Cytochrome *c* biogenesis in bacteria.

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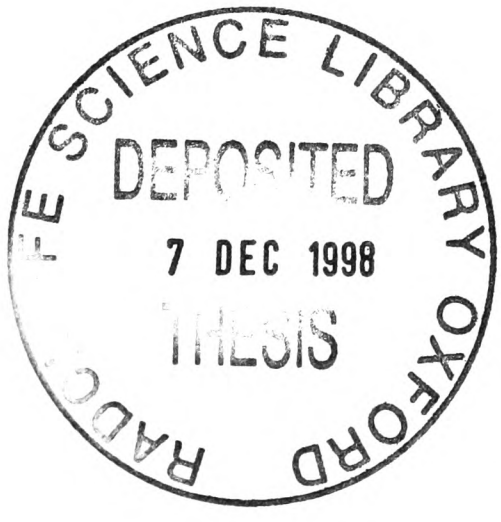

Where observation is concerned, chance favours only the prepared mind.

(Louis Pasteur)

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Abstract: Cytochrome *c* biogenesis in bacteria

Cytochromes *c* are electron transfer proteins in which haem is covalently attached to the polypeptide chain via thioether bonds formed from thiol groups of the two cysteines and the two vinyl groups of haem. This attachment is a post translational process and in many species of bacteria as many as approximately twelve gene products, the functions of which are largely unknown, are required. In Gram-negative bacteria the assembly of the *c*-type cytochromes occurs in the periplasm. Cytochrome c_{552} from the thermophilic organism *Hydrogenobacter thermophilus* is known to be expressed in the cytoplasm of *Escherichia coli*. This unique example of cytoplasmic assembly of a *c*-type cytochrome has previously been postulated to result from insertion of haem into the folded apoform of the cytochrome followed by non-catalysed attachment of the haem. This postulate is supported by the present work which has shown that the cytoplasmic assembly of this cytochrome c_{552} continues in the absence of the *E. coli ccm* genes which are needed for 'normal' *c*-type cytochrome assembly in that organism. Attempts to test the postulate of spontaneous assembly of the cytochrome c_{552} with *in vitro* experiments require large amounts of cytochrome c_{552} and its apo protein. A number of procedures for preparing these proteins were investigated. Although a T7-based expression produced lower amounts than was expected, its use led to detection of the apo form of cytochrome c_{552} in *E. coli*. It was shown that this apoform has some secondary structure, whereas mitochondrial apocytochrome *c* has a random coil conformation. This observation is consistent with, but does not prove, the postulate for cytochrome c_{552} assembly. It was unexpectedly found that a strain of *E. coli* that produces abnormally large amounts of its endogenous *c*-type cytochromes also made large amounts of cytoplasmic cytochrome c_{552}. NMR studies on this material are consistent with a single and 'normal' attachment of the

haem to the polypeptide. Thus the unusual cytoplasmic assembly was not different from the usual periplasmic assembly that occurs in the *H. thermophilus* itself.

In *E. coli* there is a periplasmic cytochrome b_{562} that is presumed to acquire its haem in the periplasm. Some of the *ccm* genes, required for *c*-type cytochrome assembly, are postulated to code for a system that transports haem to the periplasm. Cytochrome b_{562} synthesis was not blocked by the absence of these genes. This implies that haem provision for cytochrome b_{562} synthesis occurs independently of the *ccm* system. Apocytochrome b_{562} could be detected in *E. coli* with the ratio apo:holo being higher in a strain that produces *c*-type cytochromes to relatively low levels. It is suggested that the synthesis of both cytochrome b_{562} and *c*-type cytochromes is at least partly a reflection of the rate of production of haem by the cells.

Contents

Publications

Some of the work presented in this thesis has been published as follows:

a) As a paper:

1) Sinha, N. and 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 Microbiology letters* **161,** 1-6.

b) As abstracts at conferences:

1) Sinha, N. and Ferguson, S.J. Cytochrome *c* targeting and translocation in bacteria. *Bioch Soc Trans* **24,** 587S, submitted as a poster abstract in 4th IUBMB conference on the life and death of the cell held in Edinburgh in 1996.

2) Sinha, N. and Ferguson, S.J. Biogenesis of cytochrome c_{552} from thermophilic bacterium *Hydrogenobacter thermophilus*. *Conference Book of Abstracts* MO 2325, **40,** submitted as a poster abstract in 8th European Congress On Biotechnology, held in Budapest in 1997.

List of abbreviations

ATP	Adenosine triphosphate
bp	Base pairs
BSA	Bovine serum albumin
CM	Caboxy-methyl
DMSO	Dimethylsulphoxide
dNTP	Deoxynucleotide triphosphate
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
IPTG	Isopropyl-thio-β-D-galactoside
LB	Luria Bertani
MOPS	4-Morpholinepropanesulfonic acid
NMR	Nuclear magnetic resonance
O.D.	Optical density
PCR	Polymerase chain reaction
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
TAE	40 mM Tris-acetate pH 8.0, 1mM EDTA
TBE	45 mM Tris-borate pH 8.3, 1mM EDTA
TE	10 mM Tris-HCl pH 8.0, 1mM EDTA
TEMED	N, N, N', N'-tetramethylethylenediamine

X-Gal	5-bromo-4-chloro-3-indolyl-β-D-galactoside

X-Gal	5-bromo-4-chloro-3-indolyl-β-D-galactoside

Chapter I

General Introduction

1.1 General Background

Cytochromes are electron transfer proteins that carry haem as a prosthetic group. They play a central role in biological electron transport, the best known example probably being mitochondrial cytochrome *c*. However, the haem group can also participate in chemical reactions, as occurs for instance in cytochrome P_{450} which catalyses hydroxylation reactions. The haem cofactor of cytochromes absorbs light at specific wavelengths. Haem is thus readily detected by visible absorption spectroscopy which has allowed cytochromes to be classified into types a, b, c, d, d_1, f and o. Each of these types is distinguished by either the chemical structure of the haem or the mode of association of the haem with the protein. In *c*-type and *f*-type cytochromes the haem is attached to the polypeptide by thioether linkages between the two haem vinyl groups and the two cysteines in the conserved motif Cys-X-Y-Cys-His of the polypeptide (Figure 1.1). There are also unusual cytochromes *c* which have C-X-X-X-C-H (Denariaz *et al.,* 1989) and C-X-X-X-X-C-H (Ambler, 1973) sequences. Although C-X-C-H has not been found in any *c*-type cytochromes, it is present in the Rieske iron-sulphur proteins (Hauska *et al.,* 1988). On the other hand there are examples of proteins having the haem binding motif but which are not *c*-type cytochromes, for example 4Fe-4S ferredoxin subunit of *E. coli* DMSO reductase (Bilous *et al.*, 1988), Orf 2 of the *E. coli* formate hydrogenlyase (Bohm *et al.*, 1990), and the large subunit of *Alcaligenes eutrophus* hydrogenase (Kortluke *et al.,* 1992). The classification of *f*-type cytochrome is really redundant as there is no chemical difference from a *c*-type cytochrome in respect of the haem group. The name cytochrome *f* was introduced in studies of thylakoids and for reasons of tradition the name *f* (named from the Latin *frons*, for foliage) has been retained. In *a*-, *b*-, *d*-, d_1 and *o*-type cytochromes, the cofactor is bound noncovalently to the protein. The prosthetic group of

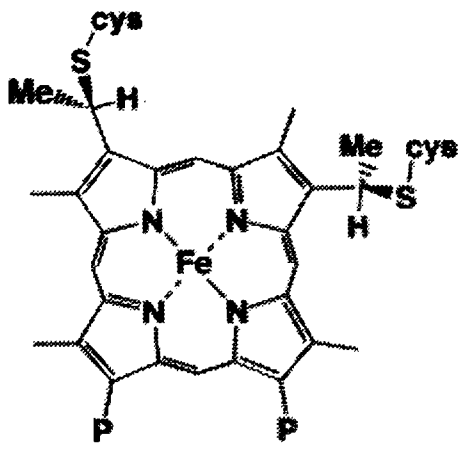


Figure 1.1. The Haem group in a *c*-type cytochrome showing the stereochemistry at the carbons of the thioether bonds formed between vinyl groups of protohaem IX and two cysteine residues of a polypeptide chain.

b- and *c*-type cytochromes is protohaem IX but the haems of other cytochromes are modified. Haems *o* and *a* both carry a farnesylhydroxyethyl side chain but haem *a* has an additional formyl group at position 8 of the tetrapyrrole ring. *d*-type haem has partial saturation of the pyrrole whilst d_1-type haem has undergone a complex modification (Wu and Chang, 1987) so as to fit it in some not fully understood way for its role in nitrite reduction in cytochrome cd_1 (Fulop *et al.*, 1995).

C-type cytochromes are subdivided into three classes mainly on the basis of amino acid sequence (Ambler 1990). In class I, the most common class, *c*-type cytochromes consist of 80-120 amino acid residues and have haem near the *N*-terminus of the protein. Class II *c*-type cytochromes have haem near the *C*-terminus, while class III *c*-type cytochromes are multihaem. These classes are further divided into subclasses on the basis of their tertiary structure (Pettigrew and Moore, 1987). Our present knowledge of the structure and physical properties of water soluble *c*-type cytochromes is quite extensive

(for review see Margoliash and Schejter 1996). Their easy availability from many sources, relative stability, solubility in water and defined functional significance has made them most interesting proteins for structural, genetic, evolutionary and biogenesis studies. *C*-type cytochromes are often low molecular weight and stable proteins that can reversibly withstand rather extreme conditions. Thus they are particularly suitable for studying the mechanisms by which such conditions affect protein structure. The depth of interest in the subject is illustrated by the publication of three monographs on the topic, by Pettigrew and Moore, 1987, Moore and Pettigrew, 1990 and Scott *et al.*, 1995.

In a *c*-type cytochrome the histidine of the C-X-X-C-H motif provides one of the axial ligands to the haem iron. The second axial ligand is most frequently methionine but there are many examples known where it is a second histidine residue. The recently determined structure of hydroxylamine oxidoreductase provides an example of the latter ligation as well as of a *c*-type cytochrome centre with only one axial ligand, the other position being available for binding of the substrate, hydroxylamine (Igarashi *et al.*, 1997). An unusual axial ligand is found in cytochrome *f* where the alpha group of the *N*-terminal amino acid is bound to the Fe of the haem group (Cramer *et al.*, 1994).

Almost all eukaryotes and many prokaryotes have *c*-type cytochromes. Eukaryotes have the soluble cytochrome *c*, located in the mitochondrial intermembrane space, and a membrane-bound cytochrome c_1, which is anchored to the inner mitochondrial membrane by a trans membrane helix but which has a substantial globular domain, containing the haem group, exposed to the inter-membrane space. A photosynthetic eukaryotic cell also contains one or two *c*-type cytochromes associated with the thylakoid membrane of the chloroplast. One of these is a membrane bound cytochrome, the already mentioned cytochrome *f*, which is associated with the cytochrome *bf* complex, found in the thylakoids

of all studied plants and algae. The second *c*-type cytochrome found in thylakoids occurs only in certain algae and is known as cytochrome c_6. In bacteria *c*-type cytochromes are topologically oriented outside the cytoplasmic membrane, such that in Gram-negative bacteria they are either water-soluble proteins in the periplasm or membrane anchored with their globular haem-containing domain located in the periplasm. In Gram-positive organisms, which formally lack a periplasm, it is likely that the *c*-type cytochromes are only of the membrane-anchored type (Pettigrew and Moore 1987; Page and Ferguson 1989). The strong similarities between certain aerobic bacteria and mitochondria and between cyanobacteria and chloroplasts suggests that the present mitochondria and the choloroplast are endosymbionts. The covalent attachment between the two cysteines and haem vinyl groups is universally present in *c*-type cytochromes with few exceptions in lower eukaryotes where the first cysteine of the haem binding motif, Cys-X-X-Cys-His, is missing and replaced by alanine; e.g. cytochromes *c* and c_1 from *Euglena* and *Crithidia* (Pettigrew, 1972, 1973; Mukai *et al.,* 1989; Priest and Hajduk, 1992). *b*-type cytochromes with their non-covalently bound haem group are generally structurally quite distinct from the *c*-type, although redox potentials covering almost the entire biologically relevant span can be found for both classes. Many *b*-type cytochromes are proteins with several transmembrane helices between which one or more haem groups can be sandwiched. This organisation, which has never been found for *c*-type cytochromes, is exemplified by the cytochrome bc_1 complex (Xia *et al.,* 1997) and the bacterial respiratory nitrate reductase enzyme (Berks *et al.,* 1995, Magalon *et al.,* 1997). A relatively rare example of close relatedness between *b* and *c*-type cytochromes is *E. coli* cytochrome b_{562} which has similarities with cytochrome *c′*; the latter is a type II class of *c*-type cytochrome that occurs, for example, in photosynthetic bacteria. This class of cytochrome has a

characteristic four α-helix structural fold which is also adopted by cytochrome b_{562}, although there is not significant sequence homology (Matthews, 1985). Cytochrome b_{562} is the only member of this family of cytochromes, none of which has an assigned function, not to have covalently bound haem and could, therefore, represent an evolutionary intermediate on a pathway to the *c*-type cytochromes. It is conceivable that there may be as yet undiscovered similar close relationships between other *b* and *c*-type cytochromes.

Our knowledge of how cytochromes are assembled *in vivo* is still quite limited. The covalent attachment between apocytochrome *c*-polypeptide and haem should make the biogenesis of *c*-type cytochromes different from the biogenesis of other type of cytochromes, at least by one step. The assembly of proteins into biologically functional structures or enzymes is a fascinating topic of research and raises several questions, including how the prosthetic groups are incorporated into proteins and where the final assembly takes place. The maturation of many proteins can be defined as the conversion of a primary gene product into a mature form by insertion of prosthetic group or other modification. In the case of multiprotein complexes the maturation process also includes the assembly of the polypeptide subunits. Where the subcellular sites of synthesis of polypeptide and cofactor differ from the functional sites of the holoprotein, the two components have to be translocated, with the help of a targeting or signal sequence, alongside coupling to the holoprotein formation. The cleavage of the targeting sequence would give the mature and functional protein. The most extensive biochemical analyses of cytochrome *c* maturation have been done in mitochondria and chloroplasts (Glick *et al.*, 1992 a,b,c; Stuart and Neupert, 1990; Howe and Merchant 1992; 1993; Zollner *et al.*, 1992). The depth of knowledge of *c*-type cytochromes biogenesis in bacteria and the components involved in this process has increased in the last few years. However, the

detailed analysis as to how cytochromes *c* in bacteria are matured, the exact steps for their formation, the role of different gene products which have been characterised to be essential for cytochrome *c* maturation, and their regulation still needs substantial investigation. This complex process raises many questions such as: is there a specific enzyme required for the haem ligation to apocytochrome *c*? where does the assembly of *c*-type cytochromes take place? how are the haem and apocytochrome *c* translocated to the assembly site? what are the conditions required for the maturation of *c*-type cytochromes? The present work is focused on some of the important aspects of cytochrome *c* maturation in bacteria and how this important process is regulated. The hypothetical flow chart of the maturation of a *c*-type cytochrome is shown in Figure 1.2.

As we shall see, the biogenesis of *c*-type cytochromes requires post-translational events of varying complexity depending on the cell type. This inevitably raises the question as to the purpose of covalently attaching the haem to the polypeptide in the *c*-type cytochromes when it is clear that a variety of functions can be achieved by cytochromes, for example the *b*-type, with non-covalently attached haem. This question appears to have been first asked explicitly by Wood (1983). He argued that *c*-type cytochromes first appeared in the bacterial periplasm, or equivalent, as an adaptation to overcome any tendency for non-covalently bound haem to dissociate and diffuse out of the cell. There are certainly few examples of haem non-covalently bound to polypeptides in the periplasm, but Wood's hypothesis cannot be regarded as proven. In contrast to the

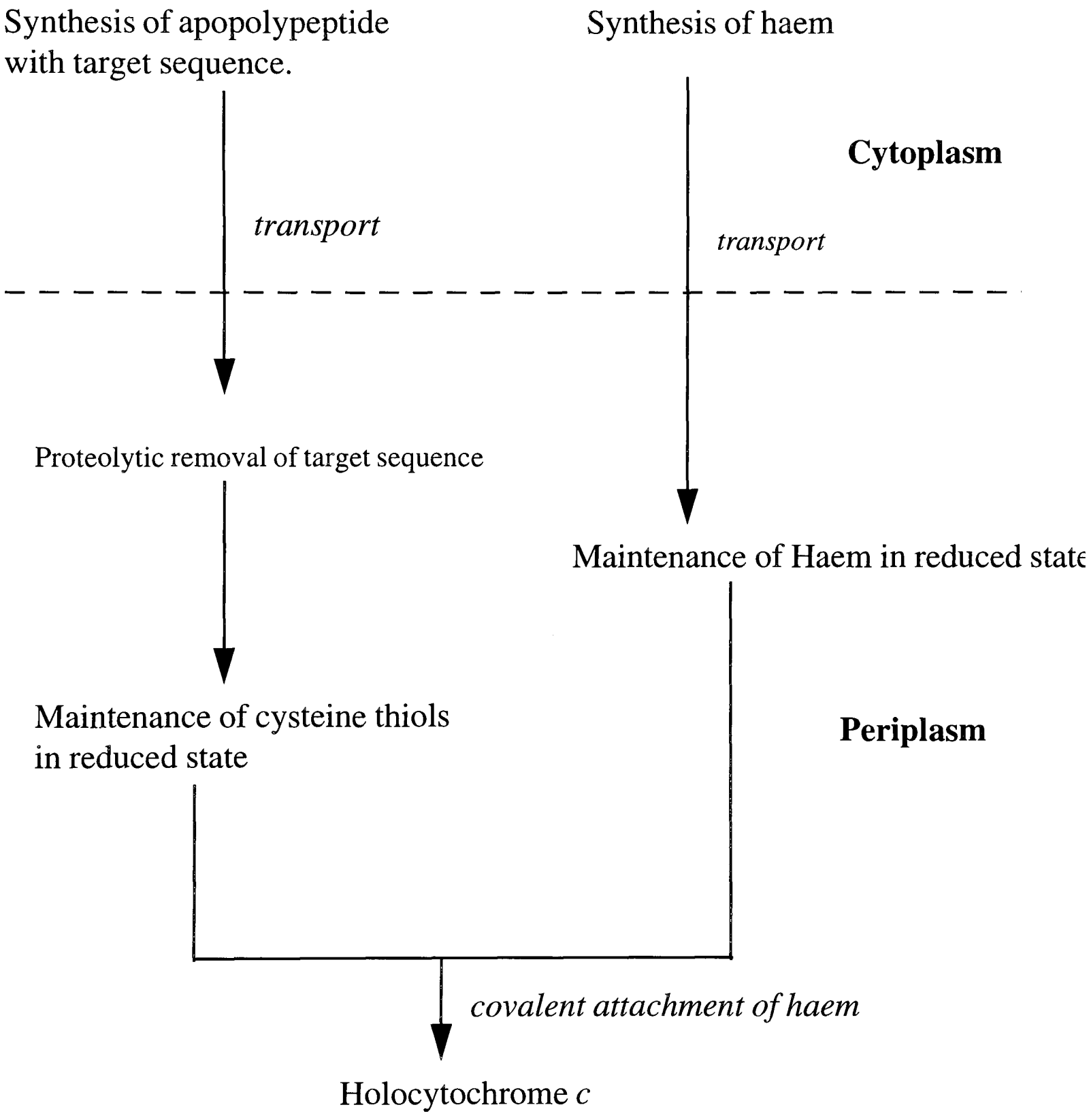


Figure 1.2. Simplified flow diagram of potential route for *c*-type cytochrome maturation in the bacterial periplasm.

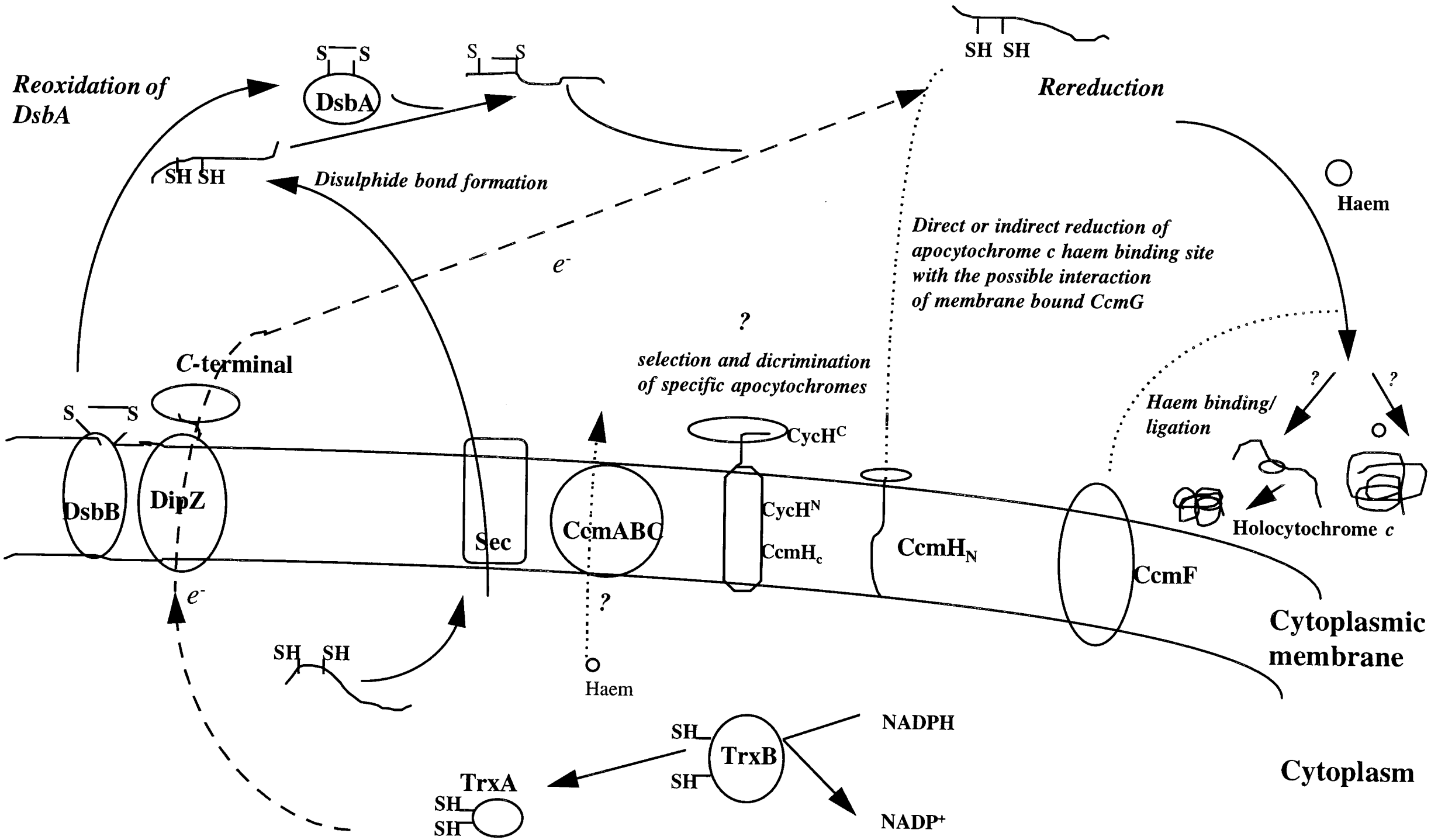


Figure 1.3 A hypothetical pathway of *c*-type cytochrome maturation. The gene products (see table 1) shown here are reported for *B. japonicum, R. capsulatus, P. denitrificans and E. coli* amongst other Gram-negative bacteria. The dotted arrows are used just for clarity, and mean the same as continous lines, While the lines with the spaces show the transport of electrons.

situation when Wood made his proposal, cytochrome b_{562} of *E. coli* is now firmly established as a periplasmic protein. The dissociation constant for the haem is estimated to be around 10^{-9} M (Granick and Beale, 1978) which suggests that dissociation of haem is not a serious problem. Furthermore, given that cytochrome b_{562} is related to cytochrome *c′* and can be converted by mutagenesis into a *c*-type cytochrome (Barker *et al.,* 1995) then if covalent attachment is so advantageous in countering dissociation of haem it is perhaps surprising that evolutionary pressure has not selected against the periplasmic cytochrome b_{562}. Wood (1983) goes on to argue that the *c*-type cytochromes in eukaryotic cells, where the haem dissociation problem may not be so acute owing to the availability of free haem, are derived from prokaryotic precursors. Again we may ask why evolutionary pressures have not then acted to cause the loss of the covalent attachment. Perhaps we are failing to recognise one or more advantages in having the haem covalently attached to the polypeptide chain in *c*-type cytochromes.

1.2 Functions of *c*-type cytochromes

1.2.1 Mitochondria

In non-photosynthetic eukaryotic cells *c* type cytochromes are usually two in number, both associated with mitochondria. A soluble *c*-type cytochrome, simply known as mitochondrial cytochrome *c*, is located on the outer surface of the inner mitochondrial membrane where its role is to transfer electrons to cytochrome aa_3 oxidase from the cytochrome bc_1 complex, associated with the inner mitochondrial membrane. The bc_1 complex contains cytochrome c_1, the second *c*-type cytochrome associated with mitochondria; this accepts electrons from the Reiske iron-sulphur protein and transfers them on to cytochrome *c*. The structure of the cytochrome bc_1 complex has recently been

reported; it confirms the expectation that cytochrome c_1 is anchored to the membrane bilayer by a *C*-terminal alpha helix (Xia *et al*, 1997). In some instances cytochrome *c* has additional electron transport roles, in transferring electrons from a sulphite oxidising system to cytochrome aa_3 (Macleod *et al.*, 1961; Cohen and Fridovich 1971; Oshino and Chance 1975), the terminal detoxification step in the biological degradation of sulphur-containing amino acids in animal tissues, or delivering electrons from the cytochrome bc_1 complex to cytochrome *c* peroxidase, in case of the yeast *S. cerevisiae* (Pettigrew and Moore, 1989). Recently a new function has been proposed for mitochondrial cytochrome *c* as a signal in a system for inducing apoptosis. It has been suggested that mitochondria may participate in apoptosis by releasing cytochrome *c*. Apoptosis is programmed cell death, during which the release of cytochrome *c* from the mitochondria, presumably as a consequence of some earlier damage to the outer membrane, is argued to trigger events on the apoptosis pathway (Yang *et al.*, 1997).

1.2.2 Thylakoids

The only universal *c*-type cytochrome in thylakoids is cytochrome *f* of the cytochrome *bf* complex and which plays an analogous role to cytochrome c_1 in mitochondria. The second *c*-type cytochrome in thylakoids, as mentioned earlier, is found only in certain algae. The molecule is known as cytochrome c_6 and is an alternative to plastocyanin as an electron carrier between the cytochrome *bf* complex and photosystem I. A good example of its occurrence is in *Chlamydomonas reinhardii*, where it has been shown to be synthesised in response to a lack of copper in the growth medium, a condition that compromises the synthesis of plastocyanin (Wood, 1978). Cytochrome c_6 belongs to a family of soluble *c*-type cytochromes which function as electron carriers in the energy

transducing electron transfer chains of mitochondria, respiring and photosynthetic bacteria, and some chloroplasts (Matthews, 1995).

1.2.3 Bacteria

In contrast to mitochondria and thylakoids, the number of different types of *c*-type cytochrome found in the bacterial world is very large. Even a single organism, for example the denitrifier *Paracoccus denitrificans*, may contain upwards of twelve such *c*-type cytochromes (for a review see Ferguson, 1991). Amongst these is cytochrome c_{550} which in many respects, especially its 3D structure, but not its pI value, is very similar to mitochondrial cytochrome *c* (Moore and Pettigrew, 1990). This type of water soluble cytochrome is widespread amongst Gram-negative bacteria where it is located in the periplasm, the part of the cell that lies between cytoplasmic membrane and the cell wall. It was one of the first types of bacterial *c*-type cytochromes to be identified, not in *P. denitrificans*, but in a photosynthetic bacterium *Rhodospirillum rubrum* (Elsden *et al.*, 1953). Its early discovery is still reflected in the use of the name cytochrome c_2 when it occurs in such photosynthetic bacteria, where its role is to transfer electrons from the cytochrome bc_1 complex (also common in many bacteria) to the photosynthetic reaction centre. In non-photosynthetic organisms cytochrome c_{550} is thought to transfer electrons to/from periplasmic reductases/oxidases and membrane bound electron carriers including the cytochrome bc_1 complex and cytochrome aa_3 oxidase. In the case of *P. denitrificans* cytochrome c_{550} is not an obligatory carrier in this respect as its absence as a consequence of mutation does not result in cessation of the electron transfer events (van Spanning *et al.*, 1990). For other examples of mono-haem *c*-type cytochromes in Gram-negative bacteria,

the reader is referred to the reviews/ books by Meyer (1996) and Pettigrew and Moore (1987).

The discovery of a *c*-type cytochrome in a sulphate-reducing bacteria by Postgate (1954, 1956) was at the time a surprise because it had been thought hitherto that such cytochromes did not occur in obligate anaerobes. This particular cytochrome was called cytochrome c_3, for obvious historical reasons, but this is a particularly unfortunate name as the polypeptide chain has four haem groups attached (Czjzek, *et al.*, 1994). The function of the periplasmically located cytochrome c_3 is in electron transfer, but the reason why four haem groups are present is still not clear even today.

Over the years since the original discoveries of Elsden *et al.* (1953) and Postgate (1954, 1956), a very large number of soluble *c*-type cytochromes have been isolated from many species of Gram-negative bacteria. Some clearly play very specific roles, for example in the pathway of periplasmic methanol or methylamine oxidation (Anthony and Long, 1991). The reader is referred to Moore and Pettigrew (1987) for a more extensive listing of these proteins. There are also examples of membrane-bound *c*-type cytochromes, in addition to cytochrome c_1, found in Gram-negative bacteria. For example, there is a membrane anchored cytochrome c_{552}, also known as CycM, that can act as an electron carrier between the cytochrome bc_1 complex and a terminal oxidase enzyme (Bott *et al.*, 1991; Turba *et al.*, 1995; Wu *et al.*, 1996). Other examples are *R. capsulatus* cytochrome c_y (Jenney *et al.*, 1993; Jenney *et al.*, 1996) and the *c*-type cytochrome subunit of the cbb_3 type of cytochrome oxidase that functions under microaerophilic conditions (Preisig *et al.*, 1996). As far as is known, all the membrane *c*-type cytochromes have an extensive globular domain that contains the haem and is exposed to the periplasm.

Unlike their counterparts in eukaryotic cells, bacterial *c*-type cytochromes are neither necessarily found as the sole redox centre in a protein nor only as electron transfer centres. A good example of the former is cytochrome cd_1 nitrite reductase for which a crystal structure was obtained in Oxford recently (Fulop *et al.,* 1995). In this protein the catalytic site for nitrite reduction is provided by the d_1 haem whilst the *c*-type cytochrome centre is regarded as a point of entry into the enzyme of electrons from donor proteins such as cytochrome c_{550}. This *c*-type cytochrome centre in nitrite reductase shows remarkable behaviour. In the oxidised form of the molecule the two axial ligands are both histidine, but upon reduction of the enzyme one of the histidines is replaced by a methionine (Williams *et al.,* 1997). There are examples where a *c*-type cytochrome centre is the active site for chemical catalysis. Two examples are a bacterial cytochrome *c*-peroxidase for which a structure was also obtained in Oxford (Fulop *et al.,* 1995) (note that this type of enzyme in bacteria is itself a *c*-type cytochrome whereas in mitochondria it is not) and hydroxylamine oxidoreductase (Igarashi *et al.,* 1997). The latter provides a dramatic example of a *c*-type cytochrome, because each polypeptide in the trimer contains eight covalently attached haems, with one of them being the site of hydroxylamine oxidation to nitrite. The activity of the latter enzyme provides an example of a metabolic activity, lithotrophy, that is thought to be found only amongst Gram-negative organisms. This is because such activities seem to be dependent on water-soluble components, including many *c*-type cytochromes, in the periplasm. Many Gram-negative bacteria, for example *P. denitrificans* and *R. capsulatus*, have diverse growth modes, each of which depends significantly on the functions of *c*-type cytochromes (Anthony, 1988; Pettigrew and Moore, 1987). Such diverse growth modes are not observed in Gram-positive bacteria, which by definition lack the periplasmic compartment (Merchant *et al.,* 1995). This

strongly suggests that the water soluble periplasmic *c*-type cytochromes in Gram-negative bacteria have an important role in the electron transport processes of the diverse growth modes (Ferguson 1988, Hooper and DiSpirito 1985). Although Gram-positive bacteria lack the range of metabolic activities found in the Gram-negatives, they nevertheless do contain *c*-type cytochromes. In *Bacillus subtilis*, a Gram-positive organism, a cytochrome c_{550} contains a hydrophobic *N*-terminal domain (Von Wachenfeldt and Hederstedt, 1990), indicating that the molecule is inserted in the cytoplasmic membrane. This suggests that, owing to the absence of the periplasmic compartment, any *c*-type cytochromes, which otherwise might be soluble in the periplasm of Gram-negative bacteria, are anchored to the cell membrane. *B. subtilis* cells, under certain growth conditions, contain two other membrane bound cytochromes *c*. These are CtaC protein, a subunit of the terminal respiratory enzyme cytochrome caa_3 (van der Oost *et al.,* 1991) and the QcrC protein, a subunit of cytochrome *c* oxidoreductase (Yu *et al*, 1995).

The bacterial world is today regarded as comprising two kingdoms, the eubacteria and the archaebacteria. Thus far the discussion has been concerned with *c*-type cytochromes in eubacteria. It is of interest to know whether this kind of cytochrome is also found in archaebacteria. To date *c*-type cytochromes have been identified in at least two species of archaebacterium (Lubber *et al.,* 1995; Schafer *et al.,* 1996). Importantly whole genome sequencing has identified cytochrome *c* biogenesis genes in at least one archaebacterium, *Archaeoglobus fulgidus*, in which the occurrence of *c*-type cytochromes has not yet been established.

The *c*-type cytochromes of *E. coli* are of particular interest for two reasons. First, the range of molecular biology methods available for use with *E. coli* means that expression of foreign *c*-type cytochromes in this host is of interest and therefore knowledge of the

endogenous cytochromes is important. Second, as will be discussed later, the biogenesis of *c*-type cytochromes can be studied in *E. coli*, not least because its whole genome has been sequenced. Thus the knowledge of the endogenous *c*-type cytochromes of *E. coli*, and their pathways of synthesis, is important. It synthesises *c*-type cytochromes only under anaerobic growth conditions. *E. coli* has the ability to use, instead of oxygen, five different anaerobic electron acceptors, nitrate, nitrite, dimethylsulfoxide, trimethylamine-*N*-oxide and fumarate and it synthesises at least five *c*-type cytochromes depending on the growth conditions. The 43 kDa TorC cytochrome, which is anchored to the cytoplasmic membrane, is formed during anaerobic growth in the presence of trimethylamine-*N*-oxide (Mejean *et al.,* 1994). Two further *c*-type cytochromes, the periplasmic 50 kDa nitrite reductase (cytochrome c_{552}) and 18 kDa pentahaem cytochrome *c* (Darwin *et al.*, 1993b; Hussain *et al.,* 1994) are induced during anaerobic growth by nitrite (Darwin *et al.,* 1993a,b; Hussain *et al.,* 1994; Page *et al.,* 1990; Rabin and Stewart, 1993). Two other *c*-type cytochromes, with molecular weights 16 kDa and 24 kDa, are also strongly induced by nitrite (Iobbi-Nivol *et al.,* 1994).

1.3 Biogenesis studies on mitochondrial *c*-type cytochromes

The biogenesis of *c*-type cytochromes was first studied in mitochondria (for review Glick *et al.*, 1992a,b; Stuart and Neupert, 1990; Gonzales and Neupert, 1990; Howe and Merchant, 1994). Most of such studies have been carried out in *Saccharomyces cerevisiae* or *Neurospora crassa*. Both soluble cytochrome *c* and membrane anchored cytochrome c_1 are synthesised on cytoplasmic ribosomes and are translocated to the intermembrane space of mitochondria by different pathways. Apocytochrome *c* does not contain an *N*-terminal signal sequence (Gonzales and Neupert, 1990) and is translocated across the outer

membrane to the intermembrane space in a pathway that is tightly coupled to haem attachment (Dumont *et al.,* 1988). Apocytochrome *c* may bind to a protease insensitive 'receptor' site on the mitochondrial outer membrane for its translocation (Lill and Neupert, 1996). In the case of cytochrome c_1 the polypeptide is originally synthesised with a bipartite target sequence. The molecular events surrounding the recognition and handling of this sequence are controversial. According to one view (Gonzales and Neupert, 1990), the *N*-terminal part of the targeting sequence directs the polypeptide to the mitochondrial matrix where this part of the sequence is cleaved. The second part of the targeting sequence then directs export to the inter membrane space, followed by the cleavage of the second targeting sequence and the haem attachment. An alternative view supposes that the bipartite target sequence acts to stop transfer of the polypeptide into the mitochondrion (Van Loon and Schatz 1987; Wachter *et al.,* 1992). The pathway in which the polypeptide is first translocated into the mitochondrial matrix means that the later steps in the maturation of cytochrome c_1 are analogous, at least topologically, to the export and maturation of a prokaryotic cytochrome c_1. For this reason this proposed pathway for mitochondrial cytochrome c_1 generation is known as conservative. The haem attachment to apocytochrome *c* and c_1 is catalysed by specific haem lyases, originally known as cytochrome *c* synthetases, in the intermembrane space. The enzyme that catalyses the haem attachment to cytochrome *c* polypeptide is known as cytochrome *c* haem lyase (CCHL) (Dumont *et al.,* 1987; Nicholson *et al.,* 1987), which is distinct from the enzyme which catalyses the haem attachment to cytochrome c_1, known as cytochrome c_1 haem lyase (CC_1HL). Mitochondrial, or solublised extracts thereof, catalyse the covalent bonding between haem and apocytochrome *c*, in the presence of NADPH (or NADH), to form holocytochrome *c* (Basile *et al.,* 1980; Veloso *et al.,* 1981; Henning and Neupert,

1981). The reason for the requirement of reductant is not known; it may reflect a need to keep the haem and the two cysteine residues of the polypeptide chain in a reduced state. It has also been possible to detect the covalent attachment of haem to a polypeptide containing only residues 1 to 25 of mitochondrial cytochrome *c* (Veloso *et al*, 1984). This observation suggests that the haem lyase recognises only a limited stretch of the apocytochrome *c* sequence. Mutations causing specific deficiency in the maturation of cytochromes *c* and c_1 have been characterised for *Saccharomyces cerevisiae* (Rothstein and Sherman, 1979). These include alterations in the structural gene, *cyc 1*, for iso-1-cytochrome *c* and those having alterations in 5 distinct loci, *cyc2, cyc3, cyc4, cyc5, cyc6, cyc8* and *cyc9*. This organism contains two iso-cytochromes *c* that perform similar functions, but differ in the primary structure. 95% of the total cytochrome *c* complement consists of iso-1-cytochrome *c*, which is encoded by *cyc1* locus (Sherman *et al.,* 1966). The remaining 5% consists of iso-2-cytochrome *c*, which is encoded by *cyc7* locus (Downie *et al.,* 1977). *cyc2* encodes a factor involved in the import of cytochrome *c* into the mitochondria (Dumont *et al.,* 1993) though the gene product is not an essential component of an import apparatus, since even in the deleted strains, there is never less then 10% of the normal level of holocytochrome *c* (Dumont *et al.,* 1993). The *cyc3* gene product encodes cytochrome *c* haem lyase (Dumont *et al.,* 1987), which functions in the intermembrane space of the mitochondria (Lill *et al.,* 1992). *cyc8* and *cyc9* mutants overproduce iso-2-cytochrome *c* (Rothstein and Sherman, 1980a,b). A *cyc4* mutant was found to be related to the porphyrin biosynthesis and mutations in *cyc5* or *cyc6* gave only a partial effect on the synthesis of the two iso-cytochrome *c*. Thus besides the structural genes *cyc 1* and *cyc7* , *cyc2* and *cyc3* are the only two genes which appears to be directly involved in the synthesis of the two iso-cytochrome *c*. Cytochrome c_1 haem lyase is

encoded by *cyt2* gene (Zollner *et al.,* 1992) and is predicted to be associated with the inner membrane, but partially exposed to the intermembrane space. The translocation of apocytochrome *c* is intrinsically coupled with the haem attachment. There are two different views regarding the role of CCHL in cytochrome *c* maturation. According to one the haem attachment is proposed to be required for translocation and the haem attachment provides the driving force to complete the translocation reaction (Dumont *et al.,* 1988; Nargang *et al.,* 1988; Nicholson *et al.,* 1988). The other view suggests that the haem attachment is not required for the translocation of apocytochrome *c* across the outer membrane. Rather, the haem attachment induces a conformational change in the polypeptide which renders the translocation of cytochrome *c* irreversible, or the holoprotein formation traps the polypeptide within the intermembrane space (Hakvoort *et al.,* 1990; Dumont *et al.,* 1991). This reaction requires NAD(P)H and a cofactor present in the cytoplasm (Nicholson and Neupert, 1989). The haem is required to be in a reduced state for this covalent bond formation and, thus, for the subsequent translocation of the apocytochrome *c* from the outer membrane space. Why only reduced haem can form a covalent bond is unknown though it might be that the cytochrome *c* haem lyase enzyme active site is specific for a reduced haem. As will be discussed later, the attachment of haem to the apocytochrome *c* in the bacterial periplasm, an oxidising environment, also requires reducing power. The haem lyase enzymes also follow the novel pathway for the transport to the intermembrane space; they do not contain cleavable pre-sequences and appear to be targeted to the intermembrane space through a non-conservative sorting pathway (Lill *et al.*, 1992; Zollner *et al.,* 1992). The genes encoding both the CCHL and the CC_1HL have been cloned from *N. crassa* and *S. cerevisiae* (Dumont *et al.,* 1987; Drygas *et al*, 1989; Zollner *et al.,* 1992). They are encoded in the nucleus. Unlike the

normal pathway for the other proteins present in the intermembrane space, which are thought to be first translocated to the matrix through the contact sites of the two membranes and then targeted to the intermembrane space by the second targeting signal present in the presequence, cytochrome *c* haem lyase is directly targeted to the intermembrane space without going to the matrix. Thus it does not first traverse the inner membrane before reaching the intermembrane space (Lill *et al.,* 1992; Zollner *et al.,* 1992). Cytochrome *c* haem lyase also does not have any targeting sequence thus resembling the soluble cytochrome *c*, but in contrast cytochrome *c* haem lyase uses a receptor complex in the outer membrane for its import (Nicholson *et al.,* 1988). These haem lyases show only 30% homologies in their amino acid sequences. These enzymes show very high specificities for their respective apoprotein substrates.

Most of what is known about *c*-type cytochromes biogenesis in mitochondria has been learned from studies with fungal systems, *S. cerevesiae* or *N. crassa*. It might be thought obvious that all mitochondria would rely on the same machinery for biogenesis of *c*-type cytochromes. Indeed there is evidence for cytochrome *c* haem lyase genes in humans (Schaefer *et al.*, 1996). Studies with rat liver mitochondria also have indicated a cytochrome *c* haem lyase activity. However, a number of instances have been reported where mitochondrial genomes contain genes which are closely related to genes, to be described in the next section, known to be involved in bacterial *c*-type cytochrome biogenesis. Most dramatic is the case of protozoan *Reclinomonas americana*, in which four such genes are present in a contiguous organisation (Lang *et al.*, 1997). Furthermore, these genes are all absent from the *S. cerevisae* genome. These observations at least raise the possibility that there are differences amongst mitochondria from different origins in respect to their *c*-type cytochrome biogenesis.

There have been numerous studies in recent years on the refolding pathway followed by denatured mitochondrial cytochrome *c* upon removing denaturing agents. A variety of biophysical techniques has been used, especially nuclear magnetic resonance. A recent example of this type of study is provided by Hammack *et al.*, (1998). In all these studies the haem remains covalently attached to the polypeptide throughout. *In vivo* it is not known whether the haem is attached to an unfolded protein which then folds to the final tertiary structure or whether the protein adopts some tertiary structure before addition of the haem. Thus given the uncertainty as to whether the *in vitro* studies of cytochrome *c* folding relate to the *in vivo*, they are not discussed further here. However, one other *in vitro* study of mitochondrial cytochrome *c* folding warrants mention. This is the work of Paschler *et al.,* (1996) who showed that cytochrome *c* shows a greater speed of refolding if the haem is reduced rather than oxidised. This may correlate with the knowledge that haem lyase activity requires reducing conditions.

As mentioned above, there is uncertainty as to whether the *in vitro* folding of mitochondrial cytochrome *c* follows the covalent attachment of the haem or whether binding of haem to the apo protein induces folding. A variety of evidence indicates that the apo form of mitochondrial cytochrome *c* has a random coil structure (Stellwagen *et al.,* 1972; Fisher *et al.,* 1973; Cohen *et al.,* 1974). Dumont *et al.,* (1994) have argued that non-covalent attachment of haem induces structure into the apopolypeptide. Whether these observations, which contradict previous views (see Dumont *et al.,* 1994) on haem binding, are related to the folding pathway is not known.

1.4 Cytochrome *c* biogenesis in bacteria

Bacterial cytochrome *c* biogenesis involves a post translational pathway for the conversion of pre-apocytochromes *c* into the mature holocytochromes *c*. It involves an unknown number of consecutive biochemical steps, including translocation of precursor polypeptide and haem from the cytoplasm into the periplasm and covalent linkage between these two molecules (Figure 1.2). As mentioned earlier, after translocation some cytochromes remain bound to the membrane by their uncleaved *N*-terminal signal sequence; these include CycM, FixO and FixP proteins in *Bradyrhizobium japonicum*, cytochrome *c*y in *Rhodobacter capsulatus* and cytochrome c_{550} of *Bacillus subtilis* (Bott *et al.,* 1991; Preisig *et al.,* 1993; Jenney and Daldal, 1993; von Wachenfeldt and Hederstedt, 1990). The evidence that haem and apo polypeptide are translocated separately to the periplasm is strong, although the conversion of apocytochrome *c* to the holocytochrome *c* in the periplasm has not been demonstrated. Experimental observations that point to the periplasmic location for haem attachment include: a) detection of apo forms of *c*-type cytochromes, with their periplasmic targeting sequences removed, in the periplasm of *P. denitrificans* (Page and Ferguson, 1989, 1990); b) expression of *c*-type cytochromes from a gene lacking the periplasmic targeting sequence results in a cytoplasmic polypeptide lacking the covalently attached haem (Sambongi and Ferguson 1994b; Thony-Meyer *et al.,* 1996); c) the *N*-terminal targeting sequence of *c*-type cytochromes are of the type, often containing positive residues at the *N*-terminus with at least 10 mostly hydrophobic residues which usually is followed by -3 to -1 leader peptidase cleavage site (Pugsley, 1989), recognised by the *sec* system (Pugsley, 1993) which has recently been shown to be needed for *c*-type cytochrome assembly (Thony-Meyer and Kunzler, 1997). This *sec* system is usually regarded as carrying proteins in an unfolded state; haem attachment might be expected to induce folding; d) as will be discussed shortly, the products of a

number of genes required for *c*-type cytochrome biogenesis appear to have their functional domains located in the periplasm.

A periplasmic location for attachment of haem to the apo polypeptide of a *c*-type cytochrome does fit with the general idea that polypeptides are translocated across membranes in a relatively unfolded state. However, it has recently been argued that some periplasmic proteins, characterised by targeting sequences that are longer then those typically used by the *sec* system, are translocated, in at least a partially folded state, with their redox cofactors (Berks, 1996). This is supported by the finding that an *E. coli* periplasmic trimethylamine-*N*-oxide reductase, which has a molybdenum cofactor, is translocated to the periplasm independently of the *sec* system (Santini *et al.,* 1998). Thus it is not necessarily general that redox group attachment to a polypeptide will occur in the periplasm.

Mutants that are pleiotropically deficient in *c*-type cytochromes have been isolated from several species of bacteria. The fact that pleiotropic deficiency is essentially always observed implies that one apparatus makes each of the different *c*-type cytochromes, that is with no discrimination between membrane-bound and soluble cytochromes *c* that are found in these bacteria. An exception is found in *R. capsulatus* where the protein CycH (described later), is essential *in toto* for the synthesis of all *c*-type cytochromes. When CycH is deleted in its periplasmic-carboxy terminal domain cytochrome c_1 synthesis alone continues, presumably therefore requiring the *N*-terminal membrane associated portion of CycH. This occurs when cells are grown in enriched medium. Synthesis of several other *c*-type cytochromes occurs when cells are grown in minimal medium, showing that the two subdomains of this protein play distinct roles for the biogenesis of membrane-associated and periplasmic *c*-type cytochromes (Lang *et al.,* 1996).

The mutants giving rise to the pleiotropic deficiency of *c*-type cytochromes raise the question as to what is recognised in a polypeptide as indicating a requirement for covalent attachment of haem. As mentioned earlier, it is unlikely that it is simply the C-X-X-C-H motif alone. The haem group in the polypeptide is attached not only by the two cysteine residues and the histidine residue of the C-X-X-C-H motif but also by a second axial ligand, the nature of which is variable. The question arises as to whether this ligand is recognised by the post-translational apparatus that attaches the haem to a polypeptide destined to become a *c*-type cytochrome. The answer appears to be no, because it has been shown that haem is covalently attached to a hybrid protein containing the part of a *c*-type cytochrome with the C-X-X-C-H motif fused to alkaline phophatase in such a way that the second axial ligand of the *c*-type cytochrome was absent (Sambongi *et al.,* 1996; Brandner *et al.,* 1991). It can then be asked as to whether there are any indications as to how many residues flanking the C-X-X-C-H motif are recognised in the biogenesis process. This cannot be answered at present, but the fact that two C-X-X-C-H motifs are separated by only five residues in hydroxylamine oxidoreductase (Igarashi *et al.,* 1997) suggests that the number of flanking residues recognised might be quite small.

Genes required for *c*-type cytochrome biogenesis have been identified largely through transposon mutagenesis. Eight or nine genes in different Gram-negative bacterial species have been identified as required for *c*-type cytochrome biogenesis. Unfortunately identified genes were given different names depending on the organism in which they were identified and sometimes on the basis of guesses as to the functions of their products (Table 1). Identification of genes for *c*-type cytochrome biogenesis in *E. coli* was achieved later than for some other organisms but when it did happen the genes were given the *ccm* name for cytochrome *c* maturation. As it seems a more rational nomenclature system than

any of the others used, it will be employed here whilst cross referencing where appropriate to the other names (Table1). Genes encoding functions that are essential for the maturation of *c*-type cytochromes have been identified in *Rhodobacter capsulates* (Beckman *et al.* 1992; Beckman and Kranz 1993), *Bradyrhizobium japonicum* (Ramseiesr *et al.* 1991; Ritz *et al.* 1993, 1995; Thony-Meyer *et al.* 1994), *Rhizobium meliloti* (Kereszt *et al.* 1995), *E. coli* (Hussain *et al.* 1994; Crooke and Cole1995; Metheringham *et al.* 1995; Grove *et al.* 1996a; Thony-Meyer *et al.* 1995) and *Paracoccus denitrificans* (Page and Ferguson 1995). A relatively detailed model has been proposed (Thony-Meyer *et al,*1994; Thony-Meyer 1997) for the biogenesis of *c*-type cytochromes in bacteria.

Genetic studies suggest that haem is likely to be transported separately by an ABC, (ATP binding cassette), type transporter. CcmABC (HelABC and CycVW(+ *orf263* gene product later called *cycZ*) as original names in *R. capsulatus* and *B. japonicum*) are suggested to form an ATP driven membrane transporter of the ABC super family (Ramseier *et at.,* 1991; Beckman *et al.*,1992). The *ccmA* gene (*cycV* and *helA* as the original names in *B. japonicum* and *R. capsulatus*, respectively) encodes for the ATP-binding subunit of the transporter, while *ccmB* and *ccmC* (*cycW* and *cycZ*, *helB* and *helC* as original names in *B. japonicum* and *R. capsulatus*, respectively) code for integral membrane components. *helC* in *R. capsulatus* was always thought to be essential for *c*-type cytochrome biogenesis (Beckman *et al.,* 1992), but initially *orf263* (*cycZ*) in *B. japonicum* was reported not to be so (Ramseier *et al.,* 1991). It has been argued that these three gene products will form a complex with stoichiometry $(CcmA)_2$ CcmB CcmC (Page *et al.,* 1997). The clear implication from the sequences of their gene products for a transport function, together with the belief that haem is transported to the periplasm separately from the apo polypeptide of a *c*-type cytochrome, naturally suggested that

CcmA, CcmB and CcmC proteins would constitute an outwardly directed haem transporter. However, there is no direct evidence for this role and indeed there is some against (Page *et al.,* 1997; Goldman *et al.,* 1996; Thorne-Holst *et al.,* 1997; this thesis Chapter VI). Haem is a relatively hydrophobic molecule and it might be argued that it could diffuse passively through the bacterial cytoplasmic membrane so as to reach the periplasm. However, the transmembrane movement of haem has been shown to be slow, rate constants of the order of 0.001 sec^{-1}, at least for the carbon-monoxide adduct of haem passing from the exterior to the interior of phospholipid vesicles (Light and Olson, 1990). It is debatable whether such rates of non-mediated movement of haem across membranes are sufficient to match the requirement for haem to be supplied to the periplasm for the synthesis of *c*-type cytochromes.

In most organisms, for example *E. coli*, *R. capsulatus*, *P. denitrificans*, *ccmD* is also clustered with *ccmA, ccmB* and *ccmC*. The function of CcmD (CycX and HelD as a original names in *B. japonicum* and *R. capsulatus*, respectively), however, is not known. It is a small protein that has been predicted to face the cytoplasm and be anchored to the membrane (Thony-Meyer, 1997). The products of *ccmEFH* have been predicted to function in haem ligation. (the homologue of *ccmE* has a original name of *cycJ* in *B. japonicum*, homologs of *ccmF* had a original names of *cycK* and *ccl1* in *B. japonicum* and *R. capsulatus*, respectively while *cycL* and *ccl2* were the original names for *ccmH* in these two species of bacteria). CcmF and CcmH of *R. capsulatus*, *Bradyrhizobium japonicum* and of *Rhizobium meliloti* (Table 1) have often been suggested as components of a periplasmic haem lyase complex but there is no direct evidence for this (Beckman *et al.,* 1992; Thony-Meyer *et al.,* 1994, 1995; Ritz *et al.,* 1995; Kerestz *et al.,* 1995). The products of *ccmF* and *ccmH* are membrane bound; this has been shown by experimental

analysis or by computational analysis. The location is in the cytoplasmic membrane and the globular domain is oriented towards the periplasm. *E. coli* exceptionally has a second set of three of these genes, *nrfE*, *nrfF* and *nrfG*, the products of which are homologous to CcmF and the *N*- and *C*- terminal domains of CcmH, respectively. NrfE, NrfF and NrfG are required for formate dependent nitrite reduction pathway (Grove *et al.,* 1996; Hussain *et al.,* 1994). There is recent evidence that these *nrf* gene products are involved in attaching a haem group to an unusual CXXCK motif which is speculated to form the active site of the nitrite reductase which also contains four other *c*-type haem centres per polypeptide chain (Eaves *et al.,* 1998). This would explain why the *nrfE* gene product is only needed for nitrite reductase activity, as opposed to attachment of haem groups to CXXCH motifs within the protein. Furthermore, this conclusion implies that NrfE is involved in protein-protein interactions with the target protein, nitrite reductase. As discussed by Pearce *et al.,* (1998) the sequence similarity between NrfE and CcmF is great and therefore subtle differences might have to account for recognition of the CXXCK in nitrite reductase on the one hand and CXXCH in several cytochromes.

The product of *cycH* might be an additional cytochrome *c* haem lyase subunit but assignment of function is difficult because nothing resembling CycH is found in the data bases (Lang *et al.,* 1996; Page and Ferguson, 1995). *E. coli ccmH* is unique; it encodes a fused protein, where the *N*-terminal portion is similar to CycL and the *C*-terminal portion is similar to CycH. Recently Ccl2 (CcmH) has been also shown to be involved in the reduction of the two cysteines of the haem attachment site in apocytochrome *c* (Monika *et al.,* 1997).

In a Gram negative bacterium the environment of the periplasm is highly oxidising, in contrast to the cytoplasm (Derman and Beckwith, 1991; Derman *et al.*, 1993).

Disulphide bond formation is important for the folding and structure of many proteins (Luckey *et al.*,1991), most of which are located in the periplasm. However, an oxidising environment of the periplasm is not itself sufficient for the formation of disulphide bonds in the exported proteins. Protein disulphide oxido-reductases, catalysing the formation or the cleavage of the disulphide bonds, have been shown to be necessary for the formation of disulphide bonds in exported proteins in *E. coli* (Bardwell *et al.,* 1991). These oxidoreductases, known as Dsb proteins (disulphide bond formation), contain a typical thiol disulphide active site Cys-X-Y-Cys and belong to the thioredoxin superfamily of protein disulphide isomerases (PDIs). The proteins DsbA and DsbB have been shown to be necessary for the formation of disulphide bonds (Bardwell *et al,* 1991, 1993; Missiakas *et al,* 1993; 1994). DsbA catalyses the formation of disulphide bonds of a variety of proteins and is reoxidised in turn by DsbB protein (Bardwell *et al.*, 1991, 1993; Akiyama *et al.*, 1992; Guilhot *et al.*, 1995; Kishigami *et al.,* 1995). Another protein DsbC has been shown to play a role in disulphide bond isomerisation (Rietsch *et al.*, 1996), probably to ensure that the disulphide bond forms between the wrong pair of cysteine residues are corrected by isomerisation. Somewhat unexpectedly it has turned out that functional DsbA and DsbB proteins are required for *c*-type cytochrome biogenesis in *E. coli* (Metheringham *et al.,* 1995; Sambongi and Ferguson, 1996). At first sight it might have been expected that the two cysteine residues of the CXXCH motif in a *c*-type cytochrome would be shielded from the machinery for making disulphide bonds in the periplasm. In principle, such shielding may occur and the requirement for the Dsb system might be because one or more of the *ccm* gene products requires a disulphide bond for its activity. However, there is no evidence for any shielding effect and so it is entirely possible that the two cysteines of the CXXCH motif are oxidised to a disulphide. This would imply that

there would be a subsequent reduction step to allow the haem attachment. This would explain why *c*-type cytochrome biogenesis requires a periplasmic protein with similarity to thioredoxin. This protein is now known as CcmG but was originally called TlpB in *B. japonicum* (Ramseier *et al.,* 1991; Thony-Meyer *et al.,* 1994), *cycY* in *R. leguminosarum* (Vargas *et al.,* 1994) and *helX* in *R. capsulatus* (Backman and Kranz, 1993) (Table 1). There is evidence that CcmG has a reductase function (Page and Ferguson, 1997; Fabianek *et al.,* 1997; Monika *et al.,* 1997) and that, as mentioned earlier, this can be active towards CcmH (formerly Ccl_2). Although the idea cannot be regarded as proven, it seems very plausible that this 'reductase' system is present to allow reductive addition of the haem into a disulphide bond.

How might a source of reducing power reach CcmG in the periplasm? This might be the role of an integral membrane protein, DipZ, also known as DsbD, which at least in *E. coli* (Crooke and Cole, 1995) is required for *c*-type cytochrome biogenesis. It has been proposed that a cytoplasmic domain of DipZ can be reduced by thioredoxin (Rietsch *et al.,* 1996) and that the periplasmic domain acts as a disulphide reductase. The reductase activity could be directed towards DsbC, the periplasmic protein thought to be involved in reshuffling of disulphide bonds (Rietsch *et al.,* 1996), or CcmG. How exactly the reductant would be passed across the cytoplasmic membrane is a matter of conjecture, but Page *et al.,* (1997) have proposed that two conserved cysteine residues, each located in the middle of a strongly predicted transmembrane α-helix, are involved. It has recently been discovered that DipZ is not necessary for *c*-type cytochrome biogenesis in *P. aeruginosa* (Page *et al.,* 1997). This, and the failure to find cytochrome *c* deficient mutants defective in DipZ for other bacteria has been explained on the basis that such bacteria have a CcdA protein, not found in *E. coli*, that can substitute for DipZ (Page *et al.,* 1997).

Most of the cytochrome *c* biogenesis gene products identified so far appear to be membrane bound. It is quite possible that the components of the putative haem transporter (CcmABC) and the proposed haem lyase activity (CcmE/F/H) form an oligomeric complex for the haem translocation and haem attachment respectively, while CcmG functions by keeping the haem binding site of the apocytochrome *c* in the reduced state (Page and Ferguson, 1997). It is likely that CcmG is also in physical contact with the other components. Thus, it might be that the *ccm* gene products function in a supercomplex and form a 'Maturase' for the biogenesis of *c*-type cytochromes (Thony-Meyer, 1997) although Pearce *et al.* (1998) argued against this. In *Rhodobacter capsulatus* all the genes required for the maturation of *c*-type cytochromes are membrane tethered (Monika *et al.*, 1997). In case of soluble periplasmic *c*-type cytochromes the signal sequence is removed by a signal peptidase present on the periplasmic side of the cytoplasmic membrane and the haem attachment and the signal cleavage are independent processes (Thony-Meyer, 1997). A diagram for a hypothetical maturation pathway of *c*-type cytochrome in a typical Gram-negative bacteria is shown in Figure 1.3.

The need for such a complex machinery (Figure 1.3) could be to overcome the oxidative power present in the periplasm, due to both the presence of DsbAB system and the oxidative environment of the periplasm. This view is supported by recent observations on a Gram negative bacteria *Helicobacter pylori*, which has both membrane bound and periplasmic cytochromes. The screening of its recently released genome (Tomb *et al.*, 1997) shows that it lacks homologs of nearly all the genes required for *c*-type cytochrome biogenesis identified in other Gram negative organisms (Page *et al.* 1997b). *H. pylori* contains only two readily identifiable genes for this biogenesis process. One is *ccdA*, recently implicated in *c*-type cytochrome biosynthesis in the Gram-positive bacteria

Bacillus subtilis (Schiott *et el.,* 1997a,b). The other gene identified is *ccsA*, formerly known as *ycf5*; this is related to an orthologue of *ccmF* that is found in plant mitochondrial and chloroplast genomes (Xie and Merchant, 1996). It has been suggested that since proteins responsible for disulphide bond formation, DsbAB, are absent from *H. pylori*, the problem of inevitable disulphide bond formation once an apocytochrome *c* carrying cysteines enters the periplasm is avoided (Page *et al.,* 1997b). Thus much of the *c*-type cytochrome biogenesis machinery, present in the periplasm, for reduction of disulphides is dispensable in *H. pyroli.* This will be discussed again later with reference to the maturation of *Hydrogenobacter thermophilus* cytochrome c_{552} in *E. coli*; this is matured in the cytoplasm, a reducing environment, of *E. coli* and is believed not to require any enzyme assistance for its maturation.

In conclusion, the assembly in the periplasm of *c*-type cytochromes clearly requires a complex apparatus in all but one of the Gram-negative bacteria studied to date. The exception is *H. pylori* in which *c*-type cytochrome assembly does not require many of the gene products needed in the other Gram-negative organisms. The assembly of *c*-type cytochromes in *H. pylori* appears to be more closely related to the process in a Gram-positive bacterium such as *B. subtilis*. The genome sequence of the latter (Kunst *et al.,* 1997) shows that in common with *H. pylori* most of the genes needed for cytochrome *c* biogenesis in Gram-negative organisms are absent. Thus, unexpectedly, it appears that there are two different systems for assembling *c*-type cytochromes amongst the eubacteria.

1.5 Organisation of cytochrome *c* biogenesis genes in bacteria

The organisation of *ccm* genes in different Gram-negative bacteria is only partly conserved, despite their coding for similar functions. *ccm* genes are organised in one

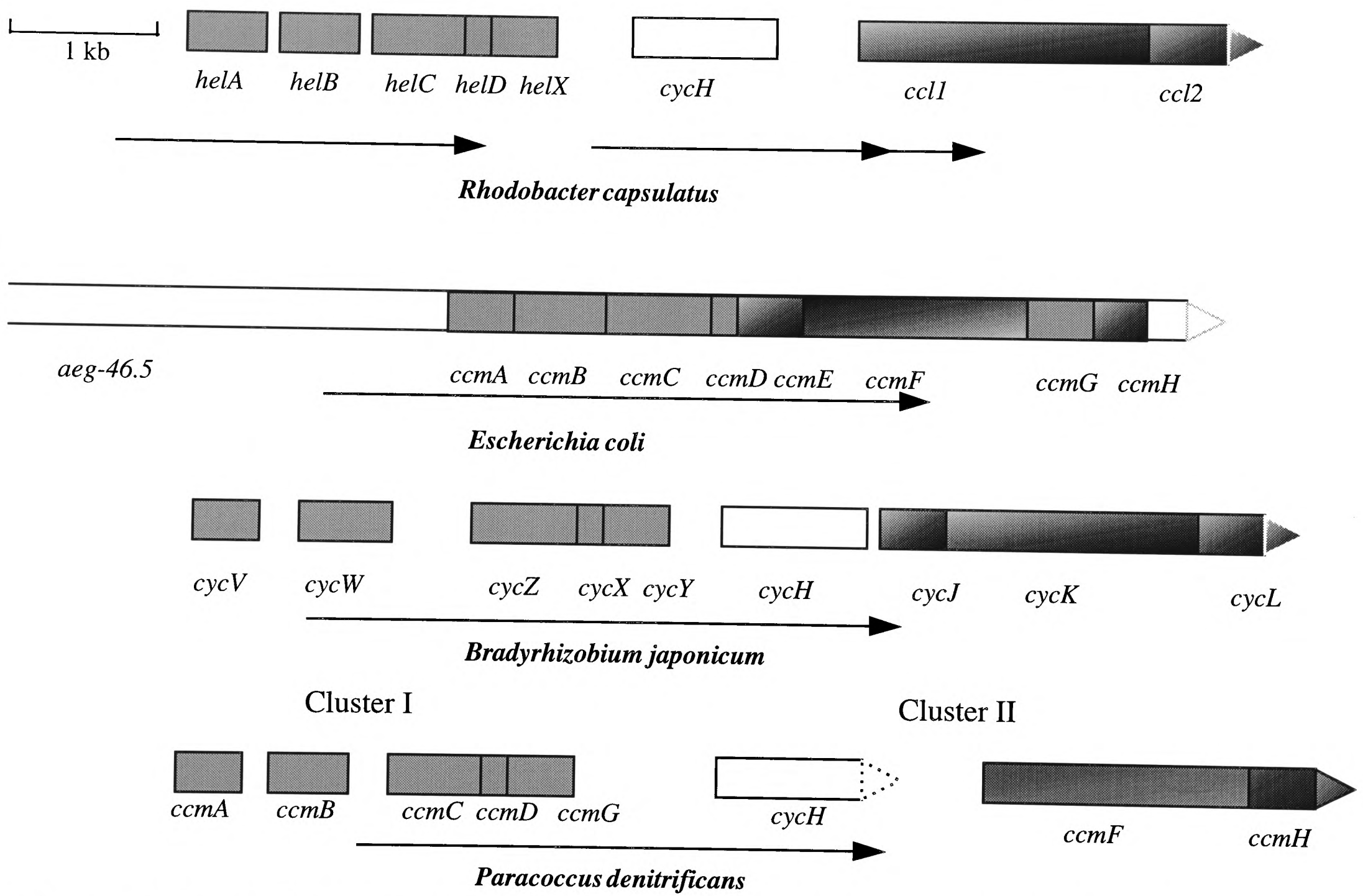


Figure 1.4 The organisation of bacterial cytochrome *c* maturation genes from *B. japonicum, R. capsulatus, E. coli* and *P. denitrificans*. Different groups of genes coding for an ABC transporter, *ccmABC*, possible cytochrome *c* haem lyase subunits, *ccmFH*, thioredoxin like protein, *ccmG*, and genes with unknown

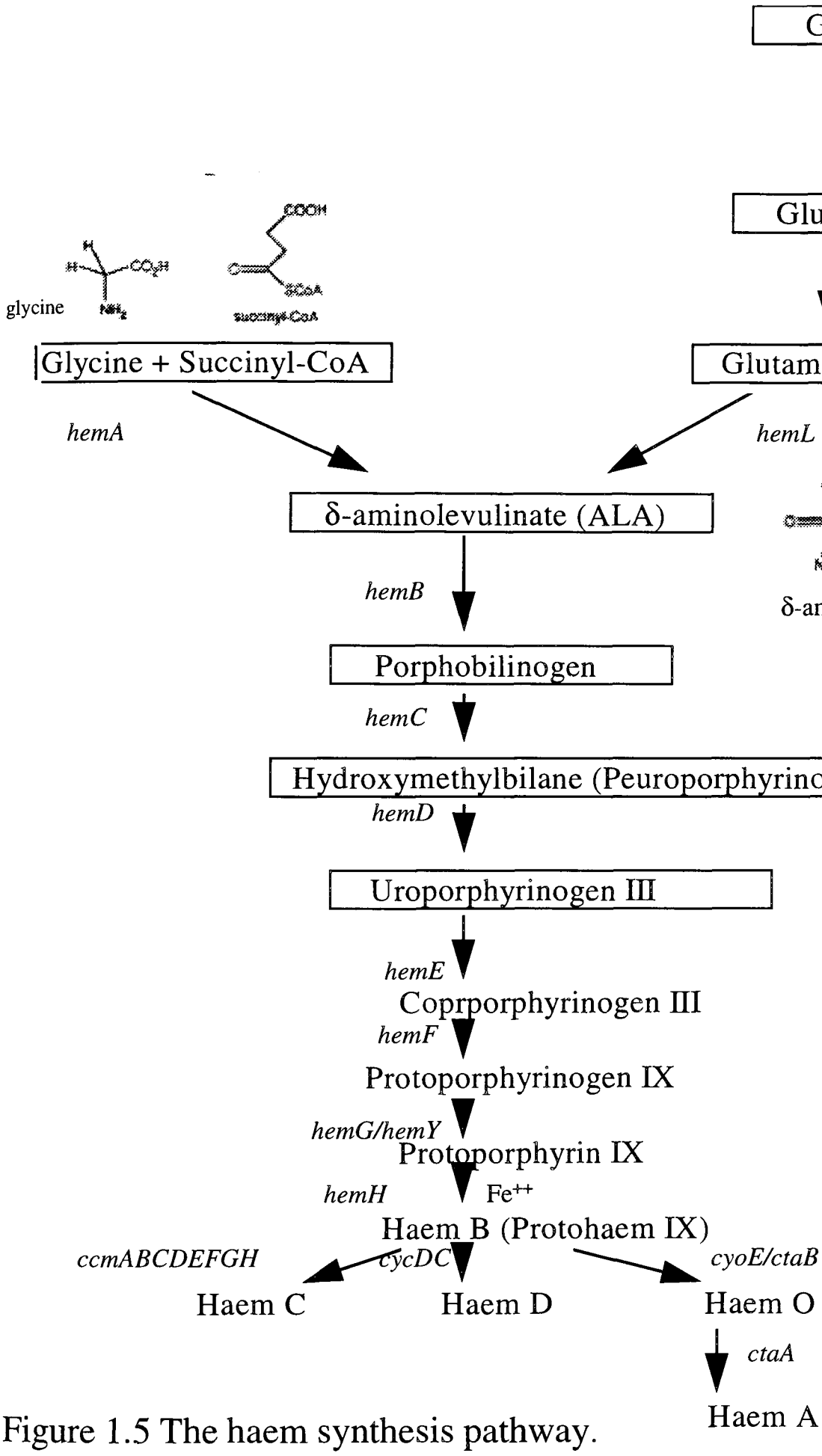


Figure 1.5 The haem synthesis pathway.

Table 1: Genes involved in bacterial cytochrome *c* biogenesis:

Organism	Gene		Reference
	Original name	**Suggested uniform nomenclature**	
Bradyrhizobi	*cycV, cycW,*	*ccmABCD*	Ramseier *et al.*,1991.
m japonicum	*cycZ, cycX,*		
	cycH	*ccm*H_C	Ritz *et al.* 1993.
	cycJKL	*ccmEFH*$_N$	Ritz *et al.* 1995
	*tlp*A, *tlp*B	*ccmG*	Loferer *et al.* 1993; Ritz *et al.* 199 Loferer and Hennecke 1994.
Rhodobacter	*helA, helB,*	*ccmABC*	Beckman *et al.* (1992)
capsulatus	*helC*		
	helX	*ccmG*	Beckman and Kranz (1993)
	ccl1, ccl2	*ccmF, ccmH*$_N$	Backman *et al.*(1992)
E.coli	*ccmA, ccmB,*	as original	Cole *et al.* (1996); Thony-
	ccmC, ccmD,		Meyer *et al.,* (1995).
	ccmE, ccmF,		
	ccmG, ccmH		
	*dsb*A	as original	Bardwell *et al.* (1991)
			Kamitani *et al.,* (1992).
	*dsb*B		Bradwell *et al.,* (1993).
	*dsb*C		Shevchick *et al.,* (1994).
	dipZ		Beck *et al,* (1994).
			Crooke and Cole (1995).
P.denitrifica	*cycH*	*ccmH*$_C$	Page and Ferguson (1995)
ns			Page and Ferguson (1996)
	ccmABC	as original	Page *et al.,* 1997
	ccmG ccmD		Page and Ferguson, 1997
	ccmF ccmH		Pearce *et al.,* 1997

cluster in *E. coli, H. influenzae and P. fluorescens,* in two clusters in *B. japonicum* and probably in *Rhizobium leguminosarum*, and in at least three in *R. capsulatus* and *P. denitrificans* (Figure 1.4). Within each cluster an operon organisation is likely. The genetic organisation of *ccmABC* genes is highly conserved, wherever reported so far. Apart from the *ccm* genes at the 47 minute region of *E. coli* chromosome, *nrfEFG* genes at the 92 minute region have also been implicated in cytochrome *c* biogenesis during anaerobic growth in *E. coli* (Hussain *et al.,* 1994). The *nrf* genes are part of an operon that codes for a respiratory nitrite reductase which includes cytochrome c_{552} (a nitrite reductase) coded by *nrfA*. CcmF is homologous to NrfE, the *N*-terminal region of CcmH is homologous to NrfF and *C*-terminal portion of CcmH is similar to NrfG. As mentioned earlier, NrfE is specifically required for the attachment of haem to an unusual CXXCK sequence motif (Eaves *et al.,* 1998; Grove *et al.,* 1996a). It is not clear why NrfF and NrfG are needed. There must presumably be some reason why the assembly of the nitrite reductase is optimised by having these contiguous *nrf* genes rather than relying on their *ccm* homologues elsewhere in the genome.

There is much less to say about the organisation of the genes for cytochrome *c* biogenesis in Gram positive organisms and *H. pylori*. The two definately identified genes *ccdA* and *ccsA* (*ycf 5*) are not contiguous.

1.6 Biogenesis of chloroplastic *c*-type cytochromes

C-type cytochrome maturation has been extensively studied in chloroplasts (Howe and Merchant, 1992, Zollner *et al.*, 1992). However, many questions concerning the step wise maturation of *c*-type cytochromes in the chloroplast have yet to be answered. The biosynthesis of photosynthetic *c*-type cytochromes seems to be a comparably complex

process to that in bacteria. Targeting of the nuclear-encoded soluble cytochrome c_6 requires translocation across three membranes (double envelope chloroplast membrane and thylakoid membrane). However, the organelle-encoded, membrane anchored cytochrome *f* crosses only one membrane for its maturation. The post translational biosynthetic steps involved in biogenesis of cytochrome *f* are i) insertion of precursor polypeptide into the thylakoid membrane, ii) proteolytic removal of the presequence iii) ligation of haem to two cysteinyl residues iv) and the assembly into the b_6f complex. The biogenesis of cytochrome c_6 involves a) the translocation across the envelop and the thylakoid membrane, b) sequential cleavage of the two targeting sequences c) the covalent attachment of haem. Cytochrome c_6 polypeptide (Merchant and Bogorad, 1987) contains a 'two domain' transit sequence, which is found in almost all nuclear encoded pre-proteins that target the thylakoid lumen (Franzen *et al.,* 1989; von Heijne *et al.,* 1989; Merchant *et al.,* 1990). It has been proposed that the translocation of pre-proteins occurs in two steps (Weisbeek *et al.,* 1989; Bauerle *et al.,* 1991). The pre-protein is transported across the envelope membrane to the stroma followed by the transport across the thylakoid membrane to the lumen. A proteolytic processing step, catalysed by the lumen-facing, thylakoid membrane-bound processing protease (Hageman *et al.,* 1986; Kirwin *et al.,* 1988), is proposed to occur either concomitant with, or subsequent to, the second translocation. The processing step is independent of haem attachment for either of the plastidic cytochrome precursors (Xie and Merchant, 1996; Anderson and Gray, 1991). This step is in contrast to the mitochondrial *c*-type cytochrome biogenesis in which the targeting is tightly coupled with the haem attachment but it is similar to that found in bacteria, where cytochrome *c* is processed in the absence of haem attachment (Page and Ferguson, 1989; Thoney-Meyer and Kunzler, 1997). The fully processed apocytochrome

c_6 is proposed to be a substrate for haem attachment. The step of the covalent attachment of haem to the apocytochrome c_6 has not yet been characterised. A chloroplastic gene product of *ccsA* is believed to perform this function based on its homology to bacterial *ccmF* genes. The inactivation of this gene results in a non-photosynthetic phenotype attributable to the absence of *c*-type cytochromes (Xie and Merchant, 1996). *ccsA* does not show any sequence similarity with the mitochondrial cytochrome *c* haem lyases. In contrast to the mitochondria, where separate haem lyases are required for soluble and membrane bound *c*-type cytochromes, the chloroplastic *ccsA* gene product, with the possible function of haem lyase, is sufficient for the haem ligation for both soluble and membrane bound *c*-type cytochromes i.e cytochrome *c6* and cytochrome *f*. *In vivo* and *in organelle* studies indicate that the association of the targeted apoprotein with its cofactor occurs in the lumen of the thylakoid membrane (Merchant and Bogorad, 1986) and the apoprotein is degraded in the absence of haem attachment (Merchant and Bogorad, 1986).

Mutants defective in the posttranslational maturation of cytochrome c_6 were associated with defects in the assembly of the cytochrome b_6f complex. The steps in the maturation of cytochrome *f* are not clearly understood, for example whether the processing or haem attachment occur co-translationally or post-translationally. A stop transfer model has been proposed for the insertion of cytochrome *f* into the thylakoid membrane where the nascent presequence mediates the binding of the ribosome to the membrane and the translocation of the *N*- terminal haem binding domain occurs co-translationally until the *C*-terminal hydrophobic domain becomes 'anchored' to the lipid bilayer (Willey and Gray, 1988). Processing of the pre-protein by cleavage on the *C* side of a conserved AXA sequence generates a mature protein with an *N*- terminal tyrosinyl residue whose alpha amino group serves as a sixth ligand to the haem iron (Cramer *et al.*, 1994). The

proteolytic cleavage is thus a prerequisite for a proper assembly of the holoprotein and is thus essential for cytochrome *f* function.

Thus in chloroplasts, as in bacteria, the multiple genes appear to specify functions that are necessary for the biosynthesis of both soluble and membrane bound *c*-type cytochromes. The proteins required for the maturation of soluble and membrane associated *c*-type cytochromes in the choloroplast, are encoded by nuclear and plastid genome (for review; Howe and Merchant, 1994). Many required genes may not have been isolated but recent analysis of chloroplast and nuclear genomes of *C. reinhardtii* (Goldschmidt-Clermont 1991; Adam *et al.,* 1993; Schnell and Lefebvre 1993) might provide the relevant information.

1.7 Pathway comparison: Mitochondria, Bacteria and Chloroplast *c*-type cytochrome maturation

Most of the research devoted to cytochromes *c* has focused on their structure and function in the biological electron transport. In past 10-12 years *c*-type cytochromes have also been exploited as model proteins for the study of the protein assembly in biological systems. Many features in the structure, function and the biogenesis of *c*-type cytochromes are conserved, making cytochrome *c* a tool to study evolutionary relatedness. X-ray crystallographic studies have shown that many of the soluble cytochromes *c* in the biological world are very similar in their 3-dimensional structure (Dickerson 1980). The conserved haem binding motif Cys-X-Y-Cys-His is present universally in all the *c*-type cytochromes, sequenced so far, around 200 in number, with very few exceptions (see General background). The process of *c*-type cytochrome biogenesis in fungal mitochondria is relatively simple and requires far fewer components than the bacterial process. The

same pathway may operate in mitochondria from higher cells because the gene for a haem lyase has been identified. As already indicated, the process in bacteria seems to require many more components and is rather complex. It might be that since the periplasm is more vulnerable to changes in the external environment like pH, temperature and osmolarity due to the presence of leaky outer membranes, the maturation of *c*-type cytochromes in this compartment thus requires far more components to counter balance the external conditions. In addition, the co-existence with disulphide bond forming systems appears to add a complication that does not occur in mitochondria. This complication may also be the reason, or at least one reason, why not all bacteria use the same apparatus for making *c*-type cytochromes. Thus *H. pylori* and Gram positive organisms may not have the disulphide bond forming system and so they differ from *E. coli* in respect of *c*-type cytochrome biogenesis and other Gram negative bacteria. However, even in a Gram positive organism the assembly of the *c*-type cytochromes will occur exposed to external conditions (see above) and this may explain why they differ from fungal mitochondria in respect of cytochrome *c* biogenesis. The targeting and processing of cytochrome pre-proteins in chloroplasts are comparable to the analogous steps in the prokaryotic systems. The *N*-terminal presequence of pea cytochrome *f* has been shown to direct β-galactosidase to the bacterial cytoplasmic membrane in a Sec-A dependent pathway (Rothstein *et al.*, 1985). Moreover, the purified leader peptidase of *E. coli* can cleave pre-apocytochrome *f* from pea and *Chlamydomonas reinhardtii* (Anderson and Gray 1991; Howe and Merchant 1993). The rest of the *c*-type biogenesis apparatus in thylakoids appears to be related to that in Gram positive bacteria rather than to those of Gram negative bacteria or fungal mitochondria. It is possible that the low pH inside illuminated thylakoid means that the mitochondrial system could not operate there. The absence of a disulphide bond forming

system, as far as is known, inside thylakoids may explain why the Gram negative cytochrome *c* biogenesis system does not occur in thylakoids.

Putative homologues of the *ccmF* gene have been identified in mitochondrial genomes of *Paramecium, Oenothera,* wheat and carrots, and in the choloroplast genomes of liverwort, tabacco, rice and *Cyanidium* on the basis of sequence identity within a short region of respective open reading frames (Beckman *et al.,* 1992; Schuster *et al.,* 1993; Gonzales *et al.,* 1993). The recent identification of ORF 287 shows 57% sequence identity with liverwort ORF320, 22% identity with *R. capsulatus ccmF* gene product. This, and other, occurrence of bacterial cytochrome *c* biogenesis genes in some plant and protozoa mitochondria, especially *Reclinomonas americana*, is surprising because it implies that these mitochondria make their *c*-type cytochromes *c* by a different route than fungal mitochondria. This remains to be proved, and if it is, the reasons for this phenomenon will have to be elucidated.

In summary, it has surprisingly emerged that there are three different systems for making *c*-type cytochromes. Why this is the case, and how each system works, are all areas of great uncertainty at the moment.

1.8 Biosynthesis of haem cofactor

Haem is the iron derivative of protoporphyrin IX. It is a rigid planar molecule comprising four pyrrole groups linked by methine bridges to form a tetrapyrole ring. Each pyrrole ring is synthesised by the condensation of the two molecules of δ-aminolevulinic acid (ALA), a universal precursor for haem synthesis. It is well established that one of the most important regulatory sites for haem biosynthesis is δ-aminolevulinic acid synthase (ALAS) and that the response of activity of this enzyme to haem and iron levels is of

major significance. Two kind of pathways exists in the biological world for the formation of δ-aminolevulinic acid (Figure 1.5). In the C4 pathway which is operative in yeast and mammalian cells (Jordan 1990), ALA is formed by the condensation of succinyl coenzyme A and glycine (Shemin and Russell 1953). Also, in alpha proteobacteria such as *Rhodobacter, Rhizobium, Bradyrhizobium* and *Agrobacterium* ALA is synthesised by the C4 pathway. This process is catalysed by an enzyme known as ALA synthase. In the other pathway, known as the C5 pathway, ALA is synthesised from glutamate (Beale *et al.*, 1975). The C5 pathway operates in many micro-organisms, such as eubacteria including *E. coli* (Avissar and Beale 1989; Li *et al.*, 1989; O' Neil *et al.*, 1989), archaebacteria and cyanobacteria as well as in the chloroplasts of algae and plants. In the C5 pathway glutamate serves as a precursor involving glutamyl tRNA. This is converted to ALA by glutamyl tRNA reductase. The genes encoding ALA synthase and glutRNA reductase are confusingly both called *hemA*. The cyclic intermediate uroporphyrinogen III is made from ALA in three steps. Two molecules of ALA are condensed by ALA dehydratase (*hemB* gene product) to yield porphobilinogen (PBG). Next four molecules of PBG are transformed into uroporphyrinogen III: PBG is deaminated by PBG deaminase (*hemC* gene product) and finally uroporphyrinogen III is formed by uroporphyrinogen III cosynthase, the *hemD* gene product. The rest of the steps require uroporphyrinogen III decorboxylase (*hemE* gene product), coproporphyrinogen III oxidase (*hemF or hemN* gene product), portoporphyrinogen IX oxidase (*hemG* or *hemY* gene product, and ferrochelatase (*hemH* gene product). The hydrophobic profile of ferrochelatase suggests that it is a peripheral membrane protein and most likely facing the cytoplasmic side of the membrane

in bacteria. Haem *b* is incorporated directly into the respiratory enzymes and serves as a precursor for the formation of haems *c*, *d*, *o* and *a*. Several comprehensive reviews on the biosynthesis of haem are available; Beale, (1993); Beale, (1996); Dailey, (1990); O' Brian, (1996). The general pathway of haem synthesis in biological systems is shown in Figure 1.5. The intracellular transport of haem has not been characterised. Haem transport is most likely achieved by an energy dependent transporter (see section 1.4 in the biogenesis of *c*-type cytochromes). However, the work of Page *et al.,* (1997), shows that CcmABC might not be a haem transporter. The regulatory effect of haem on the expression of *c*-type cytochromes has been studied in detail in mitochondria and is discussed in section 1.13.

1.9 Cytochrome c_{552}* from *Hydrogenobacter thermophilus*

Hydrogenobacter thermophilus is a thermophilic, obligatory autotrophic, hydrogen-oxidising bacterium. Cytochrome c_{552} is the major *c*-type cytochrome of this organism, which is present abundantly in the periplasm, and is believed to be important in the energy metabolism of the organism. It reacts with the hydrogenase isolated from *H. thermophilus* TK-6, which is considered to be the first reaction in the electron transport pathway from hydrogen in this micro-organism (Ishii *et al.,* 1987). Cytochrome c_{552} has been purified from the wild type strain, TK-6 (Ishii *et al.*, 1987) and its amino acid sequence was found to be 56% homologous with that of *Pseudomonas aeroginosa* cytochrome c_{551}. It is the lowest molecular weight cytochrome *c* ever reported, with 80 amino acid residues; *P. aeruginosa* cytochrome c_{551} has 82 residues. The molecular weight of *H. thermophilus*

* Cytochrome c_{552} in the rest of the text should be considered as *H. thermophilus* cytochrome c_{552} unless otherwise stated.

cytochrome c_{552} has been reported to be 7.6 kD (Sanbongi *et al.,* 1989)**. The study of the tertiary structure of *P. aeruginosa* cytochrome c_{551} shows that it is composed of several α-helical regions (Almassy and Dickerson, 1978). Most of the regions in *H. thermophilus* cytochrome c_{552} which correspond to the α-helical regions in *P. aeruginosa* c_{551} show high similarity and the observed amino acid substitutions are those which are frequently found in homologous proteins. Therefore the tertiary structure as well as the amino acid sequence of *H. thermophilus* cytochrome c_{552} are believed to be similar to those of c_{551} of *P. aeruginosa*. However, cytochrome c_{552} is a basic protein with 15 basic amino acid residues against 7 acidic ones while *P. aeruginosa* c_{551} is an acidic protein. Furthermore, *H. thermophilus* cytochrome c_{552} is an extremely stable protein (Sanbongi *et al.,* 1989a) relative to cytochrome c_{551} from the mesophilic bacterium *P. aeruginosa.* This thermophilic cytochrome c_{552} can return to its native structure even after being autoclaved at 120°C for 10 min (Sanbongi *et al.,* 1989a). Its stability has been investigated by following changes in the circular dichroism (CD) spectra on increasing the temperature up to 100°C. It remained intact up to 100°C and its melting temperature in the presence of 1.5 M guanidine hydrochloride was 34°C higher than that of *P. aeruginosa* cytochrome c_{551} (Sambongi *et al.,* 1989). Cytochrome c_{552} contains two cysteine residues, Cys-10 and Cys-13, and a histidine residue, His-14. *H. thermophilus* cytochrome c_{552} is synthesised *in vivo* as a precursor having a typical *N*-terminal signal sequence consisting of 18 amino acid residues. The cytochrome c_{552} gene lacking its own signal sequence has been cloned and it was successfully expressed in *E. coli* (Sanbongi *et al.,* 1991). The cytochrome c_{552} expressed in *E. coli* was similar to native cytochrome c_{552} in spectral properties (Sanbongi

** The present work shows this figure to be incorrect. The correct molecular weight of *H. thermophilus* cytochrome c_{552} is 9.129 kD as obtained by mass spray analysis and recalculating its molecular weight (see also later). However, its molecular weight is still lowest among *c*-type cytochromes.

et al., 1991) and the haem was attached to the apocytochrome c_{552} by a covalent linkage. It was matured in the cytoplasm of *E. coli* in the absence of its signal sequence. This was remarkable as other *c*-type cytochromes require translocation to the periplasm for their maturation (see the previous sections). Attempts to express mitochondrial cytochrome *c* in the cytoplasm of *E. coli* resulted, in contrast to cytochrome c_{552}, in the formation of inclusion bodies containing the apo protein (Tong *et al.*, 1995).

1.10 Studies on the biogenesis of *Hydrogenobacter thermophilus* cytochrome c_{552}

The remarkable formation of the holoform of cytochrome c_{552} in the cytoplasm of either *E. coli* or *P. denitrificans*, that had been transformed with a plasmid carrying the coding region of its mature gene (Sambongi and Ferguson, 1994b), is in apparent contrast to the hypothesis that haem attachment to *c*-type cytochromes occurs in the bacterial periplasm (see earlier). Moreover, this *H. thermophilus* cytochrome c_{552} was found to be matured in an *E. coli* mutant strain lacking the *dipZ* gene, coding for a disulphide isomerase or thioredoxin like protein, essential for other normal *c*-type cytochrome maturation (Crooke and Cole, 1995). The maturation of *H. thermophilus* cytochrome c_{552} in the cytoplasm might suggest that the synthesis of this particular cytochrome *c* is independent of the cellular mechanism for bacterial *c*-type cytochrome assembly. Alternatively, understanding the biogenesis of this cytochrome in the cytoplasm might show that existing ideas about how and where the covalent bonds between the polypeptide and the haem are formed to be incorrect. Perhaps, for example, enzyme catalysis is not needed *per se* to catalyse formation of the covalent bonds between the cysteine thiol groups and the vinyl groups of the haem. A Gram-negative bacterium *Helicobacter pyoli*

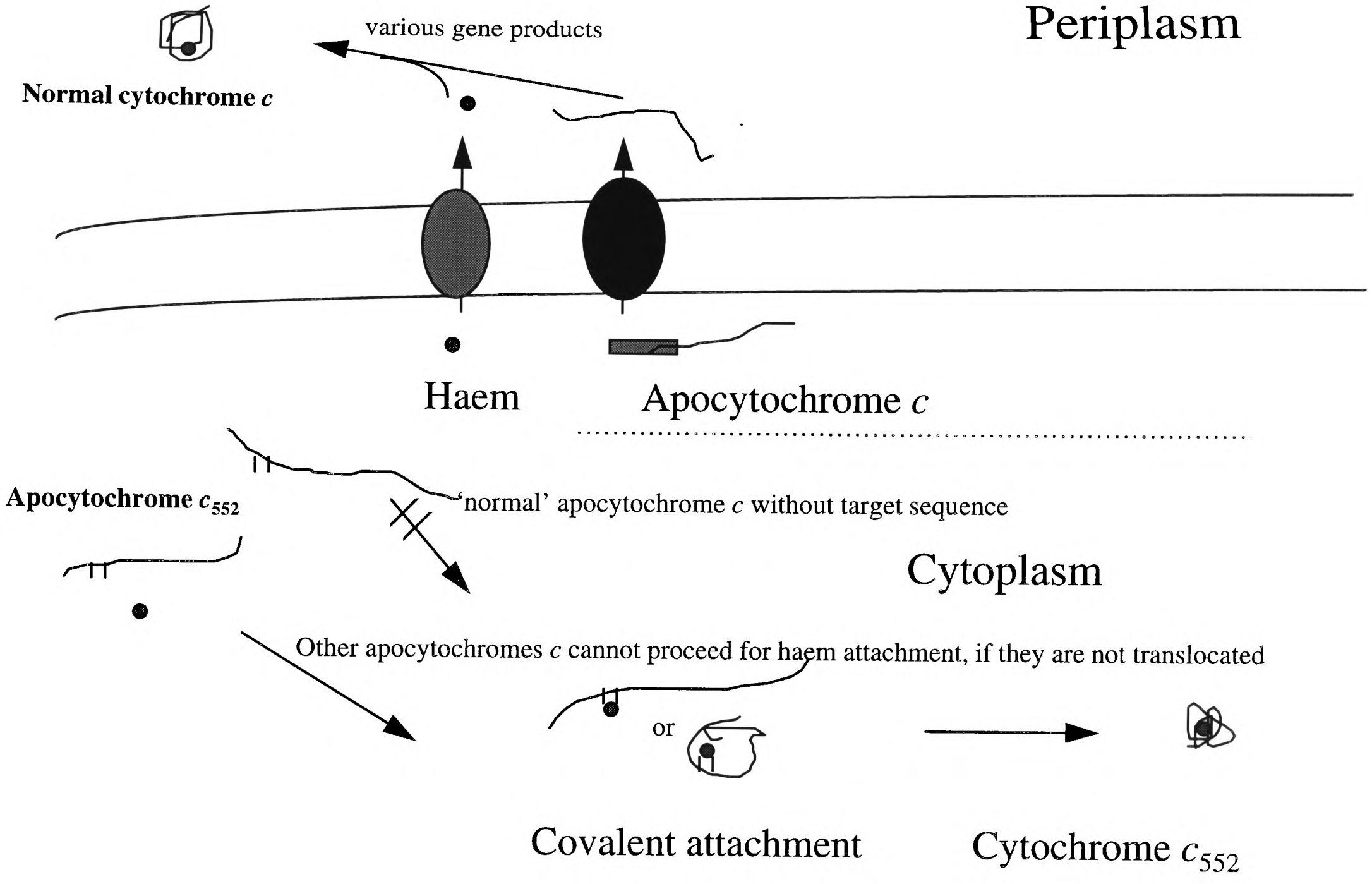


Figure 1.6. The hypothetical pathway in *E. coli* for the maturation of *H. thermophilus* cytochrome c_{552} in relation to the maturation of other *c*-type cytochromes in a Gram-negative bacteria.

lacks nearly all of the genes for *c*-type cytochrome biogenesis (Page *et al.*, 1997). As discussed before, the absence of disulphide bond forming proteins in the periplasm of this bacteria could mean that the machinery for the rereduction of the apopolypeptide is not required for the apocytochrome *c* prior to the covalent linkage. This hypothesis fits well with the maturation of cytochrome c_{552} in the cytoplasm of *E. coli*. It might be that the reducing environment of the cytoplasm means that a complex machinery for the reduction of apocytochrome *c* is dispensable. Moreover, it might be that the apocytochrome c_{552}, being a protein from a thermophile, has sufficient tertiary structure to provide a haem binding pocket for haem so that no further enzymatic power is needed for the covalent attachment, which would therefore be spontaneous, under the reducing environment of the cytoplasm of mesophilic *E. coli*. The biogenesis of *H. thermophilus* cytochrome c_{552} is studied further, using *E. coli* as an expression system, in the present work. Since *H. thermophilus* cytochrome c_{552} is matured in the cytoplasm of *E. coli*, both under aerobic and anaerobic growth conditions (discussed in chapter III), the study of its biogenesis in *E. coli* under aerobic growth conditions should be straightforward, without any background from endogenous *c*-type cytochromes which are only made under anaerobic conditions. A hypothetical model for the maturation of *H. thermophilus* cytochrome c_{552} is shown in Figure 1.6.

1.11 Protein folding and stability: with reference to *H. thermophilus* cytochrome c_{552}

Not much is known about how a cytochrome *c* reaches a conformation which allows the covalent ligation of haem or whether the apocytochrome is partially folded before haem acquisition. In the absence of haem the mitochondrial apocytochrome *c* is unfolded

and the noncovalent binding of haem induces the formation of a compact structure of polypeptide (Dumont *et al.,* 1994). The folding of cytochrome *c* polypeptide could be tightly connected with the haem binding. The cytoplasmic maturation of *H. thermophilus* cytochrome c_{552} could be merely due to the fact that the apocytochrome c_{552} is stable and thus has some tertiary structure which further is compacted on haem acquisition. *B. japonicum* cytochrome c_{550} when expressed in *E. coli*, either from the construct lacking the coding region of its signal peptide or with mutated haem binding sites, was degraded rapidly although the mutants in haem binding sites were shown to be translocated to the periplasm (Thony-Meyer and Kunzler, 1997). This might suggest that the haem binding stabilises the protein and protects it from the proteolytic degradation. Cytochrome *c* deficient mutants of *P. denitrificans* are different from other bacteria in that that they contain detectable apocytochrome c_{550}. The reason for this is unknown. It might be that after the apocytochrome gets translocated to the periplasm, potentially specific periplasmic chaperons, for endogenous *c*-type cytochromes, protect them from proteolysis. Until now chaperons specific for *c*-type cytochrome biogenesis have not been detected. It is likely that one of the well known cytoplasmic chaperons GroEL. DnaK, or SecB plays a role in cytochrome *c* biogenesis (Kumamoto. 1991). Periplasmic chaperons involved in specific pathways have been discovered recently (Hultgren *et al.,* 1993; Hultgren and Jones. 1995; Chen and Henning 1996; Jacobs *et al.,* 1993). It might be that certain proteins involved in *c*-type cytochrome biogenesis have chaperone like functions, especially when the roles of many such proteins are yet to be defined. For example CcmE CcmH and CycH, could be the periplasmic chaperons. The requirement of chaperons could be to hold the apocytchrome *c* and haem in the right conformation for the covalent attachment to occur. In the case of cytoplasmic production of cytochrome c_{552} it may be that the stability

of the apo protein is such that no assistance from, or protection by, chaperones is needed. The situation is abnormal because a thermophilic protein is being expressed in the mesophilic *E. coli*.

1.12 An introduction to *E. coli* cytochrome b_{562}; its possible importance in the study of *c*-type cytochrome biogenesis

Cytochrome b_{562} from *E. coli* is a small (12 kD) water-soluble haem protein, localised in the periplasm with an as yet undefined role. It contains an *N*-terminal leader sequence which is subsequently cleaved during or after transport through the cytoplasmic membrane (Nikkila *et al.*, 1987). It appears to be a type II cytochrome *c* which has lost, or not acquired, both of its haem binding cysteine residues (Weber *et al.*, 1981). It is very similar to cytochrome *c′* from photosynthetic species, belonging to the class of cytochromes with 4-α-helical structure (Weber *et al.*, 1982) and its electronic and structural properties are similar to *c*-type cytochromes (Mathews, 1985; Meyer and Kamen 1982; Moore *et al.*, 1982). Introducing cysteine residues into the amino acid sequence of cytochrome b_{562} in positions homologous to those found in the other members of the family results in the formation of *c*-type holocytochrome with covalently attached haem (Barker *et al.*, 1995). The exact steps of its biogenesis have yet to be defined. It is as yet unknown whether this cytochrome acquires its haem in the cytoplasm or in the periplasm. Genes for two putative haem transporters have been identified in *E. coli*. *cycDC* and *ccmABC* (Thony-Meyer *et al.*, 1995; Poole *et al.*, 1994), though *cydDC* mutants are found to be defective in a step not specifically associated with haem availability (Goldman *et al.*, 1996). Mutants defective in *cydD* and *cydC* lack both cytochrome *bd*- and *c*-type

cytochromes (Poole *et al.*, 1994; Goldman *et al.*, 1996). Possibly the b_{562} apocytochrome and haem use the Sec and CcmABC transporter respectively to be translocated to the periplasm where the haem attachment takes place. It might be that the pathways of haem acquisitions vary in between *c*-type and *b*-type cytochromes and that the CcmABC, a predicted haem transporter, is necessary for *c*-type cytochrome synthesis but a different, non-specific or diffusional, mechanism operates for haem export to periplasmic cytochrome b_{562}. Elucidation of this point would help us to understand periplasmic cytochrome *c* assembly.

1.13 The regulatory effect of haem on the expression of *c*-type cytochromes

The structure and function of several mature cytochrome *c* proteins have been well studied, but little is known about the regulation of the cytochrome synthesis and how this regulation is co-ordinated with production of the haem ligand. The response of the transcriptional control region of the *Rhodobacter sphaeroides*, cytochrome c_2 gene, *cyc A*, to the intermediates of haem biosynthesis was studied by Schilke and Donohue (1992) in order to understand if, and how, synthesis of cytochrome c_2 is tied to haem availability. It was found that the addition of δ-aminolevulinic acid, the first committed intermediate in haem biosynthesis, inhibited *cyc A* transcription initiation at both the upstream and downstream promoter regions. The *c*-type cytochromes are required for photosynthetic growth (Daldal *et al.*, 1987) and for dark, anaerobic growth (Kranz 1989) in *R. capsulatus*. Under anaerobic conditions, synthesis of the photosynthetic apparatus and δ-aminolevulinic acid production is induced to higher levels. It has been suggested that in photosynthetic cells, the tetrapyrrole biosynthetic pathway must supply both haem and

bacteriochlorophyll to support growth. The large increase in bacteriochlorophyll biosynthesis under anaerobic conditions is postulated to limit haem availability and relieve haem dependent feedback inhibition of tetrapyrrole biosynthesis (Lascelles, 1978). Coupling of cytochrome biosynthesis to ligand availability has been observed in *Saccharomyces cerevisiae*, but the mechanisms appear to be different in this organism relative to *R. sphaeroides*. In yeast, haem appears to have a positive effect on cytochrome synthesis since transcription of genes for both isoforms of mitochondrial cytochrome *c* are activated by a haem dependent DNA binding protein (Guarente and Mason, 1983; Prezant *et al.*, 1987; Winkler *et al.*, 1988). It has been shown in the mutants of *Saccharomyces cerevisiae*, deficient in first enzyme of porphyrin biosynthesis, δ-aminolevulinic acid synthase (Gollub *et al.*, 1977), the amount of haem and cytochrome c synthesis could be varied over a wide range simply by controlling the amount of δ-aminolevulinic acid in growth medium. There is an *E. coli* mutant in the gene *hemA*, encoding glutamyl t-RNA reductase, required for the synthesis of δ-aminolevulinic acid, which could likewise be used for studies of the effect of haem availability on exogenous *c*-type cytochrome biogenesis in *E. coli*.

1.14 The aims of the present work

The cytoplasmic synthesis in *E. coli* of the holo form of the cytochrome c_{552} from *H. thermophilus* is intriguing. At the outset of the present work it had been argued to be formed in a spontaneous reaction that did not require any gene products that are normally required for *c*-type cytochrome biogenesis. One of the objectives of the present work was to test this proposition. Amongst the experimental questions that could be asked were: i) Is there a single stereochemistry of haem attachment to the cytochrome c_{552} isolated from the

E. coli cytoplasm and is it the same as is found in the protein isolated from *H. thermophilus* ? ii) Can the apoform of cytochrome c_{552} be shown to have at least some of the tertiary structure which is expected if it is to bind haem in a manner that leads to the proper, but spontaneous, covalent attachment that is a characteristic of a *c*-type cytochrome; iii) Could the formation of holo cytochrome c_{552} be demonstrated *in vitro*, either through incubation of the apo protein with haem or by using an *in vitro* transcription/ translation system; iv) with the expectation that *E. coli* mutants affected in some or all of the cytochrome *c* biogenesis genes would become available during the course of the present work, the effects, if any, on the synthesis of cytoplasmic cytochrome c_{552} could be tested. v) If such mutants of *E. coli* did become available it was hoped to test for the functions of at least some of the genes required for *c*-type cytochrome biogenesis. In particular it was planned to test whether the synthesis of the periplasmic cytochrome b_{562} was dependent on any of the gene products, especially those postulated to constitute a system for transporting haem from cytoplasm to the periplasm.

The types of experiments envisaged under i) to iii) above were recognised to require substantial quantities of cytochrome c_{552}. Thus it was anticipated that a significant amount of effort would have to be directed towards identifying procedures for preparation of the required amounts of cytochrome c_{552} along with studies to identify the factors which contribute towards the cytoplasmic maturation of this cytochrome c_{552}.

Chapter II

Materials and Methods

2.1 Bacterial Strains: The *E. coli* strains used in the present study were:

Organism	Genotypes	References
Escherichia coli		
JM83	*ara* Δ(*lac-proAB*)*rspLϕ80 lacZΔM15*	Yanisch-Perron *et al.,* 1985.
JM109	e14$^-$(McrA$^-$) *recA1 endA1 gyrA96 thi-1 hsdR17*(r_K- m_{K+}) *supE44 relA1* Δ(*lac-pro AB*) [F'*traD36 proAB lacI*^{q}*ZΔM15*]	Yanisch-Perron *et al.,* 1985.
DH5α	*supE44 ΔU169* (ϕ80 *lacZΔM15*) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	Hanahan, 1983
JCB387	*E. coli* RV Δ *nir B*	Griffiths and Cole., 1987.
HMS174(DE3)	F- *recA hsdR*(r_{K12}-m_{K12}+) Rif^R (DE3)	Supplied by Novagen, originally developed by Studier and Moffatt, 1986
JCB712	*pro his trpΔlac*	M. Jones-Mortimer.
JCB71202	*Δccm(A'-'H)*::Ω	Grove *et al.,* 1996b
HU227	*hemA metB1 rel A1* haem permeable HfrCovalli.	Hingerer *et al.,* 1995

2.2 Growth media and growth conditions

2.2.1 Aerobic growth media

Media-Luria-Bertani (LB) media was used for aerobic growth. This contained 10 g Bacto-Trptone, 5 g Bacto-Yeast extract and 10 g NaCl per litre of deionised water. This was sterilised by autoclaving for 20 minutes at 15 lb/sq inch.

2.2.2 Anaerobic growth media

This contained 1 X Minimal Salts Solution,10 % L B medium, 0.4 % Glycerol, 40 mM fumarate and 25 mM potassium nitrite. All the components were sterilised separately by autoclaving as above. Anaerobic growth medium was prepared fresh from these components shortly before inoculation. Double concentrated minimal salts solution contained, per litre of deionised water, 9 g of KH_2PO_4, 21 g of K_2HPO_4, 2 g of $(NH_4)_2$ SO_4, 1 g of tri-sodium citrate, 0.1 g of $MgSO_4.7H_2O$, 2 ml of *E. coli* sulphur free salts, 2 ml of 1 mM $(NH_4)_6$ Mo_7, 2 ml of 1 mM sodium selenate. The medium was autoclaved as described above. It was then stored at the room temperature for further use.

E. coli sulphur free salts solution contained, in 100 ml of deionised H_20, 2.8 g of $MgCl_2.7H_2O$, 1.0 g of $MnCl_2.4H_2O$, 0.4 g of $FeCl_2.6H_2O$, 0.1 g of $CaCl_2$. The solution was autoclaved as above and stored at the room temperature for further use.

2.2.3 Growth of the cells

The cells were grown aerobically by inoculating either approximately 5 µl from glycerol stocks or cells taken directly from a plate using a sterile tooth pick, into 2 ml medium in 15 ml test tubes, 50 ml medium in 250 ml conical flasks or 500 ml medium in 2.5 l conical flasks, as required, and incubating the culture overnight at 37°C with vigorous shaking. For the screening of single cell colonies after transformation, or for the replication of single cell colonies, the cells were grown on Luria agar (LA) media on Petri plates containing 20-25 ml LB medium solidified by 1.5 % bacteriological agar. 50-100 µl of transformed cells were spread or streaked onto these plates. Appropriate amounts of antibiotics were added where required. 4 µl of isopropylthio-β-D-galactoside (IPTG) (from the stock of 200 mg/ml) and 40 µl of substrate 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal) (Horwitz *et al.*,

1964) (from a stock of 20 mg/ml in dimethylformamide) was spread on the top of the agar just before plating of the cells having first incubated the plates at 37°C until all their surfaces had dried, where cloning was done in those vectors which allow the histochemical identification of the recombinant clones. These vectors carry a segment of DNA derived from the *lac* operon of *E. coli* that codes for the amino terminal fragment of β-galactosidase, the synthesis of which can be induced by IPTG, which is capable of intra-allelic (α) complementation with a defective form of β-galactosidase encoded by the host. Bacteria exposed to the gratuitous inducer IPTG synthesise both the fragments of enzyme and form blue colonies when plated on media containing the chromogenic substrate X-gal Insertion of foreign DNA into the polycloning site of the plasmid inactivates the amino-terminal fragment of β-galactosidase and abolishes α-complementation. Bacteria carrying recombinant plasmids therefore give rise to white colonies.

For the anaerobic growth the cells from overnight aerobic culture were inoculated in 7 ml of anaerobic media in 7.5 ml sterile tubes, 200 ml of anaerobic media in 200 ml bottles and 5 l anaerobic media in 5 l bottles, as required, and grown at 37°C without shaking for 12-16 hrs, till the culture has reached the OD of 1.0 at 580 nm. The appropriate antibiotics were added where required.

The haem precursor, δ-aminolevulinic acid, was added in growth medium in some studies, as indicated. The final concentration of haem precursor used was 0.1 mM-1.0 mM. 1 M stock solution was prepared by dissolving δ-aminolevulinic acid, supplied by Sigma, in autoclaved deionised H_20.

2.3 Protein Biochemistry Techniques

2.3.1 Preparation of total soluble extracts

Total soluble bacterial cell extracts were prepared in order to check the expression of particular cytochrome by SDS-PAGE and spectrophotometric analysis. Usually 10 ml of the culture of OD 1.5 at 580 nm was used for this purpose. The cells were harvested by centrifugation at 12,000g for 30 seconds, using the bench microfuge. The cells were suspended in 100 µl of STE [0.1 M NaCl, 10 mM Tris. HCl (pH 8.0), 1 mM EDTA (pH 8.0)]. The suspension was frozen at -70°C and thawed at room temperature to break the cell wall. The suspension was then sonicated at maximum power using an MSE sonicator with a frequency of 20 khz with a probe size of 3 mm diameter. Two cycles of sonication were used for each sample; each cycle consisted of sonication of 1 minute followed by 1 minute on ice. The suspension was then centrifuged at 4°C and 15,000g for 5 minutes. The supernatant consisted of the components of the periplasm and cytoplasm while pellet consisted of membranes and other cell debris. This supernatant was stored at -20°C until use.

2.3.2 Purification of cytoplasm and periplasm or the preparation of the normalised crude extracts

The cytoplasmic and the periplasmic fractions were separated when the localisation of a particular cytochrome was to be studied. When comparisons in the expression of cytochromes were to be made between different strains or growth conditions normalised crude extracts or cytoplasmic and periplasmic fractions were prepared.

The normalised extracts were prepared by growing the cells in 10 ml of media until a culture had reached an OD of 1.5 at 580 nm. Total cell extracts were prepared as described above. The periplasmic and cytoplasmic fractionation was performed using the cold osmotic shock procedure described by Neu and Heppel (1965). 10 ml of overnight grown culture was

harvested by centrifugation at 4°C and at 12,000g for 10 minutes, using the rotor JA20. The cells were resuspended in 200 µl of STE and kept on ice for 10 minutes. This was centrifuged as above. These cells were quickly resuspended in ice cold deionised H_2O and centrifuged again as above. The periplasmic fraction was obtained as a supernatant. It was stored at 4°C. The pellet obtained contained 'shocked' cells that still contained cytoplasm. This pellet was resuspended in 10 mM Tris-HCl (pH 7.5), sonicated and centrifuged at 18,000g, in JA20 rotor. The cytoplasmic fraction was obtained as the supernatant.

2.3.3 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was used to analyse the purified proteins and proteins in the crude extracts and was performed as described in Sambrook *et al.* (1989). 15% acrylamide gels were prepared from a stock of acrylamide and N, N'-methylenebisacrylamide, in the ratio 30:0.8. The stacking gel contained 5% acrylamide, 130 mM Tris-HCl pH 6.8 and 0.1% w/v SDS. The resolving gel contained 15% acrylamide, 375 mM Tris-HCl pH 8.8 and 0.1 % w/v SDS. The polymerisation of the gel was initiated by adding 0.1 % w/v ammonium persulphate and 0.1 % v/v TEMED (N, N, N', N' -tetramethylethylenediamine) to the above mix just before to pouring the gel. The sample of purified proteins, normalised crude extracts or just the crude extracts, as required, were prepared in 1X sample buffer (50 mM Tris-HCl pH 6.8, 100 mM dithiothreitol, 2 % w/v SDS, 0.1 % w/v bromophenol blue, 10 % glycerol) followed by boiling for 5 minutes. When SDS-PAGE was followed by analysis for haem stain, dithiothreitol was not added to the sample buffer and the samples were only incubated at 45 °C for 10 minutes. The samples were separated at 120-180 V for 45-90 minutes, depending on the gel dimensions, at which time the tracking dye had approached the bottom of the gel.

2.3.4 Staining of PAGE gels

Gels were stained for peptide bonds by Coomassie stain as described in Sambrook *et al.*, (1989). Gels were soaked in a staining solution containing Coomassie Brilliant Blue R250 (0.5 % w/v), in 50 % v/v methanol, 10 % v/v acetic acid, for one hour at room temperature. Gels were then destained with 30 % v/v methanol, 10 % v/v acetic acid until the background was clear. For storage the gels were dried between cellulose sheets using the Promega gel drying system.

The haem staining was performed by soaking gels in 70 ml of 0.25 M sodium acetate, pH 5.2, for 15 minutes with gentle shaking. 30 mg of 3, 3', 5, 5'-tetramethylbenzidine dissolved in 30 ml of methanol was added to the gel and shaking continued for another 15 minutes. The stain was developed by adding 0.3 ml 30 % v/v hydrogen peroxide and incubating the gel for 5-10 minutes in the dark. After the bluish green stain comes, the gel was washed twice in 50 ml of 70 % v/v 0.25 M sodium acetate pH 5.2, 30 % v/v isopropanol. When required gels were stored as described above. This procedure essentially followed the method of Goodhew *et al* (1986).

2.3.5 Non-denaturing polyacrylamide gel electrophoresis

The technique is particularly useful for visualising cytochrome *b* when its detection is required by the haem staining. In *b*-type cytochromes the haem is non-covalently attached to the peptide and thus gets removed under the denaturing conditions of SDS-PAGE. This technique is in general useful for the detection of proteins where the separation is required on the basis of charge and size. Non-denaturing gel electrophoresis was performed as described by Mclellan (1982). In the present work cytochrome b_{562}, which is a basic protein, was visualised on 15 % acrylamide gels made with 25 mM histidine/30 mM MOPS, pH 6.6 by

Coomassie and haem staining, as required, after running the samples, at 60-80V for about an hour using small polyacrylamide gel apparatus supplied by Biorad, towards cathode. The sample was prepared in 25 mM histidine/ 30 mM MOPS buffer (pH 6.6) containing 0.2 % sterile glycerol and 0.1 % w/v bromophenol blue.

2.3.6 Western blotting

The S•Tag at the *N*-terminus of cytochrome c_{552}, expressed in T7 expression system as a fusion protein (see Chapter IV), was used to detect immunologically the amount of apocytochrome c_{552} and holocytochrome c_{552} by Western blotting. Protein extracts were separated on 15 % acrylamide gels and transferred to nitrocellulose membranes using semi-dry transfer buffer (50 mM Tris, 38 mM glycine pH 8.3, 0.04 % w/v SDS, 20 % v/v methanol) as an electrolyte, in a Pharmacia LKB Novablot electroblotting apparatus. After transfer, the gel was blocked overnight in PBS-A-T buffer (8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4 pH 7.4, 140 mM NaCl, 7.7 mM NaN_3, 0.3 % v/v Tween-20) plus 1 % w/v skimmed milk powder (blocking buffer). The membrane was washed in PBS-A-T plus 0.1 % w/v skimmed milk powder (wash buffer), then incubated for two hours in blocking buffer containing a rabbit antibody against the S•Tag (obtained from Novagen), at a dilution of 1/1000 in blocking buffer. The membrane was washed thrice for five minutes each time with wash buffer, then incubated for two hours with anti-rabbit antibody that was conjugated to alkaline phosphatase, also diluted to 1/1000. After three more five minutes washes, the membrane was washed four times with PBS-A-T buffer, then equilibrated for one minute in TBS buffer (100 mM Tris-HCl pH 9.5, 100 mM NaCl). Bound antibody was detected colorimetrically by incubating the membrane in TBS buffer containing 10 mM $MgCl_2$, 200 μM nitroblue tetrazolium and 20 μM 5-bromo-4-chloro-3-indolyl phosphate. Development

was stopped by several washes of distilled water and membranes were stored in TE buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA) in the dark.

2.3.7 Protein purification

H. thermophilus cytochrome c_{552} was purified as a recombinant protein and as a recombinant tagged protein by ion-exchange and affinity chromatography. The purification of cytochrome c_{552} from the *E. coli* JCB387, expressing cytochrome c_{552} from the construct pKHC12 (Figure 2.1), is described below while the purification of cytochrome c_{552} as a tagged protein, using T7 expression system is described in Chapter IV.

2.3.7.1 Growth of the cells

Approximately 7 µl from glycerol stock of *E.coli* strain JCB387 carrying the construct pKHC12, which contains the coding region of the mature cytochrome c_{552} with the coding region of its signal sequence deleted (Sanbongi *et al.*, 1991) (Figure 2.1), was inoculated in 2 ml of LB and grown at 37°C overnight with vigorous shaking. One ml of this culture was inoculated in two 200 ml aliquots of anaerobic media contained in 200 ml bottles and the culture was allowed to grow without shaking at 37°C for 10-12 hrs until an OD of 1.0 or more was attained, measured at 580 nm. 100 ml each of this was inoculated in four 5 l aliquots of anaerobic media in 5 l bottles and again grown at 37°C without shaking for 16-18 hrs until the culture has reached an OD of 1.0, or higher, measured at 580 nm.

2.3.7.2 Preparation of the extract

The cells were harvested by centrifugation at 16,000g for 10 minutes, using the rotor, JA10. The pellet was then washed by resuspending in 10 mM Tris/HCl, pH 8 followed

by centrifugation as before. Washing was repeated three times using 200 ml of buffer each time. The pellet was finally resuspended in 100 ml of STE, kept on ice for 10 min and centrifuged. The cytoplasm and the periplasm was separated by the osmotic shock method described above. After sonication of the shocked cells the suspension was centrifuged for 20 min at 40,000g and 4°C, to pellet the membranes. The cell-free extract was then ultra centrifuged at 183,000g and 4°C, to remove remaining membranes and then treated with streptomycin for 30 minutes on ice so as to precipitate nucleic acid. The nucleic acid was removed by centrifugation as above. The cytoplasmic extract was

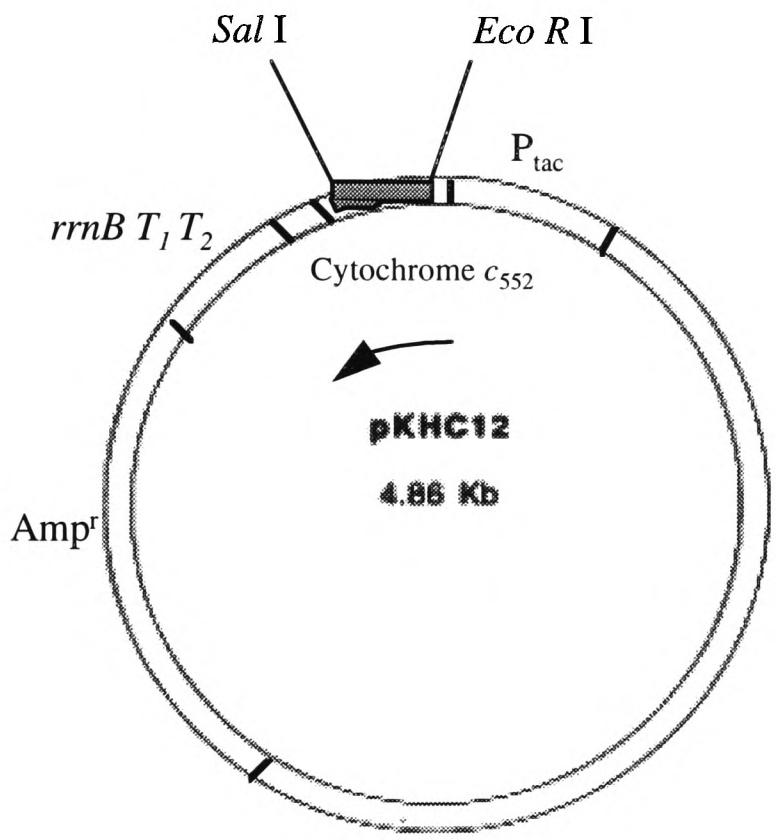


Figure 4.1 A Map of the construct pKHC12. The coding region of *H. thermophilus* cytochrome c_{552} deleted in its signal sequence was cloned at the *Eco R* I-*Sal* I site of the vector pKK223 (Sanbongi *et al.*, 1991).

dialysed against 1 l of 10 mM KH_2PO_4, pH 7.0, overnight. The extract was again centrifuged at 18,000g and 4°C for 20 min. This supernatant was used in subsequent purification steps.

2.3.7.3 Ion-exchange chromatography

The material from the previous step was loaded on to a cation exchange column, cellulose CM-52, size 1 x 10 cm, that had been equilibrated with 10 mM phosphate buffer, pH 7.5. Non-specifically bound material was washed through with 3 column volumes of 10 mM potassium phosphate buffer (pH 7.5). A continuous linear gradient of 0-500 mM NaCl, of volume 500 ml, in the same buffer was used to elute cytochrome c_{552}. 7 ml fractions were collected using an automatic fraction collector.

2.3.7.4 Spectrophotometric analysis

Visible absorption spectra for small amount of each fraction were recorded on a Perkin Elmer Lambda 2 spectrophotometer, interfaced to a Dell 316SX PC running Perkin Elmer spectroscopy software. The spectra were studied from 400-600 nm for the α, β, γ (Soret) peaks for *c*-type haem. The presence of cytochrome c_{552} was confirmed by the observing 552 nm peak in the dithionite reduced spectra (see Chapter III). The cytochrome c_{552} obtained as a reduced and oxidised forms were pooled and dialysed against 1l of 10 mM potassium phosphate buffer (pH 7.5), over night. This material was then used for second step purification step using affinity chromatography.

2.3.7.5 Affinity chromatography

The substantially purified cytochrome c_{552} obtained from the previous step was loaded on to an affinity column, Dye Matrix green A (supplied by Amicon Far East Ltd), pre-

equilibrated with 10 mM phosphate buffer (pH 7.0). The cytochrome c_{552} was eluted as described for the ion exchange chromatography. 7 ml fractions, approximately 8, containing cytochrome c_{552} were pooled, dialysed as above and concentrated, using Amicon Centricon concentrators, to approximately 1 ml.

2.3.8 Determination of the protein concentration in the extracts

The total cell protein present in the crude extracts was estimated as described by Bradford (1976) using BioRad reagent kit. 20 µl of diluted sample was mixed with 0.8 ml of distilled water and 0.2 ml of reagent. The reaction mixture was vortexed and incubated at the room temperature for 5 minutes. Absorbance at 595 nm was recorded and converted to protein concentration in mg ml^{-1} using a standard curve prepared with known concentrations of bovine serum albumin.

2.3.9 Circular Dichroism (CD) spectroscopy

CD spectra were obtained using a JASCO automatic recording spectropolarimeter, Model J20. The CD spectroscopy was also used to study the thermostability of recombinant cytochrome c_{552} (Chapter IV), as described by Sanbongi *et al.,* (1989a). The ellipticity at 222 nm was employed as the structural parameter. The sample (0.3 ml) was placed in a quartz cell with a 1-cm light path embedded in a brass jacket, through which ethylene glycol was circulated. For thermostability studies, the temperature of ethylene glycol was raised by the use of a circulating water bath and the rise in the sample temperature was directly monitored by a thermistor sensor which penetrated the sample. The temperature was continuously raised from 25°C- 95°C, and spectra were recorded after each five degree rise in temperature.

2.3.10 Electrospray ionisation mass spectroscopy

Salt free samples of proteins, required for electrospray-ionisation mass spectroscopy, were obtained by dialysis of the purified protein against deionised distilled water and then concentrating the protein to a volume of 300-500 µl using Amicon Centricon concentrators. The final protein concentration for the analysis was 0.5 mg/ml. The spectra were recorded on a Fisons Instruments Platform Mass Spectrometer with samples in 100 % water at pH 5 (pH adjusted with formic acid). The source temperature was set at 20°C and the spectra were calibrated against hen lysozyme. The instruments were operated by Dr. Carol Robinson and Dr. Robin Aplin, OCMS, University of Oxford.

2.3.11 Gel-filtration column chromatography

Gel-filtration chromatography was used to separate haem from apocytochrome *c* after removing haem from a cytochrome *c* using chemical procedures. A gel filtration column, Sephadex G-25, 10 x 2 cm, was equilibrated with 7 % acetic acid (~ 10 ml) and 2 ml of sample was loaded onto the column. The haem and the apocytochrome *c* were eluted at room temperature by continuous flow of 7 % acetic acid. One ml fractions were collected and examined at 280 nm for protein and at 410 nm for haem.

2.3.12 Alkaline phosphatase assay

The activity of the enzyme alkaline phosphatase (AP) was used as a periplasmic marker. AP enzyme activity assays were done as described by Brickmen and Beckwith (1975). One ml diluted periplasmic extract in 50 mM Tris-HCl (pH 8.0) was added to 0.1 ml of phosphatase substrate Sigma 104 (0.4 % w/v) at 37°C and the increase in the absorbance at 420 nm was measured.

2.4 DNA Techniques

2.4.1 Preparation of plasmid DNA

Plasmid DNA was prepared by the alkaline lysis method similar to what is described in Sambrook *et al.*(1989). Between 2-10 ml of an overnight grown culture was centrifuged at 4°C and at 4,000 rpm to harvest the cells. The cells were resuspended in 300-400 µl of solution I (GTE: 50 mM Glucose, 25 mM Tris/HCl pH 8.0 and 10 mM EDTA), in a microcentrifuge tube. 300-400 µl of freshly prepared solution II (0.2 M NaOH and 1 % SDS) was added followed by gentle mixing. 300-400 µl of ice cold solution III (3 M potassium acetate and 2 M acetic acid) was then added, again with gentle mixing. The suspension was left on ice for 10 minutes. It was then centrifuged at 12,000 rpm for 10 minutes to remove cell debris. The supernatant was treated with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1). This was gently mixed and the phases separated by centrifugation at 15,000 rpm for 2 minutes. This was repeated thrice. The aqueous phase was precipitated with 2 volumes of ethanol and 1/10 volume of 3 M sodium acetate (pH 5.3). The precipitated DNA was pelleted by centrifugation at 15,000 rpm for 15 minutes. The pellet was dried under vacuum suction and suspended in 20-50 µl of sterile H_2O or TE (10 mM Tris/HCl and 1mM EDTA). Plasmid DNA preparation for the purpose of sequencing, PCR and cloning, was performed using a Wizard purification kit supplied by Promaga, using the method described in the instruction manual. The first three steps were similar those described in the above protocol. A silica based resin, to which DNA binds, supplied with the kit, and was added to the clear solution obtained after centrifugation following the addition of solution III. The suspension was then applied to the minicolumn and washed with 2 ml of column wash

solution, again as supplied with the kit. A minicolumn, containing the DNA, was fixed on the top of a 1.5 ml microcentrifuge tube, as described in the instruction manual, and the column dried by centrifuging the whole assembly (minicolumn +microcentrifuge tube) at 10,000 rpm for 2 minutes. The DNA was then eluted by the addition of 50 µl of deionised H_2O to the minicolumn and centrifuging the whole assembly for 20 seconds.

2.4.2 Restriction digestion of DNA

Restriction enzymes of type II, supplied by Boerhinger-Mannheim and New England Biolabs, were used in the present study. The reaction was usually set up in 20 µl final volume. 2-5 units of restriction endonuclease was used per µg of plasmid DNA. Buffers supplied by respective companies were used as recommended. Reactions were allowed to proceed for 3-7 hrs at 37°C. Reactions were stopped by heat inactivation of enzyme as recommended by the suppliers.

2.4.3 Gel electrophoresis for separation and purification of DNA

Plasmid DNA, restriction digested DNA and PCR fragments were separated on agarose gel electrophoresis in a Pharmacia submarine gel tank. 0.8 % to 2 % agarose gels were prepared in 1X TAE buffer (40 mM Tris/acetate, pH 8.0 and 1 mM EDTA). 0.5-2 µg of DNA in a sample buffer (10mM TrisCl pH 8.0, 1 mM EDTA, 50% v/v glycerol and 0.05% bromophenol blue) in total volume of 20 µl was loaded in each well. Ethidium bromide was added to agarose gels and running buffers at a final concentration of 0.5 µg /ml. Gels were run at a constant voltage of 65 V. Electrophoresed DNA was visualised under UV illumination. DNA fragments and PCR products were purified for the purpose of cloning and

sequencing from the agarose gel, using Geneclean II kit from bio 101 (Stratech Scientific) or the Wizard PCR DNA Clean-up system supplied from Promega, respectively. A required band of DNA was excised from a agarose gel under high wavelength UV light provided by a hand held lamp. The DNA fragment was dissolved in 1 ml of 6 M sodium iodide solution for 5 minutes at 50 °C. 5 µl of glass milk (a DNA-binding silica matrix) was added and the mixture was incubated on ice for 15 minutes. The glass milk was pelleted by a brief centrifugation, then washed three times in 1 ml of NEW Wash (an ethanol-based buffer salt solution) and pelleted again. The pellet was dried in air for 10 minutes. The DNA was eluted by incubating it at 50 °C in 10-20 µl of TE buffer for 2-3 minutes. Centrifugation then pelleted the resin and the supernatant was purified DNA.

PCR fragments were purified from the gel using Promega Wizard PCR prep kit, as described in the instruction manual. An agarose slice containing the desired PCR fragment was dissolved in 1 ml of DNA binding resin by incubating it at 50 °C for 5 minutes. The resin was absorbed on to a minicolumn and washed with 2 ml of 80 % (v/v) isopropanol. The DNA was eluted with 40-50 µl of pre warmed TE buffer (70°C) during a brief centrifugation.

2.4.4 Conversion of cohesive DNA ends to blunt ends using Exonuclease III

Exonuclease III is active towards double stranded DNA ends containing unpaired 5' termini and recessed 3' termini so as to generate double stranded DNA with blunt ends. This enzyme was used to blunt end the DNA fragments digested by those restriction nucleases which produce 5' overhangs and where cloning was to be performed at the site of a blunt end cutting restriction endonuclease. The restriction digested DNA was purified by ethanol precipitation. The reaction was performed in 50 mM Tris-HCl (pH 8.0), 5 mM $MgCl_2$, using 20 units of Exonuclease/ µg of DNA in a total volume of 25 µl, at 37°C for 30 minutes.

2.4.5 Dephosphorylation of DNA

Dephosphorylation of DNA was done to prevent the self ligation of the plasmid in the ligation reaction, especially for difficult clonings such as those which did not involve the blue-white selection procedure. For dephosphorylation the volume of the reaction mixture after a restriction digestion was increased to 200 µl with sterile distilled water and 1 X shrimp alkaline phosphatase buffer (20 mM Tris-HCl pH 8.8, 10 mM $MgCl_2$). 2 Units of shrimp alkaline phophatase supplied by United States Biochemical was then added and the mixture incubated at 37 °C for 30 minutes, followed by 20 minutes at 65 °C to inactivate the enzyme. DNA was recovered by extraction with an equal volume of phenol: chloroform, as described above, followed by ethanol precipitation.

2.4.6 Ligation of DNA

The ligation reaction of DNA fragments was performed using T4 DNA ligase (supplied by New England Biolabs). Reactions were performed 1X ligation buffer (50 mM Tris-HCl pH 7.6, 10 mM $MgCl_2$, 10 mM dithiotreitol), 1 mM ATP. 0.5 -1.0 Weiss unit of T4 DNA ligase was used to ligate 200 ng-700 ng DNA. Reactions were incubated at room temperature for 3-4 hrs and stopped by heat inactivation of ligase at 65°C for 5 minutes.

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

Competent cells were prepared using the protocol standardised in the laboratory. The desired strain of *E. coli* was grown overnight in LB. Next morning 1.5 ml of this culture was inoculated into 150 ml of LB and growth allowed until the culture had reached an OD of 0.5

at 580 nm. The culture was then chilled on ice for 10 minutes. 100 ml of culture was next centrifuged at 4000 rpm at 4°C and the cells were resuspended in 40 ml of sterile 0.1 M chilled $CaCl_2$, left on ice for 20 minutes, and then centrifuged, as above. The cells were then suspended in 8 ml of sterile 0.1 M chilled $CaCl_2$ and kept on ice for at least 10-15 minutes. These competent cells were used for the transformation. For long term storage of the competent cells, an equal volume of glycerol was added to the cells which were then placed at -70°C in 1.5 ml microfuge tubes. For transformation, 200-500 ng DNA was mixed with 50 µl of competent cells and 25 µl of 0.1 M $CaCl_2$ in a sterile glass capped test tube. The mixture was treated at 37°C in a water bath for 30 seconds and then incubated on ice for 90 minutes. The mixture was then again heat shocked at 37°C. One ml of LB was next added to the mixture and the cells were allowed to grow for 90 minutes with vigorous shaking. 50 µl of this mixture was plated on an LA plate containing appropriate antibiotic. Where screening by α-complementation was possible, X-Gal and IPTG were spread on the plates 10 minutes before plating of transformants, as described above. The plates were incubated at 37°C for 8-12 hrs.

2.4.8 DNA sequencing methods

DNA sequencing was performed by dideoxy-chain termination method as described by Sanger (1977), using the DNA sequencing kit supplied by Amersham with the brand name Sequenase 2.0. 50 ng - 200 ng of the purified plasmid DNA was denatured by incubating in 0.2 M NaOH for 10 minutes at 37 °C. This was then neutralised with 0.2 M HCl and again incubated at 37°C for 10 minutes. Approximately 1 pmol of sequencing primer was annealed to the template at 37 °C for 10 minutes, in a volume of 10 µl. The DNA labelling reaction

comprised the 10 µl solution of annealed DNA template plus primer, which was made to a final volume of 16 µl containing the following components: ^{35}S-dATP (6.25 µCi), DTT (3.2 mM), Sequenase reaction buffer (40 mM Tris-HCl pH 7.5, 50 mM NaCl, 20 mM $MgCl_2$), 0.25 µM each of dCTP, dGTP and dTTP, and 1.6 units of Sequanase enzyme. The labelling reaction was incubated at room temperature for 5 minutes. Following labelling, the reaction mix was divided into four aliquots and each of these was mixed with 2.5 µl of termination mixture, containing 80 µM of each dNTP + 8 µM of appropriate dideoxy-NTP, 50 mM NaCl. The termination reactions were performed at 42 °C for 5 minutes. Finally each reaction was inactivated by the addition of 4 µl of stop solution (95 % v/v formamide, 20 mM Na_2EDTA, 0.05 % w/v each of bromophenol blue and xylene cyanol FF). 3.5-4.0 µl of the A C G and T termination reactions were electrophoresed side by side on a 6 % polyacrylamide sequencing gel, in TBE (89 mM Tris-borate pH 8.3, 2 mM Na_2.EDTA) buffer at a constant power of 70 W. Following the separation the gel was dried under vacuum at 80 °C and exposed to X-ray film.

2.4.9 Cloning in the pET expression system

The pET system, a powerful system for cloning and expression of recombinant proteins in *E. coli*, was used to clone and express *H. thermophilus* cytochrome c_{552}. The gene was cloned in the pET plasmid under the control of strong bacteriophage T7 transcription and translation signals. The expression was induced by providing a source of T7 RNA polymerase in the host cell. Further details of this system and the cloning and expression of *H. thermophilus* cytochrome c_{552} in this system is described in Chapter IV.

2.4.10 Polymerase chain reaction methods

The poymerase chain reaction (PCR) technique was used to generate DNA fragments for cloning, for site directed mutagenesis and for colony screening. 50 µl reaction mixtures contained 10-500 ng of template (usually linearised vector containing a target gene to be amplified), PCR buffer (10 mM Tris-HCl pH 9.0, 50 mM KCl, 0.1 % Glycerine), 0.25-2.0 mM $MgCl_2$, 0.2 mM each dNTP, 25 pmol of forward and reverse primers and 2.5 units of Taq DNA polymerase. The reaction was performed in 0.5 ml thin-walled Eppendorf tubes, in a programmable thermocyler (MJ Research Inc.). A typical cycle would consist of denaturation (97°C, 3 minutes), followed by 30 cycles of denaturation (94°C, 1 minute), primer annealing (55°C, 2 minutes) and extension (72°C, 2 minutes), with a final extension step of 72°C for 10 minutes. The PCR products were analysed by gel electrophoresis, as described above, on 2 % agarose gels.

Taq polmerase leaves a single 3' dATP overhang. Special linearised vectors containing single 5' dTTP overhangs are available for the direct cloning of the PCR fragments generated from the Taq polymerases. One of this kind of vector, pScreenT supplied by Novagen, has been used in the present study, Chapter VI. This vector is prepared by digesting with *Eco R* V followed by the addition of single 3' dT residues at each end. Thus the inserts having single 3' dA overhangs can be ligate directly into the vector.

2.4.11 Colony PCR was performed as follows

Individual colonies, at least 1mm in diameter, arising from single cells were picked from the agar plate using 200 µl pipette tip or sterile toothpick. A copy of each colony is made by touching the pipette tip, containing the colony, to a agar plate before transferring to the bulk of the colony to a microcentrifuge tube containing 50 µl of sterile water. This was

vortexed to dispersed the pellet. These tubes containing the resuspended colonies were placed in the boiling water for 5 minutes to lyse the cells and denature nucleases. This was then centrifuged at 12,000Xg for 1 min to remove cell debris. 10 µl from this supernatant was used for the immediate PCR reaction. The PCR reaction was performed as described above. 30 µl of the reaction was checked on 2% agarose gel.

2.4.12 Computer analysis of the DNA data

The DNA data analysis was performed using the GCG8-Open VMS and UNIX software suite (Devereux *et al.,* 1984, Genetics Computer Group, Wisconsin, USA), maintained at the William Dunn School of Pathology, University of Oxford, for the sequencing searches and for looking for the restriction endonuclease sites in a DNA sequences. The available software, Genbank and Genome databases, on the World Wide Web was also used.

2.4.13 Oligonucleotide synthesis and preparation

Oligonucleotides for sequencing and PCR mutagenesis were synthesised by Val Cooper at the Oligonucleotide Sequencing Service, Dyson Perrins Organic Chemistry Laboratory, University of Oxford. The oligonucleotides were incubated at 55°C overnight to evaporate residual ammonia. The oligonocleotides were then purified by ethanol precipitation and redissolved in sterile distilled water.

2.4.14 *In vitro* transcription/ translation

Attempts to synthesize cytochrome c_{552} *in vitro* were made by adding linearised pKHC12 to a Promega *E. coli* transcription/ translation kit, supplemented with ^{35}S-labeled methionine (10μCi). The result was analysed by SDS-PAGE followed by autoradiography. An autoradiogram of an SDS-PAGE gel did not show any polypeptide corresponding to cytochrome c_{552}. This approach is not described further in this report although its successful execution remains a goal for future.

Chapter III

Purification and some properties of *H. thermophilus* cytochrome c_{552} as a recombinant protein from *E. coli* JCB387

3.1 Introduction

As discussed in Chapter I, the maturation of *H. thermophilus* cytochrome c_{552} in the cytoplasm of *E. coli* either means that the covalent bond formation between the haem and the apocytochrome c_{552} is spontaneous or there is a *c*-type cytochrome biogenesis machinery present in the cytoplasm of *E. coli.* The latter is highly unlikely as scrutiny of the *E. coli* genome suggests the presence of only one copy of a *c*-type cytochrome biogenesis machinery. *C*-type cytochrome biogenesis genes in *E. coli* were first identified by similarity to *c*-type cytochrome biogenesis genes found in other Gram-negative bacteria (Richterich *et al.,* 1993) but have subsequently been confirmed by the finding that disruption of these genes results in the expected loss of *c*-type cytochromes (Thony-Meyer *et al.,* 1995, Grove *et al.*, 1996). The components of this machinery are predicted to be periplasmic or attached to the cytoplasmic membrane with their functional domains oriented towards the periplasm, just as has been suggested for homologous components from other Gram-negative bacteria (Lang *et al.,* 1996; Monika *et al* 1997; Thony-Meyer 1997), and thus seem unlikely (confirmed in Chapter V) to participate in the biogenesis of cytoplasmically expressed cytochrome c_{552}. The finding of Sambongi *et al.,* (1994) that cytoplasmic maturation of cytochrome c_{552} is independent of the *dipZ* gene product, required for the periplasmic *c*-type cytochrome assembly in *E. coli*, also led to the suggestion that the cytoplasmic maturation of this thermophilic cytochrome c_{552} does not need any enzymatic assistance. It was proposed that apocytochrome c_{552} has some tertiary structure and haem slots into a binding pocket and the covalent bond forms spontaneously (Sanbongi and Ferguson, 1994b). Since the structure of haem is asymmetric, the covalent bonds between the vinyl groups of the haem and cysteine residues 14 and 17 of apocytochrome c_{552} will have defined stereochemistry (Figure 1.1). The mitochondrial

cytochrome *c* haem lyase not only catalyses the formation of covalent linkages between haem and apocytochrome *c* but also selects a single chirality for the haem linkage. This thioether bond is formed in a single configuration with respect to the chiral α-carbon so that hematoporphyrin obtained from cytochrome *c* is not racemic but optically active (Paul, 1951). If the formation of the covalent bond between apocytochrome c_{552} and haem is spontaneous, only 50% of the haem linked apocytochrome *c* might be native cytochrome *c* while the other 50% of the holo protein would show the opposite chirality. The stereochemistry of the thioether attachment of the haem group to the polypeptide of a *c*-type cytochrome can be determined by NMR spectroscopy (Senn and Wuthrich, 1983). Thus application of the NMR method to recombinant *H. thermophilus* cytochrome c_{552} purified from *E. coli* should in principle show whether or not the presumed spontaneous attachment of haem to the apo polypeptide of cytochrome c_{552} occurs with a single stereochemistry. Such NMR experiments require many milligrams of protein. Similar amounts were also anticipated to be required for a second approach to understand the maturation of thermophilic cytochrome c_{552} in the cytoplasm of *E. coli*. This is the *in vitro* preparation of the apo form cytochrome c_{552} followed by analysis for tertiary structure using CD spectroscopy and, more ambitiously, attempts to form holo cytochrome c_{552} *in vitro* by incubation of the apo protein with haem. Thus this Chapter describes the preparation and characterisation of holo and apo cytochrome c_{552} from JCB387 strain of *E. coli* that had already been shown in Oxford (Sambongi and Ferguson, 1994b) to produce this recombinant protein in readily detectable amounts. The reader may at this point be asking, as many others in this field have, why the proposed uncatalysed maturation of cytochrome c_{552} is not tested by studies with an *in vitro* transcription/translation system. Calculation shows that it may be just possible to detect

the incorporation of ^{14}C labelled haem into polypeptide produced this way. In the present work a considerable amount of time was spent trying to develop such an *in vitro* system using a *E. coli* extract supplied by Promega for the purpose of *in vitro* transcription/ translation. The amounts of polypeptide obtained this way were not quite enough to be used for haem reconstitution and so the approach is not further discussed in this thesis. However, related experiments can also be performed by purifying the apocytochrome c_{552} directly from cells and reconstituting with the haem *in vitro*. Steps towards achieving this are described in Chapter IV, section 10.

3.2 Results

3.2.1 Recombinant cytochrome c_{552} was obtained as a homogenous protein after two step purification

H. thermophilus cytochrome c_{552} was purified from *E. coli* strain JCB387 using the construct pKHC12, which contains the coding region for the mature cytochrome *c* polypeptide lacking its native signal sequence (Sanbongi *et al,* 1991). The cytochrome c_{552} was obtained as a homogenous protein after a two step purification, ion-exchange chromatography followed by affinity chromatography (see Materials and Methods, Chapter II). Both the SDS-PAGE (Figure 3.1) and spectrophotometric analysis (Figure 3.2) showed that by these criteria pure cytochrome c_{552} could be attained. This purified recombinant cytochrome c_{552} was then used for further analysis.

3.2.2 The recombinant cytochrome c_{552} had two kinds of *N*-terminus

In the course of purifying recombinant cytochrome c_{552} the cell extract of *E. coli* JCB387 resolved into three bands on the ion-exchange column. A corresponding extract

from the host, *H. thermophilus*, resolves into two bands only, corresponding to the oxidised and reduced forms of the protein (Dr. Y. Sambongi, personal communication). This prompted investigation of the differences between these three bands. Spectrophotometric analysis of the three peaks eluted from the CM-52 column showed that material in two peaks was reduced whereas that in one peak was oxidised (Figure 3.3). Investigation of the *N*-terminus of each of these eluted fractions showed that of the two reduced bands one was detected with the *N*-terminal methionine uncleaved and the other with the *N*-terminal methionine cleaved (Figure 3.4). This is, for reasons explained in the discussion, an interesting observation, but the presence or absence of a single *N*-terminal residue was not expected to influence the experiments planned in the present work.

3.2.3 CD (Circular dichroism) spectroscopy of recombinant cytochrome c_{552}

In order to compare the gross structures of the native cytochrome c_{552} purified from the host *H. thermophilus* and the recombinant cytochrome c_{552} purified from *E. coli* the CD spectrum of the latter was measured. The spectrum (Figure 3.5) showed the same features, indicating significant α-helical content, as observed for the native cytochrome c_{552} (Sanbongi *et al.,* 1989a). This observation, along with the characteristic α-band absorption of the haem at 552 nm, indicated that the recombinant protein had the same overall structure as the native material.

3.2.4 Haem removal from cytochrome c_{552}: preparation of apocytochrome c_{552}

In order to investigate whether apocytochrome c_{552} has any tertiary structure, an attempt was made to remove haem from the holocytochrome c_{552} by the method described

by Ambler *et al.*, 1985. For comparative purposes the same procedure to remove the haem was also applied to the commercially available mitochondrial cytochrome *c* (Table 3.1). After treatment with $HgCl_2$, the protein was passed down a Sephadex G-25 column. The initial fraction that eluted had a higher absorbance at 280 nm than at 410 nm. The ratio A^{410}/A^{280} was 0.33, whereas the ratio for the holoprotein, the starting material, is approximately 5. This shows that apoprotein had been prepared in reasonable yield. However, complete formation of the apoprotein should have been indicated by an A^{410}/A^{280} of 0.0. This indicated that the release of haem had not been quite complete. When the same procedure was applied to recombinant *H. thermophilus* cytochrome c_{552} the first fractions to elute from Sephadex G-25 column had A^{410}/A^{280} ratios of close to zero (Table 3.2), suggesting almost complete conversion to the apoform. Thus it seems that the cytochrome c_{552} was, despite its thermal stability, more effectively converted to the apo form than mitochondrial cytochrome *c*. However, the yield of apoprotein was too small to provide material for characterisation by NMR or to attempt the *in vitro* reconstitution assay. An attempt to obtain the CD spectrum of this apoprotein was unsuccessful. The haem removal from the apocytochrome c_{552}, expressed in another system along with the purification of apocytochrome c_{552} synthesised *in vivo* was attempted and is discussed in Chapter IV.

3.2.5 The amount of recombinant cytochrome c_{552} obtained

The yield of recombinant cytochrome c_{552} was estimated, on the basis of either protein or haem determination, to be about 3.0 mg from 20 litres of culture grown to OD 1.2 at 580 nm. This amount was, at best, only marginally sufficient for NMR analysis, apocytochrome c_{552} purification and reconstitution studies. Thus other attempts were

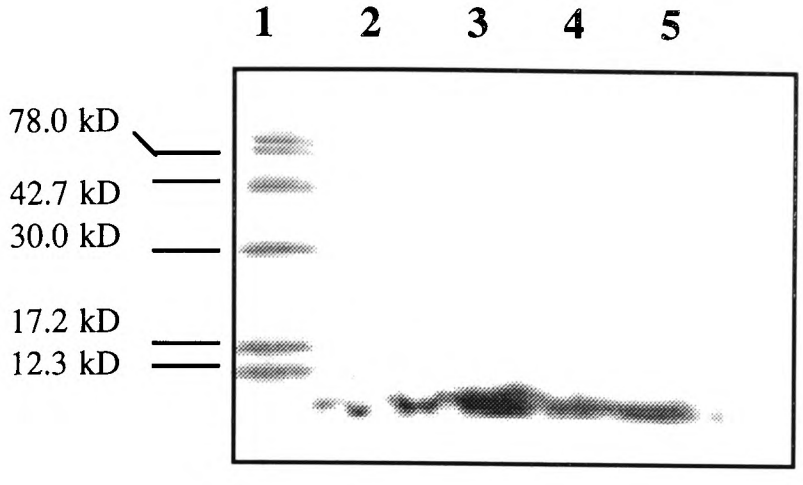


Figure 3.1. Coomassie stained SDS-PAGE gel of the purified recombinant cytochrome c_{552} from the *E. coli* strain JCB387, expressing cytochrome c_{552} from the construct pKHC12. The gel was loaded as follows:

Lane 1: Protein markers, from the top; Ovotransferrin 78 kD, Ovalbumin 42.7 kD, Carbonic anhydrase 30 kD, Myoglobin-17.2 kD Horse heart cytochrome *c*-12.3 kD.

Lane 2: Diluted early fraction from CM-cellulose column

Lane 3,4,5: Early, middle and late fractions from CM -cellulose column. 30 µl from 7 ml of each fractions were loaded onto the gel.

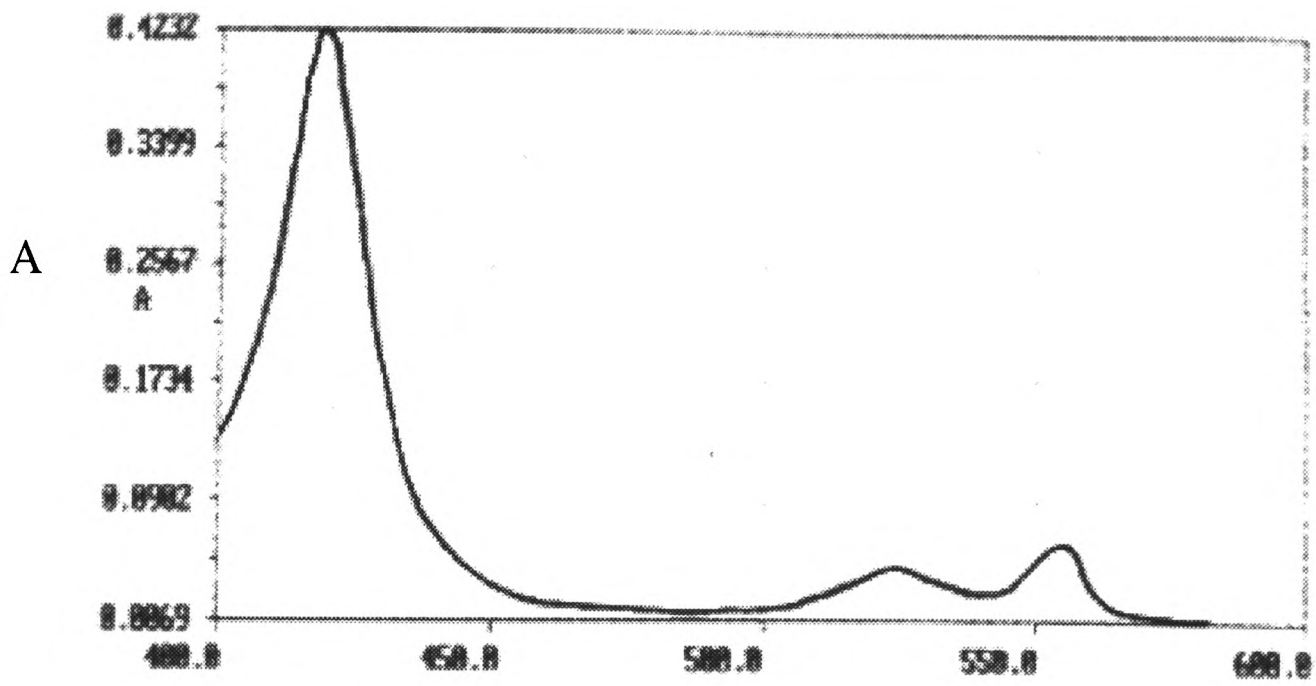


Figure 3.2. The spectrum of dithionite-reduced cytochrome c_{552} purified from *E. coli* JCB387, expressing cytochrome c_{552} from the construct pKHC12. 12 µg cytochrome c_{552} in 10 mM phosphate buffer (pH 7.5) was used. Sufficient solid sodium dithionite was added to reduce the sample completely

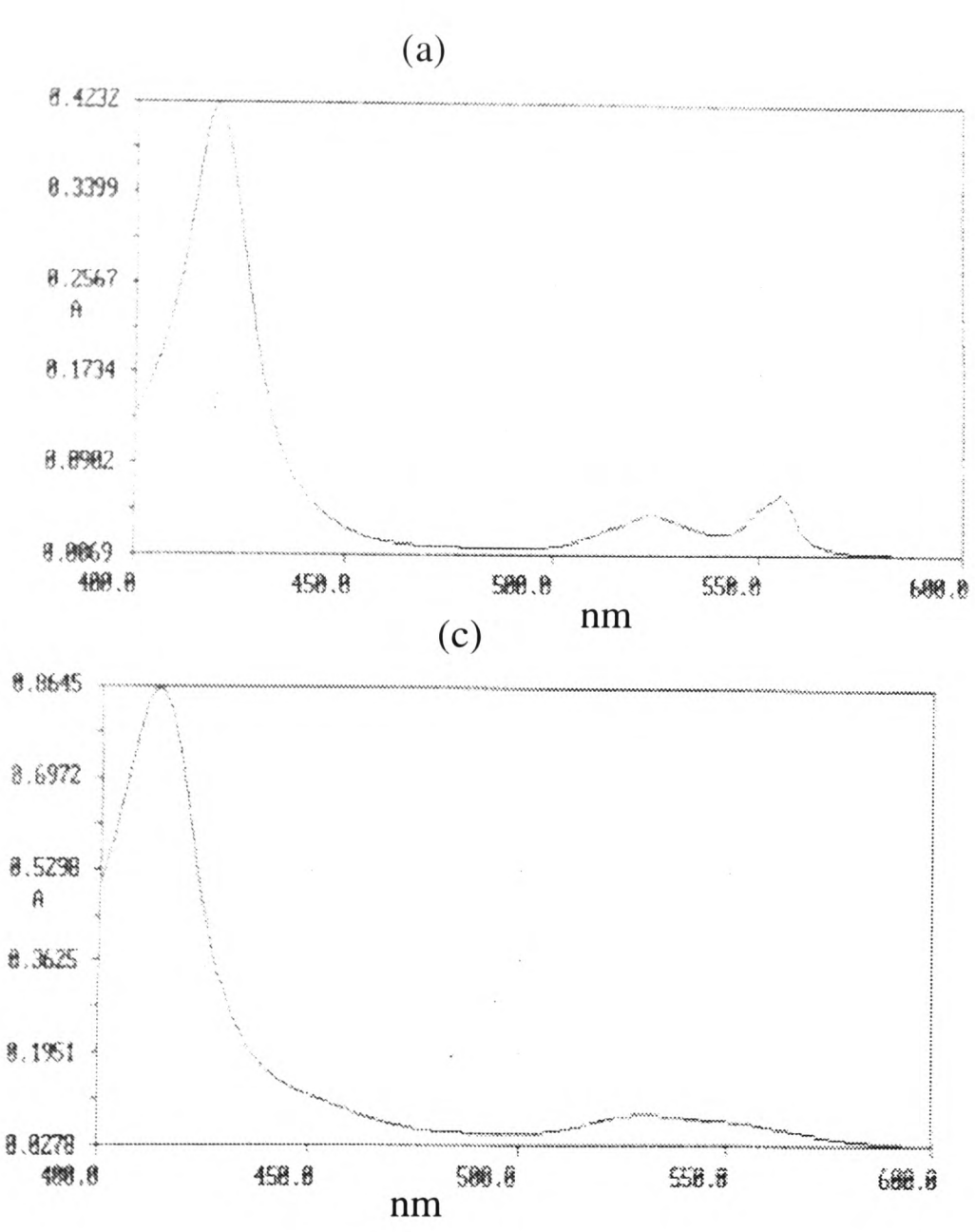


Figure 3.3a-c. The spectra of cytochrome c_{552} in the three peaks eluted from the CM-52 column while purifying cytochrome c_{552} from *E. coli* JCB387 expressing cytochrome c_{552} from the construct pKHC12. Note that the first two peaks (a and b) were found to contain reduced cytochrome c_{552} while the third (c) was oxidised. d is the column profile of cytochrome c_{552} eluted from the cellulose CM-52, upon increasing concentration of NaCl. Over the profile shown the NaCl concentration was increased from 0 mM to 500 mM.

N E Q L A K Q K G - M A - H D ....

(1)

M N E Q L A K Q K G - M A - H ....

(2)

Figure 3.4. *N*-terminal sequences of the material in two peaks of eluted reduced cytochrome c_{552} from the CM-52 column in the course of purifying recombinant cytochrome c_{552} from *E. coli* JCB387 expressing cytochrome c_{552} from the construct pKHC12. Recombinant cytochrome c_{552} eluting, see Figure 3.3d, in the first peak has its *N*-terminal methionine cleaved while in the second peak the recombinant cytochrome c_{552} has its *N*-terminal methionine unprocessed.

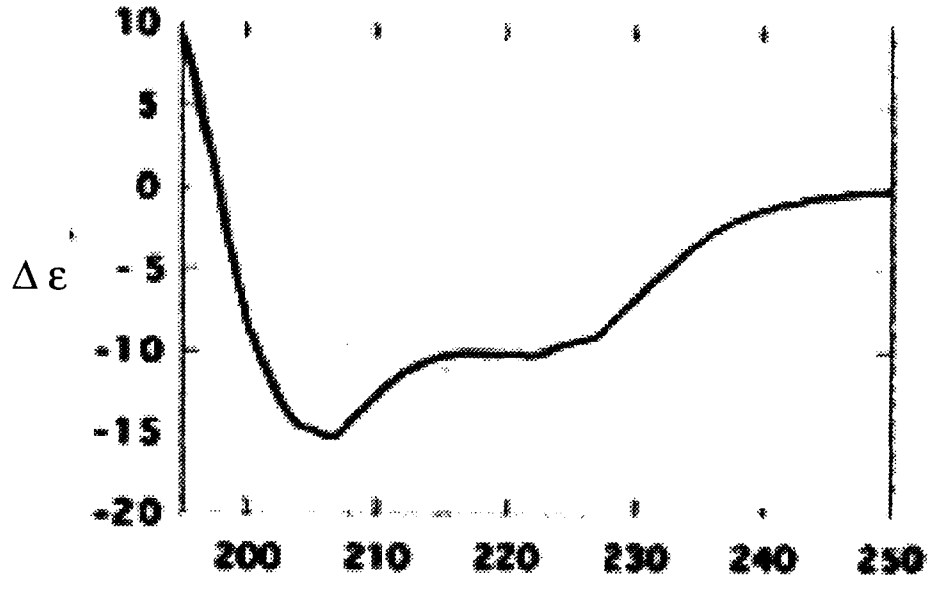


Figure 3.5. CD spectrum of purified recombinant cytochrome c_{552}. The analysis was performed as described in Materials and Methods, at room temperature. The sample, 0.2 mg protein, was dissolved in 10 mM potassium phosphate buffer (pH 7.5). The units of $\Delta\varepsilon$ are mM^{-1} cm^{-1}.

Table 3.1

Elution profile from Sephadex G-25 following treatment of horse heart cytochrome *c* with $HgCl_2$. The reaction was performed as described in Materials and Methods, Chapter II. Fractions, of the size 1 ml, were eluted by 7 % acetic acid. The presence of protein and haem was checked by reading the absorbance at 280 nm and 410 nm, respectively.

	Absorbance	
	280 nm	**410 nm**
Fraction 1	0.020	0.002
Fraction 2	0.220	0.007
Fraction 3	0.559	0.020
Fraction 4	1.100	0.060
Fraction 5	0.220	0.000
Fraction 6	0.050	0.000
Fraction 7	0.260	0.310
Fraction 8	0.390	0.720
Fraction 9	0.200	0.320
Fraction 10	0.120	0.120
Fraction 11	0.080	0.060
Fraction 12	0.071	0.050

Table 3.2

Elution profile from Sephadex G-25 following treatment of *H. thermophilus* cytochrome c_{552} with $HgCl_2$. The reaction was performed as described in Materials and Methods, Chapter II. Fractions, of the size 1 ml, were eluted by 7 % acetic acid. The presence of protein and haem was checked by reading the absorbance 280 nm and 410 nm, respectively.

	Absorbance	
	280 nm	**410 nm**
Fraction 1	0.006	0.002
Fraction 2	0.204	0.005
Fraction 3	0.509	0.007
Fraction 4	0.038	0.006
Fraction 5	0.043	0.016
Fraction 6	0.092	0.017
Fraction 7	0.042	0.018
Fraction 8	0.032	0.082
Fraction 9	0.093	0.156
Fraction 10	0.144	0.077
Fraction 11	0.115	0.033
Fraction 12	0.080	0.018

made to achieve a high level of expression of cytochrome c_{552}. The coding region of cytochrome c_{552} from pKHC12 was cloned into the high copy number plasmid pUC18 (Materials and Methods, Chapter II) resulting in pUNC2. The ribosome binding site in the construct pKHC12 is about 9 bp upstream from the start codon. Some *in vivo* experiments suggest that a spacing between a ribosome binding site and a start codon of 9 bp is optimal (Shepard *et al.,* 1982, Wood *et al.,* 1984), but there are other reports where a distance of 5-7 bp has shown to be optimum (Hartz *et al.,* 1990). Thus the optimum spacing for one specific mRNA might not be the optimum for another. A cloning cassette designed specifically for use in the construction of prokaryotic transcriptional fusions has a spacer region of 8 bp between the ribosome binding site and the start codon (Miller and Lindow, 1997). Since there is evidence that ribosome binding site sequences show an average spacing from the start codon of 7 bp and spacings of less then 5 bp and more than 9 bp are rare (Stormo *et al.,* 1982), it was thought important to test whether decreasing the distance between the ribosome binding site and the start codon in the construct pKHC12 (Materials and Methods, Chapter II) would increase the expression of cytochrome c_{552}. Thus the construct pKHC12 was digested with *EcoR* I, a site for which is present in between the ribosome binding site and the start codon, and the 5' overhangs, consisting of 4 bases, resulting from the restriction digestion, were removed by treating with Exonuclease III. The blunt end product was religated to give a construct pKHC12r, which has an expected 5 bp distance in-between the ribosome binding site and the start codon. The transformed colonies were screened for the loss of the *EcoR* I site. Both the constructs, pUNC2 and pKHC12r, did not show any increase in the expression of cytochrome c_{552} and so attempts to express from these constructs or to characterise them by sequencing were not carried further.

3.3 Discussion

The work described in the present chapter shows that *H. thermophilus* cytochrome c_{552} can be obtained as a recombinant protein from *E. coli* strain JCB387 in a form which is essentially similar to that isolated from the thermophilic organism itself. Furthermore, evidence that the apo form of the protein can be prepared *in vitro* was also obtained. Unfortunately, despite many efforts, the yields of cytochrome c_{552} in several preparations always fell short of what was required to carry out structural and reconstitution studies on the material. Consequently, and as described in subsequent chapters, different strategies were tried in order to obtain increased amounts of recombinant cytochrome c_{552}.

The finding of two populations of reduced cytochrome c_{552} in material extracted from *E. coli* JCB387 was not expected. The observation that the *N*-terminal methionine in one population was processed in *E. coli* despite being followed by an asparagine residue is interesting. Protein synthesis in bacteria is initiated with *N*-formyl methionine. The *N*-formyl moiety is subsequently removed by formylases while the methione can be removed by an aminopeptidase depending on the residue following the *N*-terminal methionine. Protein data base analysis (Driessen *et al.,* 1985, Persson *et al.,* 1985, Boissel *et al.,* 1985, Sherman *et al.,* 1985) and studies of the processing of mutant proteins (Sherman *et al.,* 1985, Tsunasawa *et al.,* 1985, Moerschell *et al.,* 1990), or proteins with systematically modified *N*-terminal sequences (Huang *et al.,* 1987, Boissel *et al.,* 1988), suggest that methionine aminopeptidases from different sources have quite similar substrate specificities. Sherman *et al* (1985) surveyed which such residues were compatible with cleavage of the *N*-terminal methionine. They concluded that in both prokaryotes and eukaryotes methionine was cleaved only if it preceded a residue with a side chain having a radius of gyration of 1.29 Å or less. Thus cleavage occurs adjacent to

e.g. alanine, cysteine or glycine but not next to residues with longer side chains. This view was supported for *S. cerevisiae* by an extensive study of variant forms of iso-1-cytochrome *c* with different *N*-terminal sequences (Sherman *et al.,* 1985). Subsequently, Ben-Bassat *et al.* (1987) cloned the gene for, and then expressed, *E. coli* methionine aminopeptidase. Studies with the purified enzyme were consistent with the specificity previously predicted by Sherman *et al.* (1985). Thus asparagine, which has a side chain radius greater then 1.29 A, as the penultimate residue at the *N*-terminus would not be compatible with cleavage of the *N*-terminal methionine by the *E. coli* methionine aminopeptidase. Hence the present finding of cleavage of methionine followed by asparagine (Figure 3.4) is surprising. Three possible explanations can be considered. First, the specificity deduced by both Sherman *et al.* (1985) and Ben-Bassat *et al.* (1987) was incorrect as a result of considering too few substrates. Second, *E. coli* JCB387 contains a form of methionine aminopeptidase with altered specificity. Third, there is a second aminopeptidase enzyme in *E. coli.* However, a recent paper shows the existence of only single gene encoding methionine-aminopeptidase in *E. coli* (Keeling and Doolittle, 1996). Thus the first two explanations have to be considered as serious possibilities. A related issue is why a mixture of species, either with or without the *N*-terminal methionine, was obtained. The reason why some of the fractions containing unprocessed methionine at the *N*-terminus could be due to the over production of cytochrome c_{552} polypeptide and the saturation of aminopeptidase capacities. Many of hyperproduced recombinant proteins in *E. coli* contain a fraction with unprocessed methionine at their *N*-terminal (Sherman *et al.,* 1985). Although the final yield of cytochrome c_{552} was quite low as compared to the expression of other recombinant proteins, it might be that the polypeptide for cytochrome c_{552} was overproduced but due to reasons such as lack of an

ample supply of haem, or rapid degradation, was not matured into the holocytochrome c_{552}. The cleavage of *N*-terminal methionine with asparagine as a penultimate residue in this thermophilic cytochrome c_{552} could perhaps be due to some structural feature of this particular thermostable protein; possibly the methionine was more exposed towards the activity of methionine aminopeptidase. The cleaved methionine with asparagine as the penultimate residue in the purified cytochrome c_{552} is unique to our knowledge. It is important to mention here that this thermostable cytochrome c_{552} normally has lysine as the penultimate residue. The recombinant cytochrome c_{552} was modified to remove the signal sequence and thus the methionine was introduced just before the coding region of the mature cytochrome c_{552} (Sanbongi *et al.*, 1991). It is, however, difficult to see how this change can be related to the unusual cleavage by aminopeptidase.

The finding that both oxidised and reduced recombinant cytochrome c_{552} were found in the extract of *E. coli* JCB387 cannot at present be explained, but may eventually provide insight into the assembly process for cytochrome c_{552}. In the case of mitochondrial cytochrome *c* assembly it is known that reductant is essential (Nicholson and Neupert, 1989) and it is thought that reduced haem is probably the species that is added to the polypeptide. A similar situation may pertain for the assembly of cytochrome c_{552} in the cytoplasm of *E. coli*, thus explaining the formation of the reduced cytochrome c_{552}. If oxidised cytochrome c_{552} were the initial product of the assembly process then a subsequent reductive step would have to account for the formation of the reduced species. This may be tentatively considered improbable because there would obviously be no physiological redox partners available for this cytochrome c_{552} in the *E. coli* cytoplasm. Initial formation of the reduced state cytochrome c_{552} would require the observation of some oxidised cytochrome c_{552} in the *E. coli* extract to be explained by auto-oxidation

following breakage of the cells. This possibility could be tested in future work by breaking cells under reducing conditions and also examining the kinetics of the auto-oxidation of cytochrome c_{552}.

The amount of cytochrome c_{552} obtained from this system was found to be inadequate for NMR studies. Also the purification of cytochrome c_{552} using anaerobic growth conditions and growing 20 l of culture was tedious and time consuming. Thus other expression systems, shown to yield higher amounts of other recombinant proteins, were tried and attempts were made to optimise the conditions for the expression of this thermophilic cytochrome c_{552} in the cytoplasm of *E. coli* (Chapters IV and V). This study nonetheless shows that the recombinant cytochrome c_{552} produced in the cytoplasm of *E. coli* was similar to the native species; identity in terms of the exact mode of haem attachment was not, however, established.

Chapter IV

The expression of *H. thermophilus* cytochrome c_{552} in a T7 expression system as a S•Tag Protein.

4.1 Introduction

Chapter III established that the purification of recombinant cytochrome c_{552} from *E. coli* JCB387, although successful, gave a yield that fell short of what was desirable if the important NMR and *in vitro* reconstitution with haem studies were to be carried out. Thus an alternative strategy for preparation of the recombinant thermophilic cytochrome c_{552} needed to be identified. Expression in T7 systems frequently yields very substantial amounts of recombinant material, and special vectors compatible with this system are available for the high expression of cytoplasmic and periplasmic proteins. This is the gene expression system based on bacteriophage T7 RNA polymerase, originally developed by Studier and colleagues (Studier and Moffatt, 1986; Rosenberg *et al,* 1987; Studier *et al.,* 1990). Further details of T7 expression system are given in section 4.2.

In the present work a translation vector was chosen for use in a T7 expression system. This vector carries a sequence adjacent to the cloning site that codes for a peptide tag, an S•Tag, which allows the quantification, detection on Western blots and affinity purification of the expressed protein. This feature is particularly useful for those studies where a comparison of the expression of cytochrome c_{552} under two different conditions needs to be made. Thus the T7 expression system was intended to be used mainly to obtain the cytochrome c_{552} in higher amounts and to study factors that may influence the biogenesis of recombinant cytochrome c_{552} (Chapter V), especially where a quantification was needed.

4.2 An introduction to the T7 expression system

The T7 expression system relies on the synthesis of the target protein from the gene cloned in a plasmid with transcription catalysed by T7 RNA polymerase, the source of

which is a host *E. coli* strain which contains a cloned copy of the T7 RNA polymerase gene, T7 gene *1*, in its chromosome. T7 RNA polymerase directs the high level transcription from its promoter which is present on a multicopy plasmid. Transcription can proceed several times around the plasmid without terminating, and can be so active that transcription by *E. coli* RNA polymerase is greatly decreased. The natural terminator from T7 DNA also works effectively in the plasmid. Both the rate of synthesis and the accumulation of mRNA produced by the action of T7 RNA polymerase can reach levels comparable with those ribosomal RNAs in a normal cell. It has been suggested that specific mRNA produced by T7 RNA polymerase can rapidly saturate the translational machinery of *E. coli*, so that the rate of protein synthesis from such an mRNA will depend primarily on the efficiency of its translation. When the mRNA is efficiently translated, a target protein can accumulate to greater than 50 % of the total cell protein in three hours or less (Studier and Moffatt, 1986). The delivery of T7 RNA polymerase into the cell is either achieved by infection with a lambda derivative that carries gene *1*, or induction of a chromosomal copy of gene *1*. For stable target plasmids induction of a chromosomal gene *1* can be a convenient way to produce large amounts of target RNA and/or protein. Such a host was developed by integrating a DNA fragment containing the gene for T7 RNA polymerase under the control of *lacUV5* promoter, which is inducible by isopropyl-β-D-thiogalactopyranoside (IPTG), into the *int* gene within the chromosome of the host strain (Studier and Moffatt, 1985). The integration into the *int* gene was essential in order to avoid the fragment from integrating into, or excising from, the chromosome without a helper phage. The T7 RNA polymerase seems to be capable of transcribing almost any DNA linked to a T7 promoter.

The pET expression system, introduced by Novagen and used in the present study, is based on the above T7 expression system and was developed for the cloning and the expression of the recombinant proteins in *E. coli*. The target genes are cloned in pET plasmids under control of strong bacteriophage T7 transcription and (optionally) translation signals.

The pET vectors were originally constructed by Studier and colleagues (Studier and Moffatt 1986; Rosenberg *et al.*, 1987, Studier *et al.*, 1990). The derivatives of pET vectors developed by Novagen have features to permit easier subcloning, detection and purification of the target protein. The translation vector, pET29(a), used in the present study, contains a highly efficient ribosome binding site from the phage T7 major capsid protein. This vector contains the coding region for the S•Tag peptide adjacent to the cloning sites, which allows the quantification of the expressed proteins by a rapid homogeneous assay, detection on Western blots and affinity purification. It is based on the interaction of 15aa S•Tag peptide with ribonuclease S protein. The specific binding of these two molecules is of high affinity (Richards and Wyckoff, 1971) and allows convenient detection and purification of any protein fused with the S•Tag sequence. Whereas neither S•Tag sequence nor the S-protein alone has enzymatic activity, the two components together generate active ribonuclease; this forms the basis of the S•Tag assay, described later. Figure 4.1 is a diagrammatic representation of the T7 expression system. The disadvantage of the S•Tag is that it adds amino acids to the *N*-terminus of the protein with which it is fused. However, as it contains only 35 amino acids, 15 aa of S•Tag plus 20 aa present between the S•Tag and the cloned gene, it was hoped that its presence would not compromise the production of the thermally stable cytochrome c_{552}.

4.3 Results

4.3.1 Cloning of cytochrome c_{552} gene in the pET expression vector pET29(a)

The *Eco* RI- *Sal* I fragment, which has 250 bp, from the vector pKHC12 (Materials and Methods), containing the coding region of mature cytochrome c_{552} deleted in its signal sequence (Sanbongi *et al.,* 1991), was cloned into the *Eco* RI- *Sal* I site of the pET29(a) expression vector. It was expected that the cloned fragment would be in the correct reading frame relative to the sequence coding for the S•Tag. The resulting construct, pENC2 (Figure 4.2), was transformed into *E. coli* strain HMS174(DE3), a host for T7 expression. Colonies were screened first on the basis of the red colour relative to cells not expressing cytochrome c_{552}. Figure 4.3 shows the red coloured cells of HMS174(DE3) containing pENC2 compared with the pale cells of HMS174(DE3) containing pET29(a) without an insert. Such red cells were subsequently shown to contain the expected 250 bp *Eco R* I- *Sal* I fragment derived from pKHC12 (Figure 4.4). SDS-PAGE, followed by haem staining, of a crude extract obtained from a colony satisfying both screening criteria showed the presence of a haem staining band with slightly lower mobility (Figure 4.5), than recombinant cytochrome c_{552} that had been obtained as described in Chapter III. This difference is a consequence of the presence of 35 additional amino acid residues at the *N*-terminus of this cytochrome c_{552} (15 aa of S•Tag plus 20 aa between S•Tag and cloned cytochrome c_{552} (Figure 4.6). As expected, a crude extract of *E. coli* HMS174(DE3) that had not been transformed with pENC2 did not contain material that haem stained (Lane 2, Figure 4.5).

4.3.2 Checking the stability of the construct pENC2

The target protein can comprise as much as 50 % of the total cell protein in the T7 expression systems, as stated in the Novagen instruction manual. Preliminary

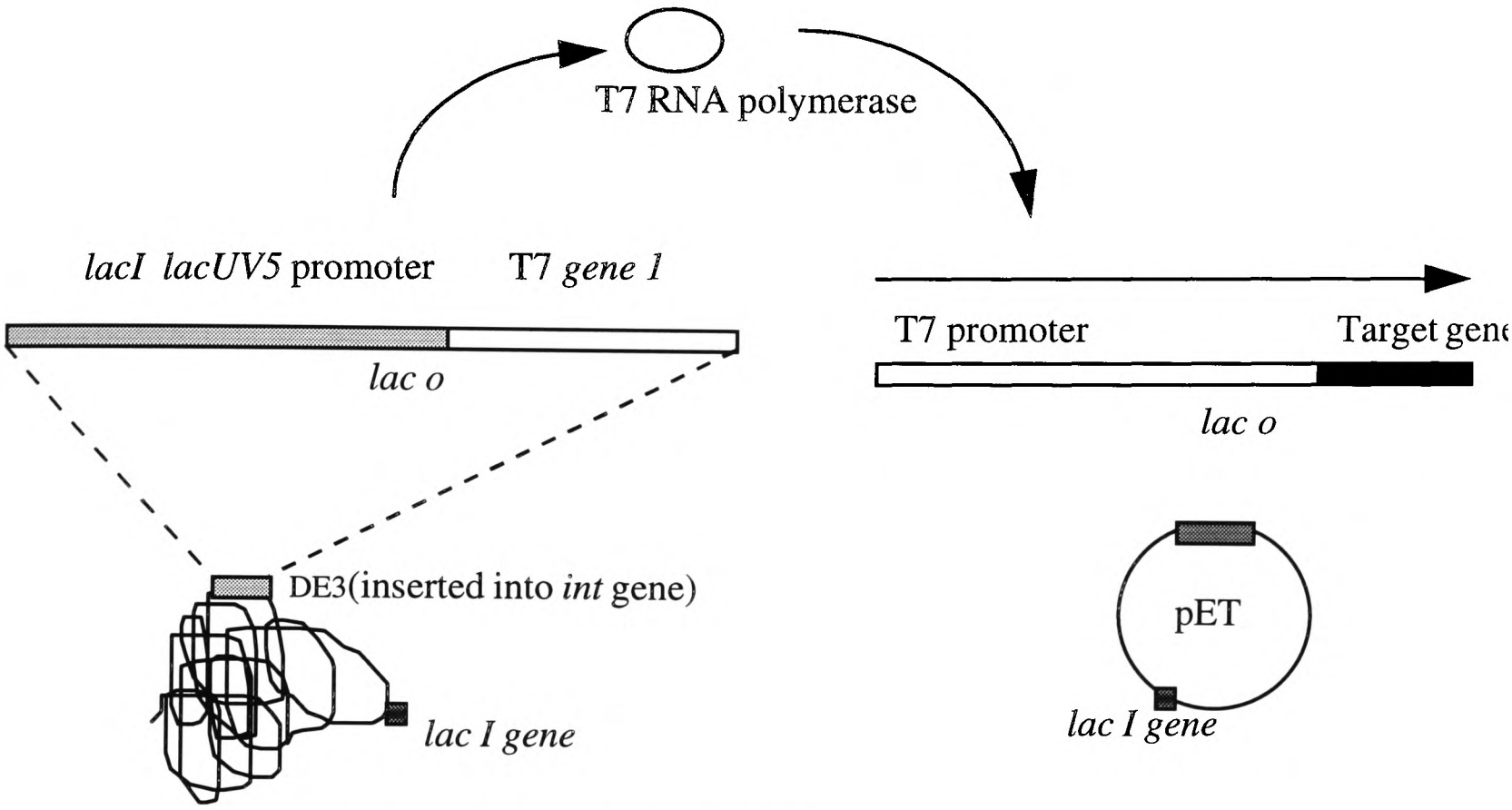


Figure 4.1. Diagrammatic representation of a T7 expression system

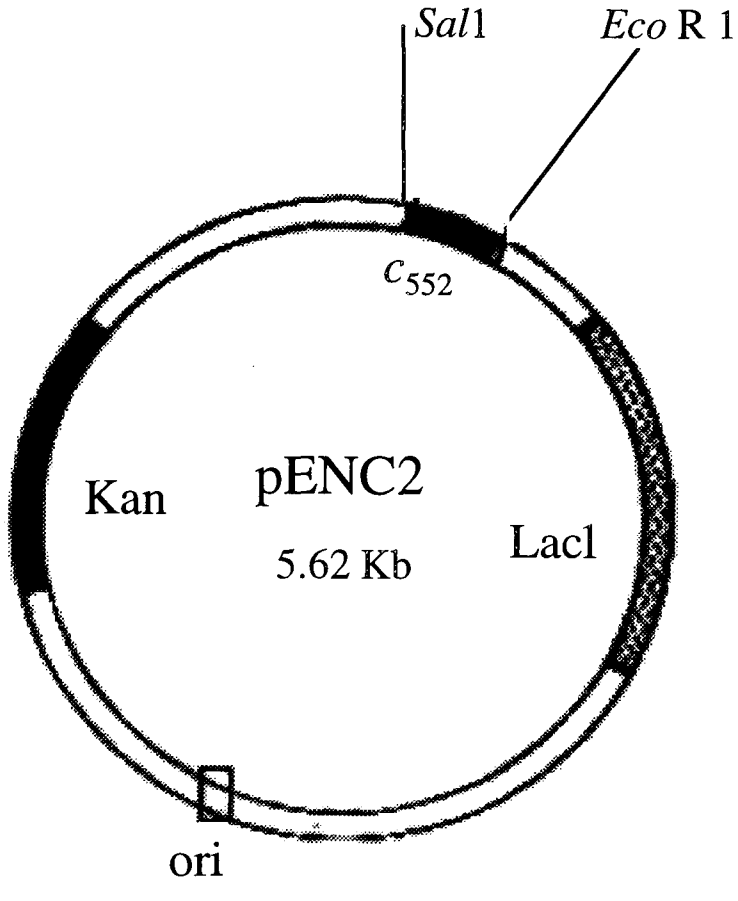


Figure 4.2. The construct pENC2. This was made by cloning the *Eco* R1-*Sal*1 fragment of pKHC12, containing the coding region for the mature cytochrome c_{552}, into the *Eco* R1-*Sal* 1 site of pET29(a). Cytochrome c_{552} is expressed from this plasmid containing 15 amino acid residues of the S-Tag and 20 amino acid residues linking the S-tag to cytochrome c_{552}. Thus there are in total 35 amino acid residues present at the *N*-terminus of cytochrome c_{552}.

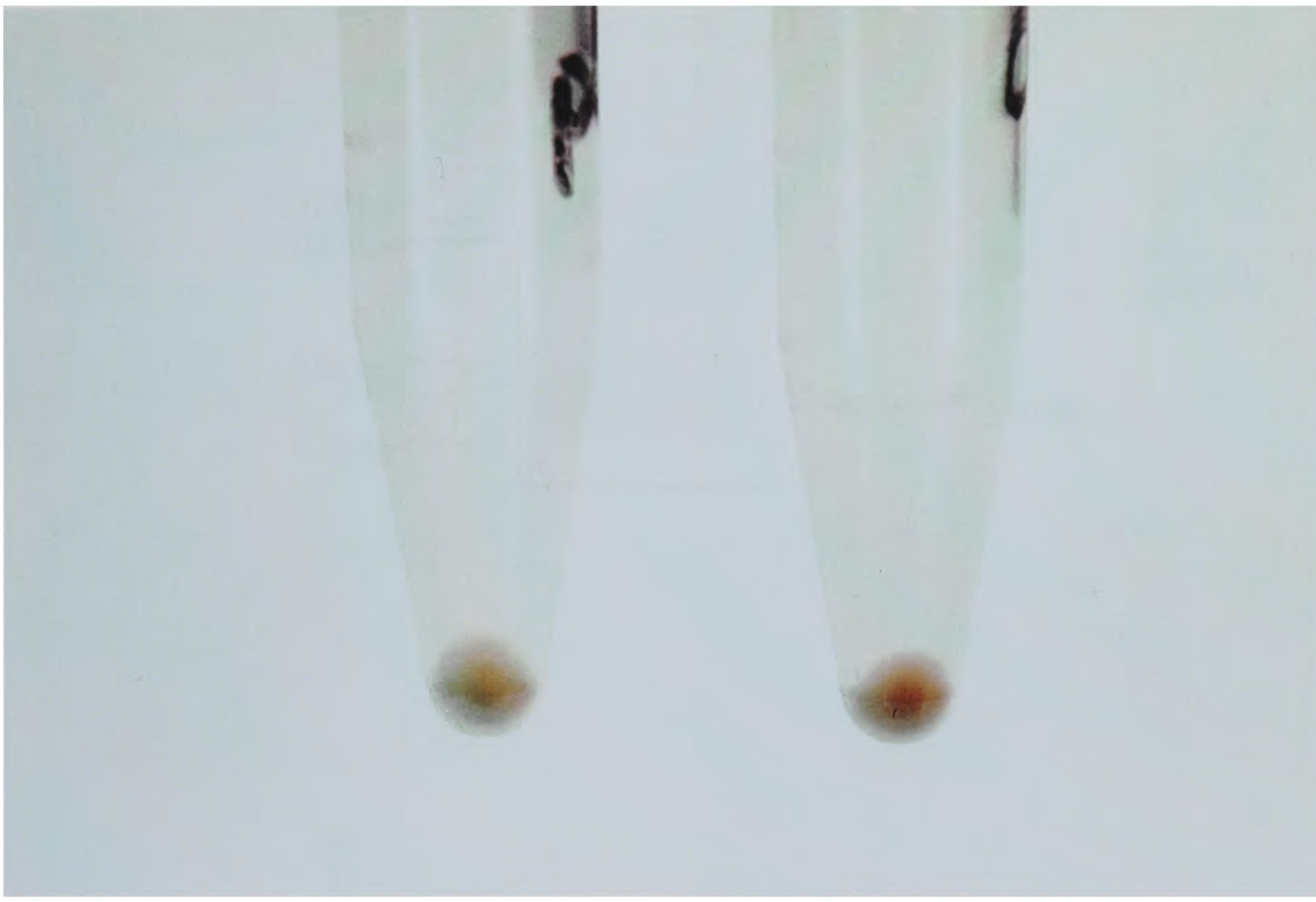


Figure 4.3. The red coloured cells expressing cytochrome c_{552} from the construct pENC2 (2) against the pale coloured cells of the host HMS174(DE3) containing pET29(a)(1).

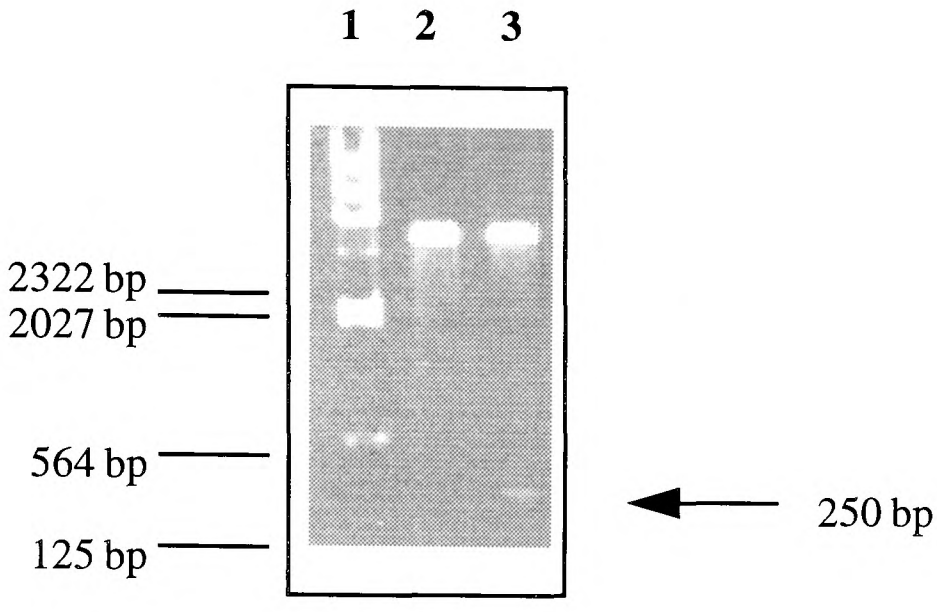


Figure 4.4. Agarose gel showing 250 bp cloned fragment in the plasmid pENC2.

Lane 1: Marker. λ DNA cleaved with *Hind* III.

Lane 2: pET29(a) digested with *E coR* 1 and *Sal* 1.

Lane 3: pENC2 digested with *E co*R1-*Sal*1.

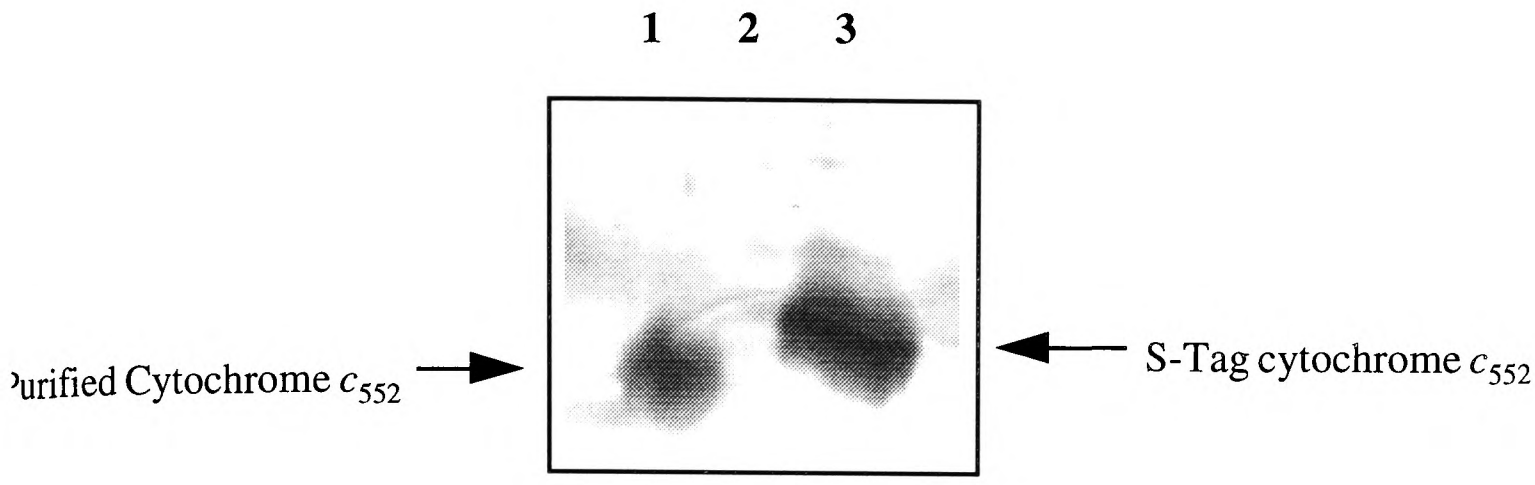


Figure 4.5. SDS-PAGE haem stain gel of the crude extract of strain HMS174(DE3), expressing cytochrome c_{552} from the construct pENC2. Crude extracts were prepared as described in the Materials and Methods (Chapter II). Approximately 80 μg of total cell protein was loaded on each lane. The gel was loaded as follows:

Lane 1: 8 μg of purified cytochrome c_{552} from the construct pKHC12 expressed in *E. coli* JCB387(Chapter III)
Lane 2: Control, Crude extract of HMS174.
Lane 3: Crude extract of HMS174 expressing the S-Tag cytochrome c_{552} from the construct pENC2.

[Note that the lower mobility of S-Tag cytochrome c_{552} as compared to cytochrome c_{552} expressed from the construct pKHC12, is due to the presence of an extra 35 amino acid residues at its *N*-terminus.]

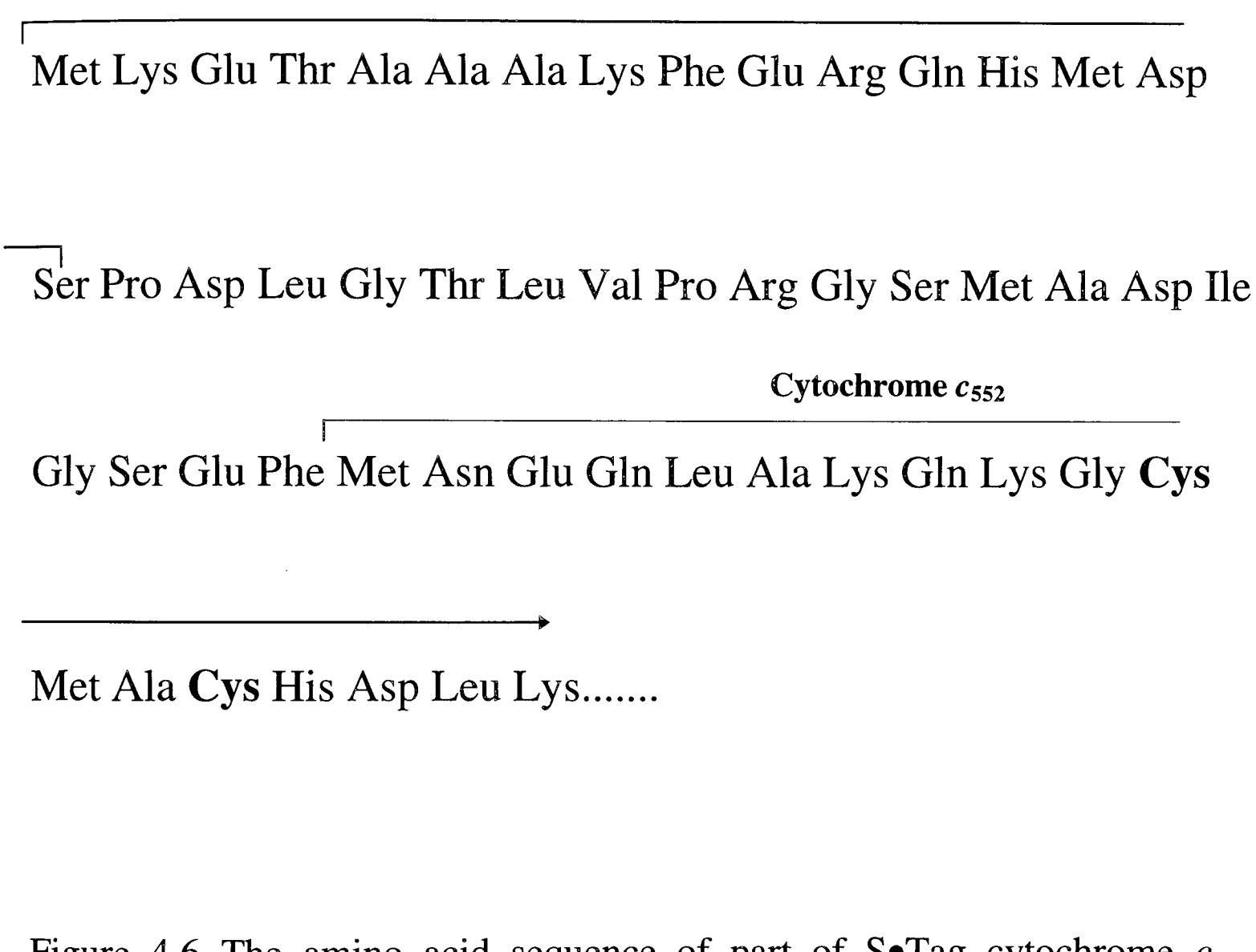


Figure 4.6 The amino acid sequence of part of S•Tag cytochrome c_{552} expressed from pENC2. The S•Tag comprises of 15 amino acid residues followed by 20 amino acid residues between the S•Tag and the start codon of cytochrome c_{552}.

analysis of the S•Tag cytochrome c_{552} content in extracts of the transformed *E. coli* HMS174(DE3) cells indicated that the expression levels were some way short of what was hoped for. One reason for relatively poor expression can be low stability of the plasmid containing the gene to be expressed. This can arise if the gene product is toxic to the cell. Consequently, the stability of pENC2 was investigated as recommended (Novagen instruction manual). *E. coli* HMS174(DE3) containing pENC2 were titrated on four plates having either: (i) antibiotic to which the plasmid conferred resistance; (ii) IPTG; (iii) the antibiotic plus IPTG; (iv) neither of these additions. All viable cells should grow on plates containing no additions, only cells that retain the plasmid should grow in the presence of antibiotic, whilst only cells that have lost the plasmid, or mutants that have lost the ability to express target DNA, should grow in the presence of IPTG. Finally, only mutants that retain the plasmid but have lost the ability to express cloned DNA for cytochrome c_{552} should grow in the presence of both antibiotic and IPTG. Overnight grown cultures of HMS174(DE3) cells containing pENC2 were plated at a dilution of 10^{-5} on plates that had i) both IPTG and antibiotic ii) just IPTG, and at a dilution of 2×10^{-6} on plates that had iii) just antibiotic iv) nothing added. The plates were incubated for 8-10 hrs and the colonies were counted on each plate. In a culture useful for producing recombinant protein, almost all cells should form colonies both on plates without any additions and on plates containing added antibiotics; less than 2% of cells should form a colony on plates containing only IPTG and less then 0.01% should form a colony of plates containing both antibiotics and IPTG. The fraction of cells that have lost plasmid will be reflected by an increase in the colonies on the IPTG plate and decrease on the antibiotic plates. The plasmid stability test for S•Tag cytochrome c_{552} clones showed that the plate without any additives and the plate containing only antibiotics formed lots of

colonies. However, the plate containing just IPTG produced 2 % of the colonies formed on plates with either no additives or just containing the antibiotic. The plate containing both the antibiotic and IPTG produced 0.02 % of the colonies formed either on the plate with no additives or on the plate which just contained the antibiotic. Thus the results showed that the construct pENC2 was stable in the host HMS174(DE3), and that therefore the disappointingly low expression of cytochrome c_{552} was not due to the plasmid instability.

4.3.3 Purification of S•Tag cytochrome c_{552}

4.3.3.1 Growth of the cells

5-6 µl of the glycerol stock of HMS174(DE3) containing pENC2 was inoculated in 2 ml of LB and cells were grown overnight. Next morning, such a culture was inoculated into 50 ml LB, in a 250 ml flask, and grown until the cells reached an OD of 1.0 at 580 nm. The following morning this was inoculated into 500 ml of LB in a 2.5 l conical flask and allowed to grow until the culture had reached an OD of 0.8. 50 ml from this was inoculated into each of ten 2.5 l conical flasks containing 500 ml LB and allowed to grow until the culture reached an OD of 0.8. At this point IPTG was added to a final concentration of 0.4 mM and the growth was allowed for another four hours. In some growths 0.1 mM δ-aminolevulinic acid was added at the same time as the IPTG. All the cultures were grown aerobically.

4.3.3.2 Preparation of the extract

The pink cells were harvested by centrifuging the culture at 4°C and 4000 x g for 15 minutes. The cells were washed twice, each time with 200 ml of 10 mM Tris-

HCl (pH 8.0). The cells were finally suspended in 100 ml of STE and frozen at -70°C for at least half an hour. After thawing the suspension it was sonicated, in batches of 20 ml using three cycles of one minute sonication separated by one minute intervals.

4.3.3.3 Chromatographic purification of S•Tag cytochrome c_{552}

The cell extract containing S•Tag cytochrome c_{552} was treated in the same way as extracts of *E. coli* JCB387 expressing cytochrome c_{552} from the construct pKHC12 (see its purification in Materials and Methods, Chapter II) before being applied to a CM-52 column. Fortunately, the additional 35 *N*-terminal amino acids provided by the S•Tag plus amino acid residues present between the S•Tag and cytochrome c_{552} (Figure 4.6) only slightly decreased the net positive charge on the protein. Thus the positively charged protein was bound to the ion-exchange column and could be eluted by applying a continuous gradient of 0-0.5 M NaCl in 10 mM potassium phosphate (pH 7.5) buffer, as described for the purification of recombinant cytochrome c_{552} (Chapter II, Materials and Methods). S•Tag cytochrome c_{552} eluted as two peaks, one of which was oxidised and the other reduced. This material appeared pure as judged by a single band that stained for protein on SDS-PAGE (Figure 4.7). Figure 4.8 shows the spectrum of S•Tag cytochrome c_{552} as eluted from CM-52 column. Nevertheless, the standard second purification step of chromatography on a Dye Matrix Green column (see Materials and Methods, Chapter II) was done in order to remove any trace impurities. The S•Tag cytochrome c_{552} was dialysed and concentrated as described for the purification of recombinant cytochrome c_{552} (see Materials and Methods, Chapter II). Material purified

in this way was found to have a *N*-terminal sequence (Figure 4.9) that agreed with expectation (Figure 4.6).

4.3.4 The yield of S•Tag cytochrome c_{552} was found to be higher than that of cytochrome c_{552} obtained from *E. coli* JCB387

The concentration of S•Tag cytochrome c_{552} was estimated by spectrophotometry as described for the cytochrome c_{552} purified from the strain JCB387 containing pKHC12 (Chapter III) and by the S•Tag assay, described in the next section. The amount of S•Tag cytochrome c_{552} for several preparations was estimated by spectrophotometry to be between 4 and 10 mg from 10 l of culture that reached an OD 1.0 at 580 nm. The amount of cytochrome c_{552} purified using the T7 system was appreciably higher than the yield of 3.0 mg/ 20 l culture obtained from JCB387, expressing cytochrome c_{552} from pKHC12 (Chapter III), but not as high as has been hoped.

4.3.4.1 S•Tag assay

The S•Tag system is a gene tagging and detection system based on the interaction of the 15aa S•Tag peptide with ribonuclease S-protein. The two components together form active ribonuclease. Therefore, the assay of ribonuclease activity following the addition of purified S-protein provides a direct measurement of the concentration of S•Tag fusion protein. The ribonuclease activity is measured by incubation with poly(C) followed by centrifugation. The absorbance of the supernatant at 280 nm, which increases as poly(C) is broken down into acid soluble nucleotides, reflects the enzyme activity. By comparing the results with a known S-peptide standard (suplied by Novagen), the molar

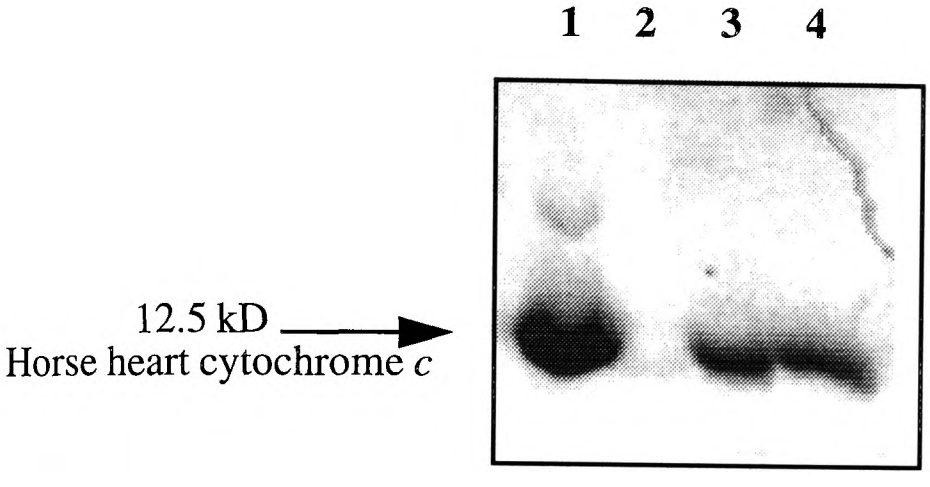


Figure 4.7. SDS-PAGE Coomassie stained gel of purified S·Tagged cytochrome c_{552}. The analysis was performed as described in Materials and Methods, Chapter II. The gel was loaded as follows:

Lane 1: 5 µg of purified horse heart cytochrome *c*
Lane 3 and 4: Approximately 1µg of one step purified S·Tag cytochrome c_{552}, eluted from CM-52 column.
(Lane 2 is empty).

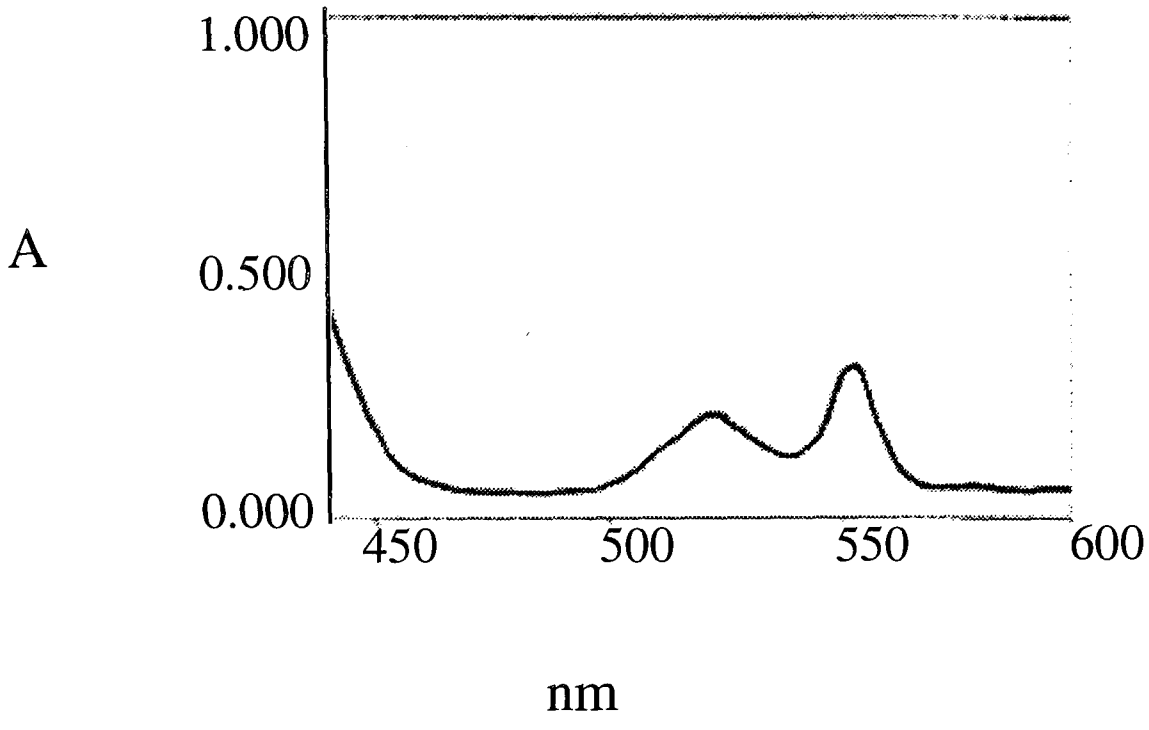


Figure 4.8. Spectrum of reduced S·Tag cytochrome c_{552} eluted from the CM-52 ion exchange column.

No.	Amnt. picomol	AA residue(s)					No.	Amnt. picomol	AA residue(s)				
		1	2	3	4	5			1	2	3	4	5
1	1714	M					26		G				
2		K					27		S				
3		E					28		M				
4		T					29		A				
5		A					30		D				
6		A					31						
7		A					32						
8		K					33						
9		F					34						
10		E					35						
11		R					36						
12		Q					37						
13		H					38						
14		M					39						
15		D					40						
16		S					41						
17		P					42						
18		D					43						
19		L					44						
20		G					45						
21		T					46						
22		L					47						
23		V					48						
24		P					49						
25		R					50						

Figure 4.9. The *N*-terminal sequence of purified S-Tag cytochrome c_{552}. 1-2µg of purified protein in 10 mM potassium phosphate buffer (pH 7.5) was used for the analysis, as described in Materials and Methods, Chapter II.

concentration of the target protein in the sample can be estimated. The reaction was performed in 1.5 ml microfuge tubes containing 1 x S•Tag assay buffer [20 mM Tris-HCl (pH 7.5), 100 mM NaCl, 100 µg/ml Poly(c)], 2µl of the bacterial crude extract of HMS174(DE3) containing the construct pENC2 (from the resuspension of 100 µl prepared from 10 ml culture of OD 1.0) and 10 µl of S•Tag grade S-protein in the final volume of 400 µl made up with sterile water. The same reaction mix was used for the S•Tag standard. A similar reaction was performed as a blank with a crude extract of HMS174(DE3) which did not contain any plasmid. The reactions were incubated at 37° C for 5 minutes and stopped by adding 100 µl ice-cold 25 % trichloroacetic acid. The reaction was vortexed and kept on ice for 5 minutes. This was then centrifuged at 14,000 x g for 10 minutes. The absorbance of the supernatant was taken at 280 nm after the blank correction. The amount of the target protein was calculated with the help of the absorbance obtained from the S•Tag standard of known concentration. The amount of purified S•Tag cytochrome c_{552} obtained from 10 l of culture was approximately 20 mg, as measured by S•Tag assay, compared with between 4 and 10 mg measured by spectrophotometric analysis (previous section). This suggests that some of apocytochrome c_{552} was not converted to the holoprotein (see later), and/or there were significant losses during purification of S•Tag cytochrome c_{552}.

4.3.5 Electrospray ionisation mass spectroscopy of S•Tag cytochrome c_{552} shows that the mass of cytochrome c_{552} is 9.3 kD.

A sample for electrospray mass spectroscopy was prepared by dialysing purified cytochrome S•Tag c_{552} against deionised H_2O and the molecular weight of

recombinant S•Tag cytochrome c_{552} was determined to be 13.129 kD (Figure 4.10). This agrees with adding the molecular weights of the *N*-terminal 35 amino acid residues (S•Tag + 20 amino acid residues between the S•Tag and cytochrome c_{552}) to the molecular weights of the 80 residues of cytochrome c_{552}, 3.9 kD and 9.21 kD respectively. This study shows the earlier reported molecular weight of cytochrome c_{552} of 7.6 kDa (Sanbongi *et al.*, 1989) to be incorrect. Nevertheless, as claimed earlier (Sanbongi *et al.*, 1989), it is still the lowest molecular weight reported for a cytochrome *c*.

4.3.6 CD (circular dichroism) spectroscopy of S•Tag cytochrome c_{552} shows that the presence of 35 amino acid residues at the *N*-terminal of cytochrome c_{552} does not affect the tertiary structure of the protein.

CD spectroscopy of the S-tagged cytochrome c_{552} was done to assess the structural changes the extra 35 amino acid residues (15aa S•Tag + 20aa present between the S•Tag and cytochrome c_{552}, Figure 4.6) might have caused. The CD spectrum (Figure 4.11) of S•Tag cytochrome c_{552} was found to be similar to that from *E. coli* JCB387 (Figure 3.5) cytochrome c_{552}, suggesting that the 35 amino acid residues at the *N*-terminus of cytochrome c_{552} do not grossly affect its tertiary structure.

4.3.7 The melting profile of S•Tag cytochrome c_{552} suggests that it retains its thermostability.

The melting profile analysis used in the present study was a modified version of that of Fujita *et al.*, 1979. The experiments were carried out in the presence of 10mM KH_2PO_4 and the ellipticity at 222 nm was employed as the structural parameter. The melting profile as a function of temperature was recorded as described in the Materials

and Methods, Chapter II. The CD spectra of S•Tag cytochrome c_{552} did not indicate any melting or change in structure even when the temperature reached 95°C (Figure 4.12). Against this behaviour can be contrasted the CD spectra of horse heart cytochrome *c*, which showed a distinct change at a temperature of 55°C (Figure 4.13). Temperatures higher than 95°C were beyond the range of the CD spectrophotometer and, thus the thermostability study of S•Tag cytochrome c_{552} at higher temperatures could not be performed. A thermostability study on the native cytochrome c_{552} shows that cytochrome c_{552} can return to its native structure even after being autoclaved at 120 °C for 10 minutes (Sanbongi *et al.,* 1989). The autoclaving of S•Tag cytochrome c_{552} was, however, not attempted. Nevertheless, the data shown in Figure 4.12 can be taken as evidence that the S•Tagged cytochrome c_{552} is a very thermally stable protein. Therefore, the addition of the *N*-terminal S•Tag has not had a very significant effect on the thermal stability.

4.3.8 Removal of haem from S•tagged cytochrome c_{552}

In order to determine whether apocytochrome c_{552} has some tertiary structure, an experiment to remove haem from S•Tag cytochrome c_{552} was performed. The reaction was performed as described for the haem removal of recombinant cytochrome c_{552} expressed in strain JCB387 (Chapter III), except that the final volume of the reaction contained 8 M rather than 16 M urea and the reaction was carried out in the presence of 0.1% SDS. These changes were made for two reasons. First, that 16 M urea, recommended for the removal of haem from this thermophilic cytochrome c_{552} (Y. Sambongi, personal communication), was not fully soluble. Second, SDS was included to help unfold the protein to facilitate haem removal. Otherwise the components and the

procedure used were same as described in Chapter III. Table 4.1 shows the readings obtained from the S•Tag cytochrome c_{552} haem removal experiment. The haem and the apocytochrome c_{552} were separated using gel filtration chromatography as described in Chapter III. Early fractions had a higher absorbance at 280 nm than at 410 nm (Table 4.1). Since the holoprotein had a A_{410}/A_{280} ratio of approximately 5, it can be concluded that S•Tag cytochrome c_{552} was effectively converted to the apoform. Unfortunately it proved difficult to remove the denaturant, whilst maintaining solubility, from material produced in this way and thus it was not possible to characterise the apo protein.

4.3.9 Attempts to increase the yield of S•Tag cytochrome c_{552}

The finding that a thermally stable S•Tagged cytochrome c_{552} could be prepared, together with evidence that the haem group could be removed *in vitro*, suggested that some of the objectives of the present project might be met if larger amounts of the fusion protein could be prepared. To this end investigation was made of some changes to the growth conditions that might lead to increased expression of the protein. The successful production of cytochrome c_{552} in *E. coli* requires not only the synthesis of the polypeptide but also an adequate supply of haem. Haem synthesis in bacteria is usually regulated, but the presence of an exogenous protein requiring haem does not necessarily remove all constraints on the rate of synthesis of haem. Thus in the present work the effect of adding the haem precursor δ-aminolevulinic acid (see Chapter I, section 1.15) on the synthesis of cytochrome c_{552} was investigated. The S•Tag described earlier was used as the assay.

4.3.9.1 The presence of δ-aminolevulinic acid in the growth medium increase the expression of S•Tag cytochrome c_{552}

4.3.9.1.1 Sample preparation

A few µl from glycerol stock of HMS174(DE3) containing the construct pENC2 was inoculated in 2ml of LB medium and grown aerobically untill the OD of 0.8 at 580 nm was reached. The culture was then stored at 4°C. Next morning the culture was centrifuged and cells were resuspended in 2ml of LB. This was inoculated in 50 ml of LB contained in a 250 ml flask. This was grown aerobically till O.D reached to 0.8 at 580 nm. 25 ml of this culture was then transferred to another 250 ml conical flask. 0.4 mM IPTG was added to both of the flasks while 0.1 mM δ-aminolevulinic acid was added to one flask. The cultures were then allowed to grow aerobically for 3-4 hrs. The cells were harvested after centrifugation and washed with buffer, 50mM Tris-HCl pH 8.0, 2mM EDTA. The cells were then suspended in 2ml of STE each. The suspension was stored at -70°C, giving sufficient time to freeze completely. Cells were next thawed and sonicated. The membranes were removed by centrifuging at 15,000 rpm for 10 mins. For the comparison of expression, with and without δ-aminolevulinate, 2µl of this was used for the S•Tag assay while 100 µl was used for SDS-PAGE analysis by the haem stain (as described in Materials and Methods, Chapter II).

The concentration of S•Tag cytochrome c_{552} was found to be 0.7 µg per ml cell culture and 2.2 µg per ml cell culture following the supplementation with δ-aminolevulinic acid. The data thus show that concentration of expressed S•Tag cytochrome c_{552} was enhanced by the presence of δ-aminolevulinic acid, as estimated by the S•Tag assay. The SDS-PAGE analysis (Figure 4.14) of the effect of δ-aminolevulinic

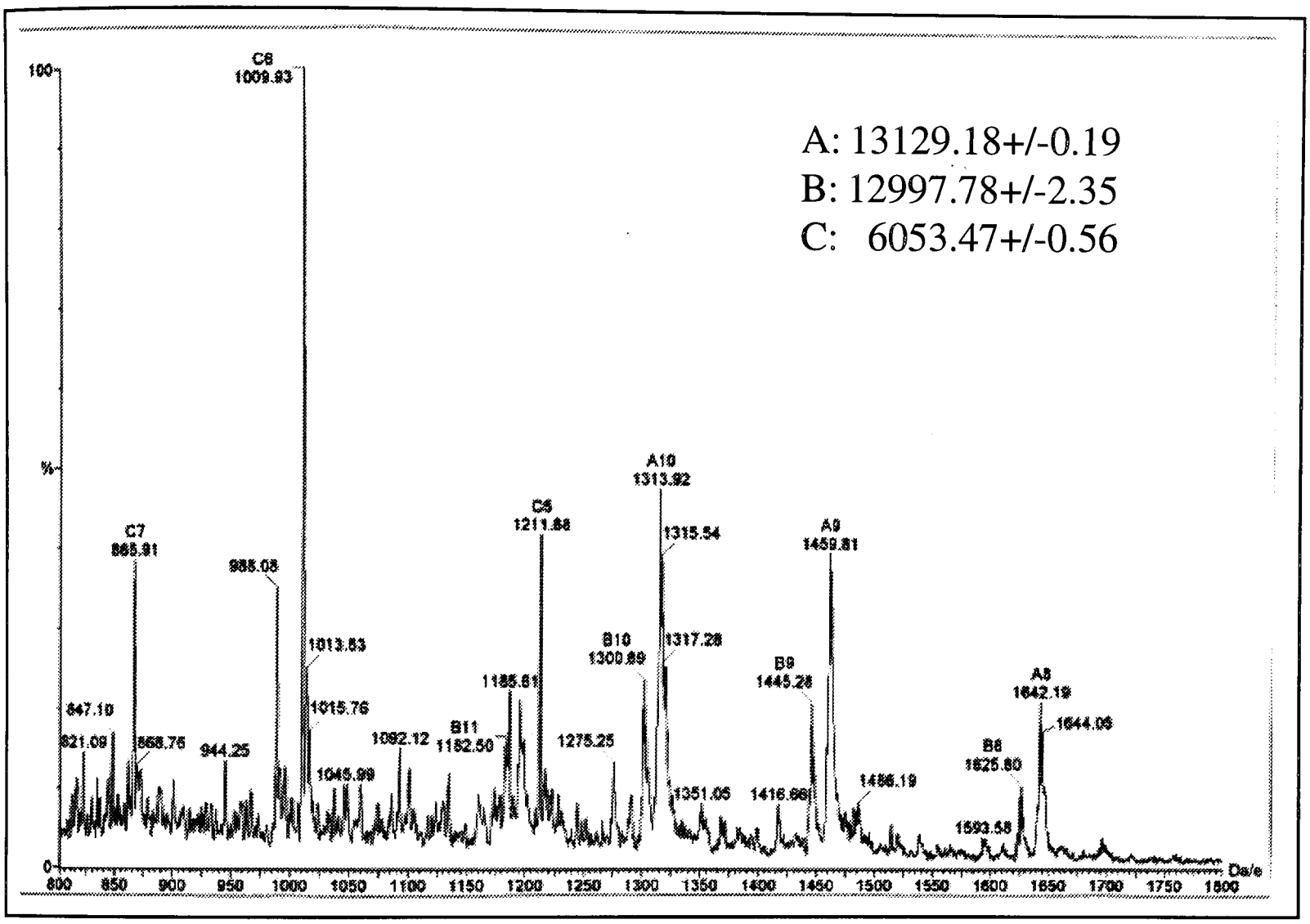


Figure 4.10. Electrospray ionisation mass spectroscopy of S-Tag cytochrome c_{552}. The individual fragmentation peaks which have different charge: mass ratios, are shown. Species A corresponds to the intact S·Tag cytochrome c_{552}. Species B is presumably a form of the protein that has lost the *N*-terminal methionine amino acid residue. Species C is probably a consequence of peptide bond breakage in the spectrometer. The large intensity of one of the peaks for C does not mean that this is a predominant species. The detection of a species molecular weight 6,053, but not of other molecular weights below 13,000, suggests that the rest of the polypeptide was fragmented into many small species or into larger ones that were not readily detected in the spectrometer.

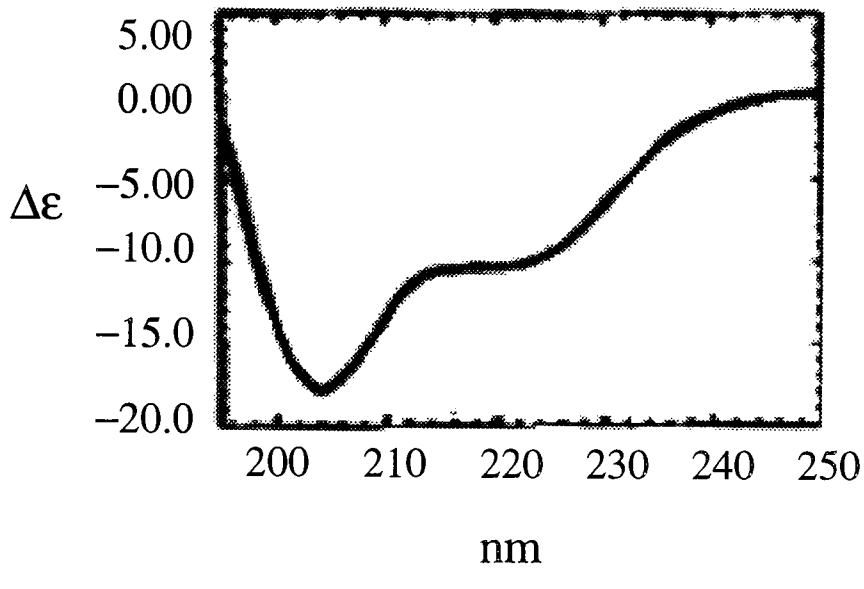


Figure 4.11. The CD spectrum of S·Tag cytochrome c_{552}, at a temperature of 25°C. The spectroscopy was performed as described in Materials and Methods, Chapter II. The sample was prepared in 10 mM potassium phosphate (pH 7.5). The concentration of purified S-Tag cytochrome c_{552} used was 400 μg/ml. Units of Δε are mM^{-1} cm^{-1}.

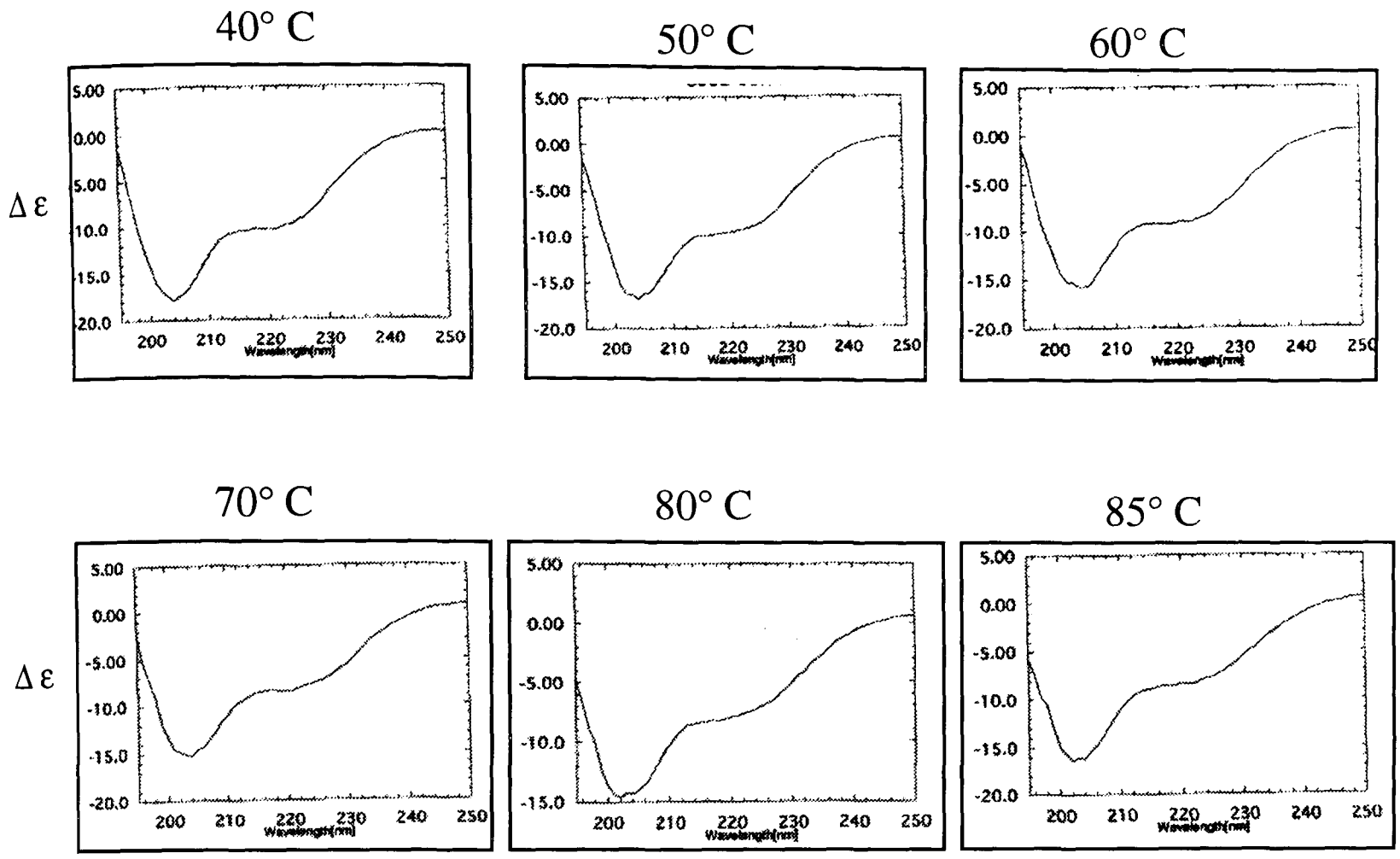


Figure 4.12. The thermostability of S-Tag cytochrome c_{552} demonstrated by CD spectroscopy. The sample (0.4 ml with the concentration of 0.5 mg/ml) in 10 mM potassium phosphate buffer (pH 7.5) was placed in a stoppered quartz cell with 1-cm light path embedded in a brass jacket, through with the water was circulated. The analysis was performed as described in Materials and Methods, Chapter II. The temperature was raised from 20° C to 95° C, recording the spectrum at the temperatures shown. Units of $\Delta\varepsilon$ are mM^{-1} cm^{-1}.

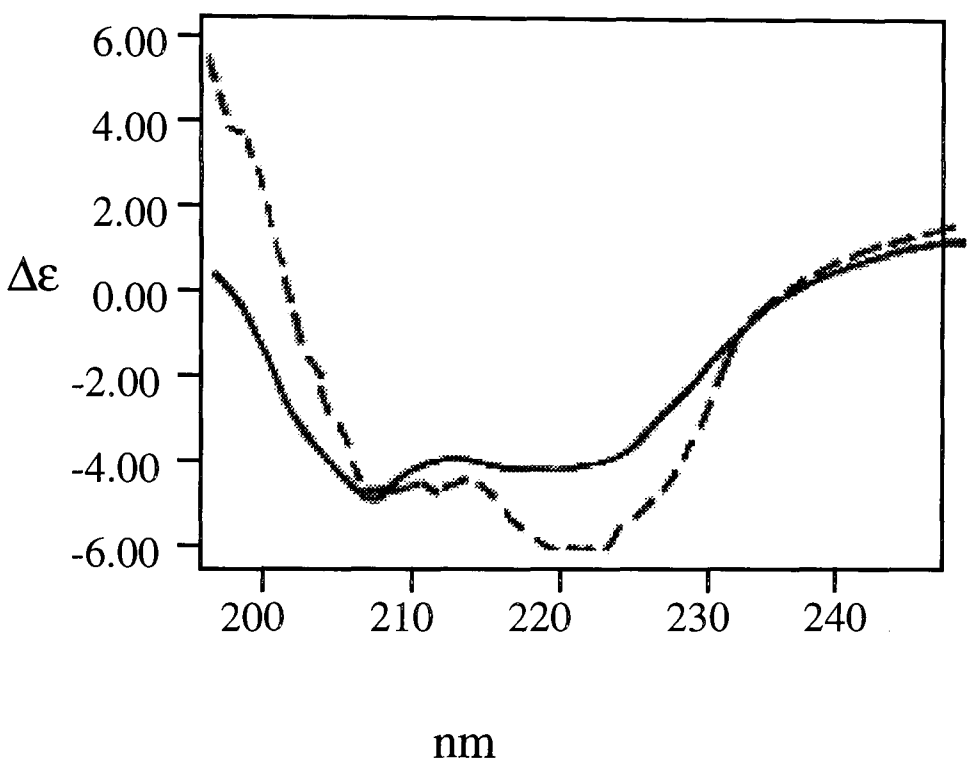


Figure 4.13. The CD spectrum of horse heart cytochrome *c* at 25° C (—) at 55° C (- -). The purified protein, supplied by Sigma, was used in a stoppered quartz cell at the concentration of 0.5 mg ml^{-1} in 10 mM potassium phosphate buffer (pH 7.5). The analysis was performed as described in Materials and Methods, Chapter II. The units of Δε are mM^{-1} cm^{-1}.

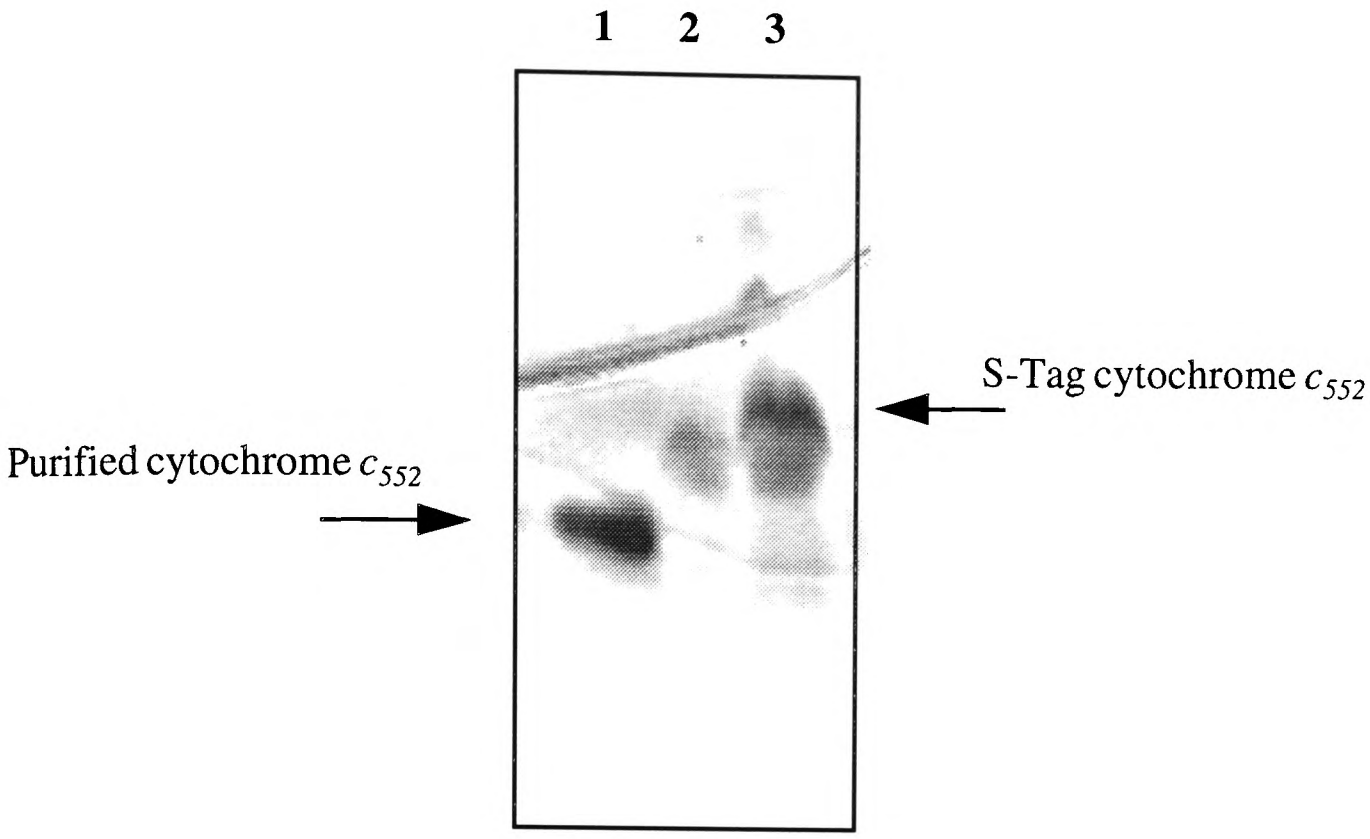


Figure 4.14. An SDS-PAGE haem stained gel to show the effect of δ-aminolevulinic acid on the expression of S-Tag cytochrome c_{552}. The crude extract was prepared as described in the present Chapter. SDS-PAGE and Haem staining was performed as described in Materials and Methods, Chapter II. The normalised crude extracts, approximately 50 μg of total cell protein, were loaded on each lane so that the comparison in expression could be made.

Lane 1: 8 μg of purified cytochrome c_{552} from the strain JCB387 expressing cytochrome c_{552} from the construct pKHC12 (Chapter III).

Lane 2: Crude extract of HMS174 expressing cytochrome c_{552} from the construct pENC2.

Lane 3 : Same as Lane 2 except that the growth medium was supplemented with 0.1 mM of δ-aminolevulinic acid.

[Note the lower mobility of S-Tag cytochrome c_{552} ; this is due to the presence of extra 35 amino acid residues at the *N*-terminus.]

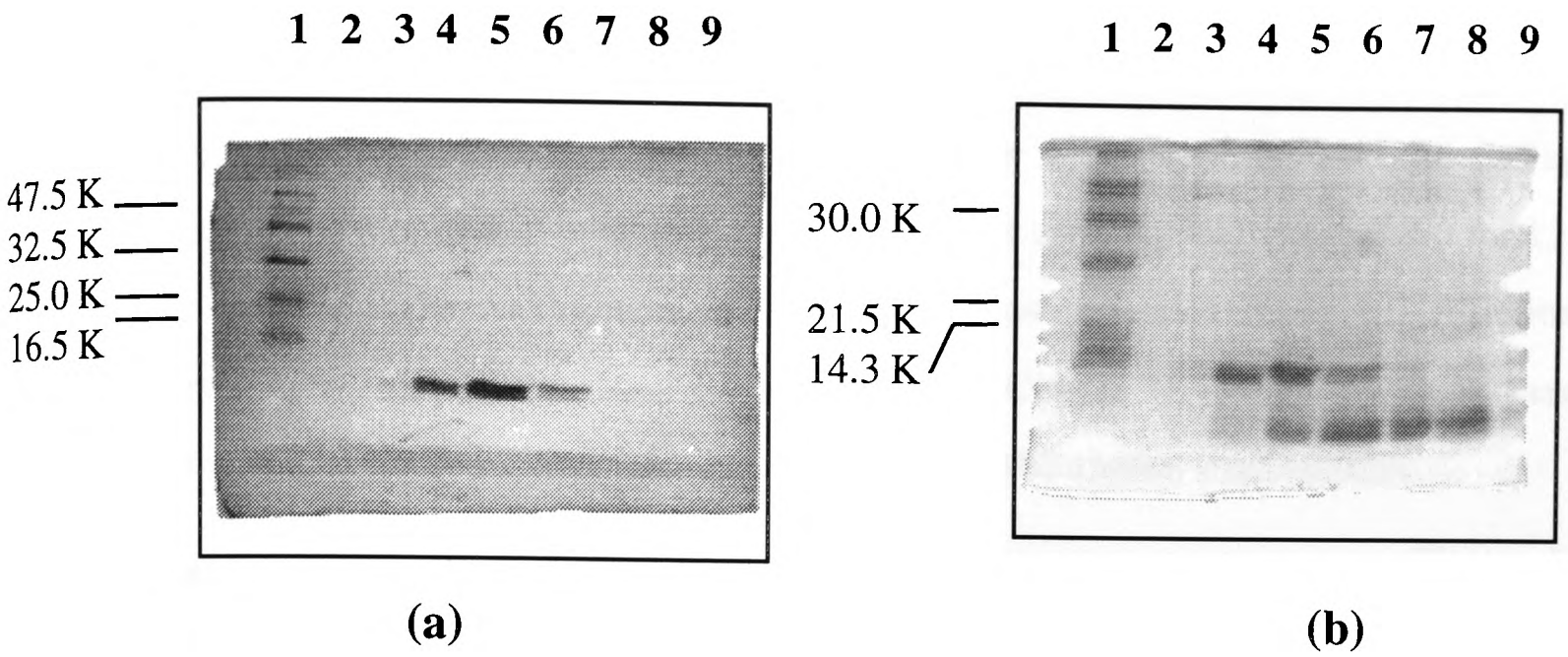


Figure 4.15. Demonstration of an apoform of S·Tag cytochrome c_{552} in *E. coli* HMS174(DE3).(a) Western blot analysis of seven consecutive (15-22) fractions eluted from a cellulose CM column. These fractions were loaded (40 µl in each case) on lanes 2-9. The primary antibody used for blotting was an alkaline phosphatase conjugate of an antibody towards the S·Tag. Lane 1 in (a) and (c) was loaded with the same prestained molecular weight markers provided by New England Biolabs. A different set of unstained markers was used in (b).
(b) Coomassie blue stained SDS-PAGE of the same fractions as loaded in (a).
(c) Haem stained SDS-PAGE gel of the same fractions as loaded in (a)
(Experiment in collaboration with Dr. E. Tomlinson)

Table 4.1 Haem removal from S•Tag cytochrome c_{552}:

The procedure used for the haem removal of S•Tag cytochrome c_{552} was a slight modification of what is described by Ambler *et al.* (1985). 2-4 μM S•Tag cytochrome c_{552} was treated with 25 mg of $HgCl_2$ in 8M urea, 0.1%SDS at pH 2.0 for 12-16 hrs at 37°C. The S•Tag apocytochrome c_{552} was separated using gel filtration chromatography, described in Materials and Methods, Chapter II. One ml fractions were measured at 410 nm and 280 nm, for haem and protein absorbance respectively.

Fractions	**Absorbance**	
	280nm	**410 nm**
1	0.004	0.007
2	0.022	0.009
3	0.990	0.014
4	0.871	0.014
5	0.933	0.023
6	0.877	0.029
7	0.511	0.023
8	0.262	0.067
9	0.099	0.020
10	0.041	0.012
11	0.043	0.010
12	0.037	0.010
13	0.008	0.010
14	0.015	0.010

acid on the expression of cytochrome c_{552}, agrees qualitatively with the S•Tag assay readings, the exact quantitation of haem stains being difficult to achieve.

4.3.9.2 Comparison in the expression of cytochrome c_{552} under aerobic and anaerobic growth conditions

E. coli synthesises its own *c*-type cytochromes only under anaerobic growth conditions but the cytoplasmic maturation of *H. thermophilus* cytochrome c_{552} should not show any difference in expression under aerobic and anaerobic growth conditions. Nevertheless, it was considered that this should be checked and thus the expression of cytochrome c_{552} was studied under aerobic and anaerobic growth conditions, with the amount of expressed cytochrome c_{552} being determined by S•Tag assay.

4.3.9.2.1 Sample preparation

A few µl glycerol stock of HMS174(DE3) containing the construct pENC2 was inoculated in 2 ml of LB and grown aerobically, overnight. Next morning one ml of this was inoculated into 7 ml of anaerobic media (see Material and Methods, Chapter II) contained in 8 ml vials and allowed to grow for about 24 hrs, without shaking, untill the OD had reached between 0.8 and 1.0 at 580 nm. 0.4 mM IPTG was then added to the vial and the culture was allowed to grow for another 8-12 hrs. Cells were centrifuged and resuspended in 100 µl of resuspension buffer, washed and again resuspended in 100 µl of resuspension buffer. Cells were frozen at -70°C. The frozen cells were thawed and sonicated, as described in Materials and Methods, and centrifuged. To determine the effect of δ-aminolevulinate on the expression of cytochrome c_{552} under anaerobic growth

condition, 0.1 mM δ-aminolevulinic acid was added to the growth medium at the same time of IPTG addition.

The concentration of S•Tag cytochrome c_{552} obtained from anaerobically grown cultures was 1.4 μg per ml cell culture, approximately double that obtained from an aerobic culture. However, the addition of δ-aminolevulinic acid did not cause any increase in the expression under the anaerobic growth conditions. The concentration of S•Tag cytochrome c_{552} obtained when the anaerobic growth was supplemented with 0.1 mM of δ-aminolevulinic acid was 1.5 μg per ml cell culture, nearly equals to the concentration when the cells were grown without the supplementation of δ-aminolevulinic acid. In contrast, the supplementation with 0.1 mM of δ-aminolevulinic acid to the aerobic growth resulted in three times enhancement in the concentration of S•Tagged cytochrome c_{552}. Tentatively, this may be because haem synthesis in *E. coli* is better able to supply the requirement of cytochrome c_{552} under anaerobic rather then aerobic conditions. Since the best yield of cytochrome c_{552} was obtained by growing the cells under aerobic growth conditions supplemented with 0.1 mM δ-aminolevulinic acid, these conditions would be optimal for the large scale purification of S•Tag cytochrome c_{552}.

Following all these screenings of growth conditions several preparations of S•Tag cytochrome c_{552} were obtained from cells grown aerobically (growth is faster than anaerobically) with 0.1 mM δ-aminolevulinic acid present. Some of this material was examined by NMR in collaboration with Dr. P. Barker (MRC Lab, Cambridge). Despite the S•Tag increasing the molecular weight, reasonably well resolved NMR spectra could be obtained, but they were always less than optimal for attempting to elucidate the

chemistry of haem attachment. Fortunately (Chapter V) an alternative route to purifying cytochrome c_{552} in higher amounts was later discovered.

4.3.10 Further investigation of the expression of S•Tagged cytochrome c_{552}

The relatively modest yields of S•Tag holocytochrome c_{552} under any of the growth conditions tested, and the effect of adding δ-aminolevulinic acid, suggested that a significant amount of the S•Tag apocytochrome c_{552} formed may have failed to acquire haem. Such material may have been present in the cell extract as the apoprotein, either as intact polypeptide or in a degraded form. This possibility was tested (in collaboration with Dr. E. J. Tomlinson) using an antibody to the S•Tag to probe for the presence of such apoprotein. Figure 4.15a shows Western blotting of material eluting from a CM-52 column that had been loaded with an extract of cells that had been expressing S•Tag cytochrome c_{552}. The later eluting fractions that contained the S•Tag did not stain for covalently attached haem on SDS-PAGE, thus indicating that the apo form of S•Tag cytochrome c_{552} was in the extract (Figure 4.15c). It was also clear that the material not recognised by the anti-S•Tag antibody, and eluting in later fractions, had a range of molecular weights. This suggested degradation of the protein. The implication of these findings is that the S•Tag apocytochrome c_{552} might be formed in significant excess over the amount that is formed as the holoprotein, and that, therefore, despite the thermal stability (Figure 4.12), a significant proportion of the S•Tagged apocytochrome c_{552} is degraded in the *E. coli* cytoplasm before haem can attach.

The identification of S•Tag cytochrome c_{552} in the fractions that eluted from CM-52 after the holoprotein suggested that it might be possible to detect apocytochrome c_{552},

without the S•Tag, in other strains of *E. coli*. It should be recalled that the S•Tag itself makes no difference to the net charge on the polypeptide and thus non-tagged apocytochrome c_{552} should chromatograph on CM-52 with the same relationship to the holoprotein as the apo and holo forms of the S•Tagged material.

When pKHC12 was transformed into *E. coli* JM109 (in collaboration with Dr. Tomlinson) and the resulting soluble cell extract chromatographed on cellulose CM-52, fractions eluting after holocytochrome c_{552}, which could be identified on a gel by haem staining, contained a prominent polypeptide on SDS-PAGE with the same mobility as holocytochrome c_{552}. Because few proteins bind to CM-52 cellulose this protein was obtained in an almost pure state. When the same procedure was applied to *E. coli* JM109 that had been transformed with a control plasmid then this polypeptide was not seen. Hence it was concluded that apocytochrome c_{552} had been purified. By pooling the fractions containing this apocytochrome c_{552} it proved possible to acquire a CD spectrum (Figure 4.16). This spectrum implies that the apo protein is not entirely random coil in conformation but contains at least some α-helical structure. This conclusion is based upon the increasingly negative band as the wavelength declines below 240 nm and the tendency to a minimum close to 208 nm.

4.4 Discussion

It is established in this Chapter that holo S•Tag cytochrome c_{552} can be synthesised by a T7 polymerase-dependent system in *E. coli* and that the *N*-terminal S•Tag does not measurably affect the thermal stability of the protein. However, the amount of material produced by this system was not as great as had been hoped. Although the reasons for this

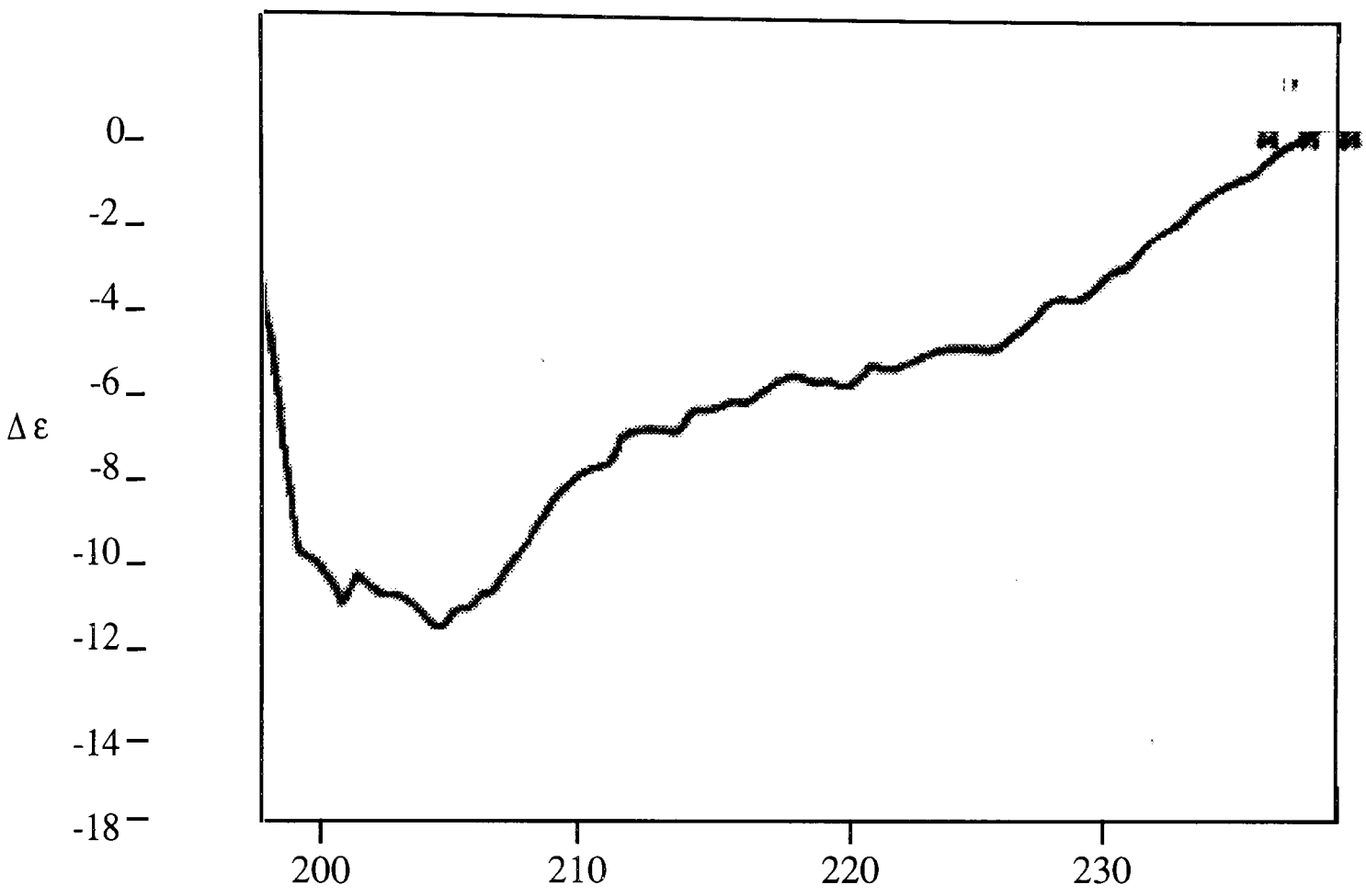


Figure 4.16 A CD spectrum of apocytochrome c_{552} purified from the extract of *E. coli* JM109, expressing cytochrome c_{552} from the construct pKHC12. The analysis was performed as described in Materials and Methods, Chapter II. The apocytochrome c_{552} was purified as described in section 4.3.11. 300 µl of 0.5 mg ml^{-1} protein in 10 mM potassium phosphate buffer (pH 7.5) was used for the analysis. (Experiment in collaboration with Dr. E. Tomlinson). The units of $\Delta\varepsilon$ are mM^{-1} cm^{-1}.

have not been analysed in detail, it seems probable that the synthesised polypeptide cannot acquire haem sufficiently quickly to prevent breakdown by proteolysis. The addition of haem precursor, δ-aminolevulinic acid, resulted in some elevation of the amount of S•Tag holocytochrome c_{552} but, possibly because of a restraint on the rate at which haem could be produced from this precursor, it was not possible to supply haem sufficiently rapidly to increase by much the amount of holo protein. In future work it may be possible to circumvent this problem by using a strain of *E. coli* that is permeable to haem. The identification of S•Tag apo cytochrome c_{552} was unanticipated but led to the important identification of apocytochrome c_{552} in another strain of *E. coli*. According to CD spectroscopy this material was not entirely in a random coil conformation, but rather had a significant secondary structure. However, the CD spectrum in Figure 4.16 was taken using a dilute buffer that is compatible with CD spectroscopy in UV region. It may be that the protein has more, or less, structure in solution with a composition, at least in terms of ionic strength, found in the cytoplasm. This finding of some structure in the apocytochrome *c* contrasts with apo mitochondrial cytochrome *c* which is generally regarded as having a random coil structure (Stellwagen *et al.,* 1972; Fisher *et al.,* 1973; Cohen *et al.,* 1974). Therefore the observation of some structure in the apo form of recombinant *H. thermophilus* cytochrome c_{552} can be regarded as preliminary support for the hypothesis (Sanbongi and Ferguson, 1994b) that this polypeptide folds so as to provide a pocket into which the haem can slot. This hypothesis will need to be developed by further work in which the interaction *in vitro* of the apo-protein with haem is investigated. This may not be straightforward as haem is relatively insoluble in water and the competence of both its oxidation states for insertion will have to be studied. Furthermore, the integrity of the two cysteine groups of the apo protein will need to be

assessed. The two thiol groups of these cysteines are needed to attach to the haem. If, for example, they are modified either to give the disulphide or some other product, then attempts to detect *in vitro* haem attachment to the apo polypeptide of cytochrome c_{552} will be unsuccessful. However, it is important to note that apocytochrome c_{552} prepared directly from the cytoplasm of *E. coli* cells is likely to be better material with which to work than material prepared by removing the haem from the holoprotein with $HgCl_2$. Unfortunately, the time available to the present author has not permitted experimental examination of these possibilities.

The minor difference of the expression of cytochrome c_{552} under aerobic and anaerobic growth conditions in *E. coli*, which synthesises its own *c*-type cytochromes only under anaerobic growth conditions (see General Introduction and Chapter V), agrees with the assumption that since this cytochrome c_{552} does not need the activities of any gene products for its maturation, normally needed for other *c*-type cytochromes, it should express to similar levels both under aerobic and anaerobic growth conditions. There are, however, examples of *c*-type cytochromes which were expressed periplasmically in *E. coli* under aerobic growth conditions. Expression and correct maturation of *B. subtilis* and *Thiobacillus versutus* *c*-type cytochromes under aerobic conditions have been observed (von Wachenfeldt and Hederstedt, 1990; Ubbink *et al.,* 1992) although their expression was always periplasmic. It is not clear why these authors succeeded in expressing these cytochromes aerobically.

In conclusion, it should be noted that there is no report yet on the conformation of the apo form of any other bacterial *c*-type cytochrome. After the observation that the thermophilic cytochrome c_{552} has some secondary structure it would be helpful to know whether or not the apo form of, for example, *P. denitrificans* cytochrome c_{550} has a

random coil structure. If, in contrast to mitochondrial apocytochrome *c*, it has significant secondary structure, this might provide clues to the periplasmic assembly pathway.

Chapter V

Biogenesis of *H. thermophilus* cytochrome c_{552} in different strains of *E. coli*.

5.1 Introduction

As explained earlier, cytochrome c_{552} from the thermophilic bacterium *H. thermophilus* is the only *c*-type cytochrome that has been shown to be synthesised as a holoprotein in the cytoplasm of *E. coli*. The synthesis of this protein occurs in a mutant of *E. coli* that lacks the *dipZ* gene which codes for a disulphide isomerase-like protein which is required for 'normal' *c*-type cytochrome maturation in *E. coli* (Sambongi and Ferguson, 1994a; Crooke and Cole, 1995). These observations suggest that the holoform of this cytochrome c_{552}* acquires its covalently bound haem group in the cytoplasm without any enzymatic assistance. However, this proposal brings with it the assumption that none of the other genes requires for *c*-type cytochrome biogenesis in *E. coli* (the *ccm* genes, Chapter I) have any unsuspected role in the synthesis of cytochrome c_{552}. It is generally assumed that the products of the *ccm* genes have their functional regions facing the periplasm but it cannot be excluded that some cytoplasmic facing regions, e.g. of CcmD (Chapter I), of these gene products may have unrecognised activities. During the course of the present work an *E. coli ccm* deletion strain (Grove *et al.,* 1996b) became available and therefore enabled testing of the prediction that cytoplasmic cytochrome c_{552} synthesis should be unaffected by the lack of *ccm* gene products. The *ccm* deleted strain, JCB71202, was confirmed to be Nrf negative and defective in the synthesis of all *c*-type cytochromes (Grove *et al*, 1996b). The maturation of endogenous *c* type cytochromes synthesised under anaerobic respiratory conditions, with nitrite, nitrate or trimethylamine *N*-oxide as the electron acceptor, was found to be defective in a comparable strain (Thony-Meyer *et al.*, 1995). Thony-Meyer *et al.,* (1995) also showed that their mutant failed to express an exogenous periplasmic cytochrome *c*. The *ccm* deletion strain used in

* The cytochrome c_{552} mentioned in the text should be considered as *H. thermophilus* cytochrome c_{552} unless otherwise stated

the present work is derived from a parent strain, JCB712, which produces, for unknown reasons, elevated levels of endogenous, periplasmic facing, *c*-type cytochromes (Grove *et al.,* 1996). It was therefore decided also to determine whether JCB712 produces elevated levels of cytoplasmic cytochrome c_{552} and, if so, to investigate a possible basis for the ability of this strain to synthesise elevated levels of *c*-type cytochromes. These experiments were done in the context that a route for producing large amounts of cytochrome c_{552} was still needed to permit NMR studies of the haem attachment.

The strain JCB387 used to purify cytochrome c_{552} (Chapter III) is among the strains specifically made to study anaerobic respiratory pathways in *E. coli*, which involves the synthesis of *c*-type cytochromes. Anaerobic cultures of *E. coli* either use NADH or formate to reduce nitrite to ammonia, via the pathways known as NADH-dependent nitrite reduction, Nir, (Cole 1988; Harborne *et al.,* 1992) and formate-dependent nitrite reduction known, Nrf, respectively. The Nrf pathway involves the synthesis of endogenous cytochrome c_{552} (Darwin *et al.,* 1993b) and exploits the presence of two major formate dehydrogenases to transfer electrons from formate to the endogenous cytochrome c_{552}. The Nir pathway is usually more active and an attempts to devise a medium to select for Nrf+ that are defective in more active Nir pathway were unsuccessful (Darwin *et al.,* 1993b). The strain JCB387 is mutant in *nirB,* encoding for NADH-dependent nitrite reductase while it is FdhF+ FdhN+ FdhO+, the three formate hydrogenases of *E. coli*, shown to transfer the electrons from formate to cytochrome c_{552} in Nrf pathway (Darwin *et al.,* 1993b). It is due to this genetic background of JCB387, that it has been used in the study of *c*-type cytochromes.

Table 5.1: Details of the constructs used in this Chapter

pKHC12	Contains coding region of cytochrome c_{552} mature protein,with the coding region for the signal sequence deleted,as described in Sanbongi *et al.*, (1991).
pKPHC12K	Contains the coding region for the signal sequence of *P. denitrificans* cytochrome c_{550} +first *N*-terminal 10 amino-acid of this cytochrome followed by the coding region for the cytochrome c_{552} mature protein, as described by Sambongi *et al.*, (1994).
pKPHC12ΔSIG	Contains the first 10 amino-acid of *P. denitrificans* cytochrome c_{550} followed by the coding region for mature protein of cytochrome c_{552},as described by Sambongi *at al.*, (1994).

5.2 Results

5.2.1 Cytochrome c_{552} was matured in an *E. coli* mutant deleted in *ccm* (cytochrome *c* maturation) genes

Expression of *H. thermophilus* cytochrome c_{552} in *E.coli ccm* deleted strain JCB71202 was readily observed from the red colour of the cells, haem staining following SDS-PAGE (Figure 5.1) and by spectrophotometry (Figure 5.2). Thus none of the *ccm* genes that are deleted in this strain are required for cytoplasmic synthesis of this cytochrome.

5.2.2 Expression of cytochrome c_{552} in *E. coli* JCB 712 strains

JCB712, the parent strain of JCB71202, for unknown reasons synthesises higher level of endogenous periplasmic or periplasmic facing *c*-type cytochromes than other strains (Grove *et al.*, 1996). The ready detection of cytochrome c_{552} in JCB71202 strain suggested that this higher level of expression may also apply to this cytoplasmically expressed protein. To test this proposal the expression of this cytochrome in strains JCB712 and JCB387 was examined. Figure 5.1 shows that whereas extent of expression of cytochrome c_{552} in JCB712 was comparable with that in JCB71202, a significantly lower level was observed for strain JCB387. Cells of JCB712 expressing cytochrome c_{552} were red compared with the pale light brown coloured cells of JCB387 expressing cytochrome c_{552} from the same construct pKHC12. Although a normalised amount of crude extract was loaded on SDS-PAGE in each case, the higher level of expression of holocytochrome c_{552} in JCB712 or JCB71202 was also demonstrated by measuring the visible absorbance of this cytochrome c_{552} (Figure 5.2). This established that the

expression of cytochrome c_{552} was many fold (approximately 10 times) higher in JCB712 strains then in JCB387.

The observations described so far suggest that the enhanced production of *c*-type cytochromes by JCB712 strains is unlikely to be due to the activity of the *ccm* gene products since the synthesis of cytochrome c_{552}, which is independent of these products, is also elevated. Synthesis of *c*-type cytochromes requires haem, and a possible explanation for the difference between JCB387 and JCB712 was that the former was less able to meet the demand for haem during the expression of cytochrome c_{552}. This idea could in principle be tested by examining the effects of adding the haem precursor, δ-aminolevulinic acid (see Chapter I) to the growth media. In case of *E. coli* JCB387, the inclusion of 0.1 mM δ-aminolevulinic acid in the growth medium resulted in noticeably redder cells and an increase in the amount of cytochrome c_{552} (Figure 5.3, 5.4). A further increase in the concentration of δ-aminolevulinic acid had less effect (Figure 5.5). The dependence on the concentration of δ-aminolevulinic acid is similar to that reported for the expression of other haem proteins in *E. coli* (Verderber *et al.*, 1997). In contrast to the effect of adding 0.1 mM δ-aminolevulinic acid to the growth medium for JCB387 cells, the same supplementation for JCB712 was without effect on the synthesis of *H. thermophilus* cytochrome c_{552} (Figure 5.6). Thus further investigation was made regarding the effect of δ-aminolevulinic acid on the expression of cytochrome c_{552} in *E. coli* JCB387 and JCB712 strains (Discussed in the next section).

5.2.3 Further investigation on the effect of the haem precursor δ-aminolevulinic acid on the expression of cytochrome c_{552}.

The effect of supplementation of the growth medium with δ-aminolevulinic acid on the expression of cytochrome c_{552} in *E. coli* JCB387 could also be clearly seen when the plasmid pKHC12ΔSig was used. This has the cytochrome c_{552} structural gene sequence preceded by the coding sequence for 10 residues from the *N*-terminus of *P. denitrificans* cytochrome c_{550} protein, but a periplasmic targeting sequence is absent, as in pKHC12. The variant form of cytochrome c_{552}, extended by ten residues at the *N*-terminus, was scarcely detectable when expression was attempted in JCB387 unless the growth media was supplemented with δ-aminolevulinic acid (Figure 5.7). In contrast, this form of cytochrome c_{552} was expressed from the same plasmid at readily detectable levels in the strain JCB712 without supplementation of growth medium with δ-aminolevulinic acid, to an extent approximately equivalent to that found in JCB387 after supplementation with δ-aminolevulinic acid (Figure 5.8). Figure 5.9 shows the expression of cytochrome c_{552} from the same construct in the *ccm* deleted strain JCB71202 to a readily detectable level without the supplementation of δ-aminolevulinic acid to the growth medium.

Sambongi (personal communication) failed to achieve expression of cytochrome c_{552} in the periplasm of *E. coli* when a plasmid containing a native periplasmic targeting sequence was used. The periplasmic expression of mitochondrial cytochrome *c* was achieved by fusing the periplasmic targeting sequence for *P. denitrificans* cytochrome c_{550} along with the first 10 *N*-terminal amino acids of that protein (Sambongi *et al.*, 1996). Consequently Sambongi (personal communication) constructed an analogous plasmid, pKPHC12K, in an attempt to detect periplasmic expression of cytochrome c_{552} in *E. coli*. Plasmid pKHC12ΔSig, which differs from pKPHC12K in lacking the periplasmic targeting sequence, was thus a control for pKPHC12K. Sambongi (personal communication) was unable to detect any expression of cytochrome c_{552} from

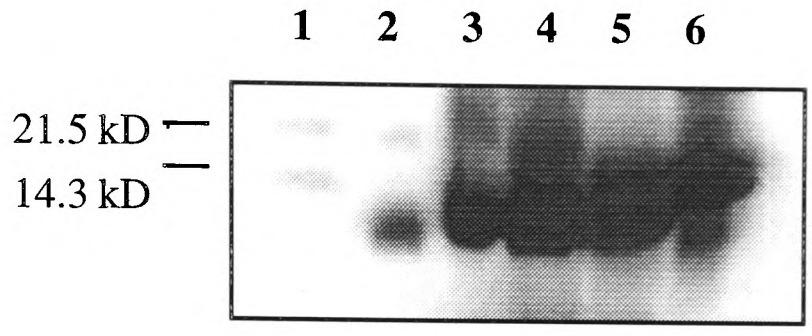


Figure. 5.1. SDS-PAGE haem stained gel of the crude extracts of three *E. coli* strains expressing cytochrome c_{552} from the construct pKHC12. The cells were grown aerobically and the crude extracts were prepared as described in Materials and Methods. The same amount of total cell protein was loaded in each lane, approximately 80 µg, so that the comparison in the expression of cytochrome c_{552} can be made.

Lane 1: 4 mg of rainbow coloured marker supplied by Amersham life sciences.

Lane 2: Purified cytochrome c_{552}.

Lane 2: Crude extract of JCB387 expressing cytochrome c_{552}.

Lane 3: Crude extract of JCB712 expressing cytochrome $c_{552.}$

Lane 4: Crude extract of JCB71202, deleted in *ccm* (cytochrome *c* maturation) genes, expressing cytochrome c_{552}.

Lane 5: 10 µg of horse heart cytochrome *c* supplied by Sigma.

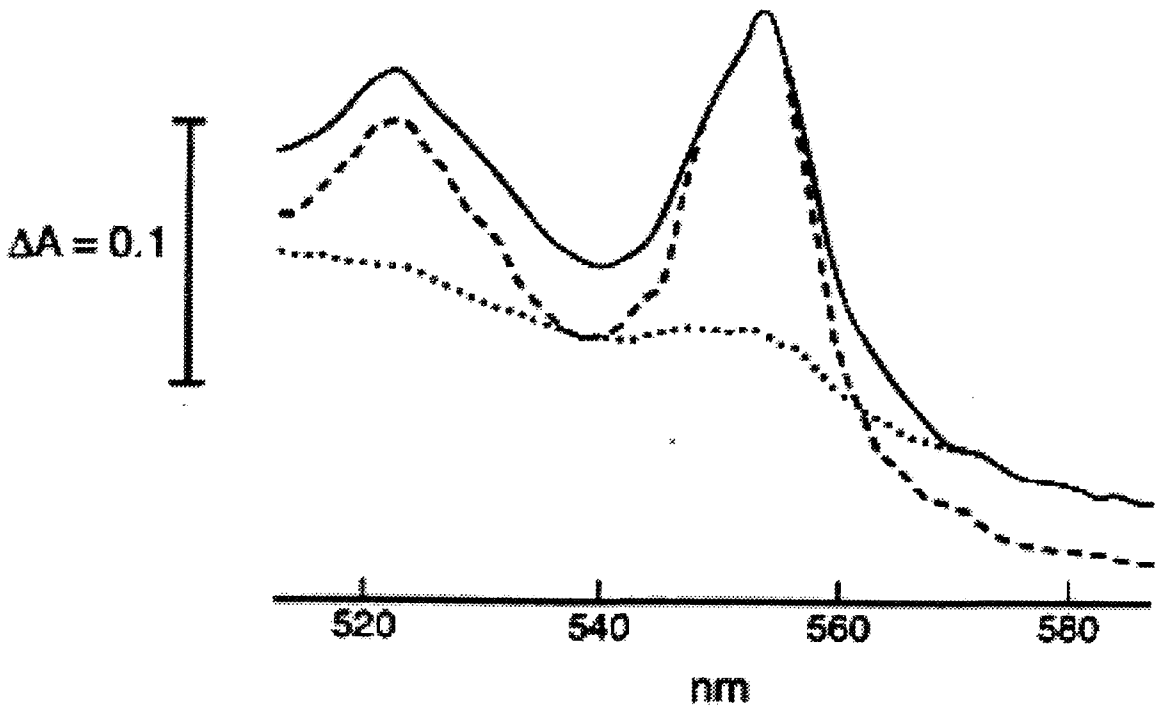


Figure. 5.2. Dithionite reduced spectrum of cytochrome c_{552} from the *E. coli* strains JCB387 (....), JCB712 (—) and JCB71202 (----), *ccm* deleted, expressing cytochrome c_{552} from the construct pKHC12. The crude extracts were prepared as described in Materials and Methods. 0.8 mg protein ml^{-1} was used for the analysis after adding sufficient solid sodium dithionite to reduce the samples completely.The spectra were measured against a buffer reference.

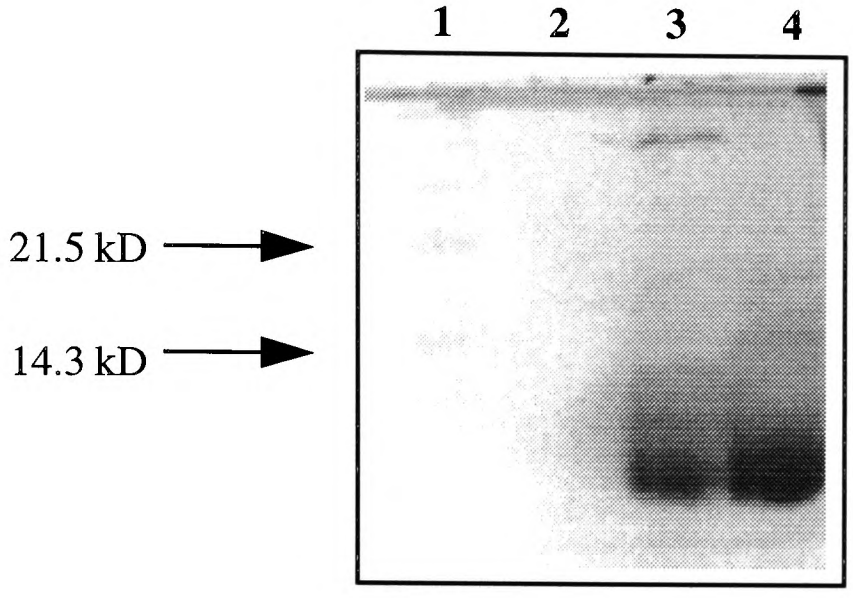


Figure 5.3. An SDS-PAGE haem stained gel showing the effect of δ-aminolevulinic acid on the expression of cytochrome c_{552} by the *E. coli* strain JCB387 expressing cytochrome c_{552} from the construct pKHC12. The SDS-PAGE analysis and haem staining was performed as described in Materials and Methods, Chapter II. Normalized crude extracts were prepared as described in Materials and Methods, Chapter II. Same amount of total cell protein, approximately 50 μg was loaded in each lane so as the comparison in the expression could be made.

Lane 1: 3 mg of the rainbow coloured markers supplied by Amersham Life Sciences.

Lane 2: Empty

Lane 3: Crude extract without the supplementation of δ–aminolevulinic acid.

Lane 4: Crude extract with the supplementation of 0.1 mM δ-aminolevulinic acid in the growth medium.

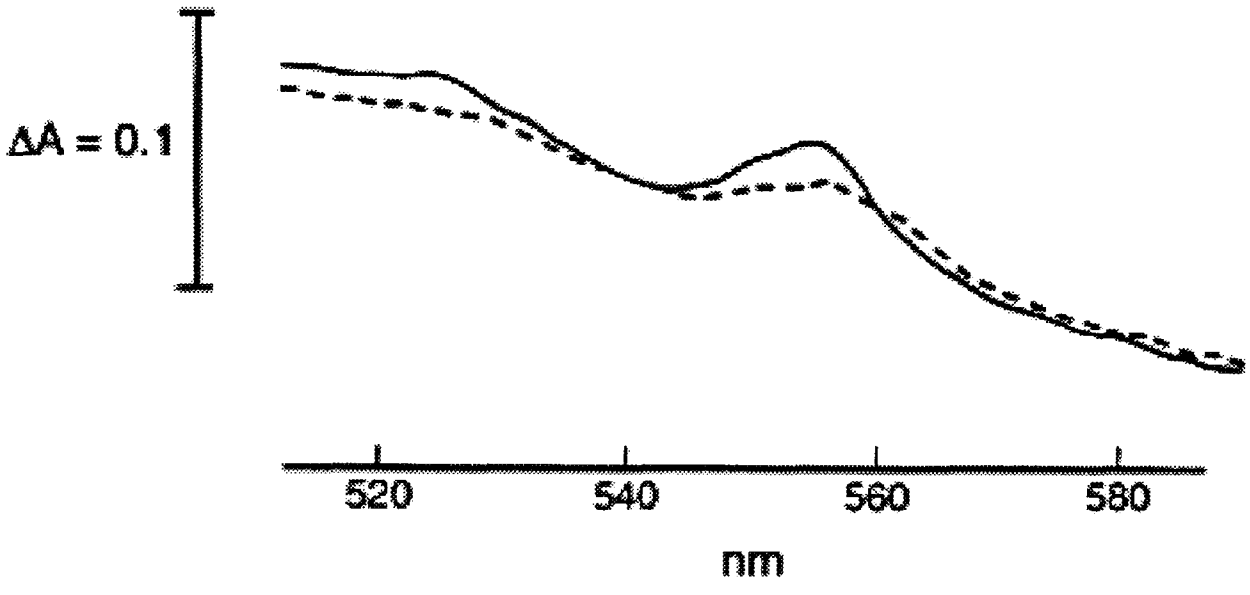


Figure 5.4. Spectra of reduced cytochrome c_{552} in crude extracts of *E. coli* strain JCB387 expressing cytochrome c_{552} from the construct pKHC12 either without (----), or with (—), 0.1 mM δ-aminolevulinic acid added to the growth medium. The crude extracts were prepared as described in Materials and Methods, Chapter II. 0.8 mg ml^{-1} of total cell protein was used for the analysis. The spectra were taken against a buffer reference.

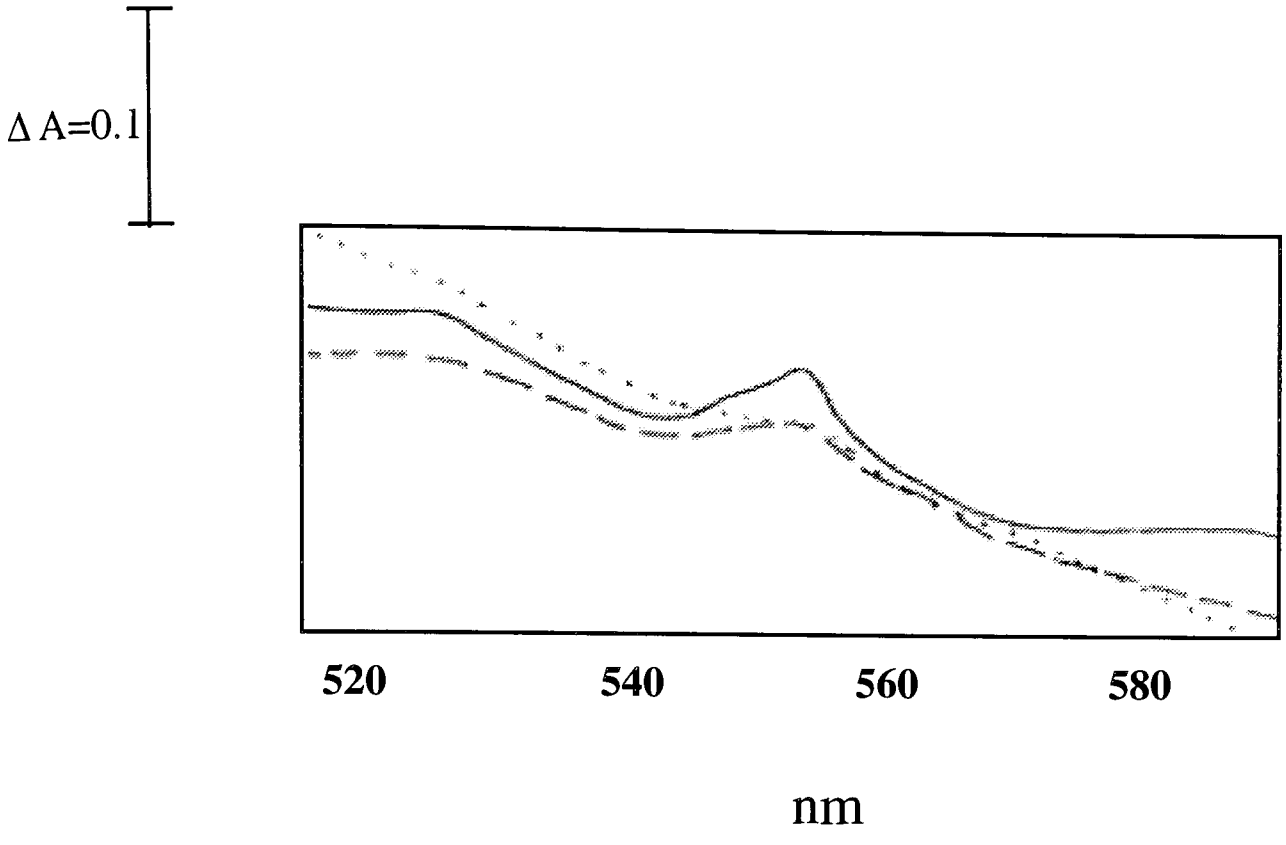


Figure 5.5. The effect of different concentrations of δ-aminolevulinic acid in the growth medium on the expression of cytochrome c_{552}.The figure shows dithionite reduced spectra of cytochrome c_{552} from the strain JCB387 expressing cytochrome c_{552} from the construct pKHC12 under 0.02 mM (....), 0.1 mM (——) and 0.5 mM (----) concentrations of δ-aminolevulinic acid. 0.8 mg ml^{-1} total cell protein was used for the analysis. The crude extracts were prepared as described in Materials and Methods. Spectra were taken against buffer reference.

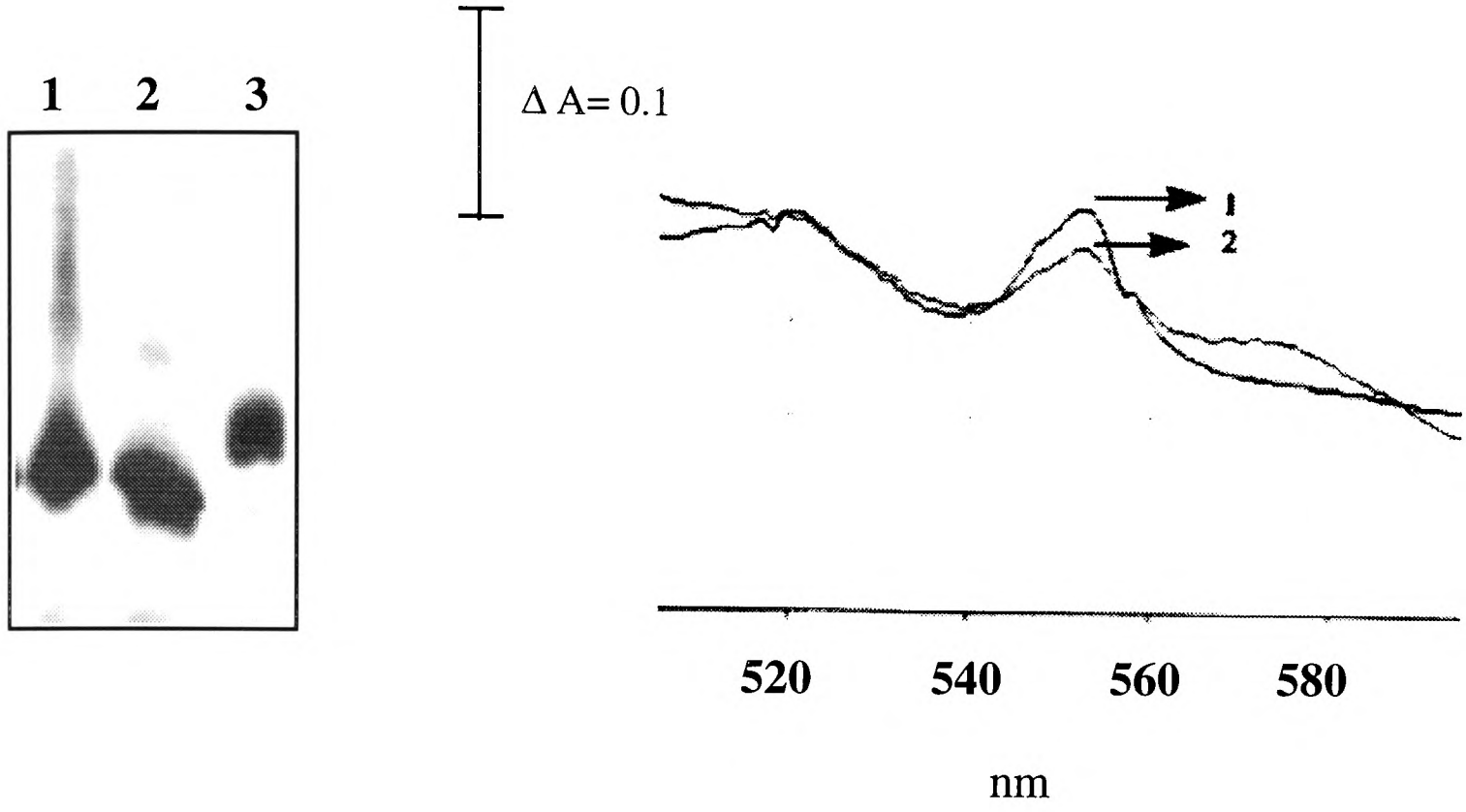


Figure.5.6. The effect of δ-aminolevulinic acid on the expression of cytochrome c_{552} in the strain JCB712.

(a) SDS-PAGE haem stained gel of the crude extract of the strain JCB712 expressing cytochrome c_{552} from the construct pKHC12. The crude extract was prepared as described in Materials and Methods. Approximately 50 μg of total cell protein was used in each lane. The gel was loaded as follows:
Lane 1: Without the supplementation of δ-aminolevulinic acid.
Lane 2: With the supplementation of 0.1 mM δ-aminolevulinic acid.
Lane 3: 2μg of purified horse heart cytochrome *c*, supplied by Sigma.
(b) Dithionite reduced spectrum of cytochrome c_{552} from the strain JCB712 expressing cytochrome c_{552} from the construct pKHC12. 0.5 mg ml^{-1} of total cell protein was used for the analysis. The spectra were taken against the buffer reference.
1) Without the supplementation of δ-aminolevulinic acid.
2) With the supplementation of 0.1 mM δ-aminolevulinic acid in the growth medium.

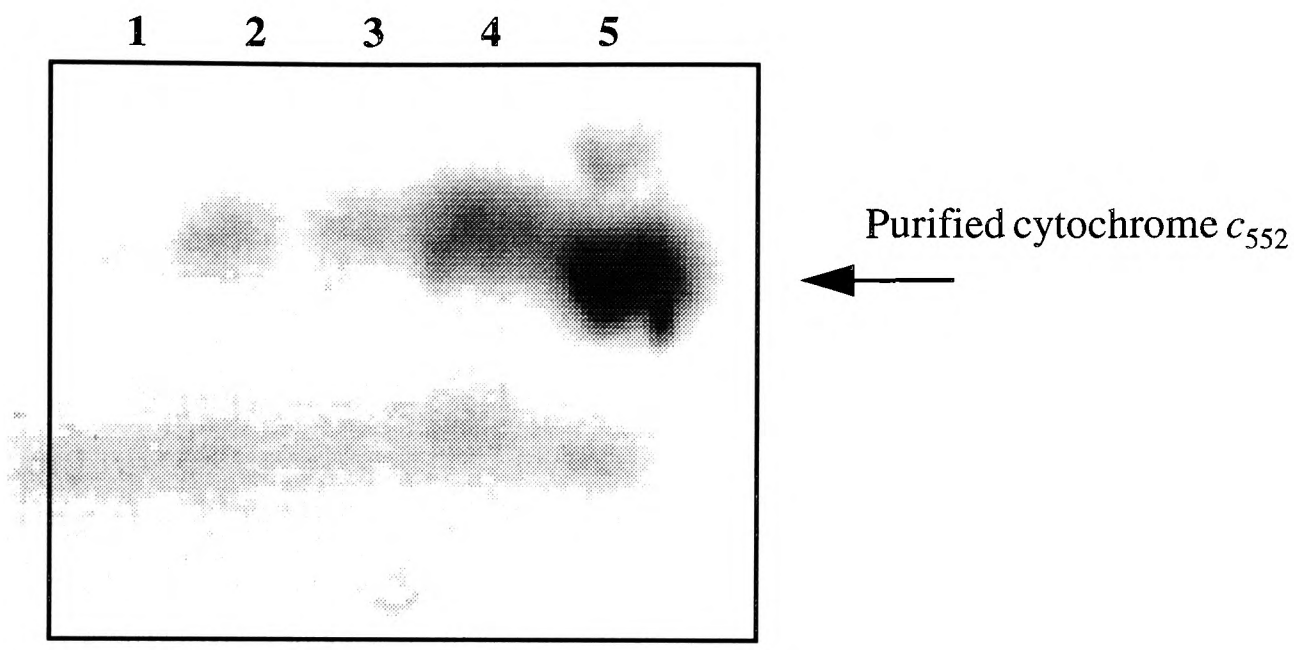


Figure 5.7 SDS-PAGE haem stained gel showing the effect of δ-aminolevulinic acid on the expression of cytochrome $c_{552.}$ in the strain JCB387 from from constructs pKHC12ΔSig and pKHC12K, respectively .The normalized crude extracts were prepared as described in Materials and Methods. The same amount of total cell protein, approximately 40 μg, was loaded in each lane so as the comparison in expression could be made. The gel was loaded as follows:

Lane 1: Cytochrome c_{552} from the construct pKPHC12K

Lane 2: Same as Lane 1 except that the growth medium was supplemented with 0.1 mM δ-aminolevulinic acid.

Lane 3: Cytochrome c_{552} from the construct pKHC12ΔSig.

Lane 4: Same as Lane 3 except that the growth medium was supplemented with 0.1 mM of δ-aminolevulinic acid.

Lane 5: 8μg purified cytochrome c_{552} (expressed from pKHC12 in *E. coli* JCB387).

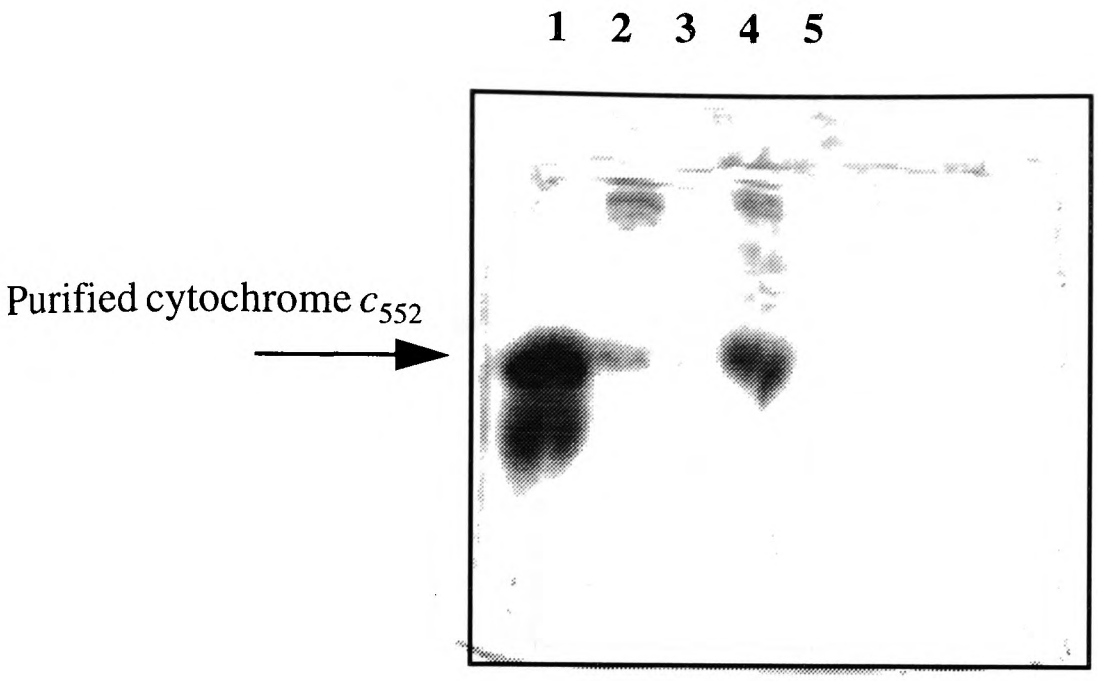


Figure 5.8. SDS-PAGE haem stained gel to showing the effect of δ–aminolevulinic acid on the expression of cytochrome c_{552} and the site of its expression in the strains JCB387 and JCB712. Normalized crude extracts were prepared as described in Materials and Methods. The same amount of total cell protein, approximately 30 μg, was loaded in each lane.SDS-PAGE gel analysis and the haem staining were performed as described in Materials and Methods, Chapter II.

Lane 1: 4μg of purified cytochrome c_{552}, from *E. coli* JCB387 containing pKHC12.

Lane 2: The cytoplasm of the JCB387 expressing cytochrome c_{552} from the construct pKHC12ΔSig when the growth medium was supplemented with 0.1mM of δ-aminolevulinic acid.

Lane 3: The periplasm of the same sample of Lane 2.

Lane 4: The cytoplasm of JCB712 expressing cytochrome c_{552} from the construct pKHC12ΔSig.

Lane 5: The periplasm of the same sample of Lane 4.

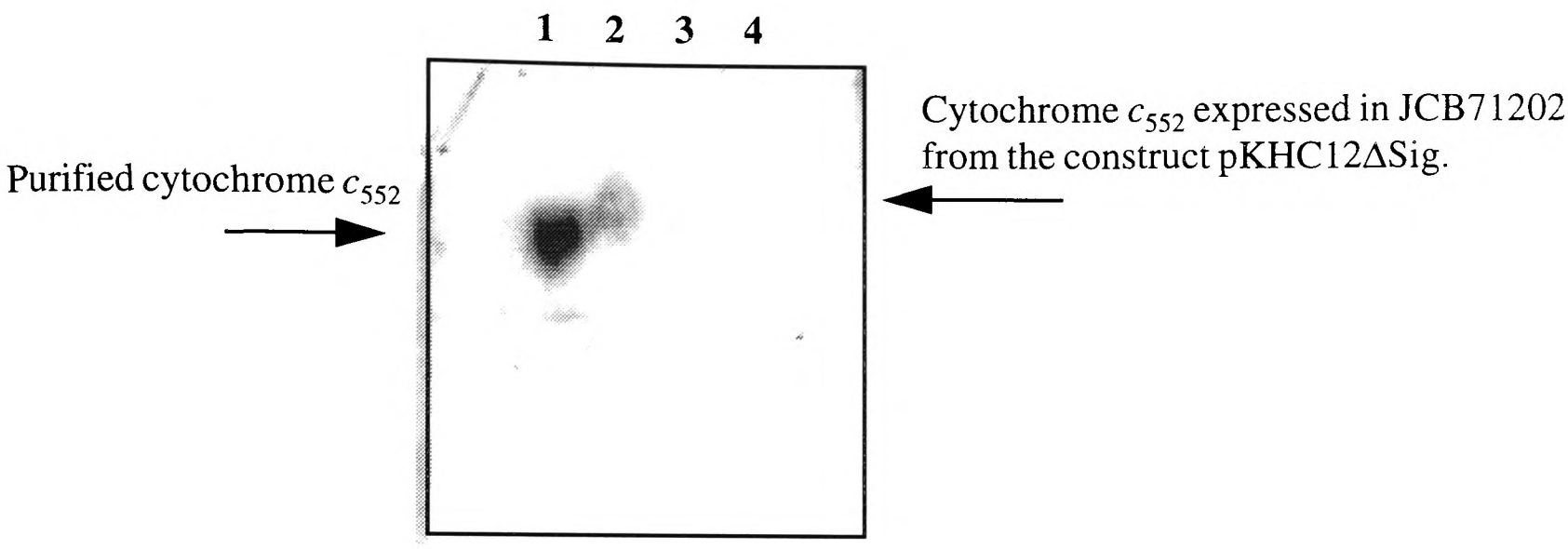


Figure. 5.9. An SDS-Page haem stained gel showing the expression of cytochrome c_{552} in the strain JCB71202 from the construct pKHC12ΔSig . The cells were grown aerobically and the normalized cytoplasmic and periplasmic extracts were prepared as described in Materials and Methods. SDS-PAGE gel analysis and haem staining were performed as described in Materials and Methods, Chapter II.

Lane 1: 4µg of purified cytochrome c_{552}, from JCB387 expressing cytochrome c_{552} from the construct pKHC12.

Lane 2: Cytoplasmic extract.

Lane 3: Empty.

Lane 4: Periplasmic extract.

pKPHC12K in *E. coli* JCB387. The findings in the present work suggested that the failure may have been connected with the relatively poor supply of haem in *E. coli* JCB387. Indeed evidence for expression from pKPHC12K could be obtained if the growth medium contained δ-aminolevulinic acid (Figure 5.7). The expression was nevertheless at a lower level then that from pKHC12ΔSig with the same amount of δ-aminolevulinic acid present (Figure 5.7). The cytochrome c_{552} produced from pKPHC12K had a similar molecular weight to that produced from pKHC12ΔSig. This is the expected result if the product from pKHC12K was translocated to the periplasm and the 20 amino acid targeting sequence cleaved off. However, attempts to establish that this material was in the periplasm were inconclusive. Therefore, the possibility remains that this cytochrome c_{552} was in the cytoplasm and that by some unknown mechanism the target sequence had been cleaved off.

It is important to mention that in all the studies done on cytochrome c_{552} in *E. coli* so far, the site of expression of this cytochrome c_{552} was always found to be cytoplasmic (see Figure 5.8 and Figure 5.9). Thus all the evidence suggests that the JCB712 strains are good producers of haem and the presence of haem might be the only requirement for the maturation of this thermophilic cytochrome c_{552}.

5.2.4 Studies on *E. coli* mutant in *hemA*, encoding for glutamyl tRNA reductase

The increase in the expression of cytochrome c_{552} on supplementation of δ-aminolevulinic acid in the growth medium of JCB387 prompted further study of the effect of δ-aminolevulinic acid on the expression of cytochrome c_{552}. An *E. coli* mutant in

hemA, encoding glutamyl tRNA reductase which catalyses the synthesis of δ-aminolevulinic acid, was used for such a study. The effect of haem concentration on the cytoplasmic expression of cytochrome c_{552} could be observed in this strain after supplementing the growth medium with a gradual increase in the concentration of δ-aminolevulinic acid. An increase in the concentration of δ-aminolevulinic acid in the growth medium increases the expression of cytochrome c_{552}. The expression of cytochrome c_{552} from the constructs pKHC12ΔSig or pKPHC12K was not observed in the strain JCB387 until the growth medium was supplemented with 0.1 mM δ-aminolevulinic acid (see the previous section). A far higher concentration, 1.6 mM, of δ-aminolevulinic acid was needed to detect forms of cytochrome c_{552} (Figure 5.10) in the *hemA* mutant. At 0.1 mM δ-aminolevulinic concentration no haem stained material could be seen. This is reasonable because the *hemA* mutant itself lacks the synthesis of haem and totally depends on the external supply of haem, while a strain such as JCB387 will also have the endogenous resources of haem. It was also found with this *hemA* mutant that expression of cytochrome c_{552} from the construct pKPHC12K was less then the expression from the construct pKHC12, under similar growth conditions (Figure 5.10). However, the saturation level of δ-aminolevulinic acid was not studied for either construct.

5.2.5. NMR analysis of cytochrome c_{552} shows a single mode of attachment of the haem to the cytochrome c_{552} polypeptide

It was unexpected that the JCB712 strain of *E. coli* produced substantial amounts of cytochrome c_{552}. It had wrongly been assumed during much of the current

work that the tendency of this strain to produce more endogenous *c*-type cytochromes than other strains was related to the higher expression of the *ccm* genes that are needed for *c*-type cytochrome biogenesis (see earlier). However, the belated realisation that *E. coli* JCB712 and JCB71202 produced relatively large amounts of cytochrome c_{552} finally proved a route for acquiring sufficient amounts of this protein to permit NMR studies. These were expected to show whether the haem attachment was normal for *c*-type cytochromes. Figure 5.11 shows part of ^{1}H TOCSY spectrum of cytochrome c_{552}. Two cross peaks, 6.13/2.32 and 6.09/1.91, could be observed that are characteristic of the $SCHCH_3$ unit of a *c*-type cytochrome. 6.13 ppm and 6.07 ppm correspond to the methine protons; 2.32 ppm and 1.91 ppm to the methyl group protons. Importantly, there is no evidence anywhere in the spectrum for a vinyl group of haem that would arise from either non-covalently bound haem or perhaps haem covalently bound through only one cysteine residue. Cross peaks from vinyl protons would be expected at 8.5/6.0, 8.5/5 and 6/5 (and the inverses) in Figure 5.11 (Barker *et al.,* 1995). Thus the NMR data provide strong support for there being only one mode, in terms of both chemistry and chirality, of attachment of the haem to the polypeptide in cytochrome c_{552}. A tentative assignment is that the 6.13/2.32 peak originates from the 4 position (Figure 5.12) and the 6.09/1.91 pair from the 2 position. This is deduced from a NOESY spectrum shown in Figure 5.13, which includes counterparts of the 6.13/2.32 and 6.09/1.91 peaks seen in the Figure 5.11. Much of these spectra can be assigned by analogy with other *c*-type cytochromes. Once this assignment has been achieved it will possible to establish the stereochemistry at the chiral centres including the $SCHCH_3$ methine carbon centre. The data collected so far, and the assignments, suggests that stereochemistry normally observed in *c*-type cytochromes is present. Figure 5.14 is a different region of the NOESY spectrum. This

Cytochrome c_{552} expressed in *E. coli hemA*$^-$ from the construct pKPHC12K, with extra residues at its *N*-terminus.

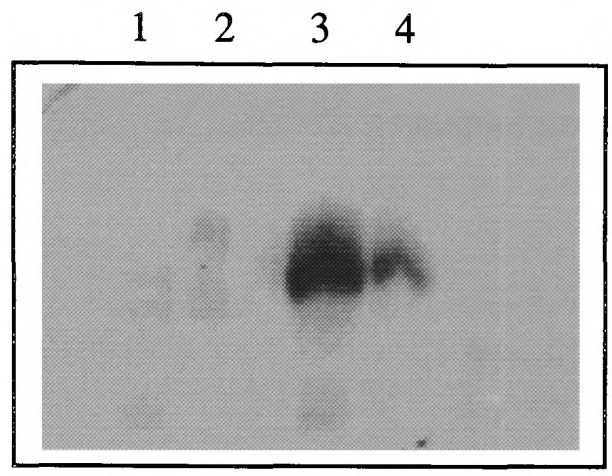


Figure 5.10. SDS-PAGE haem stained gel showing the effect of δ-aminolevulinic acid on the expression of cytochrome c_{552} from the construct pKHC12 and pKPHC12K *E. coli hemA* mutant. The normalized crude extracts were prepared as described in Materials and Methods. The same amount of total cell protein, approximately 40 μg, was loaded in lanes 2 and 3. Approximately 4μg was loaded in lane 4. SDS-PAGE gel analysis and haem staining were performed as described in Materials and Methods, Chapter II. The gel was loaded as follows:

Lane 1: Control: crude extract of *E. coli hemA* with the supplementation of 0.8 mM δ–aminolevulinic acid.
Lane 2: Expression from the construct pKPHC12K with the supplementation of 1.6 mM δ-aminolevulinic acid.
Lane 3: Expression from the construct pKHC12 with 1.6 mM δ-aminolevulinic acid.
Lane 4: Lower loading than Lane 3.

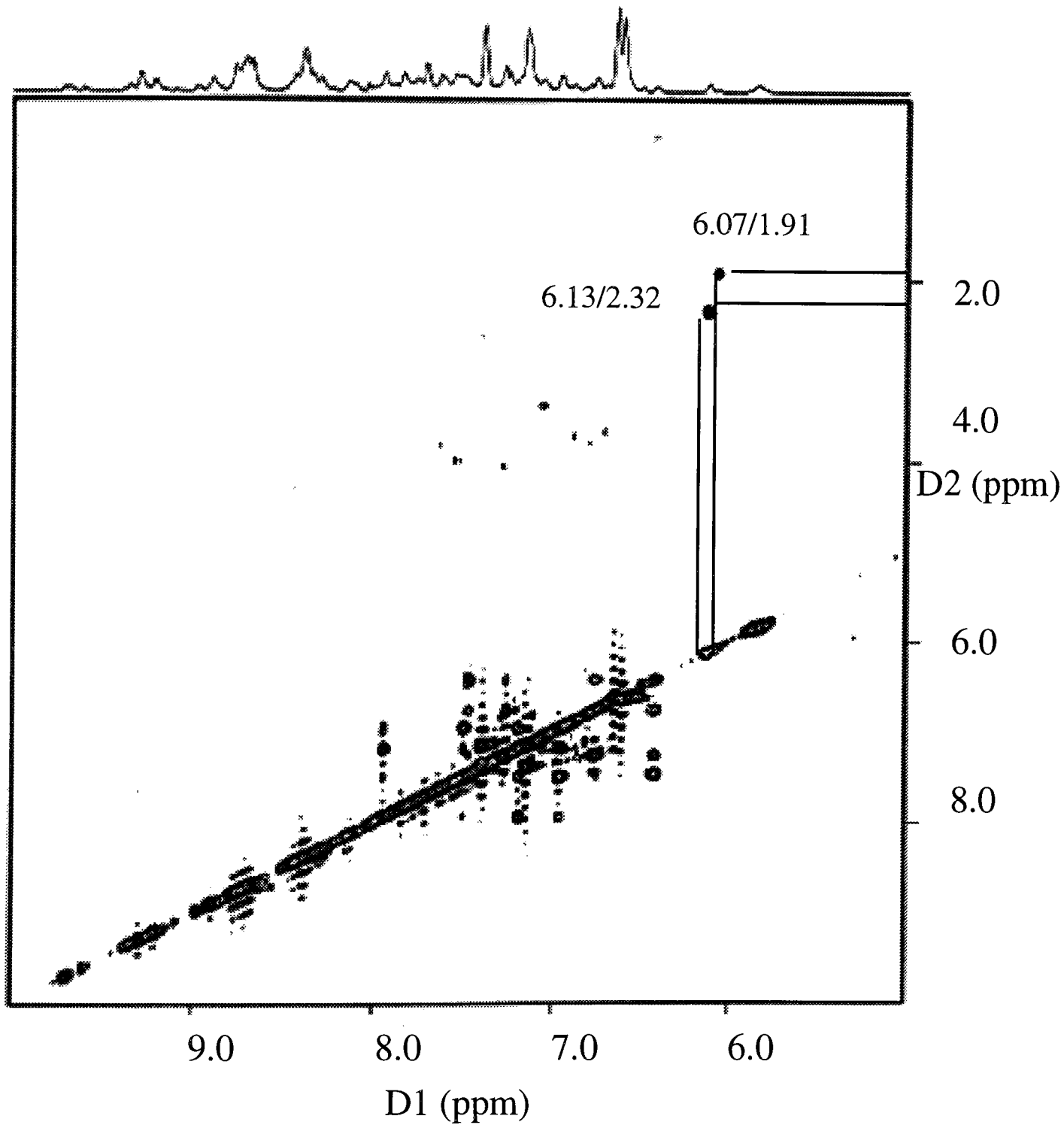


Figure 5.11. ^{1}H TOCSY spectrum of recombinant *H. thermophilus* ferrocytochrome c_{552}. The protein concentration was 3 mM dissolved in 10 mM potassium phosphate buffer (pH 7.5). The protein was reduced under argon with 5 mM dithionite. The NMR methods are as described in Barker *et al.*, (1995). (Spectrum by courtesy of Dr. P. Barker, Cambridge Centre for protein Engineering).

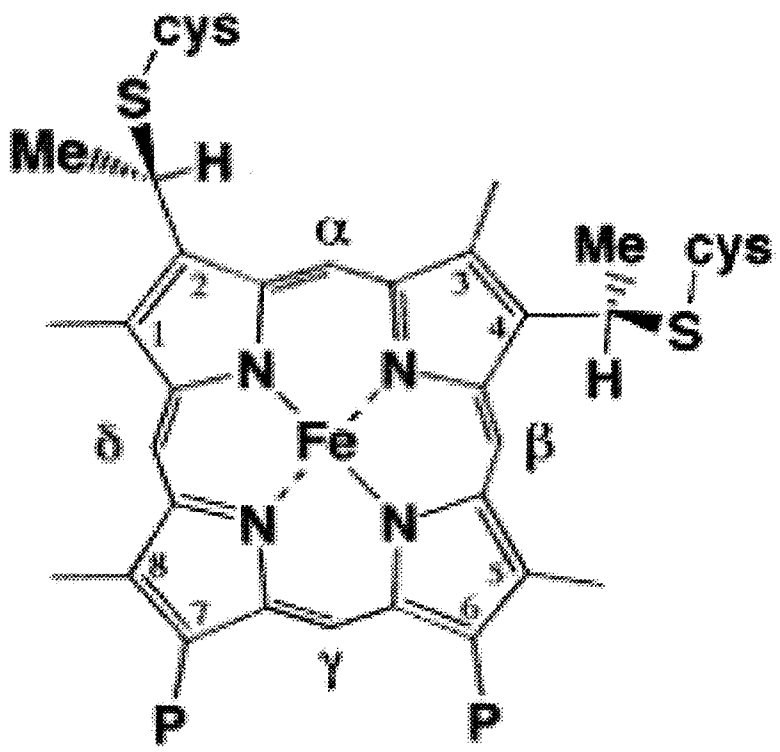


Figure 5.12 Haem *c* showing labelling of carbon atoms and expected stereochemistry of the thioether linkages.

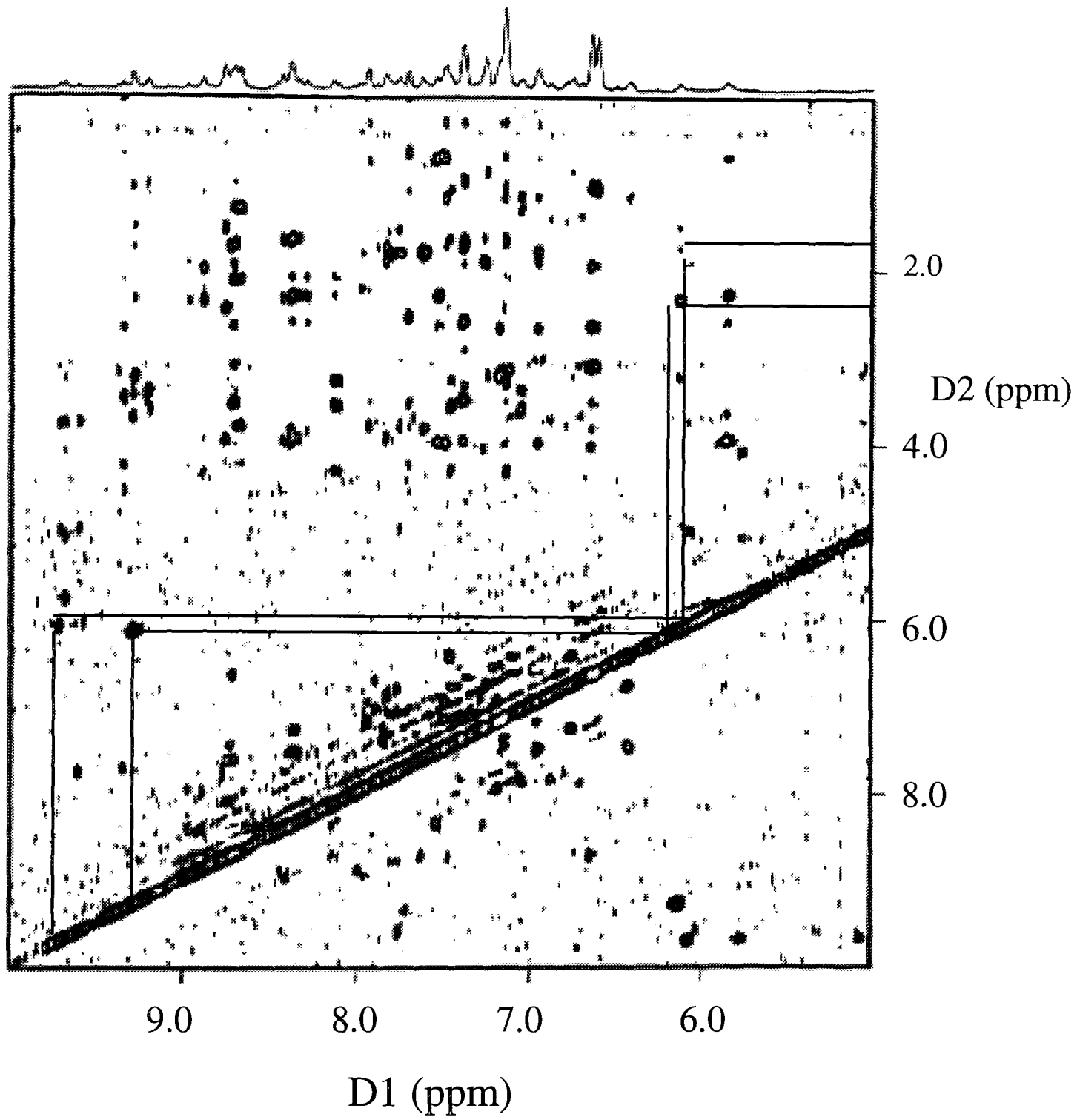


Figure 5.13 ^{1}H NOESY spectrum in the upfield region of the recombinant cytochrome c_{552}. Conditions as for Figure 5.11 except that the different pulse sequences were used to generate the spectrum. The 6.09/1.91 ppm crosspeak is very weak. This is attributed to a baseline problem (Dr. P. Barker, personal communication).
(Spectrum by courtesy of Dr. P. Barker, Cambridge Centre for Protein Engineering)

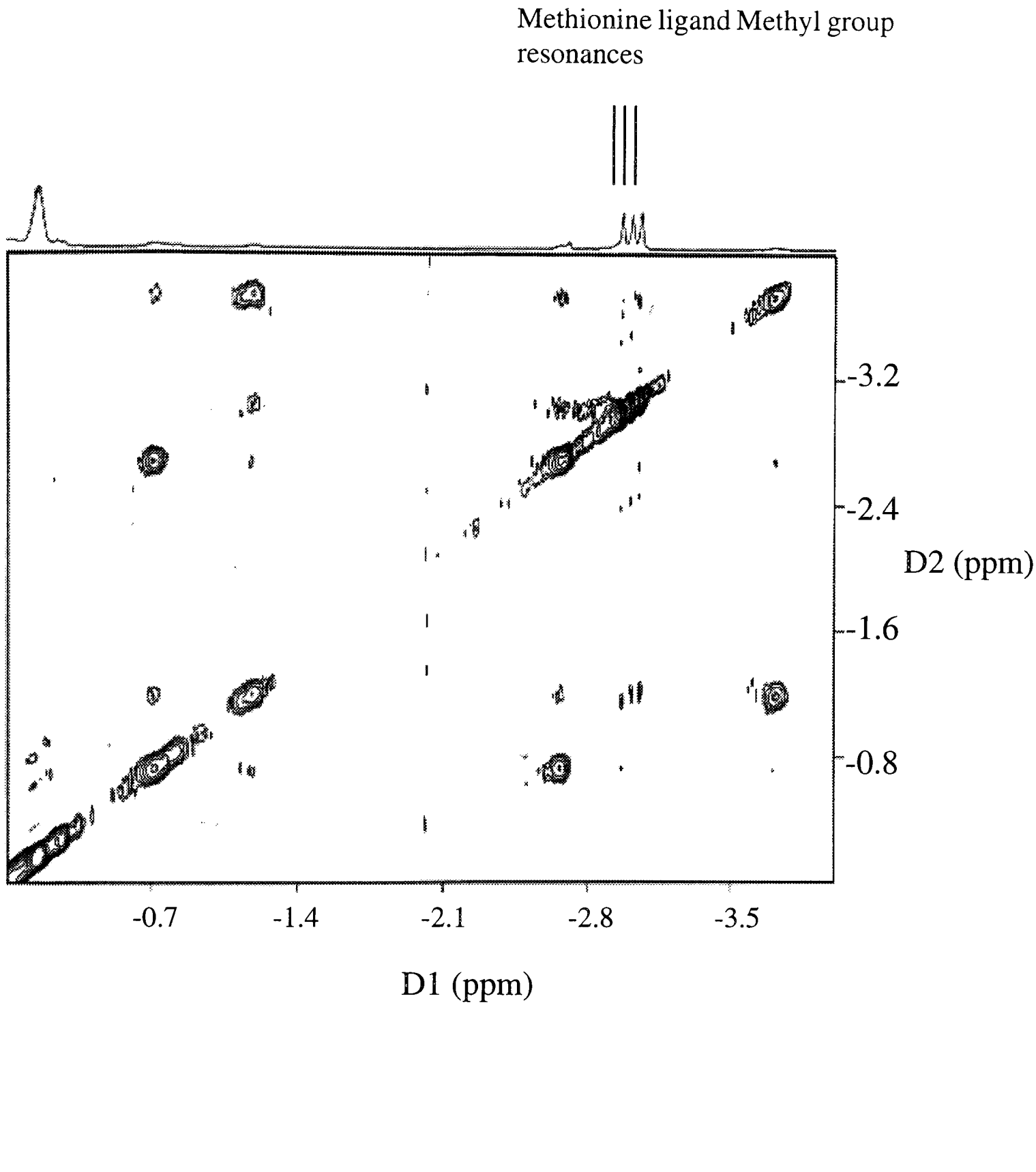


Figure 5.14 ^{1}H NOESY spectrum in the upfield region of recombinant ferrocytochrome c_{552}, conditions as for Figure 5.11. (Spectrum by courtesy of Dr. P. Barker, Cambridge Centre for Protein Engineering)

shows the unexpected feature of a heterogeneous spectrum from the axial methionine ligand to the haem iron. The signals at circa -3 ppm suggests that there are three approximately equally occupied populations of axial methionine in the protein. Normally, for a *c*-type cytochrome one would expect one peak in this region.

5.3 Discussion

The present study shows some of the unique features of the biogenesis of *H. thermophilus* c_{552}. Its maturation in *E. coli* strain deleted in the *ccm* genes (cytochrome *c* maturation) strengthens the previous hypothesis that the covalent linkage between apocytochrome c_{552} and haem is independent of enzymatic assistance (Sanbongi *et al.*, 1994). The observations that δ-aminolevulinic acid increases the expression of cytochrome c_{552} from the strain JCB387 but that there is no effect of δ-aminolevulinic acid on the expression of cytochrome c_{552} from the strain JCB712, lead one to assume that *E. coli* JCB712, and strains derived from it are, at least to some extent, producing more haem relative to the other strains e.g. JCB387. The observation of the synthesis of holocytochrome c_{552} in the absence of *ccm* genes, and its dependence on a haem precursor in *E. coli* JCB387, together imply that the only identified factor contributing to the formation of this cytochrome is availability of haem. This may be because cytochrome c_{552} is highly thermostable (Sanbongi *et al.*, 1989) with the consequence that its apocytochrome has some tertiary structure, including a binding pocket into which haem inserts. The covalent attachment between apocytochrome and haem would then take place spontaneously. Furthermore, the insertion of haem may enhance the folding of apocytochrome c_{552} and thus can retard the degradation of apocytochrome c_{552}. This proposal can explain why the expression of holocytochrome c_{552} from the construct

pKHC12ΔSig and pKPHC12K in the strain JCB387 was not readily detectable or was not possible, respectively, unless the growth medium was supplemented with δ-aminolevulinic acid. The expression of cytochrome c_{552}, however, from the construct pKHC12ΔSig in JCB712 was readily detected without the supplement of δ-aminolevulinic acid, while expression from the construct pKHC12 in the strain JCB712 does not show any effect of δ-aminolevulinic acid on the expression of cytochrome c_{552}; infact it rather shows a slight inhibitory effect. Thus the results so far suggest that the JCB712 strains are over producing haem. Furthermore the expression of cytochrome c_{552} from the construct pKPHC12K was lesser then the expression from the construct pKHC12ΔSig under the same concentration of δ-aminolevulinic acid. The extra 10 *N*-terminal amino acid residues in cytochrome c_{552} expressed from pKHC12ΔSig might retard folding of apocytochrome c_{552} and thus the availability of haem may be crucial for displacing an equilibrium to state with tertiary structure and so more haem will be required for this equilibration to express cytochrome c_{552} from the construct pKHC12K, which contains cytochrome c_{550} signal sequence along with the 10 *N*-terminal amino acid residues of cytochrome c_{550}. The observations with the *hemA* mutant background confirms the positive role of haem on the expression of cytochrome c_{552}. The requirement of minimum 1.6 mM δ-aminolevulinic acid for expressing cytochrome c_{552} from the construct pKPHC12K while the same δ-aminolevulinate gives much higher expression of cytochrome c_{552} from the construct pKHC12 could be explained that apopolypeptide c_{552} from pKPHC12K contains extra amino acid residues on its *N*-terminal then more haem would be needed to equilibrate with the inhibitory effect of extra *N*-terminal residues.

This strengthens the view that haem forces the apocytochrome c_{552} to form a tertiary structure.

It may be that in the case of normal periplasmic cytochrome *c* assembly much of the biogenesis machinery is involved in holding the apocytochrome *c* and haem in the appropriate conformation, rather than in catalysing the chemical reaction of thiol addition to vinyl groups of haem. Analysis of the recently released genome of *Helicobacter pylori* (Tomb *et al.*, 1997), which has both membrane bound and periplasmic cytochromes *c*, shows that it lacks nearly all the homologous genes for *c*-type cytochromes biogenesis identified in other studied Gram-negative organisms (Page *et al.*, 1997). It has been suggested that since proteins responsible for disulphide bond formation are absent from this bacterium the problem of inevitable disulphide bond formation once an apocytochrome *c* carrying cysteines enters the periplasm is avoided (Page *et al.*, 1997). Thus much of the *c*-type cytochrome biogenesis machinery, present in the periplasm, for reduction of disulphides is dispensable in *H. pylori*. This hypothesis can be compared with the maturation of cytochrome c_{552} into the cytoplasm of *E. coli*. Thermostable apocytochrome c_{552}, having some tertiary structure, in the reducing environment of cytoplasm, does not need any other enzymatic assistance for the covalent attachment. The same may be true in the non-oxidising environment of the *H. pylori* periplasm. *In vitro* studies will eventually demonstrate for the exact requirements for the maturation of this cytochrome c_{552}.

The NMR studies reported in this Chapter indicate that there is a single chemistry and chirality of the attachment of the haem to the polypeptide in cytochrome c_{552}. In some respects this is perhaps surprising if the attachment is not catalysed. The simplest interpretation is that in the *E. coli* cytoplasm the haem has interacted with an asymmetric binding site provided by the apoprotein. However, an alternative view (P. Barker personal

communication) is that an enzyme activity in the *E. coli* cytoplasm is able as a side reaction to catalyse the haem attachment. It remains important to confirm rigorously that the stereochemistry of haem attachment in recombinant cytochrome c_{552} is the same as in the protein from *H. thermophilus* itself. This should soon be possible as Y. Sambongi and colleagues (personal communication) have recently solved the NMR solution structure of the native protein. Their data, when available, should allow rapid assignment of the spectra in Figure 5.11 and 5.13.

Chapter VI

Probing cytochrome *c* biogenesis by studying assembly of *E. coli* cytochrome b_{562} and a cytochrome c_{552} variant with only one cysteine residue.

6.1 Introduction

General properties of *E. coli* cytochrome b_{562} and the possible importance of its use as a reporter molecule in the studies of *c*-type cytochrome biogenesis in *E. coli* have been discussed in the introductory Chapter I. It is a rare example of a protein with non-covalently bound haem in the periplasm. As the gene sequence for cytochrome b_{562} shows the presence of a typical periplasmic targeting sequence it is probable that the protein is exported to the periplasm in an unfolded state without the haem bound. Therefore, there must be a haem delivery system to the periplasm, unless passive diffusion through the bilayer is sufficient, and the question arises as to whether the putative haem transporter, coded by the *ccmA*, *ccmB* and *ccmC* genes, might be responsible for supplying haem to the cytochrome b_{562}. This possibility could be tested through use of the *E. coli* JCB71202 strain which is deleted in *ccm* genes.

Barker *et al.*, (1996) have shown that cytochrome b_{562} can be converted to a *c*-type cytochrome by introducing one or two cysteine residues into the amino acid sequence of cytochrome b_{562} in positions related to those found in a *c*-type cytochrome, specifically cytochrome *c′*, generating the ubiquitous haem binding motif C-X-X-C-H or a variant with just one cysteine. If the maturation of *b*-type cytochrome, or at least cytochrome b_{562}, in *E. coli* follows a spontaneous pathway, as proposed for the maturation of *H. thermophilus* cytochrome c_{552}, then it is possible that changing the corresponding cysteine to some other residue in the *H. thermophilus* cytochrome c_{552} C-X-X-C-H motif will result into a species related to the native protein, though lacking one point of covalent attachment and thus representing a step towards a *b*-type cytochrome. It has been shown that mutated human cytochrome *c*, in which the first cysteine of the conserved motif was changed to an alanine residue, was functionally expressed in yeast and that therefore in

this case at least cysteine 14, the first cysteine of the motif Cys-X-X-Cys, is not essential for full human cytochrome *c* function (Tanaka *et al.,* 1990).

Also, natural mitochondrial variants of cytochrome *c* are known in which the AXXCH motif replaces the normal CXXCH (Chapter I), but there is as yet no report of a similar species from a bacterial source, either naturally or as a result of expressing a protein with the AXXCH motif introduced by mutation (Sanbongi *et al.,* 1996). It has been suggested that this failure reflects a requirement of both cysteines in the CXXCH motif to participate in disulphide bonding during the biogenesis process (Sanbongi *et al.,* 1996). If cytochrome c_{552} is assembled spontaneously in the *E. coli* cytoplasm then it might be that covalent attachment of haem to an AXXCH motif can occur. To test this hypothesis an attempt was made, again using a T7 expression system in conjunction with an S•Tag, to express a mutant form of cytochrome c_{552} with an alanine replacing the first cysteine of the CXXCH motif.

6.2 Results

The constructs used in the present study are listed in Table 6.1.

6.2.1 One Step purification of cytochrome b_{562}

In order to use cytochrome b_{562} as a marker in SDS-PAGE analysis and to confirm its expression in some of the experiments, the protein was partially purified by ion-exchange chromatography. The single step purification of *E. coli* cytochrome b_{562} was performed as follows. The cytochrome b_{562} was purified from the strain JCB387 containing the constructs b_{562}W.T. and b_{562} ΔSig, respectively. The cells were grown in 2ml of LB overnight and inoculated in 500 ml of LB in a 250 ml conical flask. The

cultures were grown till the OD had reached 1.5 at 580 nm. The pink cells were harvested and resuspended in 10 ml of GTE. The cell walls were broken by freezing and thawing and the plasma membrane was removed by sonicating followed by centrifugation, as described in Materials and Methods. The nucleic acids were also removed by treating the suspension with the spectinomycin followed by centrifugation, as described in Materials and Methods. This suspension was then loaded onto a anion exchange column, DEAE-cellulose. The elution of the protein followed by dialysis was performed as described for the purification of *H. thermophilus* cytochrome c_{552} from the strain JCB387 containing pKHC12 (Chapter III). Figure 6.1 shows the spectrum of purified cytochrome b_{562}.

6.2.2 Cytochrome b_{562} was matured in the cytoplasm and the periplasm of an *E. coli* strain deleted in *ccm* (cytochrome *c* maturation) genes, using the constructs $b_{562}\Delta$Sig and b_{562} W.T. respectively

In order to determine whether the *ccm* gene products play any role in the synthesis of cytochrome b_{562}, its expression was studied in the strain JCB71202 which is deleted in all the cytochrome *c* maturation genes and shown to lack the synthesis of *c*-type cytochromes (Grove *et al.,* 1996b). Cytochrome b_{562} was successfully made in this strain. Figure 6.2 shows the red cells of JCB71202 transformed with either the construct b_{562} W. T or $b_{562}\Delta$Sig (Table 6.1). The latter construct should result in absence of cytochrome b_{562} from the periplasm. Lanes 2 and 4 in Figure 6.3 are consistent with this expectation, although nothing can be deduced about expression in the cytoplasm from lanes 3 and 5. Figure 6.4a shows the spectra of cytochrome b_{562}, expressed in the strain JCB71202 without and with the construct b_{562} W.T. Although the expression of cytochrome b_{562} was much higher when expressed from the construct, there is still a detectable amount of

cytochrome b_{562} made in the strain JCB71202 without the construct. Figure 6.4b shows that the expression of cytochrome b_{562} from the construct b_{562}W.T. is much higher in the strain JCB7123 and JCB71202 in comparison to JCB387.

The comparison of expression of periplasmic cytochrome b_{562} in *E. coli* strains JCB7123, which has *ccm* genes, and JCB71202 which does not, therefore establishes that the absence of these genes has no deleterious effect on cytochrome b_{562}. Indeed the absence of these genes seems to increase the expression of this *b*-type cytochrome (Figure 6.4b). The lower expression in *E. coli* JCB387 is comparable to what was observed for expression of cytochrome c_{552} (Chapter V). Cytochrome b_{562} was shown to be in the periplasm of *E. coli* JCB71202 because it co-fractionated with alkaline phophatase activity.

6.2.3 The Holo:Apo cytochrome b_{562} ratio was found to be larger in the strain JCB71202 then in JCB387.

Since earlier results showed that *E. coli* JCB712 and its derived strains produce higher amounts of *c*-type cytochrome (Chapter V) in comparison to other strains e.g. JCB387, and comparable findings were made for cytochrome b_{562}, it was of interest to determine whether the Holo:Apo ratio for cytochrome b_{562} is higher in the strain JCB71202 then in JCB387. It is known that apocytochrome b_{562} can be detected in extracts of *E. coli* cells (P. D. Barker and M. D. Page personal communications). The assay to determine the ratio of Holo:Apo cytochrome b_{562} was performed as follows.

Table 6.1

Constructs	Relevant information and references
Cytochrome b_{562} W.T.	The coding region on *E. coli* cytochrome b_{562} cloned in the vector pBSKS. M. K. Trower(1993)
Cytochrome $b_{562}\Delta$Sig	The coding region of *E. coli* cytochrome b_{562} deleted in the coding region of its signal peptide is cloned in vector pBSKS. M.K. Trower(1993),
pScreenT	PCR fragment cloning vector for the T7 driven expression of the target protein, supplied by Novagen.
pSTNC52	C10A cytochrome c_{552} cloned in pScreenT vector at the T cloning site, Present work.

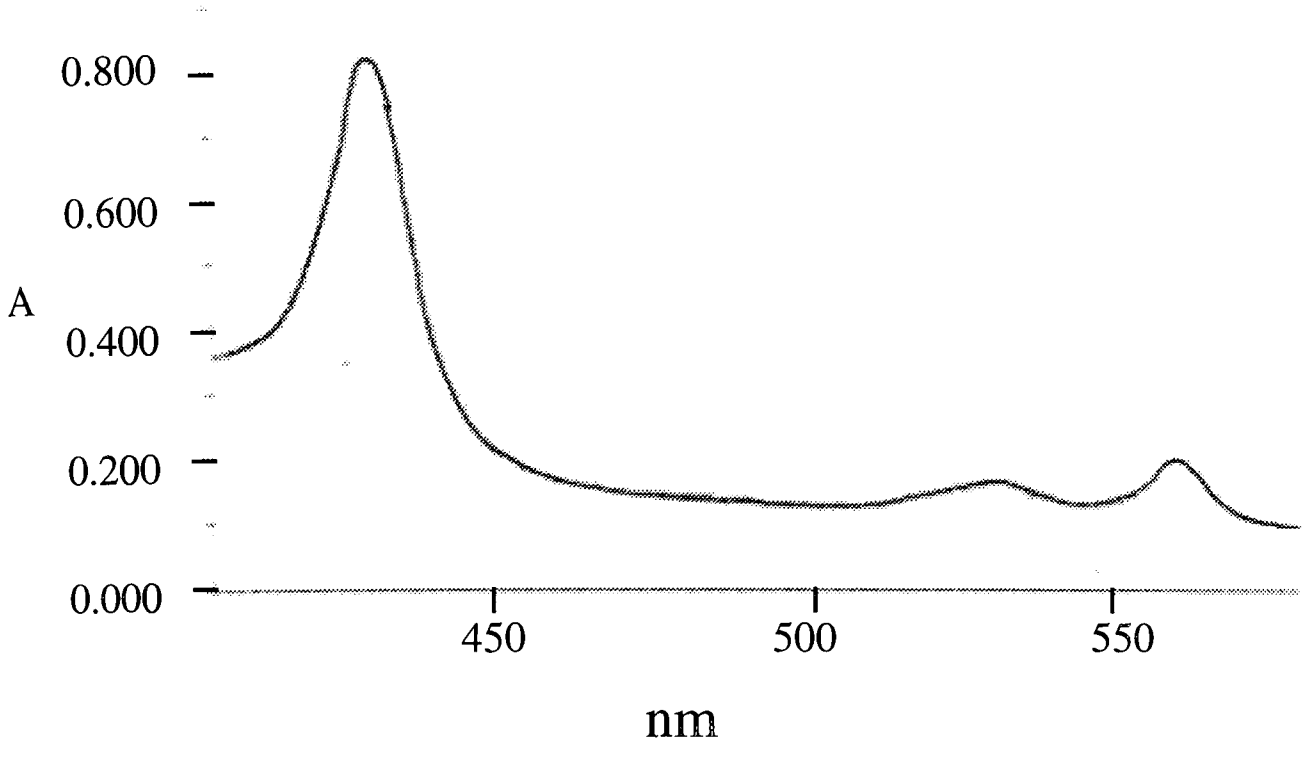


Figure 6.1. The spectrum of dithionite reduced cytochrome b_{562} purified from *E. coli* JCB387, expressing cytochrome c_{562} from the construct b_{562}W.T. 20 μg of purified protein in 10 mM potassium phosphate buffer (pH 7.5) was used. The spectrum was taken against a buffer reference.

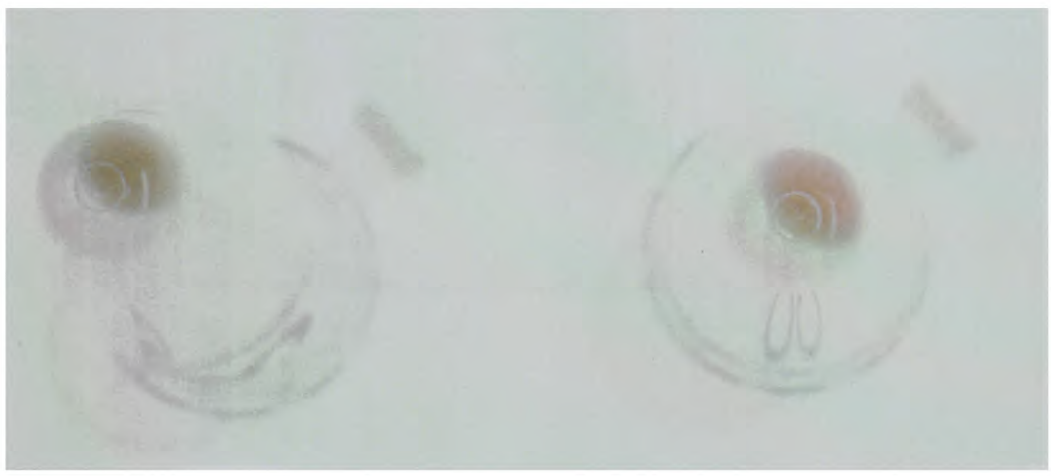


Figure 6.2. The red coloured cells of JCB71202 expressing cytochrome b_{562} from the construct $b_{562}\Delta$Sig (1) and b_{562} W.T. (2). The cells from 1 ml culture of the OD 1.5 at 580 nm were pelleted in 1.5 microfuge tube for the photo. The red cells show the high expression of cytochrome b_{562}.

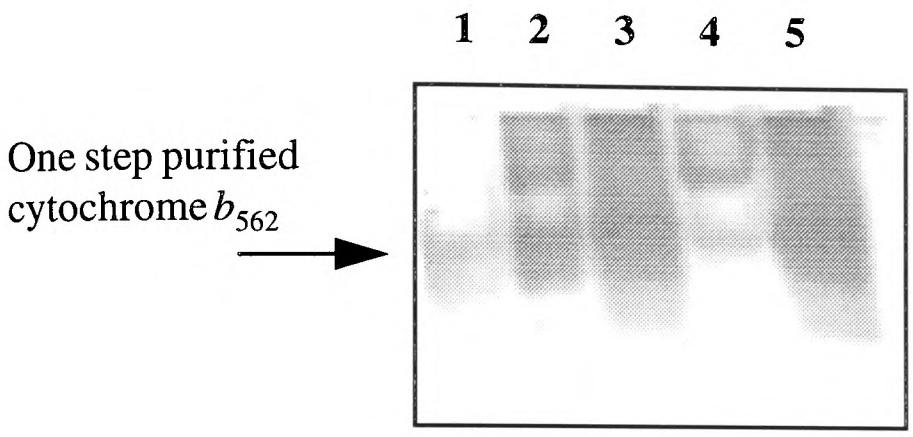
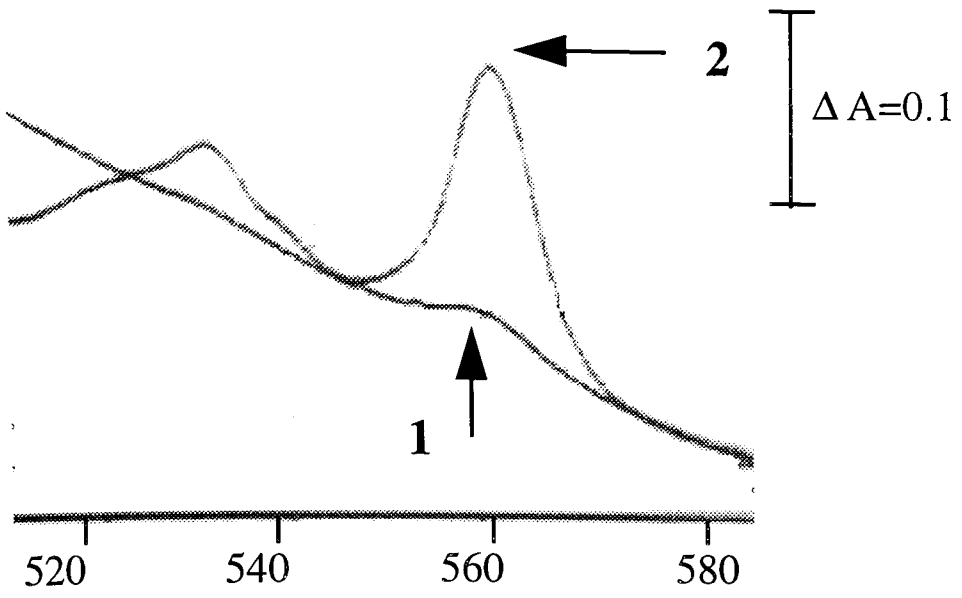
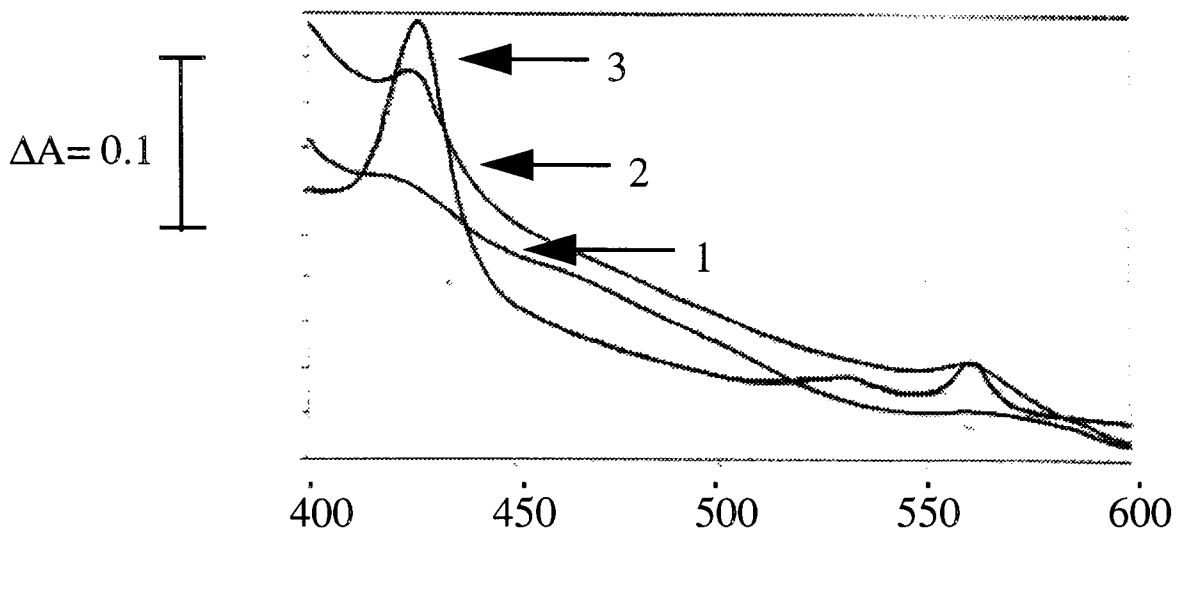


Figure 6.3. Non-denaturing Coomassie stained PAGE gel showing the expression of cytochrome b_{562} in *E. coli* JCB71202, the *ccm* deleted strain, from the constructs b_{562} W.T and $b_{562}\Delta$ Sig. The gel analysis was performed as described in Materials and Methods. The cytoplasm and the periplasm were purified as described in Materials and Methods, Chapter II. Approximately 50µg of total cell protein was loaded in each lane. The gel was loaded as follows:

Lane 1: 2-3µg of one step purified cytochrome b_{562}, as described in Section 6.3.1.
Lane 2: The periplasm of JCB71202 expressing cytochrome b_{562} from the construct b_{562}W.T.
Lane 3: The cytoplasm of the same sample loaded in lane 2.
Lane 4: The periplasm of JCB71202 expressing cytochrome b_{562} from the construct $b_{562}\Delta$Sig.
Lane 5: The cytoplasm of the same sample loaded in lane 4.



(a)



(b)

Figure 6.4. a)Dithionite reduced spectra of the crude extract of JCB71202, expressing cytochrome b_{562} without (1) and with the construct b_{562} W.T. (2). The crude extracts were prepared as described in Materials and Methods, Chapter II. 2.6 mg protein ml^{-1} was used for the analysis after adding sufficient solid sodium dithionite to reduce the samples completely. The spectra were measured against a buffer reference.

b) Dithionite reduced spectra of the crude extract of JCB387 (1), JCB7123 (2) and JCB71202 (3), expressing cytochrome b_{562} from the construct b_{562}W.T. 1.2 mg ml^{-1} of total cell protein was used for the analysis. The spectra were taken against the buffer reference.

6.2.3.1 Cytochrome b_{562}-haem titration; Holo:Apo b_{562} estimation

Cells of JCB387 and JCB71202 containing b_{562}W.T. were grown in 2 ml of LB overnight. Next morning this was inoculated in 25 ml of LB in a 250 ml conical flask and grown until the culture had reached an OD of 1.5 at 580 nm. The harvested cells, pink in colour, were suspended in 200 µl of GTE. The periplasm was purified as described in Materials and Methods. The periplasmic fraction was then used for haem titration as described by Karim *et al.* (1993). The periplasmic fraction was diluted with 10 mM Tris-HCl (pH 8.0) to 1 ml. Haem was directly added to the cuvete. The final concentrations of haem in the cuvete were increased progressively. A 1 mM haem stock was prepared as described in Karim *et al.*, 1993. For comparable amounts of extract, saturation with haem in the strain JCB71202 was achieved at 15 µM haem supplementation, while in JCB387 the saturation of the cytochrome b_{562} was achieved at 25 µM haem supplementation (Figure 6.5, 6.6). The Soret haem peak, 410nm, found in the periplasmic fraction relates to the amount of holo cytochrome b_{562} in the periplasm as isolated, while the peak obtained at the end point of haem titration represents the original holo plus the apo that was converted to the holo by titration with haem. The difference of these two measurements gives the amount of apo protein present in the periplasm before the haem supplementation. From this the ratio of Holo:Apo can be calculated. The Holo:Apo ratio of cytochrome b_{562} in JCB387 was found to be 25:75 while in JCB71202 it was found to be 55:45. (Fig. 6.5, 6.6).

6.2.4 Creating a *H. thermophilus* cytochrome c_{552} mutant containing Alanine instead the position of the first Cysteine in the haem binding motif Cys-X-X-Cys-His

If *H. thermophilus* apo cytochrome c_{552} has sufficient tertiary structure for haem to slot into a binding pocket followed by covalent haem attachment then even without the formation of two covalent bonds between apo cytochrome c_{552}, then a form of the cytochrome with noncovalently bound haem may be formed.

6.2.4.1 Site directed mutagenesis to create Ala-X-X-Cys-His mutant of cytochrome c_{552}; C10A cytochrome c_{552}

The coding region of *H. thermophilus* cytochrome c_{552} was amplified from the *Bam H* I digested fragment of pKHC12 (Materials and Methods, Chapter II), which expresses cytochrome c_{552} without its signal sequence, using specific primers (Figure 6.7). The 5' primer was made containing the coding region for alanine residue at the place of first cysteine residue of the haem binding motif, Cys-X-X-Cys-His. The 250 bp of the coding region of mature C10A cytochrome c_{552} fragment was amplified by 25 cycles of PCR. The PCR reaction was performed as described in Materials and Methods. 25µl from the reaction mixture was checked on 2% agarose gel. Reaction mixtures containing 0.75, 1.0 and 1.25 mM $Mgcl_2$ gave the correct size, 250 bp, PCR product (Figure 6.8). The fragment was purified using a Wizard kit for isolating PCR fragments and cloned into pScreenT vector, resulting into the construct pSTNC52 (Figure 6.7). This was then transformed into HMS174(DE3). The cloning and the transformation were performed as described in Materials and Methods

6.2.4.2 A brief description of the pScreenT vector

The pScreenT vector, supplied by Novagen, is designed for expression, driven by T7 RNA polymerase, of inserts as stable fusion proteins. As with other pET based vectors, the source of the T7 RNA polymerase is provided by the host cells which

contain a chromosomal copy of the gene for T7 RNA polymerase which is under the control of *lacUV5* promoter that is inducible by isopropyl-β-D-thioglactopyranoside (IPTG). The vector has been prepared by digestion with *Eco* R V followed by the addition of single 3' dT residues at each end. Inserts having 3'dA overhangs can be ligated directly into the vector. Many thermostable DNA polymerases that are used for PCR, such as *Taq* or *Tth* polymerase, naturally leave single 3' dA overhangs on their reaction products. These molecules are thus ready to ligate into the pScreenT vector. The T-cloning site in pScreen is positioned such that target sequences are expressed as a fusion containing the first 260 amino acids of the T7 gene *10* protein. The fusion ensures high level expression. When cloning PCR products it is necessary to use a 5' primer having the proper reading frame, such that the first base initiates a codon. The vector also contains His•Tag and S•Tag sequences, present adjacent to the cloning site, that simplify analysis and purification of target proteins.

6.2.4.3 Screening for the correct clones

Colony screening was done by the technique of colony PCR, as described in Materials and Methods, Chapter II. The primers used to generate the mutant were used in the colony PCR to check the insert. An S•Tag primer, a vector specific primer which binds to the coding region of S•Tag sequence (present towards the 5' end of the target sequence) was used as a 5' primer to check the right orientation of the cloned fragment togather with the 3' primer used to generate the mutant. The correct fragment when using the two primers used for creating the mutant should have 250 bp, while when using the S•Tag primer and the 3' primer (used for mutant creation), to check the correct orientation, 343 bp should be present.

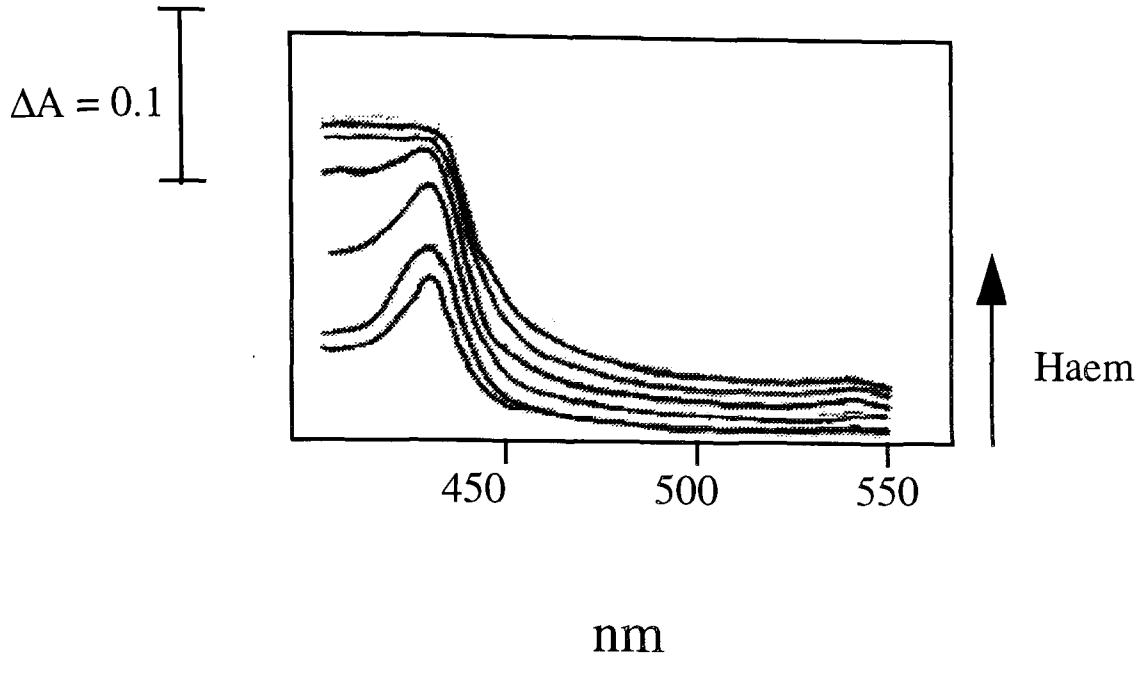


Figure 6.5. Titration of cytochrome b_{562} with haem addition to the reference cuvette, in a crude extract, 10 mg ml^{-1} of total cell protein, of *E. coli* strain JCB387, expressing cytochrome b_{562} from the construct b_{562}W.T. The assay was performed as described in the text. The added haem concentration was increased incrementally in steps of 5μM from 0 to 25 μM.

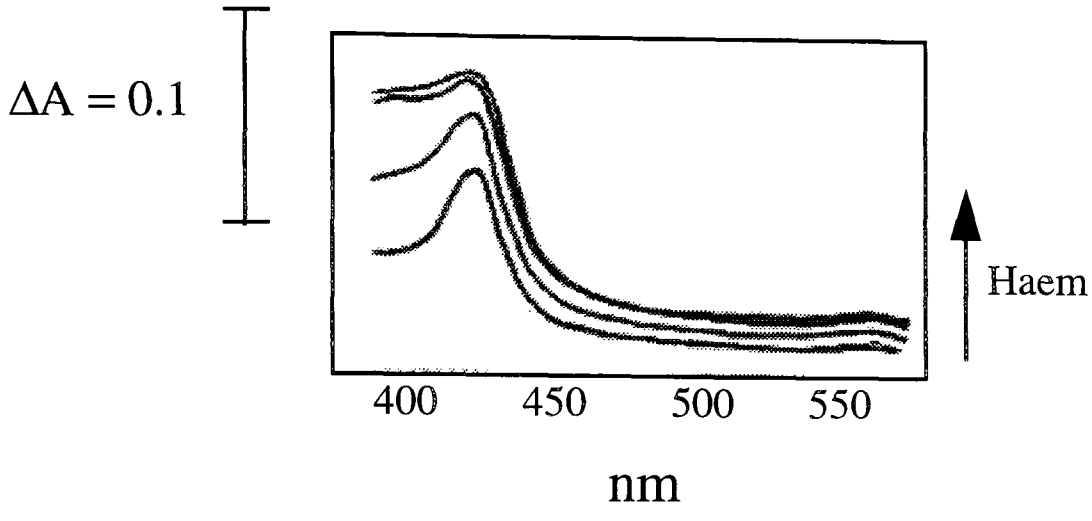


Figure 6.6. Titration of cytochrome b_{562} from the crude extract of the strain JCB71202, expressing cytochrome b_{562} from the construct b_{562}W.T. The assay was performed as described in the text. The assay was started with the supplementation of 5 μM haem directly to the cuvette. The added haem concentration was increased incermentally in steps of 5μM from 0 to 15 μM.

1st Primer

5'ATG AAT GAA CAG CTT GCC AAG CAA AAG GGC **GCT** ATG GCT TGC CAC GAT CTG AAA GCT AAG 3'

2nd Primer:
5'TTA CTT TAT GGA GAG TAT CCA CTG GGC 3'

pKHC12 *Bam* H I

PCR

pScreenT

250 bp PCR fragment

Ligation

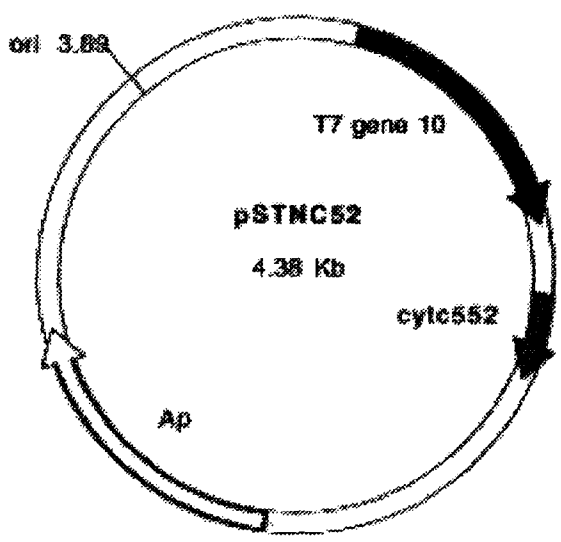


Figure 6.7. The construction of pSTNC52, which contains the coding region for C10A cytochrome c_{552}. The 250bp amplified PCR fragment from the construct pKHC12 was cloned into the vector pScreen T. The forward primer used for the PCR amplification contained the coding region for alanine instead of the first cysteine of the haem binding motif Cys-X-X-Cys-His. The PCR and the colony screening was performed as described in Materials and Methods, Chapter II.

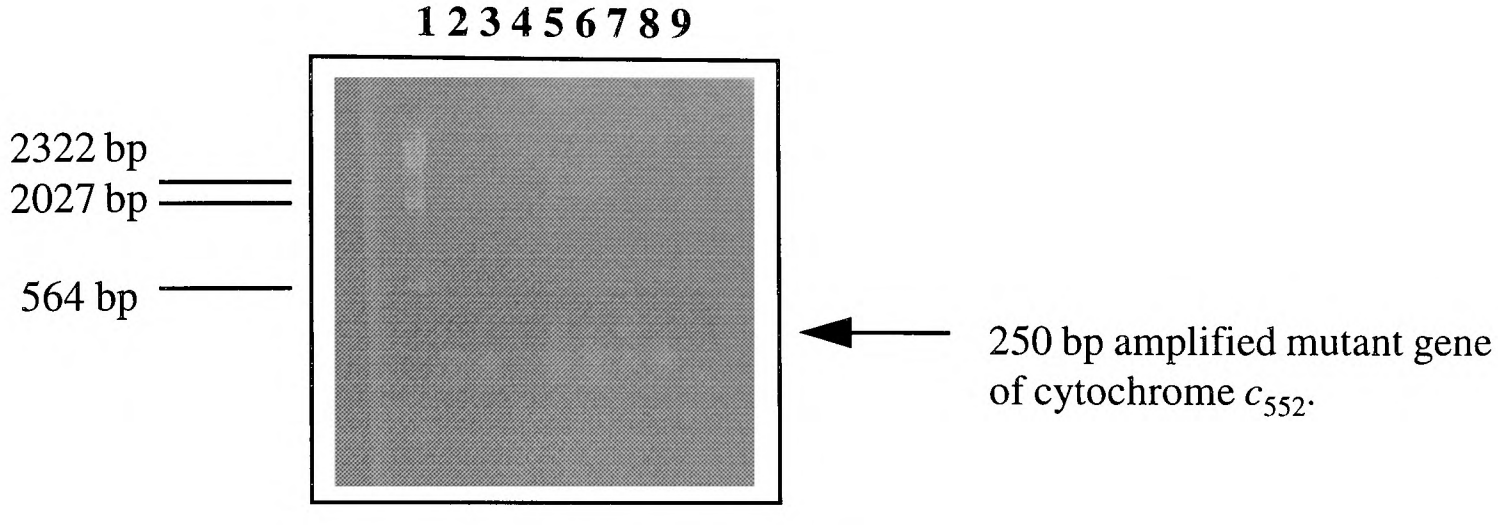


Figure 6.8. 2% agarose gel showing the amplified C10Acytochrome c_{552} from the construct pKHC12 by PCR. The PCR reaction and the agarose gel electrophoresis was performed as described.

Lane 1: λ DNA *HInd* III digest supplied by Boehringer Mannheim.

Lane 2,3,4,5,6,7,8,9: 30µl from the PCR reaction with 0.25 mM $Mgcl_2$, 0.5 mM $Mgcl_2$, 1.0 mM $Mgcl_2$, 1.25 mM $MgCl_2$, 1.5 mM $MgCl_2$, 1.75 mM $MgCl_2$, control reaction lacking the forward primer and control reaction lacking the reverse primer, respectively.

[Note: Lane 5,6,7 show the amplification of the right size fragment.]

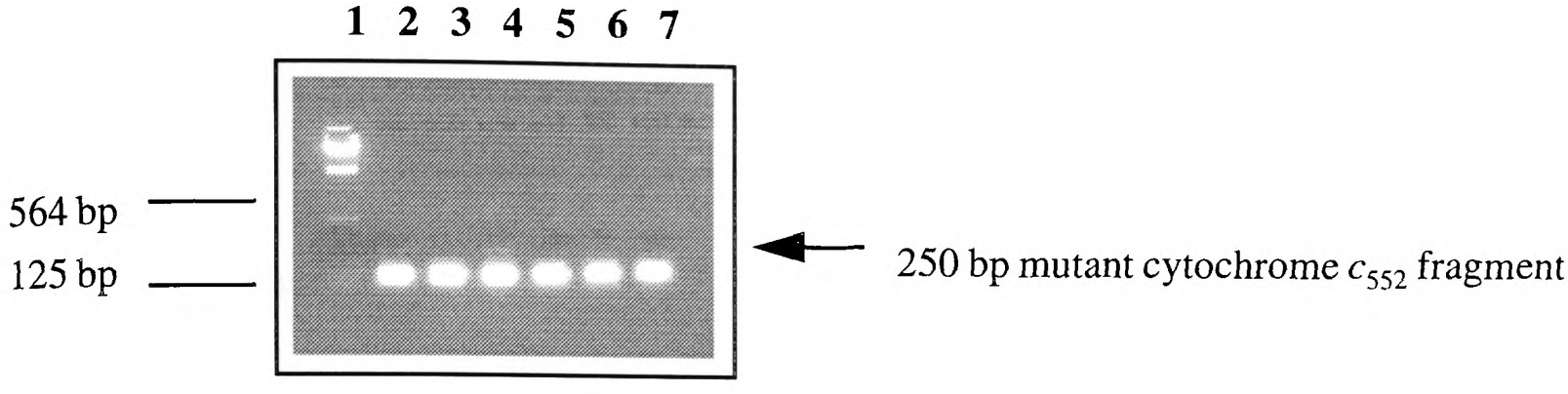


Figure 6.9. Analysis by electrophoresis on a 2.0 % agarose gel to check the right fragment using the technique of colony PCR of the transformed HMS174 with pSTNC52. The 5' and 3' primers used were same as used to create the mutant. The PCR reaction and agarose gel electrophoresis was performed as described in Materials and Methods.

Lane 1: λ DNA, *Hind* III digest supplied by Boehringer Mannheim.
Lane 2,3,4,5,6,7: 30μl from the colony PCR reactions.

[Note Lanes 4 and 6 show the right size, 250 bp, fragment.]

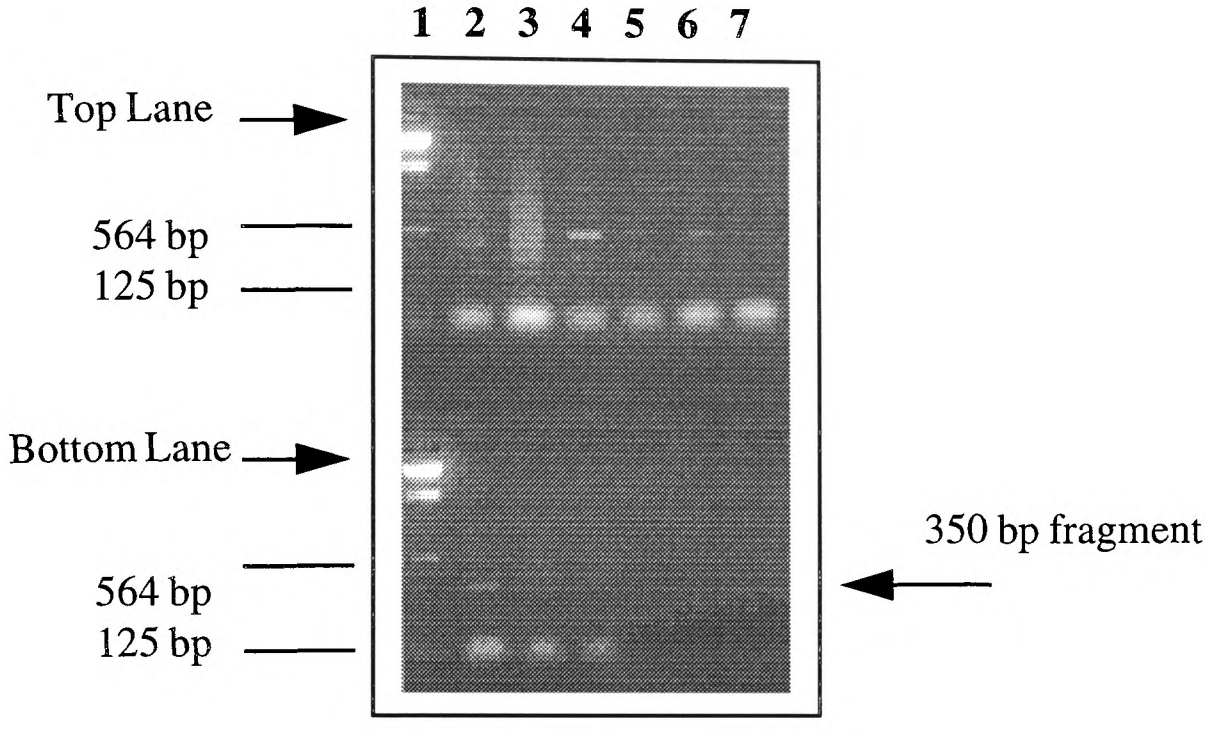


Figure 6.10. Analysis by electrophoresis on a 2% agarose gel showing the fragments obtained by colony PCR, to check for the right orientation of the cloned C10A cytochrome c_{552} while screening for pSTNC52. The PCR reaction and the agarose gel electrophoresis was performed as described in Materials and Methods, Chapter II. Note that the gel had two origins, at the top and in the middle, so that 7 top and 7 bottom lanes were run.

Lane 1(Top and Bottom): λDNA *Hind* III digests supplied by Boeringer Mannheim.
Lane 2,3,4,5,6,7(Top) Lane 2,3,4 (Bottom): 30µl from the colony PCR.

[Note the right size, 343 bp, fragment in Lane 2 (Bottom)showing that the C10A cytochrome c_{552} is in the right orientation in the vector pSTNC52.]

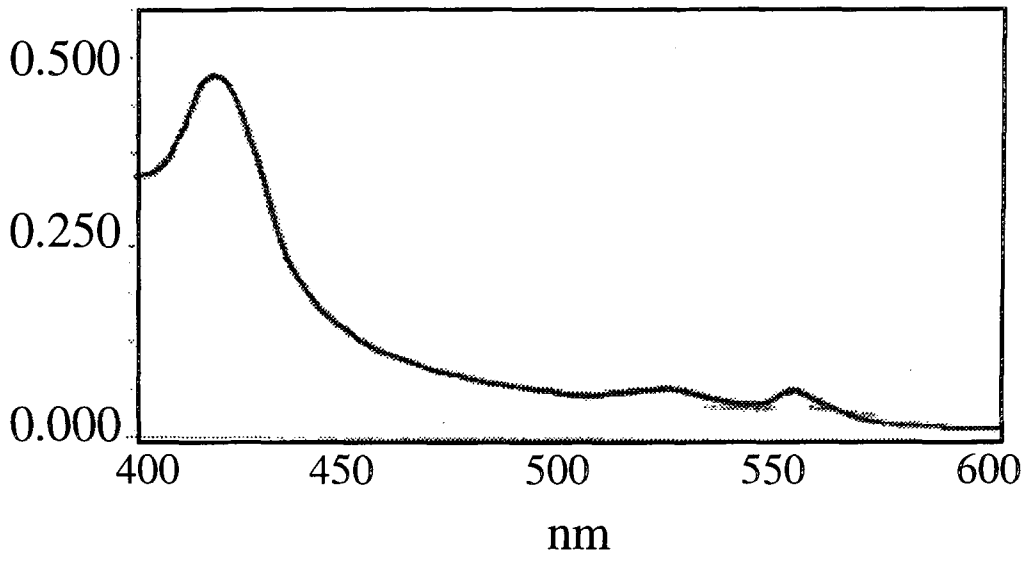


Figure 6.11. The dithionite reduced spectrum of the extract of *E. coli* HMS174(DE3) expressing C10A cytochrome c_{552} from the construct pSTNC52. 0.5 mg ml^{-1} of the total cell protein was used for the analysis. The spectrum was taken against the buffer reference.

6.2.4.4 Colony screening showed that the C10A cytochrome c_{552} is cloned and is in the right orientation.

Out of the twelve colonies screened five showed the presence of right size fragment while two out of these five showed the fragment to be in the right orientation. Figure 6.9 and Figure 6.10 show the results of the colony screening. Glycerol stocks of these colonies were made for further analysis of the putative C10A cytochrome c_{552}.

6.2.4.5 Spectrophotometric analysis of supposed C10A cytochrome c_{552} shows that cytochrome c_{552} is expressed but the spectra shows no difference from the native cytochrome c_{552} spectra

Spectrophotometric analysis for an α-band cytochrome peak was carried out as described in the Materials and Methods. An extent of pink coloured cells carrying pSTNC52 gave the spectra shown in Figure 6.11. The absorbance maximum was at 552 nm which was surprising because attachment of haem to a AXXCH motif usually results in a shift to larger wavelengths (Tanaka *et al.*, 1990).

6.2.4.6 SDS-PAGE analysis of C10A cytochrome c_{552} shows that the haem was covalently bound to the polypeptide

A haem stain SDS-PAGE analysis of the crude extract of HMS174(DE3) expressing pSTNC52 showed that the haem was bound covalently to the C10A cytochrome c_{552} polypeptide (not shown). The molecular weight of the material was larger than that of cytochrome c_{552} or indeed of mitochondrial cytochrome *c*. This was expected because of the presence of an extra 328 amino acids contributed as a consequence of the fusion described earlier. However, the haem staining material was not

obviously of molecular weight 48,000. It is suggested that it was partially degraded. The above two results together suggest that the C10A cytochrome c_{552} forms a species similar to the native cytochrome *c*, even though there is supposed to be only one covalent attachment between the polypeptide and haem.

An attempt to sequence the cloned fragment, C10A cytochrome c_{552}, in the vector pSTNC52 was, however, unsuccessful. One of the problems encountered in several attempts at cloning was that the DNA (vector containing the mutant cytochrome c_{552} gene, pSTNC52) could never be obtained sufficiently purified to be used for sequencing. Sequencing attempts for the vectors which did not have the cloned C10A cytochrome c_{552} or vector having native cytochrome c_{552} gene were always successful. The reason for the failure was not resolved, but the colony PCR, spectrophotometric and SDS-PAGE analysis, however, do suggest that the coding region of cytochrome c_{552} gene has been cloned in the right reading frame and that the presumed mutant cytochrome c_{552} was expressed in fairly high amounts. *N*-terminal sequencing of the mutant protein was not attempted because the expected AXXCH sequence would have been preceded by the long fusion protein.

6.3 Discussion

The present work shows that the *ccm* genes are not required for providing haem to periplasmic cytochrome b_{562}. Just after the experiments establishing this point had been completed, a similar conclusion was published by Goldman *et al.,* (1996). Later, Thorne-Holst *et al.,* (1997) showed that cytochrome b_{562} synthesis continues when individual genes, including *ccmA* and *ccmC*, were deleted. Thus it is clear from observations in three different laboratories that the provision of haem for periplasmic cytochrome b_{562} is not

compromised by the absence of the *ccm* genes. Thus if CcmA, CcmB and CcmC do together constitute a haem transport system, the protein complex formed from these genes must deliver the haem at a rate whose loss makes little difference to the synthesis of periplasmic cytochrome b_{562}. Another gene product needed for cytochrome *c* biogenesis, DipZ, has also been shown not to be required for cytochrome b_{562} production (Sambongi *et al.*, 1994). Although the continuing synthesis of cytochrome b_{562} was disappointing in that no insight to the function of the *dipZ* or *ccm* genes could be gained, it is perhaps not surprising given that in *E. coli* the activity of *ccm* genes is under anaerobic control (Choe and Reznikoff, 1993; Rabin and Stewart, 1993) whereas there is no evidence that the same conditions favour the production of cytochrome b_{562}. How then does haem reach the periplasmic cytochrome b_{562}? It has been suggested that *cydC* and *cydD* genes, located close to the structural genes for cytochrome *bd* oxidase, may also be haem transporters; these two genes code for an ABC transporter (Cook *et al.*, 1997). However, Goldman *et al.*, (1996) have argued against a role of CydC and CydD in haem translocation. A further possibility for the route of haem supply to the periplasm may be provided by one of the many identified reading frames, presumed to be coding for transport proteins, in the *E. coli* genome. In their study of periplasmic expression of liver cytochrome b_5 in *E. coli*, Karim *et al.* (1993) argued that the initial formation of apo cytochrome b_5 in the periplasm subsequently triggered supply of haem to the periplasm. They argued that the rate of cytochrome b_5 accumulation in the periplasm might reach 100 molecules per second per bacterial cell. For haem transport to approach this rate it seems improbable that passive diffusion will suffice (Light and Olson, 1990) and that there must be a protein system for transporting haem.

Whatever is the nature of such a transport system it can apparently not supply haem for periplasmic *c*-type cytochrome assembly. Otherwise, but contrary to observation, this system could complement a mutation in the *ccmA*, *ccmB* or *ccmC* genes that prevents *c*-type cytochrome biogenesis (Throne-Holst *et al.,* 1997) unless these genes, contrary to the general opinion do not code for a haem transporter. Provided it is accepted, but see below, that cytochrome b_{562} and cytochrome b_5 acquire haem in the periplasm of *E. coli*, then several interpretations of the failure of *c*-type cytochrome assembly in *ccmA*, *ccmB* or *ccmC* mutants can be considered. The first is that neither the products of these genes, nor any other *ccm* gene products, translocate haem to the periplasm. The fact that the Gram-negative organism *H. pylori* can make periplasmic *c*-type cytochromes without the *ccmA*, *ccmB* or *ccmC* genes is at least consistent with the possibility that these *ccm* gene products do not translocate haem. This, of course, leaves open the question of what they do translocate.

Karim *et al.,* (1993) concluded that the apo form of cytochrome b_5 was exported to the periplasm of *E. coli* where it was converted to the holo form. This conclusion was based on titrating haem into periplasmic fractions prepared at various times during cell growth. This was on the basis that it was shown that the appearance of apo protein preceded that of the holo. However, this does not prove that apo protein was converted to the holo form in the periplasm. This important point therefore remains to be proved, as it does for cytochrome b_{562}. In principle this could be done by adding chloramphenicol, a protein synthesis inhibitor, to cells of *E. coli* expressing cytochrome b_{562}. The present work shows that strain JCB387 accumulates substantial amounts of apo cytochrome b_{562}. If haem is added to the apo protein in the periplasm then it may be possible to detect the conversion of apo to holo cytochrome b_{562} in the periplasm. Until such a demonstration

has been achieved it will not be possible to exclude the possibility that cytochrome b_{562} binds with haem in the cytoplasm before export to the periplasm.

The finding that holo cytochrome b_{562} is formed in the periplasm when the *ccmABC* genes are inactivated prompts the question as to what would happen if the periplasmic expression of *H. thermophilus* cytochrome c_{552} could be studied in the same background. If this cytochrome could obtain its haem spontaneously, as is argued for its cytoplasmic assembly, then possibly in common with cytochrome b_{562} it would assemble spontaneously in the periplasm without the assistance of the *ccm* gene products. Unfortunately, this experiment could not be attempted because the full length gene for cytochrome c_{552} was not available and all attempts to synthesise it by ligating an oligonucleotide, coding for the signal sequence, to the available part of the gene were unsuccessful. However, the weak expression from pKPHC12K (Chapter V) gives a hint that such experiments may be possible.

The result that the holo:apo cytochrome b_{562} ratio was higher in the strain JCB71202 than in JCB387 adds to the previous findings suggesting that the JCB712 derived strains might be, at least relatively, over producing haem (Chapter V) and that haem is the only requirement for the maturation of cytochrome b_{562}, as was suggested for *H. thermophilus* cytochrome c_{552}. However, there is a possibility that the maturation of *b*-type cytochromes does need assistance from proteins present in the cytoplasm or periplasm. [illegible], especially when it is not yet known whether cytochrome b_{562} acquires its haem in the cytoplasm or in the periplasm. In the case of periplasm this could include a haem transporter as discussed above. However, recently Kuras *et al.*, 1997 have implicated at least one gene product as required for the assembly of cytochrome b_6 in thylakoids. Therefore *b*-type cytochrome assembly might not be always spontaneous.

To study the remarkable biogenesis of cytochrome c_{552} further and to know more about the similarity in the hypothesised pathway of the maturation of cytochrome b_{562} and cytochrome c_{552}, the first cysteine of the haem binding site, Cys-X-X-Cys-His, was altered to Alanine. The study shows that the mutant cytochrome c_{552} forms a holo protein similar to the native protein, confirmed by the spectrophotometry and SDS-PAGE haem stain analysis. The lack of the DNA sequencing data, however, does not confirm the mutation having alanine instead of cysteine. The ambiguity of the created mutant is unlikely though, as the gene was amplified from the primer which contained the mutation i.e. alanine at the place of cysteine and so the amplified fragment should contain the mutated site. However, a reversion to a cysteine codon cannot be excluded.

Chapter VII

Conclusion and Prospects for future work

7.1 Conclusion

The maturation of *H. thermophilus* cytochrome c_{552} in the cytoplasm of *E. coli* is unique. Since earlier evidence had suggested that the biogenesis of this cytochrome c_{552} might be independent of enzymatic assistance (Sambongi and Ferguson, 1994b; Sambongi *et al.,* 1994) it was thought important to study its maturation in detail both *in vivo* and *in vitro* in order to ascertain both the exact requirements for the biogenesis of this cytochrome *c* and why its maturation is different from other bacterial *c*-type cytochromes. Its thermostability seems to be an obvious reason. The suggestion that the covalent attachment between the cytochrome c_{552} and haem might be spontaneous (Sambongi and Ferguson, 1994b) could mean that in 50 % of the molecules of cytochrome c_{552}, the haem have opposite chirality, in contrast to other *c*-type cytochromes where the thioether bond is formed in a definite configuration with respect to the chiral α-carbon so that cytochrome *c* is not racemic but optically active (Paul, 1951). This haem orientation could be studied by using the technique of NMR (Senn and Wuthrich, 1983) while the proposal of spontaneous haem attachment could be tested *in vitro*. Both *in vitro* studies and NMR required high amounts of purified cytochrome c_{552}. Thus attempts were made to increase the expression of cytochrome c_{552} in the cytoplasm of *E. coli* by using different approaches and studying the factors which could increase its expression. Among the several attempts to increase the expression of cytochrome c_{552}, its gene was cloned and expressed in a T7 expression system. Reasonable yields were obtained using this system as discussed in Chapter IV. However, these yields were not as high as has often been observed with the T7 system and it proved impossible to obtain sufficient pure cytochrome c_{552} to carry out biophysical studies. However, use of the T7 expression did lead to an unexpectedly useful development. This was the recognition that apo protein

was present in T7 expressed material, which in part explains the disappointingly low yields of holo protein. The detection, via its S•Tag of this apo protein, led to the discovery of apo cytochrome c_{552} in another strain of *E. coli*. This material did not have an entirely random coil conformation, at least as judged by preliminary CD spectroscopy. This finding is consistent with, but does not prove, that the holo cytochrome c_{552} is assembled as a consequence of haem binding to a pocket already formed in the apoprotein.

The finding, described in Chapter V, that cytochrome c_{552} synthesis continues in a mutant of *E. coli* that lacks the *ccm* genes also suggests the idea that the assembly of cytochrome c_{552} is spontaneous. Another unexpected development was that this mutant actually made larger amounts of cytochrome c_{552} than had ever been observed before. This was attributed, but not fully proven, to a better provision of haem by this mutant, and the strain from which it was derived, than by other strains of *E. coli* in which the expression of cytochrome c_{552} had been previously examined. This unexpectedly substantial synthesis of cytochrome c_{552} by this *E. coli* strain permitted sufficiently large amounts of the molecule to be obtained for NMR studies. Although the work is not yet complete, the NMR studies are indicating that the haem attachment is indistinguishable from that in other *c*-type cytochromes. This also suggests that cytochrome c_{552} is assembled by haem binding to a preformed pocket provided by the apo protein. Otherwise, a heterogeneity of products may have been expected. Overall, all the observations reported in the present work support the proposal that cytochrome c_{552} assembles spontaneously.

Chapter VI describes the studies on the maturation of *E. coli* cytochrome b_{562}. It is a small (12 kD) soluble protein present in the periplasm of *E. coli* with yet unknown

function and is similar to cytochrome c' from photosynthetic species in its tertiary structure. The maturation of cytochrome b_{562} was studied in an *E. coli* strain that lacks the *ccm* genes. Along with the other components which are required for the maturation of *c*-type cytochromes this *E. coli* JCB71202 strain lacks *ccmABC* genes, which encode for a predicted haem transporter. The maturation of cytochrome b_{562} in the periplasm of this strain would mean either that the haem destined for the biogenesis of the *b*-type cytochrome is transported by some other route, or even that cytochrome b_{562} acquires the haem in the cytoplasm.

7.2 Future work

A main goal must still be to determine whether the cytochrome c_{552} from *H. thermophilus* can be assembled spontaneously. Probably the best approach will be to add haem to the apocytochrome c_{552} and try to identify conditions under which the haem will attach covalently. It may, for example, be necessary to avoid aerobic conditions which might oxidise either the haem or the thiol groups of the cysteines of the apo cytochrome c_{552}. If covalent attachment could be demonstrated, eg by haem staining on an SDS-PAGE gel it would then be necessary to try to obtain sufficient material to show, eg by visible range spectrophotometry, that the 'normal' attachment had occurred so as to give cytochrome c_{552}.

If future work fails to demonstrate the *in vitro* attachment of haem to the apo cytochrome c_{552} then reasons for failure should be investigated. The evidence provided in this thesis for the purification of apo cytochrome c_{552} is difficult to interpret in another way; recall that the material, of unusually low molecular weight, is only obtained from cells transformed with a plasmid carrying the appropriate gene. However, the final

identification of the apo cytochrome c_{552} could be achieved by *N*-terminal sequencing. Assuming this procedure gives the expected result, and no haem attachment can be demonstrated, some reasons for this could be investigated. In particular it could be checked whether the thiol groups of the two cysteine residues of the CXXCH motif are intact or whether they are part of a disulphide bridge. If they were to be found in the latter state it would be a first demonstration that such a disulphide, postulated to occur in the pathway of *c*-type cytochrome biogenesis in the bacterial periplasm, can form. A disulphide in the apo cytochrome c_{552} could be reduced and a test made of whether or not haem can then be attached covalently.

Irrespective of whether haem can be attached *in vitro* to the apo cytochrome c_{552}, more information about this apo protein should be obtained. Ideally, sufficient material should be made to allow the 3D structure to be obtained by NMR.

It will be interesting to find out if the *N*-terminus of apo cytochrome c_{552} has methionine or asparagine as found in Chapter III for the holoprotein. If it has a mixture it could be useful to incubate this protein with the *N*-terminal methionine aminopeptidase. It may be possible to determine the rate at which the unexpected (Chapter III) cleavage of methionine next to the asparagine occurs.

It should not be forgotten that *in vitro* transcription/translation may still prove a route to demonstrate the spontaneous attachment of haem to the apo cytochrome c_{552}. It is worth noting that Hakvoort *et al.* (1990) managed in this way to make sufficient amounts of apo mitochondrial cytochrome *c* to allow biochemical experiments. If this could be done for apo cytochrome c_{552} it would be a step forward.

Inconclusive attempts in the present work to demonstrate the formation of an AXXCH variant of cytoplasmically expressed cytochrome c_{552} should ideally be followed

up. Also, it would be interesting to know if haem can be attached to CXXAH or CXXCM motif as a result of spontaneous assembly. The latter case would lead to an interesting cytochrome with two methionine axial ligands to the haem iron.

The present work has confirmed that the assembly of cytochrome c_{552} in the cytoplasm of *E. coli* is very probably spontaneous. An interesting question is whether this protein could also assemble spontaneously in the periplasm. In this work some evidence of expression of cytochrome c_{552} from pKPHC12K was obtained. This plasmid has the cytochrome c_{552} gene that is fused to a coding region for a periplasmic targeting sequence. If the expression can be shown to lead to periplasmic holocytochrome c_{552} then it will be important to know if a similar expression can be achieved in *E. coli* JCB71202 which lacks the *ccm* genes. Also, it should be worth checking whether a *dsbA*$^-$ *dsbB*$^-$ *ccm*$^-$ mutant could assemble periplasmic cytochrome c_{552}. Even if the *ccm* genes are not needed the *dsb* system might prevent haem attachment by catalysing disulphide bond formation within the CXXCH motif.

More generally further research on the role of the *ccm* gene products needs to be done. From the present work, and that simultaneously of others, it is clear that periplasmic cytochrome b_{562} can be assembled without the *ccm* gene products. It will be interesting to know whether variants of cytochrome b_{562} carrying cysteines at the haem binding site (Barker *et al.*, 1995) can also be assembled in the periplasm without the activities of the *ccm*, and *dipZ*, gene products. However, it should be remembered that there is no proof that haem is added to the cytochrome b_{562} in the periplasm. This requires proving or disproving by experiment.

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