THE ROYAL SOCIETY

Published online 18 November 2002

C-type cytochromes: diverse structures and biogenesis systems pose evolutionary problems

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C-type cytochromes are a structurally diverse group of haemoproteins, which are related by the occurrence of haem covalently attached to a polypeptide via two thioether bonds formed by the vinyl groups of haem and cysteine side chains in a CXXCH peptide motif. Remarkably, three different post-translational systems for forming these cytochromes have been identified. The evolution of both the proteins themselves and the biogenesis systems poses many questions to which answers are currently being sought. In this article we review the progress that has been made in understanding the need for covalent attachment of haem to proteins in cytochromes *c* and the complex systems involved in their formation.

Keywords: *c*-type cytochromes; disulphide bond; haem; maturation systems; lyase

1. INTRODUCTION

C-type cytochromes, a widespread class of proteins essential for the life of almost all organisms, pose both a structural and an evolutionary puzzle. Cytochromes *c* are characterized by the covalent attachment of haem (iron-protoporphyrin IX) to a polypeptide chain via two (or rarely one) thioether bonds which are generated as a result of the reaction of thiol groups of cysteine residues with vinyl groups of haem (Barker & Ferguson 1999). The two cysteine residues almost always occur in the amino-acid sequence CXXCH, where the histidine is an axial ligand to the haem iron. Almost any residues (except cysteine) may be found in the XX positions; very rarely there are three or four residues between the two cysteines (Barker & Ferguson 1999). Mammalian and fungal mitochondrial cytochrome *c* is by far the best known of this type of protein, but there is a very great range of other such proteins, often with several haems attached per polypeptide chain, which are not related to these mitochondrial cytochromes *c*. Cytochromes *c* typically function in electron transfer, but *c*-type cytochrome centres are also found in the active sites of many enzymes, and in eukaryotic cells cytochrome *c* has a recently discovered and surprising role in apoptosis (Pettigrew & Moore 1987; Moore & Pettigrew 1990; Scott & Mauk 1995; Igarashi *et al.* 1997; Einsle *et al.* 1999; Martinou *et al.* 2000). The aim of this article is to review what advantages might accrue from covalent attachment of a haem group to a protein and then consider how the thioether bonds are formed, a process that curiously differs between various cell types and is far from chemically facile. This leads on to a discussion of the evolution of both cytochromes *c* and their biogenesis systems.

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One contribution of 21 to a Discussion Meeting Issue 'Chloroplasts and mitochondria: functional genomics and evolution'.

2. POSSIBLE ADVANTAGES OF FORMING THIOETHER BONDS

Barker & Ferguson (1999) sought to identify reasons that would explain the advantage(s) conferred by the thioether bonds in cytochromes *c*. One suggestion is from Wood (1983), who noted that because in Gram-negative bacteria these proteins are located in the periplasm, the compartment between the cell wall and the cytoplasmic membrane, covalent attachment of the haem would guard against a finite rate of dissociation of haem from a non-covalent complex with protein (i.e. a *b*-type cytochrome). Any such dissociation could be followed by passage of haem through the cell wall and hence loss to the medium external to the cell. It is impossible to disprove this hypothesis absolutely, but a number of considerations militate against it being the main reason for occurrence of covalent haem attachment in *c*-type cytochromes. These are: (i) although in principle haem might pass readily through a cell wall, its hydrophobic nature does mean that it would adsorb onto the wall and hence would not be lost to the cell (i.e. it could remain available for incorporation into haem-binding proteins); (ii) there are now several known extracellular proteins that have non-covalently bound haem, in effect *b*-type cytochromes (Barker & Ferguson 1999), one example being an enzyme involved in cellobiose degradation (Hallberg *et al.* 2000); and (iii) there are several periplasmic *b*-type cytochromes now recognized. Of the latter, the enigmatic (its function is uncertain and it is rare in other species) cytochrome b_{562} in *Escherichia coli* (Arnesano *et al.* (1999) and references therein) is arguably the best known. More recently discovered examples include a group of related *b*-type cytochromes that are involved in the oxidation of ethylbenzene or dimethyl sulphide and in reduction of selenate (Hanlon *et al.* 1996; Schroder *et al.* 1997; Kniemeyer & Heider 2001). Furthermore, it has proved possible to direct the formation, in substantial amounts, of stable holocytochromes b_5 and P450, and of haemoglobin, to the periplasm of *E. coli* (Karim *et al.* 1993; Goldman *et al.* 1996;

Kaderbhai *et al.* 2000); severe problems with loss of haem from the periplasm might have been expected to pose an impediment to such synthesis. A final consideration is that the specialized d_1 haem of the periplasmic respiratory enzyme cytochrome cd_1 nitrite reductase is bound non-covalently (Hill & Wharton 1978; Fülöp *et al.* 1995), despite the significant biosynthetic expense of this haem (Zumft 1997).

(a) *Comparison of the physico-chemical properties of related* b- *and* c-*type cytochromes*

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

Cytochrome *c* maturation normally requires specialized biogenesis systems. However, exceptionally, haem may be covalently attached to the cytochromes c_{552} from *Hydrogenobacter thermophilus* and *Thermus thermophilus* in the cytoplasm of *E. coli*, apparently without the action of any biosynthesis proteins (Sambongi & Ferguson 1994*b*; Keightley *et al.* 1998; Sinha & Ferguson 1998); the usual location of *c*-type cytochrome formation in bacteria is the periplasm. The cytoplasmically produced *H. thermophilus* protein folds correctly and its physical properties are in many respects indistinguishable from those of protein matured by a dedicated biogenesis apparatus in *H. thermophilus* itself (Karan *et al.* 2002). It has been proposed that cytoplasmic assembly occurs because these apoproteins, which are normally found in thermophilic organisms (optimum growth at 70 °C or more), adopt a pre-folded conformation that can bind haem when produced in the mesophile *E. coli* (Sambongi & Ferguson 1994*b*; Sinha & Ferguson 1998). This idea is consistent with the observation that replacement of both of the *H. thermophilus* cytochrome c_{552} haem-binding cysteine residues by alanines (in a C11A/C14A variant) results in cytoplasmic formation of a *b*-type cytochrome whose haem is not covalently bound to the polypeptide and can be removed reversibly *in vitro* (Tomlinson & Ferguson 2000*a*). More significantly, the ability to create such a protein has allowed a direct comparison between a *c*-type cytochrome and a *b*-type cytochrome that, in principle, differ only in that the haem is covalently attached to the polypeptide in the former but not in the latter (Tomlinson & Ferguson 2000*a*).

Biophysical studies of wild-type *H. thermophilus* cytochrome c_{552} and of the C11A/C14A variant showed that the gross secondary structures of the two proteins were the same, as judged by their virtually identical far-UV circular dichroism spectra; nuclear magnetic resonance spectroscopy and sequence analysis showed that the *b*-type variant retained the His/Met haem iron coordination of the *c*-type cytochrome (Tomlinson & Ferguson 2000*a*). However, the *b*-type (C11A/C14A) cytochrome was structurally less stable, with the guanidine hydrochloride unfolding midpoint occurring at a concentration 2 M lower than for the wild-type protein. By contrast, the reduction potential was only 75 mV lower than that of the *c*-type cytochrome (Tomlinson & Ferguson 2000*a*). This difference is extremely small given that variations of several hundreds of millivolts can arise in the reduction potential of *c*-type cytochromes owing to differences such as the extent of solvent exposure of the haem (Tezcan *et al.* 1998) and indicates that modulation of reduction potentials is probably not a key reason for the covalent attachment of haem to the polypeptide. The *b*-type variant protein was also shown to be stable with respect to loss of haem in dialysis experiments over a period of days (Tomlinson & Ferguson 2000*a*), providing a further argument against haem retention as the basis of covalent attachment.

Additional exploitation of the cytoplasmic assembly in *E. coli* of *H. thermophilus* cytochrome c_{552} and variants allowed a more thorough investigation of the significance of two thioether bonds in cytochromes *c*. Variants of the protein in which each of the cysteines that form the covalent linkages to haem had been mutated to alanines (i.e. with AXXCH or CXXAH haem-binding motifs) could be isolated and their physico-chemical properties investigated (Tomlinson & Ferguson 2000*b*). For each of these proteins, the haem is covalently attached to the polypeptide through a single thioether bond. Remarkably, the single thioether bond-containing proteins have similar properties, including thermal stability and reduction potential, to the wild-type CXXCH protein (Tomlinson & Ferguson 2000*b*). In combination with the work showing that the AXXAH variant of cytochrome c_{552} is much less stable than the CXXCH form (Tomlinson & Ferguson 2000*a*), it can be concluded that covalent attachment of haem via either of the thioether bonds is sufficient to confer considerable stability and that these bonds contribute little to the setting of the reduction potential. Other laboratories have attempted the reverse approach, i.e. selective introduction of one or two cysteine residues into the *E. coli* cytochrome b_{562} or bovine liver cytochrome b_5, positioned such that they could form covalent bonds with the vinyl groups of haem (Barker *et al.* 1993, 1995; Arnesano *et al.* 2000). An NMR characterization of one such variant (b_{562} R98C; CXXXH haem-binding motif) demonstrated formation of the thioether bond and also that the overall secondary and tertiary structures of the protein were very similar to those of the wild-type cytochrome b_{562}. The single-thioether-bonded *c*-type variant of cytochrome b_{562} was shown to have a significantly increased stability, compared with that of wild-type b_{562}, towards thermal and chemical denaturation (Arnesano *et al.* 2000). Thus, conversion of a *c*-type cytochrome to a *b*-type and both of a *b*-type to a *c*-type indicates that formation of the thioether bonds confers considerably greater stability on the product cytochrome; this holds true even with only a single thioether bond. The absence of naturally occurring ZXXCH or CXXZH haem-binding motifs from bacterial cytochromes *c* (in proteins discovered to date) in contrast to their occurrence in some mitochondria (see § 3c) may relate to the coexistence of the assembly pathway with that

for formation of disulphide bonds in the bacterial periplasm.

(b) *Packing of haems*

In the bacterial world, especially as part of the biological nitrogen and sulphur cycles, there are many *c*-type cytochromes with multiple haems per polypeptide chain. Examples include the octahaem hydroxylamine oxidoreductase (Igarashi *et al.* 1997), the tetrahaem NapC/NirT/TorC family (Roldan *et al.* 1998), the 65 kDa 16-haem-containing protein Hmc (high molecular mass cytochrome *c*) from sulphate-reducing bacteria (Florens & Bruschi 1994), and cytochrome c_3 which is, remarkably, a tetrahaem protein of only *ca*. 16 kDa molecular mass (Higuchi *et al.* 1984). Such proteins bear no resemblance to the two vertebrate/invertebrate/fungal *c*-type cytochromes (*c* and c_1), but form structurally related families (in which the positions of the haems can often be overlaid) even when there is little sequence conservation between members of the family (e.g. pentahaem nitrite reductase, hydroxylamine oxidoreductase and flavocytochrome fumarate reductase) (Barker & Ferguson 1999). It is striking that in these proteins the haems are clustered together. Barker & Ferguson (1999) have argued that only by fixing the haems spatially via their thioether bonds can such clustering be achieved. Certainly it is the case that in general the amino acid to haem ratio in this type of protein is much lower than it is in haem proteins with non-covalently bound haem. It is probably also significant that dense packing of haems allows extremely rapid electron transfer between the haem centres (Page *et al.* 1999), an essential part of the function of these cytochromes, which are often involved in multi-electron oxidoreductase reactions. This property is ideal for enzymes such as the pentahaem nitrite reductase, which catalyses the six-electron reduction of nitrite to ammonia, or hydroxylamine oxidoreductase, which catalyses the four-electron oxidation of hydroxylamine to nitrite. However, it should be noted that there are many other examples of proteins with a dense packing of non-covalently bound cofactors. One example is photosystem I of thylakoids. In this case the chlorophylls (closely related to haems) are positioned either between transmembrane helices or in loops between those helices. It has been pointed out previously (Ferguson 2001) that *c*-type cytochromes with the haem attached in a transmembrane part of the protein have not been observed; at least one outer-membrane *c*-type cytochrome is now thought to have a globular conformation on the external surface of the cell (Richardson 2000). Thus at present we can tentatively correlate multi-haem *c*-type cytochrome formation with a requirement to have a cluster of haems in a globular protein. It should also be reiterated that many cytochromes *c* (notably vertebrate/invertebrate/fungal cytochrome *c* and the bacterial respiratory electron transport cytochromes c_2) carry only a single haem. Therefore, the ability to pack many haems into economical polypeptides with conserved relative orientations cannot be the only reason for covalent haem attachment in the cytochrome *c* fashion, but it may be an evolutionary consequence of the development of systems for such attachment (or, indeed, vice versa).

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

Finally, in pondering the evolutionary origin of *c*-type cytochromes, one might note that a CXXC peptide motif is found in other proteins including the thioredoxin class, which (see § 3c,d) is intimately involved in at least one cytochrome *c* biogenesis pathway. The CXXC motif is also found in many iron–sulphur proteins (Johnson 1994), which are generally regarded as having occurred before cytochromes during evolution.

3. CYTOCHROME *C* BIOGENESIS STRATEGIES

Remarkably, three very different cytochrome *c* biogenesis systems have been identified to date. This in itself indicates that there must be one or more strong reasons for the adoption of thioether bonds for anchoring haem to protein in cytochromes *c*. The simplest system is apparently unrelated to the other two and operates in the mitochondria of fungal, invertebrate and vertebrate cells. In these organisms enzymes called haem lyases promote formation of the product cytochrome (Steiner *et al.* 1996). At least in the case of fungi, each lyase is specific for a particular cytochrome (i.e. one for cytochrome *c*, one for cytochrome c_1). However, only a single enzyme homologous to the yeast lyases is found in the genomes of the human and mouse (that enzyme having a high degree of sequence similarity to both the yeast cytochrome *c* lyase and the cytochrome c_1 lyase). Remarkably, plant mitochondria do not contain haem lyase but use instead at least part of a bacterial cytochrome *c* biogenesis system for assembly of cytochromes *c* and c_1 (see § 3c). There is no obvious haem lyase 'ancestor' in an archaeal or bacterial genome and thus the origin of this protein is puzzling. Its absence from plants may also have implications for understanding the evolution of the eukaryotic cell. It is intriguing that haem lyase is found in *Dictyostelium discoideum* and *Plasmodium falciparum* organisms that are

distinct from, and may pre-date yeast or *Neurospora* in evolutionary terms.

The ability to express yeast mitochondrial cytochrome *c* in the cytoplasm of *E. coli* if the haem lyase is coexpressed provides strong evidence that only one ancillary protein is required to promote thioether bond formation in this case (Pollock *et al.* 1998). It has also been shown that the yeast lyase can promote attachment of haem, in low yield, to a variant of human mitochondrial cytochrome *c* carrying an AXXCH haem-binding sequence (Tanaka *et al.* 1990). Additionally, a C14S variant of yeast *iso*-1-cytochrome *c* (i.e. with a SXXCH motif) has been isolated from the cytoplasm of *E. coli* following its coexpression with the yeast cytochrome *c* haem lyase (Rosell & Mauk 2002). Thus it appears that the haem lyases do not have a strict requirement for the CXXCH motif, which implies in turn that unlike for bacterial cytochrome *c* assembly (see § 3c,d) a disulphide bond is not formed within this motif during the haem lyase type of cytochrome *c* biogenesis. This idea is consistent with the successful expression of yeast cytochrome *c* (CXXCH motif) in the *E. coli* cytoplasm provided the haem lyase is coexpressed (Pollock *et al.* 1998), as disulphides would not be expected to form in the reducing environment of this compartment of the cell. The mode of action of the haem lyases is not established; while it may be catalysis of formation of the thioether bonds between haem and cysteine, other possibilities must be considered (see §§ 4 and 5).

(a) *The Ccm system*

Many Gram-negative bacteria (the α- and γ-proteobacteria) use proteins known as the Ccm system (named for the genes *ccmABCDEFGH* found in *E. coli* but also sometimes called system I) for cytochrome *c* biogenesis. Analogues of at least some of these proteins are also found in plant and protozoal mitochondria. Note that various different names are given to the equivalent genes from organisms other than *E. coli*, but here we use the Ccm nomenclature adopted by Page *et al.* (1998); see Thöny-Meyer (2000) for a survey of the (very confusing) cytochrome *c* biogenesis nomenclature. The bacterial Ccm system is complex and generally quite poorly understood; nevertheless, it is extremely flexible with regard to substrate and can catalyse the formation of cytochromes *c* of diverse prokaryotic and eukaryotic origin (Sambongi *et al.* 1996; Schlarb *et al.* 1999; Sanders & Lill 2000). The Ccm proteins are all located in the periplasm and/or cytoplasmic membrane and the Ccm system functions periplasmically; all bacterial cytochromes *c* are either periplasmic or anchored on the periplasmic face of this membrane. Among the better understood Ccm proteins is CcmE, which has been purified and partially characterized (Schulz *et al.* 1998). This protein forms a biologically novel type of covalent bond between a histidine residue and haem, although the nature of this bond has not yet been established.

It has proved possible recently to study the behaviour of CcmE *in vitro*. The soluble periplasmic domain (i.e. lacking the N-terminal transmembrane helical sequence) was produced in, and isolated from, the periplasm of *E. coli* in apo and holo forms (Daltrop *et al.* 2002*b*). The apoprotein showed an unusual preference for ferric, rather than ferrous, haem. Treatment of a non-covalent complex between ferric haem and CcmE with a suitable reductant resulted in a covalent bond being formed between the haem and protein. The spectroscopic properties of *in vitro*-produced CcmE with covalently linked haem were very similar to those of the holoprotein isolated from *E. coli* (Schulz *et al.* 1998; Daltrop *et al.* 2002*b*). We can conclude that the covalent bond does involve at least one of the vinyl groups of the haem because mesohaem (a haem analogue containing ethyl groups in the position of the vinyl moieties of Fe-protoporphyrin IX) did not give a covalent adduct. If *in vivo*-prepared holo-CcmE was incubated with the apocytochrome c_{552} from *H. thermophilus* in the presence of a reductant, then the covalent bond between CcmE and haem was broken and replaced by thioether bonds between apocytochrome *c* and haem to give holocytochrome c_{552}. Holo-CcmE did not transfer haem to the C11A/C14A variant of *H. thermophilus* apocytochrome c_{552}, which cannot form the thioether bonds of a *c*-type cytochrome. These data (Daltrop *et al.* 2002*b*) show that the Ccm system requires cysteine residue(s) in the substrate apocytochrome to release haem from CcmE. Such haem release also requires that the haem be ferrous, which might be a protective mechanism ensuring correct cytochrome *c* formation because side products are formed from the reaction of apocytochrome *c* and ferric haem (see below and Barker *et al.* 1993 and Daltrop *et al.* 2002*a*). The experiments with wild-type apocytochrome c_{552} and holo-CcmE have replicated a step in the biosynthesis of a *c*-type cytochrome, although the low reaction rate indicates that this step must be catalysed *in vivo*. Presumably our provision *in vitro* of a reductant substituted for several components of the Ccm system and DsbD (often called DipZ), which appear to have this reducing role *in vivo*. It is notable that the absence *in vivo* of DsbD (Sambongi & Ferguson 1994a) and CcmH (Fabianek *et al.* 1999) can be overcome by addition of a low molecular mass thiol-containing compound such as coenzyme M (2-mercaptoethanesulphonic acid).

(b) *Transporters associated with the Ccm system*

CcmA and CcmB form elements of an ATP-dependent ABC transporter whose substrate is not known. Several components may need to be transported from the bacterial cytoplasm for covalent attachment of haem to protein to occur in the periplasm. Apocytochrome *c* is transported by the Sec system (Thöny-Meyer & Kunzler 1997), but another obvious requirement is haem. Some evidence from protein stability and coimmunoprecipitation experiments indicates that a complex of CcmABCD exports haem from its site of biosynthesis in the cytoplasm (Goldman & Kranz (2001) review the evidence in some detail). However, alternative evidence is accumulating that CcmAB is not a straightforward haem transporter from cytoplasm to periplasm. Disruption of *ccmA* did not inhibit accumulation of holocytochrome b_{562} in the periplasm of *E. coli* (Goldman *et al.* 1996; Throne-Holst *et al.* 1997). Supplementation of the growth medium with haem did not stimulate *c*-type cytochrome formation in *ccmA* or *ccmB* mutants of *Paracoccus denitrificans* (Page *et al.* 1997*a*). Furthermore, *in vitro* haem uptake into everted membrane vesicles was neither ATP-dependent, nor was it different in a *ccmA* mutant when compared with the wild-type (Cook & Poole 2000). So if CcmAB does not

transport haem, what else might be its substrate? Mutants deficient in *ccmA* or *ccmB* could not form holo-CcmE, although very high level overexpression of CcmC complemented for this deficiency (Schulz *et al.* 1999). However, when the Δ*ccmA* mutant was complemented by overexpression of CcmC, holo-CcmE accumulated, but holocytochrome *c* did not (Schulz *et al.* 1999). These observations indicate that the substrate for CcmAB probably functions in the cytochrome *c* biogenesis process after haem attachment to CcmE, but might, depending on the precise role of CcmC, also function in that haem attachment. Such a factor may, for instance, be involved in accelerating haem transfer from holo-CcmE to apocytochrome *c* or, directly, in haem ligation to the apocytochrome *c*. Because correct attachment of haem to apocytochrome *c* (and to CcmE) requires that the haem iron be reduced (ferrous) (Barker *et al.* 1993; Daltrop *et al.* 2002*a*,*b*), another candidate substrate for CcmAB is a specific (ideally one-electron) ferric haem reductant (but see Schulz *et al.* 1999).

CcmF has a predicted topology of 11 transmembrane helices (Goldman *et al.* 1998) and thus, speculatively, seems a plausible candidate for a second transporter in the Ccm system (Pearce *et al.* 1998). CcmF is essential for this mode of cytochrome *c* maturation, functions after covalent attachment of haem to CcmE (Reid *et al.* 1998; Schulz *et al.* 1998), and has been shown to interact with CcmE and CcmH (Ren *et al.* 2002), so it seems most unlikely to be transporting haem or a catalytic agent for holo-CcmE formation. However, possible transport substrates for CcmF are reductants for haem or a catalytic agent for haem transfer from CcmE to apocytochrome. Others have proposed that CcmF is a component of a bacterial haem lyase system (Ren *et al.* 2002) (although no mechanism has been proposed) or, because most of the highly conserved residues of CcmF are periplasmic, a haem transport system along the outer surface of the cytoplasmic membrane (Goldman & Kranz 2001).

In *E. coli*, an additional ATP-dependent ABC transporter, CydDC, is also related to cytochrome *c* biogenesis. Both *cydD* and *cydC* mutants were shown to be deficient in cytochromes *c* (Poole *et al.* 1994; Goldman *et al.* 1996). This transporter is associated with maintaining the redox balance of the periplasm but seems very unlikely to transport haem (Goldman *et al.* 1996; Cook & Poole 2000). Thus the substrate of CydDC may be required for use by the Ccm system (as discussed above for CcmAB), or its absence may deleteriously affect the functions of one, or a combination of, the thioredoxin-like Dsb proteins, CcmG and CcmH (see § 3c).

(c) *The Ccm system and disulphide bonding in the apocytochrome CXXCH haem-binding motif*

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

Such proposed interactions of the Ccm and Dsb systems require substantiation in several ways. First, there has until recently been no example of an apocytochrome *c* with a disulphide bond between the apocytochrome cysteines. Second, the requirement for the Dsb system could reflect the need for a disulphide bond in one or more of the Ccm proteins; it is not clear if the phenotypes of the various Dsb mutants arose because of the inability of these mutants to process a disulphide involving apocytochrome *c*, one or more of the Ccm proteins, or indeed a combination of these proteins. In this regard it is notable that a Gram-negative bacterial protein secretion system has been shown to require a disulphide in the secretion apparatus, rather than, as previously thought, in the secreted substrate itself (Pugsley *et al.* 2001). Recently, we showed that the apo form of the cytochrome c_{552} from *H. thermophilus* will, relatively readily, form an intramolecular disulphide bond *in vitro* (Daltrop *et al.* (2002*a*) and see § 4). Thus, there is no inherent structural feature in such a protein that prevents formation of such a bond. Others (Fabianek *et al.* 2000) have proposed that formation of a disulphide in the apocytochrome *c* pre-folds the protein to facilitate haem attachment.

A recent investigation into cytochrome *c* biogenesis by the Ccm system has highlighted required roles for *both* of the cysteine residues in the apocytochrome haem-binding motif (Allen *et al.* 2002). As described above, *H. thermophilus* cytochrome c_{552}, which has a typical CXXCH haem-binding motif, and mutants with AXXCH and CXXAH motifs can, exceptionally, be expressed as stable holocytochromes in the cytoplasm of *E. coli* (see § 2a; Tomlinson & Ferguson 2000*b*). By targeting these proteins to the periplasm of *E. coli* using the signal peptide of a bacterial cytochrome *c* we have assessed the ability of the Ccm system to attach haem covalently to proteins with one or two cysteine residues in the haem-binding motif. Expression of the endogenous *E. coli* Ccm proteins was repressed by growing cells in aerobic conditions, but the Ccm proteins could be provided by their expression from a plasmid as required. Only the wild-type protein, with two cysteines, was effectively processed by the Ccm system and thus accumulated in the periplasm as a holocytochrome. With the signal sequence and the *ccm* plasmid present, essentially 100% of the wild-type cytochrome was periplasmic. By contrast, the mutant cytochromes were found in the periplasm only in small amounts even when the Ccm proteins were coexpressed (in each case less than 5% of the total cytochrome after subtractions for cytoplasmic contamination and endogenous cytochrome production by *E. coli*). Assessment of these data and the

relative yields of the cytochromes allowed estimation of the upper limit of the activity of the Ccm proteins towards the single-cysteine substrate apocytochromes as 2% of that towards the wild-type (CXXCH) apocytochrome *c*. These data (Allen *et al.* 2002), reporting at the level of the apocytochrome *c* rather than of the biogenesis proteins, are further strong evidence that an intramolecular disulphide bond involving the two cysteine residues of apocytochrome *c* is an intermediate in the Ccm type of cytochrome *c* biogenesis, and/or that the ultimate functional recognition determinant of the Ccm system requires the two cysteines in the haem-binding motif. An additional possibility is that both cysteine residues in the CXXCH motif have specific and required roles in the maturation pathway. It may be, for example, that the Ccm system is unable to process single cysteine apocytochromes *c* because two cysteine residues are required to release haem from holo-CcmE. Note, however, that any rationalization of these observations (Allen *et al.* 2002) must allow for the fact that the Ccm proteins are active with substrate apocytochromes that either have naturally, or have been mutated to have, CXXXXCH or CXXXCH binding motifs (Herbaud *et al.* 2000; Rios-Velazquez *et al.* 2001), and thus it is the two cysteines that are important, rather than their precise spatial arrangement. Also, the periplasmic protein DsbC from *E. coli* (and some other bacteria) has a CXXCH motif, but is not a *c*-type cytochrome (McCarthy *et al.* 2000); thus, the *c*-type cytochrome haem-binding motif on its own is not sufficient for covalent attachment of haem by the Ccm system. No (naturally occurring) cytochromes with haem attached through a single thioether bond have been observed to date in bacteria, which is consistent with the inability of the Ccm system to process them.

If the Ccm system is organized to handle a disulphide-bonded apocytochrome then an interesting point arises with respect to plant and some protozoal mitochondria. Unlike fungal, invertebrate and vertebrate cell mitochondria, these seem to use some components of the Ccm system (at least CcmA, CcmB, CcmC, CcmE and CcmF; Spielewoy *et al.* 2001). If the analogy is drawn with the operation of the Ccm system in bacteria, then one might expect that there would be an intramolecular disulphide in an apocytochrome *c* in the intermembrane space of plant mitochondria. There does not appear to be any evidence for or against this compartment being sufficiently oxidizing for this to occur. Note, however, that analogues of CcmG or CcmH, the potential disulphide reductases of the Ccm system, have not been identified to date in plant mitochondria (Spielewoy *et al.* 2001). The CcmG and CcmH proteins from the bacterium *Rhodobacter capsulatus* have been estimated to have $E^{\circ\prime}$ values of -300 and -210 mV, respectively, for reduction of their disulphide bonds; a peptide designed as a model for an apocytochrome *c* had an $E^{\circ\prime}$ value of -170 mV (Setterdahl *et al.* 2000). In plant mitochondria it may be that the $E^{\circ\prime}$ is such that CcmG and CcmH are not needed. Cytochromes *c* in which haem is attached to a single cysteine residue ((F/A)XXCH motif) have been isolated from some protozoan cells (Pettigrew *et al.* 1975; Brems & Stellwagen 1983; Priest & Hajduk 1992), but it is not yet known which cytochrome *c* biogenesis system is used by these organisms. If any eukaryote were found to have both the Ccm system and single-cysteine attachment of haem in a cytochrome *c* then, by implication, the Ccm system can be modified when it operates outside bacteria to cope with a single-cysteine haem-binding motif.

(d) *System II*

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

More recently Erlendsson & Hederstedt (2002) have argued that *B. subtilis* has counterparts of DsbA and DsbB, called BdbD and BdbC, respectively. It is suggested that in combination the latter proteins generate a disulphide in the CXXCH motif of a *c*-type cytochrome. Electrons would be provided subsequently by CcdA (a clear relative of DsbD) and probably a protein called ResA, which is a homologue of CcsX; the latter has been shown to be involved in *c*-type cytochrome synthesis in *B. pertussis* (above). Thus at present it appears that all bacterial *c*-type cytochrome synthesis involves formation of a disulphide within the CXXCH motif. However, this may not be obligatory in the case of the *Bacillus* system (system II); Erlendsson and Hederstedt have shown that *c*-type cytochrome synthesis can occur in the absence of both CcdA and the Bdb system. By contrast, it should be noted that the absence of both DsbA and DsbD from *E. coli* did not permit cytochrome *c* synthesis by the Ccm proteins (system I) (Metheringham *et al.* 1996). Furthermore Simon *et al.* (2002) have proposed that the *Wolinella succinogenes* system II biogenesis apparatus can covalently attach four haems to a variant of NrfH, a tetrahaem subunit of nitrite reductase, even when one of the CXXCH haem-binding motifs has been mutated to SXXCH.

It is worth making a few further remarks concerning the biogenesis of an unusual cytochrome *c*. NrfA is a periplasmic enzyme that reduces nitrite by six electrons to ammonia. It contains five covalently bound haems; four of these are bound through the typical CXXCH cytochrome *c* motif, but the active site haem is bound through a CXXCK motif. In *E. coli*, which uses the system I (Ccm) biogenesis apparatus, three additional biogenesis genes (*nrfEFG*) are dedicated to attaching the haem to this CXXCK motif (Eaves *et al.* 1998; Thöny-Meyer 2000). NrfE is an analogue of CcmF, NrfG of the C-terminal region of CcmH, and NrfF of the N-terminal region of CcmH. (Note that in some bacteria, the analogues of these two parts of *E. coli* CcmH are transcribed from separate genes (Thöny-Meyer 1997).) Interestingly, in *W. succinogenes*, which uses the system II cytochrome *c* biogenesis apparatus, it is proposed that haem attachment to NrfA requires the additional dedicated system II gene *nrfI*; this gene has no obvious homology to *nrfEFG* (Simon *et al.* 2000; Kranz *et al.* 2002; Pisa *et al.* 2002).

4. UNCATALYSED *IN VITRO* CYTOCHROME *C* FORMATION

We have recently achieved, to our knowledge, the first successful *in vitro* production of a cytochrome *c* in mild reaction conditions by reconstituting *H. thermophilus* apocytochrome c_{552} with haem (Daltrop *et al.* 2002*a*). Because this reaction occurred in the absence of any biosynthesis apparatus, it has provided much insight into the fundamental requirements for making this class of proteins and the *in vivo* processes that must occur. Apocytochrome *c* was produced by treatment of the holocytochrome with silver sulphate to remove the haem, followed by purification steps to remove the silver. *In vitro* cytochrome *c* formation occurred, provided formation of an intramolecular disulphide bond within the CXXCH motif of the apocytochrome was avoided (see § 3c,d for discussion of such a disulphide in cytochrome biogenesis by systems I and II). To our knowledge, this is the first direct observation of a disulphide bond in the haem-binding motif of an apocytochrome *c*. Formation of the disulphide bond induced additional structure in the apocytochrome as judged by circular dichroism spectra.

As well as reduction of the disulphide, reduced haem was required to ensure correct formation of the product holocytochrome *c* (i.e. to avoid side products); it is probable that haem must be reduced when any *c*-type cytochrome is made *in vivo*, as has already been deduced for fungal mitochondrial cytochrome *c* (Nicholson & Neupert 1989; Tong & Margoliash 1998). Formation of a *c*-type cytochrome in the absence of any biosynthesis apparatus (Daltrop *et al.* 2002*a*), a reaction believed to be impossible for the last 30 years, was substantiated by the characteristic absorption spectra and reduced alkaline pyridine haemochrome spectra, mass spectrometric analysis, activity staining of SDS–PAGE gels for covalently bound haem, an inability to extract non-covalently bound haem and a demonstration of the absence of free thiol groups in the protein. The success in forming holocytochrome c_{552} from apoprotein plus haem without catalysis *in vitro* makes it very likely that the original observation by Sanbongi *et al.* (1991) of the biogenesis of this protein in the cytoplasm of *E. coli* reflects the same uncatalysed reaction. Although the *in vitro* rate is relatively slow at room temperature, the *in vivo* rate is sufficient to produce cytochrome c_{552} in amounts that make the *E. coli* cells pink. Thus there would be sufficient cytochrome c_{552} for it to act functionally if required. Why then are *c*-type cytochromes not naturally made in the cytoplasm in a catalysed reaction? Folded holocytochromes *c* could be exported to the periplasm by the TAT system (Robinson & Bolhuis 2001; Sanders *et al.* 2001; Sargent *et al.* 2002). Perhaps this would work for mono-haem cytochromes *c*, but the multi-haem cytochromes *c*, with their clustered arrays of *c*-type centres, might need to be assembled via correctly formed intermediate disulphides. Otherwise, thioether bonds might form between a wrong pair of cysteines and the haem moiety. In general, disulphide bonds cannot form in the cytoplasm as it is a reducing environment. Therefore, if it is advantageous to assemble *c*-type cytochromes via disulphide bonds, this would provide a rationale for a periplasmic route (Ferguson 2001). To date, multihaem cytochromes *c* have only been found in organisms that use the Ccm and system II biogenesis systems (which as described in § 3c,d can interact with disulphide-bond-forming apparatus). By contrast, multihaem cytochromes *c* have not been identified in organisms that use the haem lyase biosynthesis system, for which the balance of evidence currently suggests that *in vivo* a disulphide bond does not form in the CXXCH haem-binding motif (see § 3).

In vitro formation of *H. thermophilus* cytochrome c_{552} (Daltrop *et al.* 2002*a*) proceeded via an intermediate in which the haem iron was coordinated by two amino-acid side chains from the protein but in which the haem was not covalently bound to the polypeptide (i.e. a *b*-type cytochrome), following which covalent (thioether) bonds between haem and protein formed spontaneously. An analogue of the *b*-type cytochrome intermediate formed on addition of reduced Fe-mesoporphyrin to reduced apocytochrome c_{552}. Mesoporphyrin has ethyl groups in the positions of the vinyl groups of protoporphyrin and therefore cannot form thioether bonds with the polypeptide. These observations imply, consistent with earlier reports (Dumont *et al.* 1994), that (at least some) apocytochromes *c* have a nascent haem-binding site. A consequence is that the eukaryotic mitochondrial haem lyase enzymes do not necessarily catalyse the covalent attachment of haem to protein. Instead, their roles could include stabilization of particular conformations of apoproteins, binding of haem and its subsequent presentation to the apocytochromes *c* (including control of the stereospecificity of haem attachment; see § 5) and maintenance of the haem-binding cysteines as free thiols. Nevertheless, the slow rates of *in vitro* cytochrome *c* formation we observed, from either haem plus apocytochrome *c* (Daltrop *et al.* 2002*a*) or haem-CcmE plus apocytochrome *c* (Daltrop *et al.* 2002*b*), indicate that catalytic agents are required *in vivo*, even if the fundamental nature of the *in vivo* reactions is the same.

(a) *Control of covalent haem attachment by cytochrome* c *biogenesis systems*

In naturally produced cytochromes *c* for which high-resolution structures have been obtained the haem is

always attached covalently to the two cysteines of the haem-binding motif with the same stereochemistry. The importance of this stereochemistry with respect to the physico-chemical properties of the cytochrome *c* is unclear. However, as discussed by Barker & Ferguson (1999), strictly controlled reaction conditions are needed to obtain a regio- and stereospecific attachment of haem to an apocytochrome *c*. This is illustrated in the work of Daltrop *et al.* (2002*a*) (described above), who reported that oxidizing conditions failed to produce the correct product cytochrome *c* not only because of the disulphide bond in the apoprotein but also because the ferric haem reacted incorrectly (an observation also made by Barker *et al.* 1993). The need for such controlled reaction conditions is also apparent from the observations of Keightley *et al.* (1998) on the cytoplasmic assembly in *E. coli* of a structurally different cytochrome c_{552}, from *T. thermophilus*. In the latter case, *in vivo* addition of haem in the cytoplasm to apoprotein lacking a periplasmic targeting sequence, which therefore prevented haem attachment by the periplasmically functioning Ccm apparatus of *E. coli*, generated a mixture of three major products. In one of these, the haem was rotated 180° around its α-, γ-axis and a single thioether bond formed between residue cysteine 14 and the two-vinyl group of haem whilst cysteine 11 formed an intermolecular disulphide bond (McRee *et al.* 2001). The latter cysteine normally bonds to the two-vinyl group. Clearly all cytochrome *c* biogenesis systems have to ensure that such misattachment does not occur. Note that when *T. thermophilus* cytochrome c_{552} was coexpressed in the periplasm of *E. coli* with the Ccm proteins, the product was essentially indistinguishable from that made by *T. thermophilus* itself (Fee *et al.* 2000).

5. MECHANISTIC INSIGHTS INTO CYTOCHROME *C* MATURATION

Mechanistically, formation of the thioether bonds in cytochromes *c* is very interesting. It requires protonation of the β-carbon of the haem vinyl group and thioether bond formation between the cysteine sulphur from the polypeptide and the haem vinyl α-carbon. Recent experiments (Daltrop *et al.* 2002*a*) indicate that protonation is followed by (nucleophilic) thioether bond formation. Activation by reduction of the haem iron is required, both to prevent formation of dangerously reactive thiyl radicals (Barker *et al.* 1993) and also, presumably, to render the β-carbon of the vinyl group more prone to protonation and the α-carbon more electropositive, and hence primed for nucleophilic attack. Another important feature of the *c*-type cytochrome thioether bonds is the regio- and stereospecificity of the haem–protein attachment (see § 4); the mechanism of how biological systems create the stereospecificity is unresolved but intriguing. In the case of the fungal, invertebrate and vertebrate mitochondrial system, the proposed mechanism of haem attachment involves binding of the haem by the haem lyase (Steiner *et al.* 1996). In the light of recent experimental findings (Daltrop *et al.* 2002*a*), it is plausible that a pre-oriented haem is presented stereoselectively by the haem lyase to the apocytochrome *c*. Given that thioether bond formation can proceed uncatalysed in the *b*-type cytochrome formed from apoprotein and haem (Daltrop *et al.* 2002*a*), we can speculate that haem lyase may also function by creating a conformation of this *b*-type cytochrome which is reactive with respect to thioether bond formation. The stereoselectivity would be achieved through the correct orientation of the prochiral vinyl groups relative to the attacking thiol moieties of the cysteine residues of the conserved binding motif.

The catalytic mechanism of the bacterial Ccm system is even more complex; the mechanism of the haem transfer to apocytochrome *c* is poorly understood. One of the key aspects of the Ccm system is the attachment of haem to the haem chaperone CcmE, which involves the formation of a novel histidine–haem bond (Schulz *et al.* 1998). A plausible mechanism is the nucleophilic addition of a histidine nitrogen to the α-carbon of a haem vinyl group, after the β-carbon has been protonated. Analogous to thioether bond formation, this reaction requires the haem iron to be ferrous (Daltrop *et al.* 2002*b*). Very recently, it has been reported that a covalent bond can form spontaneously in reducing conditions between a histidine residue and the α-carbon of the two-vinyl group of haem in haemoglobin from the cyanobacterium *Synechocystis* sp. PCC 6803 (Vu *et al.* 2002). The reaction did not occur when redox inert zinc protoporphyrin IX was used, further implicating the iron atom as crucial for such chemistry. One possible implication of covalent attachment of haem to CcmE for the Ccm process is that the haem attachment occurs stereoselectively (Schulz *et al.* 1998) and thereby the release of haem from CcmE to the apocytochrome *c* occurs stereospecifically. Release of haem from the haem chaperone to apocytochrome *c* may be favourable in terms of the stability of the reduced cytochrome *c* produced relative to holo-CcmE, as the haem chaperone favours the binding of oxidized, relative to reduced, haem (Daltrop *et al.* 2002*b*) (note that the oxidation state of haem after its translocation to the periplasm is unknown). In addition, the histidine that forms the covalent bond to haem in holo-CcmE might act as a good leaving group, accelerating the rate of holocytochrome *c* formation; in the case of the *Synechocystis* haemoglobin discussed above, the histidine–haem bond is labile (Vu *et al.* 2002).

6. PERIPLASMIC HAEM DELIVERY IN GRAM-NEGATIVE BACTERIA

Delivery of haem to the periplasm is an issue for both cytochrome *c* and periplasmic *b*-type cytochrome formation and the means of delivery could be different, and specific, for each of these classes of protein. How the cytoplasmically made haem is transported to the periplasm for bacterial cytochrome *c* biosynthesis is unknown, but, as described above, there is scarce evidence it has anything to do with the ATP-dependent transporter of the Ccm system (where that system is used) and some to the contrary. It is also probable that another ATP-dependent transporter, CydDC, does not, despite earlier proposals, transport haem across the cytoplasmic membrane of *E. coli* (Cook & Poole 2000). Cook & Poole (2000) discuss extensively the conflicting evidence about haem transport across the membrane by such mechanisms as energy-dependent transport, specific and non-specific diffusion processes and processes where periplasmic apocytochromes 'pull' haem out of the lipid bilayer. For system

II cytochrome *c* biogenesis, it has been proposed that the non-ATP-dependent protein CcsA, possibly in complex with CcsB, transports the haem across the membrane for attachment to apocytochrome *c* (Kranz *et al.* 2002). *B*-type cytochromes are rare in the bacterial periplasm; an inability of the cytochrome *c* biogenesis systems to process (or perhaps not to interfere with synthesis of) proteins with non-covalently bound haem could, in part, account for this rarity. However, cytochrome b_{562} can form in large quantities in the periplasm of *E. coli* irrespective of the extent of expression, or disruption, of the *ccm* genes (Goldman *et al.* 1996; Throne-Holst *et al.* 1997; Allen *et al.* 2002). Naturally occurring periplasmic *b*-type cytochromes (e.g. b_{562}, dimethyl sulphide oxidase) have Sec signal sequences, so the haem does not become bound to the protein in the cytoplasm, as was also the case with experiments where exogenous cytochromes were periplasmically targeted (Karim *et al.* 1993; Kaderbhai *et al.* 2000). Since they are rare, it is possible that the specific nature of the periplasmic apocytochrome *b* may be important for the process of haem acquisition. The non-covalently bound d_1 haem of the cytochrome cd_1 type nitrite reductases is transported to the periplasm by a specific transporter that uses the TAT system (Heikkila *et al.* 2001), but it should be noted that this haem is significantly more biosynthetically demanding than protoporphyrin IX and is only found in cytochromes cd_1.

7. CONCLUSIONS

Various explanations may be offered for the existence of cytochromes *c*, proteins in which haem is covalently bound to polypeptide through thioether bonds, almost always with the two cysteines of a CXXCH peptide motif. Comparisons with *b*-type cytochromes indicate that greater stability is conferred on the protein by this mode of haem attachment. Analysis of multi-haem cytochromes shows that covalent attachment allows dense packing of haems in an economical polypeptide. The complexity and diversity of cytochrome *c* biogenesis systems is intriguing and in itself indicates that there are strong evolutionary imperatives for formation of this class of proteins. An explanation of why different systems have evolved for cytochrome *c* biogenesis might, in part, be offered by analysis of the oxidizing environment of the locus of cytochrome *c* maturation. Two vital aspects are the accessibility of free thiol functionalities of the cysteine residues, which have been shown to be able to form an internal disulphide bond, and the ferrous oxidation state of the haem. The interaction of apocytochrome *c* with disulphide-bond-forming systems, particularly in bacteria, may account for the need for two haem-binding cysteine residues, even though one such bond confers almost the same biophysical advantages on the product cytochrome as two.

This work was supported by grants C11888 and C13433 from the BBSRC to S.J.F. J.W.A.A. is the W. R. Miller Junior Research Fellow, St Edmund Hall, Oxford. O.D. is in receipt of a University of Oxford scholarship in association with St Edmund Hall, Oxford. J.M.S. gratefully acknowledges funding from the BBSRC.

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GLOSSARY

Ccm: cytochrome *c* maturation
Sec: type II secretion
TAT: twin arginine translocation