Human Ferrochelatase Is an Iron–Sulfur Protein†

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ABSTRACT: Recombinant human ferrochelatase has been expressed in *Escherichia coli* and purified to homogeneity. Metal analyses revealed ≈2 mol of non-heme Fe per mol of the purified enzyme (M_r = 40 000). The UV–visible absorption spectrum of the purified enzyme consists of a protein absorption at 278 nm ($\epsilon \approx 90\,000$ M^{-1} cm^{-1}) and bands at 330 nm ($\epsilon \approx 24\,000$ M^{-1} cm^{-1}), 460 nm (shoulder, $\epsilon \approx 11\,000$ M^{-1} cm^{-1}), and 550 nm (shoulder, $\epsilon \approx 9000$ M^{-1} cm^{-1}) that are indicative of a $[2Fe\text{-}2S]^{2+}$ cluster. The spectra show an additional band at 415 nm that varied in intensity for different preparations and is attributed, at least in part, to a minor component of enzyme-associated high-spin Fe(III) heme. The presence of a single $[2Fe\text{-}2S]^{2+,+}$ cluster as a redox active component of human ferrochelatase was confirmed by variable-temperature MCD and EPR studies of the dithionite-reduced enzyme which showed the presence of a $S = {}^1/_2$ $[2Fe\text{-}2S]^+$ cluster in addition to residual high spin Fe(II) heme. The reduced enzyme exhibits a $S = {}^1/_2$ EPR signal, g = 2.00, 1.94, 1.91 accounting for 0.75 ± 0.25 spins/molecule, that readily saturates at low microwave powers below 10 K but is observable without significant broadening at temperatures up to 100 K. The Fe-S cluster is labile and gradually disappears over period of 24 h, with concomitant loss of enzyme activity, when the enzyme is stored aerobically at 4 °C. Genetically engineered C-terminal truncated human ferrochelatase, as well as the normal yeast and *E. coli* ferrochelatases, lack the [2Fe-2S] cluster. These data suggest that human ferrochelatase contains a $[2Fe\text{-}2S]^{2+,+}$ cluster that is essential for activity and is coordinated by at least one and probably all of the four cysteines that are conserved in the C-terminal region of mammalian ferrochelatases.

The terminal step of the heme biosynthetic pathway is catalyzed by the enzyme ferrochelatase (EC 4.99.1.1) [see Dailey (1990)]. This enzyme catalyzes the insertion of ferrous iron into the porphyrin macrocycle, protoporphyrin IX. The enzyme utilizes the divalent transition metals Fe^{2+}, Co^{2+}, and Zn^{2+}, it will not utilize ferric iron, and it is strongly inhibited by Hg^{2+}, Mn^{2+}, and Cd^{2+} and weakly inhibited by Pb^{2+} (Dailey, 1987, 1990). Specificity for the porphyrin substrate is more species dependent, but in all known cases the IX isomers of proto-, hemato-, meso-, and deuteroporphyrin are effective substrates (Dailey et al., 1989, 1990). *N*-Methyl and *N*-ethyl IX isomer porphyrins are tight-binding, competitive inhibitors of ferrochelatase.

Eukaryotic ferrochelatase is nuclear encoded, cytoplasmically synthesized, and translocated into the mitochondrion in an energy-requiring step that involves proteolytic cleavage of a precursor form into the mature enzyme (Karr & Dailey, 1988; Camadro & Labbe, 1988). While there is evidence to suggest that the enzyme in mammals is inducible by some drugs and that there may be differentially polyadenylated mRNAs (Andrew et al., 1990; Chan et al., 1993), there is currently no evidence to suggest that the enzyme has any regulatory function.

With the recent cloning of ferrochelatase from yeast (Labbe-Bois, 1990), mouse (Brenner & Frasier, 1991; Taketani et al., 1990), human (Nakahashi et al., 1990), *Escherichia coli* (Miyamoto et al., 1991), *Bradyrhizobium japonicum* (Frustaci & O'Brian, 1992), and *Bacillus subtilis* (Hansson & Hederstedt, 1992), it has become possible to compare the enzyme from diverse sources. The eukaryotic ferrochelatases all contain an amino-terminal mitochondrial targeting signal sequence that is not found on the prokaryotic enzymes, but an unexpected finding is that the bacterial ferrochelatases are approximately 30 amino acids shorter at their carboxyl terminus when compared to the eukaryotic enzymes. Below we provide evidence that mammalian, but not yeast or bacterial, ferrochelatase contains an iron–sulfur cluster whose presence is necessary for activity. UV–visible absorption and variable-temperature MCD[1] studies coupled with EPR measurements of recombinant human ferrochelatase identify this center as a $[2Fe\text{-}2S]^{2+,+}$ cluster. It is proposed that this [2Fe-2S] center is coordinated by at least one and possibly all four cysteine residues present in the carboxyl-terminal region of the mammalian enzyme which is lacking in the prokaryotic enzymes.

MATERIALS AND METHODS

Recombinant human ferrochelatase and the carboxyl-terminal truncated version of the enzyme were produced and purified as described previously (Dailey et al., 1994). Expression vectors for production of yeast and *E. coli* ferrochelatase in *E. coli* were based upon the tac promoter vector used for human ferrochelatase. These enzymes are also purified in the same fashion as described for human ferrochelatase. For the experiments described below all purification procedures were carried out within1 day, and spectra were recorded immediately. Ferrochelatase activity was determined

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[1] Abbreviations: Fd, ferredoxin; MCD, magnetic circular dichroism.

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via the pyridine hemochromogen assay previously described (Dailey & Fleming, 1983) using ferrous iron and mesoporphyrin IX as substrates. Mesoporphyrin was obtained from Porphyrin Products, Logan, UT. Protein was quantitated using bicinchoninic acid (BCA) procedure from Pierce Chemical Co. Iron content was determined both by plasma emission (University of Georgia Analysis Laboratory) and in solution using the ferrous iron chelating chromophore Ferrozine (Carter, 1971; Dailey & Lascelles, 1977). All spectra shown were obtained with ferrochelatase in buffer containing 20 mM Tris MOPS, pH 8.1, 20% glycerol, 1.5 M KCl, and 1.0 (wt/vol) sodium cholate.

Instrumentation. UV–visible absorption spectra were recorded using either a Varian 219 or a Shimadzu UV-3101PC spectrophotometer. CD spectra were recorded using a Jasco J-500C spectropolarimeter which was mated to an Oxford Instruments SM-3 split-coil superconducting magnet for variable-temperature MCD measurements. The experimental protocols for measuring MCD spectra and magnetization curves over the temperature range 1.5–300 K with magnetic fields up to 5 T have been described elsewhere (Johnson, 1988). EPR spectra were recorded using a Bruker ESP-300E spectrometer fitted with an Oxford Instruments ESR-9 flow cryostat (4.2–300 K). Spin quantitations were carried out under nonsaturating conditions using a 1 mM Cu(EDTA) as the standard and the procedures developed by Aasa and Vänngård (1975). EPR simulation was carried out using the Simfonia software package supplied by Bruker Instruments.

RESULTS

Recombinant human, *Saccharomyces cerevisiae*, and *E. coli* ferrochelatase and a truncated form of the human enzyme lacking the four C-terminal cysteine residues were purified, assayed, and analyzed for non-heme Fe. The human, *S. cerevisiae*, and *E. coli* enzymes had comparable activity (approximately 150 nmole of mesoheme formed min^{-1} mg^{-1}), whereas the truncated form of the human enzyme had no measurable activity. Of these four samples, only the human enzyme contained significant amounts of non-heme Fe, 1.8 ± 0.3 Fe/molecule for the purest (A_{330}/A_{278} = 0.27) and most active preparations. The UV–visible absorption spectra of these four ferrochelatase samples as prepared are compared in Figure 1. The yeast and *E. coli* enzymes showed no visible absorption and the spectra comprise solely the 278-nm protein band. Both the native and C-terminal truncated human ferrochelatase samples exhibited a band of variable intensity centered at 415 nm that arises from trace amounts of high spin Fe(III) heme (<10% of the enzyme concentration based on the Soret band intensity). This was confirmed by EPR studies at 10 K which showed a weak axial resonance, $g_\perp$ = 6 and $g_\parallel$ = 2, characteristic of trace amounts of high spin Fe(III) heme and a small isotropic resonance at g = 4.3 from adventitious (rhombic) Fe(III) (data not shown). In addition to the heme absorption bands, normal human ferrochelatase has a visible absorption band centered at 330 nm ($\epsilon \approx 24\,000$ M^{-1} cm^{-1}), with pronounced shoulders at 460 nm ($\epsilon \approx 11\,000$ M^{-1} cm^{-1}) and 550 nm ($\epsilon \approx 9000$ M^{-1} cm^{-1}). The molar extinction coefficients are for freshly prepared samples with A_{330}/A_{278} = 0.27 and are based on protein determinations and M_r = 40 000. These bands were not present in the C-terminal truncated form of the enzyme and are gradually lost over a period of 24 h with concomitant loss of activity on storing samples of human ferrochelatase aerobically at 4 °C. Such absorption characteristics are indicative of a $[2Fe\text{-}2S]^{2+}$ cluster as evidenced by comparison with the published spectra of a wide range of oxidized [2Fe-2S]-containing Fds (Palmer, 1973; Stevens et al., 1978; Ta & Vickery, 1992; Cárdenas et al., 1976) and Rieske-type proteins (Fee et al., 1984). Biological $[2Fe\text{-}2S]^{2+}$ centers typically have visible absorption bands centered around 330 nm (ϵ in the range 11 000–16 000 M^{-1} cm^{-1}), 420 nm (ϵ in the range 8000–11 000 M^{-1} cm^{-1}, not observed as a well-resolved band for Rieske-type proteins in particular), and 460 nm (ϵ in the range 6000–10 000 M^{-1} cm^{-1}), with a broad shoulder centered around 550 nm (ϵ in the range 3000–6000 M^{-1} cm^{-1}). While the extinction coefficients for ferrochelatase are approximately 50% higher that those established for biological $[2Fe\text{-}2S]^{2+}$ clusters, the EPR and MCD intensities for the reduced enzyme (see below) are consistent with no more than one [2Fe-2S] cluster per molecule.

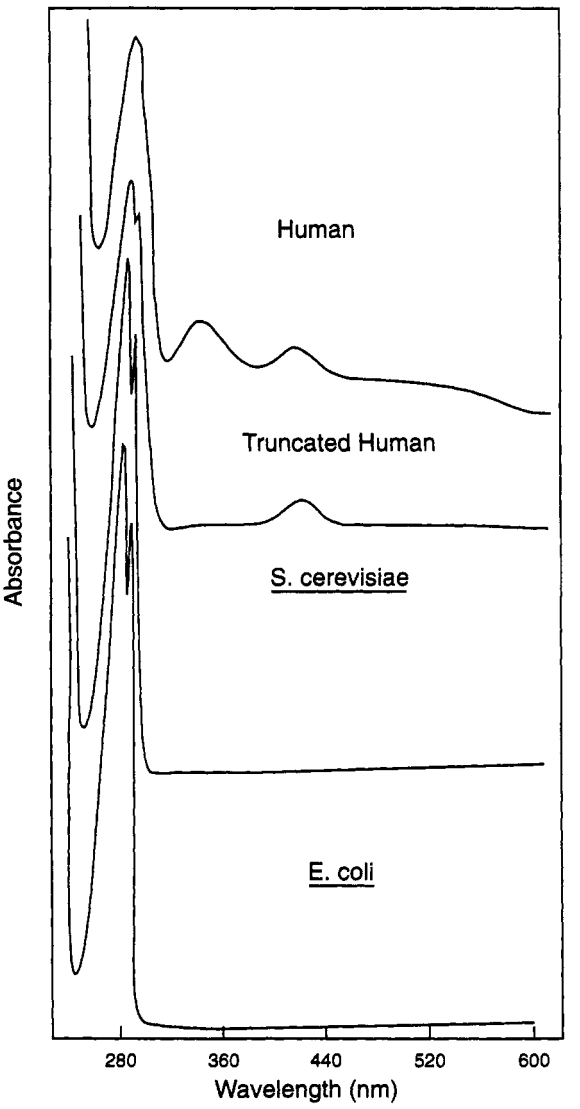

FIGURE 1: UV–visible spectra for recombinant ferrochelatases. The source of enzyme is shown on the figure. All spectra were recorded at room temperature.

Oxidized $[2Fe\text{-}2S]^{2+}$ clusters are invariably diamagnetic at low temperatures and consequently do not exhibit EPR or temperature-dependent MCD bands. However, biological [2Fe-2S] centers generally exhibit visible CD spectra that are an order of magnitude more intense than those of biological [4Fe-4S] clusters. Three distinct types of CD spectra have been reported for [2Fe-2S] proteins, and these are typified by plant Fds (Stephens et al., 1978; Ta & Vickery, 1992),

Clostridium pasteurianum 2Fe Fd (Cárdenas et al., 1976), and Rieske protein (Fee et al., 1984). The room temperature absorption and CD spectra of as prepared and dithionite-reduced human ferrochelatase in the visible region are shown in Figure 2. On the basis of the CD spectra of high spin Fe(II) and Fe(III) hemoproteins (Myer, 1978), the CD spectra of as prepared or dithionite-reduced ferrochelatase can be attributed exclusively to the [2Fe-2S] center with the possible exception of the region of the heme Soret absorption, 400–450 nm. The CD spectra are similar in intensity but quite distinct in form to those reported for oxidized or reduced [2Fe-2S] centers in Fds or Rieske-type proteins. However, CD spectra are much more sensitive to the environment of the chromophore than either the absorption or MCD spectra, and the distinctive CD compared to other biological [2Fe-2S] clusters most likely reflects differences in protein conformation in the vicinity of the cluster and/or differences in cluster ligation.

Dithionite-reduced samples of recombinant human ferrochelatase were investigated by EPR and variable temperature MCD spectroscopy which together provide a discriminating method for identifying cluster type in paramagnetic Fe-S proteins (Johnson et al., 1982). The EPR spectrum comprises a rhombic $S = {}^1/_2$ resonance which is well simulated with $g = 2.002$, 1.936, and 1.912, with line widths of 3.06, 4.06, and 1.96 mT, respectively (see Figure 3). Other than slight narrowing of the line widths, anaerobic addition of 50% (v/v) glycerol, necessary to form a low-temperature glass for variable temperature MCD studies, had no effect on the EPR signal. For freshly prepared samples that were reduced under anaerobic conditions, the resonance accounted for 0.75 ± 0.25 spins/molecule (average of three determinations on different preparations). The resonance undergoes power saturation with microwave powers >0.2 mW at 10 K and is observed without significant broadening up to 100 K. While the g values alone cannot discriminate between $S = {}^1/_2$ $[4Fe\text{-}4S]^+$ and $[2Fe\text{-}2S]^+$ clusters, the relaxation properties are only consistent with a $[2Fe\text{-}2S]^+$ center. Among the known [2Fe-2S] proteins, the EPR signal is most similar to those of the $[2Fe\text{-}2S]^+$ clusters in *C. pasteurianum* and *Azotobacter vinelandii* [2Fe-2S] ferredoxins, $g = 2.01$, 1.94, and 1.92 (for a comparison of EPR properties of biological $[2Fe\text{-}2S]^+$ centers, see Ackrell et al., 1992).

Dithionite-reduction results in bleaching of the visible absorption and the appearance of a broad band centered at 550 nm. This feature is a characteristic of $[2Fe\text{-}2S]^+$ clusters and has been attributed to a Fe(II)/Fe(III) intervalence band (Fu et al., 1992). The intense band centered at 420 nm and the shoulder at 314 nm arise from high spin Fe(II) heme and excess dithionite, respectively. Characterization of the temperature-dependent MCD bands of the $S = {}^1/_2$ Fe-S center is complicated by the presence of high spin ($S = 2$) Fe(II) heme which exhibits an extremely intense and characteristic low-temperature MCD under the Soret band (Cheesman et al., 1991) (see Figure 4). However, since large zero-field splitting leaves a singlet state lowest in energy, the MCD spectrum of high spin Fe(II) hemes are almost independent of temperature below 4.2 K. In contrast, chromophores with $S = {}^1/_2$ ground states undergo a 50% increase in intensity at 4.5 T on going from 4.2 to 1.6 K. Hence the 1.6 minus 4.2 K difference spectrum eliminates the heme MCD and provides an assessment of the form of the temperature-dependent MCD spectrum of the paramagnetic Fe-S cluster (see Figure 4). MCD magnetization data collected at 537 nm are well fit by theoretical magnetization data constructed using the EPR-determined g values and confirm a $S = {}^1/_2$ ground state for these MCD transitions (data not shown). Although the region between 400 and 450 nm in the difference spectrum is unreliable due to the intense heme MCD bands, the pattern of positive and negative MCD band in the temperature-difference spectrum is very similar to that observed for a range of reduced [2Fe-2S] ferredoxins (Fu et al., 1992) and is uniquely indicative of a $[2Fe\text{-}2S]^+$ cluster. In accord with the EPR spin quantitations, the intensity of MCD bands

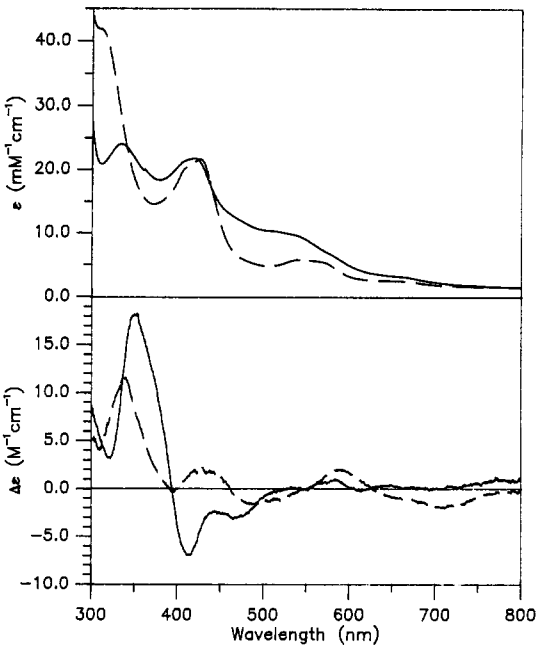

FIGURE 2: Room temperature absorption and CD spectra for recombinant human ferrochelatase. Solid line, as prepared; broken line, dithionite-reduced. The protein concentration was 45 μM, and the spectra were recorded in a 1-cm cuvette.

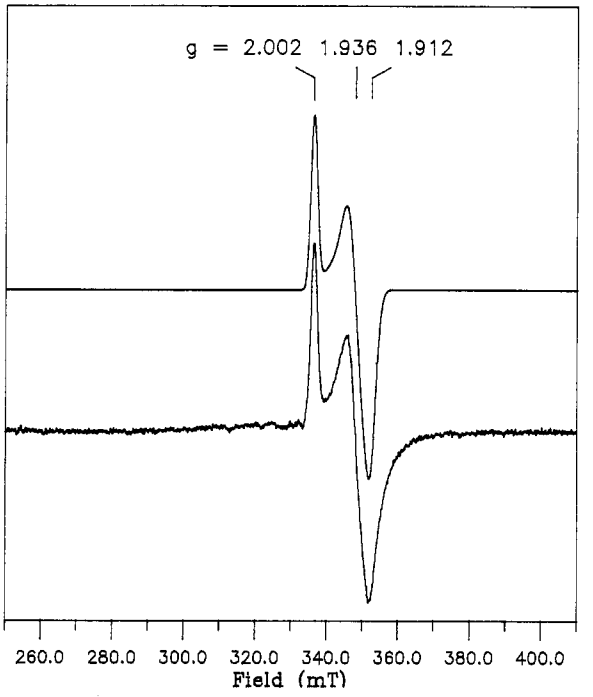

FIGURE 3: EPR spectrum of dithionite-reduced recombinant human ferrochelatase. (Lower spectrum) X-band EPR spectrum of dithionite-reduced ferrochelatase with 50% (v/v) glycerol, recorded at 35 K, with a microwave power of 1 mW, modulation amplitide of 0.63 mT, and microwave frequency of 9.432 GHz. The sample concentration was 60 μM. (Upper spectrum) Simulation with $g = 2.002$, 1.936, and 1.912 with line widths of 3.06, 4.06, and 1.96 mT, respectively.

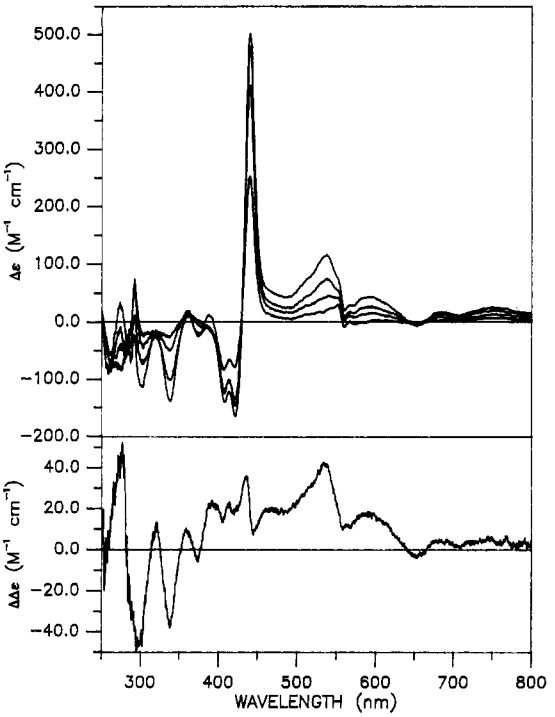

FIGURE 4: Variable-temperature MCD spectra of dithionite-reduced recombinant human ferrochelatase. (Upper panel) MCD spectra of dithionite-reduced ferrochelatase at 1.66, 4.22, 9.1, and 23 K with a magnetic field of 4.5 T. The sample was the same as that used for EPR studies (see Figure 3). All bands increase in intensity with decreasing temperature. (Lower panel) Difference spectrum, 1.66 minus 4.22 K.

Human	-	L	C	S	K	Q	L	T	L	S	C	P	L	C	V	N	-	-	P	V	C	R	E	T	K	S	F	P	T	S	Q	Q	L
Mouse	-	L	C	S	T	Q	L	S	L	N	C	P	L	C	V	N	-	-	P	V	C	R	K	T	K	S	F	F	T	S	Q	Q	L
Yeast	-	L	Y	S	N	Q	L	P	L	D	F	A	L	G	K	S	N	D	P	V	K	D	L	S	L	V	F	G	N	H	E	S	T

FIGURE 5: Derived carboxyl terminal sequence for human, mouse, and yeast ferrochelatases. Conserved residues are outlined in blocks and the four cys that are present in human and mouse are underlined. This 30/32 amino-terminal region is lacking in all currently known prokaryotic ferrochelatases.

attributed to $[2Fe\text{-}2S]^+$ cluster are consistent with between 0.5 and 1.0 clusters per molecule.

DISCUSSION

Ferrochelatase catalyzes a mechanistically interesting reaction, the chelation of iron into the porphyrin macrocycle. While the protein may then be viewed as a transient hemoprotein or a transient iron or porphyrin binding protein, a search of all available data bases reveals no significant homology with any heme, porphyrin, or iron-binding protein. The deduced amino acid sequence for ferrochelatase from human (Nakahashi et al., 1990), mouse (Brenner & Frasier, 1991; Taketani et al., 1990), yeast (Labbe-Bois, 1990), *E. coli* (Miyamoto et al., 1991), *B. subtilis* (Hansson & Hederstedt, 1992), and *B. japonicum* (Frustaci & O'Brian, 1992) have recently become available. One feature that becomes obvious when all of these sequences are compared is that the eukaryotic ferrochelatases contain an approximately 30 amino acid extension in the carboxyl terminus (Figure 5). [Interestingly this region does not represent a unique exon in mammals but makes up about 75% of human ferrochelatase exon 11 (Taketani et al., 1992.)] The importance of this region, however, was made apparent by the discovery that in some humans suffering from the genetic disease protoporphyria, where ferrochelatase activity is deficient, the defect in ferrochelatase was a F417S mutation, seven residues from the end, which results in the production of an inactive protein (Brenner et al., 1992; Dailey et al., 1994). As seen above with the carboxyl truncated human ferrochelatase, total elimination of this region to produce a protein of the same size as *E. coli* ferrochelatase results in a totally inactive protein.

The data presented above clearly demonstrate that human ferrochelatase contains a [2Fe-2S] center. The spectroscopic properties are consistent with coordination of this cluster via protein cysteine residues but do not identify the exact residues involved. However, a variety of lines of evidence suggest coordination by at least one and possibly all of the cysteine residues in the carboxyl-terminal region. The evidence in support of this is (1) the *E. coli* ferrochelatase lacks this carboxyl terminal region and has no evidence of an iron sulfur cluster, (2) an engineered mutant of the human enzyme that has been truncated on the carboxyl terminus has no iron sulfur cluster, (3) the carboxyl terminus of human and mouse ferrochelatase contains four conserved cysteines in a $C\text{-}X_7\text{-}C\text{-}X_2\text{-}C\text{-}X_4\text{-}C$ arrangement that resembles that found in plant-type Fds, $C\text{-}X_4\text{-}C\text{-}X_2\text{-}C\text{-}X_{29}\text{-}C$ except for the spacing to the more remote cysteine residue, and (4) yeast ferrochelatase, which has the carboxyl terminus but without the four Cys residues, does not have an iron–sulfur cluster. In view of the lability of the cluster, particularly in very dilute solutions, and the uncertainty in the cluster quantitation 0.5–1.0 cluster/monomer, we have not ruled out the possibility that the cluster may be bridging between identical subunits. The presence of such a homodimer could explain the observations found in the genetic disorder protoporphyria where in some patients a single defective allele for ferrochelatase results in a 75% drop in activity [see Nordmann and Deybach (1990)]. It would also be consistent with radiation inactivation studies that suggest functional ferrochelatase in situ has the target size of a homodimer (Straka et al., 1991). On-going studies with recombinant human ferrochelatase may provide an answer to this question.

An obvious question that remains unanswered is the function of the [2Fe-2S] cluster in mammalian ferrochelatase. Recent developments have served to demonstrate the functional diversity of biological Fe-S clusters [for recent reviews, see Cammack (1992) and Johnson (1994)]. While the majority are involved in electron transport, it is now evident that several redox [e.g., aconitase and other (de)hydratases] and nonredox enzymes (e.g., hydrogenases, nitrogenases, and CO dehydrogenases) utilize homometallic or heterometallic Fe-S clusters for substrate binding and activation. In addition, purely structural roles have been proposed in enzymes such as endonuclease III, and regulatory roles have been proposed in the iron-responsive element binding protein (Fe-sensing) in glutamine phosphoribosylpyrophosphate aminotransferase (O_2-sensing). The observation that Fe-S clusters are components of enzymes such as ribonucleotide reductase, lysine 2,3-aminomutase, and biotin synthase that function by radical mechanisms has raised the possibility that they may also be involved in generation and/or stabilization of radical intermediates.

Currently the only answers concerning the role of the Fe-S cluster in mammalian ferrochelatase are negative ones. Since yeast and bacterial ferrochelatases examined to date do not have iron–sulfur clusters, but have enzyme activity, it appears that this cluster is not directly involved in the catalytic mechanism. Likewise, there is no direct evidence that it could be involved in ferric iron reduction since our ferrochelatase

assays always have ferrous iron and removal of the cluster destroys enzyme activity. Also, the available evidence suggests that iron reduction *in situ* involves a system separate from ferrochelatase (Taketani et al., 1986) and the purified enzyme exhibits no ferric iron reductase activity. Of course, it may be that the cluster is involved in iron reduction but that the physiological reductant has been removed during the purification and so addition of compounds such as NADH or NADPH alone would have no effect.

Another question is why do the mammalian enzymes have the cluster but prokaryotes do not. One possibility is that in bacteria a separate iron–sulfur protein, such as a ferredoxin, may be present *in situ* with ferrochelatase and may serve a function similar to that served in the mammalian enzymes. If such a situation is true, then the mammalian enzymes may have arisen from a gene fusion event during evolution. Such a model, however, does not explain the presence of a carboxyl-terminal extension in the yeast which does not contain an iron–sulfur cluster.

The fact that mammalian ferrochelatase activity is dependent upon the presence of the iron–sulfur cluster does suggest that it must play either an important structural or perhaps regulatory role. While a regulatory role may initially seem attractive, the fact that iron regulates the first step of the pathway at ALA synthase via the iron-responsive element in erythroid cells (Dandekar et al., 1991; Cox et al., 1991) would mean dual regulation at both the first and last steps in these cell types although no iron regulation has been identified in non-erythroid cells (Ponka et al., 1990; May et al., 1990). This seems superfluous since inadequate iron supply would naturally limit ferrochelatase activity. However, by analogy to an earlier step in the pathway, porphobilinogen deaminase, where the apoenzyme is stabilized by the presence of its substrate via covalent linkage of the first two porphobilinogen molecules to the enzyme [see Jordan (1990)], apoferrochelatase may require the presence of iron, vis-à-vis the [2Fe-2S] cluster, to stabilize any newly synthesized enzyme. In such a situation, the role of the cluster would only be regulatory in the sense that it is required for proper assembly and stabilization of the active enzyme. Obviously more studies at both the cellular and molecular levels will be required to clarify the role of the [2Fe-2S] cluster in mammalian ferrochelatase.

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