The histidine of the c-type cytochrome CXXCH haem-binding motif is essential for haem attachment by the Escherichia coli cytochrome c maturation (Ccm) apparatus

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INTRODUCTION

c-type cytochromes are characterized by covalent attachment of haem to the protein by two thioether bonds formed between the haem vinyl groups and the cysteine sulphones in a CXXCH peptide motif. In Escherichia coli and many other Gram-negative bacteria, this post-translational haem attachment is catalysed by the Ccm (cytochrome c maturation) system. The features of the apocytochrome substrate required and recognized by the Ccm apparatus are uncertain. In the present study, we report investigations of maturation of cytochrome b562 variants containing CXXCR, CXXCK or CXXCM haem-binding motifs. None of them showed any evidence for correct maturation by the Ccm system. However, we have determined, for each variant, that the proteins (i) were expressed in large amounts, (ii) could bind haem in vivo and/or in vitro and (iii) were not degraded in the cell. Together with previous observations, these results strongly suggest that the apocytochrome substrate feature recognized by the Ccm system is simply the two cysteine residues and the histidine of the CXXCH haem-binding motif. Using the same experimental approach, we have also investigated a cytochrome b562 variant containing the special CWSCK motif that binds the active-site haem of E. coli nitrite reductase NrfA. Whereas a CWSCH analogue was matured by the Ccm apparatus in large amounts, the CWSCK form was not detectably matured either by the Ccm system or by the dedicated Nrf biogenesis proteins, implying that the substrate recognition features for haem attachment in NrfA may be more extensive than the CWSCK motif.

Key words: c-type cytochrome biogenesis, cytochrome b562, cytochrome c maturation (Ccm), haem-binding motif, NrfA, post-translational modification.
two thioether bonds with the correct (universally conserved) stereochemistry, the α-peak is at 556 nm (Figure 1A) [6,7]. However, as mentioned above, $b_{562}$ CXXCH can also form holocytochromes with incorrect covalent haem attachment, e.g. where the haem is inverted and attached through only one thioether bond [7]. This occurs in vivo when the Ccm apparatus is not expressed and also in vitro, i.e. when the attachment is not enzyme-catalysed and is apparently uncontrolled. In these cases, the α-peak of the absorption spectrum is significantly red-shifted, from 556 nm to 558–560 nm [6,7]. Reduced pyrindine haemochrome spectra are highly indicative of the nature of covalent haem attachment to a protein, while being independent of that protein. Thus they provide a further means of investigating cytochrome maturation. In such experiments, the protein is saturated with pyridine/hydroxide solution, resulting in haem iron with bis-pyrindyl co-ordination. Non-covalently bound haem (as in haemoglobin or wild-type cytochrome $b_{562}$) gives rise to an α-peak at 556 nm when so treated; $c$-type cytochromes with two thioether linkages (e.g. mitochondrial cytochrome $c$ and Ccm matured $b_{562}$ CXXCH) diagnostically have this peak at 550 nm (e.g. [7]), whereas natural cytochromes $c$ with one thioether bond (e.g. from mitochondria of the Euglenozoa) have the peak at 552–3 nm (e.g. [9]). Thus there are very reliable spectral characteristics that can be used to assess the nature of haem attachment and hence the maturation of $c$-type cytochromes, including variants of cytochrome $b_{562}$ (e.g. [6,7]).

In this paper, we report investigations of the expression and Ccm-dependent maturation of CXXCR, CXXCM, CXXCK and CWSCK variants of cytochrome $b_{562}$, each thus being mutated in the proximal haem iron ligand His$^{102}$ (the histidine of the CXXCH motif). Methionine is, in common with histidine, frequently a strong field ligand to the haem iron of $c$-type cytochromes. For example, in mitochondrial cytochrome $c$, the haem iron has a histidine residue from the CXXCH motif as its proximal ligand and a methionine residue as the distal ligand. Thus it is interesting and important to determine whether Met can substitute for the histidine in the CXXCH motif during $c$-type cytochrome maturation. A cytochrome $b_{562}$ variant in which His$^{102}$ was replaced by methionine, creating a bis-Met-co-ordinated $b$-type haem, has been characterized previously [18,19]. Barker et al. [18,19] also
produced a $b_{562}$ variant with bis-Met haem co-ordination and (non-Ccm-dependent) covalent attachment through one thioether bond. The present study investigates a protein capable of bis-Met haem iron co-ordination and formation of two thioether bonds from protein to haem. This is the appropriate variant to assess the ability of the Ccm system to mature a CXXCM-containing c-type cytochrome. The CXXCK and CWSCK $b_{562}$ variants in the present work are models for the active-site haem of NrfA. In that case, the covalently attached haem has a lysine as the proximal iron ligand, but there is no distal ligand bound (i.e. the haem is penta-co-ordinate and capable of binding the substrate). However, several CXXCH c-type cytochromes with penta-co-ordinate haem are matured by the Ccm apparatus and it therefore seems apparent that the covalent haem attachment process is independent of a distal ligand to the iron ([17] and references therein). Two groups have investigated *Saccharomyces cerevisiae* cytochrome *c* where the invariant axial histidine ligand has been mutated to a distal ligand to the iron ([20,21]). In one case, the cytochrome *c* H18R (His$^\beta$ Arg) variant [20] grew on non-fermentable carbon sources, implying that the maturation of the cytochrome by the c-type cytochrome biogenesis apparatus of yeast (which is different from that of *E. coli* [22]). Thus the effect of mutation of the proximal histidine residue to an arginine on cytochrome *c* biogenesis is intriguing and requires investigation in the context of the Ccm system.

**EXPERIMENTAL**

**Plasmid construction**

Site-directed mutagenesis was performed by the Quik Change method (Stratagene, Cambridge, U.K.) using Pfx DNA polymerase (Invitrogen) according to the manufacturer’s instructions. The plasmid for cytochrome *b$_{562}$* R98C/Y101C (CNACH haem-binding motif) was as described by Barker et al. [6] and was used as the template for further mutagenesis. Three His$^{102}$ mutants (CXXCH → M, CXXCH → R and CXXCH → K), a CWSCH double mutant and a CWSCK triple mutant were constructed. Mutagenesis primers were used in each case: 5′-CTGAAAACGAGCCTGAAAGCCTGTATAGCAGATATCG and 5′-AGCAACTCTTCTCTTGCATACGCGTTGTCGTTGTCGTTGTCGTTGCATTTCAG; (ii) CNACH to CNACM: 5′-CGAATCTGAAACAGTGGCAGGAGCTGTATACGTCATACGCGTTGTCGTTGTCGTTGCATTTCAG; (iii) CNACH to CNACR: 5′-CTGAAAACGAGCCTGGAAGCCTGTATACGTCATACGCGTTGTCGTTGTCGTTGCATTTCAG; (iv) CNACH to CWSCH: 5′-GAGCAACTGAAAACGAGCCTGGGTCTTCCATGAGATATCG and 5′-AGCAACTCTTCTCTTGCATACGCGTTGTCGTTGTCGTTGCATTTCAG; (v) CNACH to CWSCK: 5′-CTGAAAACGAGCCTGGAAGCCTGTATACGTCATACGCGTTGTCGTTGTCGTTGCATTTCAG.

**RESULTS AND DISCUSSION**

**Effect of proximal haem ligand residue change from histidine to arginine on cytochrome *c* biosynthesis**

Cells were transformed with the plasmid encoding cytochrome $b_{562}$ R98C/Y101C (containing the putative covalent haem attachment motif CXXCR). This variant was expressed in the presence of the Ccm apparatus expressed from plasmid pEC86. Absorption spectra of periplasmic extracts from such cells showed that there was no increase in holocytochrome production relative to expected endogenous (background) cytochrome expression levels (see the Experimental section). However, SDS/PAGE showed substantial periplasmic expression of protein of similar molecular mass to wild-type cytochrome $b_{562}$, indicating the presence of large amounts of apo (i.e. haem-free) protein. Electrospray mass spectra of the purified apoprotein showed that the observed mass of the variant (11 684 Da) was in agreement with

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the expected mass (11 684 Da) and thus confirmed the presence of the desired mutation and that the protein was intact.

Previous evidence is inconclusive as to whether a proximal arginine residue can ligate the haem iron of a c-type cytochrome in vivo [20,21]. Work on S. cerevisiae iso-1-cytochrome c showed that a variant protein with a CXXCR haem-binding motif still possessed electron-transfer function, allowing cells to grow on non-fermentable carbon sources (i.e. where functional cytochrome c is required for respiration), albeit at retarded rates, when the wild-type gene was removed [20]. This implies that the variant c-type cytochrome was matured by the cytochrome c biogenesis system of yeast, the enzyme haem lyase [22]. However, expression levels of the variant protein were low and rigorous determination of its identity, e.g. by MS, was not performed. In contrast, another study with an S. cerevisiae cytochrome c H18R variant [21] suggested that arginine cannot act as the proximal haem ligand since not only did the transformed cells exhibit no growth on a non-fermentable medium (indicating loss of function), but also showed no evidence of the product cytochrome c.

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Formation of the c-type R98C/Y101C/H102M variant of E. coli cytochrome b562

A plasmid encoding cytochrome b562 R98C/Y101C/H102M (i.e. with a CXXCM haem-binding motif) was transformed into cells with or without the ccm plasmid pEC86. Absorption spectra of the reduced periplasmic extracts from cells expressing the CXXCM protein showed the presence of a product c-type (covalent) cytochrome at well above background levels in each case. Covalent attachment of the haem to b562 CXXCM was confirmed by activity staining of SDS/PAGE gels with purified holocytochrome b562 CXXCH as a marker (results not shown). Coomassie-stained SDS/polyacrylamide gels of the periplasmic extracts from b562 CXXCM-transformed cells showed high levels of apoprotein expression. The apoprotein was purified and analysed by MS, which indicated the presence of the desired mutation (calculated and observed masses 11 659 and 11 658 Da respectively).

A major aim of the present study was to assess whether the Ccm system can mature cytochromes with variant (e.g. CXXCM) haem-binding motifs. Thus the CXXCM holocytochrome product produced in the presence and absence of pEC86 was analysed in more detail. It is insufficient to look only at the crude periplasmic extracts because cells grown with pEC86 produce more of the endogenous soluble E. coli c-type cytochromes (NrfA, NapB) than those without; naturally, this makes the spectra of the crude extracts look more like bona fide c-type cytochromes. Thus the CXXCM holocytochromes were purified by anion-exchange chromatography. Following this step, on either a DEAE-Sepharose or a Q-Sepharose column, no distinct absorption, pyridine haemochrome or electrophoretic differences, within the limits of detection and errors of the experiment, were observed in holocytochrome b562 CXXCM produced with or without the Ccm apparatus. In both cases, the product CXXCM protein has absorption and pyridine haemochrome spectra characteristic of an improperly matured c-type cytochrome derivative of b562 (Figure 1B), i.e. red-shifted maxima compared with Ccm matured cytochrome b562 CXXCH (see the Introduction section and Figure 1A). We conclude that, although the CXXCM variant of b562 can bind haem covalently to form a holocytochrome, the Ccm system is unable to facilitate its maturation.

Curiously, the Soret absorption band of the b562 CXXCM holocytochrome, purified using either DEAE-Sepharose or Q-Sepharose, is a doublet of roughly equal intensities with maxima at approx. 422 and 429 nm (Figure 1B). Resolved α- and β-absorption maxima show that the haem iron is low-spin. A bis-Met co-ordinated b-type haem in cytochrome b562 has its Soret peak at 431 nm [19]; for a c-type variant with haem attachment through two vinyl groups, one would expect a blue shift of approx. 6 nm for this peak. Thus the doublet observed for the CXXCM protein may at first sight be interpreted as a mixture of covalent and non-covalent bis-Met-co-ordinated cytochromes. Several lines of evidence indicate that this is not the case: (i) the pyridine haemochrome α-band maximum (approx. 551 nm) is not consistent with large quantities of non-covalently bound haem in the cytochrome; (ii) the absorption maxima did not change after treatment of the holocytochrome with acidified butanone, a procedure that removes non-covalently bound haem [29]; and (iii) haem-stained SDS/polyacrylamide gels of preparations of the purified holocytochrome indicate the presence of only one cytochrome (rather than two) with covalently bound haem. Thus we conclude that the doublet arises from two isoforms of holocytochrome b562 CXXCM with haem covalently bound. The pyridine haemochrome spectrum of this material has a typical (singlet) Soret band, thus the absorption band doublet may well represent mixed co-ordination of the haem iron.

Effect of mutation of the proximal axial ligand to lysine on biosynthesis of c-type cytochromes

A cytochrome b562 variant was generated containing the mutation H102K [b562 R98C/Y101C/H102K (CXXCK motif)]. Absorption spectra revealed periplasmic formation of a c-type holocytochrome of this variant (Figure 1C), which was confirmed by staining of SDS/polyacrylamide gels for purified holocytochrome b562 CXXCH as a marker (results not shown).

In the absence of oxygen, E. coli can grow anaerobically using nitrate or nitrite as terminal electron acceptors. This requires the expression of a number of proteins from the Nrf pathway, including NrfA, the pentahaem nitrite reductase enzyme. This enzyme has four haems covalently attached through typical CXXCH motifs and one active-site haem attached by an unusual CWSCK motif [16]. Covalent attachment of this active-site haem requires the presence of the CcmFH orthologues, NrfEFG, in addition to the Ccm apparatus [16], although it is not yet clear what the specific recognition factors for the Nrf biogenesis apparatus are or precisely why these proteins are required. Therefore we
also constructed a b\textsubscript{562} variant with a CWSCS haem-binding motif (b\textsubscript{562} R98C/N99W/A1005/Y101C/H102K; the motif till now described as CXXCK is actually CNACK) and, as a control, a CWSCS variant. Cells transformed with these plasmids were first grown aerobically (as for all the other variants in the present study). Apoprotein was shown to be plentiful in each case by SDS/PAGE, and MS indicated the presence of the desired mutations (for b\textsubscript{562} CWSCS, the calculated mass was 11753 Da and the observed mass was 11754 Da; for b\textsubscript{562} CWSCS, both the calculated and observed masses were 11744 Da). Cytochrome b\textsubscript{562} CWSCS expressed in the presence of the Ccm apparatus (from pEC86) was a correctly matured c-type cytochrome, indicated particularly (Figure 1D) by the absorption and pyridine haemochrome \(\alpha\)-band maxima at 556 and 550 nm respectively. These values are the same as for b\textsubscript{562} CNACH (‘CXXCH’), which has been extensively analysed (by NMR) \[7\], and are essentially diagnostic of covalent haem attachment to the b\textsubscript{562} variant through two thioether bonds. SDS/PAGE of crude periplasmic extracts stained for covalently bound haem showed the dominant band at the same molecular mass as that for purified b\textsubscript{562} CXXCH. The yield of holocytocrome b\textsubscript{562} CWSCS was approx. 4 mg/g of wet cells, using a molar absorption coefficient of 146 mM/cm at 420 nm (as reported for Ccm matured b\textsubscript{562} CNACH \[6,7\]). In contrast, cytochrome b\textsubscript{562} CWSCS behaved very similarly to the CNACK variant (see data above), i.e. there was a population of improperly matured holocytochrome and no evidence for such a holoprotein being different in the presence of the Ccm apparatus.

E. coli JCB387 cells transformed with either the ampicillin-resistance-conferring plasmid pKK223-3 (as a control/reference) or the b\textsubscript{562} CWSCS plasmid were also grown anaerobically on minimal medium containing formate and nitrate, after which periplasmic extracts were prepared. Under these conditions, the native Nrf and Ccm systems of E. coli are expressed and active, but there was no evidence for significant maturation of b\textsubscript{562} CWSCS judged by the spectral maxima in comparison with those for the control (pKK223-3) samples. In each case, the spectra of endogenous soluble c-type cytochromes (mainly NrfA \[30\]) were readily apparent, but c-type cytochromes based on cytochrome b\textsubscript{562} (such as Ccm-matured b\textsubscript{562} CWSCS) have distinct (red-shifted) absorption peaks from those of the natural E. coli c-type cytochromes. A very small population of protein with mass corresponding to that of cytochrome b\textsubscript{562} was, however, observed on haem-stained SDS/polyacrylamide gels of periplasmic extracts from large (5 litre) cultures of E. coli expressing cytochrome b\textsubscript{562} CWSCS; the apocytochrome was shown to be plentiful by Comassie staining. An attempt was made to purify the holocytochrome b\textsubscript{562} CWSCS by anion-exchange chromatography, but it was present in only minimal amounts and co-eluted with NrfA, which was by a long way the dominant cytochrome present. Thus we cannot state categorically that no properly matured holocytochrome b\textsubscript{562} CWSCS was produced by the E. coli Ccm/Nrf systems, but any such protein was present at a very low level. It is also entirely possible that the holocytochrome b\textsubscript{562} CWSCS observed on gels may have been improperly matured, as was seen when the protein was expressed under aerobic conditions (see data above).

Conclusions
A great virtue of the cytochrome b\textsubscript{562}-based experimental system used in the present study is the production of large quantities of stable apoprotein for each variant whether or not any holoprotein was also formed. Therefore, for each b\textsubscript{562} variant we have investigated, it has been shown that the protein (i) was expressed (detected by SDS/PAGE), (ii) contained the correct mutations and was intact/undigested in the periplasm (by ESI–MS), and (iii) could bind haem \textit{in vivo} and/or \textit{in vitro} (spectroscopically, e.g. Figure 1). CXXCH variants of b\textsubscript{562} form stable and spectroscopically distinguishable holocytochromes in the presence and absence of the Ccm apparatus (where ‘XX’ = WS (the present study) or NA \[7\]). Thus we can say clearly that the failure of the Ccm system to mature cytochromes with CXXCR, CXXCM or CXXCK haem-binding motifs was due to the properties of the maturation system and not due to a lack of expression of the apocytochrome substrate, proteolysis of the apocytochrome or instability/proteolysis of the holocytochrome.

\textit{In vivo}, the most likely reason for the failure of the Ccm system to mature the CXXCH, CXXCR and CXXCM b\textsubscript{562} variants is an inability to recognize the modified haem attachment site. Crow \textit{et al.} \[31\] have recently suggested that reduced ResA, a protein of the distinct (non-Ccm) cytochrome \(c\) biogenesis system of \textit{Bacillus subtilis}, may contain a specific recognition site for the histidine of the CXXCH motif of apocytochromes \(c\). They argue that “such a ‘histidine clamp’ mechanism would confer specificity to the recognition of apo-cytochrome \(c\) without compromising the capacity to recognize several different sequences”. A similar site, specifically requiring the histidine, might be present in one (or more) of the Ccm proteins. Additionally or alternatively, the CXXCH histidine may play a crucial role in the kinetics of haem attachment; it might, for example, serve as the necessary proton donor to the haem vinyl group during thioether bond formation.

Clearly, the critical specificity determinant for apocytochrome substrates of the \textit{E. coli} Ccm system is the combination of the two cysteine residues and the histidine residue of the CXXCH haem-binding motif. Both cysteine residues have been shown to be required \[9,14,32\] along with the histidine residue (the present study). However, the system will mature both natural and variant \(c\)-type cytochromes with three or four residues between the cysteine residues, rather than the usual two \[10,33,34\]. A distal ligand to the haem iron is not required for Ccm-mediated haem attachment \[17,35\]. Very recently, it has been shown that the Ccm system can covalently attach haem to a peptide of only 12 amino acids, with a CXXCH motif and a C-terminal His\(_6\) tag \[12\]. The occurrence in a multihaem cytochrome of two CXXCH motifs separated by as few as five other residues \[13\] also implies recognition of little more than the CXXCH motif by the Ccm system.

In contrast, the biogenesis (covalent haem attachment) proteins of the Nrf system seemingly detect more in NrfA than simply the CWSCS active-site haem-binding motif, e.g. other motifs or structural features in the latter protein might be recognized. The Nrf haem attachment proteins may function only after attachment of haems to some or all of the CXXCH motifs of NrfA by CmA-H. It is also possible that the Nrf biogenesis proteins were inhibited by our attempt to mature a CWSCS-containing cytochrome that would also (presumably) have a distal methionine ligand, in contrast with the CWSCS-bound haem of NrfA, which is ultimately penta-co-ordinate. A further possibility is that the Nrf apparatus is saturated by the production of the NrfA required for respiration under anaerobic growth conditions and thus does not have sufficient capacity to mature our overexpressed (exogenous) cytochrome. It appears likely that b\textsubscript{562} CWSCS could, in principle, form a stable, correctly matured c-type cytochrome because its CXXCH histidine is matured in large amounts by the Ccm apparatus (Figure 1D). The molecular basis for the attachment of haem to the CWSCS motif of NrfA nitrite reductase evidently warrants further study.

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