C-type Cytochrome Formation: Chemical and Biological Enigmas

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Received June 9, 2004

ABSTRACT

C-type cytochromes are proteins that are essential for the life of virtually all organisms. They characteristically contain heme that is covalently attached via thioether bonds to two cysteines in the protein. In this Account, we describe the challenging chemistry of thioether bond formation and the surprising variety of biogenesis systems that exist in nature to perform the difficult posttranslational heme attachment process. We show what insight has been gained into the various biogenesis systems from in vitro and in vivo experiments and highlight some forthcoming challenges in this field.

Introduction

The association of heme (Fe-protoporphyrin IX; Figure 1, structure **I**) with polypeptide has been recognized for over 100 years. The heme group provides a range of functionalities including oxygen binding, electron transfer, signaling, and catalysis. The word cytochrome (cellular pigment) was coined by Keilin in the 1920s to apply to components involved in mitochondrial respiration. Today, it is used more generally for heme proteins that either carry out an electron-transfer function or catalyze a chemical reaction involving oxidation and reduction of substrates. Heme is noncovalently bound in the majority of different types of heme proteins. Among the cytochromes, *a*-types and *b*-types (including most cytochrome P450s) are prominent examples. However, there are many different *c*-type cytochromes, the major protein group with heme covalently bound. Mitochondrial cytochrome *c* exemplifies the almost universal mode of such heme attachment. The two original vinyl groups of heme are converted into thioether bonds (Figure 1, structure **II**) following the addition of thiol sulfurs, from two cysteines of a CXXCH motif within the protein, to the α-carbons of the heme vinyls (Figure 2B).[1,2] Rare exceptions are either attachment to a CXXXCH, CXXXXCH, or CXXCK motif or attachment via a single thioether bond to an A/FXXCH motif, the latter presently known only in a group of eukaryotes, the Euglenozoa.[1] The H (or K) is always one of the axial ligands to the heme iron. The second axial ligand is usually either a methionine, as in mitochondrial cytochrome *c* and many related bacterial monoheme *c*-type cytochromes, or histidine, as in many bacterial multiheme *c*-type cytochromes; these ligands are supplied by residues distant in the sequence from the CXXCH motif. Mitochondrial cytochrome *c* functions in apoptosis,[3] but its main role is electron transport; an important feature is that the methionine ligand preferentially stabilizes Fe(II) and thus shifts the reduction potential to more positive values (ca. +250 mV) than are usually seen for bis-histidinyl coordinated *c*-type cytochromes (sometimes as low as ca. −400 mV).[4] However, the dielectric constant of the heme-binding environment is also a major influence on the potential.[4] In bacterial *c*-type cytochromes the number of heme groups per polypeptide chain varies from one to, for example, 34 in one of the 111 predicted *c*-type cytochromes in the genome of the iron-oxidizing bacterium *Geobacter sulfurreducens*. It is striking that among globular proteins such large numbers of hemes per polypeptide chain are only found in *c*-type cytochromes. The structure of mitochondrial cytochrome *c* (Figure 2A) has no resemblance to that of multiheme cytochromes *c*, while a unique structure is provided by cytochrome *f,* a *c*-type cytochrome of plant thylakoids; this is all β-sheet in contrast to the usual predominance of α-helices in such cytochromes.[2] A notable feature of many multiheme *c*-type cytochromes, for example, hydroxylamine oxidoreductase and a soluble fumarate reductase,[2] is that they have a conformationally related close packing of the hemes, despite having little or no sequence similarity. In these cases, and others including the tetraheme cytochromes c_3 of sulfate-reducing bacteria, the heme clustering may be comparably important as the polypeptide chain for determining the overall fold. Despite the variations in protein structure among the *c*-type cytochromes, it is striking that the stereochemistry of heme attachment is always the same (Figure 2B).[2]

Why Is Heme Covalently Attached in *C*-type Cytochromes?

Two recent reviews addressed the question as to why *c*-type cytochromes are formed.[1,2] Formation of the thioether bonds (see later) is not straightforward and is intriguing considering that there are so many heme proteins in intra- and extracellular environments without covalent attachment of heme. An experimental system permitting some of the issues to be addressed is based

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10.1021/ar030266l CCC: $27.50
Published on Web 10/12/2004

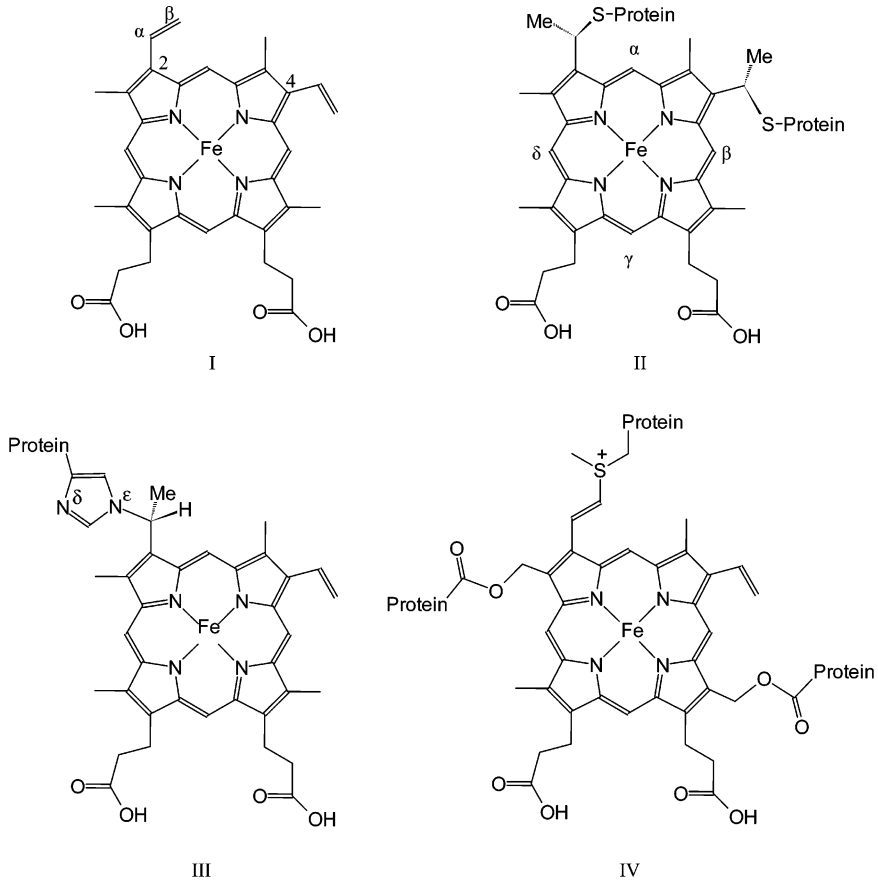

FIGURE 1. Structures of (**I**) heme (Fe-protoporphyrin IX) and (**II**−**IV**) three modes of covalent binding of heme to polypeptide chains. The 2- and 4-positions are labeled (see **I**) using the "Fischer System of Nomenclature". The vinyl groups at positions 2 and 4 are replaced by ethyl groups in mesoheme. The designation of the vinyl carbon atoms as α or β is also shown in **I**. The thioether bonds with invariant S stereochemistry found in *c*-type cytochromes are shown in **II**, as is the $\alpha,\beta,\gamma,\delta$ designation of the meso carbons of heme[2]. The novel bond with R stereochemistry between a histidine side chain and heme, recently described for a *Synechocystis* hemoglobin, demonstrated to form in vitro and assumed also to occur in vivo to some extent, is shown in **III**.[21] The covalent bonds between polypeptide, one methionine, one aspartate, and one glutamate side chain in mammalian myeloperoxidase are shown in **IV**.[20]

on cytochrome c_{552} from the thermophilic bacterium *Hydrogenobacter thermophilus*. Noncovalent heme binding, but close to wild-type tertiary structure, is observed after the loss of thioether bonds through mutation of CXXCH to AXXAH.[5,6] Such conversion of a *c*-type to a *b*-type cytochrome resulted in decreased thermal and chemical stability of both the oxidized and reduced protein but shifted the reduction potential by only −70 mV. Loss of one thioether bond, through mutation to AXXCH or CXXAH, had much smaller effects on the stability and potential.[7] It is not clear why formation of one thioether bond has such a dramatic effect on stability that could not be provided by other features (e.g., closer packing) of the polypeptide chain, although the smaller entropy gain on unfolding a *c*-type rather than a *b*-type cytochrome, when the heme completely dissociates, may be a factor (see ref 8). Other plausible reasons for covalent bonds in *c*-type cytochromes are discussed elsewhere.[1,2] The main correlation previously observed was that such bonds can allow packing of more hemes for a given number of amino acids.[2] The significance of the resulting heme clusters is not clear; in some cases, they may be an almost isotropic electron-harvesting device.[9]

In Vitro Thioether Bond Formation

The formation of *c*-type cytochrome thioether bonds in vivo is a catalyzed posttranslational modification process, the understanding of which has benefited from recent analysis of the in vitro reaction between heme vinyl groups and cysteine thiols of the CXXCH sequence in apocytochrome c_{552}.[10] Removal of heme from cytochrome c_{552} generated apoprotein, which when mixed with stoichiometric amounts of heme formed first a noncovalent complex, a *b*-type cytochrome; over several hours the latter converted to material that was indistinguishable from cytochrome c_{552}.[10] The interpretation is that this apoprotein was readily able to recognize heme and then adopt the essential structure of the holoprotein with the consequence that the thiols were close to the heme vinyls. A proximity effect thus appears to have provided substantial rate acceleration for thioether bond formation. The mechanism of the addition is not clear but the following are known: (1) The reaction is somewhat faster at lower pH, suggesting that protonation of the vinyl β-carbon, a carbocation mechanism, is followed by thiol addition and proton loss (Figure 3A), rather than initial

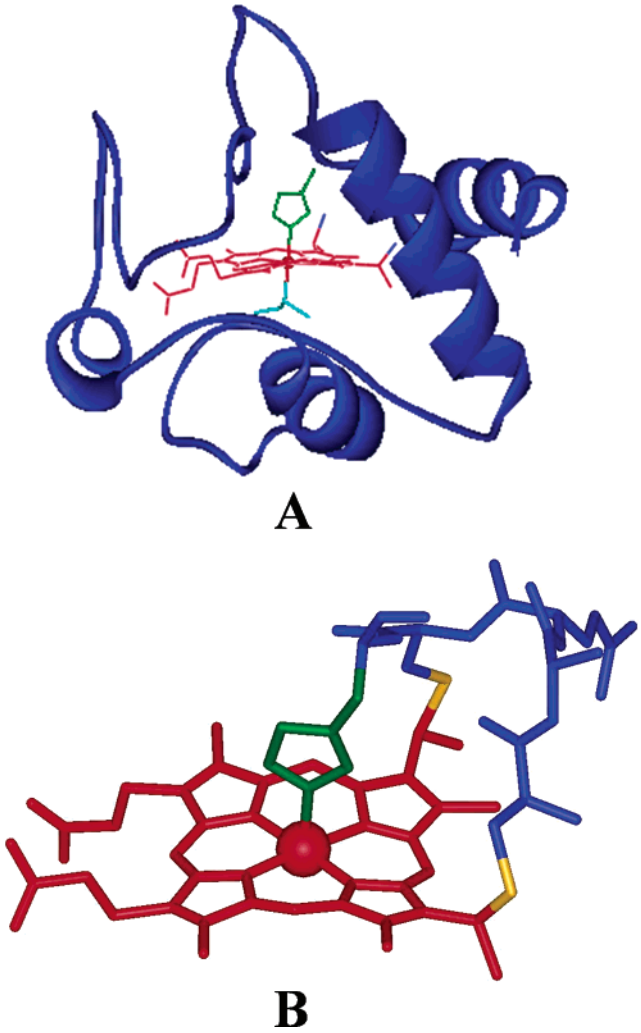

FIGURE 2. Panel A presents a schematic representation of horse heart (mitochondrial) cytochrome *c* (PDB code 1HRC), showing the heme in red and the polypeptide in blue. The axial ligands to the heme iron are histidine (green) and methionine (cyan). Note that the thioether bonds between the heme and the protein are not shown. Panel B presents the single enantiomer thioether bonds between the heme vinyl groups and the cysteine side chains (yellow) in the CXXCH motif. The histidine side chain and the remaining amino acids are shown in green and blue, respectively. Note that the organization of the CXX(XX)CH motif shown in Figure 2 is the same in all structurally characterized *c*-type cytochromes. The 2- and 4-vinyl groups of heme are always, respectively, attached to the N- and C-terminal cysteines of the CXXCH motif in native *c*-type cytochromes; thus the heme is always oriented the same way with respect to the α,γ meso axis. The methionine ligand to iron shown in panel A is not always an axial ligand in *c*-type cytochromes; histidine is often the sixth ligand, and there are examples with cysteine, an N-terminal amino group, asparagine, lysine, or a vacant coordination site. There are few restrictions on the nature of the XX residues.

attack by the cysteine thiol(ate). An amino acid side chain may contribute to protonation of the vinyl group. This mechanism of addition is supported by earlier work where thiols of free cysteine reacted at acidic pH with vinyls of protoporphyrin IX.[11,12] (2) The reaction requires ferrous heme; reaction with ferric heme leads to mixed products, none of which are bona fide *c*-type cytochromes[10] (for reasons, see Figure 3 legend). (3) A disulfide bond between CXXCH cysteines forms readily and prevents thioether bond formation.[10] This argues against any mechanism in which the breakage of a disulfide provides part of the driving force for the thioether bond formation, although steric hindrance by the disulfide may also be a factor. (4) There is no evidence that the two thiols react in an ordered fashion, as deduced from studies of reactions between single cysteine cytochrome c_{552} variants and either heme or monovinyl hemes; two isomers, 2- or 4-vinyl heme, react specifically with CXXAH and AXXCH variants, respectively.[13] The rates of these latter reactions are similar to that of the first phase of reaction of wild-type protein with heme,[10] implying that the rate of initial attack of the thiol on the vinyl group is independent of the possibility of a second thioether bond formation. Moreover, significant amounts of single-cysteine-attached products could not be detected during reaction of CXXCH protein with heme, suggesting that formation of the first thioether bond is followed more rapidly by the second.[10]

Study of the addition of cysteine to vinyl uracil[14] supports the initial vinyl protonation mechanism suggested here for *c*-type cytochrome thioether bond formation.[14] However, such a mechanistic pathway, without catalysis, is too slow to be viable as the mode of formation of *c*-type cytochromes in vivo, which is believed to occur on a time scale of a few minutes.[15] A radical mechanism (Figure 3B) has been contemplated on the basis of model studies;[16] this cannot be ruled out, even if it does bring with it the potential problem of unwanted side products.[17] It is noteworthy that proximity effects also play a role in another reaction of cysteines with heme, perhaps analogous to those we have reported in vitro for various apocytochromes *c*. Thiols of self-assembled mercaptoalkane monolayers on an electrode surface form thioether bonds with vinyls of heme adsorbed to the monolayer.[18] This arrangement presumably increased the effective concentration of reactants to yield a product with thioether bonds. Similar considerations may apply to the recent in vitro synthesis of de novo cytochromes *c*.[19]

Other Heme to Polypeptide Covalent Bonds

In myeloperoxidases, the β-carbon of the heme vinyl attaches to a methionine sulfur (Figure 1, structure **IV**); the vinyl double bond is retained. This is believed to occur in an uncatalyzed, but probably radical-based, reaction involving a proximity effect generated by the binding of the vinyl close to the methionine side chain in an initial noncovalent complex.[20] The occurrence of such chemistry emphasizes that thioether bond formation in *c*-type cytochromes requires careful control.

A surprising recent discovery is the addition of histidine side chains to heme vinyl groups. This is best characterized for an algal hemoglobin;[21] the reaction, which occurs in vitro to give the product shown in Figure 1, structure **III**, rapidly follows the addition of a small excess of dithionite to an initial noncovalent complex between ferric heme and protein. This reaction cannot be replicated with Zn-protoporphyrin IX (which might have been thought

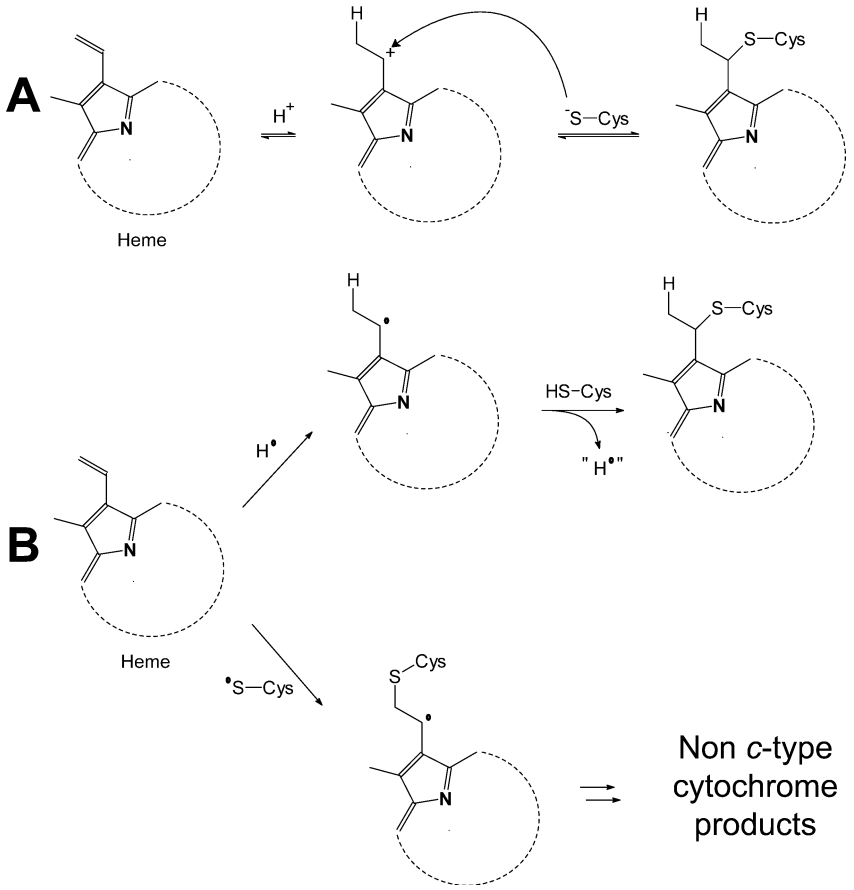

FIGURE 3. Possible mechanisms for reactions of cysteine thiol groups with vinyl groups of heme. Panel A shows the carbocation mechanism discussed in the text, with heme in the ferrous state. Note that there is no reason to advocate a Michael addition (e.g., as in reaction of a thiol with *N*-ethyl maleimide) because there is no activating group appropriately positioned. Concerted [2+2] addition of a thiol to a carbon−carbon double bond is not favored for conservation of orbital symmetry reasons. It is believed that ferric heme would not promote correct thioether bond formation because thiol coordination to the heme iron may interfere. Ferric heme might favor initial nucleophilic attack of a thiol group, but this might be directed to the β vinyl carbon so that any final product would not be a *c*-type cytochrome.[17] The mechanism shown cannot be distinguished from one in which the initial protonation is followed by attack by thiol rather than thiolate with loss of a proton as the final step. Panel B shows possible radical mechanisms for thioether bond formation.[16] These might not require ferrous heme, but this is not established.

to be equivalent to ferrous heme); thus there are indications that ferric/ferrous transitions, together with radical formation, might be important.[21] The formation of a vinyl−heme bond in a hemoglobin may relate to one of the ways in which biology makes *c*-type cytochromes. In many species of Gram-negative bacteria and plant mitochondria, the Ccm (**c**ytochrome ***c*** **m**aturation) proteins (Figure 4) catalyze this process.[22] One of the components, CcmE, forms a covalent bond between a completely conserved histidine and a vinyl group of heme.[15,23] The nature of this covalent bond has not been determined, although it can be speculated to be similar to that in the algal hemoglobin (Figure 1, structure **III**), a notion supported by the failure of mesoheme (see legend, Figure 1) to attach covalently to CcmE.[23] The covalent bond between heme and CcmE breaks in vivo, although it is not clear how, when the heme is transferred from CcmE to an apocytochrome.[15] It has been possible in vitro to both form and break, the latter through transfer to an apocytochrome *c*, the bond from heme to a soluble form of CcmE.[23] This has allowed demonstration that reductant is needed for both processes, but as yet there are no significant clues for radical involvement in CcmE−histidine−heme bond formation. In vivo and in vitro studies have shown that if the heme-binding histidine is changed to cysteine then a covalent (presumably thioether) bond forms and, at least in vivo, cannot be broken by subsequent transfer of the heme to apocytochrome *c*.[24,25]

The structures of two heme-free CcmE proteins[26,27] provide some insight into the mechanism by which the protein functions. There is a rigid core structure of six β-strands and a flexible C-terminal domain. This is similar to a family of proteins with oligo-binding folds, which function in uptake and subsequent transfer of nonprotein molecules to target proteins, consistent with the proposed function of CcmE. A modeled heme-binding site[27] for CcmE involved a patch of hydrophobic residues and two basic residues in suitable positions to interact with the heme propionate groups. The heme 2-vinyl group was in close proximity to the heme-binding histidine and in particular to N^{δ} (thus differing from Figure 1, structure **III**), which is a candidate for making the covalent bond to heme.[27] It is clear from these structures that heme binding must occur on the surface of CcmE, in contrast

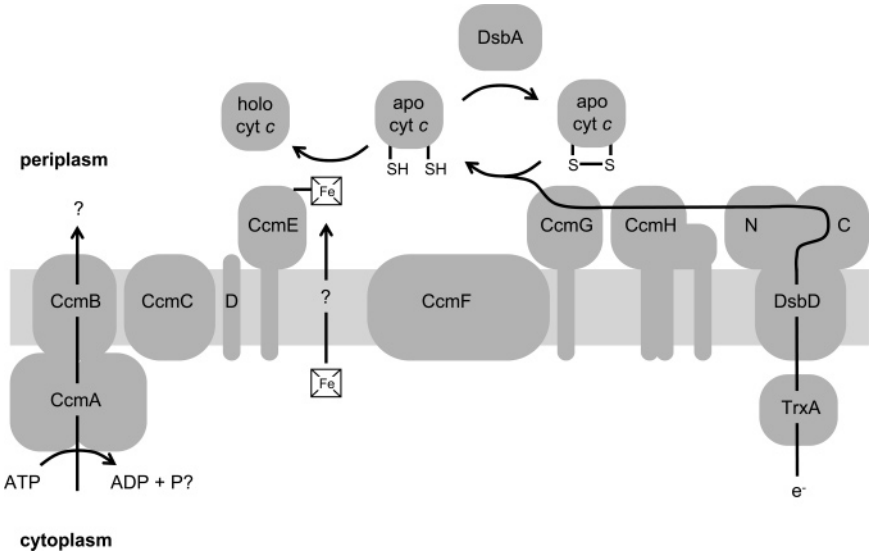

FIGURE 4. Cytochrome *c* biogenesis system I from a typical Gram-negative bacterium, in this case *E. coli*. This system, with some variations, is found in the α, β, and γ groups of proteobacteria, deinococci, and plant and red algal mitochondria. As mentioned in the text, CcmG and DsbA are apparently absent from plant mitochondria. Some bacteria, especially α-proteobacteria, have an additional component called CcmI, which is a mainly periplasmic protein, but with two predicted transmembrane helices, and has some similarity with the C-terminal part of *E. coli* CcmH. DsbA is reoxidized by an integral membrane protein called DsbB, which is itself reoxidized by quinones of the respiratory chain. Some of the many uncertainties, for example, does the CcmAB system, identified from sequence analysis as a member of the ABC group of transporters, act as an ATPase to transport a substrate across the membrane, and the pathway of heme across the membrane, are designated with a "?". The extent of complex formation between the different Ccm components is still under investigation; as explained in the text, CcmE has been suggested to shuttle between other Ccm components. DsbD is a remarkable protein with two thiols among the eight transmembrane helices that are believed to accept reducing equivalents from thioredoxin. These thiols in turn pass on the reducing power to periplasmic C- and N-terminal domains. From there reductant passes both to the *c*-type cytochrome biogenesis apparatus, tentatively by the route shown, and to an apparatus for reducing incorrectly formed disulfide bonds in periplasmic proteins. CcdA (see Figure 5) sometimes operates in conjunction with system I instead of, or as well as, DsbD. The reduction potential for the dithiol/dicysteine conversion for the CXXC of *Rhodobacter sphaeroides* cytochrome c_2 has been estimated at -170 mV. The widely used Ccm nomenclature is occasionally not followed.[22]

with other heme-containing proteins, which normally have heme in a solvent-shielded cavity. A single covalent bond from CcmE to a specific vinyl group is an attractive possibility because this would provide an obvious control point for the conserved stereochemistry of the heme attachment to apocytochromes *c*. Strikingly, all the highly conserved residues of CcmE, except for the heme-binding histidine but including the C-terminal domain,[28] can be altered without abolition of function.[29] It should be noted that the presence of a His-tag complicates the in vitro behavior of CcmE,[25] and the absence of the membrane anchor affects in vivo performance.[28]

Cytochrome *c* Biogenesis in Vivo

At present there is only limited mechanistic evidence as to how the thioether bonds of *c*-type cytochromes form, but this provides a foundation for seeking to understand how biology forms these bonds. A surprise has been the discovery of at least three distinct cytochrome *c* biogenesis systems,[1,22,30] only one of which, with rare exceptions, occurs in any particular cell type. They have been termed systems I, II, and III (Figures 4–6). System I is found most commonly in Gram-negative bacteria and plant mitochondria. System II is found in thylakoids and both Gram-negative and Gram-positive bacteria. System III is restricted to certain types of mitochondria. Until recently it was assumed that no cells had more than one biogenesis system potentially functioning in the same location; genomic analyses have revealed this to be incorrect. *Anopheles gambiae*, the malarial mosquito, remarkably has elements of all three known cytochrome *c* biogenesis systems in its genome (although system I at least is incomplete).[29] Our analysis of the β-proteobacteria *Bordetella bronchiseptica* and *Bordetella parapertussis* genomes shows that these organisms contain all the components of both systems I and II. At least for the former organism, all the maturation proteins contain every residue that has been identified from experimental studies as important; expression studies are awaited. However, there is no obvious reason why only one of these maturation systems would be functional (or preferred) in these bacteria. The genome of the Gram-positive bacterium *Desulfitobacterium hafniense* also contains both system I and II components. It remains to be demonstrated how frequently organisms contain multiple cytochrome *c* biogenesis systems and to what extent there is then functional degeneracy.

The slow observed in vitro rates of thioether bond formation, even with the aid of a proximity effect as seen in studies with cytochrome c_{552},[10] suggest that activation of either the heme or the cysteines or both in vivo (for instance, by adding a potential leaving group to the cysteines of the CXXCH motif) cannot be ruled out. Indeed, system I can currently be considered to use breakage of

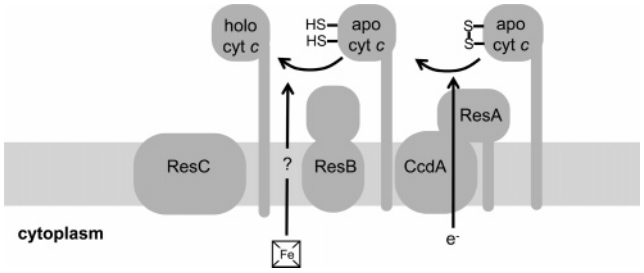

FIGURE 5. Cytochrome *c* biogenesis system II from a typical Gram-positive bacterium, in this case *Bacillus subtilis*, showing the substrate *c*-type cytochrome anchored to the membrane. This system is also found in some Gram-negative bacteria (including β-, δ-, and ϵ-proteobacteria and at least one γ-proteobacterium, *Acidithiobacillus ferooxidans*) and thus can act on cytochromes *c* that are destined to be water-soluble periplasmic proteins. The system also occurs in actinomycetes, cytophagales, aquaficales, plant, and algal chloroplasts as well as cyanobacteria. In some cases, the CcdA protein is replaced by the DsbD protein shown in Figure 1. Disulfide bond formation in at least some organisms having system II is catalyzed by the Bdb proteins, which have resemblance to the Dsb proteins usually found in organisms with system I. Presumably, CcdA also functions to transfer reductant from cytoplasmic thioredoxin to the external surface of the membrane; thus in the absence of CcdA, cytochrome *c* synthesis is possible if the Bdb proteins are also absent through mutation. The structure of the soluble domain of ResA shows it to be thioredoxin-like but with a cleft that is postulated to be tuned for binding the CXXCH motif.[51] Alternative nomenclatures are in use for system II proteins: ResA = CcsX, ResB = CcsB = Ccs1, ResC = CcsA, and CcdA = CcsC.

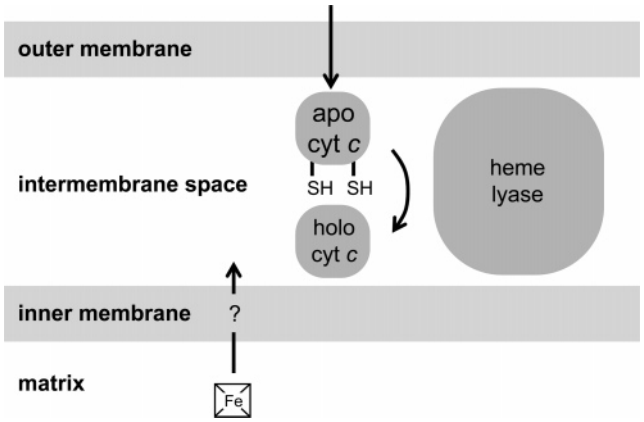

FIGURE 6. Cytochrome *c* biogenesis system III. This system has been found biochemically in mitochondria of fungi, vertebrates, and invertebrates and is present in the genomes of *Chlamydomonas reinhardtii* and *Plasmodium falciparum*. The transport route for the apocytochrome *c* through the outer mitochondrial membrane has been elucidated but is beyond the scope of this article. The exact location of the heme lyase, attached to the inner or outer membrane or unbound in the intermembrane space, is not clear. The mechanism of heme transport across the inner mitochondrial membrane from its site of synthesis in the matrix is also unknown and is shown with a "?". As explained in the text, some organisms have separate heme lyases for cytochromes *c* and c_1, whereas others have just one lyase that can act on both apocytochromes.

the CcmE–heme–histidine bond as part of the driving force for thioether bond formation, and it is possible that different chemical strategies have been adopted by the three systems.

System I

System I (Figure 4) comprises many Ccm components, the roles of most of which are far from understood. In bacteria this system acts on the periplasmic side of the membrane to attach heme to the apocytochrome. The latter is transferred from the cytoplasm by the Sec apparatus but how the heme reaches the periplasm from its cytoplasmic site of synthesis is a mystery. CcmA and CcmB, perhaps in conjunction with CcmC and CcmD, are often considered to be candidates for a heme transporter because sequence analysis indicates that at least CcmA and CcmB are components of a putative ATP-dependent transporter. However, there is no firm experimental evidence for a heme transport role and some against.[1,31] It is possible that the hydrophobic heme simply partitions into the cytoplasmic membrane and by equilibration reaches proteins in the periplasm; the potential toxicity of "free" heme militates against this proposal. While the exact roles of CcmA, -B, -C, and -D remain uncertain, heme becomes covalently attached (see above), in a CcmC-dependent manner in vivo, to CcmE.[32] Such attachment occurs in the absence of CcmFGH, excluding them from any role in heme transfer to the periplasm, but these three proteins are needed for the breakage of the covalent (assumed to be C–N) bond between heme and CcmE and its replacement by formation of two thioether bonds in a product *c*-type cytochrome. CcmE may function in a CcmCEF supercomplex or as a heme shuttle between CcmC and CcmF.[29] The seemingly novel CcmE-dependent chemistry involved here is not understood but the following are known: (i) In vivo, heme transfer will not occur to variant *c*-type cytochromes that have only one cysteine, even where such variants are stable proteins that can be experimentally made by other means.[33] This suggests that two thioether bonds compensate energetically for the breaking of one histidine to heme bond, but the Ccm system may also be organized to recognize a disulfide in, or the two thiols of, the CXXCH motif in one or more steps. (ii) All structurally characterized *c*-type cytochromes have the same orientation and stereochemistry of heme attachment (Figure 2B).[2] (iii) Use of a model system in which a four helix bundle protein cytochrome b_{562} has been converted into a *c*-type cytochrome by incorporation of a CXXCH motif in the heme binding site has shown that spontaneous thioether bond formation is possible but with heterogeneous products, whereas the action of the Ccm system ensures stereochemical fidelity, a characteristic that might ensue from one of the vinyls of heme being selected specifically for reaction with the essential histidine of CcmE.[34] (iv) System I does not function in the absence of provision of reductant, physiologically via the DsbD protein (Figure 4), a requirement that could reflect the need, as established from the in vitro studies described earlier, to reduce a disulfide bond in the CXXCH motif, to maintain the heme iron in the reduced state, or both.[1] (v) CcmG is a thioredoxin-like protein but with some structurally distinct features,[35] while CcmH also has some characteristics of a

disulfide reductase (Figure 4);[22] each is probably involved in reductant transfer from DsbD to the targets (a disulfide bond in the CXXCH motif, heme, or both).

What is recognized in the apopolypeptide that is destined to become the *c*-type cytochrome is uncertain. In some cytochromes, multiple CXXCH motifs are separated by only a few amino acid residues. Thus it appears probable that the Ccm system first recognizes, and then attaches hemes to, CXXCH motifs of extended polypeptides and that final folding of the protein follows the attachment. It is remarkable that the Ccm system of *Escherichia coli,* and presumably of other bacteria, will insert heme into almost any CXXCH motif, with rare exceptions,[1] from that in mitochondrial cytochrome *c* to those of a foreign multiheme protein.

An unexpected finding has been that whereas many mitochondria use system III, plant and red algal mitochondria use system I.[36] However, CcmG has not been found in plant genomes, and it is not clear whether, once the apoprotein has arrived in the intermitochondrial space from its site of synthesis in the cytoplasm, there is any kinetically relevant tendency to form a disulfide bond within the CXXCH motif. In the bacterial periplasm, there is a strong such tendency because of DsbA (Figure 4). There is no obvious analogue of DsbD/CcdA in the inner mitochondrial membrane and so any provision of reductant is unlikely to be from a matrix source. No thioredoxin localized to the intermembrane space of any mitochondria has yet been definitively identified.

System II

The key components in system II, and its occurrence, are in Figure 5.[30,37] System II has some resemblances to system I with respect to the presence of components for ensuring that any disulfide bond formation in the CXXCH motif is reversed, but otherwise they are very different. The provision of reductant involves either DsbD or CcdA, which is similar to the transmembrane section of DsbD. System II shares with system I a lack of definitive information as to how heme is delivered across the membrane. In addition, there is as yet no sign of a counterpart to CcmE that could form a transient covalent bond to heme. System II may differ from system I by being able to attach heme to a XXXCH motif. This important question has not been tested experimentally to the same extent as for system I, but it is reported that a tetraheme *c*-type cytochrome matured by system II can incorporate a heme at one variant AXXCH binding site.[38] Initially it appeared that system I was always used when a large number of water-soluble multiheme *c*-type cytochromes were made, e.g., in *Shewanella,* but now it is clear that system II is adopted by some such organisms, e.g., *Geobacter*. Thus the reasons for use of system I rather than II are not clear. The achievement of the same stereochemistry of heme attachment by systems I and II, despite the seeming absence of a CcmE analogue from the latter, reinforces the possibility that these two systems use different chemical pathways to the final thioether bonds.

System III

System III is striking for its relative simplicity and limited occurrence (Figure 6). Either a pair of closely related proteins known as cytochrome *c* and cytochrome c_1 heme lyase, or a single heme lyase, are the only known components necessary to attach heme to apocytochromes *c* in many mitochondria.[39] Yeast exemplifies use of two heme lyases (although there is some overlapping specificity for cytochrome *c* or cytochrome c_1[39]), whereas in mammals, one heme lyase apparently acts on both cytochromes *c* and c_1. There is evidence that yeast cytochrome *c* heme lyase is the only dedicated component needed for formation of cytochrome *c*: (i) yeast genetic screens only identified this component and (ii) expression of yeast cytochrome *c* heme lyase in the *E. coli* cytoplasm with mitochondrial apocytochrome *c* produces a good yield of holocytochrome *c*.[40] Analysis of the operation of systems I and II raises issues, for example, achievement of correct stereochemistry, which are just as pertinent to system III; on the other hand, the simplicity of system III prompts the question why are the other systems so complex. One response is that system III only deals with one type of monoheme cytochrome *c* (cytochrome *c* and the globular part of cytochrome c_1 have very similar folds) whereas the other systems have very diverse apocytochrome substrates. Substrate recognition in system III is in one respect more specific than the other systems because not only are there specific lyases for cytochromes *c* and c_1 in yeast, but attempts to demonstrate that heme lyase can attach heme to a bacterial cytochrome *c* with very similar structure to the mitochondrial protein have failed.[41] On the other hand, and in contrast to system I, system III can add heme to an AXXCH motif.[42]

The heme provision pathway for system III remains to be elucidated; heme synthesis is completed through the action of ferrochelatase in the mitochondrial matrix. There is also the issue of possible disulfide bond formation in the CXXCH motif of the apocytochrome; the uncertainties here are the same as for system I in plant mitochondria.

Given that it has been observed that *H. thermophilus* apocytochrome c_{552} will bind heme and form correct thioether bonds in vitro[10] and the same result can be substantially obtained for mitochondrial apocytochrome *c*,[43] it follows that heme lyase could be considered as a specific chaperone that achieves the best fit between heme and apopolypeptide. Then a proximity effect might drive the catalysis of thioether bond formation but at an accelerated rate compared with what has been observed uncatalyzed in vitro. In this model, thioether bond formation would occur because heme lyase could recognize sufficient features of apocytochrome *c* for the latter to adopt an optimized, in stereochemical terms, noncovalent complex between heme and apocytochrome *c*. There is evidence that the heme lyase and apocytochrome *c* can form a complex.[44] On the other hand, there is also evidence that heme lyase can interact with heme,[45] raising the possibility that it might hold the heme in position to activate reaction with the apoprotein. A further possibility

is that heme lyase acts as a classical catalyst, promoting the chemistry of the reaction between the thiols and the heme vinyl groups. Considerably more work is needed on both the mode of action of heme lyase and why it evolved to take the place, presumably, of the original system I, which for some reason plant mitochondria have retained. Finally, we note that if heme lyase will work on a mitochondrial cytochrome *c* N-terminal peptide carrying the CXXCH motif, as reported some years ago,[46] then some of the present proposals would be eliminated or at least modified to envisage recognition between heme lyase and only the N-terminal region of the apoprotein. However, the failure of heme lyase to act on a bacterial cytochrome[41] would then have to be rationalized in terms of local sequence specificity around the CXXCH motif. The action of heme lyase has to be consistent with the overlapping specificities of the two yeast heme lyases[39] in the context that there is little sequence similarity between cytochromes *c* and c_1 in this region, and also with heme attachment to an AXXCH motif.[42] Overall, recognition by the lyase of more than the immediate sequence around the CXXCH motif is indicated.

Future Prospects

We are only at the beginning of understanding thioether bond formation in heme proteins. Many unknowns in the field have been highlighted here, and there are others; for example, the biogenesis system for *c*-type cytochromes in archaea is not evident from genome analyses. The extent to which in vitro observations of apoproteins binding around heme so as to position cysteine thiol groups for reaction with heme vinyls are relevant to in vivo catalyzed formation of *c*-type cytochromes remains to be established.[10,13,43] Very recent work[47] has shown that the unusual single-cysteine mitochondrial *c*-type cytochromes of the Euglenozoa, including the pathogenic trypanosomes, must be matured by a novel biogenesis apparatus (but, consistent with earlier observations,[15,33] cannot be matured by the Ccm system unless a second cysteine is introduced by mutation[47]). There is also the striking example of an unexpected thioether bond between heme and polypeptide recently discovered in crystal structures of the cytochrome b_6f complex of thylakoids.[48] The cytochrome b_6 component contains an additional covalently bound heme attached through only one thioether bond. The heme iron is, uniquely, not coordinated by any ligands from the protein, that is, there is no histidine corresponding to that of the CXXCH motif. Four genetic mutants of *Chlamydomonas reinhardtii* that lack a properly assembled cytochrome *b* subunit can now be interpreted as the first observation of a requirement for biogenesis genes for this novel heme attachment to cytochrome b_6f.[49] Similar covalent heme attachment is also found in the *bc* complex of *Bacillus subtilis*,[50] which implies that any dedicated biogenesis genes should also be found there. In conclusion, thioether bond formation in *c*-type cytochromes is clearly an enigmatic phenomenon; the complexity of the chemistry and the diversity of the biogenesis systems that catalyze this reaction suggest that there are strong evolutionary imperatives for formation of this class of proteins that are not yet fully understood.

This work has been supported by the UK BBSRC (Grants C13343, C20071, and BB/C508118/1), the EP Abraham Trust, and Christ Church, Oxford. We thank Paul Barker and Michaël Cartron for helpful discussions. The number of references has been constrained by the strict length limit of the journal.

Note Added in Proof. In the context of apocytochrome substrate recognition by the Ccm system (system I), it has very recently been shown that the system can covalently attach heme to a peptide as short as 12 amino acids but containing a CXXCH motif and a C-terminal His_6-tag [Braun, M.; Thöny-Meyer, L. Biosynthesis of artificial microperoxidases by exploiting the secretion and cytochrome c maturation apparatuses of Escherichia coli, *Proc. Natl. Acad. Sci. U.S.A.* **2004**, *101*, 12830−12835].

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AR030266L