Interaction of Heme with Variants of the Heme Chaperone CcmE Carrying Active Site Mutations and a Cleavable N-terminal His Tag*

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Cytochrome c maturation in the periplasms of many bacteria requires the heme chaperone CcmE, which binds heme covalently both in vivo and in vitro via a histidine residue before transferring the heme to apocytochromes c. To investigate the mechanism and specificity of heme attachment to CcmE, we have mutated the conserved histidine 130 of a soluble C-terminally His-tagged version of CcmE (CcmE\textsuperscript{sol-C-His\textsubscript{6}}) from Escherichia coli to alanine or cysteine. Remarkably, covalent bond formation with heme occurs with the protein carrying the cysteine mutation, and the process occurs both in vitro and in vivo. The yield of holo-H130C CcmE\textsuperscript{sol-C-His\textsubscript{6}} produced in vivo is low compared with the wild type. In vitro heme attachment occurs only under reducing conditions. We demonstrate the involvement of one of the heme vinyl groups and a side chain at residue 130 in the bond formation by showing that in vitro attachment does not occur with either the heme analogue mesoheme or when alanine is present at residue 130. These results have implications for the mechanism of heme attachment to the histidine of CcmE. In vitro, CcmE\textsuperscript{sol} lacking a His tag binds 8-anilino-1-naphthalenesulphonate and heme, the latter both noncovalently and via a covalent bond from the histidine side chain, similarly to the tagged proteins, thus counterintuitively confirming a recent proposal that the His tag causes the heme binding. However, the His tag does appear to enhance the rate of in vitro covalent heme binding and to affect the heme ligation in the ferric b-type cytochrome form.

c-type cytochromes are important ubiquitous proteins that bind heme covalently via two thioether bonds between the cysteines in a conserved CXXCH motif in the protein and the vinyl groups of heme. In many Gram-negative bacteria, formation of these covalent bonds occurs in the periplasm in a multistep process involving the so-called Ccm (cytochrome c maturation) proteins A–H, all of which are essential (1, 2). The apocytochromes are synthesized in the cytoplasm and transported to the periplasm via the Sec pathway (3). The mechanism by which heme, which is also synthesized cytoplasmically, is transported to the periplasm is as yet uncertain (4–6). However, it has been shown that the membrane-bound protein CcmC is required for presentation of heme to the membrane-anchored periplasmic protein CcmE (7). CcmE has been identified as the heme chaperone and binds heme covalently via a conserved histidine residue (His\textsuperscript{130} in Escherichia coli) before transferring the heme to apocytochromes (8). Recently, structures of the CcmE apoproteins from two different bacterial species have been reported (9, 10), which shows that the heme-binding histidine is exposed on the protein surface but provides no clue as to the unusual properties of this residue. CcmE has been shown to be part of a complex with CcmF in vivo (11), which together with CcmH forms what is proposed to be a bacterial heme lyase. CcmH interacts with CcmG, which is part of the system that prevents heme release to the periplasm, specifically for reducing the cysteines in the CXXCH motif of the apocytochromes (12).

The nature of the novel bond between the histidine of CcmE and the heme remains unknown, although it has been shown that one of the vinyl groups of heme is involved (13). CcmE has been identified in a number of bacteria as well as in Arabidopsis and presumably other plant mitochondria (14). In vitro studies of a soluble version of CcmE (CcmE\textsuperscript{E}) have shown that the covalent attachment occurs under reducing conditions and that release of the heme to apocytochromes will only occur if heme is in its ferrous state (13). To probe further the covalent attachment of heme in vitro or in vivo, we have altered by site-directed mutagenesis the histidine 130 that has been shown previously to be the site of heme attachment in vivo (8). Replacement of this histidine by cysteine or alanine was expected to generate proteins with either a potentially reactive or an unreactive side chain at position 130. The CcmE\textsuperscript{E} protein used in previous in vitro studies (13) had a His tag at the C terminus. Very recently it has been suggested that such a His tag may significantly influence the binding of heme to CcmE\textsuperscript{E} in vitro to the extent that protein from Shewanella putrefaciens lacking a tag is reported to be unable to bind heme in vitro (9). Any possibility that a His tag might direct heme to form a nonphysiological covalent bond to other than residue 130 would also be addressed by the present mutagenesis studies. Further test of whether a His tag affects the binding of heme has been made by comparing the tagged and untagged proteins with histidine at 130. Furthermore, the mechanistic implications of our studies are discussed.

EXPERIMENTAL PROCEDURES
Plasmid Construction and Mutagenesis—E. coli strain DH5\textsubscript{w} was used for cloning, and JM109(DE3) was used for protein expression. The expression vector for CcmE\textsuperscript{sol-C-His\textsubscript{6}} (CcmE\textsuperscript{E}) was constructed as described (13) and produces the soluble periplasmic region of the protein from Ser\textsuperscript{22} with a cleavable pelB signal sequence for periplasmic targeting of the protein. Amino acid substitutions in this expression vector were performed using the ExSite PCR-based site-directed mutagenesis method (Stratagene) using the primers pET22bH130AF and -R for the H130A mutant and pET22bH130CF and -R for the H130C mutant (as listed in Table I). The vector for the cytoplasmic expression of the

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thrombin-cleavable His-tagged version of the protein (N-His<sub>6</sub>-CcmE<sup>tag</sup>) was constructed by PCR amplification of the gene from the plasmid pEC86 (which was kindly provided by L. Thony-Meyer) using the primers pET22bH130AF and -R, which include XhoI and BamHI restriction sites, respectively (Table I). The PCR products were cloned into the vector pET15-b (Novagen) using these restriction sites, producing the plasmid pE151. Mutations were made in this plasmid using the QuickChange method (Stratagene). The mutations made were H130A using the primers pET15bH130AF and -R, H130C using the primers pET15bH130CF and -R, as well as mutation of the His and Met (both to Ala) that remain on the protein following thrombin cleavage, using the primers pET15bHMAAF and -R (Table I). All of the resulting plasmids were sequenced to confirm that only the desired mutations had been incorporated.

**Protein expression and purification**—For expression of the holo-forms of the periplasmic proteins, the expression vectors pET22, pET22C, and pE223 were co-transformed with the plasmid pEC86 (15), which expresses the Ccm proteins A−H. Co-expression of these proteins is essential for production of wild-type holo-CcmE<sup>+</sup> (13). High levels of the apoproteins were expressed in the periplasm in the absence of pEC86. E. coli cultures were grown as described previously, and the proteins were purified using Ni<sup>2+</sup>-chelating Sepharose columns equilibrated with 50 mM Tris-HCl buffer, pH 7.4, as described (13). The cytoplasmically expressed proteins (from the plasmids pE151, pE152, pE153, and pE154) were purified in the same way, except that the cells were sonicated on ice three times for 30 s to prepare the cell extracts, and the buffer contained 300 mM NaCl throughout. Thrombin cleavage of N-His<sub>6</sub>-CcmE<sup>tag</sup> and mutants thereof was performed using a thrombin CleantCleave Kit (Sigma) according to the manufacturer’s instructions. Un cleaved protein was removed by re-applying the reaction mixture to the Ni<sup>2+</sup>-Sepharose column. Western blots were performed using a peroxidase conjugate of a monoclonal anti-polyhistidine antibody (Sigma) to confirm that the His tags had been completely cleaved.

**Protein characterization**—Discontinuous SDS-PAGE (15 or 17.5% acrylamide) (16) was used to analyze the proteins, and staining for covalently bound heme was performed according to the method of Goodwin et al. (17), following acidified acetone extraction to remove noncovalently bound heme. Visible absorption spectra were recorded on a Perkin-Elmer Lambda 2 spectrophotometer using between 2 and 5 µM heme-protein samples in either 50 mM sodium phosphate buffer, pH 7.0, or 50 mM Tris-HCl buffer, pH 7.4, 300 mM NaCl. Pyridine hemochromatography spectra were obtained according to the method of Barsch (18) using 5 µM protein in 19% (v/v) pyridine and 0.15 M NaOH. Electrospray ionization mass spectrometry (ES-MS) was performed using a Micromass Bio-Q II-ZS triple quadrupole atmospheric pressure mass spectrometer. ESI-MS analysis was used to confirm that the His tag had been completely cleaved from the protein (CcmE<sup>sol</sup>). The protein with the cleavable His tag showed that it has a similar ANS affinity to the protein tag, followed by reduction by disodium dithionite, no differences in the visible spectra, characteristic of a low spin state, were observed compared with the His-tag-containing protein (Table I). There was a subsequent disappearance of these spectral characteristics upon reduction, as we have observed for the wild-type protein with the C-terminal His tag (13).

**Conical heme attachment**—Disodium dithiothreitol (5 mM) was added to solutions in 50 mM sodium phosphate buffer, pH 7.0, containing protein (50 µM) and heme (10 µM) (Table II). After thrombin treatment and removal of uncleaved protein, the cleaved protein (CcmE<sup>sol</sup>) had a mass of 14,890 Da (theoretical mass, 14,890). The difference in the mass of the protein could also be seen by SDS-PAGE analysis (Fig. 1, lane 4). The absence of the His tag was also confirmed by Western blotting.

After the addition of ferric heme to apoprotein lacking a His tag, followed by reduction by disodium dithionite, no differences in the visible spectra, characteristic of a low spin state, were observed compared with the His-tag-containing protein (Table I). There was a subsequent disappearance of these spectral characteristics upon reduction, as we have observed for the wild-type protein with the C-terminal His tag (13). Interestingly, in the visible spectrum of ferric heme and CcmE<sup>sol</sup>, a species was obtained that is similar to that observed upon the addition of ferric heme to horse heart apocytochrome c (19), showing features of a high spin heme-protein species. Thus, the spectrum of the oxidized heme-protein complex obtained without a His tag (a broad Soret band around 400 nm) was different from those for the proteins with the His tag at either the C or N terminus, both of which have a Soret band at 413 nm. These data suggest that the His tag can affect the coordination chemistry of the ferric heme-CcmE<sup>sol</sup> complex leading to a high spin/low spin equilibrium. However, in light of the known effects of polyhistidine on the coordination characteristics of heme, this is arguably not surprising (20). This contrasts with the spectrum of the reduced heme-CcmE<sup>sol</sup> complex not being altered by the absence of the His tag (Table II).

In addition, further analysis of the protein without the His tag showed that it has a similar ANS affinity to the protein with the tag and the same maximum emission wavelength at 480 nm (results not shown). Also, it was possible to displace bound ANS from CcmE<sup>sol</sup> by the addition of heme, as we have shown for the tagged protein (13). The reaction of ferrous heme with the tagged protein led to a protein with covalently bound

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1 The abbreviations used are: ES-MS, electrospray ionization mass spectrometry; ANS, 8-anilino-1-naphthalenesulfonate.
heme that had characteristics indistinguishable from CcmE<sup>apo</sup>-C-His<sub>6</sub> (13) (Fig. 1, lane 2, and data not shown). After thrombin cleavage of the His tag, heme was shown to be covalently bound to CcmE<sup>apo</sup> as shown in Fig. 1 (lane 3). Reaction of CcmE<sup>apo</sup> with heme in the absence of the His tag led to the same qualitative result (Fig. 1, lane 5), but the half-life of reaction was increased by at least 10-fold relative to versions of the protein with His tags at either end.

Following thrombin cleavage of the protein expressed from pET15-b, two potential extra heme-ligating residues remain on the N terminus of the protein, namely histidine and methionine. To avoid any potential artificial heme ligation by the untagged protein obtained by thrombin treatment of the N-terminal His-tagged protein, these residues were mutated to alanines. The proteins with and without the His tag were pure as judged by SDS-PAGE analysis (data not shown) and had the expected masses as indicated by ES-MS analysis. Upon the addition of ferric heme to apoprotein without the His tag, the spectrum changed compared with the spectrum of free ferric heme (Fig. 2). The heme-protein complex appeared to be high spin in the ferric state and switched to low spin in the reduced state upon the addition of disodium dithionite (Fig. 2). Therefore, the presence of an extra methionine and histidine, derived from the linker region, had no observable effect on interactions with heme.

Characterization of the Mutants H130A and H130C—Both the H130A and H130C variants of CcmE<sup>apo</sup>-C-His<sub>6</sub> were expressed well as their apoforms in the E. coli periplasm. The masses of the proteins were confirmed by ES-MS, which also showed that the periplasmic targeting sequences had been completely cleaved. The observed masses were 15,447 Da for the H130A mutant (theoretical mass, 15,450 Da) and 15,483 Da for the H130C mutant (theoretical mass, 15,482 Da). Both proteins were also expressed in E. coli with co-expression of the other Ccm proteins, and the covalent attachment of heme to the proteins was examined by SDS-PAGE analysis followed by heme staining (Fig. 3). Fig. 3A shows that the proteins were purified to homogeneity (lanes 2 and 4 for H130A and H130C, respectively). As expected, the H130A mutant did not appear to bind heme covalently in vivo as judged by heme staining of the SDS-PAGE gel (Fig. 3B, lane 2), which is in agreement with previous experiments (8). The H130C mutant, however, was found to heme stain when expressed with the other Ccm proteins (Fig. 3B, lane 6), indicating that the protein was recognized to some extent by the Ccm system for heme delivery and attachment. To detect the stain from covalently bound heme, the gel had to be overloaded such that a broad band was seen. It was found that the apo-H130C protein formed intermolecular disulfide bonds in vitro when dialyzed extensively against oxygenated 50 mM sodium phosphate buffer, pH 7.0, as shown in Fig. 3A (lane 5). This observation was supported by analysis with Ellman’s reagent, which showed 1 and 0.2 equivalents of free thiol for reduced and oxidized protein, respectively. A significant proportion of the protein ran at a molecular mass of ~30 kDa, corresponding to a covalently linked dimer, which was also identified by ES-MS analysis. In vitro-produced H130C holo-CcmE<sup>apo</sup>-C-His<sub>6</sub> is produced at a very low level, where less than 0.5% of the protein is in the holo-form containing covalently bound heme, as determined from the relative ratio of the Soret band to the absorption at 280 nm.

In Vitro Heme Binding to the H130A and H130C Apoproteins—Upon the addition of ferric heme to the mutant CcmE<sup>apo</sup>-C-His<sub>6</sub>, both H130A and H130C formed noncovalent b-type cytochrome complexes, as determined by visible spectroscopy. The complexes formed within the mixing time and appeared to be stable in this form for several hours. The absorbance maxima for the complexes of both proteins with ferrous heme and mesoheme as well as the pyridine hemochrome spectra are shown in Table II. Mesoheme is a heme analogue that has ethyl substituents in the normal positions of the vinyl groups. The visible spectrum of the complex of H130C with ferric heme shows the characteristics of a b-type cytochrome, with an a-band at 560 nm following reduction with dithionite and immediate recording of the spectrum. Interestingly, it was not possible to record an accurate visible spectrum of the dithionite-reduced H130A mutant b-type complex because dissociation of heme from the protein was too rapid. Heme dissociation upon reduction was also observed with the wild-type heme-protein complex (13). The pyridine hemochrome spectra of the b-type complexes of both mutants with heme have absorbance maxima at 556 nm, which is characteristic of unsaturated vinyl groups and shows that covalent attachment does not occur under these conditions.

The dissociation constants (K<sub>d</sub>) of the mutant proteins for ferric heme were measured by fluorescence spectroscopy as described for the wild-type protein (13). The dissociation constants of the high affinity binding sites were compared, because these are likely to be the physiologically relevant sites. The K<sub>d</sub> of the H130A mutant was found to be 0.72 ± 0.16 μM, which shows that it has a slightly lower affinity for heme than the wild-type protein (K<sub>d</sub>, 0.2 μM (13)). This result is not unexpected because the loss of the histidine side chain is likely to have changed the conformation of the heme-binding site in the protein. The K<sub>d</sub> of the H130C mutant was found to be 0.48 ± 0.08 μM, also higher than the wild type. Both mutant proteins, however, have retained a significant affinity for heme. It should be noted, however, that the model of heme binding presented for this protein suggests that a conformational change occurs in the flexible C-terminal region upon heme binding (10).

Covalent Attachment of Heme to H130C—Upon reduction by disodium dithionite of the C-terminal His-tagged b-type H130C variant protein, with a 5-fold excess of protein over heme, the absorbance spectrum shifted toward a cytochrome spectrum corresponding to a covalent bond between heme and protein. The a-band shifted from 560 to 556 nm over several hours, and the b-band and Soret band also shifted accordingly with time (Table II). After desalting the reaction mixture, the spectrum of H130C produced in this way was very similar to the spectrum of the in vitro-produced H130C protein (Table II). The pyridine hemochrome spectrum of the in vitro produced holo-form of the protein yielded a maximum around 553 nm, which is consistent with...
with the presence of a single free vinyl group, as has been observed for single cysteine variants of c-type cytochromes (21). Interestingly, the in vivo produced holo-form of this mutant had broad absorption maxima in the reduced and pyridine hemochrome spectra. This observation suggests that as a consequence of the substitution of histidine 130 with a cysteine residue, heme attachment is not completely selective and that the formation of incorrect side products can occur. The oxidation state of heme and the cysteine thiol in vivo might not be as tightly controlled during the periplasmic attachment of heme to the protein compared with the reducing conditions of the in vitro experiments.

To prove that the spectroscopic data were indicative of in vivo and in vitro covalent bond formation between heme and the H130C mutant protein, SDS-PAGE analysis followed by heme staining was performed. Fig. 3 (lane 8) shows the reaction of the b-type complex of heme and H130C with dithionite after 14 h. The fact that the protein stains for covalently bound heme (Fig. 3B, lane 8), as does the in vivo produced holo-H130C in lane 6, indicates that in vitro and in vivo covalent attachment of heme to the protein had occurred. The controls for this experiment are shown in lanes 3, 7, and 9. These are H130A incubated with ferric heme followed by reduction with dithionite, H130C incubated with ferric heme, and the addition of ferric mesoheme to H130C protein followed by reduction with dithionite and incubation for 14 h, respectively. These controls show that covalent attachment of heme to the CcmE$^{\text{sol}}$ protein samples did not occur under these conditions, because no heme staining could be observed for CcmE$^{\text{sol}}$. The results establish that one of the vinyl groups of heme is involved in formation of the covalent bond, because attachment was not observed with mesoheme. The results also show that the bond forms with the cysteine residue of the protein, because it is not observed with the alanine mutant. Therefore, it is shown that covalent heme binding can only occur with a reactive side chain of amino acid 130. As was observed for the wild-type protein (13), these results also highlight the requirement for reduction of the heme in the covalent bond formation, because no heme staining was observed with the oxidized sample.

To remove any ambiguity regarding the effect of the His tag on the covalent heme attachment to the H130C variant and the inability of the H130A mutant to bind heme covalently in vitro, proteins with these active site mutations were also made with a N-terminal cleavable His tag. The purified proteins were shown to be pure by SDS-PAGE analysis as shown in Fig. 4 (lanes 1 and 2, for H130A and H130C, respectively). Upon the addition of heme under reductive conditions followed by removal of the His tag by thrombin cleavage, the H130C mutant was found to contain covalently bound heme as determined by SDS-PAGE analysis followed by heme staining (Fig. 4, lane 4) and visible spectroscopy (Fig. 5). The visible spectrum compares very well with the in vivo produced H130C heme-con-

<table>
<thead>
<tr>
<th>Nature of heme-CcmE$^{\text{sol}}$ complex</th>
<th>Ferrous oxidation state</th>
<th>Pyridine hemochrome (a band)</th>
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<tbody>
<tr>
<td>Wild type</td>
<td>b-type heme-CcmE$^{\text{sol}}$-C-His$_6$</td>
<td>560 nm</td>
</tr>
<tr>
<td>H130</td>
<td>Mesoheme-CcmE$^{\text{sol}}$-C-His$_6$</td>
<td>549 nm</td>
</tr>
<tr>
<td></td>
<td>b-type heme-CcmE$^{\text{sol}}$</td>
<td>555 nm</td>
</tr>
<tr>
<td></td>
<td>in vivo holo-CcmE$^{\text{sol}}$-C-His$_6$</td>
<td>560 nm</td>
</tr>
<tr>
<td>H130A</td>
<td>b-type heme-CcmE$^{\text{sol}}$-C-His$_6$</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>Mesoheme-CcmE$^{\text{sol}}$-C-His$_6$</td>
<td>549 nm</td>
</tr>
<tr>
<td>H130C</td>
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<td>560 nm</td>
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a Ref. 13.

Fig. 2. Absorption spectra of the b-type cytochrome formed after the addition of heme to apo-CcmE$^{\text{sol}}$, showing the oxidized spectrum (dashed line), the reduced spectrum obtained immediately after the addition of di-sodium dithionite (solid line), and the spectrum of free heme (dotted line). The absorption spectra were recorded by using 5 $\mu$M protein in 50 mM Tris-HCl buffer, pH 7.4, 300 mM NaCl.

TABLE II
Visible absorption maxima for various forms of CcmE$^{\text{sol}}$
taining protein. The H130A mutant failed to stain for covalently bound heme (Fig. 4, lane 3) and did not show any characteristics of a heme-containing protein in the visible spectrum (data not shown).

DISCUSSION

Effect of the His Tag—The failure of heme to bind covalently to the H130A mutant of CcmE<sup>sol</sup> establishes that the covalent binding of heme observed with the wild-type H130 and H130C proteins involves the specific participation of the imidazole or thiol groups of histidine or cysteine at this residue position. It is reasonable to assume that the initial noncovalent binding of ferric heme to any of the three proteins, i.e. H130, H130C, and H130A, positions the heme appropriately for the subsequent uncatalyzed covalent bond formation with the former two proteins. This is an important result in the context that the His tag might itself contribute to the noncovalent binding site for heme. Recently, Arnesano et al. (9) have argued that untagged CcmE from S. putrefaciens does not bind heme and that a His tag can be responsible for introducing a heme-binding site into this and other proteins. In the present work we show that the untagged CcmE<sup>sol</sup> protein from E. coli retains a heme-binding site at which a covalent bond forms between His 130 and a vinyl group in <i>vitro</i> under the same reductive conditions as described before (13). In this respect our results are at variance with those of Arnesano et al. (9). However, we have noticed that the presence of the His tag affects the visible absorption spectrum of the initial ferric noncovalent CcmE<sup>sol</sup>-heme complexes, consistent with the heme iron having a histidine ligand provided by one of the residues of the tag. In this sense the His tag appears to facilitate the <i>in vitro</i> covalent incorporation of heme into CcmE<sup>sol</sup>. A His tag at either the N terminus (present work) or at the C terminus (13) appears to act similarly in this respect. However, in the present work we show that the heme is bound covalently to CcmE<sup>sol</sup> after the addition of ferric heme to CcmE<sup>sol</sup>-His<sub>6</sub> followed by incubation under reductive conditions and thrombin cleavage. Furthermore, CcmE<sup>sol</sup>, which has had the N-terminal His tag removed by thrombin treatment, can also bind heme both covalently and noncovalently. Thus, we conclude that the presence of a His tag at either end of the protein does not alter the nature of the covalent bond formation. Any facilitation of the interaction of heme with CcmE<sup>sol</sup> by the His tag may be a coincidental partial mimicking of the histidine-rich region on the CcmC protein that is generally agreed to participate in the binding of heme to CcmE in <i>viv</i>o (5).

It is difficult to explain the differences with the results with the protein from <i>Shewanella</i> that imply that CcmE alone cannot bind heme, a view that is argued to be supported by inspection of the structure of the apoprotein which does not have a...
classic hydrophobic pocket for binding heme (9). On the other hand, Enggist et al. (10), having determined essentially the same structure for the apo-CcmE from E. coli as Arnesano et al. (9), have modeled a heme-binding site onto a hydrophobic patch on the surface of the protein. We assume that this patch provides the previously described ANS-binding site (13), which the present work establishes is also present in CcmE\textsuperscript{apo} that lacks a His tag. Judging from the fluorescence emission maximum at 480 nm, this ANS site is not as hydrophobic as can be found in some proteins, consistent with ANS binding in the relatively exposed heme-binding site advocated by Enggist et al. (10). Our previous observations that heme can displace ANS from CcmE\textsuperscript{apo} support this view. Indeed it would be surprising if the presence of a His tag did promote the binding of ANS, given that this probe has a preference for hydrophobic sites and that histidine is a hydrophilic amino acid. However, it cannot be excluded that a C-terminal His tag helps stabilize the C terminus of the protein. The effect of the His tag on the kinetic acceleration of the covalent bond formation between heme and CcmE\textsuperscript{apo} in vitro suggests that either the supplied ligand field and stabilization of the noncovalent heme-protein complex by the His tag is vital or that the His tag may even provide an acid-base catalytic effect on the heme-His\textsuperscript{130} bond formation.

**Heme Binding to H130C CcmE**—Our previous in vitro studies have shown that covalent bond formation can occur spontaneously between the histidine of the heme chaperone CcmE\textsuperscript{apo}-C-His\textsubscript{9} and ferrous heme (13). Upon the addition of ferric heme, the apoprotein binds heme noncovalently with a high affinity, and the covalent bond forms only when the heme is reduced. To investigate this process further, we have performed similar studies on this protein with the heme-binding histidine mutated to a cysteine. Surprisingly, we found that the covalent bond still formed, both in vitro and in vivo, although in the latter case to a lesser extent compared with the wild-type protein. The unusual similarity between the heme binding process occurring with the wild type and the H130C mutant leads to interesting mechanistic proposals. During revision of the present manuscript, an in vitro study was published in agreement with our findings showing that a His-tagged H130C mutant of CcmE can similarly (as judged by several criteria) bind heme covalently on this cysteine, albeit to a low level (22).

**Implications for the Roles of Other Ccm Proteins**—The results presented here also provide some insight into the role of the protein CcmC, which has been shown to bind heme and to present CcmE with heme in the periplasm (5, 7). The fact that the covalently attached form of the H130C mutant is produced in vivo in the presence of the other Ccm proteins to a considerably lesser extent than the wild-type protein, in contrast to the comparable yields in vitro, suggests that CcmC is not effective in catalyzing the attachment of the heme to the cysteine compared with the attachment to histidine. This lower efficiency may indicate that enzymatic action specifically requires the heme-binding histidine residue on the apo-CcmE protein. Ligation effects to the heme iron within the ligand field in the proximity of the heme chaperone might render the vinyl group more reactive to histidine rather than cysteine residues. The observation that the H130C mutant can readily form covalent dimers via a disulfide bridge between the cysteines at position 130 suggests that residue 130 is relatively accessible in CcmE, which is in agreement with the published structure for this protein (10). Because the heme is bound to the histidine residue in this position of the wild-type protein, it is expected that the heme is also accessible, which would allow the apocytochrome c to take up the heme from the heme chaperone. Coordination by a functional group from either the Ccm proteins or the solution leading to a weak ligand field would also enhance the heme transfer upon ligation by the apocytochrome c inducing a strong ligand field.

**Mechanistic Implications**—The successful attachment of the heme to cysteine at residue 130, both in vivo and in vitro, provides some mechanistic insight into heme attachment to CcmE. The in vitro reaction appears to resemble the uncatalyzed formation from polypeptide and heme of a c-type cytochrome, or its derivatives carrying just one cysteine in the

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**Fig. 6.** The proposed chemical process of heme binding to CcmE and heme transfer to apocytochrome c. P denotes the protein moiety of the apocytochrome c, and R abbreviates a leaving group stable as a cation species. In vitro this is presumably a proton. Alternatively, the nucleophilic attack from the apocytochrome is conducted by a thiolate group. The identity of the imidazole N atom participating in CcmE function is not known; N' has been chosen for illustration.
Heme-binding motif that was recently reported (21). The pyridine hemochrome spectra reported here suggest that CcmE<sub>sol</sub> H130C is similar to the known c-type cytochromes with a single thioether bond. At this stage we have been unable to determine the axial ligands for either the oxidized or reduced heme-protein. In <i>vitro</i> formation of thioether bonds of a c-type cytochrome presumably requires protonation of the $\beta$-carbon of the vinyl groups of heme and formation of the thioether bond between the cysteine residues and the $\alpha$-carbon of the vinyl groups of heme (23). A mechanism for this process is envisaged to involve attack of the thiol moiety of the cysteine residues on the $\alpha$-carbon. For thioether bond formation to occur, it was shown experimentally that ferrous heme is required (23). In our work, we have shown that the addition of either the histidine (13) or the cysteine residue to the heme requires ferrous heme. Given the similarities of the <i>in vitro</i> heme binding of either histidine or cysteine variant of CcmE<sub>sol</sub>, it is likely that ferrous heme is required (23). In our work, we have shown that the addition of either the histidine (13) or the cysteine residue to the heme requires ferrous heme. Given the similarities of the <i>in vitro</i> heme binding of either histidine or cysteine variant of CcmE<sub>sol</sub>, it is likely that they occur by a similar mechanism. Therefore, the mechanism of histidine-heme attachment is suggested to be analogous to the thioether bond formation in c-type cytochromes. The histidine residue of wild-type CcmE could add onto the $\alpha$-carbon of one of the vinyl groups. Recently a covalent histidine-heme bond of this nature has been reported for an unusual form of hemoglobin, which is formed <i>in vitro</i> under reductive conditions (24). If the heme attachment to CcmE occurs with a specific vinyl group and heme can bind stereoselectively to CcmE in one orientation, relative to the $\alpha$, $\gamma$ meso axis of the heme moiety, stereospecificity of the heme transfer reaction to apocytochrome c would be achieved. The proposed process of heme binding and heme release from CcmE is summarized in Fig. 6. However, a radical mechanism as proposed for <i>in vitro</i> thioether bond formation between heme and cysteine (25) cannot be excluded on the basis of the current experimental data. The release of the heme from the heme chaperone has similarities with a synthetic reaction yielding dipyrindyl sulfides whereby a quaternary pyridinium moiety acts as a leaving group upon nucleophilic attack of a thiol functionality (26). Additional studies will be required to further describe the processes involved during heme transfer, especially with respect to which vinyl moiety reacts with CcmE and, therefore, the exact nature of the histidine-heme bond. It will also be interesting to find out how the proposed disulfide intermediate with the cysteine thiolates of the apocytochrome c during cytochrome c maturation (27) will affect the heme transfer reaction. However, this work contributes to the understanding of the molecular basis of a central step in cytochrome c biogenesis.

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