Variation of the axial haem ligands and haem-binding motif as a probe of the *Escherichia coli c*-type cytochrome maturation (Ccm) system

James W. A. ALLEN and Stuart J. FERGUSON[1]

Department of Biochemistry, University of Oxford, South Parks Road, Oxford, OX1 3QU, U.K.

Cytochromes *c* are typically characterized by the covalent attachment of haem to polypeptide through two thioether bonds with the cysteine residues of a Cys-Xaa-Xaa-Cys-His peptide motif. In many Gram-negative bacteria, the haem is attached to the polypeptide by the periplasmically functioning cytochrome *c* maturation (Ccm) proteins. Exceptionally, *Hydrogenobacter thermophilus* cytochrome c_{552} can be expressed as a stable holocytochrome both in the cytoplasm of *Escherichia coli* in an apparently uncatalysed reaction and also in the periplasm in a Ccm-mediated reaction. In the present study we show that a $Met^{60} \rightarrow Ala$ variant of c_{552}, which does not have the usual distal methionine ligand to the haem iron of the mature cytochrome, can be made in the periplasm by the Ccm system. However, no holocytochrome could be detected when this variant was expressed cytoplasmically. These data highlight differences between the two modes of cytochrome *c* assembly. In addition, we report investigations of haem attachment to cytochromes altered to have the special Cys-Trp-Ser-Cys-Lys haem-binding motif, and Cys-Trp-Ser-Cys-His and Cys-Trp-Ala-Cys-His analogues, of the active-site haem of nitrite reductase NrfA.

Key words: biogenesis, cytochrome *c* maturation, haem co-ordination, *Hydrogenobacter thermophilus*, nitrite reductase.

INTRODUCTION

Cytochromes *c*, a class of proteins essential for the life of almost all organisms, are typically characterized by the covalent attachment of haem to polypeptide through two thioether bonds with the cysteine residues of a Cys-Xaa-Xaa-Cys-His peptide motif [1–3]. (Peptide motifs, after their first mention, are referred to subsequently using the single-letter amino acid code.) There are, however, a few exceptions. In some bacterial cytochromes *c*, either naturally or as the result of site-directed mutagenesis, the attachment is through a Cys-$(Xaa)_3$-Cys-His or Cys-$(Xaa)_4$-Cys-His motif [4,5]. In the mitochondria of some kinetoplastid species, the attachment is through a (Phe/Ala)-Xaa-Xaa-Cys-His motif, resulting in only one thioether bond between haem and protein [6,7]. Generally, in cytochromes *c* the haem iron is six-co-ordinate with, in the vast majority of cases, His/Met or His/His axial ligation [3,8]. However, there are exceptions here too. For example, the haem in cytochrome c', as found in denitrifying bacteria such as *Rhodobacter capsulatus*, has His/vacant co-ordination [9,10]. In the bacterial pentahaem nitrite reductase enzyme (NrfA), four haems are attached covalently to the polypeptide through typical CXXCH cytochrome *c* motifs, but the active-site haem is attached through a Cys-Trp-(Ser/Thr/Asn)-Cys-Lys motif (CWSCK in *Escherichia coli*; as determined from BLAST data, see also [11,12]).

Despite the essential roles of cytochromes *c* in biology, it is not clear either how, or why, the haem becomes attached covalently to the polypeptide; it is not covalently bound in most haemoproteins [3]. Remarkably, three different *c*-type cytochrome biogenesis systems have been identified to date [1,8,13]. Many Gram-negative bacteria use a system known as the cytochrome *c* maturation (Ccm) proteins (in *E. coli*, these proteins are CcmABCDEFGH [14]). These Ccm proteins are located in the periplasm and/or cytoplasmic membrane; all bacterial cytochromes *c* are either periplasmic or on the external face of this membrane [1,8,13]. The Ccm system has been shown to catalyse holocytochrome *c* formation from a wide variety of apocytochromes of both prokaryotic and eukaryotic origin, resulting in product cytochromes *c* with very divergent structures (e.g. [12,15–17]). The features of the apocytochrome that are recognized and required by the Ccm system remain unidentified, although it has been shown that the Ccm system does not form cytochromes *c* altered by site-directed mutagenesis to have only a single cysteine residue in the haem-binding motif (i.e. with AXXCH or CXXAH motifs) [18]. It is known that attachment of haem to the unusual CW(S/T/N)CK motif in the NrfA protein of organisms such as *E. coli*, which use the Ccm system, requires the presence of the additional proteins NrfEFG [12]. However, it is not clear precisely why the latter are required, how (functionally) they are different from their homologues CcmF and CcmH, or where the specificity arises for one set of biogenesis proteins over the other.

In the present work, we investigate the flexibility of the *E. coli* Ccm system with respect to substrate apocytochromes that have been mutated in the CXXCH motif and/or in the residue that provides the distal ligand to the haem iron. Two monohaem c_2-type cytochromes have been used in these experiments. They are *Paracoccus denitrificans* cytochrome c_{550} and *Hydrogenobacter thermophilus* cytochrome c_{552}. Both of these cytochromes *c* can be made in large amounts in the periplasm of *E. coli* when co-expressed with the *E. coli* Ccm proteins from a plasmid [18–20]. *H. thermophilus* c_{552}, a cytochrome of thermophilic origin, is remarkable in that, in addition, the haem may be covalently (and correctly) attached to the protein in the cytoplasm of *E. coli*, apparently without the action of any specialized biosynthesis proteins [20–22]. To provide additional insight into

Abbreviations used: Ccm, cytochrome *c* maturation; H15M, mutant bearing a replacement of His^{15} with methionine, etc.; IPTG, isopropyl β-D-thiogalactoside.

[1] To whom correspondence should be addressed (e-mail stuart.ferguson@bioch.ox.ac.uk).

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the fundamental nature of cytochrome *c* biogenesis, we therefore also report investigations of cytoplasmic formation of variants of *H. thermophilus* cytochrome c_{552}.

EXPERIMENTAL

Plasmid construction

Site-directed mutagenesis was carried out using the Quikchange method (Stratagene). In order to construct *H. thermophilus* cytochrome c_{552} mutants, plasmid pEST211 [18], which expresses the gene for a variant of cytochrome c_{552}, was first mutated to give the gene for the wild-type protein in a pKK223-3 (Amersham)-based expression vector (plasmid JA6-3). Thus, pJA6-3 encodes the gene for wild-type *H. thermophilus* cytochrome c_{552} fused with the periplasmic targeting sequence of *Pseudomonas aeruginosa* cytochrome c_{551}. This plasmid was used as the template for further mutations of periplasmically directed cytochrome c_{552}, specifically to produce the variants $His^{15} \rightarrow Met$ (H15M, which has a CXXCM haem-binding motif; pJA55), H15K (with a CXXCK motif; pJA59) and M60A (pJA50). Variants of *H. thermophilus* cytochrome c_{552} without a periplasmic targeting sequence (i.e. for expression in the cytoplasm) were based on pKHC12 [22]. Mutants H15A (pJA20), H15M (pJA14), H15K (pJA46) and M60A (pJA11) were constructed. *P. denitrificans* c_{550} mutants were made starting with pKPD1 as the template; this pKK223-3-based plasmid encodes the gene for wild-type cytochrome c_{550}, including its natural periplasmic signal sequence [22]. Wild-type c_{550} has a CKACH haem-binding motif. CWACH (pJA81), CWSCH (pJA87) and CWSCK (pJA90) variants were constructed. Each of the new plasmids made for this work was DNA sequenced at least twice to ensure that (where applicable) the periplasmic targeting sequence was present, that the desired mutations were present, and that there were no secondary mutations in the cytochrome gene. The ampicillin-resistance-conferring cloning vector pTZ19r was purchased from MBI Fermentas (Vilnius, Lithuania).

Note that the residue numbering used throughout this work for *H. thermophilus* cytochrome c_{552} is that for the cytoplasmically produced protein. This form carries an extra methionine residue at the N-terminus relative to the periplasmically produced protein. Thus, we refer to the haem-binding cysteine residues as Cys^{11} and Cys^{14}, and to the haem axial ligands as His^{15} and Met^{60}; in the periplasmically produced (and native) protein (see [23] for the NMR structure), these residues are Cys^{10}, Cys^{13}, His^{14} and Met^{59}.

Bacterial growth

E. coli strain JCB387 [24] was transformed with the appropriate cytochrome-encoding plasmids, and used for all growths unless stated. As appropriate, cells could also be transformed with pEC86 [25] encoding the *E. coli* cytochrome *c* maturation genes *ccmABCDEFGH* and pJG124, kindly given by Professor Jeff Cole (University of Birmingham, Birmingham, U.K.), which carries the genes for NrfEFG [26]. Transformants were initially grown on Luria Broth (LB)–agar plates with the appropriate antibiotics (100 $\mu g \cdot ml^{-1}$ ampicillin in each case, plus 34 $\mu g \cdot ml^{-1}$ chloramphenicol where pEC86 was co-transformed, and 25 $\mu g \cdot ml^{-1}$ tetracycline where pJG124 was used). Unless stated otherwise, single colonies were picked into 500 ml $2 \times$ TY medium supplemented with 1 mM IPTG (isopropyl β-D-thiogalactoside) in 2.5-litre flasks. Such cultures were grown at 37 °C with shaking at 200 rev./min for 20–28 h, before harvesting. Where *E. coli* was grown anaerobically, single colonies were picked from agar plates into 50 ml tubes filled with the minimal medium described by Sambongi and Ferguson [27], supplemented with ampicillin and IPTG as appropriate, and sealed tightly. These cultures were grown for 20 h at 37 °C without agitation.

Cell fractionation and biochemical procedures

Periplasmic and cytoplasmic fractions were obtained from cells using the procedures described previously [18]. Cytochrome content was determined by recording absorption spectra of the crude periplasmic and cytoplasmic fractions, to which a few grains of solid disodium dithionite had been added. Absorbance measurements of cell extracts were corrected because of the presence of endogenous *E. coli* cytochromes (see the Results section and [18]). Periplasmic and cytoplasmic fractions were assayed using the marker enzymes β-lactamase and malate dehydrogenase respectively [28–30]. *P. denitrificans* cytochrome c_{550} variants were purified using DEAE-Sepharose and Q-Sepharose chromatography columns, under the conditions employed by Richter et al. [19]. Electrospray-ionization MS was performed on a Micromass Bio-Q II-2S triple–quadrupole atmospheric pressure instrument equipped with an electrospray interface. Samples were introduced via a loop injector as a solution [20 $pmol \cdot \mu l^{-1}$ in a 1:1 (v/v) mixture of water and acetonitrite containing formic acid (0.2 % final concentration)] at a flow rate of 10 $\mu l \cdot min^{-1}$.

RESULTS

H. thermophilus cytochrome c_{552} variants (periplasmic expression)

In wild-type *H. thermophilus* cytochrome c_{552}, the haem is covalently attached to the polypeptide via thioether bonds to the cysteine residues of a Cys-Met-Ala-Cys-His motif [23]. In order to test the substrate flexibility of the Ccm system, *H. thermophilus* cytochrome c_{552} variants were constructed with alterations to the distal (Met^{60}) and proximal (His^{15}) haem axial ligands. An H15M variant was designed to test the strict requirement for histidine in the CXXCH cytochrome *c* motif; since methionine is, like histidine, commonly a strong field ligand to haem in cytochromes *c*, it was considered that it might be able to substitute for histidine during cytochrome *c* maturation. Barker and co-workers [31,32] have previously produced a variant of *E. coli* cytochrome b_{562} in which the proximal histidine ligand was replaced by methionine. Their variant cytochrome b_{562} product was stable, and the haem iron was *bis*-methionine-co-ordinated under certain conditions. An H15K variant of *H. thermophilus* cytochrome c_{552} was constructed as a model of the active-site haem in NrfA, which has a CXXCK motif. An M60A variant was constructed to investigate the requirement for a distal ligand to the haem iron during cytochrome *c* biogenesis. Each of these proteins was fused with a periplasmic targeting sequence: the latter enables translocation of the polypeptide to the periplasm, where the Ccm system operates.

Under the growth conditions employed in this work, *E. coli* produces endogenous *b*-type cytochromes in the cytoplasm and small amounts of endogenous *c*-type cytochromes in the periplasm (see, for example, [18]). To provide reference data for the level of this background cytochrome expression, *E. coli* JCB387 cells were transformed and grown with pTZ19r (an ampicillin-resistance-conferring plasmid) and pEC86. The absorbances of the periplasmic and cytoplasmic fractions, normalized by fraction volume and wet-cell weight and averaged over several growths, were taken to represent 'typical' endogenous (background) cytochrome expression levels. This cytochrome

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yield cannot be expressed directly, because it represents a mixture of components with different, and in some cases undetermined, molar absorption coefficients (ε). However, the endogenous cytochrome production can be expressed in terms of absorbance (which is directly proportional to the concentrations of the species present). The absorbance of the periplasmic fraction (prepared using our routine growth and fractionation procedures) was 0.026 absorbance units (mean average for the Soret band at 418 nm, with a standard deviation of 0.004 absorbance unit)/g of wet cells. Where yields of periplasmic exogenous cytochromes have been presented in the present work, this endogenous cytochrome absorbance has been subtracted from the actual observed absorbance to determine the expression level. Analogous calculations were made on the basis of the absorbance of the cytoplasmic fraction of the control samples, when investigating cytoplasmic cytochrome expression (see below).

The periplasmic fraction from cells co-transformed with plasmids for both the M60A variant of *H. thermophilus* cytochrome c_{552} and the Ccm proteins contained a significantly elevated level of cytochrome, as judged by a mean 10-fold increase in Soret band absorption, relative to the control (pTZ19r-transformed) cells. Absorption spectra of the crude (unpurified) periplasmic extract of such cells are shown in Figure 1. The main product cytochrome clearly has a high-spin haem-iron atom. As is observed for cytochrome c' [10], the spectrum of the reduced protein (Figure 1A) has a single (combined) α and β absorption band around 550 nm. (Low-spin, six-co-ordinate *c*-type cytochromes have resolved α and β absorption bands in this region.) Note that Figure 1(A) shows an aliquot of periplasmic extract to which a few grains of dithionite had been added. Comparison of this with the 'as isolated' periplasmic extract showed that the cytochrome c_{552} M60A was essentially fully reduced before the addition of dithionite. Thus the protein was probably reduced in the cell, and was not air-oxidized during the cell-fractionation procedure. In this example (Figure 1A), the endogenous *E. coli* cytochromes (see above) account for approx. 0.05 absorbance unit in the Soret band of the spectrum, i.e. < 10 % of the total absorbance. The absorption spectrum of ferricyanide-oxidized c_{552} M60A variant (Figure 1B) has its Soret absorption maximum at 401 nm (cf. 410 nm for wild-type c_{552}). On addition of cyanide, a ligand that typically binds strongly to ferric haem iron, the Soret peak of the oxidized protein shifts to 414 nm, indicative of both a co-ordination and spin-state change (Figure 1B). Dithionite reduction of the c_{552} M60A–cyanide complex results in a spectrum typical of a low-spin six-co-ordinate *c*-type cytochrome (Figure 1A), again very distinct from that of the protein in the same oxidation state in the absence of cyanide.

Addition of an aliquot of the periplasmic extract of cells transformed with the cytochrome c_{552} M60A and *ccm* plasmids to a pyridine/hydroxide solution results in a spectrum with a resolved absorption peak at 549.5 nm (Figure 2). This observation, which is independent of the normal proteinaceous ligands to the haem iron, is extremely characteristic of the formation of a *c*-type cytochrome, in which the haem is covalently attached to the polypeptide via two thioether bonds [33].

The periplasmic and cytoplasmic fractions of the cells were assayed for contamination by the other cell compartment using marker enzyme assays (see the Experimental section), which indicated that the periplasmic fraction contained approx. 95 % of the periplasmic proteins, but < 4 % of the cytoplasmic proteins. These data, in combination with the spectra of the periplasmic and cytoplasmic fractions, are a clear indication that the M60A cytochrome c_{552} was being made in the periplasm of the *E. coli* cells, rather than as the result of spontaneous (uncatalysed) formation in the cytoplasm. The molar absorption coefficient for

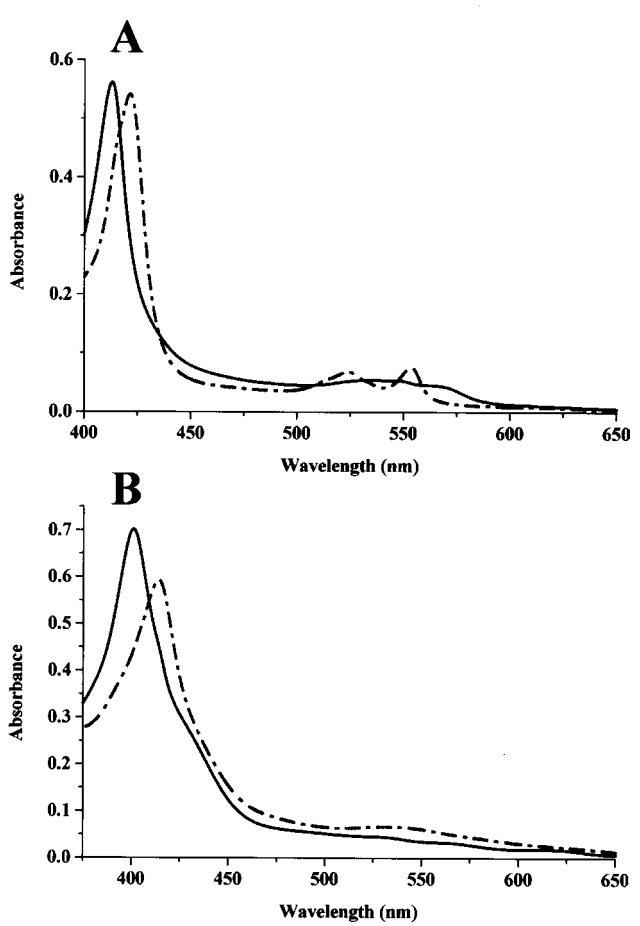

Figure 1 The periplasmic extract of cells of *E. coli* strain JCB387 transformed with plasmids for the *E. coli* Ccm system and the M60A variant of *H. thermophilus* cytochrome c_{552}

All spectra were recorded at 25 °C with the periplasmic proteins in SET spheroplasting buffer diluted 1:1 with water (final concentrations 100 mM Tris/HCl, pH 8.0, 0.5 mM EDTA and 0.25 M sucrose). (**A**) Dithionite-reduced periplasmic extract (continuous line) and dithionite reduced periplasmic extract in the presence of KCN (dot–dashed line). (**B**) Periplasmic extract oxidized with a small excess of potassium ferricyanide (continuous line) and ferricyanide oxidized periplasmic extract in the presence of KCN (dot–dashed line).

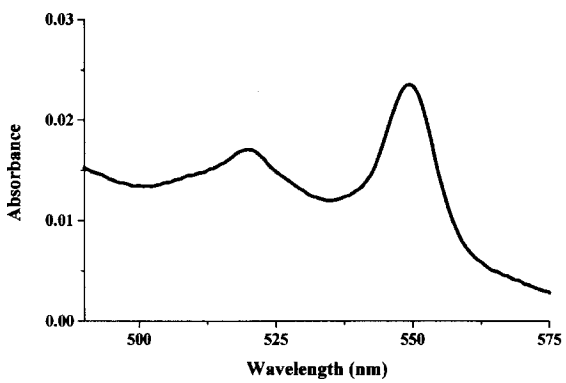

Figure 2 Reduced pyridine haemochrome spectrum of the periplasmic extract of cells of *E. coli* strain JCB387 transformed with plasmids for the *E. coli* Ccm system and the M60A variant of *H. thermophilus* cytochrome c_{552}

Final concentrations of hydroxide and pyridine were 0.2 M and 30 % (v/v) respectively.

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the M60A cytochrome c_{552} is unknown, but taking a nominal value of 100 mM · cm^{-1} for the reduced protein's Soret band (cf. cytochrome c' [10]), the yield of the cytochrome variant in crude periplasmic fractions is approx. 1 mg/g of wet cells (equivalent to approx. 8 mg per litre of cell culture).

In the absence of expression of the Ccm system (i.e. in cells grown without pEC86), the observed periplasmic yield of *H. thermophilus* cytochrome c_{552} M60A was approx. 16 % of that when pEC86 was present. This is due to a low-level background expression of the endogenous *E. coli* Ccm proteins under our growth conditions, and is quantitatively very similar to the relative yields of wild-type cytochrome c_{552} that were observed previously in the presence and absence of the plasmid-expressed Ccm apparatus [18].

Together, these data show unambiguously that the bacterial Ccm system can make periplasmically, in substantial quantities, a stable M60A variant of *H. thermophilus* cytochrome c_{552}. The product is clearly a *c*-type cytochrome with two covalent linkages between haem and protein. The haem iron is high-spin and either five-co-ordinated or six-co-ordinated by one protein ligand and a water (or hydroxide) from the solvent. The product is clearly very distinct from His/Met-co-ordinated wild-type cytochrome c_{552}.

In contrast, when *E. coli* JCB387 was transformed with plasmids with the genes for H15M (CXXCM haem-binding motif) and H15K (CXXCK) variants of *H. thermophilus* cytochrome c_{552}, either with or without the *ccm* plasmid, no statistically significant increase in cytochrome production relative to the control cells was observed. The *E. coli* nitrite reductase, NrfA, has a CXXCK haem-binding motif at its active site haem, but covalent attachment of this haem requires the proteins NrfEFG in addition to the Ccm system [12,26]. Thus cells were also co-transformed with either H15M or H15K cytochrome c_{552}, the *ccm* plasmid (pEC86) and a plasmid from which NrfEFG are constitutively expressed (pJG124) from the *galE* promoter [26]. However, even in the presence of the Ccm proteins and NrfEFG, no increase in cytochrome production relative to background could be detected for either the H15M or H15K variants of cytochrome c_{552}. Experiments were repeated multiply. Variant cytochrome genes were DNA sequenced in duplicate. Cell enzyme-marker assays were used to show that the periplasmic fractions typically contained > 90 % of the periplasmic proteins, and thus that the failure to observe cytochrome variants was not due to improper cell fractionation.

H. thermophilus cytochrome c_{552} variants (cytoplasmic expression)

In the absence of a signal sequence to direct the apoprotein to the periplasm, wild-type *H. thermophilus* cytochrome c_{552} and variants such as C11A (AXXCH haem-binding motif), C14A (CXXAH) and C11A/C14A (AXXAH) will form stable cytochromes, with thioether bonds where cysteine residues are present, in an apparently uncatalysed reaction in the cytoplasm of *E. coli* [21,34]. This characteristic is exceptional, and is believed to result from the structured nature of the c_{552} apocytochromes [21,35]. We wished to exploit this property, and so constructed plasmids with the genes for H15M, H15K, H15A and M60A variants of cytoplasmically directed cytochrome c_{552} with the intention that these would provide comparative data for our periplasmic (Ccm-dependent) expression studies. However, on growing and fractionating transformed cells, no significant increase in cytochrome expression was, within errors, observed by spectroscopic means relative to control (pTZ19r-transformed) cells for any of the variants. Pyridine haemochrome analysis of the cell extracts did not indicate any increase in the proportion of *c*-type cytochromes present relative to the control samples. In the case of the M60A variant, the soluble extract of the cells harvested from 4 litres of liquid culture was applied to a CM-52 cation-exchange column and eluted with a linear gradient from 0–0.5 M NaCl. This is the first (and most significant) purification step for wild-type cytochrome c_{552} [21]. However, no cytochrome that could be the M60A variant of c_{552} was detected spectrophotometrically in the eluent fractions.

Where wild-type c_{552} or the C11A, C14A or C11A/C14A variants were directed to the periplasm in previous studies, some accumulation of each was also observed in the cytoplasm, i.e. haem binding (and hence protein folding) in the cytoplasm and apoprotein transportation across the cytoplasmic membrane by the Sec apparatus appeared to be kinetically competitive processes [18]. However, in the present work, when CXXCK or CXXCM variants of cytochrome c_{552} were directed to the periplasm with a signal sequence there was no evidence for accumulation of holoforms of these variants in the cytoplasm.

Given the previously observed stability of c_{552} variants, and the tolerance of the protein for changes around the haem-binding motif [21,34], these data are somewhat surprising. They indicate an important difference in respect of cytochrome formation and/or stability between changes to the haem-binding cysteine residues and changes to the haem-iron ligands. Of particular note is that lack of cytoplasmic expression of the M60A variant. As described above, c_{552} M60A is stable and made in large quantities by the Ccm system in the periplasm of the same strain of *E. coli* under the same growth conditions.

Use of *P. denitrificans* cytochrome c_{550} to probe the special haem-binding motif of NrfA

NrfA, the nitrite reductase that reduces nitrite by six electrons to ammonia, contains five haems. Four of these have 'typical' CXXCH haem-binding motifs, but the fifth has a conserved C-W-(S/T/N)-C-K motif (CWSCK in *E. coli*), as determined from BLAST data (also see [36]). As described above, attachment of haem to this special motif requires the extra proteins NrfEFG in addition to the Ccm proteins [26]. However, it is not clear what causes the specificity of action, or substrate recognition, of the Nrf proteins. It may simply be the lysine residue of the CXXCK motif, but it may also be the conserved residues between the cysteine residues. It is, to date, very rare to find a tryptophan after the first cysteine in cytochromes *c* made by the Ccm system (BLAST data), although such proteins are fairly common in bacteria that use the alternative system II cytochrome *c* biogenesis apparatus. It was reasoned that this tryptophan, or a combination of this residue followed by a polar residue, might inhibit the Ccm system. For example, the bulky tryptophan side chain may prevent formation of a disulphide bond between the cysteine residues of the CWXCK motif; such a disulphide is a postulated requirement for the action of the Ccm system (for reviews, see [8,37]).

To test this hypothesis, variants of *P. denitrificans* cytochrome c_{550} were constructed. This mesophilic cytochrome was used as the 'scaffold' in case the failure to observe covalent attachment of haem to the CXXCK motif in *H. thermophilus* cytochrome c_{552} (see above) was a consequence of the particular structural rigidity of the latter, thermostable protein (e.g. if there was a steric intolerance of the lysine side chain). Wild-type *P. denitrificans* c_{550} has a CKACH haem-binding motif; the variants had CWACH, CWSCH and CWSCK motifs. As expected [19], the wild-type protein was produced in large quantities in the *E. coli* periplasm when co-transformed with the *ccm* plasmid (mean yield in the present work of 2.3 mg cytochrome c_{550}/g

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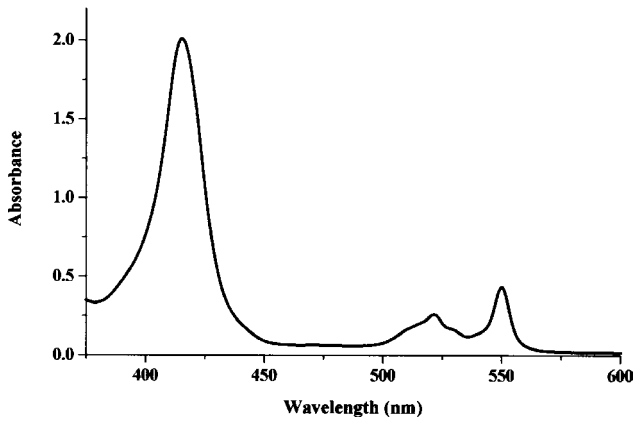

Figure 3 Absorption spectrum of the periplasmic extract of cells of *E. coli* strain JCB387 transformed with plasmids for the *E. coli* Ccm system and a CWSCH haem-binding motif variant of *P. denitrificans* cytochrome c_{550}

The spectrum was recorded at 25 °C following the addition to a few grains of dithionite to the periplasmic proteins in 100 mM Tris/HCl, pH 8.0, 0.5 mM EDTA and 0.25 M sucrose.

of wet cells). The same qualitative observation was made when the CWACH and CWSCH (Figure 3) variants were co-expressed with the Ccm proteins. Both were obtained in yields comparable with that of the wild-type protein (CWACH motif, approx. 1 mg cytochrome c_{550}/g of wet cells; CWSCH motif, approx. 2 mg cytochrome c_{550}/g of wet cells). In order to verify the presence of the desired mutations in the substrate cytochromes, they were purified to homogeneity and analysed by electrospray-ionization MS. The CWACH variant had an observed mass of 15085 Da (calculated mass from the sequence, 15086 Da; cf. calculated mass of wild-type c_{550}, 15028 Da); the CWSCH variant had a mass of 15101 Da (calculated 15102 Da).

In order to show definitively that the CWACH and CWSCH cytochrome c_{550} variants had been matured by the Ccm system, cells of *E. coli* strain JCB387 transformed with the plasmid for either of these cytochromes (but not the *ccm* plasmid) were grown under otherwise identical conditions. The mean yield of each of the exogenous cytochromes was only approx. 5% of that when the Ccm proteins were co-expressed from pEC86 (see the results described above). This low level of expression is consistent with the previously observed background activity of the endogenous *E. coli* Ccm proteins under our cell-culture conditions (see section above and [18]), which presumably arises because the growth flasks are not fully aerobic. When either of these c_{550} variants was expressed in a different (non-isogenic) *E. coli* strain, JCB71202 [38], from whose chromosome the *ccm* genes have been deleted, no *c*-type cytochrome production was observed. Thus the cytochrome c_{550} variants with CWACH or CWSCH haem-binding motifs that partially model the CWSCK motif of the active site of NrfA are matured by the *E. coli* Ccm system, but not in its absence. Expression of the cytochrome c_{550} variants was restored in cells of the *ccm*-deletion strain JCB71202 transformed with plasmids for either of the variant cytochromes and the *ccm* genes. The main product cytochrome observed in the periplasmic fractions of such cells had the spectral characteristics of the cytochrome c_{550} variants (cf. Figure 3).

In contrast with the CWACH and CWSCH variants of cytochrome c_{550}, no CWSCK holocytochrome could be detected following aerobic growth of cells co-transformed with the appropriate plasmid and the *ccm* genes expressed from pEC86. The same observation, within experimental errors, was made for cells transformed with the plasmid for the CWSCK variant of cytochrome c_{550}, the *ccm* genes and pJG124 (NrfEFG). Cells of *E. coli* strain JCB387 transformed with the plasmid for CWSCK *P. denitrificans* cytochrome c_{550} (or CXXCK *H. thermophilus* cytochrome c_{552}) were also grown anaerobically with nitrate and fumarate as terminal electron acceptors. Under such conditions, the endogenous Ccm and Nrf biogenesis systems of *E. coli* are active, and NrfA is expressed and matured [36] to enable respiration using the nitrite generated from reduction of nitrate. Under these growth conditions, no increase in the total periplasmic cytochrome expression level was observed for either of our CXXCK haem-binding motif variants relative to untransformed *E. coli* JCB387 cells. The wild-type and variant transformed cells did produce a cytochrome c_{552} spectrally similar to NrfA [36]. Owing to the elevated background cytochrome absorbance from NrfA (and also NapB), one cannot formally conclude that no CXXCK cytochrome c_{550} (or c_{552}) was present in the periplasm, having been made by the Nrf and Ccm systems, but it is clear that the extent of any such maturation of the variants was minimal and beneath the limit of detection in our experiment.

DISCUSSION

A key observation from the present study is that the Ccm system can form an atypical cytochrome *c*, in which the single haem iron is co-ordinated by only one protein ligand, without a requirement for any additional maturation or ancillary proteins. This was demonstrated by the periplasmic formation, in good yield, of an M60A variant of *H. thermophilus* cytochrome c_{552}. Met^{60} is the normal distal ligand to the haem iron; the proximal ligand is His^{15} of the CXXCH motif. Spectroscopic data showed clearly that the haem iron of the M60A product cytochrome was high spin in each oxidation state, and thus the haem had not acquired an adventitious ligand from the protein. Pyridine haemochrome analysis showed that the haem was covalently attached to the polypeptide of the cytochrome via two thioether linkages.

This result indicates that as the Ccm apparatus interacts with the apo-form of a typical monohaem cytochrome *c*, there is no need for involvement of the eventual sixth ligand of the haem iron in the haem-attachment process. Hence it is reasonable to deduce that significant steps in the acquisition of the final cytochrome fold occur after the formation of thioether bonds between haem and polypeptide. This observation complements other studies in which *c*-type cytochromes that naturally lack a sixth axial ligand were processed by the Ccm system [39–41]. It is worth reiterating that the M60A variant of c_{552} does still have a typical CXXCH cytochrome *c* haem-binding motif. Thus these data provide evidence that the substrate specificity of the Ccm system is limited to the immediate vicinity of the CXXCH motif, rather than to the overall fold of the protein itself. In the light of the present results, those of McGuirl et al. [41] and recent work with CcmE, the haem-chaperone component of the Ccm system [42,43], it is arguable that, whereas proximal ligation to the haem would be provided by the histidine of the apocytochrome CXXCH motif, the distal ligand may be provided by CcmE during the haem transfer from the latter to an apocytochrome. This idea is consistent with the observation that a range of ligands (e.g. histidine, methionine, cysteine and asparagine) are ultimately found in the distal position in various cytochromes *c* (e.g. see [10,44–47]).

A related important observation to arise from the present study is that no M60A variant of cytochrome c_{552} could be detected in the cytoplasm of *E. coli* when protein was expressed in that compartment of the cell (i.e. without a periplasmic targeting sequence). Previous studies have shown that wild-type and various

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mutants of c_{552} are made as stable holocytochromes in the cytoplasm in an apparently uncatalysed reaction [20,21,34]. The present work (e.g. Figure 1) illustrates that the M60A variant is a stable protein. Thus the contrast between its formation in the periplasm and failure to assemble via a presumed uncatalysed route in the cytoplasm implies that the mechanism of biogenesis of cytochrome c_{552} in the two cell compartments is different. Our failure to observe cytoplasmic formation of any of the c_{552} mutants studied [H15M (CXXCM haem-binding motif), H15A (CXXCA), H15K (CXXCK) or M60A] implies a crucial role for both haem ligands in our cytoplasmic mode of cytochrome *c* formation. The data are consistent with the observation that *in vitro* formation of cytochrome c_{552} on mixing haem and apoprotein proceeds via a *b*-type cytochrome intermediate (a non-covalent complex of haem and protein) in which the haem iron is six-co-ordinate [48]. It may well be that both haem ligands (His^{15} and Met^{60}) are required in order for the apoprotein to bind haem sufficiently strongly or quickly in the cytoplasm, and that in the absence of bound haem the apoprotein is susceptible to proteolysis. There may also be entropic considerations related to haem–protein complex stability that affect thioether bond formation when the haem iron is co-ordinated by only one ligand and thus associates loosely relative to the wild-type protein, which provides two co-ordinating ligands.

Several related proteins in which the distal methionine residue, analogous to Met^{60} of cytochrome c_{552}, has been replaced have been produced by various means. Bren and Gray [49] made an M80A variant of horse-heart cytochrome *c* semi-synthetically. Shortly afterwards, Lu et al. [50] produced an M80A variant of *Saccharomyces cerevsiae iso*-1-cytochrome *c*. They co-expressed, in *S. cerevisiae*, M80A cytochrome *c* and a functional variant of the cytochrome that was essentially wild-type around the haem, but modified elsewhere, to have different purification characteristics. The M80A cytochrome variant was thus apparently biosynthesized by the endogenous cytochrome *c* biogenesis enzyme of yeast, haem lyase. Silkstone et al. [51] took this approach further by co-expressing M80A (and also M80S, M80D and M80E) *S. cerevisiae iso*-1-cytochrome *c* and *S. cerevisiae* haem lyase from a single plasmid in the cytoplasm of *E. coli*. These observations are strong evidence that haem lyase is capable of, and sufficient for, synthesis of the M80A form of yeast cytochrome *c*. We have previously postulated [8,48] that uncatalysed formation of c_{552}, either in the cytoplasm of *E. coli* or *in vitro*, occurs analogously to the mode of action of the eukaryotic haem lyase enzymes. However, the fact that haem lyase can act on a distal methionine-to-alanine variant of yeast cytochrome *c* (M80A) [50,51] requires re-evaluation of this hypothesis, assuming that there are no unanticipated differential effects due to, for example, protein stability or proteolysis. The stereochemically correct attachment of haem to wild-type apocytochrome c_{552} in the *E. coli* cytoplasm is, by implication from *in vitro* experiments, preceded by a non-covalent haem–protein complex in which it is currently thought that the two vinyl groups are close to being optimally positioned for reaction with the two thiol groups of the CXXCH motif [48]. The distal methionine ligand is clearly essential for this process (as shown by the present study). In haem-lyase-mediated attachment, it is likely that the same relative positioning of thiol and vinyl groups in a non-covalent complex is required. However, the methionine ligand is dispensable [50,51]. It is plausible that at this stage of the reaction an axial haem ligand is provided by the haem lyase, analogously to the role proposed above for the CcmE protein.

A further hypothesis tested in the present work is that the conserved residues between the cysteine residues of the CW(S/T/N)CK motif of the active site haem of NrfA are responsible for the substrate specificity of the system I cytochrome biogenesis apparatus (i.e. NrfEFG relative to CcmFH). However, we have shown that the Ccm system will make, in yields comparable with the wild-type (CKACH) protein, CWACH and CWSCH haem-binding motif variants of *P. denitrificans* cytochrome c_{550}. Thus the specificity of action of, and substrate recognition by, NrfEFG cannot only come from the residues between the haem-binding cysteine residues, although their participation cannot be formally excluded. This is further evidence that the Ccm system is extremely tolerant of variation around the haem-binding motif as long as the minimal cysteine and histidine $C(X)_nCH$ (where $n = 2$ and, under rare circumstances, 3 or 4) arrangement [4,5] is maintained. Given the variety of residues in the XX positions in Ccm-produced cytochromes *c* and the range of folds found for those proteins [3], it seems increasingly likely that the simple combination of the CXXCH motif and a haem-binding pocket is all that is required for recognition and operation by the Ccm proteins. In some multi-haem cytochromes *c* there are as few as three residues between CXXCH motifs, and therefore the biogenesis apparatus seemingly cannot be recognizing long stretches of polypeptide [2,3]. The best-known anomaly is the periplasmic protein DsbC, which, for example in *E. coli* and some other bacteria, has a CXXCH motif, but is not a *c*-type cytochrome [52]. In the structure of this protein, the cysteine residues are surface-exposed and it may be consequentially that the protein has a very low affinity for haem and thus is not acted on by the Ccm system. There may also be other as-yet-unidentified aspects of DsbC, either genetic, structural or sequence-related, which inhibit the Ccm system.

A surprising outcome of this study was our failure to produce stable cytochromes *c* with CXXCK (as models for NrfA) or CXXCM haem-binding motifs. We hypothesized that since methionine is, like histidine, a common strong-field haem ligand, it may substitute for histidine in the CXXCH motif but, at least in our experimental conditions, matters are not so simple. Moreover, neither the Ccm system nor the Ccm system supplemented with plasmid-expressed NrfEFG resulted in formation of stable CXXCM *H. thermophilus* c_{552}, CXXCK *H. thermophilus* c_{552} or CWSCK *P. denitrificans* c_{550}. In the latter case, the haem-binding motif exactly replicates that of the active-site haem of *E. coli* NrfA [12,36]. The two CXXCK cytochromes were also not detectably matured when cells were grown under anaerobic conditions that naturally result in NrfA biogenesis. There are several possible explanations for the lack of production of CXXCK (and CXXCM) holocytochromes in our study. There may be special features in NrfA required for haem attachment to the CXXCK motif that are absent from our c_{550}- and c_{552}-based models. Such features could include linear polypeptide sequences (outside the five residue haem-binding motif) or secondary/tertiary structural features. It may be that CXXCK (and/or CXXCM) cytochromes were made in our system by the Ccm and Nrf biogenesis proteins, but were degraded before they could be detected. It may also be that the variant apoproteins are incapable of binding haem, or do so very slowly relative to their histidine analogues, and thus were subject to proteolysis (known to affect both apo-c_{552} and apo-c_{550} in *E. coli* [18,53]).

In summary, the present work provides further evidence on the versatility and specificity of the bacterial Ccm system (system I) and on the special case of uncatalysed *H. thermophilus* cytochrome c_{552} formation in the cytoplasm of *E. coli*. The Ccm system is shown to be tolerant of significant changes to the XX residues within the CXXCH haem-binding motif, but not to the histidine, and to function effectively on an apocytochrome *c* lacking the distal (sixth) haem iron ligand. In contrast, cytoplasmic formation of *H. thermophilus* c_{552} is shown to depend critically

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on the presence of both the proximal histidine ligand and a distal ligand to the haem.

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