Examination of the Activity of Carboxyl-Terminal Chimeric Constructs of Human and Yeast Ferrochelatases†

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ABSTRACT: Insertion of ferrous iron into protoporphyrin IX is catalyzed by ferrochelatase (EC 4.99.1.1). Human and *Schizosaccharomyces pombe* forms of ferrochelatase contain a [2Fe-2S] cluster with three of the four coordinating cysteine ligands located within the 30 carboxyl-terminal residues. *Saccharomyces cerevisiae* ferrochelatase contains no cluster, but has comparable activity. Truncation mutants of *S. cerevisiae* lacking either the last 37 or 16 amino acids have no enzyme activity. Chimeric mutants of human, *S. cerevisiae*, and *Sc. pombe* ferrochelatase have been created by switching the terminal 10% of the carboxy end of the enzyme. Site-directed mutagenesis has been used to introduce the fourth cysteinyl ligand into chimeric mutants that are 90% *S. cerevisiae*. Activity was assessed by rescue of Δ*hem H*, a ferrochelatase deficient strain of *Escherichia coli*, and by enzyme assays. UV−visible and EPR spectroscopy were used to investigate the presence or absence of the [2Fe-2S] cluster. Only 2 of the 13 chimeric mutants that were constructed produced active enzymes. HYB, which is predominately human with the last 40 amino acids being that of *S. cerevisiae*, is an active protein which does not contain a [2Fe-2S] cluster. The other active chimeric mutant, HSp, is predominately human ferrochelatase with the last 38 amino acids being that of *Sc. pombe* ferrochelatase. This active mutant contains a [2Fe-2S] cluster, as verified by UV−visible and EPR spectroscopic techniques. No other chimeric proteins had detectable enzyme activity or a [2Fe-2S] cluster. The data are discussed in terms of structural requirements for cluster stability and the role that the cluster plays for ferrochelatase.

The insertion of ferrous iron into protoporphyrin IX is catalyzed by the terminal enzyme in heme biosynthesis, ferrochelatase (EC 4.99.1.1) (*1*). This enzyme has been purified and characterized from many prokaryotic and eukaryotic sources. This enzyme from most sources can utilize ferrous iron, zinc, and divalent cobalt as its metal substrate (*2*). The IX isomers of deutoroporphyrin, hematoporphyrin, mesoporphyrin, and protoporphyrin are all acceptable porphyrin substrates (*1*). With the exception of *Bacillus subtilis* ferrochelatase (*3*), the enzyme is membrane-associated (*2*). In eukaryotes, the enzyme is nuclear encoded, synthesized in the cytoplasm, and translocated to the matrix side of the inner mitochondrial membrane (*4*).

The gene for ferrochelatase has been sequenced from a number of eukaryotic and prokaryotic sources, including *B. subtilis* (*5*), *Escherichia coli* (*6*), *Saccharomyces cerevisiae* (*7*), *Arabidopsis thaliana* (*8*), barley (*9*), *Xenopus* (*10*), chicken (*10*), *Drosophila* (*11*), mouse (*12*, *13*), and human (*14*). When the deduced amino acid sequences from these organisms are compared, two features are apparent. The first is the presence of an amino-terminal mitochondrial targeting sequence in eukaryotic ferrochelatases. The second is the presence of a carboxyl-terminal extension in eukaryotes that is 30−60 amino acids in length, with plant forms having the larger extensions (*15*). In animal sequences, this extension contains three of the four coordinating ligands of a [2Fe-2S] cluster (*10*, *11*, *15*−*17*). The cluster in animal ferrochelatases is unlike any other [2Fe-2S] cluster currently reported in its ligand spacing of Cys-X_{206}-Cys-X_2-Cys-X_4-Cys (*11*). In other eukaryotic sources, such as *S. cerevisiae* and plant forms, the carboxyl-terminal extension is present, but not the coordinating ligands and cluster (*16*). Recently, ferrochelatase from the yeast *Schizosaccharomyces pombe* was found to contain all four cysteinyl residues necessary for cluster coordination and to contain a [2Fe-2S] cluster (manuscript in preparation). As mentioned above, the other yeast form of ferrochelatase from *S. cerevisiae* does not have the [2Fe-2S] cluster but has a length similar to those of animal forms of the enzyme.

Dailey et al. (*15*) demonstrated that truncation of the carboxy terminus of the human ferrochelatase so that it resembles that of prokaryotic sequences results in the loss of both cluster and enzymatic activity. It is unknown if the corresponding extension in *S. cerevisiae* serves a functional role. This work investigates the function of the carboxy terminus of *S. cerevisiae*, human, and *Sc. pombe* ferrochelatases through the use of chimeric enzyme constructs. The data demonstrate that the carboxy terminus is necessary for enzyme activity, but that it is possible to engineer an active

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form of human ferrochelatase that lacks the [2Fe-2S] cluster and still has enzyme activity. The meaning of these results for understanding the role of the cluster in ferrochelatases that contain this feature is discussed.

MATERIALS AND METHODS

Strains and Cell Culture. Truncation, chimeric, and wild-type proteins were expressed in *E. coli* strains JM109 and Δ*hem H* (*6*, *18*). Expression of proteins for whole-cell EPR[1] was carried out in *E. coli* DW35 Δ*frdABCD* and *sdhC::kan* grown on a minimal glucose/furmarate medium as previously described (*19*).

Construction of Truncation Mutants. Two mutants of *S. cerevisiae* that lacked either 37 (YstTrun1) or 16 (YstTrun2) carboxyl-terminal amino acid residues were constructed by PCR mutagenesis. To construct YstTrun1, a stop codon was introduced at base pair 1068 with the antisense primer (5′ AAG CTT CTA GTG GCT TTT GAC GAG 3′). The underlined sequence is a *Hin*dIII restriction site that was inserted so this piece could be cloned after PCR amplification. The second truncation mutant, YstTrun2, was created with the antisense primer (5′ AAG CTT CTA ATT GGA CTT GCC AAG TGC 3′, *Hin*dIII restriction site underlined). PCR conditions were as follows: 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2 mM $MgCl_2$, dNTPs (each at 0.2 mM), 1 μM Tac forward primer, 1 μM antisense primer (listed above), 100 ng of yeast ferrochelatase expression vector, and 5 units of Taq Polymerase (Sigma). Reaction times and temperatures were as follows: 1 cycle at 95 °C for 5 min, 30 cycles at 95 °C for 1 min, 52 °C for 1 min, and 72 °C for 2 min, and 1 cycle at 72 °C for 7 min. PCR products were isolated using Geneclean Spin (Bio 101) before fragments were ligated into the T vector (Promega). The truncated yeast ferrochelatase fragments were excised using *Eco*RI and *Hin*dIII and then ligated into modified pBTac (*15*, *20*).

Construction of Chimeric Mutants. To facilitate the construction of chimeric ferrochelatases via cassette mutagenesis, a *Bgl*II restriction site was introduced into human and *Sc. pombe* ferrochelatase DNA sequences at a position equivalent to a *Bgl*II restriction site located 129 bp from the 3′ end of *S. cerevisiae* ferrochelatase. This site falls in a highly conserved region of ferrochelatase, approximately 43 amino acids from the carboxy terminus, having the amino acid sequence ADLV. The *Bgl*II site was engineered to conserve this amino acid sequence, and was constructed within the human and *Sc. pombe* sequence using the QuikChange mutagenesis protocol (Stratagene). The following sets of primers were used for human ferrochelatase and *Sc. pombe* ferrochelatase, respectively: sense, 5′ G TTC TCT AAG GCC CTG GC**A** GA**T C**TG GTG CAT TCA CAC ATC CAG 3′; antisense, 5′ CTG GAT GTG TGA ATG CAC CA**G A**TC **T**GC CAG GGC CTT AGA GAA C 3′; sense, 5′ GCT ATT CAA GGA ATG GC**A** GA**T** CTG GTT GCA GAG C 3′; and antisense, 5′ G CTC TGC AAC CAG **A**TC **T**GC CAT TCC TTG AAT AGC 3′ (*Bgl*II restriction sites underlined and mutated bases in bold). *S. cerevisiae* also has an additional *Bgl*II site located approximately 565 bp upstream of the one mentioned above. To aid in construction of mutants, this *Bgl*II site was eliminated while conserving the amino acid sequence. Once again, the QuikChange mutagenesis protocol was employed with the following set of primers: sense, 5′ GGA CTC CGA GAG **G**TC TAT ATC TTG G 3′; and antisense, 5′ CCA AGA TAT AGA **C**CT CTC GGA GTC C 3′ (mutated bases in bold). The *Bgl*II and *Hin*dIII sites were employed in cassette mutagenesis to construct HYB, YHB, HSp, SpH, and SpY (see Figure 2). SpH, SpY, and wild-type *Sc. pombe* were all expressed in pTrcHis (manuscript in preparation). The B in chimeric mutant abbreviations refers to the use of the *Bgl*II site in their construction. All other chimeric mutants were expressed using modified pBTac (*15*, *20*).

To construct HYP, HYH, YHY, and YHP, the QuikChange mutagenesis kit was used to create a *Pst*I at the site in the *S. cerevisiae* sequence corresponding to a unique *Pst*I site in human ferrochelatase located 37 bp from the 3′ termination codon. The set of primers used to create this mutation was as follows: sense, 5′ GGC AAG TCC AAT GAT CCT GT**C TGC AG**C CTT TCA TTG GTA TTT GGC AAT CAC G 3′; and antisense, 5′ C GTG ATT GCC AAA TAC CAA TGA AAG G**CT GCA G**AC AGG ATC ATT GGA CTT GCC 3′ (*Pst*I site underlined and mutated bases in bold). When this restriction site was created in the yeast sequence, it was necessary to change amino acid sequence VKDL to VCSL. The altered residues are not conserved residues, and the mutated yeast enzyme had normal enzyme activity. Chimeras that contain the amino terminus of *S. cerevisiae* thus contain this cysteine substitution, but since the *Pst*I-containing *S. cerevisiae* ferrochelatase has normal activity, we do not believe that this alteration effects protein structure. Chimeras that contain the *S. cerevisiae* carboxyl terminus do not contain additional cysteines. To construct HYP and YHP, the *Eco*RI site at the 5′ end of the genes was used along with the *Pst*I site to digest and ligate the appropriate fragments. The P in the chimeric mutant abbreviations refers to the use of the *Pst*I site in their construction. To create the HYH and YHY mutants, *Bgl*II and *Pst*I restriction digests were used. The QuikChange mutagenesis protocol was used to change tyrosine 168 of *S. cerevisiae* into a cysteine so that chimeras containing human or *Sc. pombe* carboxyl-terminal sequences would possess all four necessary cluster-liganding cysteine residues. The primers employed for this were as follows: sense, 5′ CCT CAT TTC TCT T**G**T TCC ACT ACC GGG 3′; and antisense, 5′ CCC GGT AGT GGA A**C**A AGA GAA ATG AGG 3′ (mutated bases in bold). The chimeras thus created were named YHB/Y168C, YSp/Y168C, and YHY/Y168C.

Enzyme Purification and Activity Determination. Truncated and chimeric mutants were purified using blue dye affinity columns (*15*). Several chimeric mutants, including HSp, SpH, and SpY, were expressed with a six-His tag at their amino terminus to facilitate their purification as previously described (*20*). The presence of functional in vivo ferrochelatase activity was assessed by the ability of the mutant enzyme to complement and thereby rescue Δ*hem H*, a strain of *E. coli* lacking ferrochelatase activity and requiring either a functional form of ferrochelatase or exogenous heme (*6*, *18*). In vitro activity was assayed using the pyridine hemochromagen assay (*21*). Kinetic studies of crude cell extracts were performed using Δ*hem H* containing the appropriate plasmid with a final total protein concentration

[1] Abbreviations: PCR, polymerase chain reaction; EPR, electron paramagnetic resonance.

of 5 mg/mL as assessed by BCA (Pierce). Ferrous iron and mesoporphyrin (Porphyrin Products, Logan, UT) were used as substrates. Activity from crude samples is expressed as nanomoles of heme per minute per milligram of total protein, and from purified samples as nanomoles of heme per minute per nanomole of ferrochelatase.

Western Blot Analysis. Wild-type and mutant ferrochelatases expressed in JM109 were used for Western blot analysis. Proteins from cell extracts (4 μg) were separated by polyacrylamide gel electrophoresis on a 10% Tris-HCl gel (Bio-Rad) and transferred to nitrocellulose membranes. Membranes were probed with antirecombinant human ferrochelatase antibody which cross reacted well with *S. cerevisiae* and *Sc. pombe* (*22*) following the Proto Blot Western Blot AP system (Promega).

Spectroscopy. All UV−visible spectroscopy was carried out with a Cary-G1 spectrophotometer. The extinction coefficients used to quantitate protein concentration were 46 640 M^{-1} cm^{-1} for human, 54 800 M^{-1} cm^{-1} for *S. cerevisiae*, and 47 300 M^{-1} cm^{-1} for *Sc. pombe*. EPR spectra were obtained using a Bruker ESP-300E EPR spectrometer. EPR samples of purified protein were prepared in an anaerobic chamber using sodium dithionite to reduce the samples. Whole-cell EPR was carried out as previously described (*11*).

RESULTS

Characterization of Truncation Mutants. Two truncation mutants of *S. cerevisiae* lacking 16 or 37 of the carboxyl-terminal amino acid residues were produced (Figure 1). The longer of these two truncations gives rise to an enzyme that is approximately the size of a typical prokaryotic ferrochelatase. When transformed into *E. coli* Δ*hem H*, neither truncation mutant was capable of complementing these cells. Enzyme assays of expressed YstTrun1 and YstTrun2 showed no measurable activity. Both proteins were purified and found to contain no measurable activity.

Characterization of Chimeric Mutants. A total of 13 chimeric mutants were produced (Figure 1). Eleven of the chimeric mutants had no measurable enzyme activity as shown by their inability to rescue *E. coli* Δ*hem H* and their lack of measurable in vitro enzyme activity in crude cell extracts. These proteins were HYH, HYP, YHB, YHB/Y168C, YHY, YHY/Y168C, YHP, YSp, YSp/Y168C, SpY, and SpH. Western blot analysis verified that all protein constructs were expressed (Figure 2). UV−visible spectra of the purified proteins resembled that of *S. cerevisiae* ferrochelatase, which does not contain a [2Fe-2S] cluster (data not shown). Whole-cell EPR spectra of chimeric mutants possessing all four cysteinyl ligands were obtained to determine if a cluster assembled in vivo was unstable to protein isolation techniques employed in the current study. However, none of these chimeras possessed an EPR signal from 10 to 35 K using microwave powers of 1−10 mW that would be characteristic of a [2Fe-2S] cluster (data not shown).

The two chimeras, HYB and HSp, were found to rescue *E. coli* Δ*hem H*. Purification of HYB using the blue dye affinity column resulted in recovery of inactive enzyme. In an attempt to accelerate purification and, thereby, obtain an active ferrochelatase, a six-His tag was added to the amino terminus of HYB and the protein was purified using metal chelate chromatography. This technique has been employed previously to obtain pure ferrochelatase (*20*). Unfortunately, this did not result in the recovery of active enzyme, suggesting that the HYB mutant is an unstable protein. SDS−polyacrylamide gel electrophoresis of the purified protein revealed proteolytic degradation of this chimeric protein.

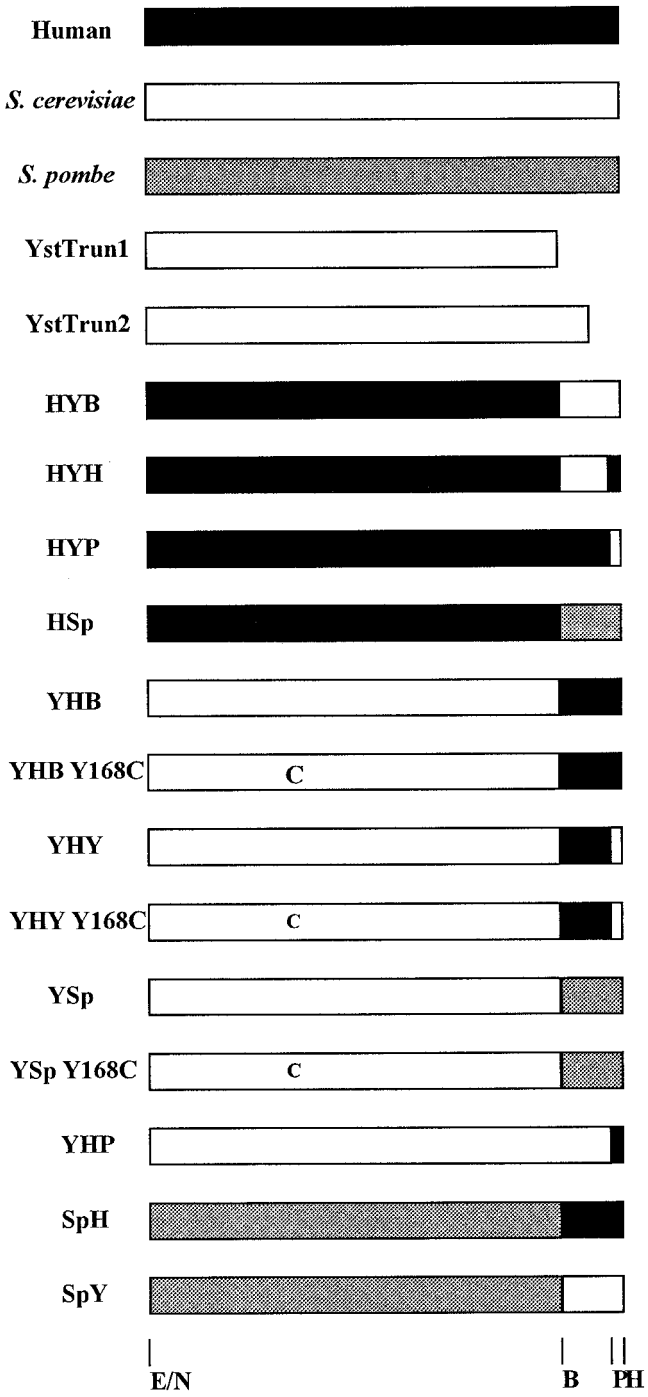

FIGURE 1: Schematic of wild-type and chimeric mutant proteins. The human protein is termed H and is represented by black boxes; the *S. cerevisiae* protein is termed Y and is represented by white boxes, and the *Sc. pombe* protein is termed Sp and is represented by gray boxes. Constructs containing the Y168C mutation in *S. cerevisiae* for re-creating the fourth cluster ligand of human and *Sc. pombe* are shown with a C. Lines at the bottom of the diagram show the comparable site in the gene sequence used in creating the mutants. Restriction sites are as follows: E, *Eco*RI; N, *Nhe*I; B, *Bgl*II; P, *Pst*I; and H, *Hin*dIII.

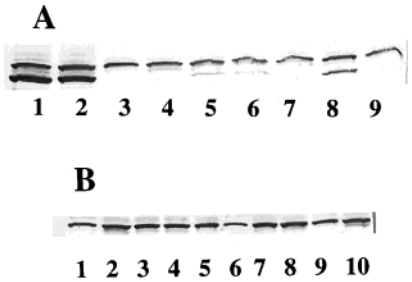

FIGURE 2: Western blot analysis of truncation and chimeric mutants. Blot A lane assignments are as follows: 1, human (non-His tag); 2, His-tagged human; 3, HYB; 4, HYP; 5, HYH; 6, His-tagged HSp; 7, His-tagged *Sc. pombe*; 8, His-tagged SpH; and 9, His-tagged SpY. Blot B lane assignments are as follows: 1, *S. cerevisiae*; 2, YstTrun1; 3, YstTrun2; 4, YHB; 5, YHB/Y168C; 6, YHY; 7, YHY/Y168C; 8, YHP; 9, YSp; and 10, YSp/Y168C.

Consequently, it was necessary to examine the enzymatic properties of HYB in *E. coli* Δ*hem H* with crude enzyme assays. Since these cells totally lack their own endogenous ferrochelatase, any enzyme activity must be attributable to the vector-encoded enzyme. It was not possible to determine a V_{max}, but kinetic data yielded an apparent K_m for Fe^{2+} of 10 μM; the apparent K_m for mesoporphyrin IX was 450 μM. These data show a similar K_m for Fe^{2+}, while the K_m for mesoporphyrin is approximately 10-fold higher than that of human or *S. cerevisiae* ferrochelatase. From crude assays, we were able to determine that HYB possesses approximately 50% of the wild-type activity. Whole-cell EPR demonstrated that the HYB mutant did not possess a [2Fe-2S] cluster.

A second chimera, HSp, also possessed ferrochelatase activity as determined by its ability to rescue *E. coli* Δ*hem H*. The HSp ferrochelatase protein is as stable as native human ferrochelatase and can be purified to homogeneity. Kinetic and spectroscopic studies demonstrate that this protein possesses properties that are more similar to those of *Sc. pombe* than human ferrochelatase. All three of these proteins have an apparent K_m for iron of 10 μM, while the K_m for mesoporphyrin was similar to that of the value obtained for *Sc. pombe*, which is approximately 10-fold higher than that of human ferrochelatase. V_{max} values for human, *Sc. pombe*, and HSp were very similar and ranged from 3.4 to 5.4 min^{-1}. UV−visible spectroscopy of the HSp mutant clearly showed the presence of a [2Fe-2S] cluster (Figure 3). The [2Fe-2S] cluster was studied further by EPR spectroscopy (Figure 4). The HSp EPR spectrum was found to be distinct from that obtained for human (*16*) and *Sc. pombe* ferrochelatase [2Fe-2S] clusters (manuscript in preparation).

Interestingly, no *Sc. pombe*−*S. cerevisiae* chimeras were obtained that had any measurable enzyme activity or possessed an intact [2Fe-2S] cluster, even when the Y168C mutation was present in the *S. cerevisiae* portion of the enzyme to provide the fourth cysteine ligand.

DISCUSSION

Following the early reports on cloning of eukaryotic and prokaryotic ferrochelatases, it was noted that the eukaryotic proteins possessed an additional 30−50-amino acid carboxyl-terminal extension that was lacking in the prokaryotic ferrochelatases (*15*). The purpose of this segment was not known, although our earlier experiments demonstrated that

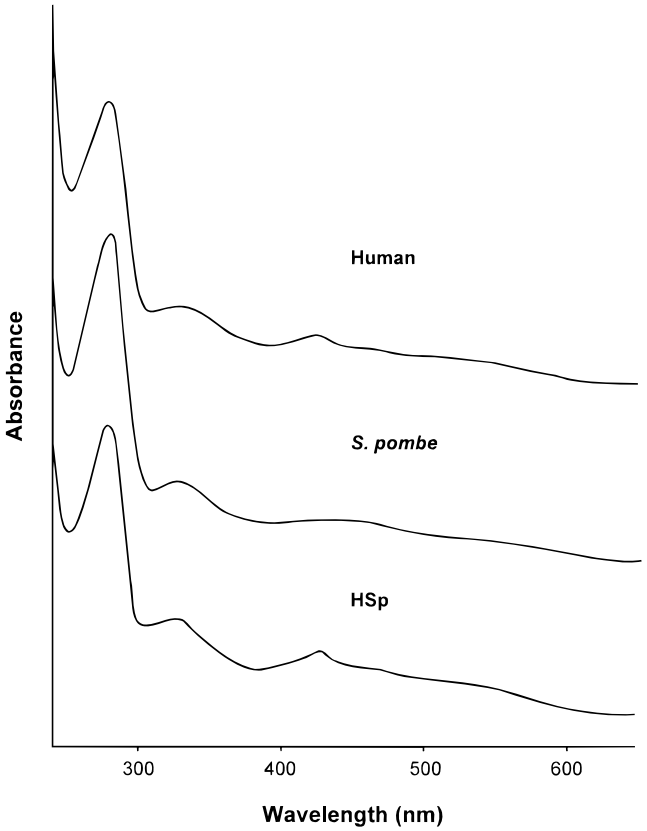

FIGURE 3: UV−visible spectra of human, *Sc. pombe*, and HSp. Protein spectra exhibit absorption at 278 nm along with absorption at ~330, ~460, and ~550 nm. These data features are characteristic of [2Fe-2S] clusters. Protein samples are in 50 mM Tris-Mops (pH 8.1), 0.1 M KCl, and 1.0% sodium cholate.

removal of this region from human ferrochelatase resulted in the loss of enzyme activity (*15*). Since the initial discovery of the [2Fe-2S] cluster in human ferrochelatase (*15*) a variety of hypotheses have been forwarded for its potential role. Since many organisms possess a ferrochelatase that lacks the cluster and still functions well, it is clear that the cluster does not play a direct role in catalysis. The sensitivity of the mammalian cluster to NO prompted the suggestion that it may play a role in host immune response (*23*), but the subsequent finding of the cluster in lower animals, including *Drosophila* (*11*), and more recently in *Sc. pombe* ferrochelatases (manuscript in preparation) diminishes support for this hypothesis. A role in the structural stabilization is a possibility, but the recent structure determination of human ferrochelatase (*24*) demonstrates that the cluster plays a minor role in dimerization of the protein, but is not involved in maintenance of the active site conformation. Still unexplored roles for the cluster may be in fine-tuning of enzyme activity via redox sensing or in serving to prevent inopportune oxidation of the substrate ferrous iron in a fashion analogous to that of the enzyme-bound NADPH of mammalian catalase (*25*).

We have found that several animal, but not *S. cerevisiae*, ferrochelatases possess a [2Fe-2S] cluster (*10*, *11*, *15*, *16*) ligated by four cysteine residues (*11*) and that three of these cysteine residues are present in the 30 carboxyl-terminal residues of the enzyme (*26*). With the recent identification of the four [2Fe-2S] cluster ligands (*11*) and the availability of additional ferrochelatases with carboxyl-terminal extensions, we have reexamined this region of ferrochelatase via

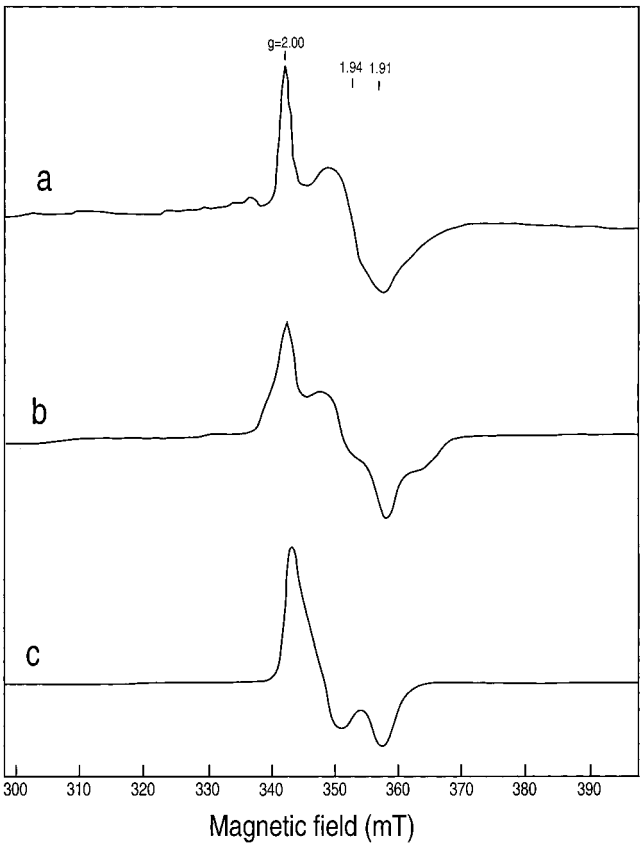

FIGURE 4: EPR spectra of human (a), *Sc. pombe* (b), and HSp (c). Samples were reduced in an anaerobic chamber with sodium dithionite. Spectra were recorded at 35 K, with a microwave power of 1 mW, a modulation amplitude of 0.63 mT, and a microwave frequency of 9.6 GHz. The *g* values that are given are those of the human protein spectrum.

the production of chimeric proteins in an attempt to elucidate the role that this structural feature plays in those ferrochelatases which possess it. Although there is a relatively high degree of sequence identity and similarity among the core regions of eukaryotic ferrochelatases, the 30 carboxyl-terminal amino acid residues of the yeasts *S. cerevisiae* and *Sc. pombe* and animal ferrochelatases have little sequence similarity (Figure 5).

For animal ferrochelatases, it is known that elimination of any one of the cysteine ligands prevents the assembly of the [2Fe-2S] cluster and causes concomitant loss of enzyme activity (*11*, *26*). However, it is clear that the cluster per se is not required for catalysis since *S. cerevisiae* ferrochelatase lacks this feature and has enzyme activity (*16*). The demonstration that two *S. cerevisiae* carboxyl-terminal truncations have no activity suggests that the carboxyl-terminal segment itself may be required to maintain activity. The X-ray crystal structure of human ferrochelatase reveals that one role for this segment is in stabilization of the enzyme homodimer (*24*). Thus, elimination of this segment will prevent dimerization, but that does not explain the loss of activity since the ferrochelatase of *B. subtilis* lacks this carboxyl-terminal segment, is monomeric, and is enzymatically active (*3*).

As an approach to characterizing the role of the carboxyl-terminal extension and the [2Fe-2S] cluster, a number of yeast−human chimeric mutants in which the carboxyl-terminal residues of the yeast and human ferrochelatases have been exchanged were produced and characterized. These chimeras consisted of all possible combinations involving human, *S. cerevisiae*, and *Sc. pombe* ferrochelatases. In addition, for chimeras that involved the *S. cerevisiae* core region with the human or *Sc. pombe* carboxyl-terminal segment, a *S. cerevisiae* Y168C mutation was produced since this residue corresponds to the fourth cysteinyl ligand in human and *Sc. pombe* (*11*). Thus, all four cysteine residues that are necessary for ligation of the [2Fe-2S] cluster are present in these chimeras.

Of the 13 chimeras produced in the current work, only two exhibited enzyme activity. No chimera with a *S. cerevisiae* or *Sc. pombe* core possessed any measurable enzyme activity. Previously, Gora et al. (*27*) reported that a yeast−mouse chimeric ferrochelatase similar in design to the YHB chimera described above had no enzyme activity, but they claimed that their enzyme contained a [2Fe-2S] cluster because of the color of the cells that were expressing the chimeric protein. None of our yeast−human chimeras, including the YHB, possessed any measurable ferrochelatase activity, and none, including the one with the engineered fourth cysteine ligand, had a [2Fe-2S] cluster as determined by spectral characterization of the purified chimeric enzymes or by whole-cell EPR measurements of chimeric ferrochelatase-producing *E. coli* cells. On the basis of these observations and the fact that their yeast−mouse chimera lacked one of the four cluster ligands, we suggest that the chimera of Gora et al. most likely did not possess a [2Fe-2S] cluster.

One of the two chimeric ferrochelatases described above that did have enzyme activity is the HYB chimeric. This is analogous to the mouse−yeast chimera constructed by Gora et al. (*27*). They reported that they were unable to obtain an active mouse−yeast chimera and that this may have been due to protein stability. This appears to be reasonable since HYB, our human−yeast chimera, exhibits good enzyme activity in crude cell extracts, but we were unable to purify active enzyme using either the blue affinity column protocol or metal matrix chromatography of the His-tagged enzyme. This HYB enzyme is interesting since we can find no evidence of a cluster (as one would expect since it lacks three of the ligating residues), yet it possesses enzyme activity similar to that of the wild-type ferrochelatases from which it was constructed. In contrast, the HYH chimera has no activity even though it differs from the HYB enzyme only in having the 10 carboxyl-terminal residues of the human enzyme replacing the corresponding yeast residues. Thus, the replacement of the 30 terminal residues with yeast residues results in an active enzyme, but substitution of 20 yeast residues located 10 residues upstream from the carboxyl end yields an inactive protein. These data suggest that this region of ferrochelatase is extremely sensitive to minor alterations in amino acid sequence even though it exists at the distal end of the protein and is not a highly conserved region. This is consistent with the previous observation that an alteration in just F417 (located seven residues from the carboxyl terminus) results in a loss of enzyme activity (*15*).

The second chimera described above that had enzyme activity was HSp. This protein has near-normal enzyme activity, is as stable as either human or *Sc. pombe* ferrochelatase, and contains a [2Fe-2S] cluster. While the EPR signal of HSp is similar to those of both human and *Sc. pombe* ferrochelatase EPR spectra, it is distinct from both, suggest-

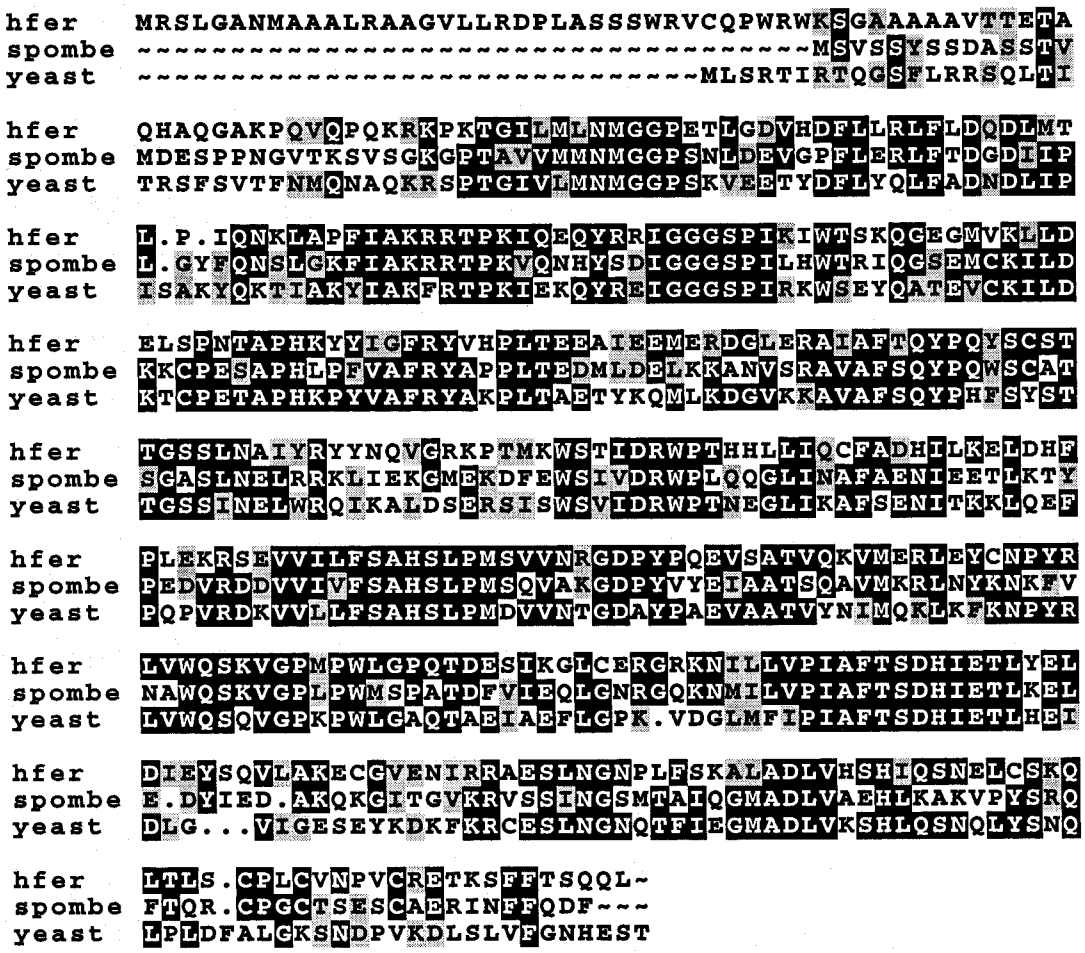

FIGURE 5: Amino acid sequence comparison of human (hfer) (*14*), *Sc. pombe* (spombe) (GenBank accession no. AL022245), and *S. cerevisiae* (yeast) (*7*) ferrochelatases. The sequence of the human ferrochelatase that is produced in the current studies differs from that of Nakahashi et al. at residue 105, which we find to be R rather than Q. Homologous residues are shaded in gray, and identical residues are shaded in black. Asterisks below sequences mark the four coordinating cysteine residues of the [2Fe-2S] cluster in the human and *Sc. pombe* sequences. Alignment was carried out using the Genetic Computer Group program pileup (*28*).

ing that the cluster environment for the chimera is altered from that found in either the human or *Sc. pombe* enzyme. As was found with the HYB enzyme, HSp ferrochelatase has a normal K_m for ferrous iron, but an elevated K_m for the porphyrin substrate. This is of interest because we now know from the crystal structure that this segment of the protein is not contiguous with the active site, yet it causes an alteration in the affinity for one of the substrates. Ongoing studies may elucidate structural features that may explain this observation.

The data presented above demonstrate two important points. First, the activity of animal ferrochelatases does not require the presence of the [2Fe-2S] cluster. This is clearly shown by the HYB chimera, which lacks the cluster, but has activity. Normal *S. cerevisiae* ferrochelatase obviously does not require a cluster for activity, but before the current data had been elucidated, it could have been argued that yeast specific residues, such as the phenolic side chain of tyrosyl residues, exist that satisfy the function served by the animal cluster. The crystal structure clearly shows that none of the carboxyl terminus is in, or physically contributes to, the active site (*24*); thus in the HYB chimera, the catalytic machinery is composed entirely of human ferrochelatase with none of the *S. cerevisiae* carboxyl-terminal segment in the active site area. Interestingly, the observation that the HYB and HSp chimeras have altered kinetic parameters for the porphyrin substrate suggests that some structural communication at a distance must exist.

The second point is that there exists a high degree of stringency in acceptable residues in the carboxyl terminus. Previous and present work has shown that truncation or amino acid alterations are not generally acceptable. The finding that only 2 out of 16 chimeras had activity, as well as the observation that even chimeras between two [2Fe-2S] cluster-possessing ferrochelatases (i.e., *Sc. pombe* and human) were inactive, except in one case, reinforces this point. One possible explanation for this is that the carboxyl terminus is centrally involved in homodimer formation and a majority of the dimer-stabilizing hydrogen bonds are between the carboxyl terminus of one monomer with core residues on the second monomer. Thus, the inability to form an active enzyme may be related to the inability to form a stable dimer and the fact that dimer formation is key to overall protein stability. Definitive answers to this question may require biochemical characterization and structure determination of additional ferrochelatases, or saturation mutagenesis studies on the carboxyl-terminal segment.

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