Probing the Active-site Residues in Saccharomyces cerevisiae Ferrochelatase by Directed Mutagenesis

IN VIVO AND IN VITRO ANALYSES*

Monica Goras†, Ewa Grzybowska‡, Joanna Rytka‡, and Rosine Labbe-Bois**

From the Institute of Biochemistry and Biophysics, Polish Academy of Sciences, 5A Pawinskiego Street, 02-106 Warsaw, Poland and the Laboratoire de Biochimie des Porphyrines, Institut Jacques Monod, Université Paris VII, 2 place Jussieu, 75251 Paris, France

Ferrochelatase is a mitochondrial inner membrane-bound enzyme that catalyzes the insertion of ferrous iron into protoporphyrin, the terminal step in protoporphyrin biosynthesis. The functional/structural roles of 10 invariant amino acid residues were investigated by site-directed mutagenesis in the yeast Saccharomyces cerevisiae ferrochelatase. The mutant enzymes were expressed in a yeast strain lacking the ferrochelatase gene, HEM15, and in Escherichia coli. The kinetic parameters of the mutant enzymes were determined for the enzymes associated with the yeast membranes and the enzymes in the bacterial soluble fraction. They were compared with the in vitro function of the mutant enzymes. The main conclusions are the following. Glu-314 is critical for catalysis, and we suggest that it is the base responsible for abstracting the pyrrole proton(s). His-235 is essential for metal binding. Asp-246 and Tyr-248 are also involved in metal binding in a synergistic manner. The Km for protoporphyrin was also increased in the H235L, D246A, and Y248L mutants, suggesting that the binding sites of the two substrates are not independent of each other. The R87A, Y95L, Q111E, Q273E, W282L, and F308A mutants had 1.2–2-fold increased Vmax and 4–10-fold increased Km values for protoporphyrin, but the amount of heme made in vivo was 10–100% of the normal value. These mutations probably affected the geometry of the active center, resulting in improper positioning of protoporphyrin.

Protoporphyrin is an important molecule used to build almost all the cytochromes and hemoproteins in the cells of practically all organisms. A key enzyme in protoporphyrin production is ferrochelatase (EC 4.99.1.1; protoporphyrin-ferro-lyase), which catalyzes the insertion of ferrous iron into the tetrapyrrolic nucleus of protoporphyrin IX, at the terminus of the heme biosynthetic pathway. In eukaryotes, ferrochelatase is a peripheral protein associated with the matrix side of the inner mitochondrial membrane. Protoporphyrin is supplied by protoporphyrinogen oxidase, the preceding enzyme in the pathway, also located in the inner mitochondrial membrane. But the nature and location of the iron pool that serves as substrate for ferrochelatase are not known. Yeast and murine ferrochelatases are synthesized as high molecular weight precursors that are proteolytically processed to their mature forms during their import into the mitochondria (see Refs. 1–4 for reviews).

The ferrochelatases isolated from various sources all have very similar catalytic properties, indicating that the major features of the reaction are conserved (1–4). Ferrochelatase genes and cDNAs have now been isolated and sequenced from bacteria (5–7), yeast (8), mammals (9–11), and plants (12, 13). The deduced amino acid sequences exhibit 10% identity. The prokaryotic enzymes are 30 amino acids shorter at the C terminus than the eukaryotic ones. A 2Fe-2S cluster was recently found in mammalian ferrochelatases, probably bound to a cysteine-rich motif in the carboxyl-terminal extension of the mammalian enzymes, but absent from the extensions of the yeast and plant enzymes (14, 15). The biological role of this cluster remains to be established.

In spite of many studies, the catalytic mechanism of ferrochelatase is still unknown. Models have been proposed on the basis of kinetic studies, substrate specificity, and chemical modifications of certain amino acid residues and from analysis of the inhibition by N-alkylporphyrins (1–4, 16). The active site is believed to be an enclosed hydrophobic pocket (17), with cysteiny1 (18) and arginy1 (19) residues implicated in the binding of metal and porphyrin propionate(s). But the fact that no cysteine is conserved in the known ferrochelatase sequences makes it very unlikely that cysteine is involved in metal binding. Whether the binding of the two substrates is random or ordered, the metal binding prior to porphyrin, is not yet clear. Also unclear is the fate of the two protons released from the pyrrolic nitrogen atoms. Distortion of the porphyrin ring has been suggested as a transition-state intermediate facilitating metalation, and aromatic residues might aid in and/or stabilize the bending of a pyrrole ring (1, 16).

Mutations have been identified that cause amino acid substitutions in human and yeast ferrochelatases, and their effects on the enzyme structure and function have been described (20–22). These mutations seem to affect structural aspects of the enzyme, rather than active-site residues specifically involved in the binding of either substrate or in catalysis. Site-directed mutagenesis in human ferrochelatase has recently shown that one strictly conserved histidine residue plays a significant role in metal ion binding (23). We have now constructed, by directed mutagenesis, single- and double-residue substitutions of 10 invariant amino acids in the yeast ferrochelatase. The mutant enzymes were expressed in a yeast strain from which the ferrochelatase gene HEM15 had been deleted.

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** To whom correspondence should be addressed. Tel.: 33-1-43-54-04-79; Fax: 33-1-44-27-57-16; E-mail: plabbe@cr.jussieu.fr.

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and in Escherichia coli. The functional consequences of these mutations for in vivo heme synthesis were compared with the enzyme defects measured in vitro. The results indicate that the four invariant residues His-235, Asp-246, Tyr-248, and Glu-314 are part of the active center, and their roles in ferrochelatase function are discussed.

MATERIALS AND METHODS

Yeast Strains, Plasmids, and Growth Conditions—The haploid Saccharomyces cerevisiae strain AS1 (12), a derivative of strain S150-2B (MATa leu2-3,112 his3Δ1 trp1-289 ura3-52) in which the HEM15 gene had been deleted (hem15Δ), was used as the host to study the mutant ferrochelatases. Plasmid pMG1 (Fig. 1), carrying the wild-type HEM15 gene or its mutant alleles, was constructed as follows, using routine DNA manipulation procedures (24). Plasmid pBluescript SK/HEM15 (Fig. 1), containing the 2.5-kb BamHI fragment from pBluescript SK/HEM15 (25) was digested with NdeI and BamHI and used for detailed biochemical analysis. Site-directed Mutagenesis—Site-directed mutagenesis was carried out using the Quick change site-directed mutagenesis kit (Stratagene) following the manufacturer’s instructions. All mutagenized codons were introduced into pMG1 by exchanging the entire hem15Δ sequence with the corresponding sequence of the wild-type HEM15 allele. The resulting DNA fragment was cut with Sall and BamHI and ligated into the pCASS3 expression vector (30) digested with Sall and BamHI. The entire hem15Δ sequence was verified by sequencing. The in vitro mutagenized codons were introduced into pMG2 by exchanging the 0.49-kb BglII fragments. The pMG2 plasmids carrying the wild-type and mutant ferrochelatases were transformed into E. coli strain DH5α (Life Technologies, Inc.). The bacteria were grown to saturation in LB/ampicillin medium, diluted 1:100 into complete low phosphate/MOPS medium containing 100 mg/liter ampicillin (29), and incubated for 18–20 h at 37°C. The cells were harvested by centrifugation, resuspended in 0.1 M Tris-HCl buffer (pH 7.6) containing 20% glycerol and 10 μg/ml phenylmethylsulfonyl fluoride, and disrupted by sonication. The lysed cells were centrifuged (100,000 × g, 1 h, 4°C) to separate the membrane and soluble fractions. The membrane pellets were resuspended in the above buffer. All fractions were analyzed immediately or stored at −20°C.

Ferrochelatase Activity Measurements—Ferrochelatase activity was monitored spectrofluorometrically by directly recording the rate of zinc-protoporphyrin formation as described previously in detail (3, 20, 31). The reaction mixture (3-ml final volume) contained 0.1 M Tris-HCl (pH 7.6), 0.2 mg/ml Tween 80, 0.03–15 μM protoporphyrin (in 0.1 M Tris-HCl (pH 7.6), 1% Tween 80), and 0.2–25 μM zinc (SnSO₄·H₂O dissolved in water); the reaction was initiated by adding the enzyme. The activity was expressed as nmol of zinc-protoporphyrin/h/mg of protein. The kinetic data were analyzed graphically and using the random Bi Bi fit of the EZ-FiT curve-fitting program (32). Heme and Porphyrin Content—The whole cell heme content was estimated by low temperature (liquid nitrogen) spectrophotometry of cells (40 mg, dry weight) filtered from YPGTe liquid cultures (34). This rapid and reliable method allows the direct quantitative comparison of the cytochrome (and therefore heme) contents of different strains. Porphyrins accumulated in the cells and extracted into the growth medium were estimated spectrophotometrically (22, 35); they were identified by high performance liquid chromatography (35).

RESULTS

Construction of HEM15 Mutant Ferrochelatases—The alignment of the amino acid sequences of ferrochelatases from various species indicates an overall 8% identity extending over the entire length of the proteins (3). The 28 invariant amino acids common to all sequences are shown in Fig. 2. To identify the residues involved in substrate binding and catalysis, we mutated those invariant amino acids that were a priori candidates for these functions. They were Arg-87, Tyr-95, Gln-111, His-235, Asp-246, Tyr-248, Gln-273, Trp-282, Phe-308, and Glu-314 (Fig. 2). We also made double mutations, changing both Asp-246 and Tyr-248. The nucleotide changes introduced and the corresponding amino acid substitutions are listed in Table I. Centromeric, low copy number plasmids (pMG1; Fig. 1) carrying the mutated hem15 alleles under the control of the HEM15 promoter and terminator were transformed into the host strain AS1 from which the chromosomal copy of HEM15 had been deleted (hem15Δ). Five or more individual transformants for each plasmid were scored for their growth phenotypes, and one was retained and used for detailed biochemical analysis.

![Fig. 1. Construction of the pMG1 and pMG2 plasmids.](image)

1 The abbreviations used are: kb, kilobase; MOPS, 4-morpholinopropanesulfonic acid.
In Vivo Activity of Mutant Ferrochelatases—The yeast strain AS1 (hem15Δ) makes no ferrochelatase and is therefore totally heme-deficient and accumulates large amounts of protoporphyrin. It cannot grow on glucose medium unless supplemented with heme (YPGheme) or Tween 80 + ergosterol (YPGTε), which supply essentially unsaturated fatty acid and sterol, the synthesis of which requires the functioning of microsomal cytochromes b₅ and P450, respectively. Also, it cannot grow on glycerol or ethanol medium because it lacks mitochondrial respiratory cytochromes. The catalytic activities of the mutant ferrochelatases, when expressed in the AS1 strain, should correlate with the ability of the transformants to grow on unsupplemented glucose (YPG) medium and glycerol (YPGly) medium. They should also correlate with the amount of total heme made by the cells grown in liquid medium and with the amount of protoporphyrin accumulated and excreted by the cells. Thus, we examined the growth phenotypes and the amounts of total hemes and porphyrins made by the cells carrying the various hem15Δ mutations; strain AS1 with wild-type HEM15 was used as a positive control, and AS1 without wild-type HEM15 was the negative control. The results are summarized in Table I and illustrated in Fig. 3. None of the mutants exhibited temperature-sensitive growth phenotypes when tested at 30 and 37°C. Two mutant ferrochelatases, H235L and E314A, appeared to be totally inactive in vivo. The double mutant ferrochelatases D246A/Y248L and D246V/Y248F had residual activities. They provided enough heme for the synthesis of cytochromes b₅ and P450 to allow some growth in the absence of Tween 80 + ergosterol, but that amount of heme was not sufficient to make a functional respiratory chain. The F308A mutant ferrochelatase made ~10% wild-type heme, which permitted the cells to grow, but poorly, on glycerol medium. All the other mutants showed no growth defects on either YPG or YPGly plates, although the amount of heme made ranged from 20 to 100% of the normal value.

There was a good inverse relationship between the amounts of hemes and those of total porphyrins (intracellular + excreted in the medium) synthesized by the mutant ferrochelatases in vivo: the less hemes, the more porphyrins, mainly protoporphyrin (>90%). However, two mutants departed from this, R87A and Y95L, which accumulated porphyrins in spite of almost normal heme synthesis.

Immunological Detection of Mutant Ferrochelatase Proteins—Whole cell protein extracts and membrane fractions were prepared from cells expressing wild-type and mutant ferrochelatases and analyzed for their content of ferrochelatase protein by immunodetection. Western blot analysis of membrane proteins showed that the cells made normal amounts of normal mature-sized ferrochelatases, except for mutants R87A and F308A (Fig. 4, row Y). Identical results were obtained with total cell proteins (data not shown). The steady-state level of the R87A mutant ferrochelatase protein was ~3-fold lower than normal, suggesting some instability of the protein. The F308A mutant protein migrated more slowly than the others, with a difference in apparent molecular weight of 1000–1500. The entire hem15 allele was resequenced to ensure against a cloning artifact. One possible reason for the abnormal mobility is discussed below. These results indicate that the absence of or decreased activity of the mutant ferrochelatases is not due to a defect in their expression and that all membrane preparations contained roughly the same amount of enzyme protein, except for mutant R87A.

Kinetic Parameters of Mutant Ferrochelatases—The activities of the membrane-bound mutant ferrochelatases were studied in the same membrane preparations as used for immunodetection of the enzyme protein. A sensitive assay that permitted direct spectrofluorometric recording of the initial velocity of zinc-protoporphyrin formation in the presence of physiological concentrations of substrates was used. The validity of this assay, and especially the use of Zn²⁺ in place of Fe²⁺, has been discussed elsewhere (3, 20, 31, 36). The maximal velocities and the Kₘ values for both Zn²⁺ and protoporphyrin of the mutant enzymes are given in Table II.

The H235L, D246V/Y248F, and E314A mutant ferrochelatases had barely detectable activities. Even when measured with high substrate concentrations (15 µM protoporphyrin, 50 µM Zn²⁺), the activity was <1% of the wild-type value. The kinetic parameters could not be determined because of this very low activity. The D246A/Y248L mutant (which carried less conservative amino acid substitutions than the D246V/Y248F mutant) had 13% of the wild-type activity, but the Kₘ for both substrates was increased ~40-fold. All the other mutant enzymes, including the single mutants D246A and Y248L, had higher activities than the wild-type enzyme and higher Kₘ values for protoporphyrin (4–12-fold the normal value). The Kₘ for Zn²⁺ of these mutant enzymes could not be measured precisely be-
cause they were already saturated with the endogenous Zn$^{2+}$ present in the assay (reaction mixture + membranes), as was the wild-type ferrochelatase. Therefore, the $K_m$ for Zn$^{2+}$ of these enzymes was considered to be similar to that of the wild-type enzyme (a 2-fold increase in the $K_m$ for Zn$^{2+}$ could have been estimated if this had been the case).

Activities of Mutant Ferrochelatases Overexpressed in E. coli—The mutant enzymes, especially those having very low activities, were further examined by overexpressing the wild-type and mutant forms of yeast ferrochelatase in E. coli. The mature form of the enzyme, lacking its mitochondrial targeting sequence (hem15p), was expressed under the control of the alkaline phosphatase promoter (plasmid pMG2; Fig. 1). The E. coli cells transformed with pMG2 or with its derivatives carrying the different mutations were grown at 37 °C in low phosphate medium and assayed for ferrochelatase by Coomassie Blue staining of fractionated whole cell proteins, immunodetection, and activity assays. All the ferrochelatase proteins, except the D246A/Y248L mutant, were expressed at similar high levels (7–8% of the total cell protein) and were found mostly (~70%) in the soluble cytosolic fraction (Figs. 4 and 5). Expression of the D246A/Y248L mutant protein was restored to the wild-type high level by growing the cells at a lower temperature (30 °C), suggesting that this mutant protein is unstable at 37 °C in E. coli. All but one of the ferrochelatases in the bacterial soluble fractions migrated similarly and as the mature-sized enzyme present in yeast membranes (Fig. 4, row E). The F308A mutant enzyme migrated more slowly, with a mobility identical to that reported for the yeast membrane-bound protein. This indicates that the abnormally slow migration of both the yeast and bacterially expressed F308A mutant enzymes is probably due to some intrinsic structural change in the protein brought about by the mutation. Adding 4 M urea to both the yeast and bacterial protein extracts did not alter the relative electrophoretic mobility of the mutant enzyme.

The kinetic parameters of the wild-type and mutant ferrochelatase activities measured in the bacterial soluble fractions are reported in Table II. As for the yeast membrane-bound enzymes, reliable measurements of $K_m$ for Zn$^{2+}$ were not possible for the wild-type and some mutant enzymes because they were almost saturated with endogenous Zn$^{2+}$. However, we estimated that they were close to 0.2 μM, the value of the purified ferrochelatase (31). The two H235L and D246V/Y248F mutant ferrochelatases had $K_m$ values for Zn$^{2+}$ that were dramatically increased compared with that of the wild-type enzyme; their $K_m$ values for protoporphyrin were also greatly increased, and their $V_m$ values were 25 and 2% of the wild-type value, respectively. The D246A single substitution caused an increase in the $K_m$ values for both substrates, while the Y248L single substitution affected only the affinity for protoporphyrin; they did not much affect the maximal velocity. In contrast, introducing these two mutations together caused a >90% decrease in the activity, without further affecting the $K_m$ value for either substrate. Interestingly, the E314A mutant had $K_m$ values identical to those of the wild-type enzyme, although the mutant displayed only ~2% of the wild-type activity. The three other mutations, Q273E, W282L, and F308A, caused 8–23-fold increases in the $K_m$ for protoporphyrin without impairing the activity much.

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**TABLE I**

| Enzyme | Growth on | Total hemes | Total porphyrins |
|--------|-----------|-------------|-----------------|
|        | Glu | Gly | % | nmol/g, dry wt |
| hem15Δ | WT | + | - | 0 | 180 |
|         |       |       |    |   |    |
| Wild-type | WT | + | + | 100 | 7 |
| CGT → gCT | R87A | + | + | 80 | 65 |
| TAT → Tta | Y95L | + | + | 100 | 32 |
| CAA → QAA | Q111E | + | + | 90 | 7 |
| CAT → CTT | H235L | + | + | 0 | 180 |
| GAT → Gca | D246A | + | + | 20 | 115 |
| TAC → Taa | Y248L | + | - | 100 | 10 |
| GAT → Gca | D246A/Y248L | +/- | -/+ | <5 | 100 |
| TAC → Taa | Y248L | +/- | -/+ | <5 | 100 |
| GAT → GtT | D246V/Y248L | +/- | -/+ | <5 | 100 |
| TAC → T1C | Y248F | + | + | 70 | 20 |
| CAA → QAA | Q273E | + | + | 60 | 46 |
| TGG → T1G | W282L | + | + | 10 | 105 |
| TTT → gT | F308A | +/+ | -/- | 0 | 160 |
| GAA → Gcg | E314A | - | - | - | - |

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**FIG. 3.** Growth phenotypes associated with the expression of the mutant ferrochelatases. The yeast strain AS1 carrying the null allele hem15Δ (-) was transformed with plasmid pMG1 expressing wild-type (WT) or mutant forms of ferrochelatase. The AS1 strain and the different transformants were grown in YPGTe. Drops (3 μl) of each culture were spotted onto different media in 10-fold serial dilutions, and the plates were incubated at 30 °C for 2 days (YPG supplemented or not) or 6 days (YPGly). YP plates contained 2% glucose (YPG) or 2% glycerol (YPGly), supplemented with hemin (YPGhemin) or Tween 80 + ergosterol (YPGTe).
Palmitic acid has been reported to be required for full activity of the purified yeast ferrochelatase (31). Therefore, we tested to see if it had any effect on the enzyme overexpressed in a soluble form in E. coli. Addition of 1 mM palmitic acid (20 μM/assay of 20 mg of palmitic acid/10 mL of dimethyl sulfoxide) did not change the maximal velocity, but did decrease the Km for protoporphyrin (0.06 μM) of the wild-type enzyme 3–4-fold. Mutants H235L, D246A, F308A, and E314A and the double mutants were totally inhibited, in a dose-dependent manner, by 1 mM palmitic acid. The activities of mutants Y248L and Q273E were increased 1.5–2-fold, and their Km for protoporphyrin decreased 1.5–3-fold. Palmitic acid had no effect on mutant W282L. Similar results were obtained with the mutant enzymes expressed in yeast in a membrane-bound form. 1 mM palmitic acid inhibited mutants D246A, D246A/Y248L, and F308A, while it increased the Vm of mutants R87A, Y95L, Q111E, Y248L, and Q273E 2–3-fold without greatly affecting their Km for protoporphyrin (a 2-fold increase at the most). The role of fatty acids in the activity of ferrochelatase is unclear (1–4). Our results favor the idea that they might interfere with the delivery of exogenous substrates to the active site, rather than promoting the proper active conformation of the enzyme.

**DISCUSSION**

This study describes the replacement by site-directed mutagenesis of 10 invariant amino acids in the yeast ferrochelatase. The function of the mutant enzymes was analyzed in vivo by expressing them in a yeast ferrochelatase-deficient strain and in vitro both in their physiological environment when bound to yeast mitochondrial membranes and as soluble mature-sized proteins overexpressed in E. coli. There was in general a good correlation between the results obtained with these three approaches despite some variations that are discussed below with the analysis of each mutation.

Glu-314—Mutation E314A led to an enzyme with a very low residual activity, but with Km values for both substrates almost identical to the wild-type values. This strongly suggests that Glu-314 is critical for catalysis. We propose that this glutamyl residue acts as a base involved in the abstraction of the two protons from the pyrrolic nitrogen atoms. There may even be an initial hydrogen bonding between the glutamic carboxyl group and the pyrrole NH groups of the porphyrin that may contribute significantly to the stabilization of the porphyrin transition-state intermediate. Then, as metal binds, the protons released from the porphyrin would be accepted by this glutamic acid residue and transferred to other residues. Two other acid residues are conserved, as aspartyl or glutamyl, at positions 311 and 318 (Fig. 2). They could provide a pathway for further transferring the two protons from the buried porphyrin ring to the solvent or the final physiological proton acceptor. Secondary structure predictions suggest that this highly conserved region surrounding Glu-314 is organized into an amphiphilic α-helix with one side lined with these conserved acidic residues (3).

### Table II

| Enzyme       | In S. cerevisiae membranes | In E. coli soluble fractions |
|--------------|---------------------------|-------------------------------|
|              | Vm  | Km,PP | Km, Zn2⁺ | Vm  | Km,PP | Km, Zn2⁺ |
| None         | 0.00 |        |          | 0.00⁺ |        |          |
| Wild-type    | 11.5 | 0.05   | 0.15     | 1800 | 0.2    | 0.25     |
| R87A         | 16.5⁺ | 0.32  | wt       |      |        |          |
| Y95L         | 14.0 | 0.21   | wt       |      |        |          |
| Q111E        | 22.5 | 0.20   |          |      |        |          |
| H235L        | 0.1  | ND     | ND       | 460  | 1.7    | 48       |
| D246A        | 25.2 | 0.62   | wt       | 1870 | 4.2    | 3.5      |
| Y248L        | 20.0 | 0.20   | wt       | 4040 | 1.2    | wt       |
| D246A/       | 1.5  | 2.0    | 5.0      | 140⁺ | 8.2    | 3.1      |
| H235L        | 0.05 | ND     | ND       | 40   | 8.5    | 32       |
| D246V/       |     |        |          |      |        |          |
| Y248F        |     |        |          |      |        |          |
| Q273E        | 13.6 | 0.30   | wt       | 1520 | 1.4    | wt       |
| W282L        | 15.7 | 0.55   | wt       | 1380 | 0.7    | 0.5      |
| F308A        | 14.3 | 0.20   | wt       | 1910 | 0.5    | wt       |
| E314A        | 0.07 | ND     | ND       | 37   | 0.05   | 0.3      |

*All the activity was associated with the membrane fraction (0.2 nmol of Zn-protoporphyrin/mg of protein).*

*The membranes contained 3-fold less immunodetectable enzyme (Fig. 4).*

*The bacteria were grown at 30 °C.*
Active-site Mutations in Yeast Ferrochelatase

Fig. 5. Overexpression of yeast ferrochelatase in E. coli. E. coli strain DH5α was transformed with the pCAS3 vector (lane 1) or the pMG2 plasmid carrying the wild-type yeast ferrochelatase (lane 2), and the cells were grown for 20 h in low phosphate medium. Total cellular proteins (10 μg) were resolved by SDS-polyacrylamide gel electrophoresis and visualized by Coomassie Blue staining (panel a) or transferred, after electrophoresis on a longer gel, for Western blotting with yeast ferrochelatase antiserum (panel b). The arrow indicates the position of yeast ferrochelatase. The major band in lane 1 in panel a is E. coli alkaline phosphatase.

Trp-282 and Phe-308—Two hydrophobic aromatic residues, Trp-282 and Phe-308, are invariant and are candidates for a role in promoting or stabilizing pyrrole ring tilting in the transition-state complex, according to Lavallee (16) and Dailey (1). But the characteristics of the enzymes mutated at these positions make it rather unlikely that either of these residues plays such a role. The W282L mutant ferrochelatase seems to function nearly normally in vitro, but with a lower affinity for protoporphyrin, which might be the cause of its 40% lower in vivo activity. We previously identified two mutations causing replacement of the invariant residues Gly-47 by Ser and Ser-102 by Phe; both mutations led to phenotypes almost identical to that of the W282L mutant (22).

The F308A mutant ferrochelatase had a puzzling phenotype. Its capacity to make heme in vivo was considerably decreased (90%), while it had a normal zinc chelatase activity measured in vitro, albeit with slightly (2.5–4-fold) lower affinity for protoporphyrin. The protein migrated a little slower on electrophoresis than the wild-type and all other mutant ferrochelatases. One possible explanation is that the wild-type enzyme retained some local residual microstructure stabilized by Phe-308 that prevented complete unfolding. The changing of Phe-308 to Ala would destroy this microstructure, promoting full denaturation of the protein, which would then migrate more slowly. This structure may be important in vivo for the proper access of protoporphyrin to the active site when this substrate is generated enzymatically within the mitochondrial membrane or for the correct and efficient release of heme probably also in the membrane.

His-235, Asp-246, and Tyr-248—These three amino acids are located in a highly conserved region of the protein. They contain side chain groups known to chelate metal ions in various metalloproteins. Our results show that, indeed, these three residues serve as ligands for ferrous ion. The H235L mutant ferrochelatase was practically inactive in yeast, whether estimated in vivo or in vitro. In contrast, the mutant enzyme expressed in E. coli exhibited 25% of the wild-type Vₘ, its Kₘ for Zn²⁺ was dramatically increased (190-fold), and that for protoporphyrin was also slightly increased (8-fold). This clearly identifies His-235 as a residue essential for metal binding and ferrochelatase function, in agreement with the recent report of Kohno et al. (23). These investigators changed the four histidine residues in human ferrochelatase that are conserved in the mammalian and yeast enzymes. They found that only the mutation H263A (corresponding to yeast His-235) led to increased Kₘ values for both ferrous iron and zinc in the mutant enzymes expressed in E. coli.

The D246A mutant ferrochelatase was ~5-fold less active than normal in vivo, and the in vitro analysis suggested that this was due to a 12-fold lower affinity for protoporphyrin. However, when expressed in E. coli, the mutant enzyme displayed increased Kₘ values for both protoporphyrin (21-fold) and metal (14-fold). The reason(s) for this discrepancy is not clear at present. The replacement of the neighboring Tyr-248 with Leu was benign: the function of the mutant enzyme was normal in vivo, and its catalytic efficiency (Vₘ/Kₘ) measured in vitro was decreased (2–3-fold) only for protoporphyrin. Therefore, both of the mutations D246A and Y248L seemed to affect mainly the affinity of ferrochelatase for protoporphyrin.

But introducing the two mutations together was highly detrimental to ferrochelatase function both in vivo and in vitro. The catalytic efficiency of the D246A/Y248L double mutant enzyme was now considerably reduced for both substrates: 300-fold for protoporphyrin and 250-fold for zinc for the enzyme expressed in yeast; similar values were obtained for the enzyme expressed in E. coli. The defects of the enzyme were still aggravated when Asp-246 and Tyr-248 were replaced by Val and Phe, respectively, although these substitutions should introduce less steric constraint on the protein (the D246A/Y248L mutant protein was less stable in E. coli grown at 37 °C compared with D246V/Y248F). Thus, it appears that it is the combined effects of the two mutations that caused a decrease in the activity of ferrochelatase and in its affinity for both substrates. The basis for this synergistic action is not understood.

The finding that His-235, Asp-246, and Tyr-248, which are clustered in the same region, serve as metal ligands is in complete agreement with the results of Franco et al. (37), who used Mössbauer spectroscopy to characterize the ferrous ion-binding site. The data indicated an ionic coordination environment for the high spin ferrous ions, consisting of nitrogen- and/or oxygen-containing ligands. Other residues, i.e. the invariant residues Ser-233 and Ser-167, could also be part of the metal-binding site.

The fact that the Kₘ values for both metal and protoporphyrin were increased in these mutants is not unprecedented. The mutations S169F, S174P, and L62F also caused an increase in the Kₘ values for both substrates, together with an increase in Vₘ (20, 22). We suggested that the binding sites of the two substrates are not independent of each other. One possibility is that one (or both) flexible propionate side chain of the porphyrin is hydrogen-bonded to some residues of the metal-binding site. Altering one of these residues or the geometry of the active site might destabilize the propionate hydrogen-bonding network and weaken the porphyrin-enzyme interactions. It is conceivable that the propionate carboxylic group(s) might play a role in the metal binding process.

Arg-87, Tyr-95, Gln-111, and Gln-273—Substitutions at these four amino acids caused very similar phenotypes: little or no decrease in heme synthesis in vivo and higher Kₘ (4–6-fold) for protoporphyrin and Vₘ. This rules out the direct involvement of Arg-87 in the binding of the porphyrin propionate group and the participation of Tyr-95 in metal binding. These two residues are located in a 50-amino acid sequence bordered by two conserved glycine-rich regions. This long sequence is rather well conserved, except in the ferrochelatase from Bacillus subtilis (3, 22). Since the B. subtilis enzyme is the only ferrochelatase to have been purified as a water-soluble protein (38), it is tempting to speculate that this segment of the protein plays a role in its association with the membrane. If this were the case, then the two mutations R87A and Y95L may have impaired the “uptake” of protoporphyrin generated within the membrane, which would explain why they caused an abnor-
mally large accumulation of protoporphyrin in vivo. The Q111E and Q237E replacements were designed to create a negative charge for perturbing any eventual hydrogen-bonding interactions with porphyrin propionate. Since the mutant enzymes were not greatly affected, we conclude that these residues are not crucial for porphyrin binding. In fact, these results, along with the observation that the R87A mutant enzyme is unstable in vivo, suggest that these four mutations have affected the geometry of the active center, disturbing the hydrophobic interactions and/or hydrogen-bonding network of the porphyrin nucleus and propionate, respectively, with the protein.

In conclusion, this initial mutational survey of the active-site amino acid residues in the yeast ferrochelatase has answered some questions about the residues implicated in substrate binding and catalysis. Further detailed kinetic and inhibition studies with purified mutant enzymes are needed before a more refined model of the active center can be proposed. However, understanding the catalytic mechanism of ferrochelatase will have to wait for the elucidation of its three-dimensional structure. Fortunately, this is in sight since a preliminary x-ray analysis of B. subtilis ferrochelatase crystals was recently reported (39).

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