Heme Binding to a Conserved Cys-Pro-Val Motif Is Crucial for the Catalytic Function of Mitochondrial Heme Lyases*

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Covalent attachment of heme to the apoforms of mitochondrial cytochromes c and c₁ requires the activity of cytochrome c heme lyase (CCHL) and cytochrome c₁ heme lyase (CC₁HL), respectively. The two enzymes differ in their cytochrome specificity, but they are related in sequence, and both contain conserved Cys-Pro-Val (CPV) motifs. By using various in vitro assays we investigated whether heme can bind directly to heme lyases and whether the CPV motif may be involved in heme binding. Heme stabilized CC₁HL, as a model protein, in a folded, protease-resistant conformation, stimulated the refolding of CC₁HL after urea denaturation, and inhibited the import of the CC₁HL precursor into mitochondria. These effects were not observed with a point mutant, CC₁HL<sub>SPV</sub>, in which cysteine was replaced by serine, and with CC₁HL<sub>ACPV</sub>, in which the motif was deleted. These results show that heme lyases can bind heme directly, and they identify the CPV sequence as a structural element important for this interaction. The phenotype of a yeast mutant expressing CC₁HL<sub>SPV</sub> is in good agreement with such a role of the CPV motif. The mutant cells accumulate the heme-free intermediate form of cytochrome c₁ and display a severe deficiency in the holo form. We suggest that the CPV motif forms a crucial part of the substrate binding site for heme.

Mitochondrial heme lyases catalyze the covalent attachment of heme to the apoforms of c-type cytochromes (1, 2). In this reaction two thioether bonds are formed between two cysteiny1 residues in the cytochromes and the vinyl group of heme. Only the reduced form of heme is a suitable substrate for the heme lyase enzymes (3). At present, three examples of heme lyases have been studied in some detail (see below), namely the cytochrome c₁ heme lyase (CC₁HL) of the yeast Saccharomyces cerevisiae (4) and the cytochrome c heme lyases (CCHL) of both yeast (5) and Neurospora crassa (6). The enzymes are distinct in their substrate specificity that in each heme lyase can only convert its own apocytochrome substrate into the holo form.

Our knowledge of the participation of heme lyases in the biogenesis of the cytochromes is limited to their role in the import of the apoproteins into mitochondria. CCHL, in addition to facilitating covalent heme attachment (7, 8), plays an important role in driving the import of apocytochrome c from the cytosol (9). During or immediately after its reversible passage across the mitochondrial outer membrane, apocytochrome c associates with CCHL (10). The energy gained from this specific complex formation is believed to trap apocytochrome c in the intermembrane space.

CC₁HL is involved in the biogenesis of cytochrome c₁ in a rather different manner. The precursors of the latter protein is synthesized in the cytosol with a bipartite signal sequence that, upon import into mitochondria (11), is processed in two proteolytic steps (12). Removal of the matrix-targeting sequence by the matrix-processing peptidase is followed by the cleavage of its intermembrane space sorting signal. The second processing step is catalyzed by inner membrane protease subunit 2 and has been shown to be strictly dependent upon heme attachment (12–14), which is mediated by CC₁HL (15). Accordingly, yeast cells lacking CC₁HL accumulate the heme-free intermediate size form of cytochrome c₁ and are deficient in the mature holo form (4).

The molecular mechanism of how heme lyases participate in covalent heme attachment is poorly understood (see Ref. 16). For instance, it has never been shown that heme lyases catalyze the heme attachment reaction themselves. Moreover, heme binding to heme lyases has not been demonstrated. No information is available about the chemical nature of the binding sites for heme and for the apoproteins. Furthermore, it is not known whether the two thioether bonds are formed in a sequential or simultaneous manner.

The heme lyases known to date share about 35% identical amino acid residues (50% similarity; see Ref. 4 and Fig. 4A). Especially in the COOH-terminal two-thirds of these proteins, there are a number of conserved sequence blocks, suggesting that this part is particularly important for their function. The NH₂ termini of the heme lyases do not display striking sequence conservation with the exception of CPX-sequences (X = V, M, I, L; hereafter termed CPV motif) present in all of these enzymes. This CPV motif is found two or three times at the amino termini of CCHL proteins and once at the amino terminus of yeast CC₁HL (see Fig. 4A). The functional role of these CPV motifs in heme lyases has not been investigated so far. In two other proteins, similar motifs have been implicated to act as heme regulatory motifs. The transcriptional activator HAP1 (CYP1) contains seven CPV motifs (17, 18). Heme binding to these motifs causes dissociation of a HAPI-bound repressor factor, allowing HAP1 to bind to DNA thereby activating transcription (19, 20). Two copies of a similar motif (CPF) are present in the prerequisites of several mammalian δ-aminole-
ulinate synthase (ALAS) precursors. They were shown to confer heme inhibition of import of the ALAS precursor into mitochondria (21), emphasizing the importance of such a sequence in heme binding to proteins.

Using CC1HL as a model protein we show by several in vitro approaches that heme lyases can interact directly with heme. Thus, heme lyases are able to bind both substrates of the heme attachment reaction, namely the apoform of the cytochromes (10) and heme (this study). A mutant CC1HL, in which the cysteinyl residue of the CPV motif has been converted to a seryl residue (termed CC1HLSPV) failed to interact with heme in these in vitro experiments, suggesting that the CPV sequence serves as part of the heme binding site. The crucial role of the CPV sequence in recruiting heme to CC1HL is supported further by the phenotype of a yeast strain expressing CC1HLSPV. Strongly reduced levels of mature holocytochrome served as part of the heme binding site. The crucial role of the CPV sequence in recruiting heme to CC1HL is supported further by the phenotype of a yeast strain expressing CC1HLSPV. Strongly reduced levels of mature holocytochrome, showed an intactness of the outer membrane of strain W303-1A as reported previously (4). Construction of a CC1HL null mutant (strain YS10, Table 1), the genomic CYT2 locus coding for CC1HL was disrupted in strain W303-1A as reported previously (4). Construction of the mutant heme lyase CC1HLSPV, carrying a mutation replacing a cysteinyl residue in position 10 (Cys10) by a seryl residue, was achieved by directed mutagenesis using a two-step polymerase chain reaction protocol and appropriate primers (25). Following the polymerase chain reaction, the resulting EcoRI fragment carrying the point mutation was used to replace the corresponding fragment of the wild type CYT2 gene harbored in plasmid pHS104 to create the pGEM4-based plasmid pHS122 used for in vitro transcription/translation of CC1HLSPV. The correct replacement of the codon for Cys10 was verified by DNA sequencing. To allow for expression of CC1HLSPV in vivo, an ApII/HindIII fragment of the wild type CYT2 gene present in the yeast expression vector pHS16 (26) was replaced by the corresponding fragment carrying the Cys10 to serine mutation thereby creating plasmid pHS143. Deletion of 15 base pairs in the CYT2 gene corresponding to the amino acid residues 10–14 of CC1HL (CPDVE) was achieved by inverse polymerase chain reaction using appropriate primers. The whole CYT2 gene was sequenced to confirm the deletion and to rule out changes in other nucleotide positions. The insert encoding the mutated protein (termed CC1HLspv) was subcloned further either as a blunt ApII/HincI fragment into the SmaI site of pGEM4 transcription/translation vector (Stratagene) or as an XbaI/XhoI fragment into pHS16 (26) to create plasmids pHs46 and pHs47, respectively.

Biochemical Procedures—The following published methods were used: in vitro transcription/translation of CC1HL, CC1HLspv, and CC1HLspv proteins in the presence of 35S-labeled methionine (27); isolation of mitochondria (28; cells harboring plasmids were pregrown in synthetic complete medium lacking uracil; only preparations were used which, according to the protease sensitivity of intermembrane space components, showed an intactness of the outer membrane of greater than 90%; use of protease-sensitive markers for either the intermembrane space (cytochrome b6) or the matrix space (Tim44; 30); in vitro protein import of heme lyase precursors into mitochondria (31); osmotic shock treatment to generate mitoplasts (29); SDS-PAGE and fluorography of resulting gels and quantitation of fluorographs by densitometry (32); preparation of hemin stock solutions (33; throughout the study, the nonreduced form of heme was used); staining of heme-containing proteins after nonreducing SDS-PAGE (34). Immunoblotting and detection by chemiluminescence using the ECL system (Amersham International, Amersham UK) and protein determination using bovine serum albumin as a standard (Bio-Rad) were performed according to the instructions of the suppliers.

RESULTS

CC1HL Binds Heme—We examined the potential of heme lyases to bind heme. Direct heme binding studies are not possible with heme lyases, since attempts to overexpress and purify these proteins in a biochemically active form have failed. Therefore the heme binding capacity of S. cerevisiae CC1HL as a model protein was tested by various indirect in vitro methods. We first asked whether heme has an influence on the conformation of the protein and would change its sensitivity to proteolytic attack. Mitochondria were isolated from wild type strain YS88 (termed CPV, see Table 1) and converted into mitoplasts (i.e. mitochondria with a disrupted outer membrane) by osmotic swelling in the presence of increasing amounts of heme. The mitoplasts were immediately treated with trypsin. In the absence of heme, CC1HL became degraded to a 25-kDa fragment (Fig. 1A). As reported recently, this fragment represents a large folded domain of CC1HL (31). In the presence of heme, CC1HL did not become cleaved and rather was shielded efficiently against the proteolytic attack. Apparently, heme induces or stabilizes a conformation of CC1HL which is largely resistant to trypic digestion. This finding indicates a direct association of heme with CC1HL.

As a control we tested whether the observed protection of CC1HL by heme against trypic digestion was dependent on a preexisting, folded conformation and was not caused by a possible unspecific inhibition of the protease. Mitochondria were incubated in buffer containing 8 M urea to cause unfolding of the heme lyase (see Ref. 31). After 20-fold dilution with buffer, samples were treated immediately with trypsin in the presence of increasing amounts of heme. In the absence of heme, the urea-treated CC1HL did not give rise to the 25-kDa fragment and became completely degraded, indicating that CC1HL had become unfolded (Fig. 1B, left panel). Addition of heme to the unfolded CC1HL did not stabilize the protein against trypic degradation, ruling out that heme inhibited the protease. To control for the effect of the residual amount of the denaturant in these experiments, 0.4 M urea was added to nondenatured mitochondria during the proteolysis step. No significant difference in the findings obtained in the absence of urea was observed (Fig. 1B, right panel; cf. Fig. 1A). These data provide evidence that heme can bind to a folded structure of CC1HL, thereby stabilizing the protein against proteolytic digestion.

In a second approach to demonstrate heme binding to CC1HL, we took advantage of the observation that unfolded CC1HL, after removal of the denaturing conditions, slowly
Heme Binding to CC1HL Occurs via the CPV Motif—Where is the heme binding site located in the heme lyase molecule? We considered it a likely possibility that, at least in part, heme binding to CC1HL is mediated by the CPV sequence, a structural motif that has been implicated in heme binding to HAP1 and the precursors of ALAS. This motif is highly conserved in the single CPV motif of yeast CC1HL. The conserved cystei-
FIG. 4. Heme binding to CC1HL is mediated by the conserved CPV sequence. Panel A, amino acid sequence alignment of mitochondrial heme lyases. The alignment was generated using the Clustal program. The conserved CPX motifs are highlighted in boldface. Stars or colons indicate sequence identity or chemical similarity, respectively, between at least five of the seven heme lyase sequences. Given are the sequences for CCHL from S. cerevisiae (Sc; accession number A26162), N. crassa (Nc; A34365), Candida albicans (Ca; U62148), man (Hu; U36787), mouse (Mu; U36788), and Caenorhabditis elegans (Ce; Z49130) as well as for CC1HL from S. cerevisiae (S24365). Panel B, a mutant heme lyase, CC1HL<sub>SPV</sub>, fails to bind heme. Purified mitochondria from CPV and SPV cells were reisolated by centrifugation and subjected to hypotonic treatment in the presence of trypsin (12.5 μg/ml) and heme (25 μM) as described in Fig. 1A. Further analysis was as in Fig. 1A. Panel C, the inhibition by heme of the mitochondrial import of CC1HL depends on the presence of the CPV motif. Radiolabeled precursors of CC1HL and CC1HL<sub>SPV</sub> were imported into mitochondria for 15 min at 25 °C in the absence or presence of heme. Further analysis was as in Fig. 3. The average of three experiments is shown; the standard error is given by the bars. Import efficiency in the absence of heme was set to 100% in each case.
nyl residue was changed to a seryl residue by site-directed mutagenesis of the CYT2 gene encoding Cc1HL. A yeast strain (YS60, termed SPV, see Table I) was generated expressing the mutant protein (Cc1HLSPV) from a centromeric plasmid under control of its endogenous promoter in a Cc1HL null mutant background. Heme binding to the mutant protein was then assayed in vitro. Mitochondria were isolated from strain SPV, converted into mitoplasts, and treated with trypsin in the absence or presence of heme. Like the wild type protein, Cc1HLSPV became clipped to a 25-kDa fragment in the absence of heme (Fig. 4B). The addition of heme during the proteolysis did not alter the sensitivity of Cc1HLSPV to trypsin, i.e. heme did not confer resistance of the mutant protein to proteolytic attack, in contrast to the finding for Cc1HL (cf. Fig. 1A). The failure of heme to stabilize Cc1HLSPV suggests that this protein does not interact or does so less strongly with heme. Importantly, the generation of the tryptic 25-kDa fragment from Cc1HLSPV also shows that the point mutation did not significantly alter the conformation of the protein even though the protein is very sensitive to structural changes in this region (not shown).

We next asked whether heme could inhibit the import of Cc1HLSPV into mitochondria. When the precursor of Cc1HLSPV was imported into mitochondria in the presence of heme, only a weak inhibition was observed compared with the strong influence of heme on the wild type protein (Fig. 4C). A similar result (30% inhibition at 10 μM heme) was obtained with a mutant heme lyase precursor, Cc1HLCVPV, in which the CPV motif was deleted (not shown). Also with urea-denatured precursors, heme had no significant influence on the import efficiency of the two mutant heme lyases (not shown). These observations suggest that heme specifically bound to the CPV motif in Cc1HL and thereby prevented the passage of the precursor protein across the outer membrane. Import inhibition occurred in a similar range of heme concentration as was found recently for ALAS precursors (21). In summary, the in vitro experiments identify the CPV motif as a site that increases the affinity of heme binding to mitochondrial heme lyases.

The CPV Motif Is Important for the in Vivo Function of Cc1HL.—Mutation of the heme binding site in heme lyases should have a profound effect on their function during covalent attachment of heme to the apocytochromes. To investigate the effect of the SPV mutation on the enzymatic properties of Cc1HL in vivo, we made use of yeast strains CPV and SPV that expressed either wild type or mutant heme lyases in a Cc1HL null mutant background (see Table I). We first examined the consequences of the cysteine to serine mutation in Cc1HL on the growth of the mutant yeast cells. Because of a deficiency in cytochrome c1, Cc1HL null mutant cells display a growth phenotype during covalent linkage of heme to the intermediate form of cytochrome c1 (cf. Fig. 1A). The mature form of cytochrome c1 was absent in Cc1HL null mutant cells grown in YPG medium at 25 °C (Fig. 5). However, for cytochrome c1, an accumulation of the intermediate form and 3–4-fold reduced amounts of the mature form were observed in SPV cells, indicating that Cc1HLSPV apparently failed to replace the function of the wild type enzyme at equal efficiency. According to heme staining experiments, the low amounts of the mature form of cytochrome c1 in mitochondria of SPV cells carried a covalently attached heme group, whereas the intermediate form did not (Fig. 6B). On the other hand, no alteration in the levels of heme-containing cytochrome c was observed in response to the mutation in Cc1HL, demonstrating that the observed effects are specific for heme attachment to cytochrome c1. Similar results were obtained for ΔCPV cells after growth at 25 °C (not shown). This analysis shows that mutation of the CPV motif in Cc1HL considerably impairs the covalent linkage of heme to the intermediate form of cytochrome c1. Nevertheless, cells with these mutated Cc1HL enzymes maintain synthesis of holocytchrome c1 at levels sufficient to allow the formation of respiratory competent mitochondria. Therefore, the CPV motif plays an important but not essential role during the catalytic function of Cc1HL. In summary, the CPV motif can be re-
Thioether formation and therefore may contain the active site lyases themselves perform the catalytic function during the heme lyase protein. This makes it very likely that heme substrates of the heme attachment reaction can interact specifically inhibited by heme. Recently, it was shown that into the intermembrane space of isolated mitochondria was forms of thionation of the molecular mechanism of heme attachment to the apo forms of e-type cytochromes. Using CC1HL as a model protein, we provide several lines of evidence that mitochondrial heme lyases bind heme. CC1HL was stabilized by heme against tryptic digestion; heme accelerated the refolding of denatured CC1HL severalfold, and the in vitro import of CC1HL precursor into the intermembrane space of isolated mitochondria was specifically inhibited by heme. Recently, it was shown that heme lyase forms a complex with the apoprotein. Thus, both substrates of the heme attachment reaction can interact with the heme lyase protein. This makes it very likely that heme lyases themselves perform the catalytic function during thioether formation and therefore may contain the active site that promotes heme attachment.

From our in vivo and in vitro studies, the conserved CPV sequence appears to represent an important yet not essential structural element required for proper function of mitochondrial heme lyases. CC1HL with an altered CPV motif no longer interacts strongly with heme in our biochemical experiments and is largely impaired in converting the intermediate apoform of cytochrome c1 to the holo form. Thus, as found with other CPV-containing proteins, this motif may be required for efficient binding of heme. How might heme binding to the CPV motif of heme lyases influence the process of covalent heme attachment to the cytochromes? One possibility is that the CPV motif forms part of the substrate binding site for heme. The higher affinity for heme will lead to an increased concentration of heme close to the active site, thereby explaining the observed higher specific activities of enzymes carrying the CPV motif. Another not mutually exclusive view for the function of the CPV motif is that the association of heme with this structural element properly positions the two vinyl groups of heme in the active site of the protein, thereby increasing the efficiency of the thioether formation with the cytochromes. In an attractive alternative model, the NH2 terminus of heme lyases containing the CPV motifs could serve as a flexible arm collecting heme and moving it into the active site of the enzyme. This model would readily explain why this part of heme lyases is important yet not essential for their catalytic function.

A conformational alteration upon heme binding to CC1HL is evident from the stabilization of the protein against tryptic digestion in the presence of heme. This structural rearrangement is mediated through the CPV sequence, since proteins with alterations in this motif do not acquire resistance to trypsin. Changes in the conformation of heme lyases may be of importance for the catalytic action during heme attachment, e.g., by moving the reactive sulphydryl and vinyl groups close to each other to allow the nucleophilic addition. It will be interesting to see whether conformational changes as a result of heme binding may be a general mechanism that is utilized by CPV-containing proteins to influence various biological processes involving regulatory circuits or substrate binding.

The CPV consensus sequence can be regarded as a commonly used structural motif for noncovalent binding of heme to various proteins. Two such proteins have been investigated already. A particularly well studied case is the heme-dependent regulation of transcription by the HAP1 protein, which contains seven CPV consensus motifs (17, 18). By the use of short peptides corresponding to such a motif, the heme binding to the CPV sequence could be directly demonstrated in a spectroscopic study (36). A second case of heme binding was reported for the precursors of ALAS which contain two copies of similar motifs in their mitochondrial targeting sequence. These CPF motifs were shown to confer heme inhibition of ALAS import into the organelles (21), similar to our observations with CC1HL. For the ALAS proteins this inhibitory effect by heme was suggested to play an important function in vivo and allow the fine tuning of the erythroid heme biosynthesis. It thus is thought to add another level of regulation to heme biosynthesis by inhibiting the mitochondrial uptake of the key enzyme of this process. Therefore the CPV motif was termed the heme regulatory motif (21). In yeast it has been shown that expression of CC1HL is independent of heme (37). Thus, it seems unlikely, yet not impossible, that in fungi heme exerts a comparable regulatory function in the biosynthesis of e-type cytochromes by controlling the import of heme lyases into mitochondria.

A common feature of heme-binding proteins containing a CPV motif is the transient character of the interaction. In the case of heme lyases, binding of heme was too labile to allow retention of the proteins on heme-agarose resins (not shown). The loose association of heme with these proteins is quite different from the situation observed for heme binding to cytochromes, globin, or other heme-binding proteins (see e.g., 38), where a stable interaction is needed to ensure proper biochemical function. In the light of the findings presented here, a general view of the function of a CPV sequence may be that of a reversible heme binding site allowing for the transient interaction with this hydrophobic compound. Structural studies are now needed to understand the molecular nature of how heme is specifically recognized by the CPV sequence.

The property of reversible association with heme via the CPV or a similar motif may have been utilized in a variety of proteins participating in diverse biological processes. Some examples of these proteins such as catalase, HRI kinase, and heme oxygenase-2 have already been discussed in some detail (36). Further interesting CPV-motif-containing proteins are found to be involved in the biogenesis of bacterial e-type cytochromes (for review, see Refs. 39 and 40) or in nitrite reduction (41). The Cc2 gene product of Rhodobacter capsulatus and its other bacterial homologs also contain a CPV motif. It is therefore tempting to speculate that these proteins represent the functional counterparts of mitochondrial heme lyases. Except for this short consensus motif, the mitochondrial and bacterial proteins do not exhibit significant similarity. This raises the interesting possibility that mitochondrial and bacterial heme lyases have evolved from different ancestral proteins yet use a similar mechanism to recruit heme to the vicinity of the active site of the enzyme. Direct biochemical studies will be necessary to test this important aspect of e-type cytochrome biogenesis.

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