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Structure and Function of Ferrochelatase

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Ferrochelatase is the terminal enzyme of the heme biosynthetic pathway in all cells. It catalyzes the insertion of ferrous iron into protoporphyrin IX, yielding heme. In eukaryotic cells, ferrochelatase is a mitochondrial inner membrane-associated protein with the active site facing the matrix. Decreased values of ferrochelatase activity in all tissues are a characteristic of patients with protoporphyria. Point-mutations in the ferrochelatase gene have been recently found to be associated with certain cases of erythropoietic protoporphyria. During the past four years, there have been considerable advances in different aspects related to structure and function of ferrochelatase. Genomic and cDNA clones for bacteria, yeast, barley, mouse, and human ferrochelatase have been isolated and sequenced. Functional expression of yeast ferrochelatase in yeast strains deficient in this enzyme, and expression in *Escherichia coli* and in baculovirus-infected insect cells of different ferrochelatase cDNAs have been accomplished. A recently identified (2Fe-2S) cluster appears to be a structural feature shared among mammalian ferrochelatases. Finally, functional studies of ferrochelatase site-directed mutants, in which key amino acids were replaced with residues identified in some cases of protoporphyria, will be summarized in the context of protein structure.

KEY WORDS: Heme; porphyrin; mitochondria; iron-sulfur cluster; heme metabolism.

INTRODUCTION

Heme is essential in almost every single facet of cell function, since it is required as a prosthetic group in a multitude of proteins of diverse functions (e.g., hemoglobin, myoglobin, cytochromes, catalases, nitric oxide synthase). These proteins are involved in processes as distinct as oxygen and electron transport and drug metabolism. Heme, in addition, has a role in regulating the initiation of translation of some eukaryotic mRNAs and controls the DNA binding of some transcription factors. The biosynthesis of heme occurs in all cells. The presence of the different hemoproteins in cells depends on the type of the cell and the respective physiological status. However, the activity of the enzymes of the heme biosynthetic pathway, at least in mammals, is highest in differentiating erythrocytes and liver, where heme demands are high due to the synthesis of hemoglobin and cytochrome P450, respectively. Ferrochelatase (protoheme ferrolyase, EC 4.99.1.1) is the terminal enzyme of the heme biosynthetic pathway; it catalyzes the chelation of ferrous iron into the protoporphyrin IX ring to form protoheme (Fig. 1). This enzyme is ubiquitous in nature, found in organisms ranging from bacteria to man. In this review we will focus on structural and functional aspects of ferrochelatase and their relationship to the molecular mechanism of heme biosynthesis.

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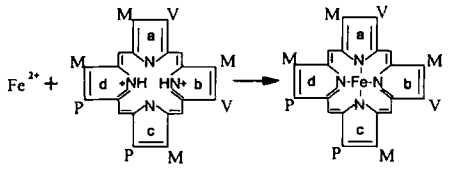

Fig. 1. Scheme of the Ferrochelatase-catalyzed reaction. M, methyl; P, propionyl; V, vinyl. a, b, c, and d indicate the four pyrrole rings.

EARLY INVESTIGATIONS ON FERROCHELATASE: IDENTIFICATION, PURIFICATION AND CHARACTERIZATION

Ferrochelatase activity was first identified in extracts of chicken erythrocytes in 1956 (Goldberg *et al.*, 1956). Subsequently, ferrochelatase was reported in a wide variety of organisms and tissues: rat liver (Nishida and Labbe, 1959; Yoneyama *et al.*, 1962), pig liver (Porra and Jones, 1963), spinach chloroplasts (Jones, 1963), yeast, and bacteria (Porra and Ross, 1965; Porra and Lascelles, 1965). The biological importance of ferrochelatase was established in 1974, when Dailey and Lascelles reported that a bacterial heme auxotroph, lacking ferrochelatase activity, had an absolute requirement for protoheme. However, it was not until 1981 that the enzyme was first purified (Taketani and Tokunaga, 1981). The low amount of protein in the starting material, the difficulty in its solubilization, its instability, and the propensity of the enzyme to form aggregates have been the major factors that hindered the development of a purification method for ferrochelatase. The different steps taken for successful solubilization and purification have been reviewed recently (Dailey, 1990; Taketani, 1993; Labbe-Bois and Camadro, 1994). The general purification scheme involves solubilization with a nonionic detergent and chromatography on reactive blue-dye-Sepharose (i.e., Cibracon blue F3GA crosslinked to Sepharose). Using small variations of this general method, ferrochelatase has been purified from diverse sources: *Rhodobacter spheroides* (Dailey, 1982), *Saccharomyces cerevisiae* (Camadro and Labbe, 1988), bovine liver (Dailey and Flemming, 1983), chicken erythrocytes (Hanson and Dailey, 1984), rat liver (Taketani and Tokunaga, 1981), mouse liver (Dailey *et al.*, 1986), baboon liver (Posnett *et al.*, 1988), and human liver (Mathews-Roth *et al.*, 1987). More recently, Hansson and Hederstedt (1994), due to the different hydropathic nature of the *Bacillus subtilis* ferrochelatase (see below), purified this bacterial protein following conventional techniques used in the purification of soluble proteins (e.g., ammonium sulfate precipitation of the bacterial soluble fraction and ion-exchange chromatography).

Except for the *B. subtilis* enzyme (Hansson and Hederstedt, 1994), ferrochelatase has been reported to be a membrane-associated protein (with the cytoplasmic membrane in prokaryotes and with the inner mitochondrial membrane in eukaryotes). All of the purified eukaryotic ferrochelatases and two bacterial ferrochelatases (*B. subtilis* and *Escherichia coli*) have similar molecular masses (35–42 kDa) (Taketani and Tokunaga, 1981; Dailey *et al.*, 1986; Camadro and Labbe, 1988; Hansson and Hederstedt, 1994; Miyamoto *et al.*, 1994), whereas the *R. spheroides* enzyme has been reported to have a very different molecular mass (115 kDa) (Dailey, 1982). Subsequent sequencing of the bacterial ferrochelatase genes indicate that the deduced protein molecular mass is in the range of 35–40 kDa (see section "Structural Studies: Cloning, Sequencing, Primary and Secondary Structure"). The determination of the ferrochelatase molecular mass by gel filtration chromatography is dependent on the concentration of sodium cholate in the column elution buffer. The elution of ferrochelatase (i.e., from yeast, rat, and bovine livers) with low concentrations of sodium cholate (i.e., 0.2%) yields a protein with a molecular mass of greater than 200 kDa, whereas the elution with buffers containing high concentrations of sodium cholate (i.e., $\geq$ 0.5%) yields a protein with a molecular mass of approximately 40 kDa. This suggests that ferrochelatase is active as a monomer and that its aggregation is prevented with high sodium cholate concentration (Dailey and Fleming, 1983; Bloomer *et al.*, 1987; Labbe-Bois and Camadro, 1994). Nevertheless, the oligomeric state of functional ferrochelatase remains to be unequivocally established. In most instances ferrochelatase has been reported to be a monomeric enzyme (Dailey *et al.*, 1986; Camadro and Labbe, 1988; Hansson and Hederstedt, 1994). However, using radiation inactivation, Straka *et al.* (1991) proposed that bovine liver ferrochelatase is functional as a dimer in the membrane environment.

Biochemical examinations of ferrochelatase have mainly focused on kinetic studies from different organisms and on the determination of activity with different substrates and inhibitors, using both native and chemically modified enzyme. While Fe^{2+}, Co^{2+}, and Zn^{2+} are used as substrates (Jones and Jones, 1969; Camadro and Labbe, 1982; Camadro, *et al.*,

1984), Fe^{3+} is not (Porra and Jones, 1963). Further, other divalent metals, i.e., Mn^{2+}, Cd^{2+}, and Hg^{2+}, are inhibitors (Taketani and Tokunaga, 1981; Dailey, 1987). Although protoporphyrin IX is the phsyiological porphyrin substrate, a diverse range of IX isomer porphyrins, with substituents at the 2,4 positions of A and B rings, can function as substrates as well (Yoneyama *et al.*, 1962; Jones and Jones, 1969; Taketani and Tokunaga, 1981; Dailey *et al.*, 1989). However, only those porphyrins with uncharged 2,4 substituents, equal in size or smaller than hydroxyethyl, can be used as substrates (Dailey *et al.*, 1989). In addition, the presence of the propionate side-chains, at positions 6 and 7 on the C and D rings respectively, seems to be an essential requirement for the porphyrin to be used as a substrate (Dailey *et al.*, 1989). Protoporphyrin I and mesoporphyrin I, which have the propionate groups at positions 6 and 8, are not substrates (Honeybourne *et al.*, 1979). These group specificities for the 2, 4, 6, and 7 positions, led to the proposal that they are required for proper orientation of the porphyrin and the alignment of the central nitrogens at the enzyme's iron-binding site (Dailey *et al.*, 1989).

Results of kinetic studies on bovine ferrochelatase appear to be consistent with an ordered bi-bi sequential mechanism, in which iron-binding occurs prior to porphyrin binding; after insertion of the metal, heme is released before the release of two protons (Dailey and Fleming, 1983; Dailey *et al.*, 1989). In contrast, Rossi *et al.* (1990) and Labbe-Bois and Camadro (1994) proposed a random bi-bi mechanism, in which each substrate binds randomly to the enzyme and the binding of the first substrate does not affect the binding of the second one. More recently, Abbas and Labbe-Bois (1993) kinetically characterized yeast ferrochelatase variants in which defined amino acids of an evolutionary conserved region were mutated. When compared to the wild-type yeast ferrochelatase, the site-directed mutants exhibited higher K_m values for both substrates (porphyrin and metal), which led the authors to conclude that the two substrates' binding events are not independent from each other. The investigators suggested that these results could be interpreted either as supporting an ordered sequential model (Dailey and Fleming, 1983) or indicating that the introduction of the mutations was in a region involved in the binding of both substrates and therefore affected the structure of the binding sites. Whether the ferrochelatase kinetic mechanism follows an ordered sequential or a random bi-bi model remains an open question.

To probe the substrate-binding sites, investigators have used chemically modified ferrochelatase. By using sulfhydryl group-specific reagents which inhibit ferrochelatase activity, Dailey (1984) proposed that sulfhydryl groups are essential for enzyme activity. One of the substrates, ferrous iron, was found to have a protective effect against thiol-reagent inactivation, whereas the other substrate, porphyrin, had no protective effect. From the kinetic analysis of the inactivation of bovine ferrochelatase with sulfhydryl-specific reagents, it was proposed that one, or perhaps two, ferrochelatase cysteine residues are involved in the binding of the substrate ferrous ion (Dailey, 1984, 1990). However, more recently, a comparison of the ferrochelatase sequences of *E. coli, B. subtilis, Bradyrhizobium japonicum, S. cerevisiae, Arabidopsis thaliana*, mouse, and man indicates no conserved cysteine residues (Fig. 2). This leaves only two possibilities: either cysteine residues are not present in the substrate iron-binding site or ferrochelatase does not have a conserved iron-binding coordination environment. Given the similar physical and catalytic properties for ferrochelatases from different organisms, the latter explanation is unlikely. Detailed studies of the spectroscopic and kinetic properties of the wild-type and site-directed mutant variants of ferrochelatase should help to define the ligand environment for the substrate iron-binding site. Arginyl, but not lysyl, residues have been implicated in binding of the porphyrin substrate via charge-pair interactions with the porphyrin propionate side chains. Arginyl residues were also proposed to have a role in the orientation of the porphyrin substrate in the active site of ferrochelatase (Dailey and Fleming, 1986).

Based on the chemical modification and inhibition studies cited above, a model for the ferrochelatase mechanism was proposed, in which ferrous ion binds to the enzyme through interactions with sulfhydryl groups of two nearby cysteines, releasing the sulfhydryl protons (Dailey *et al.*, 1989). This is followed by the binding of the porphyrin through hydrophobic interactions. Proper "alignment" of the porphyrin is ensured through charge-pair interactions with protein arginine residues. Once heme is released from the active site of ferrochelatase, the sulfhydryl groups of the cysteine ligands are reprotonated, leaving the enzyme ready for another round of catalysis (Dailey *et al.*, 1989). However, in view of the recent developments,

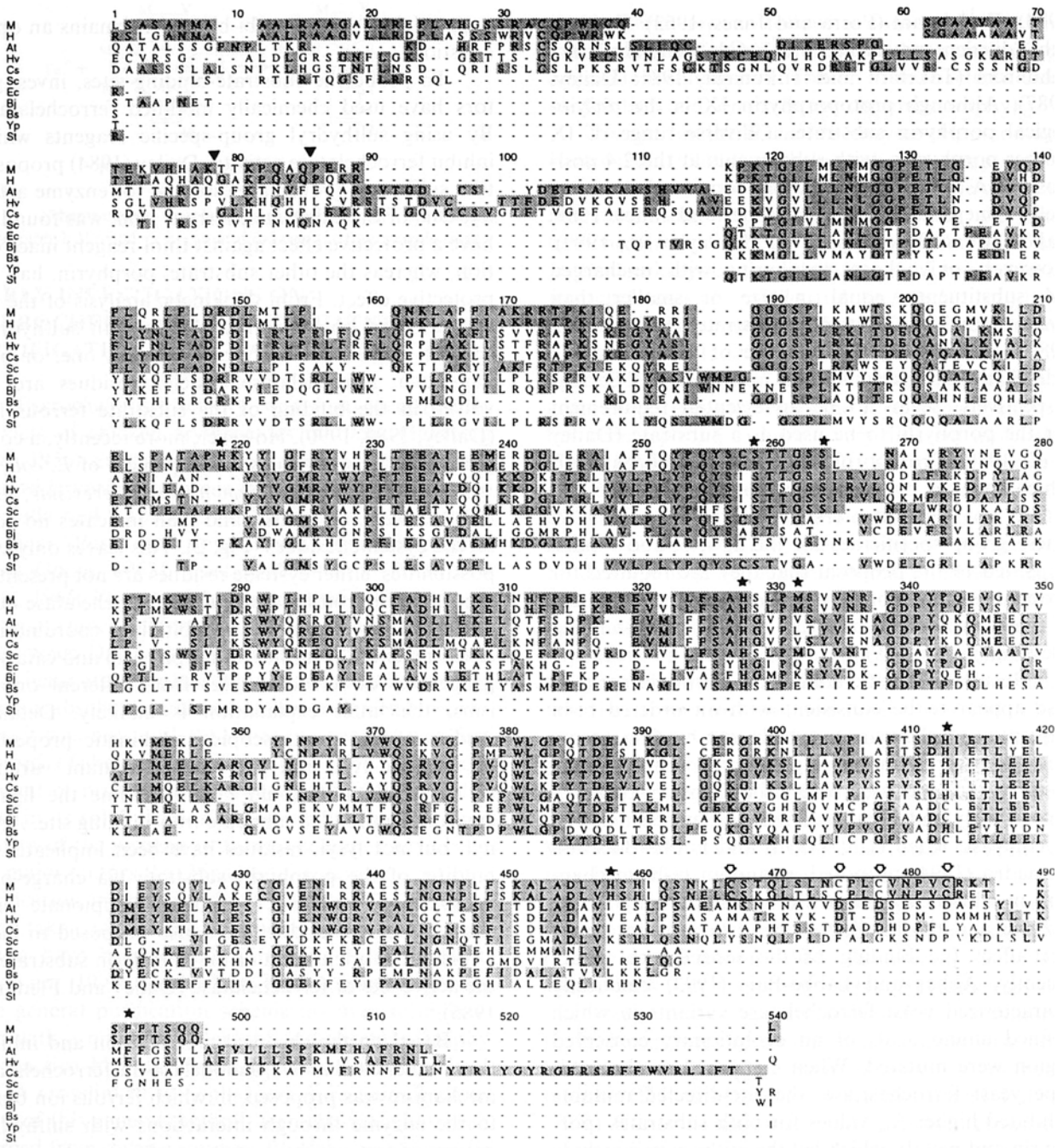

Fig. 2. Alignment of Ferrochelatase amino acid sequences. Sequences correspond to mouse (M) (Taketani *et al.*, 1990; Swiss-Prot. Accession No. P22315), human (H) (Nakahashi *et al.*, 1990; Swiss-Prot. Accession No. P22830), *A. thaliana* (At) (Smith *et al.*, 1993; Accession No. X73417), *Hordeum vulgare* (Hv) (Miyamoto *et al.*, 1994a; Accession No. D26105), *Cucumis sativus* (Cs) (Miyamoto *et al.*, 1994a; Accession No. D26106), *S. cerevisiae* (Sc) (Labbe-Bois, 1990; Swiss-Prot. Accession No. P16622), *E. coli* (Ec) (Miyamoto *et al.*, 1991; Swiss-Prot. Accession No. P23871), *B. japonicum* (Bj) (Frustaci and O'Brian, 1992; Swiss-Prot. Accession No. P28602), *B. subtilis* (Bs) (Hansson and Hederstedt, 1992; Swiss-Prot. Accession No. P32396), *Yersinia pseudotuberculosis* (Yp) (Kessler *et al.*, 1993; Accession No. Q05338) and *Salmonella typhimurium* (St) (Swiss-Prot. Accession No. P37408) ferrochelatases. The [2Fe-2S] cluster-binding motif (i.e., C-X_7-C-X_2C-X_4-C) is boxed and the cysteine residues are identified with arrows (Ferreira *et al.*, 1994; Dailey *et al.*, 1994). Residues conserved in all sequences are shaded. Site-directed mutants referred to in the section "Functional Studies:Expression and Site-Directed Mutagenesis" are indicated with closed stars. Closed arrowheads indicate the proteolytic site between the sequences encoding the presequence and the mature form of yeast (Q-N) and human ferrochelatase (Q-G).

namely the absence of conserved cysteines among ferrochelatase from different species, this working model does not seem to hold. Certainly, with the determination of the ferrochelatase residues involved in the binding of the substrates, more plausible working models will become available.

It has been assumed that the porphyrin ring is distorted during the ferrochelatase-catalzyed reaction. N-alkylporphyrins exhibit large distortion of the porphyrin macrocycle, by having the N-alkylated pyrrole ring tilted from the planar conformation of the macrocyclic ring. This bending facilitates the exposure of the "nitrogen lone pair electrons to an incoming metal ion" (Lavallee, 1988). N-alkylporphyrins have, therefore, been thought of as transition analogs of the ferrochelatase-catalyzed reaction (Lavallee, 1988). This assumption was recently tested. Cochran and Schultz (1990) reasoned that if N-methylporphyrins were transition state analogs for porphyrin metallation, antibodies elicited to alkylporphyrins should catalyze metal ion complexation by distortion of the substrate. In fact, these investigators showed than an antibody elicited to N-methylporphyrin catalyzed metal chelation by the planar porphyrin (Cochran and Schultz, 1990).

BIOGENESIS, IMPORT, AND LOCALIZATION IN MITOCHONDRIA

Eukaryotic ferrochelatase, like most of the mitochondrial proteins, is synthesized in the cytosol and imported into the mitochondria. Murine and yeast ferrochelatases are synthesized as larger precursor forms (43 and 44 kDa, respectively), and subsequently processed to the mature protein forms during translocation to the mitochondria (Karr and Dailey, 1988; Camadro and Labbe, 1988). Karr and Dailey (1988) showed that the *in vitro* translation of mouse RNA yielded a 43-kDa ferrochelatase precursor, which in the presence of mitochondria was imported and processed to the mature ferrochelatase form. The import reaction required a membrane potential (Karr and Dailey, 1988). Camadro and Labbe (1988) used antibodies raised against purified yeast ferrochelatase to immunoprecipitate the 44-kDa precursor form of the enzyme, which was generated by *in vitro* translation of total yeast mRNA, and to demonstrate that the precursor is rapidly processed *in vivo* to the 40-kDa mature form of the enzyme. Whether the ferrochelatase presequence is essential to the import process, and whether the mitochondrial targetting information resides solely in the presequence or, in contrast, in the mature region of the protein as well as the presequence, are questions that have not yet been addressed. In addition, the significance of the ferrochelatase mitochondrial import in relation to its final localization will shed light on the biological role of ferrochelatase in maintaining synthesis of heme according to the cell requirements. The early work of Jones' group suggested that ferrochelatase is located on the matrix side of the mitochondrial membrane (Jones and Jones, 1969; Barnes *et al.*, 1972). In 1985, Harbin and Dailey examined the orientation of ferrochelatase in the inner mitochondrial membrane, using a membrane-impermeable sulfhydryl-specific reagent and an antibody which inhibited the enzyme activity. From these studies, the authors proposed that ferrochelatase spans the inner mitochondrial membrane and is oriented with its active site facing the mitochondrial matrix. This membrane-associated nature of ferrochelatase corroborates the findings that the enzyme activity is greatly increased in the presence of lipids (Labbe *et al.*, 1968; Sawada *et al.*, 1969; Simpsom and Poulson, 1977; Taketani and Tokunaga, 1981).

However, present evidence support a peripheral, rather than a transmembrane, arrangement for ferrochelatase (see "Structural Studies: Cloning, Sequencing, Primary and Secondary Structure"). To explain the ferrochelatase requirement for ferrous iron in relation to its location in the mitochondria, Taketani *et al.*, (1986) proposed that ferrochelatase is associated with complex I of the mitochondrial electron transport chain. These investigators suggested that ferrous ion was produced upon the NADH oxidation in complex I (Taketani *et al.*, 1986). In yeast, iron reduction seems to take place on the outer surface of the cytoplasmic membrane, where iron is reduced by a ferrireductase (Lesuisse *et al.*, 1987). The reduced iron is then translocated into the cell by a transporter, which is common to other divalent cations (Lesuisse *et al.*, 1987; Lesuisse and Labbe, 1989; Labbe-Bois and Labbe, 1990), and finally used as ferrochelatase substrate. Despite these advances, ferrochelatase mitochondrial membrane localization and its relevance in maintaining heme biosythesis remain intriguing topics for future research.

STRUCTURAL STUDIES: CLONING, SEQUENCING, PRIMARY AND SECONDARY STRUCTURE

In the late 1980's it became clear that the understanding of the mechanism of the ferrochelatase-catalyzed reaction at the molecular level would require elucidation of the structure of the protein. With this in mind several investigators initiated research programs to isolate ferrochelatase cDNA or genes of different species, so that expression systems could be developed and larger amounts of ferrochelatase protein could be made available. In addition, changes of ferrochelatase amino acids could be possible, by mutagenesis at the DNA level, and proposed molecular mechanisms could then be tested in relation to the structure of the enzyme. Using functional complementation of a *hem 15 S. cerevisiae* mutant (Urban-Grimal and Labbe-Bois, 1981), Labbe-Bois (1990) isolated the *S. cerevisiae* ferrochelatase gene, *HEM15*. The protein predicted from the nucleotide sequence indicated that the mature ferrochelatase is preceded by a peptide of 31 amino acids. The predicted mature protein has 362 amino acids with an inferred molecular mass of 40.9 kDa (Labbe-Bois, 1990; Labbe-Bois and Camadro, 1994). Also in 1990, Taketani *et al.* isolated two full-length mouse ferrochelatase cDNA clones from a λgt11 library constructed from MEL cells mRNA. Similar to the yeast protein, an import sequence of 53 amino acids precedes the mature 367 amino acid-long mouse ferrochelatase. Subsequently, ferrochelatase genes and cDNAs have been isolated and sequenced from *E. coli* (Miyamoto *et al.*, 1991), *B. japonicum* (Frustaci and O'Brian, 1992), *B. subtilis* (Hansson and Hederstedt, 1992), *A. thaliana* (Smith *et al.*, 1994), barley (Miyamoto *et al.*, 1994a), cucumber (Miyamoto *et al.*, 1994a), mouse (Taketani *et al.*, 1990; Brenner and Frasier, 1991), and human (Nakahashi *et al.*, 1990). (An evaluation of the ferrochelatase gene structure is reviewed elsewhere in this volume by Taketani and Fujita). Comparison of the amino acid sequences of the different ferrochelatases revealed extensive similarity, particularly in the carboxyl-terminus half (Fig. 2). However, eukaryotic ferrochelatases have an extra 30 to 50 amino acid stretch at the carboxyl-terminus (see section "Presence of an Iron-Sulfur Cluster in Mammalian Ferrochelatase"). The overall similarity between ferrochelatase sequences from mouse and human is 88%, whereas the human and yeast sequences are 46% similar (Labbe-Bois, 1990; Taketani *et al.*, 1990; Nakahashi *et al.*, 1990; Labbe-Bois and Camadro 1994). In contrast, amino acid sequence comparisons of ferrochelatase with heme-binding proteins does not reveal significant sequence homology, making the assignment of putative amino acid residues, involved either in the binding of porphyrin or ferrous ion substrates, difficult.

The hydropathy profiles of the eukaryotic and prokaryotic ferrochelatases predict no transmembrane segments. This observation,along with the fact that ferrochelatase can be extracted with 0.1 M Na_2CO_3 at pH 11–12 (Volland and Urban-Grimal, 1988; Labbe-Bois, 1990), suggests that ferrochelatase, while a membrane-bound protein is not an integral membrane protein. The effect of lipids in increasing ferrochelatase activity (see section, "Biogenesis, Import, and Localization in Mitochondria") might be interpreted as a way of facilitating the substrates' access to the active site or of promoting a change of the enzyme's structure to a more favorable conformation for the catalytic event to occur (Labbe-Bois, 1990). The requirement for detergents for solubilization of ferrochelatase in the developed purification methods (see above) might be interpreted as due to additional, non-ionic, interactions between the ferrochelatase protein and the membrane or to interactions with other membrane proteins (Taketani *et al.*, 1986; Ferreira *et al.*, 1988; Labbe-Bois, 1990).

FUNCTIONAL STUDIES: EXPRESSION AND SITE-DIRECTED MUTAGENESIS

The expression of a full-length yeast ferrochelatase cDNA in baculovirus-infected insect cells yielded a functional membrane-associated enzyme; however, the expressed recombinant yeast precursor protein was not processed to its final mature form (Eldridge and Dailey, 1992). An *E. coli visA* mutant, which requires exogenous heme for growth (Nakahigashi *et al.*, 1992), acquired heme prototrophy upon the transformation with either the ferrochelatase gene from *S. cerevisiae* or a full-length mouse ferrochelatase cDNA (Frustaci and O/Brian, 1993). These investigators also demonstrated that the deleted *visA* gene in the *E. coli* mutant corresponded to a deletion of the ferrochelatase gene, by placing the *visA* coding region under the transcriptional control of T7 RNA polymerase in an *E. coli* overexpression system. The overexpressed protein had a molecular mass of 38 kDa and exhibited ferrochelatase activity; however, most of the

overexpressed protein aggregated in inclusion bodies (Frustaci and O'Brian, 1993). Both human and mouse ferrochelatases have also been produced in *E. coli*; cDNAs encoding the mature form of these proteins were placed under the transcriptional control of a *lac* promoter in an *E. coli* expression system. The recombinant proteins were functional, although they remained associated with the bacterial membrane fraction (Brenner *et al.*, 1992; Dailey *et al.*, 1994a). Ferreira (1994) cloned the mouse mature ferrochelatase-encoding region under the control of the alkaline phosphatase promoter and grew *E. coli* cells harboring this construct under phosphate starvation conditions. The recombinant ferrochelatase, in contrast to the previously cited expression systems, remained associated with the soluble bacterial fraction, facilitating the purification procedure and increasing considerably its yield. The physical and kinetic properties of the recombinant mouse ferrochelatase were identical to those of the ferrochelatase isolated from mouse livers (Ferreira, 1994). Subsequently, *B. subtilis*, human, and *E. coli* ferrochelatases have been overproduced in *E. coli* as "water-soluble" proteins, by having the DNA fragments encoding these proteins placed under the transcriptional control of *lac*, T7 RNA polymerase, and bacteriophage λ (P_R and P_L) promoters, respectively (Hansson and Hederstedt, 1994; Okuda *et al.*, 1994; Miyamoto *et al.*, 1994b). Except for the *B. subtilis* ferrochelatase, all of the other recombinant ferrochelatase proteins have been purified using minor modifications of the purification method developed for ferrochelatase isolated from natural sources (i.e., involving the use of blue dye-Sepharose, as described in the section "Early Investigations on Ferrochelatase: Identification, Purification, and Characterization").

Expression of the recombinant F417S ferrochelatase mutant, in which phenylalanine-417 is substituted with serine, as has been found in some cases of human protoporphyria, yielded an enzyme with decreased ferrochelatase activity (Brenner *et al.*, 1992). Subsequently, the effects of the F417S (Brenner *et al.*, 1992) and M267I (Lamoril *et al.*, 1991) mutations, which correspond to two distinct mutations found in patients afflicted with protoporphyria, were evaluated by expressing and characterizing these ferrochelatase variants. The F417S mutant exhibited less than 2% of the wild-type ferrochelatase activity; further, when phenylalanine-417 was replaced with leucine, tyrosine, or tryptophan, the ferrochelatase variants (F417L, F417Y and F417W) had 5% or less of the wild-type ferrochelatase activity. The M267I mutant, although having K_m and V_{max} values similar to those of the wild-type ferrochelatase, displayed increased thermolability in relation to the wild-type enzyme (Dailey *et al.*, 1994). Abbas and Labbe-Bois (1993) examined the effects of two mutations (S169F and S174P) on the yeast ferrochelatase function. Substitution of serine-169 with phenylalanine induced a 10-fold increase in V_{max} and a 35- and 45-fold increase in the K_m values of the metal and porphyrin substrates, respectively. Given the close proximity of the two amino acids to each other (S169 and S174) and their similar effects on ferrochelatase kinetic properties, albeit to a different extent, the authors concluded that the region covering the two amino acids contributes to the binding domains of metal and porphyrin substrates (Abbas and Labbe-Bois, 1993). More recently, Kohno *et al.* (1994) investigated the role of ferrochelatase histidine residues as putative ferrous ion substrate ligands. Human ferrochelatase H263, which is conserved among all ferrochelatase sequences, and H157, H341, and H388, which are conserved among mammalian ferrochelatase (Fig. 2), were mutated to alanine. While H341A exhibited similar enzyme activity to that of wild-type ferrochelatase, H157A and H388A had lower enzyme activities than that of the wild-type enzyme (albeit with similar K_m values for both substrates). The mutation at H263 affected both the V_{max} and the K_m values for ferrous and zinc ions; the K_m value for the porphyrin substrate remained the same. Kohno *et al.* (1994) concluded that "the binding site for metal ions in ferrochelatase is distinct from that for the porphyrin" and suggested that H263 "contributes significantly to the binding of metal ions."

PRESENCE OF AN IRON-SULFUR CLUSTER IN MAMMALIAN FERROCHELATASE

Ferreira *et al.* (1994), while concentrating the recombinant mouse ferrochelatase, noticed that the protein had a reddish brown color, a characteristic feature generally observed for iron-containing metalloproteins. The investigators examined the purified enzyme using EPR and Mössbauer spectroscopy, and metal analysis. No EPR resonances were observed in the aerobically isolated recombinant ferrochelatase, which is expected for proteins containing a diamagnetic $[2Fe\text{-}2S]^{2+}$ cluster (Sand and

Dunham, 1975; Orme-Johnson and Orme-Johnson, 1982). Upon reduction by dithionite, however, an axial-type EPR signal was detected at the $g \approx 1.94$ region. Further, the spectrum could be simulated with g values of 2.00, 1.93, and 1.90, which are very similar to those reported for other [2Fe-2S]-containing proteins ($g = 2.06–2.01$ and 1.94–1.84) (Münck *et al.*, 1972; Sand and Dunham, 1975; Orme-Johnson and Orme-Johnson, 1982). Significantly, EPR measurements performed on ^{57}Fe-enriched ferrochelatase demonstrated that the signal arises from an Fe-containing species.

To prove that an iron-sulfur cluster is present in the natural murine ferrochelatase, and not only in the recombinant ferrochelatase, the enzyme was also purified from mouse livers, and the EPR spectra of the natural and recombinant ferrochelatases were compared (Ferreira *et al.*, 1994). The EPR spectra were found to be identical, thereby establishing that the natural murine ferrochelatase also contains a [2Fe-2S] cluster. Finally, to provide definitive evidence for the presence of a [2Fe-2S] cluster in ferrochelatase, these investigators performed Mössbauer spectroscopy, since it detects each and every iron nucleus forming the paramagnetic system. The Mössbauer measurements on ^{57}Fe-enriched recombinant ferrochelatase and the obtained Mössbauer parameters were consistent with the presence of a [2Fe-2S] cluster and indicated a typical tetrahedral, sulfur-coordination of the high-spin ferric ions (Ferreira *et al.*, 1994).

Comparison of the amino acid ferrochelatase sequences indicates that the human and mouse enzymes contain a putative Fe-S binding site at the C-terminus (Fig. 2). The putative binding site is a 30-residue region that contains four cysteine residues arranged in a sequence ($C\text{-}X_7\text{-}C\text{-}X_2\text{-}C\text{-}X_4\text{-}C$), which is a fingerprint for a [2Fe-2S] binding motif (Ta and Vickery, 1992). This sequence is absent in nonmammalian ferrochelatases (Fig. 2). Dailey *et al.* (1994b) have also identified a [2Fe-2S] cluster in recombinant human ferrochelatase. In addition, these investigators showed that a recombinant C-terminal truncated human ferrochelatase, and the yeast and *E. coli* ferrochelatases, which are devoid of the [2Fe-2S] cluster binding motif (Fig. 2), had no iron-sulfur center. The role of the [2Fe-2S] center in mammalian ferrochelatase remains to be established, although Dailey *et al.* (1994b) proposed that the iron-sulfur cluster is essential for enzyme activity. Possible other functions include a redox role, a structural role, a regulatory role, or a combination of some of these possibilities.

FUTURE DIRECTIONS

During the past few years significant advancements have now positioned investigators to address questions concerning the structure and the catalytic mechanism of ferrochelatase at the molecular level. Genomic and cDNA clones for ferrochelatase from a wide range of sources have been isolated, sequenced, and functionally expressed. In addition, the recombinant ferrochelatase proteins have been successfully purified (i.e., with greater recoveries than previous purification methods for ferrochelatase from natural sources). A [2Fe-2S] cluster has been identified in mammalian ferrochelatase, although its biological role remains to be established. The impact of point mutations, found in some cases of erythropoietic protoporphyria, have been evaluated, by physically and kinetically characterizing recombinant ferrochelatase variants carrying the protoporphyric mutations. While this field has advanced relatively rapidly over the past few years, it appears that structural answers are now necessary for the interpretation of the ferrochelatase catalytic mechanism. The ferrochelatase amino acid residues involved in binding of the substrates and the amino acid residues which dictate the specificity of the enzyme remain to be established. Certainly, we are left with real challenges for the future, among them to obtain crystals of sufficient quality to permit three-dimensional structures to be obtained at high resolution, which should help to interpret the catalytic mechanism of the enzyme in relation to its molecular architecture.

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