PERSPECTIVE

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Why isn't 'standard' heme good enough for *c*-type and d_1-type cytochromes?†

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This perspective seeks to discuss why biology often modifies the fundamental iron-protoporphyrin IX moiety that is the very versatile cofactor of many heme proteins. A very common modification is the attachment of this cofactor *via* covalent bonds to two (or rarely one) sulfur atoms of cysteine residue side chains. This modification results in *c*-type cytochromes, which have diverse structures and functions. The covalent bonds are made in different ways depending on the cell type. There is little understanding of the reasons for this complexity in assembly routes but proposals for the rationale behind the covalent modification are presented. In contrast to the widespread *c*-type cytochromes, the d_1 heme is restricted to a single enzyme, the cytochrome cd_1 nitrite reductase that catalyses the one-electron reduction of nitrite to nitric oxide. This is an extensively derivatised heme; a comparison is drawn with another type of respiratory nitrite reductase in which the active site is a *c*-type heme, but the product ammonia.

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1 Introduction

It has often been remarked that the combination of heme with polypeptide generates a great range of functionalities, from, for instance, gas transport and sensing to electron transport and catalysis. All these functions can be achieved with heme (Fe-protoporphyrin IX) (Fig. 1(a)) non-covalently associated with the polypeptide. However, there are circumstances where the demands of function require modification of the heme. Two such examples, which will be the subject of this discussion, are

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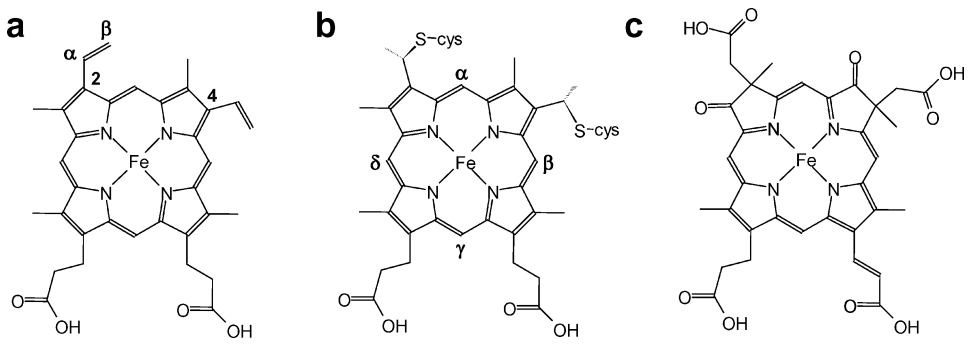

Fig. 1 The structures of the hemes found in (a) *b*-type cytochromes, (b) *c*-type cytochromes and (c) of the d_1 heme of cytochrome cd_1. The 2- and 4-positions are labelled (panel a) using the "Fischer system of nomenclature"; the designation of the vinyl carbon atoms as α or β is also shown. The thioether bonds found in *c*-type cytochromes with invariant S stereochemistry are shown in panel (b), as is the α,β,γ,δ designation of the meso carbons of heme. The methine proton is that on the α vinyl carbon which is also bonded to sulfur in the thioether linkage.

the *c*-type cytochromes, in which the heme is covalently attached to the protein by one or two thioether bonds (Fig. 1(b)), and the d_1 heme (Fig. 1(c)) which occurs, non-covalently bound, at the active site of a nitrite reductase for which nitric oxide is the reaction product. The principal aim of this discussion is to analyse to what extent we understand the purpose, and the biosynthesis, of the *c*-type and d_1-type cytochromes.

2 The purpose of *c*-type cytochromes

The general definition of a *c*-type cytochrome is that of a protein with one or more heme groups covalently attached *via* thioether bonds. The latter bonds are, with very rare exceptions, formed between the two cysteines of a CxxCH motif and the original vinyl groups of the heme (Fig. 1). In all cases but one[1] the histidine becomes the proximal ligand to the heme iron. Although the *c*-type cytochromes all share essentially the same covalent heme attachment motif, and always have the same heme attachment stereochemistry, their three dimensional structures are far from homogeneous.[2–4] The best known is the mono-heme cytochrome *c* of the mitochondrial respiratory chain. Its usual function is to transfer electrons from the cytochrome bc_1 complex to the cytochrome oxidase; the cytochrome c_1 of the former is structurally similar to mitochondrial cytochrome *c* but is argued to have evolved from a bacterial di-heme cytochrome *c*.[5] Many bacteria have an equivalent protein to mitochondrial *c*; these bacterial orthologues function in the periplasm where they often mediate electron transfer to and from a variety of dehydrogenases and reductases, as well as between the bc_1 complex and an oxidase or a photosynthetic reaction centre. This type of *c*-type cytochrome has at least two requirements, a standard reduction potential in the range of +250 to +300 mV and an ability to accept and donate electrons from/to a variety of partners. Do either of these requirements explain why the covalent attachment of the heme has been adopted? The setting of the potential is substantially a consequence of the use of one methionine axial ligand to the heme iron, a feature which provides relative stabilisation of the Fe^{2+} state. There is little reason to suppose that the thioether bonds of a *c*-type cytochrome play much role in the setting of the potential. This proposition has been tested in the only study where a folded protein has been obtained following loss of both the thioether bonds from a *c*-type cytochrome. The resulting *b*-type cytochrome, in which CxxCH was replaced by AxxAH (the heme is thus non-covalently bound), had a reduction potential only 70 mV different from the wild type protein,[6] a difference that could surely be compensated, if needed, by adjustment of the micro-environment of the heme group. The protein which underwent this *c*-type to *b*-type conversion comes from an extremely thermophilic bacterium and it is not possible to isolate

readily its partner proteins. Therefore, it is not known if the loss of the thioether bonds has altered the functional properties of the *c*-type cytochrome. As far as we can tell at present the overall structure of the cytochrome is little perturbed,[7] although the extreme thermodynamic stability of the original protein is lost.[6] Nevertheless, a *b*-type cytochrome of 'normal' stability, and which retains heme for extended periods, is obtained. It is not obvious that heme retention is the reason for the thioether covalent bonds of a *c*-type cytochrome, although it has to be recognised that by extrapolation the loss of these bonds from mitochondrial cytochrome *c* can be predicted to result in a rather unstable protein.

A feature of the mono-heme *c*-type cytochromes is that an edge of the heme is exposed and surrounded by amino acid residues that offer a surface whose properties are broadly complementary to the partner proteins.[8] Given that these are often multiple partners, it may be that the thioether bonds help fix the position of the heme edge in an easier fashion than tailoring a non-covalent binding pocket. This may therefore be a reason for the occurrence of mono-heme *c*-type cytochromes. It has been argued that the characteristic His/Met axial coordination of the Fe in this type of cytochrome is critically dependent on the two covalent bonds between polypeptide and heme.[9] The core of the argument is that the ferric Fe-methionine bond is so weak that it needs to be re-inforced by the covalent attachment of heme to protein. However, this argument overlooks the occurrence of several *b*-type heme proteins which have His/Met coordination. These include the periplasmic proteins cytochrome b_{562} of *E. coli* and the *b*-type cytochrome subunit of a dimethyl sulfide oxidase.[3] The existence of such proteins suggests that stability of the iron-methionine bond does not need to be reinforced by the thioether bonds, as does an extracellular protein involved in cellobiose degradation which also has non-covalently bound heme with His/Met coordination of the Fe.

Barker and Ferguson,[4] as well as Allen *et al.*,[3] have recently reviewed some of the other advantages that may be conferred by the presence of covalent bonds in *c*-type cytochromes. We will not rehearse all the arguments here, but multi-heme *c*-type cytochromes, which are found in many species of bacteria, do warrant further attention. In overall structural terms, these bear neither resemblance to mitochondrial cytochrome *c* nor, often, to each other. However, they are related by the presence of a low amino acid residue to heme ratio compared with other hemoproteins, and common three-dimensional clustering patterns of the hemes.[4] It is difficult to imagine how such close clustering of hemes could be achieved without the anchoring provided by the thioether bonds. As will be explained later, the heme is attached to an unfolded polypeptide chain by a post-translational modification process and thus the heme–heme associations are an important determinant in the acquisition of the final folded structure. This can help explain why, in multi-heme cytochromes of known structure, several of the hemes in a particular cluster in one protein can be overlaid on clusters observed in other proteins with which virtually no primary sequence homology is shared. None of these considerations explains why such heme clustering is advantageous. Provision of a relatively isotropic electron accepting/donating system has been suggested as a reason.[10] Clearly, the multi-heme arrangement permits a fast multi-electron transition from the all ferrous to all ferric state, and many of these proteins, *e.g.* hydroxylamine oxidoreductase and a periplasmic *c*-type nitrite reductase that catalyses the six-electron reduction of its substrate to ammonium, are involved in catalysing reactions in which more than one electron has to be abstracted from/donated to the substrate. A simple rationale for multi-heme clusters is that they would provide a nearby reservoir of either ferric or ferrous hemes so as to optimise electron delivery to, or acceptance from, the active site. Very rapid removal, or donation, of several electrons from/to the catalytic site may thus be an advantage in possessing the clusters of covalently attached heme.

On the other hand, it should be noted that a type of soluble fumarate reductase that is found in some bacteria also has such a cluster of four hemes and yet catalyses only a two-electron reduction, of fumarate to succinate.[4] Other types of fumarate reductase do not have any heme cofactors but have multiple iron–sulfur centres. In fact, the occurrence of multiple redox centres adjacent to an active sites is widespread, suggesting perhaps that there is no correlation between the number of electrons that in principle can be stored on metal centres within an enzyme and the number of electrons needed for the reaction that is catalysed. This point is reinforced by considering, for example, the two types of respiratory nitrate reductase enzymes which catalyse the two-electron reduction of nitrate to nitrite.[11] The periplasmic nitrate reductase has an FeS centre adjacent to the Mo cofactor at the active site on the catalytic NapA subunit and two *c*-type hemes on the adjacent NapB subunit. The latter receives electrons from the tetra-heme NapC protein;[11] thus overall, seven electrons can be 'stored' outside of the active site. The membrane-bound nitrate reductase enzyme has five FeS centres on two globular subunits, one of which harbours the active site Mo; why are so many redox centres needed to conduct electrons from the heme-containing transmembrane quinol oxidase subunit to the active site? Even cytochrome cd_1 (see later), which catalyses the one-electron reduction of nitrite to nitric oxide, has one heme centre at which to receive electrons from donor proteins and a second heme centre at which the reaction is catalysed. A copper protein capable of the same reaction has the same pattern; one type of copper centre receives the electrons and then they are transferred to the active site copper. There seems to be a puzzling and recurring pattern, not restricted to the examples of heme clusters in certain of the *c*-type cytochromes, of an excess of electron 'capacity' in redox enzymes, or at least in the enzyme plus its immediate redox partners. Thus the clustering of the hemes in some of the *c*-type cytochromes can be seen as part of a general picture. There is usually a considerable biosynthetic effort involved in inserting these centres into proteins; we are left with the impression that an important point of principle remains to be established.

3 Uncatalysed cytochrome *c* synthesis *in vitro* and *in vivo*

The thioether bonds of *c*-type cytochromes are, in cells, not formed spontaneously. They are made post-translationally by, in different cell types, at least four distinct systems of varying complexity.[2,3] Only in rare instances is there evidence for more than one system in a particular cell type. The existence of so many biogenesis systems, to which we return below, underlines the importance of *c*-type cytochromes to biology. However, under certain conditions *c*-type cytochromes can be formed without enzymatic assistance. In the belief that defining these conditions, as well as the nature of unwanted side products, will help us understand the *in vivo* process, we first discuss these occurrences. Initially, it is important to remember that the vinyl groups of heme are not particularly activated and thus addition of a thiol group, with the orientation as expected from Markovnikov's rule (see legend to Fig. 4), is not chemically facile.

The best example of uncatalysed cytochrome *c* formation is cytochrome c_{552} from the thermophilic bacterium *Hydrogenobacter thermophilus.* This protein, with heme attached, can, following expression from a suitable plasmid,[12] be purified from the cytoplasm of *E. coli* where there is not a *c*-type cytochrome biogenesis system. It is currently assumed that this protein is made as a consequence of the apo (*i.e.* heme free) protein adopting sufficient tertiary structure so as to provide a heme binding pocket in which the vinyl groups of heme are located next to the thiol groups of the two cysteines in the CxxCH motif. *In vitro* data to support this proposal will be summarised shortly. But what is the evidence that the covalent bonds are formed correctly? In principle, a variety of wrong products

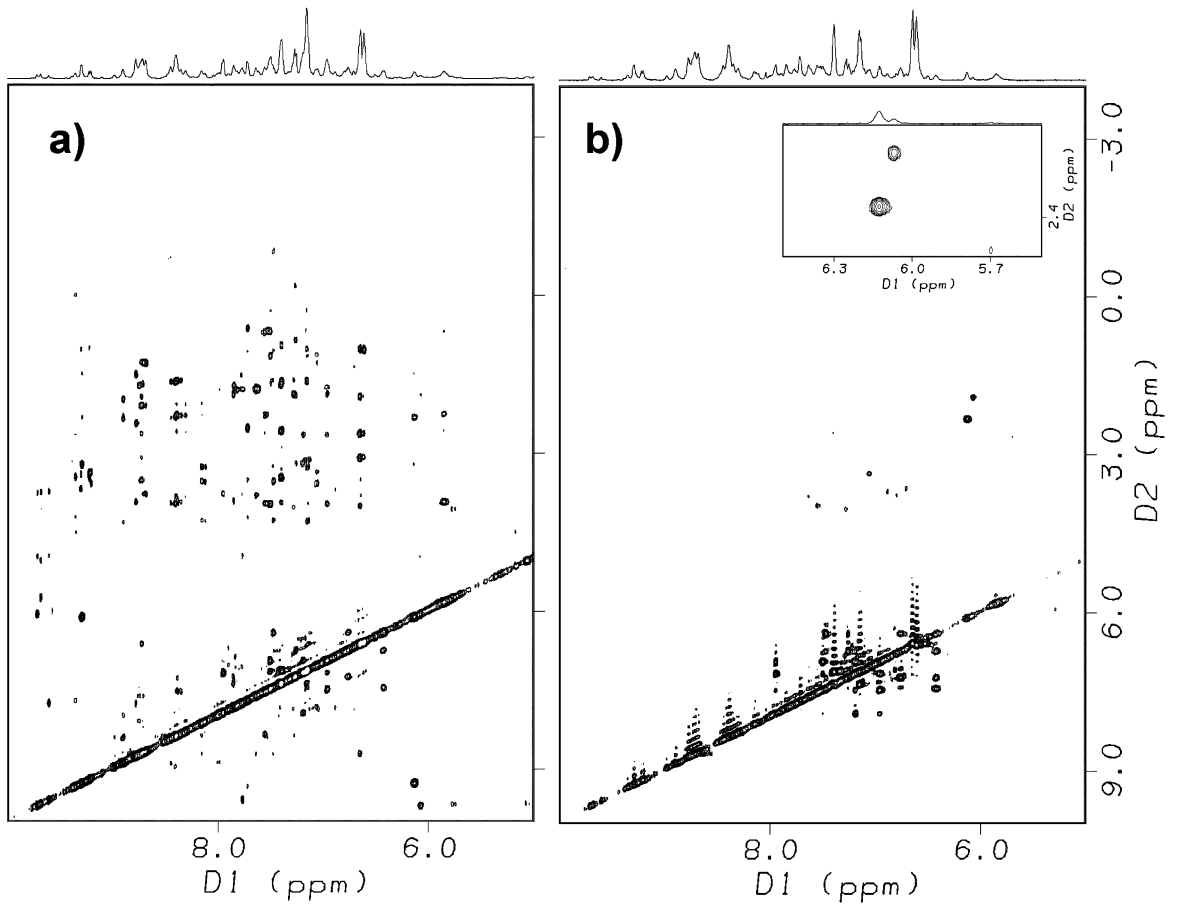

Fig. 2 Downfield regions of (a) NOESY and (b) TOCSY spectra of recombinant *H. thermophilus* ferrocytochrome c_{552} obtained from the cytoplasm of *E. coli* strain JM109. Inset to (b): Expanded region of the TOCSY spectrum showing the only crosspeaks due to the coupling between the thioether linkage 2 and 4 position methine protons and the 2 and 4 position methyl protons. ^{1}H Homonuclear two-dimensional NOESY experiments were performed as previously described[43] and acquired as 2D matrices of 2048 × 512 complex points with 32 or 64 transients per increment. The sweep width in all experiments was set to 10204 Hz. Data were processed (by two-fold zero filling and apodization with a 45°-shifted sine-bell window function) and analysed using FELIX (Molecular Simulations Inc). ^{1}H Homonuclear two-dimensional clean-TOCSY experiments were also performed as previously described[43] and processed as above. The protein concentrations were in the range 1.5–2 mM. Spectra were recorded at 300 K using a Bruker AMX 500 spectrophotometer with operating frequency of 500.13 MHz.

might form. For example, anti-Markovnikov addition, and/or attachment of the 'wrong' cysteine to one of the vinyl groups that become the thioether bonds in *c*-type cytochromes, may occur. Whatever the overall protein structures in naturally matured *c*-type cytochromes, the 2-vinyl and 4-vinyl groups of heme are always, respectively, attached to the N- and C-terminal cysteines of the CxxCH motif.[4]

We subjected ferrous *H. thermophilus* cytochrome c_{552} obtained from the *E. coli* cytoplasm to analysis by NMR, aided by the availability[13] of an assigned spectrum from the same protein that had been produced by the native cytochrome *c* biogenesis apparatus in *H. thermophilus*. The spectrum of protein isolated from *E. coli* clearly contained a predominant species that was extremely similar to the protein isolated from the thermophile itself. Crucially, only two methine protons from the thioether linkages could be observed in the NOESY (Fig. 2(a)) and TOCSY spectra (Fig. 2(b)), with chemical shifts (6.27 and 6.22 ppm), very similar to those observed in the non-recombinant material. Furthermore, the NOESY connectivity between signals assigned to the methine and methyl protons of the thioether linkage and the heme α- and β-meso protons indicated that the stereochemistry at the carbon atom of these linkages is *S*, as observed in the structure of the non-recombinant material. In addition, the NMR data indicate that the stereochemistry at the sulfur of the methionine ligand to the heme dominantly had *R* configuration (Fig. 3). Again this is the same as in non-recombinant material. It can be safely concluded that the mode of the heme attachment, including *S* stereochemistry at both thioether linkages and the orientation

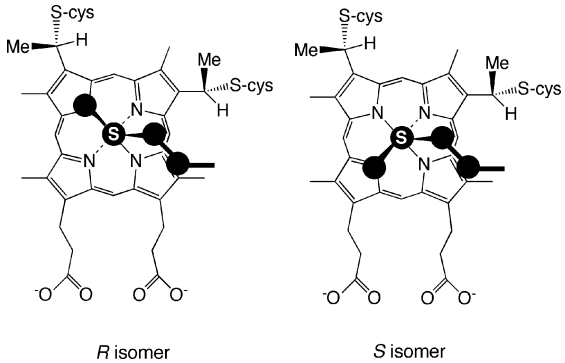

Fig. 3 The conserved *S* stereochemistry at the α vinyl carbons of heme which form the *c*-type cytochrome thioether bonds, and the two stereochemistries, *R* and *S*, that are found at the sulfur of the methionine ligand to heme iron.

of the heme, are, remarkably, the same in both recombinant and native protein. The structure of the recombinant protein in this region is essentially identical to the native protein.

The stereochemistry at the sulfur of the methionine ligand to the heme iron is one aspect that does vary between *c*-type cytochromes. For bacterial mono-heme *c*-type cytochromes with essentially the same 3D structure as mitochondrial cytochrome *c*, the stereochemistry can be either *R*, as typically found for mitochondrial cytochromes *c* and bacterial counterparts, or *S* as reported for the cytochromes c_{551} from *Pseudomonas aeringinosa* and *Pseudomonas stutzeri* (Fig. 3). The finding by

NMR of mainly *R* stereochemistry for the *H. thermophilus* ferrocytochrome c_{552}, recombinant or native, was a little surprising given the close structural similarity between this cytochrome and cytochrome c_{551} from *P. aeringinosa*. Recent studies have shown the methionine coordination to be fluxional in oxidized *H. thermophilus* cytochrome c_{552};[14] both stereochemistries are observed and are interconverting. It is less clear that the ligand is fluxional in the reduced state; both stereochemistries were observed in our NMR experiments although the *R* form clearly predominated. However, only one chemical shift is observed for the ε-CH_3 resonance. The coordination of methionine to iron is usually relatively stabilised in the ferrous state owing to enthalpic effects; such stabilisation should slow down any rate of exchange between the two stereochemistries. Thus the observation of one resonance might mean that both conformers give rise to the same chemical shift. Alternatively, the exchange between the conformers may be rapid enough for the NMR timescale to result in a single resonance.

The NMR spectra of the recombinant *H. thermophilus* cytochrome c_{552} were not completely identical to those for the native protein and gave evidence for some overall structural heterogeneity. This was most readily observed for the axial methionine ligand to the heme iron. Three distinct chemical shift values were seen (Fig. 4) for protons of this side chain with the relative intensities varying from 8 : 1 : 1 to approximate equality, depending upon the strain of *E. coli* that was used for expression. The structural basis for this heterogeneity was not established, but such is the sensitivity of the NMR technique that only very minor structural differences, which are not interconverting on the NMR timescale, are indicated. We do not believe that ligand fluxionality is the origin of this heterogeneity.

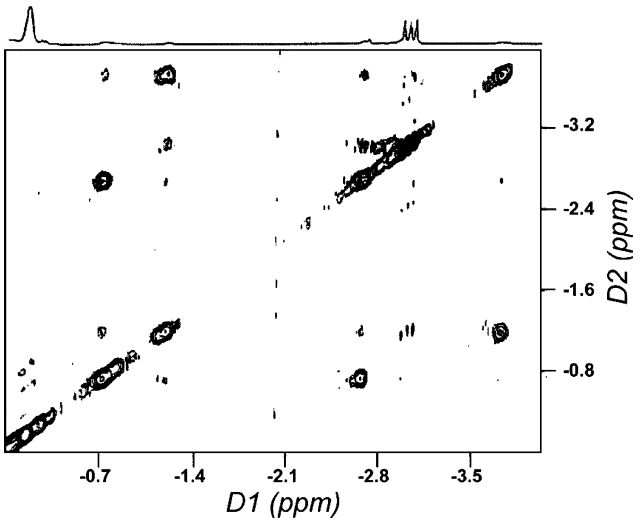

Fig. 4 Upfield region of the homonuclear NOESY spectrum of recombinant *H. thermophilus* ferrocytochrome c_{552} obtained from the cytoplasm of *E. coli* strain JCB71202, highlighting the resonances of the protons of the side chain of the methionine 59 ligand residue. Three resonances from a ε-methyl group are clearly seen at −2.92, 2.95 and 3.01 ppm. One γ-methylene proton is also clearly degenerate giving resonances at −1.15 and −1.20 ppm.

In parallel to our work,[15] Karan *et al.*[16] also studied the NMR spectrum of *H. thermophilus* ferrocytochrome c_{552} obtained *via* cytoplasmic expression from a different plasmid in a different strain of *E. coli*. They observed that most of the resonances had very similar chemical shifts to those in the native protein, implying a very similar structure, although they did not specifically analyse the resonances that are diagnostic of the 'correct' covalent heme attachment. They noted two populations of cytoplasmically expressed protein, the difference being the presence, in one population, of the N-terminal methionine. Removal of this residue by animopeptidase digestion showed that the methionine caused structural perturbations. However, this methionine appeared, from mass spectrometry analysis, to be absent from our preparations and thus could not be the cause of the heterogeneous environments observed for the heme axial ligand methionine (Fig. 4).

The most straightforward, but nonetheless remarkable, conclusion from the NMR studies is that the cytochrome c_{552} of *H. thermophilus* had, in terms of covalent heme attachment, been correctly assembled in the cytoplasm of several strains of *E. coli*. This is surprising when one considers what could have gone wrong. For example, the heme could have become attached the wrong way around, *i.e.* inversion of the normal attachment of 2- and 4-vinyl groups to the CxxCH motif, or the thiols may have added to the 'wrong' carbons of the vinyl groups, etc (*cf*. Fig. 5). Such 'wrong' attachment has been observed for cytochrome c_{552} of *Thermus thermophilus*,[17] also expressed cytoplasmically in *E. coli*, *i.e.* without the aid of biogenesis proteins. The simplest model to explain the correct assembly of *H. thermophilus* cytochrome c_{552} in the *E. coli* cytoplasm is that the apoprotein takes up sufficient tertiary structure (there is evidence that this protein is not a random coil[7]) such that thioether bonds can form *only* when the heme binds correctly. A proximity effect, possibly with the assistance of protonation of the vinyl groups, would then result in thioether bond formation *via* Markovnikov addition. In this context, the successful production in the *E. coli* cytoplasm of a *b*-type (AxxAH) analogue of c_{552} can be understood; replacement of the two cysteines by alanines would allow heme to bind snugly in its pocket. Heme often adopts both its possible orientations (related

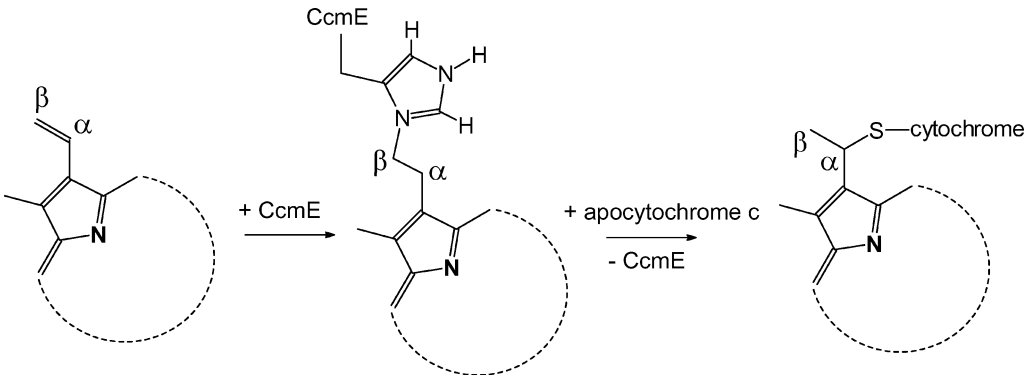

Fig. 5 The structure of the covalent bond between an essential histidine sidechain of CcmE and heme as determined by NMR[25] (showing only one pyrrole ring for simplicity), and subsequent transfer of the heme to one cysteine of an apocytochrome *c* (*i.e.* thioether bond formation). Markovnikov addition to a heme vinyl group would involve initial protonation of the β carbon such that a carbocation would be generated at the α carbon. This carbocation is expected to be more stable than the alternative that would follow from protonation of the α carbon; the two carbon–carbon bonds at the α carbon provide more stabilisation than the single bond at the β carbon. Hence it can be seen that the overall addition of a cysteine thiol to give the thioether bond of a *c*-type cytochrome follows the Markovnikov rule whilst the formation of the histidine–heme bond in CcmE does not. As in Fig. 1, the methine proton is that on the α carbon which is also bonded to sulfur in the thioether linkage.

by rotation around the α/γ axis, see Fig. 1) in such pockets but the absence of incorrect products for non-catalysed production of *H. thermophilus* cytochrome c_{552} suggests that the requirement of optimisation of fit is so precise for the formation of thioether bonds in this protein that the 'wrong' bonds do not form. It is notable that the kinetics of *in vitro* cytochrome c_{552} formation (see below) suggest that heme bound in the 'wrong' orientation must dissociate and rebind 'properly' before thioether bond formation can occur.[18]

Our observations on cytoplasmic assembly in *E. coli* of *H. thermophilus* cytochrome c_{552}, in an environment where no catalyst is expected, indicated that thiol addition to the heme vinyl groups must occur at least on the timescale of the bacterial growth, and that in principle incubation of the apoprotein with heme *in vitro* should lead to the formation of thioether bonds. This expectation has been realised and although insufficient material has been obtained for NMR analysis a variety of lines of evidence indicate correct assembly of holoprotein.[18] Importantly, this *in vitro* work has permitted the definition of factors that promote thioether bond formation. These are: (i) the ferrous state of heme, which is obligatory in the sense that the ferric state is incompetent for proper reaction; (ii) lower pH values; (iii) prevention of disulfide formation between the two cysteines of the CxxCH motif. There is evidence that the two cysteine thiol groups react independently with the heme vinyls.[18] The first *in vitro* observations of uncatalysed cytochrome *c* formation involved use of thiol reagents to avoid oxidation of the cysteine thiols, conditions which, however, could not rigorously exclude the possibility that the thiols formed mixed disulfides with the reagents before reaction with heme. However, subsequent work has shown that replacement of thiol reagents as reductants by a water-soluble phosphine has no effect on the formation of thioether bonds, thus arguing against any involvement of mixed disulfides.[19] These studies also showed that zinc protoporphyrin IX reacted similarly to ferrous protoporphyrin IX, suggesting that a divalent cation, but not any specific chemical properties of the metal, are important.[19] As mentioned earlier, the formation of the thioether bonds in cells is, as far as we know, always catalysed. These *in vitro* conditions thus set some parameters for the chemical reaction pathways that are likely to be followed by the *in vitro* thioether bond forming mechanisms. Note, however, that there is currently very little data relating to the mechanisms of any of the *in vivo* cytochrome *c* biogenesis systems.

Before leaving this section it should be pointed out that there are now a number of examples of uncatalysed thioether bond formation *in vivo* where the products are not *bona fide* cytochromes *c*, for example because only one thioether bond is formed or 'incorrect' attachment of the thiols to the vinyls has occurred. For example, such outcomes have been observed for the cytoplasmic production in *E. coli* of *Thermus thermophilus* cytochrome c_{552},[17] where single and 'wrong way around' attachment occurs, and also for cytochrome b_{562} containing an engineered CXXCH group after expression in either the cytoplasm or in the periplasm of *E. coli* in the absence of activity of the biogenesis system in that organism.[20] Thus one of the essential requirements for cellular systems catalysing the formation of cytochromes *c in vivo* is that they must discriminate against the wrong bonds being formed.

Finally we note that thioether bond formation, but not necessarily with the organisation characteristic of *bona fide* *c*-type cytochromes, has also been achieved *in vitro* within designed 4-helix bundles. As with such bond formation in natural proteins, a proximity effect and reductive conditions promoted the reactions.[21]

4 Cellular systems for *c*-type cytochrome assembly

Surprisingly, three different systems, of varying complexity, have been identified as facilitating *in vivo* thioether bond formation in *c*-type cytochromes.[2,3] Furthermore, a fourth system, as yet unidentified, is implicated in some cell types.[22] Mechanistically, the systems may all be different; this important point is far from being resolved. The first assembly or biogenesis system to be identified was that operating in fungal cells; it was later found in metazoans. In these species, proteins known as heme lyases are responsible for the attachment of heme to the apo forms of mitochondrial cytochromes *c* and c_1. Expression of the yeast cytochrome *c* heme lyase in the cytoplasm of *E. coli* along with the gene for mitochondrial cytochrome *c* (from several sources) results in cytoplasmic production of fully assembled cytochrome *c*.[23] Thus we can conclude that only one dedicated biogenesis/assembly protein is needed for this type of cytochrome *c* biogenesis. Essentially nothing is known about how mitochondrial cytochrome *c* heme lyase functions, but it is notable that it is not active towards bacterial orthologues of mitochondrial cytochrome *c*, suggesting that more is recognised than the CxxCH motif and its immediate context. It is, therefore, not surprising that the mitochondrial assembly system, arbitrarily known as system III for cytochrome *c* assembly, is not found in bacteria. In the latter group of organisms two systems have been described, known as systems I and II. With rare exceptions, only one of these systems is present in a particular organism. At the current level of understanding, system I is the most complex. It occurs in many Gram-negative bacteria, including the biochemists' paradigm *E. coli*, where most is known. System I in *E. coli* comprises eight Ccm proteins, for which precise functions remain unknown.

The production of a *c*-type cytochrome in *E. coli* starts with the synthesis of the heme and the polypeptide in the cytoplasm. The polypeptide is translocated by a relatively well defined process to the periplasm where the covalent bonds are formed to heme, which reaches the periplasm by an unknown route.[2] Several of the Ccm proteins (the presumed CcmAB complex, CcmC and CcmF) each have several transmembrane helices suggestive of transport functions, but evidence for a role in heme transport has not been obtained. The Ccm protein for which most is known is CcmE which is a membrane-anchored periplasmic protein. Heme, *en route* to being incorporated into a *c*-type cytochrome, is, remarkably, covalently attached to a histidine residue of the periplasmic domain of CcmE.[24] Very recently the chemical structure of the histidine–heme adduct has been elucidated[25] (Fig. 5). As anticipated from *in vitro* experiments with heme analogues, the covalent bond is formed from one of the original vinyl groups of the heme. The NMR method used to determine this structure could not identify which vinyl group, but a resonance-Raman study of in *vivo* produced heme-bound CcmE suggests that it is the 2-vinyl group.[26] Unanticipated was the finding that the histidine is attached to the β carbon of the vinyl group, *i.e.* the carbon that becomes the methyl group in the thioether linkage (Fig. 5).[25] Therefore the addition has taken place by an anti-Markovnikov reaction. How this occurs is not clear, although it would be consistent with a radical mechanism. Arguably even less clear is how the histidine–heme bond breaks again, a process that must involve replacement of a carbon–hydrogen by a carbon–sulfur bond at the α carbon of the saturated heme vinyl (Fig. 5). Transfer of heme from CcmE to an apocytochrome *c* has been observed *in vitro* and so seemingly a specialised protein is not obligatory for this transfer.[27] However, the *in vitro* rate is very slow and thus other Ccm proteins are required kinetically. Although the mechanism of heme transfer from CcmE to apocytochrome *c* is not easy to understand, the energetics may pose less of a problem. Two thioether bonds are formed, one by addition to a previously underivatised vinyl group and the other by displacement of the CcmE histidine sidechain. In this context it is notable that the Ccm system cannot attach heme to an AxxCH motif in an apocytochrome. The latter, with a resulting single thioether bond mode of heme attachment to protein, does occur in the mitochondria of a group of eukaryotes called the

Euglenozoa, including trypanosomes. These organisms do not have an identifiable cytochrome *c* biogenesis system as judged by the outcome of bioinformatic analysis, strongly suggesting that they have a novel assembly system ('system IV').[22] When the AxxCH motif of *Trypanosoma brucei* cytochrome *c* was converted to CxxCH, the Ccm system of *E. coli* was able to process it, thus clearly demonstrating the need for two cysteines to be available for the Ccm-dependent thioether bond formation.

The key role of the histidine–heme bond in CcmE is supported by mutagenesis experiments in which the conserved histidine of CcmE is replaced by other residues. Substitution by alanine results in a protein that is no longer functional *in vivo*. Purified alanine mutant CcmE protein binds heme non-covalently, suggesting a heme binding pocket of some kind. This is, however, a matter of some controversy. There are two NMR structures of the apo-form of CcmE.[28,29] These are generally very similar to each other but opposite conclusions have been drawn concerning a heme binding pocket. An interesting amino acid substitution is that of the essential histidine of CcmE by cysteine. The cysteine variant protein is essentially inactive *in vivo* in *c*-type cytochrome biogenesis. The purified cysteine-holoprotein, produced only in low yield, appears from absorption spectroscopic criteria to be a *c*-type cytochrome with one thioether bond.[30,31] However, this evidence is based on pyridine hemochrome spectra which cannot distinguish between thioether bond formation at the α and β vinyl carbon atoms. It would be interesting to discover if the cysteine variant of CcmE has the usual (Fig. 1) thioether bond or attachment at the same β carbon to which histidine attaches in CcmE (Fig. 5). Determination of this point might provide a vital clue as to the mechanism of covalent bond formation between heme and CcmE.

The other chemical aspect of the Ccm system into which we have some insight is that of thiol chemistry. As explained above on the basis of *in vitro* studies, the two cysteines of the CxxCH motif have to be prevented from forming a disulfide bond if heme attachment is to be successful. The CcmG protein, a variant of thioredoxin, is particularly implicated in this role, although there is no direct evidence for this. A final puzzling point about system I is that it is found in plant mitochondria, implying that the heme lyases (system III) seen in many other eukaryotes, but not recognised so far in either eubacteria or archaea, are a late evolutionary acquisition.

System II is less characterised and understood than system I; it is found in the thylakoids of green plants, where its main function is to mature cytochrome *f* (a *c*-type cytochrome despite the name), as well as in many bacteria, for example *Bacillus subtilis*. Some of the components identified for this system (ResA/CcsX and CcdA) are involved in disulfide/thiol interconversions, but the roles of the others are enigmatic. A very recent paper, describing extensive mutagenesis experiments on a bacterium containing this system,[32] suggests that only two proteins, CcsA and CcsB are specific for the system II pathway. The other components participate more widely in thiol-disulfide interchange. It is striking, therefore, that there appears to be no orthologue of the remarkable CcmE protein in the type II assembly system. However, the exact roles of CcsA and CcsB remain enigmatic.

An important point to understand for a post translationally modified protein is at what stage during the progression from an unfolded polypeptide leaving the ribosome to the acquisition of the final functional state, does the modification occur? For the type I (Ccm) cytochrome *c* biogenesis system we can now be essentially certain that the covalent heme attachment occurs before there is substantial folding of the polypeptide. This view was first suggested from the observations that heme could be attached to a protein that had been substantially truncated to the C-terminal side of the CxxCH motif.[33,34] More recently it has been shown[35] that very short CxxCH-containing-peptides can be processed by the cytochrome *c* biogenesis apparatus of *E. coli*. In one case only two residues were present at the N-terminal side of CxxCH and six histidines (which included the heme iron histidine ligand and a pentahistidine tag) at the C-terminal side (a total of 12 amino acids). Attempts to demonstrate Ccm-dependent attachment of heme to proteins containing CxxCM or CxxCK motifs have been unsuccessful.[36] Such data accords well with the presence of as few as five residues between successive CxxCH motifs in the sequences of some Ccm-matured multi-heme *c*-type cytochromes. Thus we can conclude with a fair degree of certainty that this Ccm biogenesis system recognises only local CxxCH regions of a polypeptide chain, and folding to reach the final, native 3-D structure must only occur after the covalent bonds between heme and protein have been formed. In this context, it is worth noting that the Ccm system will handle a wide range of *c*-type cytochromes ranging from mono-heme proteins, including mitochondrial cytochrome *c*, to those with many hemes. As system II makes many multi-heme proteins in *Geobacter* species (a 34 heme *c*-type cytochrome is predicted from the genome sequence), with in at least one case just two amino acids separating successive CxxCH motifs, then one can conclude that a similar mode of recognition applies as for system I.

5 The d_1 heme

One of the puzzles about the *c*-type cytochromes is why the covalent mode of heme attachment has been adopted for a range of proteins with different structures and functions. A second type of modified heme, d_1, occurs only in one enzyme, which means there is only one function to address alongside the many uncertainties as to why and how this specialised heme is made. The enzyme is known as cytochrome cd_1;[37] it is a nitrite reductase that is located in the bacterial periplasm. The reaction catalysed is the one-electron reduction of nitrite to nitric oxide. Electrons are transferred from mono-heme *c*-type cytochromes or cupredoxins to the *c*-type cytochrome domain of cytochrome cd_1. The electrons then pass on to the non-covalently bound d_1 heme of the enzyme where the reaction takes place. Fig. 1(c) shows the structure of the d_1 heme alongside the 'standard' *b*-type heme (Fig. 1(a)). The main differences are the saturation of two of the pyrrole rings, the presence of two electron withdrawing carbonyl groups and an acrylate group conjugated with the porphyrin. What are the advantageous features of this structure for the one-electron reduction of nitrite to nitric oxide? The following have been suggested:[38] (i) this ring structure allows the ferrous state of heme to be tuned to bind nitrite. This is believed to be a step in the mechanism and contrasts with the 'usual' binding of nitrite, and anions generally, much more strongly to ferric, rather than ferrous, heme; (ii) the d_1 structure may offer potential advantages for NO release in the sense that the normal strong binding of NO to heme in either oxidation state may be weakened by the special features of the d_1 heme; (iii) the reduction potential of the d_1 heme, particularly in the context of its environment within the protein, is poised such that reduction of NO to hydroxylamine, but not of hydroxylamine to ammonia, is blocked for thermodynamic reasons. Thus cytochrome cd_1 contrasts with the pentaheme *c*-type cytochrome nitrite reductase (NrfA) that functions, for example, in the *E. coli* electron transport system. In the latter case the active site, essentially a *c*-type cytochrome centre except the attachment is *via* a unique CxxCK motif, is thermodynamically poised to take nitrite *via* bound nitric oxide and hydroxylamine to ammonia. A detailed reaction scheme has been proposed for NrfA;[39] the comparison with cytochrome cd_1 is instructive.

In the scheme developed for the cytochrome *c*-type nitrite reductase (NrfA),[39] nitrite is envisaged to bind, with concomitant displacement of water, to the ferrous state of the heme. It is believed (as for cytochrome cd_1) that nitrite binds strongly to the ferrous state of the heme in NrfA. It is advocated that an important factor is a back-bonding effect from the t_{2g} orbitals

of the ferrous heme iron into the π* orbital of the nitrite anion; the 3d orbitals are not similarly available in the ferric state. Delivery of protons, one each from a tyrosine and histidine sidechain, results in loss of water and formation of nitric oxide bound to ferric heme ({FeNO}^{6}, where '6' refers to the number of d electrons plus the number of NO π* electrons), which can alternatively be considered as a low-spin Fe(II) and a bound NO^+. This heterolysis of an N–O bond in nitrite is promoted by the back-bonding described above. It is also enhanced by transfer of electron density into an antibonding orbital. A further factor is the presence in the active site of amino acid sidechains capable of forming hydrogen bonds to the oxygen atom destined to become water. The delivery of an additional electron to the {FeNO}^{6} species would generate the very stable ferrous heme–nitric oxide {FeNO}^{7} complex. It is suggested that this is avoided by a rapid two-electron delivery down to the {FeNO}^{8} species. This could provide an explanation for the clustering of hemes near the active site (see above). Protonation and further delivery of two electrons would lead to bound hydroxylamine *en route* to eventual formation of ammonia.

A reaction scheme for cytochrome cd_1 (Fig. 6) might follow closely the first two or three steps proposed for the NrfA-catalysed mechanism. Thus initial binding of nitrite to the ferrous state of the d_1 heme is consistent with the higher affinity, relative to ferric, of this state for anions, an affinity that may be enhanced by the electron withdrawing groups in the d_1 heme ring. As for NrfA, the active site of cd_1 possesses two amino acid side chains, in this case histidines, poised to hydrogen bond, and donate protons, to the nitrite oxygen destined to become water. These histidines have been implicated through both crystallography and mutagenesis.[37,40] Whether heterolysis of the N–O bond is also promoted by back-bonding is less certain as the energy levels of the t_{2g} orbitals in ferrous d_1 heme have not been analysed. Loss of water would, as for NrfA, generate the {FeNO}^{6} species that has been detected by FTIR spectroscopy.[41] At this point it is generally assumed that further

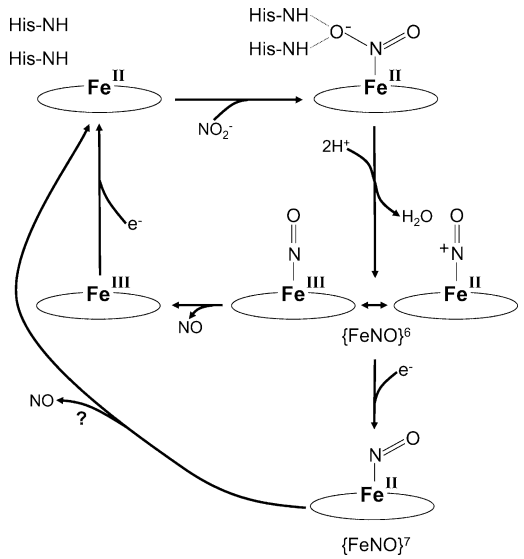

Fig. 6 Simplified scheme showing the reaction catalysed by cytochrome cd_1 nitrite reductase. Only events at the d_1 heme are considered. Nitrite is presumed to bind to ferrous d_1 heme which has an unusually strong affinity for anions. Two essential histidines hydrogen bond to the substrate, which is reduced and dehydrated to form NO. At this point, the {FeNO}^{6} species can be considered to be Fe(III)–NO or Fe(II)–NO^+, which are isoelectronic. It is generally assumed that NO dissociates from this heme-NO complex. This has not been proven experimentally, but it would yield ferric heme d_1 which could then be reduced by an electron from the *c*-heme of cytochrome cd_1 to complete the catalytic cycle. If {FeNO}^{6} is reduced, the resulting heme NO species will be Fe(II)–NO (*i.e.* {FeNO}^{7}). If NO is released from this species (which is plausible but an intuitively unlikely alternative), the catalytic cycle will be completed.

reduction should be avoided as NO release is expected to be easier from the {FeNO}^{6} rather than the {FeNO}^{7} species. The energy levels of ferric iron in d_1 heme are inverted relative to the usual pattern[42] and this may be an important factor in weakening the Fe–NO bond in {FeNO}^{6}. However, definite evidence as to which oxidation state NO is released from is still awaited. What is quite clear is that further reduction to {FeNO}^{8} and beyond *en-route* to bound hydroxylamine must be avoided as this would short circuit the nitrogen cycle. Reduction of nitric oxide to hydroxylamine is inconsistent with the operating potentials of the hemes in this molecule (cd_1) which are thought to be in the range of +250 to +300 mV. Reduction of nitric oxide to hydroxylamine needs a reductant with potential *ca.* −40 mV, consistent with the cytochrome *c*-type nitrite reductase accepting electrons from an electron transfer chain at close to the quinol level, in contrast to the cytochrome cd_1-type enzyme which receives electrons at a higher potential after they have passed through the proton translocating cytochrome bc_1 complex.[38] Thus we can argue that the cytochrome cd_1 type nitrite reductase is thermodynamically adjusted to be unable to reduce nitric oxide further.

If release of NO is from the {FeNO}^{6} state, implying that the {FeNO}^{7} state should be avoided, then we are brought back to the puzzle, discussed earlier, of why these redox enzymes appear generally to have both an 'electron receiver centre' as well as a catalytic centre. On an intuitive basis one might have thought that the d_1 heme group would be isolated within the enzyme so as to ensure delay in arrival of an 'unwanted' electron at the d_1 heme that could produce ferrous d_1 heme–NO. Such electron transfer could, however, be kinetically gated by conformational events in cytochrome cd_1 which is known to be a very flexible enzyme.

As inspection of Fig. 1 would suggest, a substantial biosynthetic effort is needed to produce the d_1 heme structure. It is known from mutagenesis studies that approximately six gene products are needed but apart from one, an orthologue of proteins in other pathways that generate pre-corrin 2, nothing is known about the heme biosynthesis reactions catalysed by these proteins; particularly intriguing is the question as to how oxygen atoms are introduced under anaerobic conditions. The biosynthetic dimension brings again into focus the point that the chemistry of the d_1 heme has evolved so as to optimise the catalysis of reduction of nitrite to nitric oxide. Much remains to be understood as to why all the features of the d_1 heme shown in Fig. 1 have been adopted. As the example of NrfA shows, the *c*-type cytochrome centre suffices for the conversion of nitrite to ammonia.

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