to the heme pocket in apomyoglobin (Stryer 1965), bound to the same site(s) as heme, the bound ANS was shown to be displaced upon heme addition. The observation that ANS binds at two sites shows that the presence of a low-affinity heme-binding site is unlikely to be due to non-specific heme stacking in a single binding site, although the low affinity suggests that it may not be physiologically relevant.

The ability of heme to displace ANS from its binding site(s) was used by Dr. Julie Stevens to confirm the preference of the protein for ferric heme. The fluorescence emission spectrum of ANS (excitation at 380 nm) in the presence of apo-$CcmE^{sol}$-C-His_6 showed a maximum at 480 nm, which is characteristic of the dye bound to a non-polar binding site (Stryer 1965). The addition of ferric heme resulted in displacement of the bound ANS from the protein, as shown in Figure 6.3, at a maximum molar equivalence of around 0.5 heme per protein. The displacement curve is consistent with the presence of two binding sites, with the ANS on the high-affinity site being displaced at lower heme concentrations. It was found that ferrous heme was also able to displace bound ANS but to a significantly lower extent than ferric heme (Figure 6.3), again showing the preference of $CcmE^{sol}$-C-His_6 for ferric heme.

When ferric mesoheme (Figure 1.1) was added to apo-$CcmE^{sol}$-C-His_6, uptake was observed by measuring the visible absorption spectrum, which gave rise to bands at 522 nm and 403 nm (Table 6.1). Upon addition of disodium dithionite to the

quantitatively formed mesoheme-protein complex, the absorption spectrum of the reduced mesoheme-CcmE^{sol}-C-His$_6$ complex was observed (Table 6.1). Subsequently, just as when heme itself was studied, mesoheme dissociated from the protein, with a half-life of the mesoheme-protein complex of approximately 30 seconds, as judged by the same criteria as for heme (see above). The subsequent addition of potassium ferricyanide led to the reformation of the oxidized mesoheme-CcmE^{sol}-C-His$_6$ complex, showing the reversibility of this process, which is dependent on the oxidation state of the porphyrin iron.

Table 6.1. Absorption maxima obtained from visible spectra of various forms of CcmE. Reduced spectra were recorded following addition of disodium dithionite.

	Wavelength/nm					
	Oxidized		**Reduced**			**Pyridine hemochrome (α band)**
b-type heme-CcmE^{sol}-C-His$_6$ complex	533	413	560	530	425	556
b-type CN-heme-CcmE^{sol}-C-His$_6$ complex	543	421	562	533	429	-
mesoheme-CcmE^{sol}-C-His$_6$ complex	522	403	549	521	412	546
in vivo holo-CcmE^{sol}-C-His$_6$	528	408	555	526	421	550.7
in vivo holo-CcmE (Schulz et al. 1998)			554.5	524.5	426.5	550.5
in vitro holo-CcmE^{sol}-C-His$_6$[†]	529	408	554	525	419	551
b-type heme-CcmE^{sol}			560	530	426	556

[†] These data were collected for samples produced by three different methods as described in the text. Oxidised absorption maxima were obtained by treatment of the ferric heme-CcmE complex with either cysteine or 2-mercaptoethanesulfonic acid. Reduced absorption maxima were acquired by addition of ferrous heme to apo-CcmE or treatment of the ferric heme-CcmE complex with either DTT or propane-1,3-dithiol.

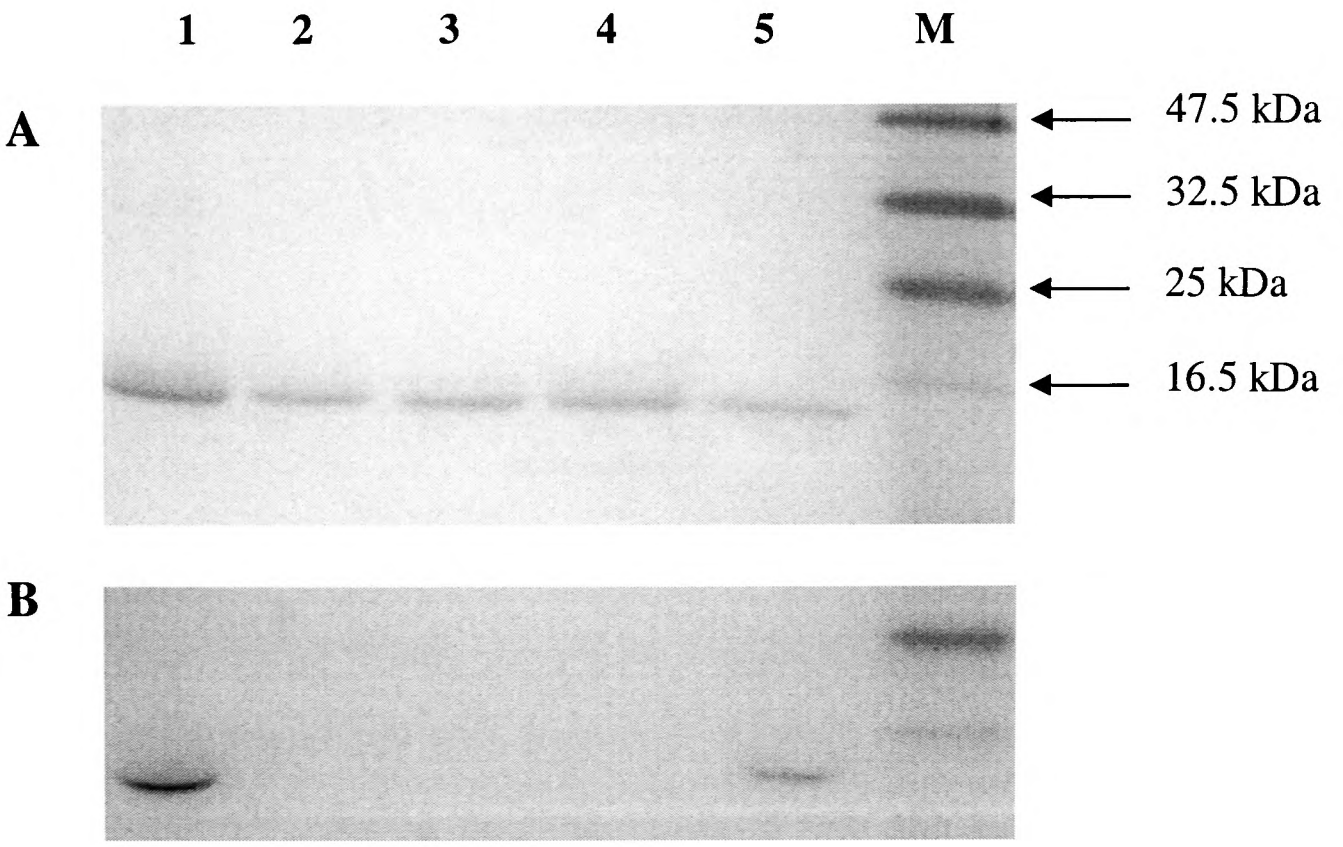


Figure 6.1. SDS-PAGE analysis of CcmE^{sol}-C-His$_6$ with covalently or non-covalently bound heme, or mesoheme. **A.** Coomassie Blue stained. **B.** Activity stained for covalently bound heme. Lane (1) the *in vivo*-produced holo form of CcmE^{sol}-C-His$_6$ from *E. coli* produced in the presence of plasmid pEC86. (2) Apo-CcmE^{sol}-C-His$_6$ from *E. coli* produced in the absence of plasmid pEC86. (3) The oxidised *b*-type cytochrome obtained upon addition of ferric heme to apo-CcmE^{sol}-C-His$_6$. (4) The *b*-type cytochrome analogue formed from apoprotein and Fe-mesoporphyrin, shown after incubation with DTT (5 mM) for 14 hours. (5) *In vitro* produced holo-CcmE^{sol}-C-His$_6$ obtained from the reaction of the *b*-type cytochrome form with DTT (5 mM) after 14 hours. (M) Pre-stained molecular mass marker. 200-300 pmoles of protein were applied to each lane of the gel for Coomassie staining, and 20-30 pmoles were applied for heme staining.

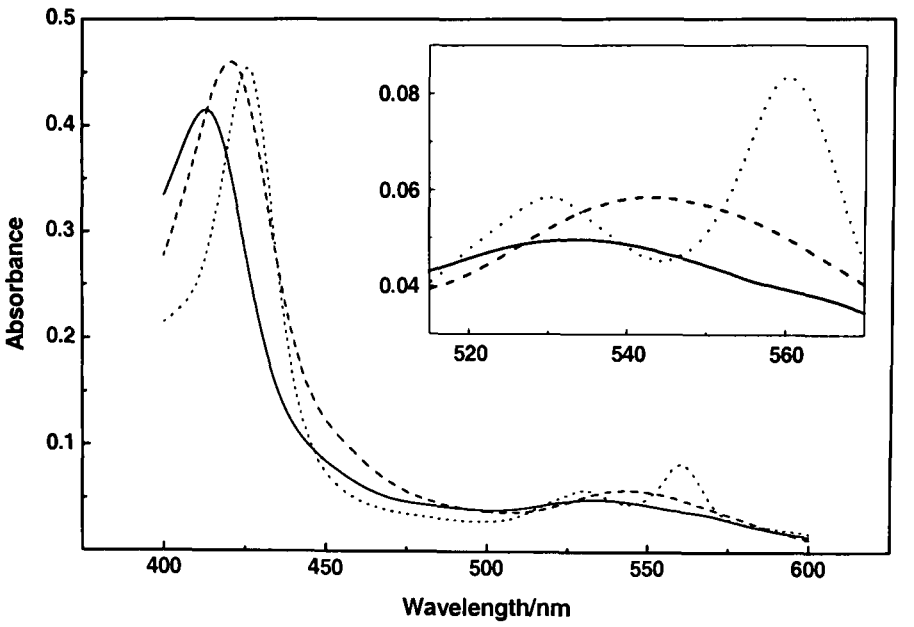


Figure 6.2. Absorption spectra of the *b*-type cytochrome formed following addition of heme to *E. coli* apo-CcmE^{sol}-C-His$_6$, showing the oxidised spectrum (———), the reduced spectrum obtained immediately after addition of disodium dithionite (- - - -) and the spectrum obtained after treating the ferric *b*-type complex with a two-fold excess of potassium cyanide (— — —). Absorption spectra were recorded using 5 μM *b*-type cytochrome in 50 mM sodium phosphate buffer, pH 7.0.

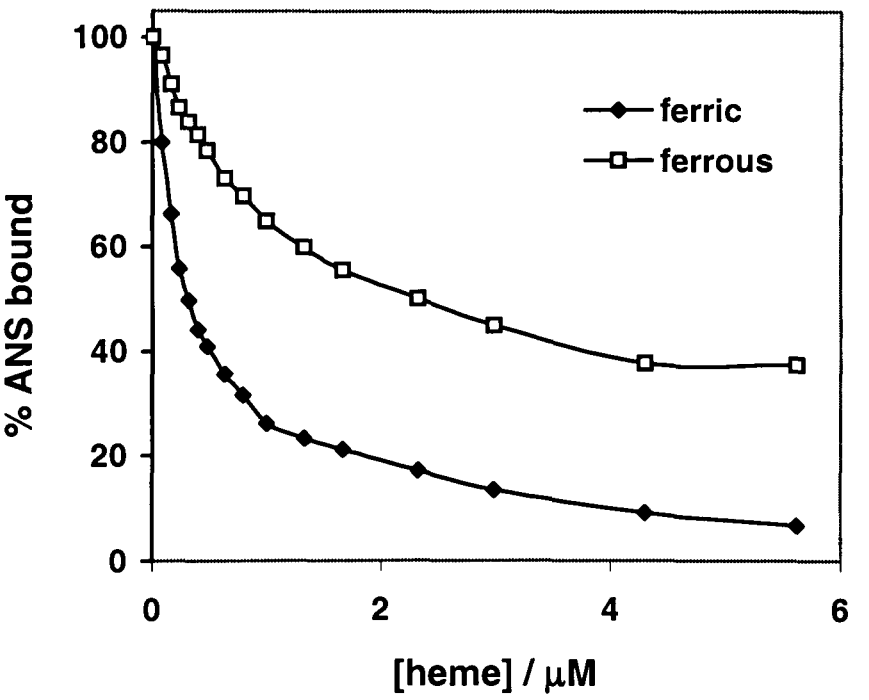


Figure 6.3. Displacement of ANS from its complex with apo-CcmE^{sol}-C-His$_6$ by ferric and ferrous heme. The percentage displacement was calculated from the decrease in ANS fluorescence upon addition of heme to a solution of protein and ANS (excitation wavelength 380 nm; emission wavelength 480 nm). Data courtesy of Dr. Julie Stevens.

6.2.3. Covalent attachment of heme to $CcmE^{sol}$-C-His_6

In the presence of a five-fold excess of apo-$CcmE^{sol}$-C-His_6 protein relative to ferrous heme, a condition that increased the fraction of heme in this oxidation state bound to the protein, the α-band shifted from 560 nm to 554 nm in the visible spectrum of the *b*-type cytochrome over a time period of several hours. A shift in the β- and Soret bands was also observed, resulting, finally, in spectral characteristics similar to those of *in vivo*-formed reduced holo-$CcmE^{sol}$-C-His_6 (Table 6.1).

Upon addition of dithiols (DTT or propane-1,3-dithiol) to the quantitative (1:1) complex of ferric heme and $CcmE^{sol}$-C-His_6, reduction of the *b*-type $CcmE^{sol}$-C-His_6 occurred without apparent dissociation of the heme-protein complex. Furthermore, the characteristic cytochrome bands shifted over time, just as observed following addition of ferrous heme to an excess of $CcmE^{sol}$-C-His_6. The change in the spectral features was complete after ca. 14 hours; again, similarity to the spectrum of reduced *in vivo*-formed holo-$CcmE^{sol}$-C-His_6 was evident (Table 6.1).

The addition of monothiols (2-mercaptoethanesulfonic acid or cysteine) to the quantitative ferric heme-$CcmE^{sol}$-C-His_6 complex resulted, over time, in a shift in the visible spectrum toward that of the (oxidized) *in vivo*-formed holo-$CcmE^{sol}$-C-His_6 (Table 6.1). Neither compound was able to yield a sufficiently reducing environment to give rise to visible spectra of a reduced species.

The reaction mixtures containing any of the monothiol or dithiol compounds, as described above, were passed through a desalting column after 14 hours of

incubation, and any weakly associated heme was removed by dialysis against imidazole. This confirmed that the spectral features, including the pyridine hemochrome spectrum, in either oxidation state, of the proteins prepared *in vitro*, were identical to those of the *in vivo*-produced $CcmE^{sol}$-C-His_6 (Table 6.1). In addition, SDS-PAGE analysis followed by staining for covalently attached heme, showed the heme to be covalently attached to the $CcmE^{sol}$-C-His_6 protein (Figure 6.1, lane 5), whereas the *b*-type $CcmE^{sol}$-C-His_6 and the mesoheme-$CcmE^{sol}$-C-His_6 complex were shown not to bind heme covalently (Figure 6.1, lanes 3 and 4 respectively). HPLC analysis showed that up to 50% of the initial *b*-type heme-$CcmE^{sol}$-C-His_6 complex could be converted into the covalent holo-$CcmE^{sol}$-C-His_6 form.

$CcmE^{sol}$-C-His_6 does not form a covalent bond with mesoheme with either reduced mesoheme or in the presence of the low molecular weight thiol-containing compounds used. These data show that at least one vinyl group of Fe-protoporphyrin IX is involved in forming the covalent bond between heme and $CcmE^{sol}$-C-His_6.

6.2.4. Characterization of $CcmE^{sol}$ lacking a His tag

Recently, it was suggested that His tags can cause artefactual non-covalent binding of heme to proteins and that non-physiological heme binding may be observed (Arnesano et al. 2002). In order to address this point, a construct of CcmE was made by Dr. Julie Stevens which can be studied with and without the His tag because there is a protease cleavage site between the His tag and the soluble CcmE domain. The protein with the cleavable His tag (N-His_6-CcmE) was analysed by ES-MS and had a mass of 16 641 Da (calculated mass: 16 641 Da); the protein ran as a single band during SDS-PAGE analysis (Figure 6.4, lane 1). After thrombin

treatment and removal of uncleaved protein, the cleaved protein ($CcmE^{sol}$) had a mass of 14 890 Da (theoretical mass: 14 890). The difference in the mass of the cleaved and uncleaved protein could also be seen by SDS-PAGE analysis (Figure 6.4, lane 4). The absence of the His tag was also confirmed by Western blotting with an antibody against hexahistidine tags by Dr. Julie Stevens (data not show).

After addition of ferric heme to apoprotein lacking a His tag, followed by reduction with disodium dithionite, no differences in the immediately obtained visible spectra, characteristic of a low spin state, were observed compared with the His tag-containing protein (Table 6.1). There was a subsequent disappearance of these spectral characteristics upon reduction, as was observed for the protein with the C-terminal His tag. Interestingly, in the visible spectrum of the complex of ferric heme and $CcmE^{sol}$, a species was obtained that is similar to that observed upon addition of ferric heme to horse heart apocytochrome *c* (Dumont et al. 1994), with features of a high-spin heme-protein complex. Thus, the spectrum of the oxidized heme-protein complex obtained without a His tag (a broad Soret band around 400 nm) was different from those for the proteins with the His tag at either the C- or N-terminus which both have a Soret band at 413 nm. These data suggest that the His tag can affect the coordination of the ferric heme-$CcmE^{sol}$ complex leading to a high-spin/low-spin equilibrium. However, in light of the known effects of polyhistidine on the coordination characteristics of heme, this is arguably not surprising (Tohjo & Shibata 1963), but contrasts with the spectrum of the reduced heme-CcmE complex not being altered by the absence of the His tag (Table 6.1).

Further analysis of the protein without the His tag ($CcmE^{sol}$) by Dr. Julie Stevens showed that it had a similar ANS affinity to the protein with the tag, and the same

maximum emission wavelength at 480 nm (data not shown). Also, it was possible to displace bound ANS from $CcmE^{sol}$ by the addition of heme, as was shown for the tagged protein. Reaction of ferrous heme with N-His_6-$CcmE^{sol}$ led to a protein with covalently bound heme that had characteristics indistinguishable from those of $CcmE^{sol}$-C-His_6 (Figure 6.4, lane 2 and data not shown). After thrombin cleavage of the His tag, heme was shown to remain covalently bound to $CcmE^{sol}$ as shown in Figure 6.4, lane 3. Reaction of $CcmE^{sol}$ with heme in the absence of the His tag led to the same qualitative result (Figure 6.4, lane 5), but the half-life of formation of the covalent complex was increased by at least ten-fold relative to versions of the protein with His tags at either end.

Following thrombin cleavage of the protein expressed from pET15-b (N-His_6-$CcmE^{sol}$), two potential extra heme-ligating residues remain on the N-terminus of the protein, namely histidine and methionine. In order to avoid any potential artefactual heme ligation by the untagged protein obtained after thrombin treatment of the N-terminal his-tagged protein, these residues were mutated to alanines. The proteins with and without the His tag were pure as judged by SDS-PAGE analysis (data not shown), and had the expected masses as indicated by ES-MS analysis. Upon addition of ferric heme to the apoproteins without the His tag, the spectra changed compared with the spectrum of free ferric heme (Figure 6.5). The heme-protein complexes were high-spin in the ferric state and switched to low-spin in the reduced state upon addition of disodium dithionite (Figure 6.5). Thus the presence of an extra methionine and histidine, derived from the linker region, had no observable effect on interactions with heme.

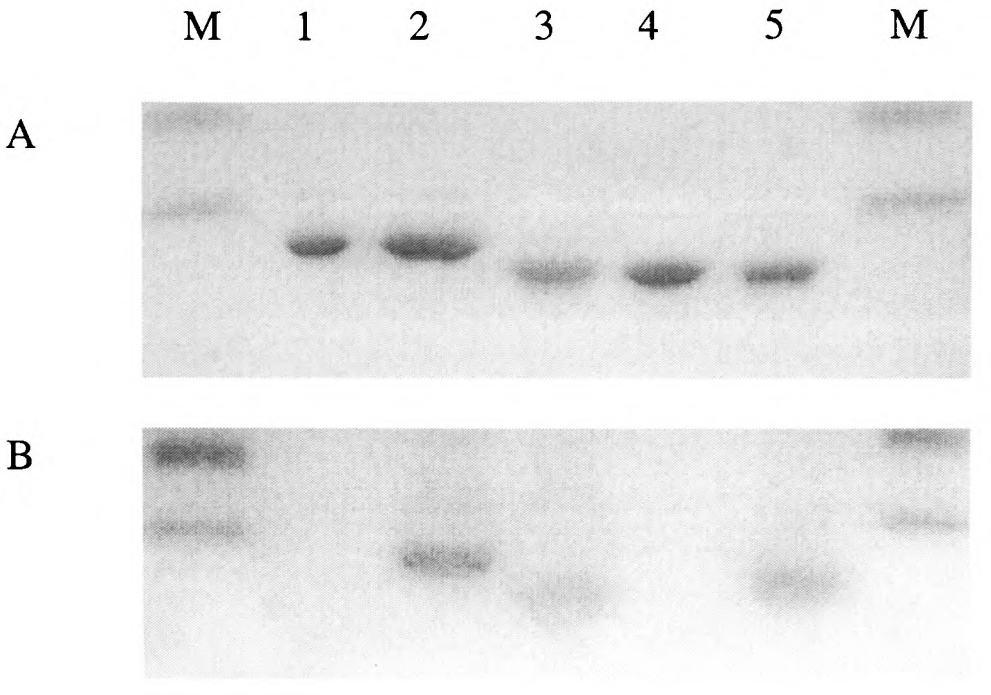


Figure 6.4. SDS-PAGE (17.5%) analysis of various forms of N-His_6-$CcmE^{sol}$ and $CcmE^{sol}$. **(A)** Coomassie Blue stained and **(B)** activity stained for covalently bound heme. (1) Apoform of N-His_6-$CcmE^{sol}$. (2) Heme-N-His_6-$CcmE^{sol}$ obtained after reaction of N-His_6-$CcmE^{sol}$ with reduced heme for 14 h. (3) Protein obtained after incubation of the protein sample shown in lane 2 with thrombin. (4) Apoform of $CcmE^{sol}$ obtained following treatment of N-His_6-$CcmE^{sol}$ with thrombin. (5) $CcmE^{sol}$ reacted with reduced heme for 40 h. M is prestained molecular mass marker, corresponding to 25 and 16.5 kDa from top to bottom.

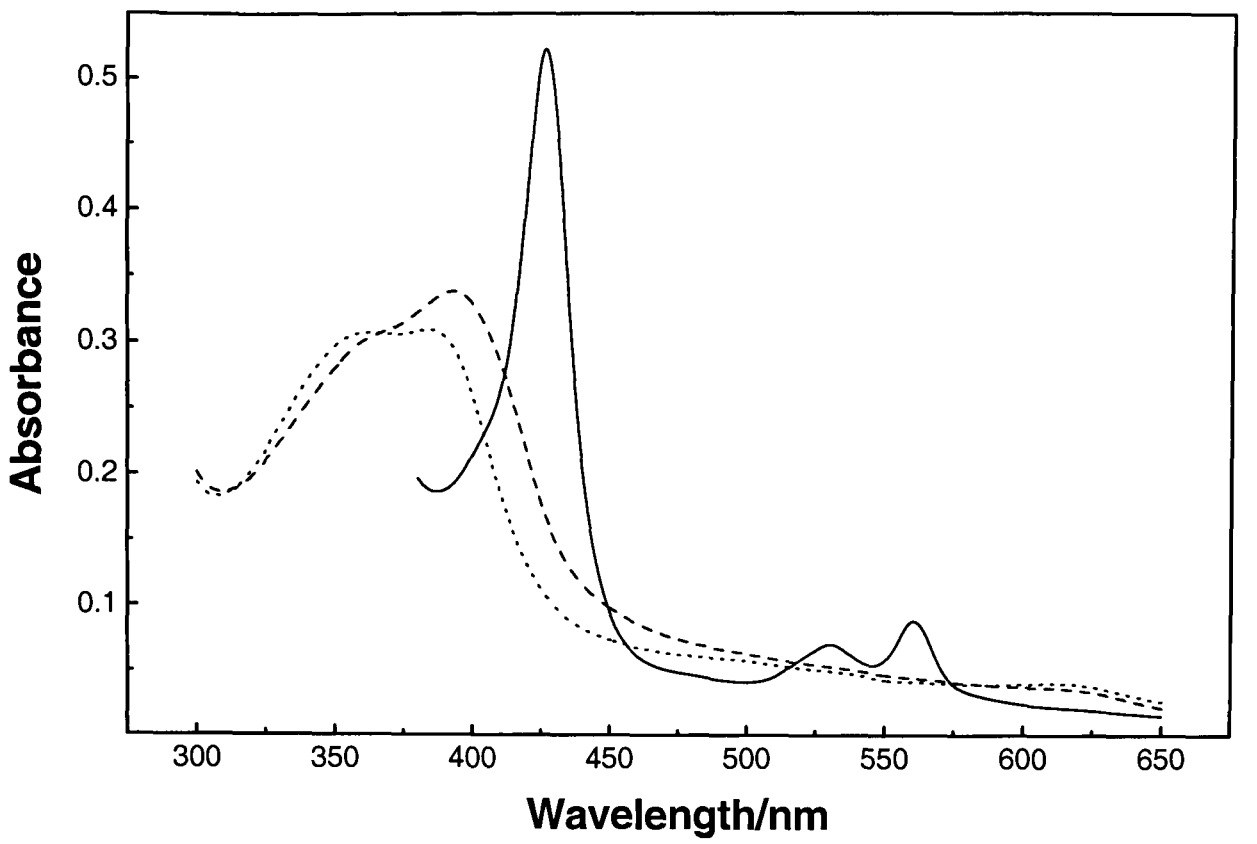


Figure 6.5. Absorption spectra of the *b*-type cytochrome formed after the addition of heme to apo-CcmE^{sol}, showing the oxidized spectrum (— — —), the reduced spectrum obtained immediately after the addition of disodium dithionite (———), and the spectrum of free heme (- - - -). Absorption spectra were recorded using 5 μM protein and 5 μM heme in 50 mM Tris-HCl buffer, pH 7.4, 300 mM NaCl.

6.3. DISCUSSION

It is shown in the present chapter that apo-CcmE^{sol}-C-His$_6$ can take up heme to form a complex resembling a *b*-type cytochrome. Heme and ANS binding data show that apo-CcmE^{sol}-C-His$_6$ has a structured ligand-binding pocket. ANS has been shown to bind to heme-binding pockets in other proteins (Stryer 1965). The observation that apo-CcmE^{sol}-C-His$_6$ is more efficient at taking up ferric rather than ferrous heme, contrasts with the usual behaviour of heme proteins, and suggests that heme could be presented to CcmE by CcmC in the ferric state *in vivo*.

Covalent attachment of heme to $CcmE^{sol}$-C-His_6 was shown to occur between ferrous heme and $CcmE^{sol}$-C-His_6 in the presence of a five-fold excess of protein. Interestingly, these data correlate with the observation that only 10-25 % of the CcmE protein is isolated in the holo form, i.e. with covalently attached heme, *in vivo* (Thöny-Meyer 1999). Alternatively, the addition of low molecular weight thiol-containing compounds to the ferric heme-$CcmE^{sol}$-C-His_6 complex was found to facilitate attachment of the heme to this heme chaperone. Since the final product of this *in vitro* reaction is identical to the *in vivo*-produced holo-$CcmE^{sol}$-C-His_6, it is clear that in the presence of an appropriate reductant, covalent bond formation between heme and CcmE can occur. This result suggests that either the periplasm or the Ccm proteins have to supply an appropriate reductant, plausibly in the form of a thiol-containing species, to the heme-CcmE complex, so that attachment of the heme to the heme chaperone can occur. Furthermore, it is apparent that *in vivo* formation of holo-CcmE occurs by the reaction of protein with Fe-protoporphyrin IX, which has not been modified prior to attachment to the protein. Covalent attachment of mesoheme to $CcmE^{sol}$-C-His_6 did not take place when the non-covalent complex between $CcmE^{sol}$-C-His_6 and mesoheme was treated with the appropriate compounds. The implication of these data is that the covalent attachment of the heme-binding protein residue, which was shown to be a histidine by Thöny-Meyer and co-workers (Schulz et al. 1998), requires a vinyl group of the Fe-protoporphyrin IX. In this context the absorbance maximum at 551 nm in the pyridine hemochrome spectrum of holo-$CcmE^{sol}$-C-His_6 can be interpreted as arising from the presence of one intact vinyl group and one vinyl group that has reacted in a novel way. The alternative of both vinyl groups having reacted with one histidine residue does not seem very plausible. Thus the maximum at 551 nm

is only coincidentally similar to that for the pyridine hemochrome spectrum of a *c*-type cytochrome in which both vinyl groups have been saturated.

The implications from these *in vitro* experiments are that ferric heme can be taken up by CcmE in the periplasm *in vivo*, following which reduction of the heme yields the covalently bound holo-heme-CcmE complex. A central aspect of future investigations will be the determination of the nature of the covalent bond between the CcmE protein and the heme vinyl group.

The control experiments on the CcmE construct with a cleavable His tag argue against the possibility that the His tag might itself cause the non-covalent heme binding observed for CcmE. Recently, Arnesano *et al.* (Arnesano et al. 2002) have argued that untagged CcmE from *Shewanella putrefaciens* does not bind heme and that a His tag can be responsible for introducing a heme binding site into this and other proteins. In the present work it is shown that the untagged $CcmE^{sol}$ protein from *E. coli* retains a heme-binding site in which a covalent bond forms between protein and a heme vinyl group, *in vitro*, under the same reductive conditions as for the tagged form. In this respect the results given in this CHAPTER are at variance with those of Arnesano *et al.* (2002). However, the presence of the His tag affects the visible absorption spectrum of the initial ferric non-covalent $CcmE^{sol}$-heme complexes, consistent with the heme iron having a histidine ligand provided by one of the residues of the tag. In this sense the His tag appears to facilitate the *in vitro* covalent incorporation of heme into $CcmE^{sol}$. A His tag at either the N-terminus or at the C-terminus appears to act similarly in this respect. However, it has been shown that the heme is bound covalently to $CcmE^{sol}$ after

addition of ferric heme to $CcmE^{sol}$-C-His_6 followed by incubation under reductive conditions and thrombin cleavage. Furthermore, $CcmE^{sol}$, which has had the N-terminal His tag removed by thrombin treatment, can also bind heme both covalently and non-covalently. Thus it is concluded that the presence of a His tag at either end of the protein does not alter the nature of the covalent bond formation. Any kinetic facilitation of the interaction of heme with $CcmE^{sol}$ by the His tag may be a coincidental partial mimicking of the histidine rich region on the CcmC protein which is generally agreed to participate in the binding of heme to CcmE *in vivo* (Schulz et al. 1999).

It is difficult to explain the differences between the present results and those for the protein from *Shewanella putrefaciens*. The latter imply that CcmE alone cannot bind heme, a view which is argued to be supported by inspection of the structure of the apoprotein which does not have a classic hydrophobic pocket for binding heme (Arnesano et al. 2002). On the other hand, Enggist *et al.* (2002), having determined essentially the same structure for the apo-CcmE from *E. coli* as Arnesano *et al.* (2002), have modelled a heme-binding site onto a hydrophobic patch on the surface of the protein. It is assumed that this patch provides the ANS binding site described in this chapter, which is also present in $CcmE^{sol}$ that lacks a His tag. Judging from the fluorescence emission maximum at 480 nm, this ANS site is not as hydrophobic as is found in some proteins, consistent with ANS binding in the relatively exposed heme-binding site advocated by Enggist *et al.* The observations that heme can displace ANS from CcmE support this view. It would be surprising if the presence of a His tag promoted the binding of ANS, given that this probe has a preference for hydrophobic sites and that histidine is a hydrophilic amino acid. However, it cannot be excluded that a C-terminal His tag helps stabilize the C-

terminus of the protein. The effect of the His tag on the kinetic acceleration of the covalent bond formation between heme and CcmE *in vitro* suggests that either the ligand field enhancement of the tag and/or stabilization of the non-covalent heme-protein complex by the His tag is important or that the His tag may even provide an acid-base catalytic effect on the heme-histidine bond formation.

CHAPTER 7

INTERACTIONS OF HEME WITH VARIANTS OF THE HEME CHAPERONE CCME CARRYING ACTIVE SITE MUTATIONS

SUMMARY

To investigate the mechanism and specificity of heme attachment to CcmE, the conserved heme-binding residue histidine 130 of a soluble C-terminally His-tagged version of *Escherichia coli* CcmE ($CcmE^{sol}$-C-His_6) was mutated to alanine or cysteine. Covalent bond formation with heme occurred for the protein with the cysteine mutation both *in vivo* and *in vitro*. The yield of holo-H130C $CcmE^{sol}$-C-His_6 produced *in vivo* was low compared with the wild-type. *In vitro* heme attachment occurred only under reducing conditions. The involvement in the covalent bond formation of one of the heme vinyl groups and a side chain at residue 130 was demonstrated by showing that *in vitro* attachment did not occur either with the heme analogue mesoheme or when alanine was present as residue 130. These results have implications for the mechanism of heme attachment to histidine 130 of CcmE.

7.1. INTRODUCTION

The nature of the novel bond between histidine residue 130 of CcmE and the heme remains unknown, although it has been shown that at least one of the vinyl groups of heme is involved (CHAPTER 6). *In vitro* studies of a soluble version of CcmE have shown that the covalent attachment occurs under reducing conditions (CHAPTER 6). In order to probe further the covalent attachment of heme *in vitro* or *in vivo,* the histidine 130 that has been shown previously to be the site of heme attachment *in vivo* (Schulz et al. 1998) has been altered by site-directed mutagenesis. Replacement of this histidine by cysteine or alanine was expected to generate proteins with either a potentially reactive or an unreactive side chain at position 130.

7.2. RESULTS

7.2.1. Characterisation of the mutants H130A and H130C

Both the H130A and H130C variants of $CcmE^{sol}$-C-His_6 expressed well as their apoforms in the *E. coli* periplasm. The masses of the apoproteins were confirmed by ES-MS, which also showed that the periplasmic targeting sequences had been completely cleaved. The observed masses were 15,447 Da for the H130A mutant (calculated 15,450 Da) and 15,483 Da for the H130C mutant (calculated 15,482 Da). These proteins were also expressed in *E. coli* with coexpression of the other Ccm proteins (CcmA-H), and the covalent attachment of heme to the proteins was examined by SDS-PAGE analysis followed by heme staining (Figure 7.1). Figure 7.1 (A) shows that the proteins were purified to homogeneity (lanes 2 and 4 for H130A and H130C, respectively). As expected, the H130A mutant did not appear to bind heme covalently *in vivo* as judged by the lack of any heme staining of the SDS-PAGE gel (Figure 7.1 (B), lane 2), which is in agreement with previous

experiments (Schulz et al. 1998). The H130C mutant, however, was found to heme stain when expressed with the other Ccm proteins (Figure 7.1 (B), lane 6), indicating that the protein was recognized to some extent by the Ccm system for heme delivery and attachment. In order to detect the stain from covalently bound heme, the gel had to be overloaded such that a broad band was seen. It was found that the apo-H130C protein formed intermolecular disulfide bonds *in vitro* when dialyzed extensively against oxygenated 50 mM sodium phosphate buffer pH 7.0. This is clear from Figure 7.1 (A), lane 5. A significant proportion of the protein ran at a molecular mass of approximately 30 kDa, corresponding to a covalently-linked dimer, which was also identified by ES-MS analysis. This observation was supported by analysis with Ellman's reagent, which showed one and 0.2 equivalents of free thiol for reduced and oxidized protein, respectively. *In vivo*-produced H130C holo-$CcmE^{sol}$-C-His_6 binds heme at a very low level *in vivo*. Less than 0.5 % of the protein is isolated in the holo-form containing covalently bound heme, as determined from the relative ratio of the Soret band to the absorption at 280 nm.

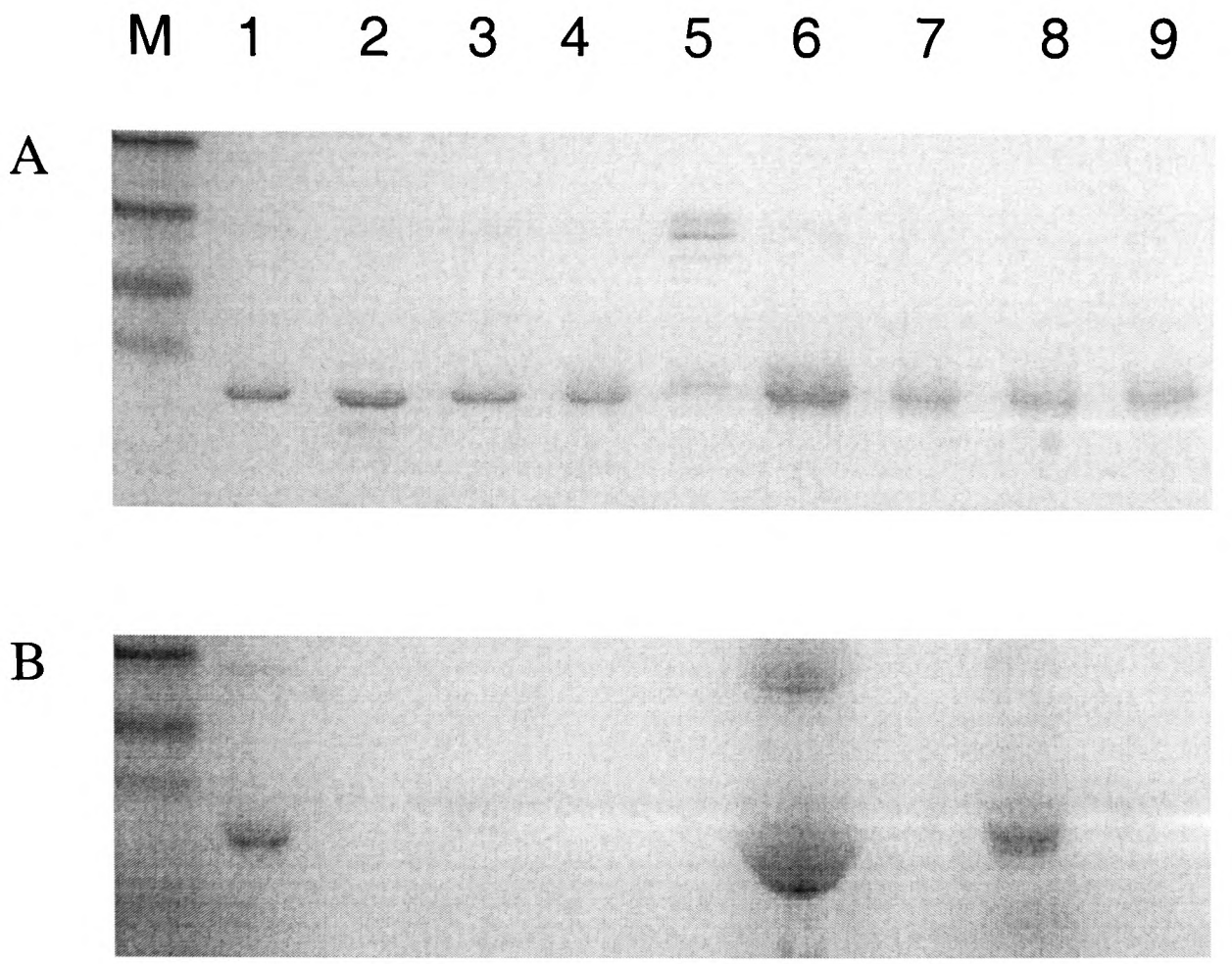


Figure 7.1. SDS-PAGE (15%) analysis of various forms of CcmE^{sol}-C-His$_6$. **(A)** Coomassie Blue stained and **(B)** activity stained for covalently bound heme. M shows molecular weight markers (16.5, 25, 32.5 and 47.5 kDa (only in panel **(A)**) from bottom to top). Lane (1) corresponds to wild-type holo-CcmE^{sol}-C-His$_6$ produced *in vivo*. (2) H130A mutant produced in the presence of pEC86. (3) H130A reacted with reduced heme *in vitro* for 14 h. (4) is the apoform of H130C isolated from the periplasm of *E. coli*. (5) apo-H130C extensively dialyzed against 50 mM aerated sodium phosphate buffer, pH 7.0. (6) holo-H130C produced *in vivo* in the presence of pEC86. 300 pmoles and 1.5 nmoles of protein were loaded in panels (A) and (B), respectively. The protein was overloaded so that the heme-staining band could be clearly observed. (7) apoform of H130C reacted with oxidized heme *in vitro* for 14 h. (8) apo-H130C reacted with reduced heme for 14 h *in vitro*. (9) the reaction product of incubation apo-H130C and reduced mesoheme *in vitro* for 14 h. 100-200 pmoles of protein were loaded into each lane, unless otherwise stated.

7.2.2. *In vitro* heme binding to the H130A and H130C apoproteins

Upon addition of ferric heme to the variant $CcmE^{sol}$-C-His_6 apoproteins, both H130A and H130C formed non-covalent *b*-type cytochrome complexes, as determined by visible spectroscopy. The complexes formed within the mixing time and appeared to be stable in this form for several hours. The absorption spectra of the ferric complexes were identical to the spectrum obtained from mixing ferric heme with wild-type $CcmE^{sol}$-C-His_6 apoprotein with a Soret band at 413 nm. The absorbance maxima for the complexes of both proteins with ferrous heme and mesoheme, as well as the pyridine hemochrome spectra, are shown in Table 7.1. Mesoheme is a heme analogue that has ethyl substituents in the normal positions of the vinyl groups. The visible spectrum of the complex of H130C with ferric heme shows the characteristics of a *b*-type cytochrome, with an α-band at 560 nm following reduction with dithionite and immediate recording of the spectrum. It was not possible to record an accurate visible spectrum of the dithionite-reduced H130A mutant *b*-type complex because dissociation of heme from the protein was too rapid. Heme dissociation upon reduction was also observed with the quantitatively formed (1:1) wild-type heme-protein complex (CHAPTER 6). Dissociation of heme from the quantitatively formed complex between heme and the H130C variant was similar to the wild-type protein-heme *b*-type cytochrome (CHAPTER 6). The pyridine hemochrome spectra of the *b*-type complexes of both mutants with heme have absorbance maxima at 556 nm, which is characteristic of unsaturated vinyl groups and shows that covalent attachment of heme had not occurred under these conditions.

The dissociation constants (K_d) of the mutant proteins for ferric heme were measured by fluorescence spectroscopy, as described for the wild-type protein

(CHAPTER 6), by Dr. Julie Stevens. The dissociation constants of the high affinity binding sites were compared, as these are likely to be the physiologically relevant sites. The K_d of heme from the H130A mutant was found to be 0.72 (± 0.16) μM, which shows that it has a slightly lower affinity for heme than the wild-type protein (K_d 0.2 μM, (CHAPTER 6)). This result is not unexpected as the loss of the histidine side-chain is likely to have changed the conformation of the heme-binding site in the protein. The K_d for the H130C mutant was found to be 0.48 (± 0.08) μM, also larger than for the wild-type. Both mutant proteins have, however, retained a significant affinity for heme.

Table 7.1. Visible absorption maxima for various forms of $CcmE^{sol}$.

Nature of heme-CcmE complex		*Absorption maxima/nm*			
		Reduced heme-protein complex			Pyridine hemochrome (α band)
H130A	b-type heme-$CcmE^{sol}$-C-His_6	nd	nd	nd-	556
	mesoheme-$CcmE^{sol}$-C-His_6	549	521	410	546
H130C	b-type heme-$CcmE^{sol}$-C-His_6	560	530	425	556
	mesoheme-$CcmE^{sol}$-C-His_6	550	522	411	546
	In vivo holo-$CcmE^{sol}$-C-His_6	555	525	419	~552
	In vitro holo-$CcmE^{sol}$	556	526	423	552.5

nd: could not be determined (see text)

7.2.3. Covalent attachment of heme to H130C *in vitro*

Upon reduction of the C-terminal His-tagged *b*-type H130C protein-heme complex with disodium dithionite in the presence of DTT, with a five-fold excess of protein

over heme, the absorption spectrum shifted toward the spectrum of a cytochrome with a covalent bond between heme and protein. The α-band shifted from 560 to 556 nm over several hours, and the β-band and Soret band also shifted accordingly with time (Table 7.1). After desalting the reaction mixture after 14 h of reaction time, the spectrum of heme-bound H130C produced in this way was very similar to the spectrum of the *in vivo*-produced H130C protein (Table 7.1). The pyridine hemochrome spectrum of the *in vitro*-produced holo-form of the protein yielded a maximum of *ca.* 553 nm, which is consistent with the presence of a single free vinyl group, as has been observed for single cysteine variants of *c*-type cytochromes (Tomlinson & Ferguson 2000b and references therein). Interestingly, the *in vivo*-produced holo-form of this mutant had broad maxima in the reduced absorption and pyridine hemochrome spectra. This observation suggests that as a consequence of the substitution of histidine 130 with a cysteine residue, heme attachment is not completely selective and that the formation of side products can occur. The oxidation state of heme and the cysteine thiol *in vivo* might not be as tightly controlled during the periplasmic attachment of heme to the protein as it is in the reducing conditions used for the *in vitro* experiments.

To confirm that the spectroscopic data were indicative of *in vivo* and *in vitro* covalent bond formation between heme and the H130C mutant protein, SDS-PAGE analysis followed by heme staining was performed. Figure 7.1, lane 8 shows the reaction of the *b*-type complex of heme and H130C with dithionite in the presence of DTT after 14 h. The fact that the protein stains for covalently bound heme (Figure 7.1 (B), lane 8), as does the *in vivo*-produced holo-H130C in lane 6, indicates that *in vitro* and *in vivo* covalent attachment of heme to the

protein had occurred in the respective cases. The controls for this experiment are shown in lanes 3, 7 and 9. They are: H130A incubated with ferric heme followed by reduction with dithionite; H130C incubated with ferric heme; and addition of ferric mesoheme to H130C protein followed by reduction with dithionite and incubation for 14 h, respectively. These data show that covalent attachment of heme to the $CcmE^{sol}$ protein samples did not occur in these conditions, as no heme staining could be observed for the $CcmE^{sol}$. The results establish that one of the vinyl groups of heme is involved in formation of the covalent bond, because attachment was not observed with mesoheme. They also show that the covalent bond forms with the cysteine residue of the protein, as it was not observed with the alanine mutant. Therefore, covalent heme binding can only occur with a reactive side chain of amino acid 130 (Cys or His). As was observed for the wild-type protein (CHAPTER 6), these results also highlight the requirement for reduction of the heme in the covalent bond formation, as no heme staining was observed when ferric heme was added to the H130C apoprotein.

In order to remove any ambiguity regarding the effect of the His tag on the covalent heme attachment to the H130C variant, and the inability of the H130A mutant to bind heme covalently *in vitro*, proteins with these active site mutations were also made with a N-terminal cleavable His tag. The purified proteins were shown to be pure by SDS-PAGE analysis as shown in Figure 7.2 (lanes 1 and 2, for H130A and H130C, respectively). Upon addition of heme under reductive conditions followed by removal of the His tag by thrombin cleavage, the H130C mutant was found to contain covalently bound heme as determined by SDS-PAGE analysis followed by heme staining (Figure 7.2, lane 4) and visible absorption spectroscopy (Figure 7.3). The visible spectrum compares very well with that of

the *in vivo*-produced H130C heme-containing protein. The H130A mutant failed to stain for covalently bound heme (Figure 7.2, lane 3) and did not show any characteristics of a heme-containing protein in the visible spectrum (data not shown).

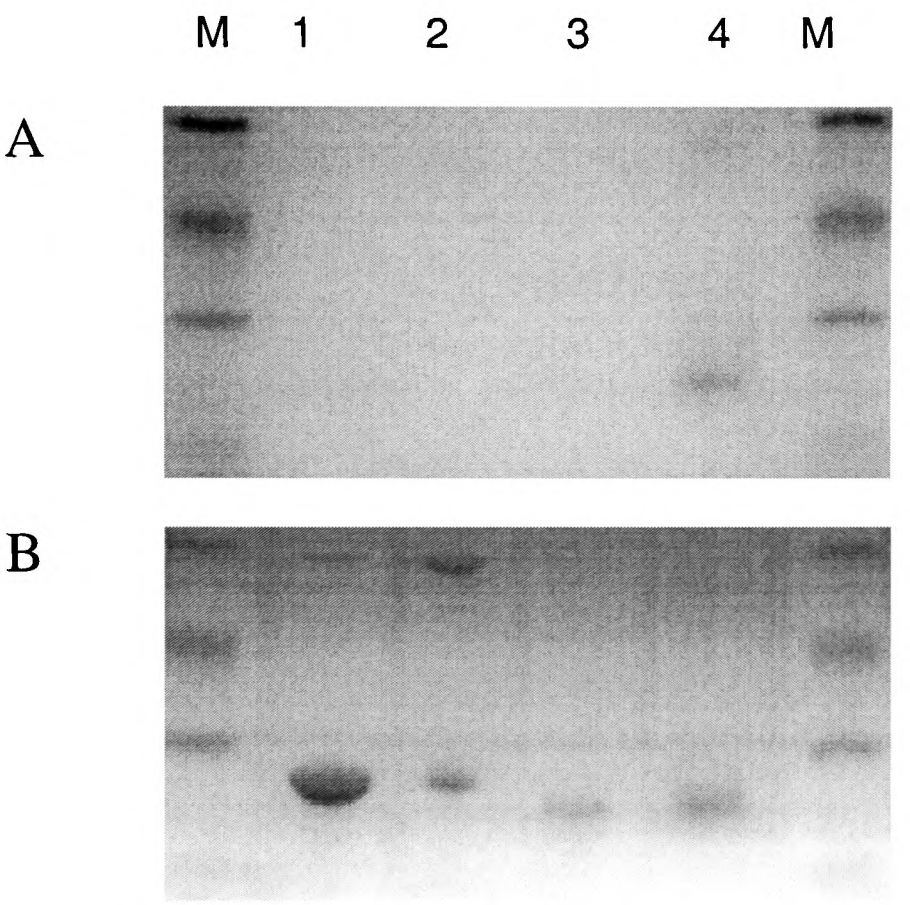


Figure 7.2: SDS-PAGE (17.5%) analysis of heme attachment to H130A and H130C $CcmE^{sol}$-C-His_6. **(A)** activity stained for covalently bound heme followed by **(B)** Coomassie Blue stained. M shows molecular weight markers (6.5 (faint), 16.5, 25, and 32.5 kDa from bottom to top). (1) apo-H130A $CcmE^{sol}$-C-His_6. (2) apo-H130C $CcmE^{sol}$-C-His_6. In the Coomassie-stained gel it is clear that some dimerisation has occurred via disulfide bonds between the cysteines at position 130. The dimer in lane 1 is assumed to be a non-covalent dimer due to over-loading of the gel. (3) H130A $CcmE^{sol}$-C-His_6 reacted with reduced heme for 14 h followed by thrombin cleavage of the His tag. (4) H130C $CcmE^{sol}$-C-His_6 reacted with reduced heme for 14 h followed by cleavage of the His tag.

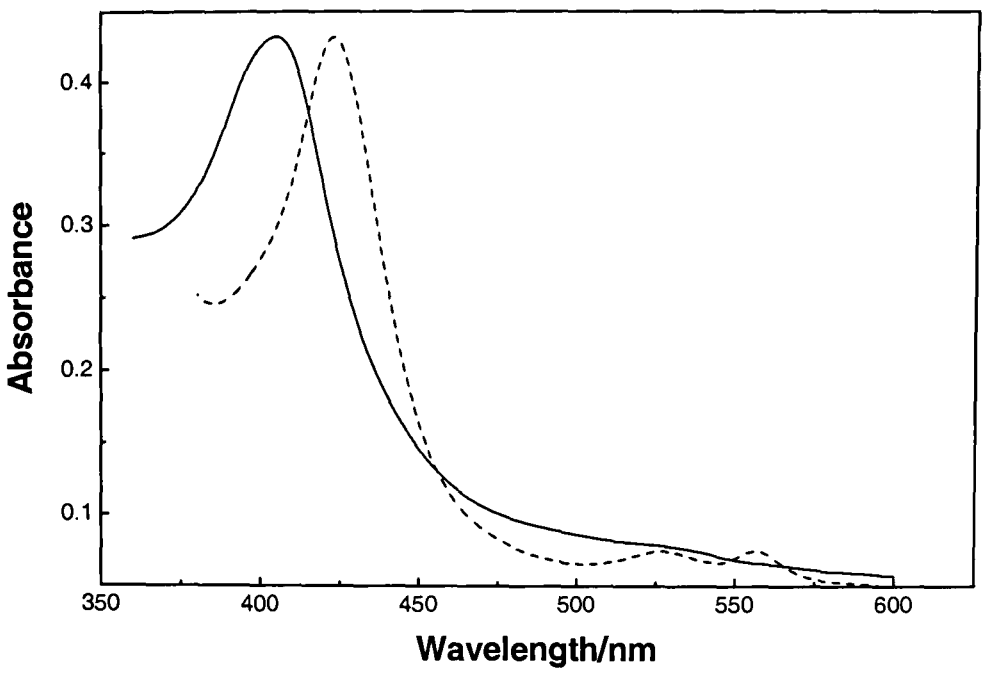


Figure 7.3. Absorption spectra of the covalently heme-bound forms of holo-H130C CcmE^{sol} produced *in vitro*, showing the oxidised spectrum (———) and the reduced spectrum obtained immediately after the addition of disodium dithionite (— — —). 4 μM protein samples in 50 mM Tris-HCl buffer, pH 7.4, 300 mM NaCl were used.

7.2.4. Cysteine to histidine variants of *H. thermophilus* cytochrome c_{552}

The approach of mutating a heme-binding cysteine residue from a *c*-type cytochrome into a potentially heme-binding histidine residue to model CcmE was probed by mutating the cysteine residues of the CXXCH motif of *H. thermophilus* cytochrome c_{552} into either AXXHH or HXXAH. It was hoped that a covalent bond between the introduced histidine and heme in *H. thermophilus* cytochrome c_{552} could form and by elucidating the nature of this novel covalent bond more understanding of the chemistry of histidine-heme bonds could be gained. This protein seemed promising for the experiment as the wild-type *c*-type cytochrome has been successfully converted, using expression and spontaneous assembly in the cytoplasm of *E. coli*, into a *b*-type cytochrome derivative with an AXXAH motif (Tomlinson and Ferguson 2000a). Plasmids pOli1 and pOli2 were constructed with AXXHH and HXXAH sequences, respectively (Table 2.3). However, *E. coli* JM109 cells

transformed with either of these plasmids and grown under identical conditions as *E. coli* cells expressing wild-type *H. thermophilus* cytochrome c_{552}, did not yield a cytoplasmic protein with covalently bound heme as judged by visible spectroscopy and SDS-PAGE analysis staining for covalently bound heme (data not shown). *In vitro* addition of heme to the lysed cell suspensions in reducing conditions did not give rise to detectable amounts of cytochrome derivatives.

These experiments indicate that heme is not bound covalently by the apoprotein of *H. thermophilus* cytochrome c_{552} derivatives with variations of the CXXCH motif to either AXXHH or HXXAH. This observation may arise from steric alterations introduced in the mutant proteins, which prevent heme from binding. A very tight packing of the wild-type cytochrome around the heme is apparent from the structure of this thermostable protein (Hasegawa et al. 1998). It has been suggested that the apopotein may be subject to proteolysis, if the heme-binding of the apoprotein is slow (Allen et al. 2002). It also cannot be formally excluded that problems in the expression of the mutant proteins caused the failure to detect any *H. thermophilus* heme-containing protein in the cytoplasm of the cells expressing either plasmid pOli1 or pOli2. It was not possible to prove that the apoprotein had been expressed due to the present lack of an antibody against *H. thermophilus* cytochrome c_{552}.

7.3. DISCUSSION

The failure of heme to bind covalently to the H130A mutant of $CcmE^{sol}$ establishes that the covalent binding of heme observed with the wild-type H130 (CHAPTER 6) and H130C proteins involves the specific participation of the imidazole or thiol groups of histidine or cysteine at this residue position. It is reasonable to assume

that the initial non-covalent binding of ferric heme to any of the three proteins, *i.e.* H130, H130C and H130A, positions the heme appropriately for the subsequent uncatalysed covalent bond formation with the former two proteins.

7.3.1. Heme binding to H130C CcmE^{sol}

Previous *in vitro* studies have shown that covalent bond formation can occur spontaneously between the histidine of the heme chaperone CcmE^{sol}-C-His$_6$ and ferrous heme (CHAPTER 6). Upon addition of ferric heme the apoprotein binds heme non-covalently with a high affinity, and the covalent bond forms only when the heme is reduced. In order to investigate this process further, similar studies on CcmE were performed with the heme-binding histidine mutated to a cysteine. It was found that the covalent bond still formed, both *in vitro* and *in vivo*, although in the latter case to a lesser extent compared with the wild-type protein. The unusual similarity between the heme binding process occurring with the wild-type and the H130C mutant, namely reaction conditions requiring reduced heme and a time scale of *ca.* 10 h for the reaction of heme and protein to form a covalent bond, leads to interesting mechanistic proposals. Recently, an *in vivo* study was published in agreement with the findings described here, showing that a His-tagged H130C mutant of CcmE can similarly (as judged by various criteria) bind heme covalently on this cysteine, albeit to a low level (Enggist et al. 2003).

7.3.2. Implications for the roles of other Ccm proteins

The results presented here also provide some insight into the role of the protein CcmC, which has been shown to bind heme and to present CcmE with heme in the periplasm (Ren & Thöny-Meyer 2001; Schulz et al. 1999). The fact that the covalently attached form of the H130C mutant is produced *in vivo* in the presence

of the other Ccm proteins to a considerably lesser extent than the wild-type protein, in contrast to the comparable yields *in vitro*, suggests that CcmC is not effective in catalysing the attachment of heme to the cysteine compared with its attachment to histidine. This lower efficiency may indicate that such enzymatic action specifically requires the heme-binding histidine residue apo-CcmE. The coordination of the heme iron in CcmE might render the heme-vinyl group more reactive to histidine 130 than cysteine 130. The observation that the H130C mutant can readily form covalent dimers via a disulfide bridge between the cysteines at position 130, suggests that the residue 130 is relatively accessible in *E. coli* CcmE, which is in agreement with the published structure of the apoprotein (Figure 1.8; Enggist et al. 2002). As the heme is bound to the histidine residue in this position of the wild-type protein (Schulz et al. 1998), it may be expected that the heme is also accessible, which would allow the apocytochrome *c* to take up the heme from the heme chaperone. Coordination of the heme iron by a functional group from either the Ccm proteins or the solution, and leading to a weak ligand-field, would also enhance the heme-transfer upon ligation because the apocytochrome *c* may provide a strong ligand-field.

7.3.3. Mechanistic implications

The successful covalent attachment of heme to a cysteine at residue 130, both *in vivo* and *in vitro*, provides some mechanistic insight into heme attachment to CcmE. The *in vitro* reaction appears to resemble the uncatalysed formation from polypeptide and heme of a *c*-type cytochrome, or its derivatives carrying just one cysteine in the heme-binding motif that was recently reported (CHAPTER 3-5, Tomlinson & Ferguson 2000b). The pyridine hemochrome spectra reported here suggest that $CcmE^{sol}$ H130C is similar to the known *c*-type cytochromes with a

single thioether bond (Tomlinson & Ferguson 2000b). At this stage the axial ligands for either the oxidized or reduced heme-protein have not been determined. *In vitro* formation of the thioether bonds of a *c*-type cytochrome presumably requires protonation of the β-carbon of the vinyl groups of heme and formation of the thioether bond between the cysteine residues and the α-carbon of the vinyl groups of heme (CHAPTER 3). A mechanism for this process is envisaged to involve attack of the thiol moiety of the cysteine residues on the α-carbon. For thioether bond formation to occur, it was shown experimentally that ferrous heme is required (CHAPTER 3). It was shown that the addition of either the histidine (CHAPTER 6) or the cysteine residue of CcmE to the heme also required ferrous heme. Given the similarities of the *in vitro* heme binding of either the histidine or cysteine variant of CcmE, it is likely that they occur by a similar mechanism. Therefore, the mechanism of histidine-heme attachment is suggested to be analogous to that of thioether bond formation in *c*-type cytochromes. The histidine residue of wild-type CcmE could add onto the α-carbon of one of the vinyl groups. Recently a covalent histidine-heme bond of this nature has been reported for an unusual form of hemoglobin, which is formed *in vitro* under reductive conditions (Vu et al. 2002). If the heme attachment to CcmE occurs via a specific vinyl group and heme can bind stereoselectively to CcmE in one orientation, relative to the α, γ meso axis of the heme moiety, stereospecificity of the heme transfer reaction to apocytochrome *c* would be facilitated, as is observed in all structurally characterised cytochromes *c* (Barker & Ferguson 1999). The proposed process of heme binding and heme release from CcmE is summarized in Figure 7.4. However, a radical mechanism as proposed for *in vitro* thioether bond formation between heme and cysteine (Kojo 1981) cannot be excluded on the basis of the current experimental data. The release of the heme from the heme chaperone has

similarities with a synthetic reaction yielding dipyridyl sulfides whereby a quaternary pyridinium moiety acts as a leaving group upon nucleophilic attack of a thiol functionality (Boduszek & Wieczorek 1980).

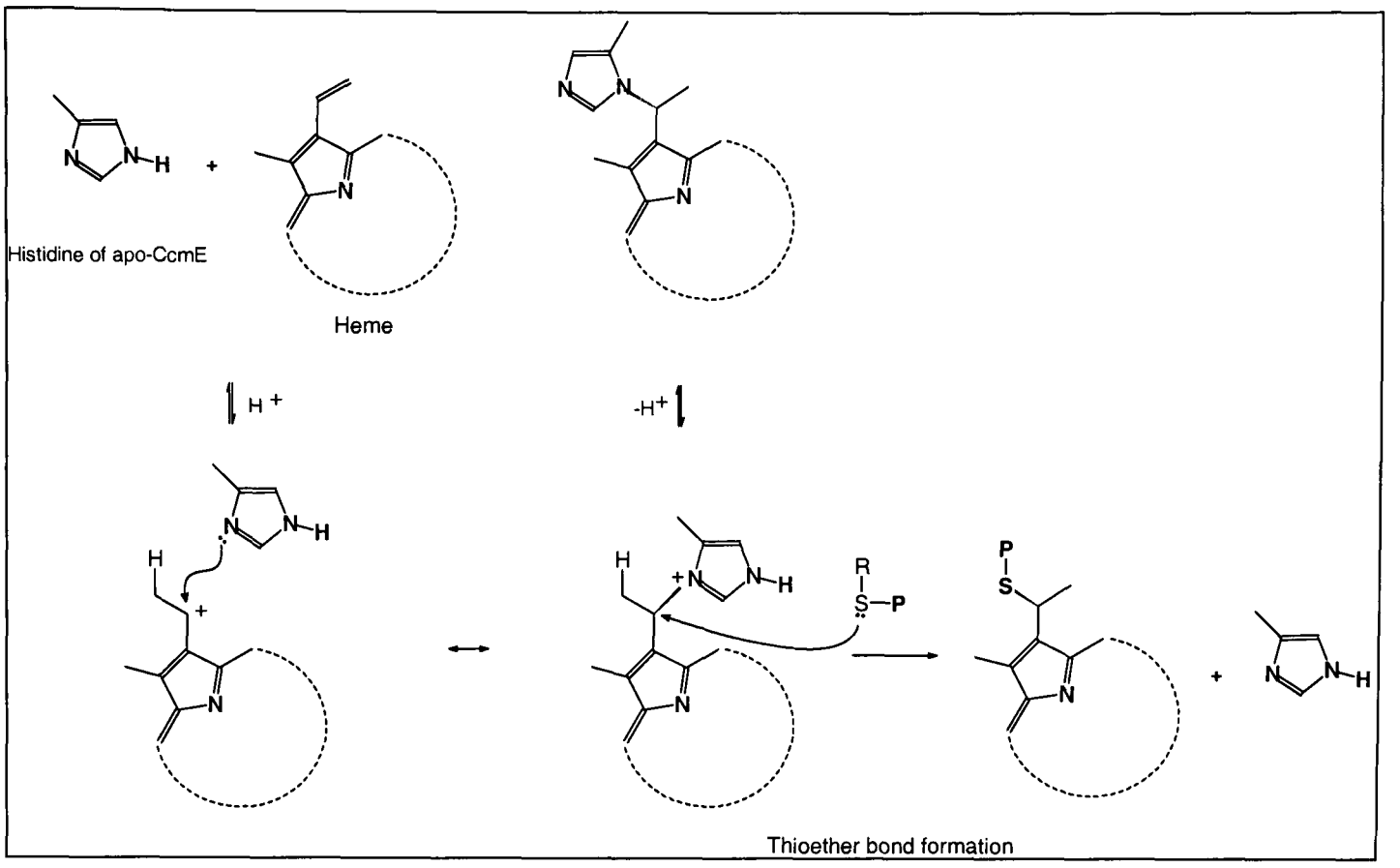


Figure 7.4: The proposed chemical mechanism of heme binding to CcmE and heme transfer to apocytochrome *c*. **P** denotes the apocytochrome *c*, **R** abbreviates a leaving group stable as a cation species. *In vitro* this is presumably a proton. Alternatively, the nucleophilic attack from the apocytochrome could be conducted by a thiolate group. Note that the reactive nitrogen in the histidine residue is unidentified. For the given reaction, N^{π} was chosen to illustrate the mechanism.

Additional studies will be required to describe further the mechanistic processes involved during heme transfer, especially with respect to which vinyl moiety reacts with CcmE, and therefore, the exact nature of the histidine-heme bond. It will also be interesting to find out how the proposed disulfide intermediate of the cysteine thiols of the apocytochrome *c* during cytochrome *c* maturation (Allen et al. 2002) will affect the heme transfer reaction. However, the work presented in this chapter contributes further to the understanding of the molecular basis of a central step in cytochrome *c* biogenesis.

CHAPTER 8

HEME TRANSFER FROM HOLO-CCME TO APOCYTOCHROME *C*

SUMMARY

Heme transfer from holo-CcmE^{sol}-C-His$_6$ to wild-type *H. thermophilus* apocytochrome *c* was observed *in vitro*, provided the heme was reduced. A variant of the apocytochrome *c* with an AXXAH motif, rather than the wild-type CXXCH heme-binding motif, was unable to accept heme from holo-CcmE^{sol}-C-His$_6$. Furthermore, single cysteine variants of *H. thermophilus* c_{552} were not capable of releasing heme from CcmE^{sol}-C-His$_6$ *in vitro*, in agreement with previous *in vivo* studies. These *in vitro* results mimic, and thus have implications for, the molecular pathway of heme transfer during *c*-type cytochrome maturation *in vivo* in many species of bacteria and in plant mitochondria.

Additionally, horse heart apocytochrome *c* and yeast iso-1-apocytochrome *c* accepted heme from holo-CcmE^{sol}-C-His$_6$ *in vitro*. These results show that CcmE can recognise a broad range of apocytochromes *c* as substrates, provided they contain two cysteine residues in the heme-binding motif.

8.1. INTRODUCTION

One of the central aspects of the Ccm system is the release of heme from the heme chaperone CcmE to apocytochrome *c*, which has been shown to occur *in vivo* (Schulz et al. 1998). Earlier studies have also shown that mitochondrial cytochromes *c* are recognised by the Ccm system (Sanders & Lill 2000). In this chapter, the heme transfer reaction from CcmE to various apocytochromes *c* is investigated *in vitro*.

8.2. RESULTS

8.2.1. Transfer of heme from *in vivo*-produced holo-CcmE^{sol}-C-His$_6$ to *H. thermophilus* apocytochrome *c*

Apocytochrome c_{552} from *H. thermophilus*, which is known to be a substrate for the Ccm system (Allen et al. 2002; Karan et al. 2002), was shown to be pure by SDS-PAGE (Figure 8.1, lane 2). The presence of free cysteines in the CXXCH motif of the apocytochrome was confirmed by analysis with Ellman's reagent (Riddles et al. 1983), which showed two free thiol moieties per protein. Upon incubation of *in vivo*-produced *E. coli* holo-CcmE^{sol}-C-His$_6$ (CHAPTER 2 & 6) with *H. thermophilus* apocytochrome c_{552}, holocytochrome c_{552} characteristics were observed on addition of dithionite. Under these reducing conditions the visible spectrum shifted towards that of the cytochrome c_{552} spectrum over a period of 18 hours. The visible absorption spectra of holo-CcmE^{sol}-C-His$_6$, holocytochrome c_{552} and the product obtained from incubation of apocytochrome *c* and holo-CcmE^{sol}-C-His$_6$ with dithionite for 18 hours are shown in Figure 8.2. SDS-PAGE analysis of samples incubated for 18 hours showed that heme transfer occurred in the presence of dithionite as reductant, leading to the formation of holocytochrome c_{552} (Figure 8.1, lane 5), but this was not the case in the absence of reductant

(Figure 8.1, lane 4). Under oxidising conditions no change in the spectra was observed. Incubating holo-CcmE^{sol}-C-His$_6$ alone under those reductive conditions showed that heme remained bound to the heme chaperone as observed by visible spectroscopy and SDS-PAGE (Figure 8.1, lane 1). Incubation of the C11A/C14A variant of apocytochrome c_{552} with CcmE^{sol}-C-His$_6$, under the same reducing conditions, did not lead to a transfer of heme from holo-CcmE^{sol}-C-His$_6$ as determined from the visible absorption spectrum and retention of heme by CcmE^{sol}-C-His$_6$ according to SDS-PAGE (Figure 8.1, lane 6). The C11A/C14A apocytochrome variant is not able to form thioether bonds to heme, as it lacks the necessary cysteine residues that are involved in forming the covalent bonds to the heme prosthetic group. However, it is known that this mutant is capable of forming a stable *b*-type cytochrome (Tomlinson & Ferguson 2000a).

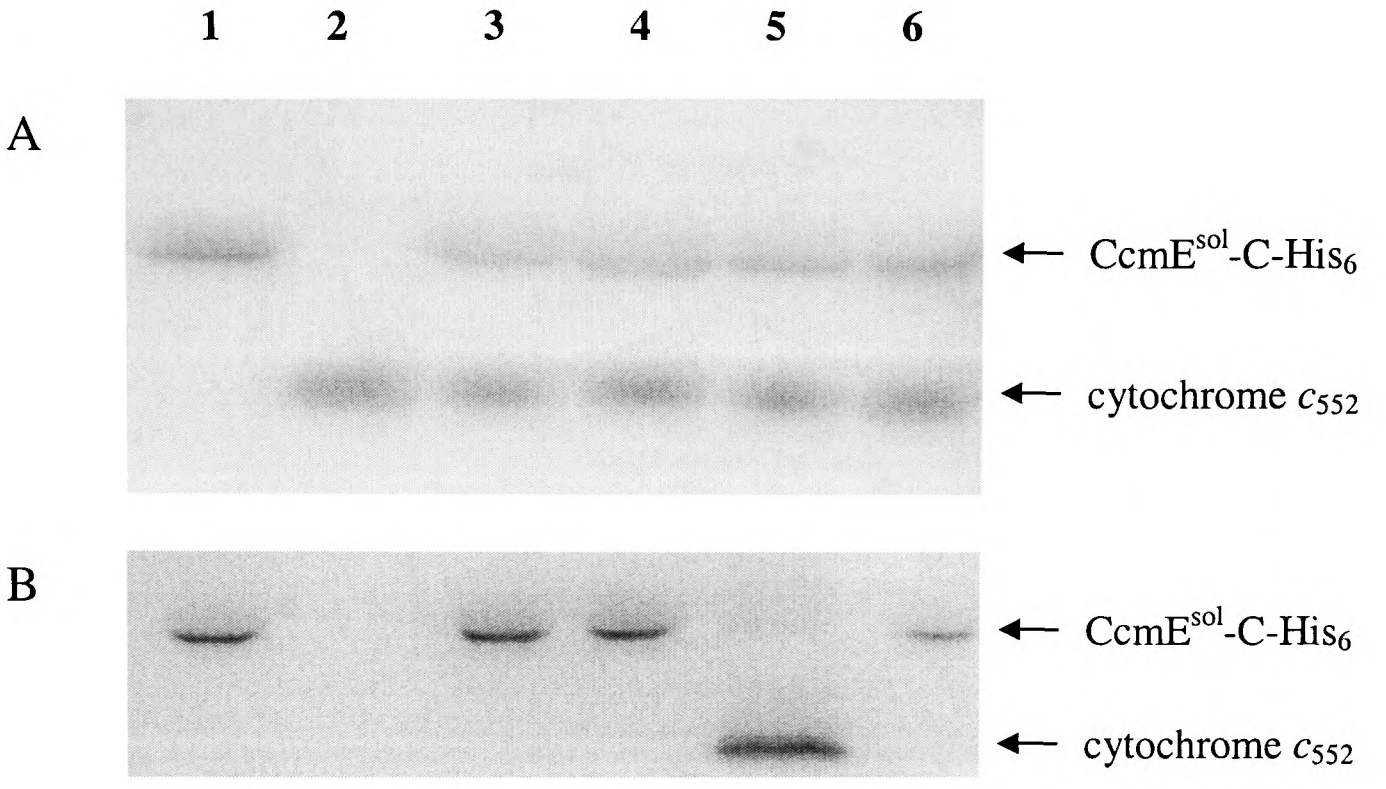


Figure 8.1. SDS-PAGE analysis of the transfer of heme from *in vivo*-produced holo-CcmE^{sol}-C-His$_6$ to *H. thermophilus* apocytochrome c_{552}. **A** Coomassie Blue stained. **B** Activity stained for covalently bound heme. Lane (1) the *in vivo*-produced holo form of *E. coli* CcmE^{sol}-C-His$_6$ after 18 hours of incubation without addition of apocytochrome c_{552} under reducing conditions. (2) Wild-type apocytochrome c_{552} (with the covalently bound heme removed by treatment with silver sulphate). (3) *In vitro* incubation of holo-CcmE^{sol}-C-His$_6$ with apocytochrome c_{552}, immediately after mixing. (4) Incubation of holo-CcmE^{sol}-C-His$_6$ with apocytochrome c_{552} in the absence of reductant for 18 hours. (5) Reaction of holo-CcmE^{sol}-C-His$_6$ with apocytochrome c_{552} in the presence of dithionite for 18 hours. (6) Incubation of holo-CcmE^{sol}-C-His$_6$ with the C11A/C14A mutant of *H. thermophilus* apocytochrome c_{552} for 18 hours in the presence of reductant. 300-400 pmoles of protein were applied to each lane of the gel for Coomassie staining, and 30-40 pmoles were applied for heme staining.

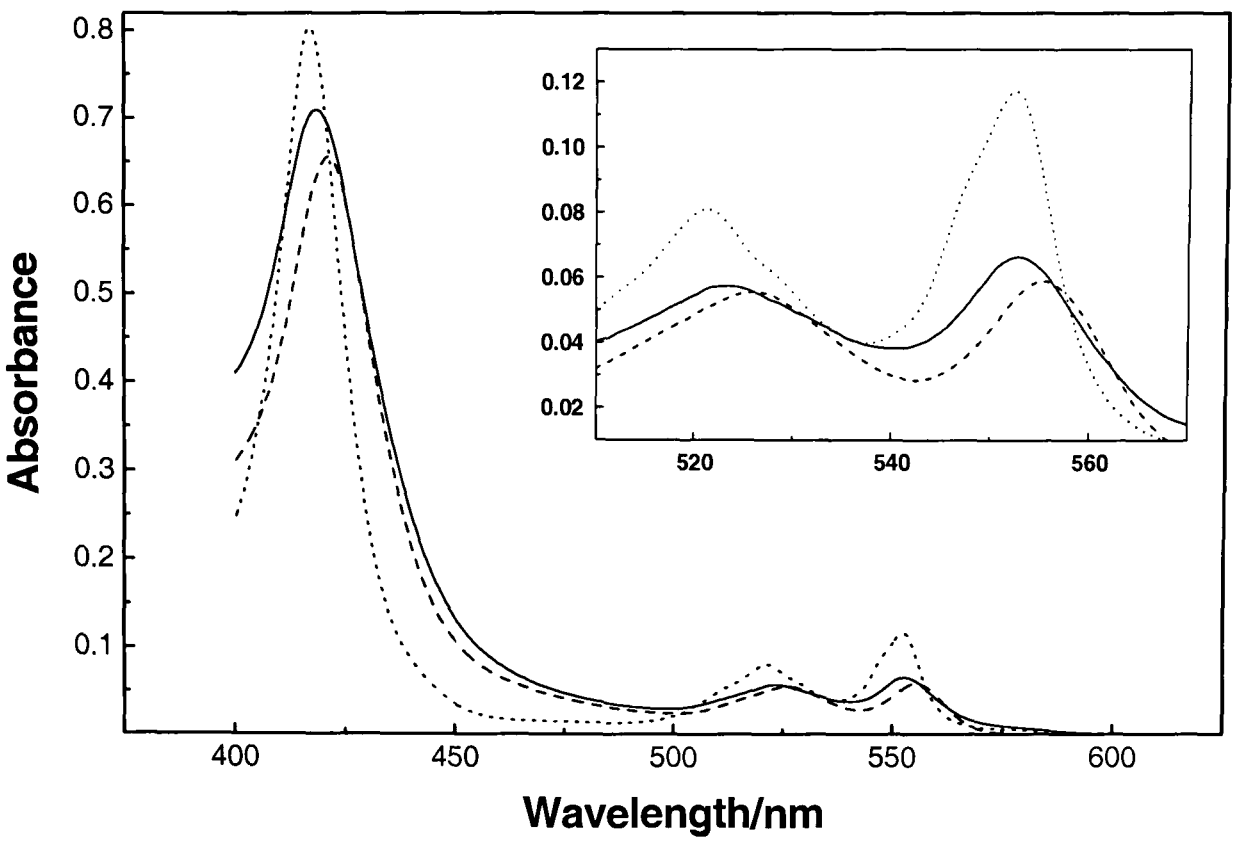


Figure 8.2. The visible spectra of holo-CcmE^{sol}-C-His$_6$ (— — —), *H. thermophilus* holocytochrome c_{552} (- - - -) purified from *E. coli* and the product obtained following incubation of *H. thermophilus* apocytochrome *c* and holo-CcmE^{sol}-C-His$_6$ with dithionite (—— ——) for 18 h.

8.2.2. Attempted transfer of heme from *in vivo*-produced holo-CcmE^{sol}-C-His$_6$ to *H. thermophilus* apocytochrome *c* variant with only a single cysteine in the heme-binding motif

C11A and C14A variants of *H. thermophilus* apocytochrome c_{552} were shown to be pure and devoid of heme as previously described (data shown in CHAPTER 4). Holo-CcmE^{sol}-C-His$_6$ from *E.coli* was shown to be of reasonable purity and to contain covalently bound heme (Figure 8.3, lane 1). It was observed that a slightly lower molecular weight protein was present in the CcmE preparation that was used in this study. This protein band may be partially degraded CcmE, which was also detected and identified by Enggist et al. (2003). When C11A and C14A variants of *H. thermophilus* apocytochrome c_{552} were incubated with *E. coli* holo-CcmE^{sol}-

C-His_6 under either oxidative or reductive reaction conditions, no heme transfer was observed as judged by SDS-PAGE analysis (Figure 8.3, lanes 2 and 3 for C11A and C14A variants, respectively) and visible absorption spectra, which showed no change during the course of incubation. These observations are analogous to the inability of the C11A/C14A apo variant of *H. thermophilus* apocytochrome c_{552} to accept heme from holo-$CcmE^{sol}$-C-His_6 (Figure 8.3, lane 4 as Figure 8.1, lane 6). Wild-type *H. thermophilus* apocytochrome c_{552} (CXXCH) did incorporate heme from CcmE under these conditions (Figure 8.1, lane 5).

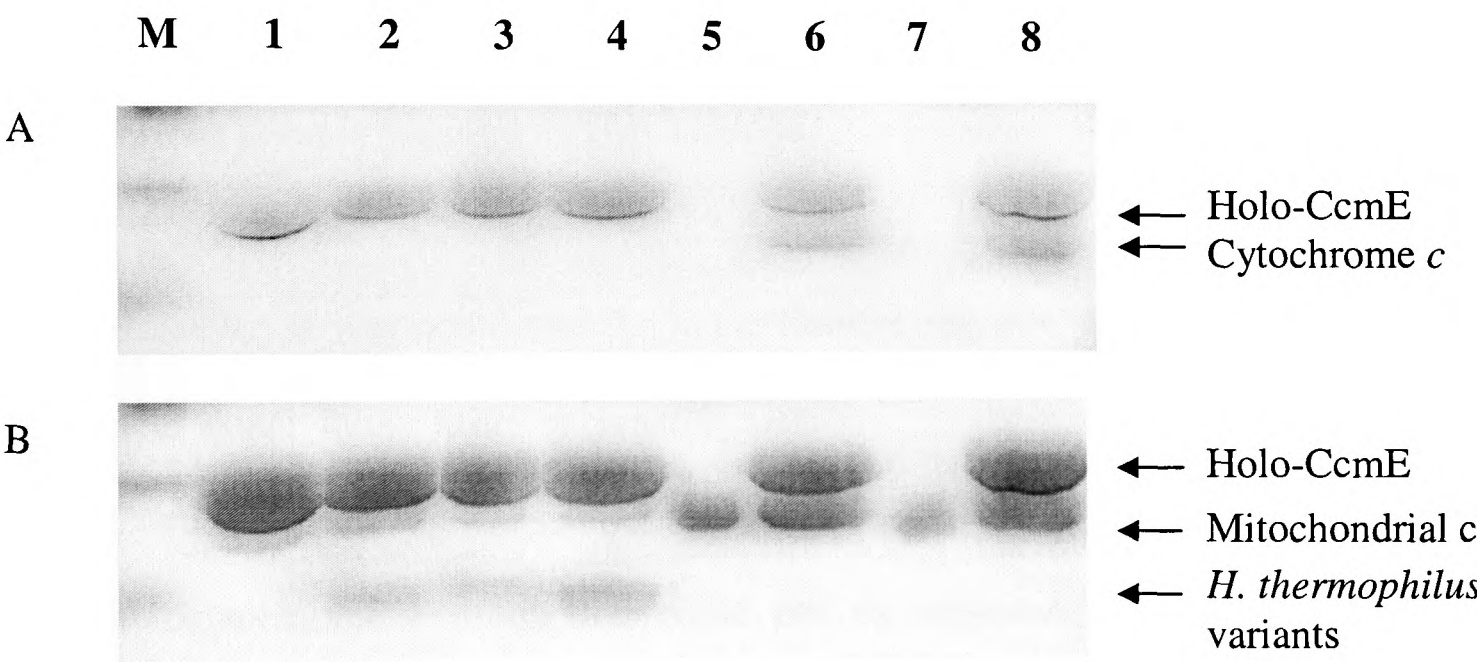


Figure 8.3. SDS/15% PAGE of the products of the heme transfer reaction from holo-$CcmE^{sol}$-C-His_6 to various apocytochromes. **A** activity stained for covalently bound heme followed by **B** Commassie Blue staining. (1) Wild-type holo-$CcmE^{sol}$-C-His_6. (2) Holo-$CcmE^{sol}$-C-His_6 and the C11A variant of *H. thermophilus* apocytochrome c_{552}. (3) Holo-$CcmE^{sol}$-C-His_6 and the C14A variant of *H. thermophilus* apocytochrome c_{552}. (4) Holo-$CcmE^{sol}$-C-His_6 and the C11A/C14A mutant of *H. thermophilus* apocytochrome c_{552}. (5) Horse heart apocytochrome *c*. (6) Holo-$CcmE^{sol}$-C-His_6 and horse heart apocytochrome *c*. (7) Yeast apocytochrome *c*. (8) Holo-$CcmE^{sol}$-C-His_6 and yeast apocytochrome *c*. All transfer reactions were carried out for 10 hours in the presence of 5 mM dithionite in 50 mM potassium phosphate buffer, pH 7.0.

8.2.3. Heme transfer reaction from holo-CcmE to horse heart and yeast apocytochromes *c*

When $CcmE^{sol}$-C-His_6 was incubated with horse heart (Figure 8.3, lane 5) or yeast (Figure 8.3, lane 7) apocytochrome *c* under reductive conditions, heme was transferred to the apocytochromes *c* as judged by SDS-PAGE (Figure 1, lane 6 and 8 for equine and yeast cytochrome *c*, respectively). The visible absorption spectra of the products were too closely similar to those of holo-$CcmE^{sol}$-C-His_6 and were, therefore, analytically not sensitive enough to show heme transfer from holo-$CcmE^{sol}$-C-His_6 to apocytochromes *c*. Without the addition of dithionite, no heme transfer was observed (data not shown), again highlighting the observation that ferrous heme is required for this heme transfer to occur.

However, it was noted that the heme transfer reaction did not go to completion with either horse heart or yeast cytochrome as there was heme still bound to $CcmE^{sol}$-C-His_6 to a substantial extent (Figure 8.3, lanes 5 and 7). An increase in holocytochrome *c* production could not be achieved by altering the reaction conditions, including the addition of apocytochrome *c* aliquots to holo-$CcmE^{sol}$-C-His_6 at one hour intervals to minimise apocytochrome *c* aggregation. These data are interpreted as being due to the lower stability of the horse heart and yeast apocytochromes *c* compared with the *H. thermophilus* apocytochrome c_{552}. The lower stability and/or solubility of the eukaryotic apocytochromes *c* became apparent as protein precipitation was observed after the time allowed for the heme transfer reaction. The amount of precipitate increased with incubation time. No such precipitate was obtained when *H. thermophilus* apocytochrome c_{552} and variants were incubated under otherwise identical conditions.

8.3. DISCUSSION

The observation that heme can be transferred from holo-CcmE^{sol}-C-His$_6$ to an apocytochrome *c* *in vitro* is in agreement with previous *in vivo* studies (Schulz et al. 1998). However, more detailed conclusions can be drawn from the *in vitro* experiments on the behaviour of two principal proteins involved in cytochrome *c* maturation, CcmE and apocytochrome *c* itself. Firstly, the oxidation state during the heme transfer process is crucial. As previous work has shown, ferrous heme is required for attachment to a thiol group, as side products can be formed between ferric heme and a cysteine residue in a heme-binding motif of an apocytochrome (Barker et al. 1993). Mitochondrial cytochrome *c* assembly has also been shown to require reducing conditions (Nicholson & Neupert 1989; Tong & Margoliash 1998). The data in this chapter indicate that *in vitro* transfer of heme from holo-CcmE^{sol}-C-His$_6$ to apocytochrome *c* also requires reduced heme. Therefore, one role of CcmE might be to prevent ferric heme from reacting with apocytochrome by taking up ferric heme itself and storing it in a covalently bound form. Secondly, as release of heme from CcmE to apocytochrome can only occur upon reduction of heme, it is possible that the Ccm protein(s) involved in the transfer have to supply reductant. Thirdly, the *in vitro* heme transfer between the two proteins is much slower than *in vivo* (Schulz et al. 1998). Therefore, one or more of the Ccm gene products may be catalysing the transfer of the heme from holo-CcmE to apocytochrome. The most likely candidates for this role are CcmF and CcmH, as previously suggested (Ren et al. 2002). However, it is clear from the data in the present work that the heme transfer can occur unaided by any other gene product(s). The validity of the quantitative *in vitro* result regarding the time scale of the heme transfer reaction is also discussed below. Fourthly, the fact that the AXXAH mutant of the CXXCH heme-binding motif in the cytochrome c_{552} was

not capable of taking up heme from holo-CcmE^{sol}-C-His$_6$, highlights the requirement for the CXXCH binding motif of *c*-type cytochromes for this process. The mechanism of the heme transfer is therefore concluded to involve the reaction of the cysteine thiols of the apocytochrome with the α-carbon of the vinyl-groups of protoporphyrin IX. Furthermore, the data with the AXXAH mutant are in agreement with the observation that cytochrome b_{562} in the periplasm incorporates heme from a source other than CcmE (Throne-Holst et al. 1997; Goldman et al. 2001; Allen et al. 2002), *i.e.*, there must be another source of heme in the periplasm for *b*-type cytochromes. In addition it was shown that the C11A and C14A variants of *H. thermophilus* cytochrome *c* could not release heme from CcmE^{sol}-C-His$_6$. These data are in agreement with the observation that neither of these protein variants are recognised effectively by the Ccm system *in vivo* (Allen et al. 2002).

The observation that the apocytochromes *c* from horse heart and yeast can release heme from holo-CcmE^{sol}-C-His$_6$ implies that this heme transfer reaction is applicable to a wide range of apocytochrome *c* substrates. These *in vitro* results are in accordance with the *in vivo* finding that Ccm system is capable of recognising apocytochromes from various sources including eukaryotes (Sanders & Lill 2000; Barker et al. 2001; Kellogg & Bren 2002).

A point to be considered is that the *in vitro* studies reported in this chapter may be affected by the use of the soluble domain of CcmE with a His tag. Unfortunately, no CcmE construct was available which produced holo-CcmE *in vivo* without such a His tag. The use of *in vitro* produced holo-CcmE, *i.e.*, where the heme was covalently attached to purified apo-CcmE, for studies on the interaction of holo-

CcmE with apocytochrome *c* was complicated by the fact that non-covalently bound heme was difficult to remove completely from CcmE. This may lead to the release of non-covalently bound heme from CcmE to yield holocytochrome *c*. To date it has been assumed that cytochrome *c* maturation by the Ccm system requires the covalently heme-bound form of CcmE as an intermediate of the pathway (Schulz et al. 1998). However, no conclusive proof of this assumption has been presented.

From the studies on the effects of His tags on CcmE variants (CHAPTERS 6 & 7) there is evidence that the His tag can provide a ligand to the heme-iron, at least in the ferric state. In addition, a His tag was seen to increase the solubility and stability of holo-CcmE (Dr. Julie Stevens, unpublished results). Therefore, it has to be possible that the His tag may interfere with the heme transfer reaction from CcmE to apocytochrome *c*. It seems unlikely that the His tag affects the qualitative result that apocytochromes with a CXXCH motif can break the covalent bond between heme and CcmE to yield holocytochrome *c*. However, the kinetic aspects of this reaction may be altered in the absence of a His tag. As the His tag increases the stability of the holo-CcmE protein, it may shield the heme from interacting with the apocytochrome *c* substrate and may lead to a more strongly coordinated heme-iron. Thus it could be that such a tag slows down the heme transfer reaction. Furthermore, the negative results of the heme release from holo-CcmE^{sol}-C-His$_6$ to *H. thermophilus* apocytochrome variants with only one or no cysteine residues in the heme-binding motif have to be regarded as preliminary data, although they are consistent with *in vivo* results (Schulz et al. 1998; Allen et al. 2002). More rigorous analysis of the heme transfer reaction from a holo-CcmE variant without a His tag is required to provide definite results *in vitro*. Additionally, it has recently been

shown that the membrane anchor of wild-type *E. coli* CcmE plays an important role in the *in vivo* formation of holocytochrome *c*, as variants of CcmE lacking the N-terminal membrane anchor had a reduced activity in cytochrome *c* maturation *in vivo* (Enggist & Thöny-Meyer 2003). Therefore, *in vitro* studies with the soluble domain of holo-CcmE on the heme transfer reaction from holo-CcmE to apocytochromes *c* have to be compared with complementary *in vivo* experiments.

It is clear that the complete pathway of *c*-type cytochrome biogenesis in Gram-negative bacteria can only be elucidated by more detailed studies involving more of the Ccm apparatus, and the combined function of the Ccm gene products. However, the data presented in this chapter provide insight into the heme release from CcmE to the apocytochrome *c*. The purpose of the covalent bond between heme and the heme chaperone, CcmE, remains unclear, although breaking of this bond might be mechanistically coupled to the formation of the thioether bonds between heme and apocytochrome *c*.

CHAPTER 9

CONCLUSIONS AND FUTURE RESEARCH PERSPECTIVES

9.1. CONCLUSIONS

Despite the fact that cytochrome *c* is one of the most studied of all proteins, and plays an essential role in cellular respiration, the assembly process whereby the heme becomes attached covalently to the polypeptide is scarcely understood. Indeed, it has surprisingly been discovered in the last ten years that this assembly process is distinct in different cell types (Page et al. 1998; CHAPTER 1). In vertebrate, invertebrate and fungal mitochondria a single gene product known as a heme lyase catalyses the heme incorporation, whilst in many Gram-negative bacteria such as *Escherichia coli*, as well as in plant mitochondria, a much more complex system involving at least twelve gene products is needed (Thöny-Meyer 2002; CHAPTER 1). The complexity in bacteria may reflect the occurrence in these organisms of many different *c*-type cytochromes.

Before this project, some advances in the understanding of cytochrome *c* maturation had been made by studying the reaction of heme and apocytochromes *c*. It was observed that horse heart mitochondrial apocytochrome *c* was sufficiently structured to yield a non-covalent heme-protein complex, *i.e.* a *b*-type cytochrome (Dumont et al. 1994).

Further insights were gained from the unusual cytoplasmic formation of *Hydrogenobacter thermophilus* cytochrome c_{552} and site-directed variants with mutations in the CXXCH heme-binding motif. The apoprotein with a double mutation to give an AXXAH motif resulted in formation of a stable *b*-type cytochrome *in vivo* (in the cytoplasm of *E. coli*) and *in vitro* (Tomlinson & Ferguson 2000a).

Understanding the assembly of *c*-type cytochromes undoubtedly requires the establishment of *in vitro* systems. In this thesis two such systems were developed and studied. One of these systems was the apparently uncatalysed reaction of apocytochromes *c* with heme *in vitro* to give a product with thioether bonds between heme and protein. These findings are described in Chapters 3-5. Such *in vitro* thioether formation between heme and cysteine residues is a process which is chemically poorly understood and it was necessary to identify optimal reaction conditions.

In Chapter 3, it is described how, upon addition of heme, *H. thermophilus* apocytochrome c_{552} initially forms a *b*-type cytochrome, which is very similar to the heme-protein species obtained from the AXXAH mutant of this protein. However, it was observed that the wild-type protein was capable of *in vitro* thioether bond formation from this *b*-type cytochrome intermediate. It emerged that optimal reaction conditions required both the heme and the cysteine residues in the CXXCH motif to be reduced. An issue, which was also addressed, was the potential formation of a disulfide bond between the two cysteine residues in the apocytochrome that bind the heme in the holoprotein. Such a disulfide bond was found to be able to form in oxidising conditions. The observations in Chapter 3 very strongly imply that the unusual cytoplasmic formation of *H. thermophilus* holocytochrome c_{552} in *E. coli* is essentially an uncatalysed process analogous to the *in vitro* reaction studied here.

In Chapter 4, two aspects of the *in vitro* thioether bond formation were investigated. Firstly, it was shown that thioether bonds can form independently of one another in cytochrome *c* derivatives obtained from reaction of the apoprotein

with heme derivatives with only one vinyl group, or reaction of heme with apocytochromes containing only one heme-binding cysteine residue. Secondly, it was shown that thioether bond formation in the thermostable *H.thermophilus* cytochrome c_{552} variants was stereoselective with respect to the α, γ mesoaxis of heme.

Chapter 5 describes the general applicability of the observations on *in vitro* thioether bond formation in cytochromes *c* to the folding pathway of apocytochromes *c* in general, as well as to their affinity for hydrophobic ligands. It was found that horse heart and yeast mitochondrial apocytochromes *c* had a very similar reactivity towards heme as *H. thermophilus* cytochrome c_{552}. It was also shown that disulfide bonds can form in the CXXCH motif of both horse heart and *Paracoccus denitrificans* apocytochromes *c*. The latter was also capable of forming thioether bonds to heme *in vitro*, but not to the same extent as *H. thermophilus* cytochrome c_{552}. These data have implications for the relative stability of different apocytochromes and for the catalytic mechanism of heme lyase. Furthermore, as a general trend, it is apparent that apocytochromes *c* can form *b*-type cytochromes upon addition of heme. The positioning of the cysteine thiols of the protein in proximity to the vinyl groups of heme can then lead to *in vitro* thioether bond formation.

A second *in vitro* system for studying cytochrome *c* maturation, involving the *E. coli* heme chaperone CcmE, was investigated. Heme transfer to apocytochromes *c* in the periplasms of many bacteria involves this protein. Previous work with this protein has focussed on *in vivo* experiments (Schulz et al. 1999; Schulz et al.

1998). Chapters 6-8 of this thesis describe *in vitro* studies of the interaction of heme with the apoform of CcmE and of holo-CcmE with apocytochromes *c*.

Chapter 6 describes how apo-CcmE interacts with heme to yield a heme-protein complex. The presence of a hydrophobic region on the protein capable of binding hydrophobic ligands such as heme was substantiated by binding studies with ANS, which bound to apo-CcmE. It was observed that CcmE has a preference for ferric rather than ferrous heme. The presence of a His tag affected the heme binding properties of the protein. However, heme binding was still observed with the protein lacking a His tag. The addition of low molecular weight thiol-containing compounds or dithionite to the non-covalent heme-protein complex led to the formation of a covalent heme-protein bond. This covalent bond formation was aided by the presence of the His tag. Mesoheme was not capable of forming this covalent bond implying that the heme-vinyl group(s) are required for the bond formation.

Chapter 7 describes the interaction of heme with CcmE variants containing site-directed mutations of the heme-binding histidine residue H130. This work established that this histidine residue of the heme chaperone is involved in the *in vitro* covalent bond formation to heme. The observation that replacement of the histidine by a cysteine yields a protein capable of binding heme covalently *in vivo* and *in vitro* led to mechanistic insights.

In Chapter 8, *in vitro* heme transfer from *in vivo* produced holo-CcmE to apocytochrome *c* is described. Under reductive conditions, heme transfer from CcmE to an apocytochrome *c* containing two cysteine residues in the CXXCH

motif was observed. Therefore, the CcmE heme-histidine bond was broken upon addition of the apocytochrome *c*, which in turn bound heme via thioether bonds to yield a holocytochrome *c*. Preliminary data are presented that suggests that single cysteine variants or cysteine-lacking forms of apocytochrome *c* cannot release heme from holo-CcmE. However, the presence of a His tag in the form of CcmE used for this work may affect these observations.

Interestingly, there are some similarities between the *in vitro* reaction of apocytochrome *c* with heme and *in vitro* heme transfer from holo-CcmE to apocytochrome *c*. Both reactions can only occur when the cysteine residue(s) of the apocytochromes *c* are reduced, *i.e.*, they contain a free thiol functionality. Furthermore, the heme iron needs to be reduced for thioether bond formation between the heme and the apocytochrome to occur.

Surprisingly, bond formation between the heme-binding histidine of CcmE and heme also requires the heme iron to be ferrous. As it was shown that the heme-vinyl group(s) react with the cysteine or histidine residues of apocytochrome *c* or CcmE, a similar mechanism for these covalent bond formations was suggested. The data were substantiated by the fact that the heme-binding histidine H130 could be mutated to a heme-binding cysteine residue in CcmE.

9.2. Future Research Perspectives

This project has shed further light on some aspects of cytochrome *c* maturation by investigating *in vitro* systems to yield information on chemical aspects of such reactions. However, a variety of experiments remain to be carried out in future

work. Three different directions are highlighted here. These are studies on the folding pathway of apocytochromes *c* and their reaction with heme derivatives, a more detailed analysis of the CcmE protein and an extension of the *in vitro* studies to the interactions between heme lyase, apocytochromes *c* and heme.

9.2.1. Characterisation of apocytochromes *c* and studies of *in vitro* thioether bond formation

Key aspects for thioether bond formation in *c*-type cytochromes are the folding state of the apocytochrome and the reactivity of the cysteine thiols toward heme *in vivo*. One approach to characterising an apocytochrome was to measure the hydrodynamic radius of the apocytochrome *c* of the C11A/C14A variant of *H. thermophilus* cytochrome c_{552} (Wain et al. 2001). Similar experiments could be performed with the apocytochromes *c* obtained in the present work, for example horse heart apocytochrome *c*. A more ambitious project would be to study the 3-D structure of an apocytochrome *c* in the absence and presence of a hydrophobic ligand like ANS. However, this type of experiment may be limited by the lack of a sufficiently organised structural domain in the protein.

In an extension of this work, novel derivatives of heme that could react faster with the apoprotein could be studied, which might provide mechanistic insights into the reaction. These heme derivatives could contain reactive side chains, for example acetylene or α-bromoethyl functionalities, in place of the vinyl groups. Furthermore, extensive kinetic studies, for example the effects of pH, temperature, relative reactant concentrations, may assist in defining, in molecular detail, the reaction between heme and apocytochrome *c*.

9.2.2. Characterisation of CcmE and its heme binding and heme release properties

The availability of soluble CcmE variants, which contain heme either non-covalently or covalently bound, should allow extensive characterisation of the heme binding and heme release by this protein. Aspects of interest for cytochrome *c* maturation are the nature of the axial ligands to the heme iron in both the *b*-type form and the covalently bound heme form of CcmE. To characterise heme proteins a variety of techniques is available including electron paramagnetic resonance (EPR), magnetic circular dichroism (MCD) and Resonance Raman spectroscopies.

Another important feature of holo-CcmE is the novel histidine-heme linkage. Interesting insights might be gained by studying the bond formation between CcmE and heme derivatives including mono-vinyl hemes. A preference for one mono-vinyl heme over the other may have important implications for heme recognition by the heme chaperone and subsequent stereospecific heme transfer to apocytochrome *c*.

Nuclear magnetic resonance (NMR) experiments may help to elucidate the exact nature of the heme-histidine bond, which, presumably, plays a key role in heme binding and release by this protein. However, the observation that the ferric heme-CcmE complex is high-spin in the absence of a His tag may lead to difficulties in interpreting NMR data. NMR spectra of the heme-CcmE complex could also yield information on the heme-binding region of CcmE and thus characterise the heme-protein interaction.

As discussed in Chapter 8, a detailed analysis of the effect of a His tag on the heme transfer from holo-CcmE to apocytochromes *c* needs to be carried out. This could lead to experiments, which allow very specific questions such as the sequence of events during heme transfer to be answered using the *in vitro* heme transfer system (see CHAPTER 8).

9.2.3. *In vitro* studies of cytochrome *c* formation in the presence of heme lyase

An investigation of the interaction between the mitochondrial cytochrome *c* biogenesis enzyme heme lyase and apocytochrome *c* could improve the presently limited understanding of the catalytic action of this enzyme. Characteristics of its interaction with the apocytochrome could be defined using a range of biophysical techniques. Such experiments should be carried out both in the presence and absence of heme in order to obtain fundamental information such as whether the heme is initially bound to the lyase before transfer to the apoprotein. Heme analogues that lack the reactive side chain vinyl groups that form the covalent bonds may play an important role here, as well as the availability of cytochrome *c* variants lacking one or both of the heme binding cysteine residues.

Furthermore, the *in vitro* studies in this thesis, along with previous work, have shown that chemical conditions during the thioether bond formation need to be strictly controlled to avoid side product formation and allow formation of properly matured cytochrome *c*. By exploring at the experimental conditions required for maximum enzymatic action of heme lyase and by studying the final product obtained and its potential side products, major insights into this cellular process could be obtained. Overall, more light can be shed on the understanding of cytochrome *c* maturation by studying it using the methodologies of *in vitro* cytochrome *c* formation.

BIBLIOGRAPHY

Abraham, B. D., Sono, M., Boutaud, O., Shriner, A., Dawson, J. H., Brash, A. R. & Gaffney, B. J. 2001 Characterization of the coral allene oxide synthase active site with UV- visible absorption, magnetic circular dichroism, and electron paramagnetic resonance spectroscopy: evidence for tyrosinate ligation to the ferric enzyme heme iron. *Biochemistry* **40**, 2251-9.

Abramson, J., Svensson-Ek, M., Byrne, B. & Iwata, S. 2001 Structure of cytochrome *c* oxidase: a comparison of the bacterial and mitochondrial enzymes. *Biochim Biophys Acta* **1544**, 1-9.

Allen, J. W., Tomlinson, E. J., Hong, L. & Ferguson, S. J. 2002 The *Escherichia coli* cytochrome *c* maturation (Ccm) system does not detectably attach heme to single cysteine variants of an apocytochrome *c*. *J Biol Chem* **277**, 33559-63.

Arnesano, F., Banci, L., Barker, P. D., Bertini, I., Rosato, A., Su, X. C. & Viezzoli, M. S. 2002 Solution structure and characterization of the heme chaperone CcmE. *Biochemistry* **41**, 13587-94.

Arnesano, F., Banci, L., Bertini, I., Ciofi-Baffoni, S., Woodyear, T. L., Johnson, C. M. & Barker, P. D. 2000 Structural consequences of *b*- to *c*-type heme conversion in oxidized *Escherichia coli* cytochrome b_{562}. *Biochemistry* **39**, 1499-514.

Arnesano, F., Banci, L., Bertini, I., Faraone-Mennella, J., Rosato, A., Barker, P. D. & Fersht, A. R. 1999 The solution structure of oxidized *Escherichia coli* cytochrome b_{562}. *Biochemistry* **38**, 8657-70.

Arslan, E., Schulz, H., Zufferey, R., Kunzler, P. & Thöny-Meyer, L. 1998 Overproduction of the *Bradyrhizobium japonicum* *c*-type cytochrome subunits of the cbb_3 oxidase in *Escherichia coli*. *Biochem Biophys Res Commun* **251**, 744-7.

Banci, L., Bertini, I., Gray, H. B., Luchinat, C., Reddig, T., Rosato, A. & Turano, P. 1997 Solution structure of oxidized horse heart cytochrome *c*. *Biochemistry* **36**, 9867-77.

Banci, L., Bertini, I., Rosato, A. & Scacchieri, S. 2000 Solution structure of oxidised microsomal rabbit cytochrome b_5. Factors determining the heterogeneous binding of the heme. *Eur J Biochem* **267**, 755-66.

Barker, P. D., Bertini, I., Del Conte, R., Ferguson, S. J., Hajieva, P., Tomlinson, E., Turano, P. & Viezzoli, M.S. 2001 A further clue to understanding the mobility of mitochondrial yeast cytochrome *c*: a (15)N T1rho investigation of the oxidized and reduced species. *Eur J Biochem* **268**, 4468-76.

Barker, P. D. & Ferguson, S. J. 1999 Still a puzzle: why is haem covalently attached in *c*-type cytochromes? *Structure Fold Des* **7**, R281-90.

Barker, P. D., Ferrer, J. C., Mylrajan, M., Loehr, T. M., Feng, R., Konishi, Y., Funk, W. D., MacGillivray, R. T. & Mauk, A. G. 1993 Transmutation of a heme protein. *Proc Natl Acad Sci U S A* **90**, 6542-6.

Barker, P. D., Nerou, E. P., Freund, S. M. & Fearnley, I. M. 1995 Conversion of cytochrome b_{562} to *c*-type cytochromes. *Biochemistry* **34**, 15191-203.

Bartsch, R. G. 1971 Cytochromes: Bacterial. *Methods Enzymol* **23**, 344-63.

Basile, G., Di Bello, C. & Taniuchi, H. 1980 Formation of an iso-1-cytochrome *c*-like species containing a covalently bonded heme group from the apoprotein by a yeast cell-free system in the presence of hemin. *J Biol Chem* **255**, 7181-91.

Baysse, C., Budzikiewicz, H., Uria Fernandez, D. & Cornelis, P. 2002 Impaired maturation of the siderophore pyoverdine chromophore in *Pseudomonas fluorescens* ATCC 17400 deficient for the cytochrome *c* biogenesis protein CcmC. *FEBS Lett* **523**, 23-8.

Beckett, C. S., Loughman, J. A., Karberg, K. A., Donato, G. M., Goldman, W. E. & Kranz, R. G. 2000 Four genes are required for the system II cytochrome *c* biogenesis pathway in *Bordetella pertussis*, a unique bacterial model. *Mol Microbiol* **38**, 465-81.

Berks, B. C., Ferguson, S. J., Moir, J. W. & Richardson, D. J. 1995 Enzymes and associated electron transport systems that catalyse the respiratory reduction of nitrogen oxides and oxyanions. *Biochim Biophys Acta* **1232**, 97-173.

Boduszek, B. & Wieczorek, J. S. 1980 Synthesis of Dipyridinyl Sulfides from Pyridyl-Pyridinium Halides. *Monatsh Chem* **111**, 1111-6.

Bullock, W. O., Fernandez, J. M. & Short, J. M. 1987 XL1-Blue - A high-efficiency plasmid transforming reca *Escherichia coli* strain with β-galactosidase selection. *Biotechniques* **5**, 376-8.

Bushnell, G. W., Louie, G. V. & Brayer, G. D. 1990 High-resolution three-dimensional structure of horse heart cytochrome *c*. *J Mol Biol* **214**, 585-95.

Cartron, M. L., Roldan, M. D., Ferguson, S. J., Berks, B. C. & Richardson, D. J. 2002 Identification of two domains and distal histidine ligands to the four haems in the bacterial *c*-type cytochrome NapC; the prototype connector between quinol/quinone and periplasmic oxido-reductases. *Biochem J* **368**, 425-32.

Colleran, E. M. & Jones, O. T. 1973 Studies on the biosynthesis of cytochrome *c*. *Biochem J* **134**, 89-96.

Cook, G. M. & Poole, R. K. 2000 Oxidase and periplasmic cytochrome assembly in *Escherichia coli* K-12: CydDC and CcmAB are not required for haem-membrane association. *Microbiology* **146**, 527-36.

Crooke, H. & Cole, J. 1995 The biogenesis of *c*-type cytochromes in *Escherichia coli* requires a membrane-bound protein, DipZ, with a protein disulphide isomerase-like domain. *Mol Microbiol* **15**, 1139-50.

Cutler, R. L., Pielak, G. J., Mauk, A. G. & Smith, M. 1987 Replacement of cysteine-107 of *Saccharomyces cerevisiae* iso-1- cytochrome *c* with threonine: improved stability of the mutant protein. *Protein Eng* **1**, 95-9.

Czjzek, M., ElAntak, L., Zamboni, V., Morelli, X., Dolla, A., Guerlesquin, F. & Bruschi, M. 2002 The Crystal Structure of the Hexadeca-Heme Cytochrome Hmc and a Structural Model of Its Complex with Cytochrome *c*(3). *Structure (Camb)* **10**, 1677-86.

Dailey, H. A. 2002 Terminal steps of haem biosynthesis. *Biochem Soc Trans* **30**, 590-5.

Daltrop, O., Stevens, J. M., Higham, C. W. & Ferguson, S. J. 2002 The CcmE protein of the *c*-type cytochrome biogenesis system: unusual *in vitro* heme incorporation into apo-CcmE and transfer from holo-CcmE to apocytochrome. *Proc Natl Acad Sci U S A* **99**, 9703-8.

Deshmukh, M., Turkarslan, S., Astor, D., Valkova-Valchanova, M. & Daldal, F. 2003 The Dithiol:Disulfide Oxidoreductases DsbA and DsbB of *Rhodobacter capsulatus* Are Not Directly Involved in Cytochrome *c* Biogenesis, but Their Inactivation Restores the Cytochrome *c* Biogenesis Defect of CcdA- Null Mutants. *J Bacteriol* **185**, 3361-72.

Dickerson, R. E., Takano, T., Eisenberg, D., Kallai, O. B., Samson, L., Cooper, A. & Margoliash, E. 1971 Ferricytochrome *c*. I. General features of the horse and bonito proteins at 2.8 A resolution. *J Biol Chem* **246**, 1511-35.

Diekert, K., Kispal, G., Guiard, B. & Lill, R. 1999 An internal targeting signal directing proteins into the mitochondrial intermembrane space. *Proc Natl Acad Sci U S A* **96**, 11752-7.

Dumont, M. E., Corin, A. F. & Campbell, G. A. 1994 Noncovalent binding of heme induces a compact apocytochrome *c* structure. *Biochemistry* **33**, 7368-78.

Eaves, D. J., Grove, J., Staudenmann, W., James, P., Poole, R. K., White, S. A., Griffiths, I. & Cole, J. A. 1998 Involvement of products of the nrfEFG genes in the covalent attachment of haem *c* to a novel cysteine-lysine motif in the cytochrome c_{552} nitrite reductase from *Escherichia coli*. *Mol Microbiol* **28**, 205-16.

Einsle, O., Messerschmidt, A., Stach, P., Bourenkov, G. P., Bartunik, H. D., Huber, R. & Kroneck, P. M. 1999 Structure of cytochrome *c* nitrite reductase. *Nature* **400**, 476-80.

Enggist, E. & Thöny-Meyer, L. 2003 The C-terminal flexible domain of the heme chaperone CcmE is important but not essential for its function. *J Bacteriol* **185**, 3821-7.

Enggist, E., Thöny-Meyer, L., Güntert, P. & Pervushin, K. 2002 NMR Structure of the Heme Chaperone CcmE Reveals a Novel Functional Motif. *Structure* **10**, 1551-7.

Enggist, E., Schneider, M. J., Schulz, H. & Thöny-Meyer, L. 2003 Biochemical and mutational characterization of the heme chaperone CcmE reveals a heme binding site. *J Bacteriol* **185**, 175-83.

Englander, S. W., Sosnick, T. R., Mayne, L. C., Shtilerman, M., Qi, P. X. & Bai, Y. 1998 Fast and slow folding in cytochrome *c*. *Acc Chem Res* **31**, 737-44.

Erlendsson, L. S., Acheson, R. M., Hederstedt, L. & Le Brun, N. E. 2003 *Bacillus subtilis* ResA Is a Thiol-Disulfide Oxidoreductase involved in Cytochrome *c* Synthesis. *J Biol Chem* **278**, 17852-8.

Erlendsson, L. S. & Hederstedt, L. 2002 Mutations in the thiol-disulfide oxidoreductases BdbC and BdbD can suppress cytochrome *c* deficiency of CcdA-defective *Bacillus subtilis* cells. *J Bacteriol* **184**, 1423-9.

Fabianek, R. A., Hennecke, H. & Thöny-Meyer, L. 2000 Periplasmic protein thiol:disulfide oxidoreductases of *Escherichia coli*. *FEMS Microbiol Rev* **24**, 303-16.

Fabianek, R. A., Hofer, T. & Thöny-Meyer, L. 1999 Characterization of the *Escherichia coli* CcmH protein reveals new insights into the redox pathway required for cytochrome *c* maturation. *Arch Microbiol* **171**, 92-100.

Falk, J. E. 1964 *Porphyrins and metalloporphyrins; their general, physical and coordination chemistry, and laboratory methods*. B.B.A. library ; v. 2. Amsterdam, New York,: Elsevier Pub. Co.

Falk, J. E. & Smith, K. M. 1975 *Porphyrins and metalloporphyrins : a new edition based on the original volume by J. E. Falk*. Amsterdam ; New York: Elsevier Scientific Pub. Co.

Fee, J. A., Chen, Y., Todaro, T. R., Bren, K. L., Patel, K. M., Hill, M. G., Gomez-Moran, E., Loehr, T. M., Ai, J., Thöny-Meyer, L., Williams, P. A., Stura, E., Sridhar, V. & McRee, D. E. 2000 Integrity of *Thermus thermophil*us cytochrome c_{552} synthesized by *Escherichia coli* cells expressing the host-specific cytochrome *c* maturation genes, ccmABCDEFGH: biochemical, spectral, and structural characterization of the recombinant protein. *Protein Sci* **9**, 2074-84.

Ferguson, S. J. 1991 The Functions and Synthesis of Bacterial *C*-Type Cytochromes with Particular Reference to *Paracoccus denitrificans* and *Rhodobacter capsulatus*. *Biochim Biophys Acta* **1058**, 17-20.

Ferguson, S. J. 2001 Keilin's cytochromes: how bacteria use them, vary them and make them. *Biochem Soc Trans* **29**, 629-40.

Fisher, W. R., Taniuchi, H. & Anfinsen, C. B. 1973 On the role of heme in the formation of the structure of cytochrome *c*. *J Biol Chem* **248**, 3188-95.

Florens, L. & Bruschi, M. 1994 Recent advances in the characterization of the hexadecahemic cytochrome *c* from *Desulfovibrio*. *Biochimie* **76**, 561-8.

Fontana, A., Veronese, F. M. & Boccu, E. 1973 Reaction of sulfenyl halides with cytochrome *c*. A novel method for heme cleavage. *FEBS Lett* **32**, 135-8.

Fulop, V., Moir, J. W., Ferguson, S. J. & Hajdu, J. 1995 The anatomy of a bifunctional enzyme: structural basis for reduction of oxygen to water and synthesis of nitric oxide by cytochrome cd_1. *Cell* **81**, 369-77.

Garrard, W. T. 1972 Synthesis, assembly, and localization of periplasmic cytochrome *c*. *J Biol Chem* **247**, 5935-43.

Gill, S. C. & von Hippel, P. H. 1989 Calculation of protein extinction coefficients from amino acid sequence data. *Anal Biochem* **182**, 319-26.

Goldberg, M. E., Schaeffer, F., Guillou, Y. & Djavadi-Ohaniance, L. 1999 Pseudo-native motifs in the noncovalent heme-apocytochrome *c* complex. Evidence from antibody binding studies by enzyme-linked immunosorbent assay and microcalorimetry. *J Biol Chem* **274**, 16052-61.

Goldman, B. S., Beck, D. L., Monika, E. M. & Kranz, R. G. 1998 Transmembrane heme delivery systems. *Proc Natl Acad Sci U S A* **95**, 5003-8.

Goldman, B. S., Gabbert, K. K. & Kranz, R. G. 1996 Use of heme reporters for studies of cytochrome biosynthesis and heme transport. *J Bacteriol* **178**, 6338-47.

Goldman, B. S. & Kranz, R. G. 2001 ABC transporters associated with cytochrome *c* biogenesis. *Res Microbiol* **152**, 323-9.

Goodhew, C. F., Brown, K.R. & Pettigrew, G.W. 1986 Haem staining in gels, a useful tool in the study of bacterial *c*-type cytochromes. *Biochim Biophys Acta* **852**, 288-94.

Hallberg, B. M., Bergfors, T., Backbro, K., Pettersson, G., Henriksson, G. & Divne, C. 2000 A new scaffold for binding haem in the cytochrome domain of the extracellular flavocytochrome cellobiose dehydrogenase. *Structure Fold Des* **8**, 79-88.

Hanahan, D. 1983 Studies on transformation of *Escherichia coli* with plasmids. *J Mol Biol* **166**, 557-80.

Hanlon, S. P., Toh, T. H., Solomon, P. S., Holt, R. A. & McEwan, A. G. 1996 Dimethylsulfide:acceptor oxidoreductase from *Rhodobacter sulfidophilus*. The purified enzyme contains *b*-type haem and a pterin molybdenum cofactor. *Eur J Biochem* **239**, 391-6.

Hasegawa, J., Yoshida, T., Yamazaki, T., Sambongi, Y., Yu, Y., Igarashi, Y., Kodama, T., Yamazaki, K., Kyogoku, Y. & Kobayashi, Y. 1998 Solution structure of thermostable cytochrome *c*-552 from *Hydrogenobacter thermophilus* determined by ^{1}H-NMR spectroscopy. *Biochemistry* **37**, 9641-9.

Heikkila, M. P., Honisch, U., Wunsch, P. & Zumft, W. G. 2001 Role of the Tat ransport system in nitrous oxide reductase translocation and cytochrome cd_1 biosynthesis in *Pseudomonas stutzeri*. *J Bacteriol* **183**, 1663-71.

Hennig, B. & Neupert, W. 1981 Assembly of cytochrome *c*. Apocytochrome *c* is bound to specific sites on mitochondria before its conversion to holocytochrome *c*. *Eur J Biochem* **121**, 203-12.

Herbaud, M. L., Aubert, C., Durand, M. C., Guerlesquin, F., Thöny-Meyer, L. & Dolla, A. 2000 *Escherichia coli* is able to produce heterologous tetraheme cytochrome *c*(3) when the ccm genes are co-expressed. *Biochim Biophys Acta* **1481**, 18-24.

Higuchi, Y., Kusunoki, M., Matsuura, Y., Yasuoka, N. & Kakudo, M. 1984 Refined structure of cytochrome c_3 at 1.8 A resolution. *J Mol Biol* **172**, 109-39.

Hill, K. E. & Wharton, D. C. 1978 Reconstitution of the apoenzyme of cytochrome oxidase from *Pseudomonas aeruginosa* with heme d_1 and other heme groups. *J Biol Chem* **253**, 489-95.

Hulse, C. L., Tiedje, J. M. & Averill, B. A. 1988 A spectrophotometric assay for dissimilatory nitrite reductases. *Anal Biochem* **172**, 420-6.

Hunte, C. 2001 Insights from the structure of the yeast cytochrome bc_1 complex: crystallization of membrane proteins with antibody fragments. *FEBS Lett* **504**, 126-32.

Igarashi, N., Moriyama, H., Fujiwara, T., Fukumori, Y. & Tanaka, N. 1997 The 2.8 A structure of hydroxylamine oxidoreductase from a nitrifying chemoautotrophic bacterium, *Nitrosomonas europaea*. *Nat Struct Biol* **4**, 276-84.

Iobbi-Nivol, C., Crooke, H., Griffiths, L., Grove, J., Hussain, H., Pommier, J., Mejean, V. & Cole, J. A. 1994 A reassessment of the range of *c*-type cytochromes synthesized by *Escherichia coli* K-12. *FEMS Microbiol Lett* **119**, 89-94.

Jones, A. S., Slater, M. J. & Walker, R. T. 1987 Some Chemical Reactions of 5-Vinyluracil and 2'-Deoxy-5-vinyluridine. *J Chem Soc Perkin Trans* **6**, 1325-9.

Kaderbhai, M. A., Ugochukwu, C. C., Lamb, D. C. & Kelly, S. L. 2000 Targeting of active human cytochrome P4501A1 (CYP1A1) to the periplasmic space of *Escherichia coli*. *Biochem Biophys Res Commun* **279**, 803-7.

Kang, X. & Carey, J. 1999 Role of heme in structural organization of cytochrome *c* probed by semisynthesis. *Biochemistry* **38**, 15944-51.

Karagianis, G., Reiss, J. A. & Scourides, P. A. 1993 Preparation and Characterisation of porphyrin *c* Analogues as Agents for Photodynamic Therapy. *Aust J Chem* **46**, 1755-62.

Karan, E. F., Russell, B. S. & Bren, K. L. 2002 Characterization of *Hydrogenobacter thermophilus* cytochromes *c*(552) expressed in the cytoplasm and periplasm of *Escherichia coli*. *J Biol Inorg Chem* **7**, 260-72.

Karim, A., Kaderbhai, N., Evans, A., Harding, V. & Kaderbhai, M. A. 1993 Efficient bacterial export of a eukaryotic cytoplasmic cytochrome. *Biotechnology (N Y)* **11**, 612-8.

Kalsbeck, W. A., Robertson, D. E., Pandey, R. K., Smith, K. M., Dutton, P. L. & Bocian, D. F. 1996 Structural and electronic properties of the heme cofactors in a multi- heme synthetic cytochrome. *Biochemistry* **35**, 3429-38.

Keightley, J. A., Sanders, D., Todaro, T. R., Pastuszyn, A. & Fee, J. A. 1998 Cloning and expression in *Escherichia coli* of the cytochrome c_{552} gene from *Thermus thermophilus* HB8. Evidence for genetic linkage to an ATP-binding cassette protein and initial characterization of the cycA gene products. *J Biol Chem* **273**, 12006-16.

Kellogg, J. A. & Bren, K. L. 2002 Characterization of recombinant horse cytochrome *c* synthesized with the assistance of *Escherichia coli* cytochrome *c* maturation factors. *Biochim Biophys Acta* **1601**, 215-21.

Kluck, R. M., Ellerby, L. M., Ellerby, H. M., Naiem, S., Yaffe, M. P., Margoliash, E., Bredesen, D., Mauk, A. G., Sherman, F. & Newmeyer, D. D. 2000 Determinants of cytochrome *c* pro-apoptotic activity. The role of lysine 72 trimethylation. *J Biol Chem* **275**, 16127-33.

Kniemeyer, O. & Heider, J. 2001 Ethylbenzene dehydrogenase, a novel hydrocarbon-oxidizing molybdenum/iron-sulfur/heme enzyme. *J Biol Chem* **276**, 21381-6.

Kojo, S. & Sano, S. 1981 Mechanism of a Novel Synthesis of Haemin *c* from Protohaemin and L-Cysteine. A Markownikoff-type Radical Addition Reaction. *J Chem Soc Perkin Trans* **1**, 2864-70.

Kojo, S., Fukunishi, K. & Tsukamoto, I. 1989 Retention of Configuration in the Non-Darwinian Evolution of Cytochrome *c*. *Angew Chem Int Ed Engl* **28**, 71-2.

Koppenhöfer, A. 1998 Functional Studies on the cytochrome-type nitrite reductase from *Thiosphaera pantotropha*. D. Phil. thesis, Oxford; University of Oxford.

Kranz, R. G., Beckett, C. S. & Goldman, B. S. 2002 Genomic analyses of bacterial respiratory and cytochrome *c* assembly systems: *Bordetella* as a model for the system II cytochrome *c* biogenesis pathway. *Res Microbiol* **153**, 1-6.

Kranz, R., Lill, R., Goldman, B., Bonnard, G. & Merchant, S. 1998 Molecular mechanisms of cytochrome *c* biogenesis: three distinct systems. *Mol Microbiol* **29**, 383-96.

Ladokhin, A. S. 2000 Fluorescence Spectroscopy in Peptide and Protein Analysis. In *Encyclopedia of Analytical Chemistry* (ed. R. A. Meyers), pp. 5762-79. Chichester: John Wiley & Sons Ltd.

Laemmli, U. K. 1970 Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680-5.

Laloi, C., Rayapuram, N., Chartier, Y., Grienenberger, J. M., Bonnard, G. & Meyer, Y. 2001 Identification and characterization of a mitochondrial thioredoxin system in plants. *Proc Natl Acad Sci U S A* **98**, 14144-9.

La Mar, G. N., Toi, H. & Krishnamoorthi, R. 1984 Proton NMR investigation of the rate and mechanism of heme rotation in sperm whale myoglobin - evidence for intramolecular reorientation about a heme twofold axis. *J Am Chem Soc* **106**, 6395-401.

Lange, C., Nett, J. H., Trumpower, B. L. & Hunte, C. 2001 Specific roles of protein-phospholipid interactions in the yeast cytochrome *bc*(1) complex structure. *Embo J* **20**, 6591-600.

Lawson, D. M., Stevenson, C. E., Andrew, C. R. & Eady, R. R. 2000 Unprecedented proximal binding of nitric oxide to heme: implications for guanylate cyclase. *Embo J* **19**, 5661-71.

Manting, E. H. & Driessen, A. J. 2000 Escherichia coli translocase: the unravelling of a molecular machine. *Mol Microbiol* **37**, 226-38.

Martinez, S. E., Huang, D., Szczepaniak, A., Cramer, W. A. & Smith, J. L. 1994 Crystal structure of chloroplast cytochrome *f* reveals a novel cytochrome fold and unexpected heme ligation. *Structure* **2**, 95-105.

Martinou, J. C., Desagher, S. & Antonsson, B. 2000 Cytochrome *c* release from mitochondria: all or nothing. *Nat Cell Biol* **2**, E41-3.

Matias, P. M., Coelho, A. V., Valente, F. M., Placido, D., LeGall, J., Xavier, A. V., Pereira, I. A. & Carrondo, M. A. 2002 Sulfate respiration in *Desulfovibrio vulgaris* Hildenborough. Structure of the 16-heme cytochrome *c* HmcA at 2.5-A resolution and a view of its role in transmembrane electron transfer. *J Biol Chem* **277**, 47907-16.

McCarthy, A. A., Haebel, P. W., Torronen, A., Rybin, V., Baker, E. N. & Metcalf, P. 2000 Crystal structure of the protein disulfide bond isomerase, DsbC, from *Escherichia coli*. *Nat Struct Biol* **7**, 196-9.

McLachlan, S. J., La Mar, G. N., Burns, P. D., Smith, K. M. & Langry, K. C. 1986 ^{1}H-NMR assignments and the dynamics of interconversion of the isomeric forms of cytochrome b_5 in solution. *Biochim Biophys Acta* **874**, 274-84.

McRee, D. E., Williams, P. A., Sridhar, V., Pastuszyn, A., Bren, K. L., Patel, K. M., Chen, Y., Todaro, T. R., Sanders, D., Luna, E. & Fee, J. A. 2001 Recombinant cytochrome rC_{557} obtained from *Escherichia coli* cells expressing a truncated *Thermus thermophilus* cycA gene. Heme inversion in an improperly matured protein. *J Biol Chem* **276**, 6537-44.

Means, G. E. & Feeney, R. E. 1971 *Chemical modification of proteins*. San Francisco,: Holden-Day.

Metheringham, R., Griffiths, L., Crooke, H., Forsythe, S. & Cole, J. 1995 An essential role for DsbA in cytochrome *c* synthesis and formate- dependent nitrite reduction by *Escherichia coli* K-12. *Arch Microbiol* **164**, 301-7.

Metheringham, R., Tyson, K. L., Crooke, H., Missiakas, D., Raina, S. & Cole, J. A. 1996 Effects of mutations in genes for proteins involved in disulphide bond formation in the periplasm on the activities of anaerobically induced electron transfer chains in *Escherichia coli* K12. *Mol Gen Genet* **253**, 95-102.

Metzger, S. U., Cramer, W. A. & Whitmarsh, J. 1997 Critical analysis of the extinction coefficient of chloroplast cytochrome *f*. *Biochim Biophys Acta* **1319**, 233-41.

Moore, G. R. & Pettigrew, G. W. 1990 *Cytochromes c : evolutionary, structural, and physicochemical aspects*. Springer series in molecular biology. New York: Springer-Verlag.

Moser, J., Schubert, W. D., Heinz, D. W. & Jahn, D. 2002 Structure and function of glutamyl-tRNA reductase involved in 5- aminolaevulinic acid formation. *Biochem Soc Trans* **30**, 579-84.

Munro, A. W., Taylor, P. & Walkinshaw, M. D. 2000 Structures of redox enzymes. *Curr Opin Biotechnol* **11**, 369-76.

Neilands, J. B. & Tuppy, H. 1960 Crystalline synthetic porphyrin *c*. *Biochim Biophys Acta* **38**, 351-3.

Nicholson, D. W. & Neupert, W. 1989 Import of cytochrome *c* into mitochondria: reduction of heme, mediated by NADH and flavin nucleotides, is obligatory for its covalent linkage to apocytochrome *c*. *Proc Natl Acad Sci U S A* **86**, 4340-4.

O'Brian, M. R. & Thöny-Meyer, L. 2002 Biochemistry, regulation and genomics of haem biosynthesis in prokaryotes. *Adv Microb Physiol* **46**, 257-318.

Page, C. C., Moser, C. C., Chen, X. & Dutton, P. L. 1999 Natural engineering principles of electron tunnelling in biological oxidation-reduction. *Nature* **402**, 47-52.

Page, M. D., Pearce, D. A., Norris, H. A. & Ferguson, S. J. 1997a The *Paracoccus denitrificans* ccmA, B and C genes: cloning and sequencing, and analysis of the potential of their products to form a haem or apo- *c*-type cytochrome transporter. *Microbiology* **143**, 563-76.

Page, M. D., Sambongi, Y. & Ferguson, S. J. 1998 Contrasting routes of *c*-type cytochrome assembly in mitochondria, chloroplasts and bacteria. *Trends Biochem Sci* **23**, 103-8.

Page, M. D., Tomlinson, E. & Ferguson, S. J. 1997b Unexpected implications from the *Helicobacter pylori* genome for understanding periplasmic *c*-type cytochrome assembly in gram-negative bacteria in coexistence with disulphide bond formation. *Mol Microbiol* **26**, 413-5.

Patel, C. N., Lind, M. C. & Pielak, G. J. 2001 Characterization of horse cytochrome *c* expressed in *Escherichia coli*. *Protein Expr Purif* **22**, 220-4.

Paul, K. G. 1950 The Splitting with Silver Salts of the Cysteine-porphyrin Bonds in Cytochrome *c*. *Acta Chem Skand* **4**, 239-244.

Paul, K. G. 1951 The Prophyrin Component of Cytochrome *c* and its Linkage to the Protein. *Acta Chem Skand* **5**, 389-405.

Pearce, D. A., Page, M. D., Norris, H. A., Tomlinson, E. J. & Ferguson, S. J. 1998 Identification of the contiguous *Paracoccus denitrificans* ccmF and ccmH genes: disruption of ccmF, encoding a putative transporter, results in formation of an unstable apocytochrome *c* and deficiency in siderophore production. *Microbiology* **144**, 467-77.

Pedrajas, J. R., Kosmidou, E., Miranda-Vizuete, A., Gustafsson, J. A., Wright, A. P. & Spyrou, G. 1999 Identification and functional characterization of a novel mitochondrial thioredoxin system in *Saccharomyces cerevisiae*. *J Biol Chem* **274**, 6366-73.

Pettigrew, G. W., Aviram, I. & Schejter, A. 1975 Physicochemical properties of two atypical cytochromes *c*, *Crithidia* cytochrome *c*-557 and *Euglena cytochrome c*-558. *Biochem J* **149**, 155-67.

Pettigrew, G. W. & Moore, G. R. 1987 *Cytochromes c : biological aspects.* Springer series in molecular biology. New York: Springer-Verlag.

Pilloud, D. L., Chen, X., Dutton, P. L. & Moser, C. C. 2000 Electrochemistry of self-assembled monolayers of iron protoporphyrin IX attached to modified gold electrodes through thioether linkage. *J Phy Chem B* **104**, 2868-77.

Pilloud, D. L., Moser, C. C., Reddy, K. S. & Dutton, P. L. 1998 Surface-promoted thioether linkage between proto- or hemato porphyrins and thiol-silanized quartz: Formation of self-assembled monolayers and interaction with imidazole and carbon monoxide. *Langmuir* **14**, 4808-18.

Pisa, R., Stein, T., Eichler, R., Gross, R. & Simon, J. 2002 The nrfI gene is essential for the attachment of the active site haem group of *Wolinella succinogenes* cytochrome *c* nitrite reductase. *Mol Microbiol* **43**, 763-70.

Pittman, M. S., Corker, H., Wu, G., Binet, M. B., Moir, A. J. & Poole, R. K. 2002 Cysteine is exported from the *Escherichia coli* cytoplasm by CydDC, an ATP-binding cassette-type transporter required for cytochrome assembly. *J Biol Chem* **277**, 49841-9.

Pollock, W. B., Rosell, F. I., Twitchett, M. B., Dumont, M. E. & Mauk, A. G. 1998 Bacterial expression of a mitochondrial cytochrome *c*. Trimethylation of lys72 in yeast iso-1-cytochrome *c* and the alkaline conformational transition. *Biochemistry* **37**, 6124-31.

Poole, R. K., Gibson, F. & Wu, G. 1994 The cydD gene product, component of a heterodimeric ABC transporter, is required for assembly of periplasmic cytochrome *c* and of cytochrome *bd* in *Escherichia coli*. *FEMS Microbiol Lett* **117**, 217-23.

Priest, J. W. & Hajduk, S. L. 1992 Cytochrome *c* reductase purified from *Crithidia fasciculata* contains an atypical cytochrome c_1. *J Biol Chem* **267**, 20188-95.

Pugsley, A. P., Bayan, N. & Sauvonnet, N. 2001 Disulfide bond formation in secreton component PulK provides a possible explanation for the role of DsbA in pullulanase secretion. *J Bacteriol* **183**, 1312-9.

Reid, E., Cole, J. & Eaves, D. J. 2001 The *Escherichia coli* CcmG protein fulfils a specific role in cytochrome *c* assembly. *Biochem J* **355**, 51-8.

Reid, E., Eaves, D. J. & Cole, J. A. 1998 The CcmE protein from *Escherichia coli* is a haem-binding protein. *FEMS Microbiol Lett* **166**, 369-75.

Ren, Q., Ahuja, U. & Thöny-Meyer, L. 2002 A bacterial cytochrome *c* heme lyase. CcmF forms a complex with the heme chaperone CcmE and CcmH but not with apocytochrome *c*. *J Biol Chem* **277**, 7657-63.

Ren, Q. & Thöny-Meyer, L. 2001 Physical interaction of CcmC with heme and the heme chaperone CcmE during cytochrome *c* maturation. *J Biol Chem* **276**, 32591-6.

Richardson, D. J. 2000 Bacterial respiration: a flexible process for a changing environment. *Microbiology* **146**, 551-71.

Richter, C. D. 2002 Kinetic studies of cytocrome cd_1 from *Paracoccus denitrificans*. M. Phil. thesis; Oxford: University of Oxford.

Richter, C. D., Allen, J. W., Higham, C. W., Koppenhöfer, A., Zajicek, R. S., Watmough, N. J. & Ferguson, S. J. 2002 Cytochrome cd_1, reductive activation and kinetic analysis of a multifunctional respiratory enzyme. *J Biol Chem* **277**, 3093-100.

Riddles, P. W., Blakeley, R. L. & Zerner, B. 1983 Reassessment of Ellman's reagent. *Methods Enzymol* **91**, 49-60.

Rios-Velazquez, C., Coller, R. & Donohue, T. J. 2003 Features of *Rhodobacter sphaeroides* CcmFH. *J Bacteriol* **185**, 422-31.

Rios-Velazquez, C., Cox, R. L. & Donohue, T. J. 2001 Characterization of *Rhodobacter sphaeroides* cytochrome *c*(2) proteins with altered heme attachment sites. *Arch Biochem Biophys* **389**, 234-44.

Ritz, D. & Beckwith, J. 2001 Roles of thiol-redox pathways in bacteria. *Annu Rev Microbiol* **55**, 21-48.

Robinson, C. & Bolhuis, A. 2001 Protein targeting by the twin-arginine translocation pathway. *Nat Rev Mol Cell Biol* **2**, 350-6.

Roldan, M. D., Sears, H. J., Cheesman, M. R., Ferguson, S. J., Thomson, A. J., Berks, B. C. & Richardson, D. J. 1998 Spectroscopic characterization of a novel multiheme *c*-type cytochrome widely implicated in bacterial electron transport. *J Biol Chem* **273**, 28785-90.

Rosell, F. I. & Mauk, A. G. 2002 Spectroscopic properties of a mitochondrial cytochrome *c* with a single thioether bond to the heme prosthetic group. *Biochemistry* **41**, 7811-8.

Sambongi, Y. & Ferguson, S. J. 1994a Specific thiol compounds complement deficiency in *c*-type cytochrome biogenesis in *Escherichia coli* carrying a mutation in a membrane-bound disulphide isomerase-like protein. *FEBS Lett* **353**, 235-8.

Sambongi, Y. & Ferguson, S. J. 1994b Synthesis of holo *Paracoccus denitrificans* cytochrome c_{550} requires targeting to the periplasm whereas that of holo *Hydrogenobacter thermophilus* cytochrome c_{552} does not. Implications for *c*-type cytochrome biogenesis. *FEBS Lett* **340**, 65-70.

Sambongi, Y., Stoll, R. & Ferguson, S. J. 1996 Alteration of haem-attachment and signal-cleavage sites for *Paracoccus denitrificans* cytochrome c_{550} probes pathway of *c*-type cytochrome biogenesis in *Escherichia coli*. *Mol Microbiol* **19**, 1193-204.

Sanbongi, Y., Yang, J. H., Igarashi, Y. & Kodama, T. 1991 Cloning, nucleotide sequence and expression of the cytochrome *c*-552 gene from *Hydrogenobacter thermophilus*. *Eur J Biochem* **198**, 7-12.

Sanders, C. & Lill, H. 2000 Expression of prokaryotic and eukaryotic cytochromes *c* in *Escherichia coli*. *Biochim Biophys Acta* **1459**, 131-8.

Sano, S. & Granick, S. 1961 Mitochondrial Coproporphrinogen Oxidase and Protoporphyrin Formation. *J Biol Chem* **236**, 1173-80.

Sano, S. & Tanaka, K. 1964 Recombination of Protoporphyrinogen with Cytochrome *c* Apoprotein. *J Biol Chem* **239**, PC3109-10.

Sargent, F., Berks, B. C. & Palmer, T. 2002 Assembly of membrane-bound respiratory complexes by the Tat protein-transport system. *Arch Microbiol* **178**, 77-84.

Schlarb, B. G., Wagner, M. J., Vijgenboom, E., Ubbink, M., Bendall, D. S. & Howe, C. J. 1999 Expression of plastocyanin and cytochrome *f* of the cyanobacterium *Phormidium laminosum* in *Escherichia coli* and *Paracoccus denitrificans* and the role of leader peptides. *Gene* **234**, 275-83.

Schroder, I., Rech, S., Krafft, T. & Macy, J. M. 1997 Purification and characterization of the selenate reductase from *Thauera selenatis*. *J Biol Chem* **272**, 23765-8.

Schubert, H. L., Raux, E., Matthews, M. A., Phillips, J. D., Wilson, K. S., Hill, C. P. & Warren, M. J. 2002 Structural diversity in metal ion chelation and the structure of uroporphyrinogen III synthase. *Biochem Soc Trans* **30**, 595-600.

Schulz, H., Fabianek, R. A., Pellicioli, E. C., Hennecke, H. & Thöny-Meyer, L. 1999 Heme transfer to the heme chaperone CcmE during cytochrome *c*

maturation requires the CcmC protein, which may function independently of the ABC- transporter CcmAB. *Proc Natl Acad Sci U S A* **96**, 6462-7.

Schulz, H., Hennecke, H. & Thöny-Meyer, L. 1998 Prototype of a heme chaperone essential for cytochrome *c* maturation. *Science* **281**, 1197-200.

Schulz, H., Pellicioli, E. C. & Thöny-Meyer, L. 2000 New insights into the role of CcmC, CcmD and CcmE in the haem delivery pathway during cytochrome *c* maturation by a complete mutational analysis of the conserved tryptophan-rich motif of CcmC. *Mol Microbiol* **37**, 1379-88.

Scott, R. A. & Mauk, A. G. 1995 *Cytochrome C : a multidisciplinary approach*. Mill Valley, CA: University Science Books.

Scourides, P. A., Morstyn, G. & Ngu, M. 1986 An Improved Synthesis of Porphyrin *C*. *J Chem Soc Chem Commun*, 1817-8.

Shedbalkar, V. P., Modi, S. & Mitra, S. 1988 Preparation and Characterisation of Iron(IV) Porphyrin in Aqueous Solution at Room Temperature: a Proposed Model for Peroxidase Compound II. *J Chem Soc Chem Commun*, 1238-9.

Shoolingin-Jordan, P. M., Spencer, P., Sarwar, M., Erskine, P. E., Cheung, K. M., Cooper, J. B. & Norton, E. B. 2002 5-Aminolaevulinic acid dehydratase: metals, mutants and mechanism. *Biochem Soc Trans* **30**, 584-90.

Simon, J., Gross, R., Einsle, O., Kroneck, P. M., Kroger, A. & Klimmek, O. 2000 A NapC/NirT-type cytochrome *c* (NrfH) is the mediator between the quinone pool and the cytochrome *c* nitrite reductase of *Wolinella succinogenes*. *Mol Microbiol* **35**, 686-96.

Sinha, N. & Ferguson, S. J. 1998 An *Escherichia coli* ccm (cytochrome *c* maturation) deletion strain substantially expresses *Hydrogenobacter thermophilus* cytochrome c_{552} in the cytoplasm: availability of haem influences cytochrome c_{552} maturation. *FEMS Microbiol Lett* **161**, 1-6.

Spielewoy, N., Schulz, H., Grienenberger, J. M., Thöny-Meyer, L. & Bonnard, G. 2001 CCME, a nuclear-encoded heme-binding protein involved in cytochrome *c* maturation in plant mitochondria. *J Biol Chem* **276**, 5491-7.

Steiner, H., Kispal, G., Zollner, A., Haid, A., Neupert, W. & Lill, R. 1996 Heme binding to a conserved Cys-Pro-Val motif is crucial for the catalytic function of mitochondrial heme lyases. *J Biol Chem* **271**, 32605-11.

Stevens, J. M., Daltrop, O., Higham, C. W. & Ferguson, S. J. 2003 Interaction of heme with variants of the heme chaperone CcmE carrying active site mutations and a cleavable N-terminal His tag. *J Biol Chem* **278**, 20500-6.

Stryer, L. 1965 The interaction of a naphthalene dye with apomyoglobin and apohemoglobin. A fluorescent probe of non-polar binding sites. *J Mol Biol* **13**, 482-95.

Tanaka, Y., Kubota, I., Amachi, T., Yoshizumi, H. & Matsubara, H. 1990 Site-directedly mutated human cytochrome *c* which retains heme *c* via only one thioether bond. *J Biochem (Tokyo)* **108**, 7-8.

Tezcan, F. A., Winkler, J. R. & Gray, H. B. 1998 Effects of ligation and folding on reduction potentials of heme proteins. *J Am Chem Soc* **120**, 13383-8.

Theorell, H. 1939 Über die chemische Konstitution des Cytochroms *c*. Darstellung von Porphyrin-Cystein-Addukten. *Biochem Z* **301**, 201-9.

Thöny-Meyer, L. 1997 Biogenesis of respiratory cytochromes in bacteria. *Microbiol Mol Biol Rev* **61**, 337-76.

Thöny-Meyer, L. 2002 Cytochrome *c* maturation: a complex pathway for a simple task? *Biochem Soc Trans* **30**, 633-8.

Thöny-Meyer, L. 2000 Haem-polypeptide interactions during cytochrome *c* maturation. *Biochim Biophys Acta* **1459**, 316-24.

Thöny-Meyer, L., Fabianek, R.A., Schulz, H., Enggist E., Henneke, H. 1999 Biosynthese von Cytochrom *c* in *Escherichia coli*. *Biospektrum* **5**, 185-191.

Thöny-Meyer, L. & Kunzler, P. 1997 Translocation to the periplasm and signal sequence cleavage of preapocytochrome *c* depend on sec and lep, but not on the ccm gene products. *Eur J Biochem* **246**, 794-9.

Throne-Holst, M., Thöny-Meyer, L. & Hederstedt, L. 1997 *Escherichia coli* ccm in-frame deletion mutants can produce periplasmic cytochrome *b* but not cytochrome *c*. *FEBS Lett* **410**, 351-5.

Tohjo, M. & Shibata, K. 1963 Absorption spectra of hematin complexes with polyhistidine and copoly-(histidine, glutamic acid). *Arch Biochem Biophys* **103**, 401-8.

Tomlinson, E. J. & Ferguson, S. J. 2000a Conversion of a *c* type cytochrome to a *b* type that spontaneously forms *in vitro* from apo protein and heme: implications for *c* type cytochrome biogenesis and folding. *Proc Natl Acad Sci U S A* **97**, 5156-60.

Tomlinson, E. J. & Ferguson, S. J. 2000b Loss of either of the two heme-binding cysteines from a class I *c*-type cytochrome has a surprisingly small effect on physicochemical properties. *J Biol Chem* **275**, 32530-4.

Tong, J. & Margoliash, E. 1998 Cytochrome *c* heme lyase activity of yeast mitochondria. *J Biol Chem* **273**, 25695-702.

Veloso, D., Basile, G. & Taniuchi, H. 1981 Formation of a cytochrome *c*-like species from horse apoprotein and hemin catalyzed by yeast mitochondrial cytochrome *c* synthetase. *J Biol Chem* **256**, 8646-51.

Vestweber, D. & Schatz, G. 1988 Mitochondria can import artificial precursor proteins containing a branched polypeptide chain or a carboxy-terminal stilbene disulfonate. *J Cell Biol* **107**, 2045-9.

Voet, D. & Voet, J. G. 1995 *Biochemistry*. New York ; Chichester: J. Wiley & Sons.

Vollhardt, K. P. C. & Schore, N. E. 1994 *Organic chemistry*. New York: W.H. Freeman.

Vu, B. C., Jones, A. D. & Lecomte, J. T. J. 2002 Novel-Histidine-Heme Covalent Linkage in a Hemoglobin. *J. Am. Chem. Soc.* **124**, 8544-5.

Wain, R., Pertinhez, T. A., Tomlinson, E. J., Hong, L., Dobson, C. M., Ferguson, S. J. & Smith, L. J. 2001 The cytochrome *c* fold can be attained from a compact apo state by occupancy of a nascent heme binding site. *J Biol Chem* **276**, 45813-7.

Wallis, R. & Drickamer, K. 1997 Asymmetry adjacent to the collagen-like domain in rat liver mannose- binding protein. *Biochem J* **325**, 391-400.

Warren, M. J., Cooper, J. B., Wood, S. P. & Shoolingin-Jordan, P. M. 1998 Lead poisoning, haem synthesis and 5-aminolaevulinic acid dehydratase. *Trends Biochem Sci* **23**, 217-21.

Whitham, G. H. 1995 *Organosulfur chemistry*. Oxford chemistry primers; 33. Oxford, Oxford University Press.

Wilkins, P. C. & Wilkins, R. G. 1997 *Inorganic chemistry in biology*. Oxford chemistry primers; 46. Oxford, Oxford University Press.

Wu, J. Z., La Mar, G. N., Yu, L. P., Lee, K. B., Walker, F. A., Chiu, M. L. & Sligar, S. G. 1991 H-1- NMR study of the solution molecular and electronic-structure of *Escherichia coli* ferricytochrome-b_{562}- Evidence for S=1/2 reversible S=5/2 spin equilibrium for intact his met ligation. *Biochemistry* **30**, 2156-65.

Yamamoto, Y. & La Mar, G. N. 1986 H NMR Study of Dynamics and Thermodynamics of Heme Rotational Disorder in Native and reconstituted Hemogloin A. *Biochemistry* **25**, 5288-97.

Yanisch-Perron, C., Vieira, J. & Messing, J. 1985 Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. *Gene* **33**, 103-19.

Yeh, S., Han, S. & Rousseau, D. L. 1998 Cytochrome *c* Folding and Unfolding: A Biphasic Mechanism. *Acc Chem Res* **31**, 727-36.

Zumft, W. G. 1997 Cell biology and molecular basis of denitrification. *Microbiol Mol Biol Rev* **61**, 533-616.

APPENDIX

Nucleotide sequences

Hydrogenobacter thermophilus cytochrome c_{552} nucleotide sequence

```
ATGAATGAAC AGCTTGCCAA GCAAAAGGGC TGTATGGCTT GCCACGATCT
GAAAGCTAAG AAGGTGGGAC CTGCTTACGC AGATGTAGCT AAGAAGTATG
CGGGAAGAAA GGATGCTGTT GATTATCTGG CTGGCAAGAT AAAGAAGGGC
GGTTCTGGTG TGTGGGGTTC TGTTCCCATG CCTCCTCAAA ATGTAACCGA
TGCGGAAGCA AAACAGCTTG CCCAGTGGAT ACTCTCCATA AAG
```

Escherichia coli N-His_6-$CcmE^{sol}$ nucleotide sequence

```
ATGGGCAGCA GCCATCATCA TCATCATCAC AGCAGCGGCC TGGTGCCGCG
CGGCAGCCAT ATGCTCGAGT CGAATATCGA TCTCTTTTAT ACGCCGGGGG
AAATTCTCTA CGGCAAGCGT GAAACTCAGC AAATGCCGGA AGTCGGTCAG
CGTCTGCGCG TTGGCGGGAT GGTGATGCCG GGTAGTGTGC AGCGCGATCC
CAATTCGCTG AAAGTGACCT TCACCATTTA CGATGCTGAA GGCTCAGTGG
ATGTCTCTTA CGAAGGCATT TTGCCGGATC TGTTCCGTGA AGGGCAGGGC
GTTGTGGTGC AGGGCGAACT GGAAAAAGGC AATCATATCC TCGCGAAAGA
AGTGCTGGCG AAACACGATG AAAACTATAC GCCGCCAGAA GTTGAGAAAG
CGATGGAAGC TAACCACCGT CGCCCGGCGA GTGTTTATAA GGACCCAGCA TCA
```

E. coli $CcmE^{sol}$-C -His_6 nucleotide sequence

```
ATGGATTCGA ATATCGATCT CTTTTATGCG CCGGGGGAAA TTCTCTACGG
CAAGCGTGAA ACTCAGCAAA TGCCGGAAGT CGGTCAGCGT CTGCGCGTTG
GCGGGATGGT GATGCCGGGT AGTGTGCAGC GCGATCCCAA TTCGCTGAAA
GTGACCTTCA CCATTTACGA TGCTGAAGGC TCAGTGGATG TCTCTTACGA
AGGCATTTTG CCGGATCTGT TCCGTGAAGG GCAGGGCGTT GTGGTGCAGG
GCGAACTGGA AAAAGGCAAT CATATCCTCG CGAAAGAAGT GCTGGCGAAA
CACGATGAAA ACTATACGCC GCCAGAAGTT GAGAAAGCGA TGGAAGCTAA
CCACCGTCGC CCGGCGAGTG TTTATAAGGA CCCAGCATCA CTCGAGCACC
ACCACCACCA CCAC
```

Protein sequences

Escherichia coli N-His_6-$CcmE^{sol}$ amino acid sequence

```
MGSSHHHHHH SSGLVPRGSH MLESNIDLFY TPGEILYGKR ETQQMPEVGQ
RLRVGGMVMP GSVQRDPNSL KVTFTIYDAE GSVDVSYEGI LPDLFREGQG
VVVQGELEKG NHILAKEVLA KHDENYTPPE VEKAMEANHR RPASVYKDPA S
```

E. coli $CcmE^{sol}$-C-His_6 amino acid sequence

```
MDSNIDLFYT PGEILYGKRE TQQMPEVGQR LRVGGMVMPG SVQRDPNSLK
VTFTIYDAEG SVDVSYEGIL PDLFREGQGV VVQGELEKGN HILAKEVLAK
HDENYTPPEV EKAMEANHRR PASVYKDPAS LEHHHHHH
```

Protein sequences of cytochromes *c*

Hydrogenobacter thermophilus cytochrome c_{552}

```
1           11          21          31          41
|           |           |           |           |
MNEQLAKQKG CMACHDLKAK KVGPAYADVA KKYAGRKDAV DYLAGKIKKG
GSGVWGSVPM PPQNVTDAEA KQLAQWILSI K
```

Horse heart cytochrome *c*

```
GDVEKGKKIF VQKCAQCHTV EKGGKHKTGP NLHGLFGRKT GQAPGFTYTD
ANKNKGITWK EETLMEYLEN PKKYIPGTKM IFAGIKKKTE REDLIAYLKK
ATNE
```

Yeast iso-1-cytochrome *c*

```
TEFKAGSAKK GATLFKTRCL QCHTVEKGGP HKVGPNLHGI FGRHSGQAEG
YSYTDANIKK NVLWDENNMS EYLTNPKKYI PGTKMAFGGL KKEKDRNDLI
TYLKKACE
```

Paracoccus denitrificans cytochrome c_{550}

```
QDGDAAKGEK EFNKCKACHM IQAPDGTDII KGGKTGPNLY GVVGRKIASE
EGFKYGEGIL EVAEKNPDLT WTEADLIEYV TDPKPWLVKM TDDKGAKTKM
TFKMGKNQAD VVAFLAQNSP DAGGDGEAAA EGESN
```

Spinach cytochrome *f*

```
MQTINTFSWI KEQITRSISI SLILYIITRS SIANAYPIFA QQGYENPREA
TGRIVCANCH LANKPVDIEV PQAVLPDTVF EAVVRIPYDM QLKQVLANGK
KGGLNVGAVL ILPEGFELAP PDRISPEMKE KMGNLSFQSY RPNKQNILVI
GPVPGQKYSE ITFPILAPDP ATKKDVHFLK YPIYVGGNRG RGQIYPDGSK
SNNTVYNSTA TGIVKKIVRK EKGGYEINIA DASDGREVVD IIPRGPELLV
SEGESIKLDQ PLTSNPNVGG FGQGDAEVVL QDPLRIQGLL FFFASVILAQ
IFLVLKKKQF EKVQLSEMNF
```

Phormidium laminosum soluble domain of cytochrome *f*

```
YPFWAQQNYA NPREATGRIV CANCHLAAKP AEIEVPQAVL PDSVFKAVVK
IPYDHSVQQV QADGSKGPLN VGAVLMLPEG FTIAPEDRIP EEMKEEVGPS
YLFQPYADDK QNIVLVGPLP GDQYEEIVFP VLSPNPATNK SVAFGKYSIH
LGANRGRGQI YPTGEKSNNA VYNASAAGVI TAIAKADDGS AEVKIRTEDG
TTIVDKIPAG PELIVSEGEE VAAGAALTNN PNVGGFGQKD TEIVLQSPNR
```