A cytochrome $b_{562}$ variant with a $c$-type cytochrome CXXCH heme-binding motif as a probe of the *Escherichia coli* cytochrome $c$ maturation (Ccm) system

James W. A. Allen‡, Paul D. Barker† and Stuart J. Ferguson‡*

‡Department of Biochemistry, University of Oxford, South Parks Road, Oxford, OX1 3QU, United Kingdom and †Chemistry Department and Centre for Protein Engineering, University of Cambridge, Lensfield Road, Cambridge CB2 1EW, United Kingdom

*To whom correspondence should be addressed. Telephone +44 (0) 1865 275240; Fax +44 (0) 1865 275259; Email stuart.ferguson@bioch.ox.ac.uk

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Running title: Expression of a $c$-type cytochrome variant of cytochrome $b_{562}$
Cytochrome $b_{562}$ is a periplasmic *Escherichia coli* protein; previous work has shown heme can be attached covalently *in vivo* as a consequence of introduction of one or two cysteines in the heme-binding pocket. In such work, a heterogeneous mixture of products was obtained and it was not established whether the covalent bond formation was catalyzed or spontaneous. Here we show that co-expression from plasmids of a variant of cytochrome $b_{562}$ containing a CXXCH heme-binding motif with the *E. coli* cytochrome *c* maturation (Ccm) proteins results in an essentially homogeneous product that is a correctly matured *c*-type cytochrome. Formation of the holocytochrome was accompanied by substantial production of its apo form, in which, for the protein as isolated, there is a disulfide bond between the two cysteines in the CXXCH motif. Following addition of heme to reduced CXXCH apoprotein, spontaneous covalent addition of heme to polypeptide occurred *in vitro*. Strikingly, the spectral properties were very similar to those of the material obtained from cells in which presumed uncatalyzed addition of heme (*i.e.* in the absence of Ccm) had been observed. The major product from uncatalyzed heme attachment was an incorrectly matured cytochrome with the heme rotated by 180° relative to its normal orientation. The contrast between Ccm-dependent and independent covalent attachment of heme indicates that the Ccm apparatus only presents heme to the protein in the orientation that results in formation of the correct product, and also that heme does not become covalently attached to the apocytochrome $b_{562}$ CXXCH variant without being handled by the Ccm system in the periplasm. The CXXCH variant of cytochrome $b_{562}$ was also expressed in *E. coli* strains deficient in the periplasmic reductant DsbD or the oxidant DsbA. In the DsbA− strain, in aerobic conditions, *c*-type cytochromes were made abundantly and correctly when the Ccm proteins were expressed. This contrasts with previous literature reports, which indicate that DsbA is essential for cytochrome *c* biogenesis in *E. coli*. 
Introduction

Cytochrome $b_{562}$ is a hemoprotein expressed in the periplasm of the Gram-negative bacterium *Escherichia coli* (1). It is the only $b$-type cytochrome (a complex of protein and heme in which the heme is not attached covalently to the polypeptide) known to occur naturally in that compartment of the *E. coli* cell. Cytochrome $b_{562}$ has a four $\alpha$-helix bundle structure (2-4) and its apoprotein (the heme free form) is also stable and structurally relatively ordered (5). The other periplasmic cytochromes in *E. coli* are of the $c$-type, *i.e.* they contain heme covalently attached to polypeptide through thioether bonds between the vinyl groups of heme and the cysteine sulfurs of a Cys-Xxx-Xxx-Cys-His peptide motif (6-9). Biogenesis of $c$-type cytochromes in *E. coli* and many other Gram-negative bacteria is a complex process thought to require at least twelve proteins. These are the cytochrome $c$ maturation (Ccm$^1$) system, CcmABCDEFGH, and the disulfide bond oxidizing and reducing proteins DsbA, DsbB, DsbD and TrxA (6, 9-11). A disulfide bond is commonly thought to form between the cysteines of the CXXCH heme-binding motif and this has been shown to occur *in vitro* in the apoproteins of several monoheme cytochromes $c$ (12, 13). The Dsb and Ccm systems are believed to work in series during cytochrome $c$ biogenesis such that as the unfolded apocytochrome is exported to the periplasm it is first oxidized by DsbA (whose oxidant is DsbB). The apocytochrome disulfide bond must be reduced before heme can be attached and this reduction is achieved by CcmG and/or CcmH, which are, in turn, reduced by the transmembrane protein DsbD whose electron donor is TrxA (14, 15 and reviewed in refs 8 and 10).

It has been shown that *E. coli* cytochrome $b_{562}$ can be altered by site directed mutagenesis such that it will form $c$-type cytochromes in the periplasm (16). In cytochrome $b_{562}$, a histidine, residue 102, is the proximal ligand to the heme iron; an analogous histidine is a virtually universal feature of
both $b$ and $c$-type cytochromes. Barker and co-workers (16) constructed R98C, Y101C and R98C/Y101C variants of $b_{562}$ resulting in CXXXH, XXXCH and CXXCH heme-binding motifs respectively. Each of these proteins could be detected in the periplasm of $E. coli$ as covalent complexes of apoprotein and heme. The R98C/Y101C variant did not form as a homogenous $c$-type cytochrome product, but rather as two major and two minor products. From these studies it was unclear whether the covalent attachment of heme was catalyzed or spontaneous. For each variant, large amounts of apoprotein were also detected (16).

When these previous experiments (16) were conducted, little was known about the cytochrome $c$ biogenesis apparatus used by $E. coli$. Subsequent work identified the Ccm system (CcmABCDEFGH) (17, 18), which is expressed physiologically only under certain anaerobic growth conditions. However, these gene products, overexpressed constitutively from a plasmid lacking anaerobic control elements, are now widely used for overproduction of $c$-type cytochromes when co-expressed with a cytochrome $c$ gene (17, 19-21). The Ccm apparatus has been shown to process cytochromes $c$ from diverse prokaryotic and eukaryotic origins (e.g. refs 17, 19-21). In the light of these recent data, we have investigated the action of the Ccm system on the R98C/Y101C (CXXCH) variant of $E. coli$ cytochrome $b_{562}$. To our knowledge, this is the first study of the Ccm system in conjunction with an "artificial" cytochrome $c$. Further, we have investigated the effect of mutations in the Dsb system with respect to production of this cytochrome, and apocytochrome, in the $E. coli$ periplasm.
Experimental Procedures

Bacterial strains, plasmids and growth conditions.

*Escherichia coli* strain JCB387 (22) was used in all experiments unless stated. Other *E. coli* strains used had mutations in the genes for the periplasmic disulfide oxidizing and reducing proteins DsbA and DsbD; these strains were JCB3510 (*dsbA*) (23), JCB571 (*dsbA*) (24) and JCB606 (*dsbD*) (14, 25). Note that JCB387, JCB606 and JCB3510 are isogenic; all were the kind gift of Professor Jeff Cole, University of Birmingham, UK. JCB571 was the gift of Professor James Bardwell, University of Michigan. Plasmids for wild type *E. coli* cytochrome *b*$_{562}$ and the R98C/Y101C (CXXCH heme-binding motif) variant were as described (16). Plasmid pKPD1 encoding the gene for *Paracoccus denitrificans* cytochrome *c*$_{550}$ was as described by Sambongi and Ferguson (26). As required, cells were also transformed with plasmid pEC86 (17) encoding the *E. coli* cytochrome *c* maturation genes, *ccmABCDEFGH*. Transformants were initially grown on LB-agar plates with the appropriate antibiotics (100 µg ml$^{-1}$ ampicillin in each case, plus 34 µg ml$^{-1}$ chloramphenicol where pEC86 was co-transformed and 25 µg ml$^{-1}$ kanamycin for the *dsbA* strains). Single colonies were picked into 500 ml 2xTY medium (16 g l$^{-1}$ peptone, 10 g l$^{-1}$ yeast extract and 5 g l$^{-1}$ NaCl), supplemented with 1 mM IPTG, in 2.5 l flasks. Cultures were grown at 37 °C with shaking at 200 rpm for 20-24 h, before harvesting.

Cell fractionation and biochemical procedures.

Periplasmic fractions were obtained from cells using procedures described previously (27). Cytochrome content was determined by recording absorption spectra of the crude or purified periplasmic fractions. *E. coli* produces small amounts of endogenous *c*-type cytochromes in the periplasm in our growth conditions (see, for example, reference 27). To provide reference data to
correct for the level of this background cytochrome expression, *E. coli* JCB387 cells were transformed and grown with pTZ19r (an ampicillin resistance conferring cloning vector; Fermentas) and pEC86. These cells were fractionated and the absorbances of the periplasmic fractions, normalized in relation to volume and wet cell weight and averaged from several cultures, were taken to represent "typical" endogenous (background) cytochrome expression levels. This cytochrome yield cannot be expressed directly because it represents a mixture of components with different, and in some cases undetermined, extinction coefficients. 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 from such cells (prepared using our routine growth and fractionation procedures) was 0.026 absorbance units (mean average for the maximum at 418 nm, standard deviation 0.004 AU) per gram of wet cells. When yields of periplasmic exogenous cytochromes are expressed in this work, this endogenous cytochrome absorbance has been subtracted from the actual observed absorbance to determine the expression level.

Apo and holocytochrome $b_{562}$ R98C/Y101C were purified on a DEAE-Sepharose anion exchange column. Chromatography was conducted at 4 °C in 50 mM Tris-HCl buffer, pH 8.0. The column (Amersham Pharmacia XK26/20 with a resin bed volume of 70 ml) was eluted with a 0 - 0.5 M NaCl gradient at a flow rate of 9 ml min$^{-1}$ and 7 ml fractions were collected. Apocytochrome $b_{562}$ R98C/Y101C clearly separated from the holocytochrome following this chromatographic step. Apocytochrome-containing fractions were identified from their absorption spectra following the addition of dithiothreitol (DTT) and heme, and were nearly pure as judged by SDS-PAGE. The spectrophotometrically purest holocytochrome fractions were pooled, diluted four-fold and loaded
onto a Q-Sepharose column (XK26/20), which was eluted with a 0 - 0.25 M NaCl gradient in the
same buffer and conditions. After this step, the holocytochrome $b_{562}$ R98C/Y101C was essentially
pure. Wild type cytochrome $b_{562}$ was purified using a protocol modified from that of Nikkila et al
(28). The crude periplasmic extract of fractionated *E. coli* cells containing the cytochrome was
taken to pH 4.55 with concentrated phosphoric acid, stirred on ice for 30 min and centrifuged at
40,000 x g for one hour. The cytochrome-containing solution was then adjusted to pH 8.0 and
purified on DEAE-Sepharose and Q-Sepharose columns as described above for the R98C/Y101C
variant. Wild type apocytochrome $b_{562}$ was prepared by extraction of heme from the holoprotein
with imidazole, using the procedure described by Tomlinson and Ferguson (29).

Hemin (Fe-protoporphyrin IX-Cl) was added to protein solutions from a stock solution in
DMSO. NMR spectra were collected as described previously (16, 30, 31) using a BRUKER AMX
500 spectrometer, or a BRUKER Avance 700 spectrometer. NMR methods were similar on the
different instruments except that WATERGATE sequences were used on the 700 MHz instrument
to remove the residual water signal. All spectra were acquired at 300 K. Data processing was as
previously described (16, 30, 31) except that it was performed using XWINNMR (Bruker). All
samples were between 0.4 and 1 mM protein in 20 mM potassium phosphate buffer in D$_2$O, pH 6.6,
0.5 M KCl. Where appropriate, the proteins were reduced by the addition of sodium dithionite to ca.
5 mM from anaerobic stock solutions in the same buffer. Electrospray ionization mass spectrometry
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 µl$^{-1}$ in 1:1 water:acetonitrile/0.2% (v/v) formic acid) at a flow rate of 10 µl min$^{-1}$. Data
were analyzed using the maximum entropy based component of the MassLynx suite of software (Micromass) (32). Ellman’s reagent was used according to Riddles et al (33).

Results and Discussion

Formation of the R98C/Y101C variant of E. coli cytochrome b$_{562}$ with and without expression of the cytochrome c maturation (Ccm) proteins.

E. coli strain JCB387 was transformed with a plasmid encoding the R98C/Y101C variant of cytochrome b$_{562}$ (16) which has the CXXCH heme-binding motif that is the general characteristic of cytochromes c. This construct includes the natural signal sequence of cytochrome b$_{562}$ which directs the apoprotein to the periplasm where both wild type b$_{562}$ and all Gram-negative bacterial cytochromes c are naturally assembled. Cells were co-transformed with plasmid pEC86 which encodes the E. coli cytochrome c maturation (ccm) genes (17). They were grown aerobically (which represses expression of the endogenous E. coli Ccm proteins (and hence also production of the endogenous c-type cytochromes)) (34) and periplasmic fractions prepared. The absorption spectrum of the dithionite-treated periplasmic extract from such cells had resolved peaks characteristic of a low-spin, six-coordinate cytochrome. The absorbance in the Soret band region, normalized in relation to fraction volume and wet cell weight, was 5 – 30 fold in excess of that in control (background expression) experiments (see Experimental for details of these controls). The $\alpha$-band of the dominant observed cytochrome was at 556 nm (Figure 1; Table 1). This value is red-shifted relative to most cytochromes c (e.g. 35, 36), but is blue-shifted by 6 nm relative to the $\alpha$-band observed for wild type cytochrome b$_{562}$ at 562 nm. Such a shift to higher energy is consistent with saturation of the heme vinyl groups and hence covalent attachment of heme to polypeptide. Cytochrome c$_{556}$, which shares the same fold and heme coordination as cytochrome b$_{562}$ but is a
natural $c$-type cytochrome (16, 37), also has its $\alpha$-band at 556 nm. The other main absorption peaks in the spectrum of the dithionite reduced periplasmic extract of cells expressing $b_{562}$ R98C/Y101C in the presence of the Ccm proteins were at 526 and 421 nm (Figure 1; Table 1). Treatment of an aliquot of a cytochrome with an alkaline pyridine solution and dithionite produces a spectrum that is highly characteristic of the nature of attachment of heme to protein (38). In the present case, the $\alpha$-band of such a spectrum of the pyridine and hydroxide treated crude periplasmic extract was at 550 nm (Figure 1, inset), the wavelength expected for covalent attachment of heme to protein through two thioether linkages to a CXXCH peptide motif, as found in a typical cytochrome $c$.

To assess the homogeneity of the product, cytochrome-containing fractions were purified from the crude periplasmic extract of cells that had expressed $b_{562}$ R98C/Y101C in the presence of the Ccm proteins. The periplasmic extract was chromatographed on a DEAE-Sepharose column (see Experimental for details). Cytochrome-containing fractions, identified by their red color, eluted in a continuum that on spectral analysis consisted of three parts. The first group of these fractions had absorption and pyridine hemochrome maxima characteristic of $c$-type cytochromes and similar to those expected for the endogenous soluble $E. coli$ cytochrome $c$ NrfA (39). The second and clearly spectroscopically dominant group was the $c$-type cytochrome form of $b_{562}$ R98C/Y101C, with its $\alpha$-band absorption maximum at 556 nm and its pyridine hemochrome $\alpha$-band maximum at 550 nm. The third (small) set of fractions contained a mixture of at least two species, one of which was the $b_{562}$ R98C/Y101C $c$-type cytochrome. The absorption spectra of these later fractions were red-shifted by 1-1.5 nm, and the pyridine hemochrome spectra by up to 1 nm, relative to the uncontaminated $b_{562}$ R98C/Y101C $c$-type cytochrome fractions. Thus, in addition to the major $b_{562}$ R98C/Y101C $c$-type cytochrome product, these fractions may contain some malformed (e.g. single
cysteine attached) cytochrome \(c\) and/or some \(b\)-type cytochrome from lyzed cytoplasm contaminating the periplasmic cell extract, either of which would have the observed effect on the spectra. Quantitatively, our chromatographic analysis showed that at least 95% of the holocytochrome \(b_{562}\) R98C/Y101C produced in the presence of the Ccm proteins was in the major cytochrome-containing band (\(i.e\). in the second group of fractions) and thus was a \(c\)-type cytochrome with spectra wholly characteristic of covalent attachment of heme to protein through two thioether bonds.

Barker et al (16) purified “component II” of the mixture of two major and two minor products that was observed when \(b_{562}\) R98C/Y101C was expressed in the periplasm of \(E. coli\) under different growth conditions and with little control over the expression of the Ccm system which had not then been identified (16). The purified holocytochrome was studied by NMR spectroscopy; it was shown to be homogeneous and that the heme was attached covalently to the cytochrome with the same stereochemistry as is observed for all naturally occurring cytochromes \(c\) (7, 16). Since the absorption spectra of component II were essentially identical to those observed in the current work for cytochrome \(b_{562}\) R98C/Y101C produced in the presence of the Ccm proteins, we have also analyzed the Ccm matured holocytochrome by NMR. The main cytochrome species, accounting for > 95% of the total holocytochrome \(b_{562}\) R98C/Y101C produced (see previous paragraph), was further purified to remove any non-cytochrome contaminants. It was found by NMR to be homogeneous (\(i.e\). a single cytochrome species), identical in both the oxidized and reduced states to component II from the earlier work (16) and thus a properly matured \(c\)-type cytochrome. The chemical shifts of the heme, aromatic and distal methionine heme ligand protons were within 0.01 ppm of those previously reported (see Supplementary Figures S1 – S3 for the NMR data). We therefore conclude that component II was holocytochrome matured by the Ccm system operating at
a background level in the growth conditions that were used earlier (16). When apparently uncatalyzed covalent heme attachment to $b_{562}$ R98C/Y101C occurred, three products that did not have the correct heme attachment were apparent (reference 16 and see also data below). In the similarly well-characterized case of uncatalyzed covalent heme attachment to *Thermus thermophilus* cytochrome $c_{552}$, at least two distinct products have been obtained (40, 41).

In contrast to the experiments where the Ccm proteins were expressed from the plasmid pEC86, in the absence of this plasmid we detected a heterogeneous mixture of forms of holocytochrome $b_{562}$ R98C/Y101C in the periplasmic fraction. These observations were, therefore, similar to those reported previously (16). The absorption spectrum of our dithionite reduced crude periplasmic extract was notably red-shifted relative to the case with the Ccm system present (Figure 1; Table 1). The $\alpha$-band maximum of the pyridine hemochrome spectrum of the product obtained in the absence of Ccm was at *ca.* 552.5 nm, consistent with the presence of improperly matured forms of this $c$-type cytochrome. A $b$-type cytochrome, such as wild type $b_{562}$ in which the heme and protein form a non-covalent complex, has a pyridine hemochrome maximum at 556 nm, a properly matured $c$-type cytochrome with double cysteine attachment (through two thioether bonds) has it at 550 nm (38) and a $c$-type cytochrome with only one thioether bond between heme and peptide, such as the naturally occurring *Euglena gracilis* cytochrome $c_{558}$, at 553 nm (42).

Our data show unambiguously that the *E. coli* Ccm system can act on a variant of cytochrome $b_{562}$ with a CXXCH heme-binding motif and produce a $c$-type cytochrome with the correct covalent attachment of heme to protein. The Ccm proteins act as a very effective quality-control system for the product cytochrome (clearly illustrated by its homogeneity and NMR spectra). The yield of
holocytochrome $b_{562}$ R98C/Y101C produced in the presence of the plasmid-expressed Ccm proteins varied from 0.3 - 1.8 mg per gram of wet cells. To our knowledge, this is the first convincing evidence that the Ccm apparatus can act on an “artificial” apocytochrome $c$. The Ccm system, found in some Gram-negative bacteria and in plant mitochondria (6, 8, 43), has previously been shown to act on eukaryotic cytochromes $c$ (19, 21) and thus to be versatile in terms of substrate. However, there is no reason why apocytochrome $b_{562}$ R98C/Y101C, based on the scaffold of a $b$-type cytochrome, should have any recognition factors required for cytochrome $c$ biogenesis (i.e. the action of the Ccm proteins) apart from, simply, an artificially created CXXCH peptide motif and a heme-binding pocket.

The yield of the homogeneous $c$-type holocytochrome $b_{562}$ R98C/Y101C produced in the presence of the Ccm system (i.e. with pEC86) varied between cell growths from 25% to 100% of that of the combined yield of all the holocytochrome species produced without expression of the Ccm proteins (given that the Soret band extinction coefficients of all the relevant species are the same to within 10% (16)). Thus despite increasing the quality of the product, the Ccm system did not catalyze the formation of increased levels of cytochrome with covalently bound heme and often lowered the overall holocytochrome yield. However, one cannot formally that exclude other factors, for instance the nature of heme provision (see below), the presence of a second antibiotic in the growth media, or additional competition for the cellular transcription/translation machinery, affected the relative cytochrome yields.

*The presence of apocytochrome $b_{562}$ R98C/Y101C.*

In the previous study of expression of R98C, Y101C and R98C/Y101C variants of cytochrome $b_{562}$ in the *E. coli* periplasm, a significant amount of apocytochrome (i.e. heme free protein) was
observed in each case (16). We therefore assessed whether the same was true in our (different) growth conditions. Aliquots of periplasmic extract were treated with DTT and dithionite to reduce any potential disulfide bond formed between the cysteines of the CXXCH motif of apocytochrome $b_{562}$ R98C/Y101C. The periplasmic material was then titrated with heme and the change in the absorption $\alpha$-band (around 560 nm) was observed. A sharp $\alpha$-band is characteristic of a reduced, six-coordinate cytochrome heme iron, but not of free heme. In order to normalize for the spectral contribution of free and non-specifically bound heme, we analyzed the change in intensity of the $\alpha$-band (i.e. peak maximum minus trough minimum). Heme was added until this value did not change significantly, regarded as the point at which the apocytochrome $b_{562}$ R98C/Y101C was saturated with heme. As reported (16), addition of heme to this variant of apocytochrome $b_{562}$ results initially in a spectrum very similar to that of wild type cytochrome $b_{562}$; thus for quantitation purposes we used the wild type extinction coefficients for such protein (16). Extinction coefficients used for the initial (untitrated) periplasmic cytochromes were as published (16). Analysis of this type showed that when $b_{562}$ R98C/Y101C was expressed either in the presence or absence of the Ccm proteins (from pEC86), at least 75% of the $b_{562}$ R98C/Y101C polypeptide was present as apoprotein (i.e., without heme bound). Apocytochrome $b_{562}$ R98C/Y101C could be purified from the periplasmic extract with relative ease; the apo form eluted from an anion exchange column well before the holo form and was retained for use in experiments when it was desirable to avoid the complication of background cytochrome absorbance.

The presence of a large amount of apocytochrome $b_{562}$ R98C/Y101C in the *E. coli* periplasm raises issues about the provision of heme to that compartment of the cell. It is not known how heme is transported across the cytoplasmic membrane (from its site of synthesis in the cytoplasm) for
formation of either cytochromes c or cytochrome \( b_{562} \) in the periplasm (see reference 8 for discussion). However, our results indicate, as do previous observations (16, 44), that heme synthesis and transport across the membrane is not governed (only) by the availability of a periplasmic receptor apocytochrome. The similar ratio of apo to holocytochrome \( b_{562} \) R98C/Y101C both in the presence and absence of the Ccm proteins in this work, particularly when considered with the generally lower total holocytochrome yield when the Ccm proteins are expressed, adds further weight to the argument that the Ccm system does not act as (or include) a heme transporter from cytoplasm to periplasm for cytochrome c production. However, the central question of how heme gets to the periplasm at all remains extant, and is a crucial issue for cytochrome c biogenesis.

A disulfide bond in the CXXCH heme-binding motif of the apocytochrome.

In the light of recent data showing that apocytochromes c can \textit{in vitro} form a disulfide bond in the CXXCH motif (12, 13), it was important to determine, at least qualitatively, the oxidation state of apo \( b_{562} \) R98C/Y101C in the periplasmic fractions from our cells. To obtain reference spectra, we first analyzed purified \( b_{562} \) R98C/Y101C apoprotein (Figure 2). On addition of oxidized (Fe\(^{3+}\)) heme to such protein handled in oxidizing (atmospheric) conditions without the addition of reductant, there were increases in the absorption bands in the region 350-390 nm. These wavelengths are where free heme, and heme non-specifically bound to protein in a high-spin complex, absorb. However, upon subsequent addition of DTT to reduce any disulfide bond in the apocytochrome, the spectrum characteristic of a reduced, low-spin, six-coordinate \( b \)-type cytochrome appeared rapidly (Figure 2). In contrast, when oxidized heme was added to pure wild type apocytochrome \( b_{562} \) in oxidizing conditions, heme bound readily resulting in the characteristic spectrum of an oxidized low-spin cytochrome (Figure 2). Clearly no disulfide bond can form in the wild type protein as it does not have any cysteine residues (2). The present results are indicative of the presence of a disulfide
bond, which may be reduced by DTT, in the \( b_{562} \) R98C/Y101C apoprotein. They were replicated with freshly prepared crude periplasmic extracts, indicating that a disulfide bond had formed in the periplasmic apocytochrome \( b_{562} \) R98C/Y101C, either in the cell, or during the cell fractionation procedure. The purified apocytochrome handled in oxidizing conditions showed no reaction with Ellman’s reagent (5, 5’-dithio-bis(2-nitrobenzoic acid)), also indicating the absence of free thiol groups (and hence the presence of a disulfide bond between the cysteines of the CXXCH motif).

Electrospray mass spectrometric analysis of the \( b_{562} \) CXXCH apoprotein in oxidizing conditions indicated that the protein was present entirely (> 99%) as a monomer, rather than as a dimer or oligomer (with intermolecular disulfides). The mass observed for the protein was 11664.54 ± 0.02 Da compared with a value of 11665.1 Da calculated from the peptide sequence for apocytochrome \( b_{562} \) R98C/Y101C with the two thiols linked in a disulfide bond. When a sample of such protein reduced overnight with DTT was analyzed by ES-MS and compared immediately and in identical conditions with a sample of the oxidized protein, both calibrated against the same equine myoglobin standard, a mass increase of 1.73 ± 0.07 Da was observed. These data are highly indicative of reduction of a disulfide bond in apocytochrome \( b_{562} \) R98C/Y101C to produce free thiol groups and thus rigorously confirm the presence of an intramolecular disulfide bond in the oxidized material. The accuracy and errors of such ES-MS methodology have been established recently in a study of human hemoglobin heterozygotes differing by as little as 1 Da (32).

Taken together, these different types of experiment show that apocytochrome \( b_{562} \) R98C/Y101C can form between its cysteines a disulfide bond that prevents heme from binding correctly. This could be due to the disulfide sterically blocking the heme-binding pocket or, alternatively, due to restriction of the conformation of the C-terminal helix altering the side chain packing, including that
of the important proximal heme ligating residue His-102. On addition of a suitable reducing agent, the disulfide is broken and heme may be quickly bound to the apocytochrome, just as it is in oxidized wild type holocytochrome $b_{562}$. Note that it is necessary for the disulfide to be reduced for correct heme binding to occur irrespective of the oxidation state of the heme iron. After fractionation of cells over $ca. 1 - 2$ hours following initial harvesting, the apocytochrome $b_{562} \text{R98C/Y101C}$ obtained is essentially oxidized. Given this, we assessed the rate of formation of the disulfide bond in vitro.

Samples of purified apoprotein were reduced by incubation overnight at room temperature with a large molar excess of dithionite and either DTT or tris (carboxyethyl) phosphine (TCEP). The excess reductant was removed using a desalting column containing P6-DG resin (Biorad). This column had been calibrated with a mixture of horse heart cytochrome $c$ and ferricyanide, which were observed by their distinct colors to separate clearly. The column was pre-equilibrated and eluted with SET buffer (200 mM Tris-HCl, pH 7.3, 1 mM EDTA and 0.5 M sucrose) under normal (atmospheric) oxygen tension, in order to replicate the conditions used for cell fractionation in this work. The eluted protein was assayed by addition of ferric heme; the first spectrum was recorded immediately after the protein eluted from the desalting column, i.e., approximately 20 min after the reduced protein was loaded on the column. In each case, the resulting spectrum was essentially characteristic of non-bound heme. These data suggest that the disulfide bond in the CXXCH motif of $b_{562} \text{R98C/Y101C}$ forms, almost completely, in $< 20$ min in normally aerated solution. As shown above (Figure 2), addition of oxidized heme to (DTT) reduced apo $b_{562} \text{R98C/Y101C}$ does, initially, give rise to a cytochrome spectrum with absorbance maxima characteristic of cytochrome $b_{562}$. That the eluted apocytochrome $b_{562} \text{R98C/Y101C}$ was mainly reoxidized after $ca. 20$ min was also demonstrated with Ellman’s reagent. The spectra obtained after reduction of protein with DTT,
passage down the P6-DG column and treatment with Ellman’s reagent indicated that not more than 25% of the thiols in the sample remained in the reduced state after ca. 20 min (i.e. $\geq$ 75% of the protein sample was oxidized).

Incorrect holocytochrome formation in vitro from the reaction of apocytochrome $b_{562}$ R98C/Y101C with heme.

It is apparent that in the absence of the Ccm proteins expressed from pEC86 some correct product cytochrome $c$ may be formed from the reaction between $b_{562}$ R98C/Y101C and heme (16, this work). This might be due to a low-level background activity of the endogenous $E. coli$ Ccm proteins, for instance if the cell cultures are not fully aerobic (e.g. 27). Alternatively, it may be that this is the result of spontaneous (uncatalyzed) thioether bond formation, as has been observed recently in vitro for three cytochromes $c$ (12, 13). Thus we investigated the extent of the in vitro (i.e. non enzyme catalyzed) reaction between the apo form of $b_{562}$ R98C/Y101C and heme. An aliquot of purified apoprotein (ca. 20 $\mu$M) was mixed with dithionite and a large excess of DTT in a sealed, nitrogen sparged cuvette. Heme was added to a final concentration of ca. 5 $\mu$M. A spectrum, characteristic of that expected for a $b$-type cytochrome, was recorded (absorption maxima 560.8, 530.4 and 425.3 nm; Table 1; Figure 3) and then the mixture was left to incubate at room temperature, in the dark, for 48 hours. In similar conditions, Hydrogenobacter thermophilus cytochrome $c_{552}$, and to some extent, horse heart mitochondrial cytochrome $c$, form the apparently correct product cytochromes $c$ with two thioether bonds between heme and protein (12, 13). After 48 hours the spectrum of the apo $b_{562}$ R98C/Y101C and heme mixture had changed; all the absorption bands had blue-shifted relative to their starting positions (maxima now at 559.4, 529.0 and 424.1 nm; Table 1; Figure 3). The pyridine hemochrome $\alpha$-band maximum of the product after
48 h was at 552 nm (compared with 556 nm for noncovalently bound heme). These data indicate that covalent attachment of heme to protein had occurred, but that the product was not a properly matured c-type cytochrome. Qualitatively, the spectral characteristics of the product mixture were similar to those of the crude periplasmic extract obtained when \( b_{562} \) R98C/Y101C was expressed without the plasmid expressed Ccm proteins (Table 1; Figure 3 c/f Figure 1).

The products of the spontaneous \textit{in vitro} reaction, prepared on a larger scale, were purified by ion exchange chromatography. One major fraction containing covalently attached heme could be identified but small amounts of other such species were observed in the purification. The major species eluted from anion exchange resin earlier than expected (based upon the elution of wild type holocytochrome \( b_{562} \)) and had optical spectra essentially indistinguishable from component I of the \( b_{562} \) R98C/Y101C mixture in the earlier work (16); it was analyzed by \(^1\)H NMR spectroscopy as previously described (16, 30, 31). The NMR spectra of the reduced (Fe(II)) form of the \textit{in vitro} prepared cytochrome were qualitatively identical to those of the \textit{in vivo} derived component I as reported previously (16) and thus we conclude that this \textit{in vitro} protein fraction is identical to component I. Thus the non-enzymatic reaction between heme and apoprotein that occurs in the cuvette is similar to the mode of (presumably uncatalyzed) covalent attachment of heme to apo \( b_{562} \) R98C/Y101C in the \textit{E. coli} periplasm when the Ccm proteins are not appreciably expressed. The NMR spectra of the reduced \textit{in vitro} produced holoprotein are consistent with the presence of at least two species that are very closely related and cannot be separated from each other. They may represent conformational isomers that are not interchanging on the NMR timescale or may be related to the presence of species with additional mass (+16 Da or +32 Da) that have been observed previously in samples of such protein (16). Notably (Figure S4 in Supplementary Data), there were...
two signals from the heme 4-vinyl α-proton, two from the α-meso proton, three signals from the β-meso proton and three from the Met-7 methyl resonance. However, only one NMR signal was observed from each of the methine and methyl groups generated by the covalent (thioether) attachment at the 2 position of heme to protein, suggesting that the conformational differences between the different species were remote from the point of attachment.

Despite heterogeneity in the NOESY and TOCSY spectra, analysis of the Fe(II) form of component I (16) revealed the presence of signals consistent with one thioether covalent linkage as the heme 2 substituent and one intact heme vinyl group as the 4 substituent in all of the species present. Since component I is spectroscopically identical to the major product with covalently attached heme formed in vitro from the reaction of heme and apocytochrome b562 R98C/Y101C, these signals are also apparent in the spectra of the latter material (Figure S4). The loss of only one vinyl group is consistent with the result from the pyridine hemochrome spectra (16 and Table 1). More strikingly, the NMR analysis clearly indicated that the heme was in an alternative (so-called “minor”) orientation, i.e. rotated 180° about its α,γ−meso axis (e.g. 12, 45), within the protein in all of the species present. Thus, both in vivo in the absence of the ccm gene products and in vitro, cysteine residue 101 has reacted with the 2-vinyl group of heme. In contrast, in all structurally characterized examples of the products of heme attachment catalyzed by any known biogenesis machinery, including by the Ccm system in this work, the 2-vinyl group reacts with the first cysteine in the CXXCH motif (in the present case residue 98).

Two main sets of paramagnetically-shifted signals were also observed in the 1H NMR spectra of the oxidized (Fe(III)) in vitro derived protein with covalently bound heme (Figure S5). Again this is
in stark contrast to the holocytochrome matured \textit{in vivo} by the Ccm apparatus that was found to be homogeneous by NMR (Figure S2).

The present work establishes that whereas $\geq 95\%$ of the holocytochrome $b_{562}$ R98C/Y101C produced \textit{in vivo} in the presence of the Ccm proteins was a properly matured $c$-type cytochrome, nearly all of the holocytochrome with covalently bound heme that was formed \textit{via} an uncatalyzed reaction (\textit{in vitro}) had the heme misattached. These data highlight the requirement for, and the importance of, the very strict, universally conserved, stereo- and regio-specific control of heme attachment that must be enforced by nature’s cytochrome $c$ biogenesis systems, including the Ccm apparatus. Recent work has shown that cytochromes $c$ can sometimes be formed without the assistance of enzymatic catalysis either \textit{in vitro} or in the cytoplasm of \textit{E. coli}. The apparent stereospecificity of heme attachment in the product(s) varied with the (apo)cytochrome studied. In the case of \textit{Hydrogenobacter thermophilus} cytochrome $c_{552}$ the product was, as far as may be determined from the available data, all properly matured (12, 46, 47; SJF and PDB unpublished observations). In the present case, essentially none of the \textit{in vitro} prepared holocytochrome $b_{562}$ R98C/Y101C was correctly formed. Horse heart mitochondrial cytochrome $c$ (13), \textit{Paracoccus denitrificans} cytochrome $c_{550}$ (13) and \textit{Thermus thermophilus} cytochrome $c_{552}$ (40, 41) were all intermediate. Presumably the observed differences between these proteins reflect differences in the structures of the apocytochromes, in the respective heme binding pockets and possibly (for the \textit{in vitro} work) in the method of preparation of the apocytochrome $c$. The stability of the cytochrome $b_{562}$ R98C/Y101C heme-protein complex prepared \textit{in vitro}, with heme bound in the alternative "minor" (rotated) orientation, clearly provides an efficient reaction pathway that could, in principle, compete with the Ccm catalyzed reaction pathway. The fact that this alternative product is not observed when the Ccm gene products are expressed suggests that (i) the Ccm apparatus only
presents heme to the protein in the orientation that results in formation of the correct product and (ii) that heme does not become covalently attached to the apocytochrome $b_{562}$ R98C/Y101C without being handled by the Ccm system in the periplasm. Both these factors are likely to be applicable to, and important for, the maturation of all Ccm made c-type cytochromes.

*The effect of mutations in the E. coli periplasmic disulfide bond oxidizing/reducing apparatus.*

Cytochrome $b_{562}$ R98C/Y101C was also expressed in *E. coli* mutants in which the periplasmic strong disulfide oxidant DsbA or the reductant DsbD had been deleted by genetic manipulation. The $dsbA$ strain is JCB3510 (23) and the $dsbD$ strain JCB606 (14), both isogenic with the parental strain JCB387 used throughout this work. A different (non-isogenic) $dsbA$ strain, JCB571 (24), was used to verify several of the qualitative results. Both DsbA and DsbD are regarded as essential for biogenesis of c-type cytochromes by the *E. coli* Ccm system. In mutants with either of these proteins deleted *E. coli* was unable to synthesize any of its endogenous cytochromes c (8, 10, 14, 23).

In the DsbD deletion mutant, in the absence of the Ccm proteins expressed from pEC86, periplasmic expression of $b_{562}$ R98C/Y101C resulted in a mean 50% decrease in holocytochrome formation relative to the DsbD$^+$ (parental) strain. Qualitatively, the absorption and pyridine hemochrome spectra were similar to those obtained for the periplasmic extract of the DsbD$^+$ strain, indicating that the mixture of product cytochromes formed was probably similar (Table 1). Note, however, that there may be a small contribution to this holocytochrome production from the endogenous *E. coli* Ccm proteins, the expression of which is not absolutely repressed in our growth conditions (see for example refs 27 and 48). In the DsbD$^-$ strain, the ratio of apo $b_{562}$ R98C/Y101C to holocytochrome increased, typically, four-fold relative to the parental (Dsb$^+$). DsbD provides
reductant to the periplasm; its absence correlates with a lower yield of holocytochrome and a higher ratio of apocytochrome to holocytochrome than is observed for the parental strain. These data are consistent with the idea that covalent attachment of heme to apocytochrome \( c \) requires that the cysteine thiols of the CXXCH motif be reduced. In the absence of DsbD, less of the apocytochrome \( c \) can be reduced and so less holocytochrome forms. However, the observation that a still significant amount of holocytochrome can form even in the absence of DsbD suggests that, at least in our growth conditions, there is an alternative source of reductant in the periplasm. These electrons may be relayed by a protein electron transport chain, or may, for example, be from a component of the rich growth media that was used. It has been shown that addition of exogenous thiols to growth media can complement for DsbD deletion in cytochrome \( c \) biogenesis (49). Alternatively, heme acquisition by apocytochrome \( b_{562} \) R98C/Y101C and oxidation of the cysteine thiols to a disulfide bond may be a kinetically competitive process in the \( E. coli \) periplasm.\(^2\)

In the \( dsbA \) deletion mutant JCB3510, in the absence of the Ccm proteins, periplasmic expression of \( b_{562} \) R98C/Y101C resulted in a similar total holocytochrome expression to that of the parental (DsbA\(^+\)) strain. Qualitatively, the absorption and pyridine hemochrome spectra were similar to those obtained for the periplasmic extract of the DsbA\(^+\) strain, indicating that the mixture of product cytochromes formed was probably similar (Table 1). More remarkably, when \( b_{562} \) R98C/Y101C was co-expressed with the Ccm proteins in \( E. coli \) JCB3510 (\( dsbA \)), properly matured \( c \)-type cytochrome formed with a yield comparable to that in the DsbA\(^+\) (parental) strain (0.4 – 1.0 mg of holocytochrome per gram of wet cells). The product was indistinguishable from that when \( b_{562} \) R98C/Y101C was made in the parental \( E. coli \) strain by the Ccm system, as judged by the absorption and pyridine hemochrome spectra (Table 1 and c/f Figure 1). We did not anticipate this, since it is commonly believed that DsbA is an essential protein for \( c \)-type cytochrome biogenesis in
E. coli (10, 14). However, the data were, qualitatively, the same for both of our (non-isogenic) DsbA− strains (JCB3510 and JCB571). One might argue that the result arose because \( b_{562} \) R98C/Y101C is not a “real” c-type cytochrome. Thus, as a control, the experiments were replicated with the plasmid for \( b_{562} \) R98C/Y101C replaced by pKPD1 (25) encoding the gene for Paracoccus denitrificans cytochrome \( c_{550} \), a typical bacterial monoheme cytochrome \( c_2 \). Once again, large amounts of correctly formed cytochrome \( c \) were made in the DsbA− mutant JCB3510 (Figure 4; Table 1); the mean holocytochrome \( c_{550} \) yield was ca. 65% of that in the isogenic parental DsbA+ strain (JCB387) (mean 1.5 and 2.3 mg of cytochrome produced per gram of wet cells for the variant and wild type cells respectively).

These observations, although unexpected, can be rationalized. Sambongi and Ferguson (50) showed that a DsbA deletion could be chemically complemented for cytochrome \( c \) biogenesis by addition of exogenous oxidant to the growth media. In their experiments cells were grown anaerobically (necessary to induce expression of the endogenous E. coli Ccm system) and cystine or glutathione were added to complement for the \( dsbA \) mutation. In the present work, our cells are grown aerobically (with shaking of the growth flasks). The Ccm proteins in our conditions are constitutively expressed from the plasmid pEC86. It may be that in our case it is oxygen, provided by the growth conditions, that is acting as the exogenous oxidant to complement for the DsbA deletion. Alternatively, in our growth conditions, an additional oxidant may be transported to, or made in, the periplasm. In either case, these observations have potentially important implications for \( c \)-type cytochrome biogenesis in Gram-negative bacteria.

E. coli is atypical in that its endogenous \( c \)-type cytochromes are expressed only anaerobically. It does not have a cytochrome \( bc_1 \) complex (respiratory complex III), nor does it have a cytochrome \( c \)
implicated in electron transport to a cytochrome oxidase (51). However, other Gram-negative bacteria such as Pseudomonas aeruginosa and Shewanella putrefaciens do make c-type cytochromes aerobically and have more typical aerobic respiratory chains. The implication of the present work is that DsbA may not be needed in these organisms for such aerobic cytochrome c formation. It is presumed that the role of DsbA in cytochrome c biogenesis in E. coli is to form a disulfide bond between the cysteines of the apocytochrome CXXCH motif. At least in the present case it appears that an exogenous oxidant can substitute in this role (or, in principle, that the disulfide bond is not required at all). However, it must be considered that in the case of multiheme c-type cytochromes (such as pentaheme NrfA and tetraheme NapC), a further role of DsbA is to ensure disulfide bond formation within each particular apocytochrome c CXXCH motif. At present, it is not clear whether an oxidant such as exogenous oxygen would be sufficient for this, i.e. whether the tendency for cysteines to form a disulfide within a CXXCH motif (references 12 and 13; this work) is so strong (and fast) as to make formation of mixed disulfides with the cysteines of other CXXCH motifs very unlikely. Even if such mixed disulfides do form quite readily, it may be that DsbA is still not required if the disulfide isomerase DsbC is present and active and thus able to readily “correct” any inter-CXXCH motif disulfide bonds. Nevertheless, a requirement for DsbA in the biogenesis of multiheme cytochromes c may account for many of the previous observations on its function in E. coli, all of whose natural c-type cytochromes have multiple hemes.

Our observations with the E. coli DsbA mutants in some respects complement those of Deshmukh et al (52) published during preparation of this manuscript. They showed that in Rhodobacter capsulatus, CcdA, a homolog of DsbD is essential for c-type cytochrome biogenesis. However, neither DsbA nor DsbB were essential, at least for the biosynthesis of monoheme cytochromes c, in any growth conditions they investigated.
It is, in fact, becoming apparent that the Ccm/Dsb c-type cytochrome biogenesis system as a whole is somewhat modular, i.e., those components used vary with the particular organism. In E. coli, by far the best-studied case, CcmABCDEFGH are all required, along with DsbD and the thioredoxin TrxA; DsbA and DsbB are also required in some conditions, but see the results above (6, 10). In Bradyrhizobium japonicum and others, an additional protein, CcmI, albeit a homologue of the C-terminus of E. coli CcmH, is required (6). In land plant mitochondria, CcmA, B, C, E, F and possibly H have been identified (43) but CcmD and G have not been to date. CcmG and H are thioredoxin like components of the Ccm system and we have suggested that the apocytochrome oxidation state in the intermembrane space of the plant mitochondrion may be such that they are not necessary in that case (8). Rhodobacter capsulatus and some other organisms use CcdA, an analog of the core transmembrane domain of DsbD, as a periplasmic reductant rather than the whole of DsbD (53) and R. capsulatus does not require DsbA or DsbB (52). Thus, flexibility is clearly apparent in the type I c-type cytochrome biogenesis machinery.

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Footnotes

1. Abbreviations
DEAE: diethylaminoethyl; DTT: dithiothreitol; Ccm: cytochrome c maturation; b_{562}: E. coli cytochrome b_{562}.

2. c-type cytochrome biogenesis in a dsbD strain of E. coli when the Ccm proteins are expressed is being addressed currently as part of a wider study (J. M. Stevens, E. H. J. Gordon and SJF, unpublished observations).

3. Note, however, that the pyridine hemochrome α-band maximum for free (noncovalently bound) heme (e.g. as in a b-type cytochrome) is at 556 nm (38).
Figure Legends

Figure 1. The effect of expression of the ccm genes on the absorption spectra of unpurified periplasmic extracts from cells of *E. coli* strain JCB387 transformed with the plasmid for the R98C/Y101C (CXXCH heme-binding motif) variant of cytochrome *b*$_{562}$. A periplasmic extract from cells also transformed with the plasmid for the *E. coli* cytochrome *c* maturation (Ccm) proteins (———). A periplasmic extract from cells not transformed with the plasmid for the Ccm system (▪ —▪ —▪ —▪). These two spectra have been normalized by extinction coefficients based on the data in reference 16. A spectrum of the periplasmic extract of cells transformed with the ampicillin resistance-conferring cloning vector pTZ19r and the Ccm plasmid grown which indicates typical endogenous (background) cytochrome expression levels (— — — —). These data have been normalized by wet cell weight relative to the data presented for cytochrome *b*$_{562}$ produced in the presence of the Ccm proteins (see Experimental for further details of the use of such control data). All spectra were recorded at 25 °C with the periplasmic proteins in SET buffer diluted 1:1 with water (100 mM Tris-HCl, pH 8.0, 0.5 mM EDTA and 0.25 M sucrose). Sodium dithionite had been added to the cuvettes. Inset: The reduced pyridine hemochrome spectrum of the periplasmic extract of the cells transformed with the plasmids coding for both *b*$_{562}$ R98C/Y101C and the Ccm proteins. Final concentrations of sodium hydroxide and pyridine were 0.2 M and 30% (v/v) respectively.

Figure 2. Spectroscopic detection of the *in vitro* heme-binding properties of different forms of apocytochrome *b*$_{562}$. Purified apocytochrome *b*$_{562}$ R98C/Y101C handled in oxidizing conditions with added oxidized (Fe$^{3+}$) heme (— — — —). Apocytochrome *b*$_{562}$ R98C/Y101C in the presence of dithionite and dithiothreitol (DTT) with an equal amount of added (initially oxidized) heme (▪ —▪ —▪ —▪ —▪). Pure wild type apocytochrome *b*$_{562}$ handled in oxidizing conditions with added oxidized
(Fe$^{3+}$) heme (———). Note that due to the presence of the reductants required to reduce the disulfide bond in apocytochrome $b_{562}$ R98C/Y101C, the resulting cytochrome is reduced (▪ — ▪ — ▪ —) giving rise to a distinct spectrum from that of the wild type cytochrome $b_{562}$ (———), which is oxidized. These spectra are normalized based on the extinction coefficients given in reference 16 for oxidized and reduced wild type holocytochrome $b_{562}$. Spectra were recorded approximately two minutes after addition of heme to the cuvette with the proteins in 50 mM potassium phosphate buffer, pH 7.0.

Figure 3. The in vitro reaction of apocytochrome $b_{562}$ R98C/Y101C with heme in the presence of dithionite and dithiothreitol. The absorption spectrum of the mixture a few minutes after adding heme (———) and after incubation for 48 hours at room temperature, in the dark, in a sealed cuvette (▪ — ▪ — ▪ —). The spectra were recorded with the protein in 50 mM potassium phosphate buffer, pH 7.0. The spectrum of the sample at the beginning of the experiment (———) is offset from the baseline by 0.03 absorbance units for improved clarity. Inset: The reduced pyridine hemochrome spectrum of the mixture after 48 hours.

Figure 4. The absorption spectrum of the unpurified periplasmic extract of cells of E. coli strain JCB3510, a $dsbA$ mutant, transformed with plasmids for the E. coli cytochrome c maturation (Ccm) system and Paracoccus denitrificans cytochrome $c_{550}$. Sodium dithionite had been added to the cuvette. The spectrum was recorded at 25 °C with the periplasmic proteins in 100 mM Tris-HCl, pH 8.0, 0.5 mM EDTA and 0.25 M sucrose.
Table 1:  Wavelengths of main peaks in the absorption and pyridine hemochrome spectra of cytochromes produced in this work. Values for samples from *in vivo* experiments are averaged over several repetitions of the experiment and rounded to the nearest 0.5 nm to account for small differences between samples and inaccuracies in the spectrophotometer. n.d. means values were not determined, n.a., not applicable.

| Protein                          | Genetic background of cells | Ccm expression from plasmid pEC86 | Wavelength maxima of absorption bands (nm) | Wavelength of pyridine hemochrome α-band maximum (nm) |
|----------------------------------|-----------------------------|-----------------------------------|---------------------------------------------|---------------------------------------------------|
| **b**<sub>562</sub> R98C/Y101C  | wild type                  | +                                 | 421, 526, 556                              | 550                                               |
| **b**<sub>562</sub> R98C/Y101C  | wild type                  | -                                 | 424, 529, 559.5                            | 552.5                                             |
| Apocytochrome **b**<sub>562</sub> R98C/Y101C mixed with heme and reductant *in vitro*, after 5 min | n.a. (*in vitro*)           | n.a. (*in vitro*)                     | 425.3, 530.4, 560.8                           | n.d. <sup>3</sup>                                  |
| Apocytochrome **b**<sub>562</sub> R98C/Y101C mixed with heme and reductant *in vitro*, after 48 h | n.a. (*in vitro*)           | n.a. (*in vitro*)                     | 424.1, 529.0, 559.4                           | 552                                               |
| **b**<sub>562</sub> R98C/Y101C  | *dsbD*                      | -                                 | 424, 529, 559.5                            | 552.5                                             |
| **b**<sub>562</sub> R98C/Y101C  | *dsbA*                      | -                                 | 423.5, 529, 559                            | 552                                               |
| **b**<sub>562</sub> R98C/Y101C  | *dsbA*                      | +                                 | 421, 526, 556                              | 550                                               |
| P. denitrificans cytochrome c<sub>550</sub> | wild type                  | +                                 | 415.5, 521.5, 550                          | n.d.                                              |
| P. denitrificans cytochrome c<sub>550</sub> | *dsbA*                      | +                                 | 415.5, 521.5, 550                          | n.d.                                              |
Figure 1

[Graph showing absorbance against wavelength (nm)]

Absorbance

Wavelength (nm)

400 450 500 550 600

0.0 0.1 0.2 0.3 0.4 0.5 0.6 0.7 0.8 0.9

[Inset graph showing absorbance against wavelength (nm)]

Absorbance

Wavelength (nm)

500 510 520 530 540 550 560 570

0.0 0.01 0.02 0.03

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Figure 2
Figure 4

![Graph showing absorbance versus wavelength (nm). Absorbance peaks at approximately 425 nm and 525 nm.]
Supplementary Data

Figure S1. Reference 1D $^1$H NMR spectrum at 700.03 MHz of reduced (Fe(II)) cytochrome $b_{562}$ R98C/Y101C produced by the Ccm system. The protein was isolated and purified as described in the Experimental section after expression in *E. coli* with co-expression of the *ccm* genes from plasmid pEC86. The water signal was suppressed using the WATERGATE sequence.

Figure S2. Reference 1D $^1$H NMR spectrum at 700.03 MHz of oxidized (Fe(III)) cytochrome $b_{562}$ R98C/Y101C produced by the Ccm system. The protein sample is the same as in Figure S1. The residual water signal was irradiated by low-power pre-saturation. The inset shows the upfield region with the y-scale expanded by 4 times, highlighting two protons from Met-7 and His-102, the ligands to the heme iron.

Figure S3. A section of the $^1$H-TOCSY spectrum of the protein in Figure S1. Data were acquired as a 2D matrix of 2048x256 complex points with a mixing time of 30 ms. 128 transients were collected per increment. The TOCSY experiment was performed as previously described (16) with an 8.5 kHz MLEV spin-lock sequence, except that WATERGATE suppression of the water signal was used. Highlighted in the expanded region are the cross-peaks from the 2 and 4 methine and methyl heme substituents that result from the reaction of the cysteine residues of the apocytochrome CXXCH heme-binding motif with the heme 2 and 4 vinyl groups. There is no heterogeneity revealed in this region of the spectrum and the chemical shifts of the relevant protons are identical to those previously reported for component II of the mixture of products described in our earlier work (16).

Figure S4. A section of (A) the $^1$H-TOCSY spectrum and (B) the $^1$H-NOSEY spectrum at 500 MHz of the purified major product with covalently bound heme of the *in vitro* reaction of apocytochrome
b_{562} R98C/Y101C and heme, prepared as described in the main text. The protein was reduced with
dithionite to generate the Fe(II) form. Data were acquired as a 2D matrix of 2048x300 (TOCSY)
and 2048x512 (NOSEY) complex points with mixing times of 25 ms and 130 ms respectively. 64
transients were collected per increment. Only one cross peak arising from coupling of the methine
(5.8 ppm) and methyl (1.2 ppm) protons is observed in the TOCSY spectrum (labeled 2). A vinyl
group spin system is observed downfield (labeled 4). The $\alpha$-vinyl proton is observed as a multiplet
at 8.45 ppm, with the two $\beta$-vinyl proton doublets at 6.2 and 6.4 ppm. Close inspection reveals at
least two sets of signals from this vinyl spin-system. Similar data were obtained from component I
of the in vivo prepared holocytochrome $b_{562}$ R98C/Y101C described in reference 16. The NOSEY
spectrum, in addition to demonstrating that the heme is in the “minor” orientation (i.e. rotated 180°
about its $\alpha,\gamma$-meso axis), shows the heterogeneity of the cytochrome. Multiple peaks (labeled H) are
observed for the $\alpha$ and $\beta$-meso protons as well as for protons of the Met-7 heme ligand methyl group
and residue Phe-65.

Figure S5. $^1$H NMR spectrum of the oxidized (Fe(III)) state of the same protein sample as described
in Figure S4. Data were collected at 500 MHz. The spectrum reveals a completely different pattern
of hyperfine shifted resonances when compared with that of the correctly (Ccm) matured protein
(Figure S2). Note the higher dispersion of low-field signals compared with those in Figure S2 and
also that the broad signals from the ligand protons (Figure S2) were not observed in the upfield
region of this spectrum. Although we have not assigned the resonances in this spectrum, the pattern
is consistent with heme attached in the so called “minor” orientation, i.e. rotated by 180° about its
$\alpha,\gamma$-meso axis (45).
Figure S2
Figure S3
A cytochrome b562 variant with a c-type cytochrome CXXCH heme-binding motif as a probe of the Escherichia coli cytochrome c maturation (Ccm) system

James W. A. Allen, Paul D. Barker and Stuart J. Ferguson

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