C-type cytochromes are characterized by having the heme moiety covalently attached via thioether bonds between the heme vinyl groups and the thiol of conserved cysteine residues of the polypeptide chain. Previously, we have shown the in vitro formation of *Hydrogenobacter thermophilus* cytochrome *c*$_{552}$ (Daltrop, O., Allen, J. W. A., Willis, A. C., and Ferguson, S. J. (2002) *Proc. Natl. Acad. Sci. U. S. A.* 99, 7872–7876). In this work we report that thioether bonds can form spontaneously in *vitro* between heme and the apocytochromes *c* from horse heart and *Paracoccus denitrificans* via b-type cytochrome intermediates. Both apocytochromes, but not the holo forms, bind 8-anilino-1-naphthalenesulfonate, indicating that the apoproteins each have an affinity for a hydrophobic ligand. Furthermore, for both apocytochromes a intramolecular disulfide can form between the cysteines of the CXCH motif that is characteristic of c-type cytochromes. In *vitro* reaction of these apocytochromes with heme to yield holocytochromes *c*, and the tendency to form a disulfide, have implications for the different systems responsible for cytochrome *c* maturation in *vivo* in various organisms.

C-type cytochromes are found in almost all organisms, being mainly involved in electron transport (1, 2). They contain a characteristic CXCH motif, whereby the conserved cysteine residues are involved in forming a covalent thioether bond between the thiol functionalities and the vinyl groups of the prosthetic heme moiety (3). Remarkably, three different systems have evolved to facilitate cytochrome *c* formation in *vitro* (4, 5). In some eukaryotes, one enzyme, designated heme lyase, catalyzes the attachment of the heme moiety to the apocytochrome *c* (6). These enzymes have a high specificity and do not act on bacterial apocytochromes *c* (7). Mammalian organisms make use of this system as exemplified by horse (*Equus caballus*) heart mitochondria.

Many Gram-negative bacteria use a system that involves more than ten gene products to ensure correct cytochrome *c* maturation (ccm) (8) in the periplasm (5, 8, 9). An example of a cytochrome *c* that is matured in *vivo* using this biosynthesis apparatus is cytochrome *c*$_{550}$ from *P. denitrificans* (10).

Following our report of the in *vitro* formation of holocytochrome *c*$_{552}$ from *H. thermophilus* (11), an important point to be established was whether the spontaneous attachment of heme to apocytochrome *c* is a reaction applicable to other cytochromes. Because holocytochrome *c*$_{552}$ from *H. thermophilus* forms exceptionally in the cytoplasm of *Escherichia coli* (12), its spontaneous *in vitro* formation might not necessarily be anticipated to apply generally to all c-type cytochromes. The previously reported thioether bond formation in holocytochrome *c*$_{552}$ from of *H. thermophilus* involved a b-type cytochrome intermediate (11). Prompted by the previously reported similar ability of the horse heart apocytochrome to bind heme to yield a species characteristic of a b-type cytochrome (13), the reaction of horse heart apocytochrome *c* with heme has been reinvestigated.

The formation of c-type cytochromes is especially interesting in light of the different biosynthetic pathways, which are reviewed extensively elsewhere (4, 5, 9), by which heme is attached to the apoproteins in *vivo*. Therefore, a second candidate for studying the reaction of heme with apocytochrome was *P. denitrificans* cytochrome *c*$_{550}$. The two cytochromes used in this study are matured by two different cytochrome *c* maturation systems in *vivo*. A comparison of the reactions with respect to the attachment mechanism is anticipated to have important implications for the molecular basis of heme attachment in different organisms in *vivo*.

**EXPERIMENTAL PROCEDURES**

**Protein Production**—Horse heart cytochrome *c* was purchased form Sigma, and *P. denitrificans* *c*$_{550}$ was produced as described by Richter et al. (14). Apocytochromes *c* were prepared analogously to the method of Fisher et al. (15). After the removal of the Ag$^+$ by addition of dithiothreitol (DTT), the proteins were dialyzed extensively in sodium phosphate buffer (pH 7.0, 20 mM). Reduced apocytochrome was obtained by dialysis in deoxygenated sodium acetate buffer (pH 5.0, 25 mM).

**Protein Characterization**—SDS-PAGE analysis was carried out using the buffer system described by Laemmli (16), and heme activity staining was performed according to the method of Goodhew et al. (17) including non-covalent heme extraction with acidified acetone. Purified protein marker (New England Biolabs, Beverly, MA) was used when heme staining was performed. Ellman’s reagent was used according to Riddles et al. (18). Thiol modification with 4-acetamido-4’-maleimidyl-stilbene-2,2’-disulfonate (AMS) (Molecular Probes Inc., Leiden, Netherlands) (19) was carried out by incubating either reduced or oxidized apoprotein (20 µM) in freshly prepared AMS solution (15 mM AMS, 100 mM Tris-HCl, pH 7.5, 1 mM EDTA, 100 mM NaCl, 1% SDS) for 45 min at 37 °C with occasional agitation. Buffer exchange to sodium phosphate buffer (50 mM, pH 7.0) was achieved with Centricon Centrifugal filter units (YM-10; Millipore, Bedford, MA) before analysis of the incubated proteins. Electrospray ionization mass spectra were recorded on a Micromass Bio-Q II-2S triple quadrupole atmospheric pressure mass spectrometer equipped with an electrospray interface. Samples (10 µl) were introduced into the electrospray source via a loop injector.

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The abbreviations used are: ccm, cytochrome *c* maturation; DTT, dithiothreitol; AMS, 4-acetamido-4’-maleimidyl-stilbene-2,2’-disulfonate; ANS, 8-anilino-1-naphthalenesulfonate; CD, circular dichroism; ES-MS, electrospray mass spectrometry.
In Vitro Interaction of Apocytochrome c with Heme

**RESULTS**

**Apocytochrome Production and Characterization**

Horse Heart Cytochrome c—Horse heart cytochrome c was shown to be pure by SDS-PAGE analysis (Fig. 1, lanes a, left hand panels) and had a mass of 12,359 (calculated 12,360) as shown by ES-MS analysis. The in vitro produced apocytochrome was shown to be devoid of heme as judged by SDS-PAGE analysis followed by heme staining procedures (Fig. 1, lanes b, left hand panels), the absence of cytochrome characteristics in the visible spectra, the disappearance of the cytochrome c fold as shown by CD analysis (Fig. 2) and ES-MS analysis, which resulted in a mass of 11,746 (calculated 11,742 and 11,744 for the oxidized and reduced protein masses, respectively). It is noteworthy that addition of oxygen to the apoprotein occurred, as judged by the increase of a mass peak in the ES-MS analysis at 11,760, if the reaction time for the heme removal was not kept to a maximum of 2 h.

When the apoprotein was dialyzed extensively overnight, the cysteines within the CXXCH motif (CAQCH for equine cytochrome c) formed a disulfide as shown by Ellman’s reagent (18), which accounted for 0.1 equivalents of thiol per mole of protein instead of the 2 equivalents that were detected in the starting material. This disulfide is shown to be internal because the ES-MS analysis gave the monomeric protein mass and the polypeptide migrates corresponding to the monomeric weight of 12,359 (calculated 12,360) as shown by ES-MS analysis (Fig. 1, lanes a, left hand panels).

**Circular dichroism spectra of various forms of horse heart cytochrome c (A) and P. denitrificans cytochrome c (B).** Oxidized holocytochrome c (—) and apocytochrome c (——) are presented for proteins from both organisms. For horse heart the reaction product of apocytochrome and heme is shown (———) and compared with the simulated CD spectrum obtained by adding the spectra of holoprotein and apocytochrome in the ratio 2:3 (⋯⋯⋯), which overlaps with the former spectrum for most of the spectral range. [θ]_290 is mean molar ellipticity per residue. Spectra were recorded using 20 μM protein in 20 mM sodium phosphate buffer, pH 7.0.

**In Vitro Thioether Bond Formation—Reconstitution of the cytochromes was achieved by the addition of apoprotein to (typically 2–5 μM) heme (or mesoheme) in sodium phosphate buffer (pH 7.0) unless otherwise stated, 50 mM at 25 °C. Fe-porphyrins were reduced with disodium dithionite. Apoprotein was kept reduced by the addition of 5 mM DTT. The presence of oxygen was avoided by thoroughly sparging all solutions with humidified argon. Reactions were carried out in the dark. Free heme and aggregated protein was removed by passing the solution through PD-10 desalting columns (Amersham Biosciences).**
The affinity of the apocytochrome for the hydrophobic ligand ANS was measured as described under "Experimental Procedures," and the binding isotherms showed one transition corresponding to a dissociation constant of 60 (± 20) μM for a single binding site on oxidized (containing the disulfide) protein. The absence of the disulfide did not alter the ANS affinity substantially (Kₐ: 70 (± 20) μM for reduced protein in the presence of 2 mM DTT). Interestingly, the protein fluorescence emission maximum was around 355 nm, indicating that the tryptophan is exposed to the aqueous environment. This observation suggests that the apoprotein is largely unfolded (25), which is in agreement with the CD data (Fig. 2). It was shown that the original holocytochrome neither bound ANS nor gave rise to fluorescence features of tryptophan residue(s) exposed to a polar environment. The ANS fluorescence in the presence of apocytochrome had a maximum around 475 nm, with excitation at 380 nm, indicating that the ANS moieties was in a relatively hydrophobic environment (23). Addition of ANS to either holoprotein or buffer gave rise to an ANS fluorescence maximum around 515 nm, which is in agreement with ANS being exposed to the aqueous environment and not bound to a site on the holoprotein (23). The structure of the protein, which shows that the tryptophan is in an hydrophobic environment close to the heme (26), is consistent with the observation of the tryptophan being in a non-polar environment in the holocytochrome as judged by the protein fluorescence experiments.

It was shown that ANS, which is known to bind to the heme pocket in apomyoglobin (23), was displaced from the protein upon addition of just over one equivalent of heme per equivalent of protein. This suggests that the hydrophobic ligand ANS binds to the same site as heme.

P. denitrificans c₅₅₀—This was shown to be pure (some runs as a polymeric form) by SDS-PAGE analysis (Fig. 1, lanes a, right hand panels), and ES-MS analysis resulted in a mass of 15,028 (calculated 15,028). The prepared apocytochrome was shown to be devoid of heme as determined by using the same techniques described above for horse heart apocytochrome. SDS-PAGE analysis (Fig. 1, lanes b, right hand panels) and CD analysis (Fig. 2) of the obtained apocytochrome are shown. To the best of our knowledge there has not been a previous report of the preparation and characterization of the apoform of a mono-heme c-type cytochrome from a mesophilic bacterium. The ES-MS data showed a mass of 14,409 (calculated 14,410 and 14,412 for oxidized and reduced cysteines, respectively).

Extensive dialysis overnight of the apoprotein resulted in the formation of a disulfide between the cysteines within the CXXCH motif (CKACH for P. denitrificans cytochrome c₅₅₀) as shown by Ellman’s reagent (18), which accounted for zero equivalents of thiol per mol of protein compared with two equivalents in the initially produced apoprotein. The monomeric state of the protein shown by ES-MS and SDS-PAGE analyses under non-reducing conditions (Fig. 1, lanes b, right hand panels) show this disulfide to be internal. AMS labeling was also performed with reduced and oxidized protein. Aproprotein containing a disulfide was unable to bind any label, whereas reduced apocytochrome reacted with two AMS moieties as shown by ES-MS and SDS-PAGE analyses (Fig. 3B). The oxidized protein incubated in AMS solution had a mass of 14,410, indicating that no alkylation had taken place. Reduced apocytochrome had a mass peak at 15,420, which is interpreted as the covalent binding of two AMS molecules (0.5 kDa) and one sodium adduct. The increased mass also becomes apparent on the SDS-PAGE analysis (Fig. 3B, lane 2), relative to unresolved (Fig. 3B, lane 1) and non-reacted protein (Fig. 3B, lanes 3 and 4).

An ANS dissociation constant of 55 (± 15) μM for oxidized apocytochrome was obtained by fluorescence experiments, proving that the apoprotein has a pronounced affinity for hydrophobic ligands. The absence of the disulfide did not affect the ANS affinity (Kₐ: 55 (± 20) μM). The exposure of the tryptophan residues and the overall fold of the apocytochrome as judged by CD spectroscopy (Fig. 2) are analogous to horse heart apocytochrome and suggest a largely unfolded structure. Holoprotein was incapable of binding ANS. It was shown that heme could displace ANS from apocytochrome, indicating the competition for the same binding site of both hydrophobic molecules.

**Reaction of Apocytochromes with Heme**

**Horse Heart Cytochrome c**—Mixing reduced horse heart apocytochrome c (no disulfide bond) with ferrous (Fe(II)) heme in the presence of 5 mM DTT at pH 7.0 resulted in a decrease in the absorption around 424 nm relative to that of heme alone. The same trend was observed around 528 and 559 nm, which are wavelengths characteristic of the presence of a reduced b-type cytochrome (Fig. 4A and Table 1). The pyridine hemochrome spectrum of this mixture had its α-band at 556 nm (Table 1), indicating that the heme contained two unreacted vinyl groups (22). These results show that the apoprotein and heme initially form a species that resembles a b-type cytochrome, in which the heme is not covalently attached to the peptide but in which its iron atom is coordinated by two amino acid side chains from the protein. These results are consistent with previous studies (13).

Following nearly quantitative formation of the b-type cytochrome from the mixture of horse heart apocytochrome c and heme under reducing conditions, the maximum at 559 nm in the absorption spectrum shifted to 550 nm with time (Table 1). After 48 h, the visible spectrum of protein, following gel-filtration chromatography to remove any unbound heme and protein aggregates, showed features both of a b- and c-type cytochrome in a ratio of 40:60 (Fig. 4A). After 72 h, the visible spectrum contained broad peaks, but showed absorption maximum very similar to the original holoprotein (Fig. 4B and Table 1). The pyridine hemochrome spectrum of the reduced protein after gel-filtration chromatography had a resolved band at 549.5 nm (Table 1), characteristic of saturation of both vinyl groups of the heme (22). CD spectra of the desalted, oxidized cytochrome c produced in vitro after 72 h of incubation showed a mixture of spectral features of holoprotein and apoprotein (Fig. 2). It was
Heme is assumed to have dissociated from 60% of the initial heme, and 60% of the protein was present in the apoform. This was calculated knowing that in the original (oxidized) holoprotein the ratio of \( A_{380}/A_{410} \) is 1:4. The reconstituted cytochrome \( c \) prepared in the present work had a CD spectrum that was almost identical to a spectrum that was simulated by adding the spectra of apocytochrome and original, purchased holocytochrome in the calculated ratio of 3:2 (Fig. 2). It was shown that CD spectra of these two components are additive, i.e., the presence of either form of the protein did not affect the fold or, therefore, the CD spectrum of the other.

Consequently, it was concluded that 60% of the \( b \)-type cytochrome intermediate was incapable of forming a \( c \)-type cytochrome. Why this is the case is unclear. Possible reasons for this difference in reactivity may arise from the orientation of the heme relative to its rotation around the \( \alpha, \gamma \)-meso-axis or the incorrect fold of the apocytochrome around the prosthetic group.

A noteworthy point is that the CD spectrum of the cytochrome \( c \) generated in \textit{vitro} had a great similarity with not only that of the previously reported \( b \)-type cytochrome complex (13), but also an apocytochrome derivative, where the cysteines had been reacted with a hydrophobic molecule (27). Hence a definite conclusion whether the full holocytochrome \( c \) fold is gained upon reaction of apocytochrome with heme cannot be reached. Separation by addition of ammonium sulfate (28) was not achieved to a satisfactory extent with our putative mixture of reconstituted cytochrome \( c \) and unreacted apoprotein.

The observations from the spectral analysis clearly indicate the formation of a \( c \)-type cytochrome, an interpretation that was substantiated further by heme staining of the proteins on SDS-PAGE gels (Fig. 1, lanes \( d \), left hand panels), including treatment of the heme-containing protein with acidified-acetone (17); in these experiments, non-covalently bound heme dissociates from protein but covalently bound heme does not. The data show \textit{in vitro} formation of horse heart cytochrome \( c \); the latter has not been previously reported to form from the \( b \)-type intermediate.

An analogue of the \( b \)-type cytochrome intermediate formed upon addition of reduced Fe-mesoporphyrin to reduced apocytochrome \( c \). The product had visible absorption maxima at 549, 519 and 416 nm and did not heme stain on an SDS-PAGE gel (Fig. 1, lanes \( c \), left hand panels). Mesoporphyrin has ethyl groups in the positions of the vinyl groups of protoporphyrin and therefore cannot form thioether bonds with the polypeptide. There was no evidence for any other type of covalent attachment of mesoporphyrin to apoprotein.

The requirement for both the heme and the apoprotein to be reduced for holocytochrome \( c \) formation was substantiated. Oxidized apoprotein (containing a disulfide), when reacted with ferric heme, did not give rise to characteristic visible spectra of a cytochrome.

\textit{P. denitrificans} \( c_{550} \)—Similar to the observation with horse heart cytochrome \( c \), when reduced \textit{P. denitrificans} \( c_{550} \) was reacted with ferrous heme, spectral features of a \( b \)-type cytochrome intermediate were observed (Fig. 5 and Table I). Over a time period of several hours spectral bands appeared at 552, 523, and 417 nm. A spectrum of the heme-protein solution after 5 h of incubation under reductive conditions is shown (Fig. 5). SDS-PAGE analysis of the reaction product showed that heme was covalently attached to the protein (Fig. 1, lanes \( d \), right hand panel). Incubation of ferrous mesoheme with reduced apocytochrome did not result in covalent attachment (Fig. 1, lanes \( c \), right hand panel), suggesting that in the reaction with heme thioether bonds had formed between the protein and the heme vinyl groups. This interpretation was substantiated by the pyridine hemochrome spec-

![Fig. 4. A, visible absorption spectra of horse heart apocytochrome c (5 \( \mu \)M) following mixing with an equimolar amount of heme under reductive conditions. The solid line shows a spectrum, corresponding to a \( b \)-type cytochrome, obtained after 10 min after mixing. The dashed line was obtained after 48 h of incubation. B, visible spectra of horse heart cytochrome c (5 \( \mu \)M) produced either \textit{in vivo} (dashed line) or \textit{in vitro} (solid line) after 72 h incubation under reductive conditions and subsequent purification.](image-url)

**TABLE I**

Absorption maxima of various forms of cytochrome species

| Protein species | Wavelength/nm |
|-----------------|---------------|
| Reduced protein | Pyridine hemochrome (alpha band) |
| Horse heart Cytochrome | 415 520 550 549.6 |
| Holo produced \textit{in vitro} | 416 520 550 549.5 |
| b-type complex | 424 528 559 556 |
| Mesoheme complex | 416 519 549 546 |
| \textit{P. denitrificans} \( c_{550} \) | 415 521.5 550 549.7 |
| Holo produced \textit{in vitro} | 417 523 552 552.4 |
| b-type complex | 423 528.5 558.5 556 |
| Mesoheme complex | 414 519.5 548.5 546 |

deduced from the ratio of the Soret band relative to the absorption at 280 nm in the absorption spectrum of oxidized protein that 40% of the apoprotein had formed thioether bonds to heme, and 60% of the protein was present in the apoform. Heme is assumed to have dissociated from 60% of the initial \( b \)-type cytochrome complex and unbound heme to have been removed upon gel-filtration chromatography. This was calculated knowing that in the original (oxidized) holoprotein the ratio of \( A_{380}/A_{410} \) is 1:4. The reconstituted cytochrome \( c \) prepared in the present work had a CD spectrum that was almost identical to a spectrum that was simulated by adding the spectra of apocytochrome and original, purchased holocytochrome in the calculated ratio of 3:2 (Fig. 2). It was shown that CD spectra of these two components are additive, i.e., the presence of either form of the protein did not affect the fold or, therefore, the CD spectrum of the other.

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**Fig. 5. Comparison of visible spectra of various forms of P. denitrificans cytochrome (5 μm).** The spectra of the b-type cytochrome complex between apocytochrome c and heme obtained after 10 min of incubation under reductive conditions (——); the reaction product of heme and apocytochrome after 5 h (∧∧); the in vivo produced protein (—−—).

trum of the reaction product, which had an α-band at 552.4 nm, which, together with the visible spectrum of reduced protein, shows that more than half of the vinyl groups have been saturated. This data is interpreted as reflecting a mixture of b- and c-type cytochrome present in the reaction mixture. The pyridine hemochrome spectrum of the reaction product of mesoheme and apoprotein, which also yields a b-type cytochrome (Table I), remained unchanged from the pyridine hemochrome of mesoheme itself. Again, the reaction of cytochrome c formation from the b-type intermediate was not quantitative. A similar ratio as in the horse heart cytochrome c experiments described above remained as b-type cytochrome. However, the fraction of apocytochrome c that could form a b-type cytochrome in this case was very low (5%), suggesting that the heme extraction procedure had adversely affected the ability of the majority of the apoprotein to form a hydrophobic pocket. However, it is clear from the data that reaction of heme and P. denitrificans apocytochrome e550 can lead to formation of holoprotein with covalently attached heme *in vitro.*

**DISCUSSION**

In this work we show the data obtained from the production of apocytochromes c from a bacterial and a mammalian organism and characterize the reaction of the cysteine thiols with the vinyl moieties of heme. Both proteins have very similar properties with regard to their apoforms and their reaction with heme. We show that the cysteines of the conserved CXXCH motif can form an internal disulfide. This observation raises the question if this oxidation occurs *in vivo* for the respective proteins in either organism. For the bacterial system it has been suggested that the disulfide is an important intermediate during cytochrome c maturation due to the presence of the disulfide bond forming (Dab) proteins in the periplasm, the location of cytochrome c maturation (2). Furthermore, it has been proposed that proteins (CcmG/H and DsbD) required for cytochrome c biogenesis are involved in the reduction of the internal disulfide bond in the apocytochrome (9). Why this *in situ* reduction of an apocytochrome has evolved is unclear, but an internal protection mechanism against metallaition of the coordination site of the reduced apocytochrome might offer an explanation.

Heme attachment to mitochondrial apocytochrome c occurs in the intermembrane space (29). The *in vivo* oxidation state of the cysteines in mitochondrial apocytochromes c has not been investigated to the best of our knowledge. Whether, as we show here for the first time *in vitro*, a disulfide bond occurs during the *in vivo* protein maturation process, will depend on both the reduction potential of its locus and kinetic factors. If formation of a disulfide must be avoided, a thioredoxin-like protein could be required in the intermembrane space (30). A thioredoxin (Trx3) and its reductase (Trx2) are known to be present in yeast mitochondria, but submitochondrial localization to the matrix is suspected rather than to the intermembrane space (31). These proteins can in any case be deduced to be dispensable for cytochrome c biogenesis in yeast, as mutants carrying specific disruptions in the corresponding genes were able to grow normally under aerobic, respiratory conditions, which require participation of cytochrome c in the mitochondrial respiratory chain. However, in the case of Arabidopsis thaliana, it has been recognized, on the basis of the genome sequence, that there may be a yet to be characterized thioredoxin that is targeted to the intermembrane space (30). Alternatively, the formation of holocytochrome c may happen sufficiently fast that the two thiols of apocytochrome c cannot form a disulfide during its lifetime in the intermembrane space following delivery from the reducing environment of the cytoplasm. Such a kinetic constraint would suffice if the rate of disulfide bond formation in the intermembrane space was to occur on a similar timescale to that *in vitro* (hours as observed in the present work).

The presence of the CcmABCEF components of one cytochrome c biogenesis pathway, but not of the thioredoxin-like CcmG component, in plant mitochondria might support the latter view.

The binding of heme to the apoform of mitochondrial cytochrome c clearly involves some ordering of the polypeptide chain. This is evident from several biophysical studies (13, 28). A non-covalent complex between heme and apoprotein of H. thermodilicthius cytochrome c550 generates a protein structure that is very similar to that of the native protein with covalently bound heme (32). Although it appears that the structure of the non-covalent complex between heme and mitochondrial apocytochrome c does not fully resemble the native, holo structure (13, 28), studies with two monoclonal antibodies that scarcely recognize the apoprotein, but bind with comparable affinities to both the non-covalent heme-apoprotein complex and the holoprotein, indicate that the complex must be similar to the holoprotein in respect of quite specific structural features at the epitope regions (28). Such similarities are evidently sufficient to permit the vinyl groups of heme to be oriented appropriately, in at least a fraction of the molecules, for reaction with the thiol groups, which have previously been shown to exhibit nucleophilic reactivity (27). The effect of covalently bound hydrophobic moieties on the folding of cytochrome c has also been discussed (27).

The affinity of the apoproteins for hydrophobic ligands and the ability of both apoproteins to ligate heme in a b-type cytochrome complex has important implications for the catalytic strategy of the enzymes involved during cytochrome c maturation. It is unclear from previous studies how the heme lyase functions in mitochondria, except for the proposal that it binds heme via a conserved CPX motif (6). For the bacterial Ccm system, two proteins have been proposed to function as a heme lyase (33), but the heme attachment is more complex due to the presence of a heme chaperone, CcmE (34). However, in view of our data showing that the b-type cytochrome can spontaneously react to give the other bond formation yielding holocytochrome c, two key conditions have to be achieved by the catalytic systems. Firstly, the heme group has to be kept reduced to give optimal reactivity for cysteine-thiol and heme-vinyl group reaction partners. This is in agreement with previous studies (35, 36). And secondly, the orientation of the heme relative to
its rotation around the α,γ meso-axis has to be correct to yield the required stereochirality at the prochiral α-carbon of the vinyl substituents of the heme moiety (3). McRee et al. (37) showed that a recombinant cytochrome c of Thermus thermophilus could be improperly matured in vivo, but without any catalytic assistance in the cytoplasm of E. coli, because of both heme inversion and an intermolecular disulfide bond between cysteine residues, which are usually involved in heme binding (37). The latter behavior was clearly reflected in the visible absorption spectrum, which was different from the reported, properly matured holocytochrome c, and the pyridine heme-iron is reduced. Indeed, we suspect that had Dumont et al. (13) extended their duration, and/or modified the reaction condition for instance by exclusion of oxygen, of incubation of heme with apocytochrome c, they too might have seen covalent bond formation to heme.

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