Biochemistry

 Volume 42, Number 45 November 18, 2003

Current Topics

A Heme Chaperone for Cytochrome *c* Biosynthesis†

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Received September 5, 2003; Revised Manuscript Received September 30, 2003

The phrase molecular chaperone is normally used to describe a group of proteins that recognize and bind nonnative polypeptides and assist them in folding and assembly into the correct three-dimensional structure. The concept that the biosynthesis of metallocenters in enzymes may also require chaperone type accessory proteins that bind the metal ion or metal complex and subsequently transfer it into the apo-proteins was formulated already in the early nineties by Robert Hausinger (*1*). Today, the term chaperone is used in the literature also in the context of metal cofactor delivery (*2*). For example, copper chaperones deliver copper ions to specific physiological partners by direct protein−protein interactions (*3*−*6*). The copper chaperones are believed to lower the activation barrier for metal transfer into specific protein-binding sites by a docking of the metal donor and acceptor sites in close proximity to each other. Two copper chaperone families have been described: Atx1-like chaperones transfer copper to intracellular copper transporters, and CCS chaperones shuttle copper to copper and zinc superoxide dismutase. Nickel chaperones have been described with UreE for nickel insertion into urease (*7*) and HypB for maturation of NiFe hydrogenases allowing delivery of a nickel atom to maturing pre-HycE (large subunit) in a GTP-hydrolysis-dependent reaction (*8*). The assembly and insertion of iron sulfur clusters into FeS proteins is a complex process requiring assembly factors and chaperone type proteins (*9*−*11*). Other metal chaperones have been proposed (*2*), and the field is growing as biosynthesis of metalloenzymes is studied in more detail.

Discovery of a Heme Chaperone. Heme is a redox-reactive, hydrophobic iron chelate that readily associates with membranes, and it is toxic to cells due to its peroxidase activity. Cells have developed strategies to protect themselves from free heme by sequestering it to specific proteins and carriers. However, heme also needs to be delivered onto many enzymes that contain it as a cofactor, for example, cytochromes.

In my laboratory, we are studying cytochrome *c* maturation (i.e., the posttranslational process needed to target apocytochrome *c* to the bacterial periplasm and insert the heme by covalent ligation to the protein). Because of a number of bacterial mutants with respiratory or photosynthetic deficiencies, genes were found in the late eighties that coded for unknown proteins, yet were essential for the biosynthesis of holo *c*-type cytochromes (*12*). Some of these factors had been postulated to be heme chaperones (*13*), meaning that they could interact specifically with heme, transferring it and docking it to the C-X-X-C-H heme-binding site of the apocytochrome. At the same time, a transient binding of heme by such factors would protect the cell from the toxic heme peroxidase activity. Despite the logic of these ideas, heme chaperones had not been identified until the *Escherichia coli* CcmE protein was discovered. The protein was found accidentally, when cells were grown under anaerobic respiratory conditions, under which *c*-type cytochromes can be synthesized by this organism. In some cytochrome *c* biosynthesis-deficient strains, a novel heme-binding membrane protein was found, and it turned out to be the gene product of *ccmE* (*14*, *15*). This protein apparently bound heme covalently because the cofactor did not dissociate from the protein in denaturing SDS−polyacrylamide gels and retained peroxidase activity in the so-called heme strain (*14*,

† This work was supported by the Swiss National Foundation for Scientific Research.

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10.1021/bi035598c CCC: $25.00
Published on Web 10/23/2003

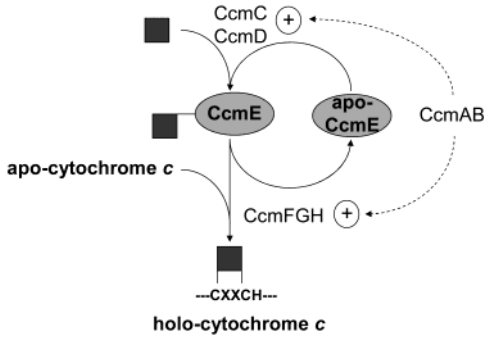

FIGURE 1: Transient binding of heme by the periplasmic heme chaperone CcmE of *E. coli*. Apo-CcmE binds heme with the help of CcmC and CcmD by the formation of a covalent bond to H130. In the presence of CcmFGH, heme can then be delivered from CcmE to the heme-binding site of apo-cytochrome *c*, whereby two covalent thioether bonds are formed between the heme vinyl groups and the cysteines of the heme-binding motif C-X-X-C-H. The ABC transporter CcmAB stimulates holo-CcmE as well as holo-cytochrome *c* formation. Heme is shown as a filled square.

15). In addition, it was shown that CcmE incorporated the radiolabeled heme precursor δ-amino levulinic acid (*14*). These findings indicated a covalent heme binding in a novel context because covalent heme binding had been described only for proteins with a C-X-X-CH motif. However, CcmE did not contain a sequence similar to this motif, and when all known CcmE protein sequences were aligned, not even a single cysteine was found to be conserved. Hence, CcmE represented a novel class of covalent heme-binding proteins.

CcmE Characteristics. The *ccmE* gene is the fifth gene in the cytochrome *c* maturation operon *ccmABCDEFGH* (*16*, *17*). Expression of the *ccm* operon is induced under anaerobic respiratory conditions. The Ccm proteins are needed for the covalent attachment of heme to *c*-type cytochromes (*18*, *19*) (Figure 1). An in-frame deletion of the *ccmE* gene prevented the production of all cellular *c*-type cytochromes, indicating that *ccmE* is essential for cytochrome *c* maturation (*14*).

E. coli CcmE is a 159 amino acid protein with an N-terminal membrane anchor (residues 1−29) and a soluble C-terminal domain (residues 30−159) that was shown to reside on the periplasmic side of the membrane. It binds heme covalently at a single histidine (H130) in the highly conserved signature motif L-A-K-H-D-E-N-Y (residues 127−134). When only the soluble part of CcmE was expressed in *E. coli*, heme binding occurred when the protein was directed to the periplasm by a cleavable signal sequence but not when the protein was expressed in the cytoplasm (*14*). This finding suggested that heme binding to CcmE is a periplasmic reaction. The periplasmic soluble CcmE was barely able to complement a Δ*ccmE* mutant, which stresses the importance of its membrane anchor (*20*). When *ccmE* was expressed in an *E. coli* strain lacking the entire *ccm* operon, the CcmE protein was produced exclusively in its apo-form and did not bind heme (*21*). However, when the *ccmABCD* genes were coexpressed, about 10−20% of the CcmE molecules bound heme covalently (*21*−*23*) (Figure 1).

Soluble apo-CcmE with a C-terminal histidine tag was purified in milligram amounts. For the purification of holo-CcmE, the apo- and holo-proteins were separated by hydrophobic interaction chromatography. CcmE has characteristic spectral properties (*14*, *22*, *23*). In visual absorption spectra, the maxima of reduced holo-CcmE with an α-band of 555−556 nm, a β-band of 526 nm, and a Soret (γ-) band of 421−422 nm are typical for cytochromes; however, the α-bands of *c*-type cytochromes are usually in the range of 550−555 nm and of *b*-type cytochromes in the range of 555−560 nm. The intermediary spectral properties of CcmE are well in agreement with a single covalent bond between H130 and one of the two heme vinyls, in comparison with two covalent bonds to both vinyls in *c*-type cytochromes (Figure 2) and no covalent bonds in *b*-type cytochromes. The spectrum of the pyridine hemochrome with an α-maximum of 551 nm was closer to that of *c*-type cytochromes (∼550 nm) and provided one of the evidences for a covalent bond. Purified soluble holo-CcmE was a monomer in solution; however, a small portion of the preparation always runs at the position of a dimer after SDS−PAGE[1] (*22*), which perhaps is the result of heme stacking.

CcmE Function. The binding of heme by CcmE was an unexpected and intriguing result and allowed insight into the function of this novel protein. Amino acid sequence and comparison with other proteins deposited in databases had not provided any clues as to what its function might be. The fact that heme binding occurred exclusively when the protein was expressed in the periplasm implied that CcmE was required after heme translocation across the membrane and that its function was connected to heme delivery to cytochrome *c*. An experiment was designed to show that heme binding by CcmE, even though covalent, was only transient. When CcmE was overproduced in wild-type cells to levels that allowed detection of the holo form, together with apocytochrome *c* whose expression was under the control of an inducible promoter, the fate of holo-CcmE over time with increasing amounts of apocytochrome *c* could be followed. As apo-cytochrome *c* was converted to holo-cytochrome *c*, a concomitant loss of heme bound to CcmE was observed, indicating a parallel event that could be best explained by a competition for heme by the cytochrome. Interestingly, this competition was successful for the cytochrome only in the presence of the CcmFGH proteins, suggesting that they were also involved in heme delivery from CcmE to cytochrome *c* (Figure 1). Moreover, a mutant cytochrome with an inactive heme-binding site was unable to incorporate the heme; under this condition, the heme remained bound to CcmE. Thus, the transient binding of heme to CcmE was clearly established (*14*). The data were in agreement with the idea of a heme chaperone that binds heme to shield it from adsorption to the membrane due to its hydrophobic nature, to prevent toxic interactions of heme with proteins and lipids, and to present it in an appropriate spatial arrangement to the apocytochrome to facilitate the stereospecific chemistry of thioether bond formation between the heme vinyl groups and the cysteines of the heme-binding motif C-X-X-C-H in apocytochrome *c*. The dual aspect of a protective as well as a delivery function is expected for a chaperone type of protein.

Structure of CcmE. The solution structures of the *E. coli* and *Shewanella putrefaciens* soluble apo-CcmE fragment have been solved recently by NMR (*24*, *25*). Besides a few

[1] Abbreviations: SDS−PAGE, SDS−polyacrylamide gel electrophoresis; HPLC, high-pressure liquid chromatography; MS/MS, tandem mass spectrometry.

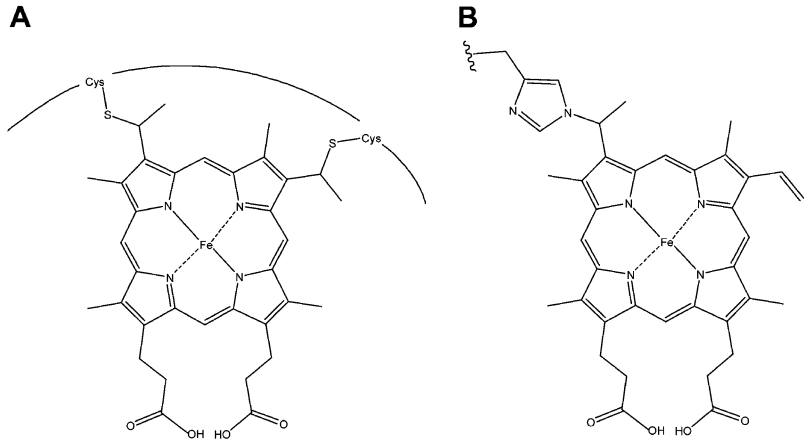

FIGURE 2: Covalent heme binding. (A) Thioether bonds formed between the heme vinyls 2 and 4 to the cysteines of the C-X-X-C-H motif of the cytochrome. (B) Postulated C−N bond between a heme vinyl (here vinyl 2) and a nitrogen (here the N^{ϵ}) of the histidine imidazole. This structure is known from a cyanobacterial hemoglobin.

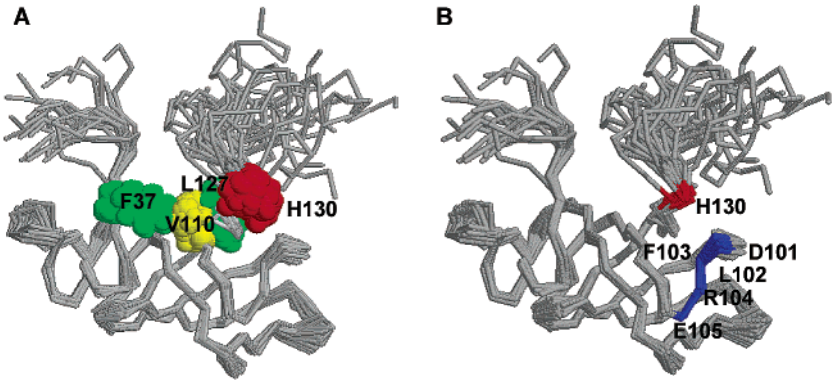

FIGURE 3: NMR structure of the soluble domain of the heme chaperone CcmE. The backbones of a bundle of the 20 best calculated structural conformers are presented (amino acids 34−143). (A) Hydrophobic-binding site at the surface of the β-barrel. The heme binding H130 (red) and the conserved hydrophobic residues (green for residues F37 and L127 with strong mutant phenotypes, yellow for V110) are shown in a space-filling mode. (B) Besides H130 (red), the second-best conserved domain D101-E105 is shown in blue forming a surface-exposed ridge that might be a docking site for an interaction partner.

unstructured amino acids at the artificial N-terminal end, the CcmE fragment revealed two domains that are flexibly oriented relative to each other: (i) a six-stranded β-barrel (residues I34−K129, *E. coli* numbering) that forms a rigid core resolved with high atomic precision and is capped by a short α-helix and (ii) a structurally less well-defined C-terminal domain (D132-S159) with a short single helical turn followed by a unstructured tail of 16 amino acids. At the interface of these two domains, the strictly conserved H130 is exposed to the surface where it is ideally suited to interact with heme (Figure 3). A putative heme-binding site at the surface of the β-barrel in the vicinity of H130 has been proposed due to the clustering of the hydrophobic, surface-exposed residues F37, V110, and L127 that might form a hydrophobic platform (Figure 3A) and two basic residues R61 and K129 that could contact the heme propionates. CcmE belongs to a family of proteins that adopt the so-called OB (oligo binding) fold, in which a α-helical domain is linked to a β-barrel for transient binding and delivery of a substrate (*24*, *25*). The dynamic properties of CcmE are reflected in the unstructured C-terminal tail that might either be mobile or form a stable conformation by physiological interaction with other proteins.

Heme Binding of CcmE. One of the most exciting findings during the discovery of CcmE was the covalent nature of the heme binding in a novel situation where no cysteine was a candidate heme-binding residue. The only known proteins binding heme covalently had been the *c*-type cytochromes, with the covalent thioether bonds formed at the cysteines of a mostly conserved motif C-X-X-C-H (Figure 2A). Even though some heme-binding sites were known to contain only one cysteine or to have a slightly different spacing between the two cysteines, the chemical cross-link between heme and peptide was always via one or two thioether bonds. In CcmE, however, it was recognized very early that this was simply not possible because soluble CcmE does not contain a single cysteine. The covalent nature of the heme−peptide had to be rigorously proved and the heme-binding residue identified. A direct approach to solve this problem was taken by isolating a tryptic peptide of CcmE that retained heme, as shown by several methods including separation by SDS−PAGE under denaturing conditions, boiling, treatment with acidic acetone that extracts the noncovalently bound heme from peptides into the organic phase, peptide sequencing, and mass spectroscopy. Combining the results from the latter two, the conclusion that heme was bound to H130 of CcmE could be drawn: digestion with trypsin revealed a dodecamer peptide H130-K141 that showed heme absorption and had a molecular mass corresponding precisely to the sum of the mass of heme plus peptide. MS/MS analysis resulted in the

identification of daughter fragments with masses corresponding to heme-H-D-E-N, heme-H-D-E-N-Y, and heme-H-D-E-N-Y-T representing the first four to six amino acids of the tryptic peptide. Atomic nitrogen bombardment had therefore led to fragmentation at peptide bonds when heme was still retained, which was a strong argument for a covalent bond. N-terminal sequencing of the heme peptide revealed a first unknown amino acid that eluted with the most hydrophobic amino acids after separation by HPLC and then the expected sequence of the peptide. Thus, only the first amino acid was abnormal, most likely due to a modification with heme (*14*). Taken together, these results confirmed the covalent nature of the heme adduct at H130 in CcmE. In agreement with this conclusion is the finding that a H130A mutant in CcmE of *E. coli* as well as *Rhodobacter capsulatus* was unable to bind heme and support cytochrome *c* maturation (*14*, *26*).

How can we imagine this novel type of heme binding? In principle, H130 might either be an extra strong axial ligand to the heme iron or form a covalent adduct at the porphyrin ring. Several lines of evidence point to the second hypothesis. (i) It seems unlikely that an axial ligand would have survived the nitrogen collisions during MS/MS determination of the mass of the heme peptide. (ii) It should be possible to extract heme from CcmE by acidic acetone if H130 was only an axial ligand. This was not the case. (iii) The H130A mutant is the only CcmE point mutant described so far that leads to a completely heme-less, inactive CcmE. Even though a H130C mutant can bind heme covalently to a low extent, it is inactive in heme delivery (*22*). In this situation, a cysteine thiolate can form a thioether bond with heme as it is seen in cytochrome *c*. (iv) A significant spectral shift in the α-region from 560 nm of the noncovalent CcmE-heme complex was observed relative to 554−555 nm of holo-CcmE (*23*). (v) Modeling of the heme into the putative heme-binding site of CcmE placed H130 close to the heme vinyl 2, where a covalent bond could form (*24*) (Figure 2B). (vi) In vitro heme ligation to CcmE could be achieved with proto-heme, but not with meso-heme, the latter lacking the vinyl groups (*23*). (vii) Covalent heme modifications at the porphyrin ring have been described in the literature. They occur at the α-, *β*-, and *δ*-meso protons (*27*−*29*), at methyl (*29*−*31*) and at vinyl groups (*32*, *33*). The linkage between a heme vinyl group and a histidine in truncated hemoglobin of the cyanobacterium *Synechococcus* sp. PCC 7002 (*32*) is of particular interest because it represents precisely what seems to be the best model for the CcmE-heme histidine bond. This model is presented in Figure 2B. It predicts that the α-carbon of vinyl 2 is connected to the sterically better accessible N^{ϵ} of the histidine imidazole. However, a final proof for this type of bond is not yet available.

The mechanism of heme binding to CcmE has been investigated both in vitro and in vivo. The hydrophobic platform at the surface of the *β*-barrel (Figure 3A) suggested that apo-CcmE might bind heme initially in a noncovalent form. In fact, when heme was added to apo-CcmE, it specifically associated with the protein, resulting in a spectrum corresponding to a *b*-type cytochrome with an α-band of 559−560 nm and a detectable heme stain after SDS−PAGE (*23*, *24*). Recent work of Ferguson's group has shown that apo-CcmE had a high affinity to ferric heme with a K_d of 200 nM. Upon an 18-h incubation with dithionite, heme was bound covalently to CcmE, yielding a spectrum of holo-CcmE (*23*).

In vivo, holo-CcmE can only be formed in the presence of CcmC, an integral membrane protein with six transmembrane helices and a characteristic tryptophan-rich motif in a periplasmic domain (*21*, *34*, *35*). CcmC is the only factor required strictly for the covalent incorporation of heme into CcmE, but the reaction is stimulated by the small membrane protein CcmD, and maximal amounts of holo-CcmE are produced when in addition the ABC transporter CcmAB is present (*21*, *34*) (Figure 1). Currently, the substrate of the ABC transporter is not known, and it is neither excluded nor proven that CcmAB transport heme. Also, the precise function of CcmC and CcmD are not established. It is possible that CcmC transports heme to the periplasm. Alternatively, CcmC is a CcmE-heme lyase involved in a correct spatial arrangement of heme and CcmE and/or catalysis of the heme-histidine bond. It has been shown by coimmunoprecipitation that CcmC and CcmE interact physically. This interaction is disturbed in particular point mutants in the tryptophan-rich motif of CcmC and seems to be essential for heme delivery to CcmE (*35*).

CcmE Mutants. Various mutants have been constructed to characterize the heme binding and delivery functions of the heme chaperone in more detail. When the known CcmE homologues were aligned, two mainly conserved patches of amino acids were found with P-D-L-F-R-E (positions 100−105) and L-A-K-H-D-E-X-Y (positions 127−134), where each defined amino acid was >90% conserved. The first motif maps to a surface-exposed loop in the *β*-barrel structure close to H130, which is in the middle of the second conserved region (Figure 3B). Before the structure was known, site-directed alanine scanning mutagenesis of prominent amino acids within these motifs (D101A, F103A, R104A, E105A, L127A, K129A, H130A, D131A, E132A, and Y134A) as well as some other highly conserved amino acids (F37A, S70A, D86A, and Y95A) were constructed with the idea to find mutants that were either blocked in heme binding or could bind heme but not deliver it to cytochrome *c* (*22*). Interestingly, mutations in acidic or basic residues affected heme binding and cytochrome *c* maturation less than mutations in other residues. The only mutant that was completely blocked in heme binding and delivery was H130A; all other mutants retained at least some heme-binding activity. However, mutant H130A produced almost normal amounts of CcmE polypeptide. While single mutations of the charged residues around H130 had almost no phenotypic consequences, those of the hydrophobic residues L127 and Y134 clearly affected heme binding. Y134A also showed low levels of *c*-type cytochromes suggesting that heme delivery was hampered. Interestingly, less heme binding was also observed in mutant F37A. All CcmE homologues have phenylalanine or another hydrophobic amino acid at this position. When the upper surface of the *β*-barrel was inspected for exposed residues, it was found that F37, L127, and H130 are arranged in one line. Another residue, V110, that was not included in our mutant collection but is highly conserved (100% V or I) resides between F37 and L127 (Figure 3A). F37, V110, and L127 appear to form a hydrophobic platform on top of the *β*-barrel next to H130 where heme can easily bind. TOCSY spectra of reduced holo-CcmE showed perturbed side chain but not backbone

resonances for F37 and V110, which is in agreement with the idea of a heme-binding site (*24*). In addition, the Y134A mutant was affected in heme binding and cytochrome *c* maturation. This residue is part of the second conserved motif and resides in the flexible C-terminal extension of CcmE. It may act as an axial heme ligand that can help to move heme in and out of the hydrophobic pocket. Among the other mutants, the most drastic effect on heme binding was seen with F103A and the triple mutant F103A/R104A/E105A within the first conserved motif. These amino acids reside on the edge of a loop of the β-barrel that is adjacent to H130 (Figure 3B). However, F103 points inward, which might be the reason mutant F103A is less stable than the wild type. It is possible that this part of CcmE provides a site for specific interaction with another protein such as CcmC or CcmF (*36*).

H130 was also changed to a cysteine to investigate its heme-binding properties. In membrane-bound CcmE-H130C, low but significant levels of holo-CcmE were found (*22*). The soluble version of the mutant protein was purified, and the tryptic heme peptide was isolated to prove covalent heme binding. In vitro heme binding to the H130A and H130C mutant proteins occurred with somewhat lower affinity (K_d = 0.72 and 0.48 μM, respectively). As expected, the H130A mutant could not bind heme covalently upon addition of dithionite, whereas heme ligation was possible in the case of H130C (*37*). Hence, covalent attachment of heme to CcmE requires a reactive side chain and reducing conditions. The mechanism for heme binding to H130 is likely to involve a nucleophilic addition to one of the vinyl groups (*37*). However, in vivo it is clear that at least CcmC is necessary for heme transfer and delivery to CcmE (Figure 1) (*36*).

The NMR structure determination of apo-CcmE revealed a highly flexible C-terminal tail of this protein relative to the rigid β-barrel. This made perfect sense in view of of the dynamic heme delivery function of the chaperone. On the other hand, the C-terminal ~20 amino acids are the least conserved of the entire protein. In some species, they are even missing. Therefore, the question arose whether this domain was of importance at all. A series of C-terminal truncations were constructed and tested for both heme binding and heme delivery. In essence, for covalent heme binding all 29 amino acids of *E. coli* CcmE beyond the heme-binding histidine could be deleted without a complete loss of heme binding. Yet, wild-type levels of heme binding were achieved only with constructs containing at least eight more residues. A similar behavior was found for the heme transfer function from CcmE to cytochrome *c* (*20*). These results suggest that the C-terminal extension is not essential for CcmE function but increases the efficiency of heme transfer. Perhaps the C-terminal tail has some role in docking CcmE to CcmC for efficient heme transfer. It would be interesting to know if this domain is structured in a CcmE-CcmC complex.

Heme Delivery for Cytochrome c *Maturation.* In vivo, the minimal requirement for heme binding to CcmE is provided by CcmC (*21*), whereas the delivery of heme to cytochrome *c* depends on the presence of CcmFGH (*14*). Coimmunoprecipitation of CcmE was demonstrated with CcmC and CcmF but was not achieved with CcmH (*35*, *38*). Both CcmC and CcmF have a periplasmic tryptophan-rich motif (W-G-X-X-W-X-W-D) that has been suggested to be a heme-binding site in conjunction with conserved periplasmic histidines, and these sequence motifs were shown to be important for cytochrome *c* maturation. The idea that CcmC is needed for heme delivery to and CcmF for heme delivery from CcmE is in agreement with the reported coprecipitations (*36*, *39*).

In vitro, the transfer of heme from holo-CcmE to apocytochrome *c* was observed without adding additional Ccm proteins (*23*). Even cytochrome *c* formation could be achieved in vitro without any additional protein, provided that the concentration of stable apocytochrome and heme with free vinyl groups and the appropriate reducing conditions were maintained (*40*−*42*). This suggests that the Ccm proteins provide a cellular environment where the heme ligation reaction can occur rather than catalyze the required chemistry.

CcmE Homologues. Type *c* cytochrome biosynthesis is exerted in nature by three different systems I, II, and III (*36*, *43*−*46*) depending on the organism. CcmE has been viewed as a hallmark of system I with most of the genes *ccmABCDEFGHI* required for efficient biosyntheis of *c*-type cytochromes. Neither in system II nor in system III has a heme chaperone been described. In system I, heme seems to be relayed via two tryptophan-rich motif-proteins CcmC and CcmF with the CcmE heme chaperone inbetween (*36*), perhaps being a sink for equilibration of extracytoplasmic heme. System II has only one W-rich protein for heme delivery, and its role and interaction partner(s) are not well-investigated (*47*). Here, a candidate for a heme chaperone is missing, as it is also the case in system III, where a single enzyme, the cytochrome *c* heme lyase, is responsible for covalent heme attachment in the mitochondria.

CcmE is found in many bacteria belonging to the α- and γ-subgroup of the proteobacteria, *Deinococcus*, and plant mitochondria (*46*). In γ-proteobacteria and *Deinococcus*, *ccmE* is found upstream of *ccmF* and downstream of *ccmC(D)*, whereas in α-proteobacteria, it is separted from *ccmCD*. In *R. capsulatus*, the *ccmE* gene is isolated from the other cytochrome *c* maturation genes (*26*).

Sequences with clear homology to CcmE can be found among the microbial genome sequences in certain β-proteobacteria (*Ralstonia metallidurans* with 41% and *Bordetella parapertussis* with 39% identity, respectively), where the other *ccm* genes are also present and in *Desulfitobacterium hafniense* (42% identity) belonging to the Gram-positive *Clostridiales*, where in addition to the CcmE homologue (GI: 23116552), a CcmF homologue (23116551) also seems to be present. Surprisingly and with a high homology score (64% identity), a CcmE homologue seems to be encoded in the mosquito *Anopheles gambiae* (GI: 30179536). The *Anopheles ccmE* gene is most likely the product of horizontal gene transfer from an α-proteobacterium because its derived product shares the highest degree of sequence similarity with the CcmE proteins of this class of bacteria. Interestingly, *Anopheles* is the first organism known in which genes for proteins of all three systems of cytochrome *c* maturation can be found: CcmE (64% identity), CcmG (38% identity), and CcmH (31%identity) of system I; ResB (23% identity) and ResC (43% identity) of system II; and CCHL (40% identity) of system III. Functionally, CcmE was shown to be involved in cytochrome *c* maturation directly in many proteobacteria such as *E. coli* and several *Rhizobium*, *Rhodobacter*, and *Pseudomonas* strains. Heme binding of CcmE was shown

for CcmE proteins expressed in *E. coli* from *R. capsulatus* (*26*), *Bradyrhizobium japonicum* (*48*), *Arabidopsis thaliana* (*49*), and *Vibrio cholerae* (M. Braun and L. Thöny-Meyer, unpublished). Besides the *E. coli*, also the *Shewanella putrefaciens* soluble part of apo-CcmE was purified and used for NMR structure analysis; however, in this case, not even noncovalent heme binding was detected (*25*) as it had been found for *E. coli*. So far, CcmE has not been found to be encoded in archaeal, fungal, or nonarthropod metazoal genomes nor has it been detected in other bacterial groups than the ones mentioned previously.

Other Heme Chaperones? The absence of CcmE homologues in many organisms does not exclude the existence of other heme chaperones. In fact, if a heme chaperone is defined as a protein that transiently binds heme to shield it from reactive interaction partners thereby preserving it for assembly with its cognate apoprotein and perhaps even participating in the protein−cofactor assembly, it seems probable that more chaperones for heme delivery are yet to be discovered. One example is that of the recently identified cytoplasmic ShuS heme sequestering protein that also interacts with DNA (*50*). In this case, however, it is not known where heme is delivered to from ShuS. An open question is also how heme is targeted to and incorporated into *b*-type cytochromes and hemoglobins. It should be stressed that the role of a heme chaperone is distinct from that of a simple heme capture or heme carrier protein as, for example, the hemophore HasA of many pathogenic bacteria, which excrete this protein to catch heme and transfer it to a specific heme receptor at the cell surface for heme acquisition (*51*, *52*). Even though HasA functions in heme delivery, a role in assembly (i.e., the specific incorporation of a heme cofactor into a protein for enzymatic function) is absent.

Perspectives. Why would a protein bind heme covalently if in a subsequent step the cargo is delivered to another protein? This question is perhaps the most striking in the context of the heme chaperone function. It has challenged our view of the biology of CcmE many times and probably will continue to be a mystery until we understand the details of the heme delivery mechanism. The idea of a reservoir for reduced heme in a place (the bacterial periplasm) where heme would be oxidized and could potentially react with many proteins and lipids in a damaging way is attractive. Mechanistically, CcmE could be involved in the stereoselective attachment to cytochrome *c*, which always occurs in only one orientation. The covalent bond formation would be helpful to select and fix one but not the other heme-protein arrangement at an intermediate state of the process. Another interesting aspect that has not been studied in detail is the regulation of the capacity and need for transient heme storage and how it is coupled to the biosynthesis and degradation of heme. The initial concept of metal chaperones has been expanded to metal−organic molecules by the discovery of the heme chaperone CcmE. In the future, it may be extended to understand more generally the specific delivery of metal or nonmetal cofactors to their target enzymes.

ACKNOWLEDGMENT

I thank Martin Braun for his valuable comments on the manuscript and Elisabeth Enggist for the preparation of Figure 2.

REFERENCES

1. Hausinger, R. P. (1990) *Biofactors 2*, 179−84.
2. Finney, L. A., and O'Halloran, T. V. (2003) *Science 300*, 931−6.
3. Rosenzweig, A. C. (2001) *Acc. Chem. Res. 34*, 119−28.
4. Solioz, M., and Stoyanov, J. V. (2003) *FEMS Microbiol. Rev. 27*, 183−95.
5. Huffman, D. L., and O'Halloran, T. V. (2001) *Annu. Rev. Biochem. 70*, 677−701.
6. Harrison, M. D., Jones, C. E., Solioz, M., and Dameron, C. T. (2000) *Trends Biochem. Sci. 25*, 29−32.
7. Soriano, A., Colpas, G. J., and Hausinger, R. P. (2000) *Biochemistry 39*, 12435−40.
8. Casalot, L., and Rousset, M. (2001) *Trends Microbiol. 9*, 228−37.
9. Lill, R., and Kispal, G. (2000) *Trends Biochem. Sci. 25*, 352−6.
10. Frazzon, J., Fick, J. R., and Dean, D. R. (2002) *Biochem. Soc. Trans. 30*, 680−5.
11. Park, S., Gakh, O., O'Neill, H. A., Mangravita, A., Nichol, H., Ferreira, G. C., and Isaya, G. (2003) *J. Biol. Chem. 278*, 31340−51.
12. O'Brian, M. R., and Thöny-Meyer, L. (2002) *Adv. Microb. Physiol. 46*, 257−318.
13. Thöny-Meyer, L. (1997) *Microbiol. Mol. Biol. Rev. 61*, 337−76.
14. Schulz, H., Hennecke, H., and Thöny-Meyer, L. (1998) *Science 281*, 1197−1200.
15. Reid, E., Eaves, D. J., and Cole, J. A. (1998) *FEMS Microbiol. Lett. 166*, 369−75.
16. Grove, J., Tanapongpipat, S., Thomas, G., Griffiths, L., Crooke, H., and Cole, J. (1996) *Mol. Microbiol. 19*, 467−81.
17. Thöny-Meyer, L., Fischer, F., Kunzler, P., Ritz, D., and Hennecke, H. (1995) *J. Bacteriol. 177*, 4321−6.
18. Darwin, A. J., and Stewart, V. (1995) *J. Mol. Biol. 251*, 15−29.
19. Tanapongpipat, S., Reid, E., Cole, J. A., and Crooke, H. (1998) *Biochem. J. 334*, 355−65.
20. Enggist, E., and Thöny-Meyer, L. (2003) *J. Bacteriol. 185*, 3821−7.
21. Schulz, H., Fabianek, R. A., Pellicioli, E. C., Hennecke, H., and Thöny-Meyer, L. (1999) *Proc. Natl. Acad. Sci. U.S.A. 96*, 6462−7.
22. Enggist, E., Schneider, M. J., Schulz, H., and Thöny-Meyer, L. (2003) *J. Bacteriol. 185*, 175−83.
23. Daltrop, O., Stevens, J. M., Higham, C. W., and Ferguson, S. J. (2002) *Proc. Natl. Acad. Sci. U.S.A. 99*, 9703−8.
24. Enggist, E., Thöny-Meyer, L., Güntert, P., and Pervushin, K. (2002) *Structure 10*, 1551−7.
25. Arnesano, F., Banci, L., Barker, P. D., Bertini, I., Rosato, A., Su, X. C., and Viezzoli, M. S. (2002) *Biochemistry 41*, 13587−94.
26. Deshmukh, M., Brasseur, G., and Daldal, F. (2000) *Mol. Microbiol. 35*, 123−38.
27. Igarashi, N., Moriyama, H., Fujiwara, T., Fukumori, Y., and Tanaka, N. (1997) *Nat. Struct. Biol. 4*, 276−84.
28. Catalano, C., Choe, Y., and Ortiz de Montellano, P. (1989) *J. Biol. Chem. 264*, 10534−41.
29. Ator, M., and Ortiz de Montellano, P. (1987) *J. Biol. Chem. 262*, 1542−51.
30. Hoch, U., and Ortiz de Montellano, P. R. (2001) *J. Biol. Chem. 276*, 11339−46.
31. Fiedler, T. J., Davey, C. A., and Fenna, R. E. (2000) *J. Biol. Chem. 275*, 11964−71.
32. Vu, B. C., Jones, A. D., and Lecomte, J. T. (2002) *J. Am. Chem. Soc. 124*, 8544−5.
33. Osawa, Y., Highet, R. J., Bax, A., and Pohl, L. R. (1991) *J. Biol. Chem. 266*, 3208−14.
34. Schulz, H., Pellicioli, E. C., and Thöny-Meyer, L. (2000) *Mol. Microbiol. 37*, 1379−88.
35. Ren, Q., and Thöny-Meyer, L. (2001) *J. Biol. Chem. 276*, 32591−6.
36. Thöny-Meyer, L. (2000) *Biochim. Biophys. Acta 1459*, 316−24.
37. Stevens, J. M., Daltrop, O., Higham, C. W., and Ferguson, S. J. (2003) *J. Biol. Chem. 278*, 20500−6.
38. Ren, Q., Ahuja, U., and Thöny-Meyer, L. (2002) *J. Biol. Chem. 277*, 7657−63.
39. Thöny-Meyer, L. (2002) *Biochem. Soc. Trans. 30*, 633−8.
40. Daltrop, O., Allen, J. W. A., Willis, A. C., and Ferguson, S. J. (2002) *Proc. Natl. Acad. Sci. U.S.A. 99*, 7872−6.
41. Daltrop, O., and Ferguson, S. J. (2003) *J. Biol. Chem. 278*, 4404−9.

42. Daltrop, O., Smith, K. M., and Ferguson, S. J. (2003) *J. Biol. Chem. 278*, 24308−13.
43. Kranz, R., Lill, R., Goldman, B., Bonnard, G., and Merchant, S. (1998) *Mol. Microbiol. 29*, 383−96.
44. Page, M. D., Sambongi, Y., and Ferguson, S. J. (1998) *Trends Biochem. Sci. 23*, 103−8.
45. Xie, Z., and Merchant, S. (1998) *Biochim. Biophys. Acta 1365*, 309−18.
46. Enggist, E., and Thöny-Meyer, L. (2004) in *Respiration in Archaea and Bacteria. Vol 1: Diversity of Prokaryotic Electron Transport Carriers* (Zannoni, D., Ed.) Kluwer Academic Publishers, Dordrecht, The Netherlands, in press.
47. Kranz, R. G., Beckett, C. S., and Goldman, B. S. (2002) *Res. Microbiol. 153*, 1−6.
48. Schulz, H., and Thöny-Meyer, L. (2000) *J. Bacteriol. 182*, 6831−3.
49. Spielewoy, N., Schulz, H., Grienenberger, J. M., Thöny-Meyer, L., and Bonnard, G. (2001) *J. Biol. Chem. 276*, 5491−7.
50. Wilks, A. (2001) *Arch. Biochem. Biophys. 387*, 137−42.
51. Stojiljkovic, I., and Perkins-Balding, D. (2002) *DNA Cell Biol. 21*, 281−95.
52. Wandersman, C., and Stojiljkovic, I. (2000) *Curr. Opin. Microbiol. 3*, 215−20.

BI035598C