Evidence That the Fourth Ligand to the [2Fe-2S] Cluster in Animal Ferrochelatase Is a Cysteine

CHARACTERIZATION OF THE ENZYME FROM DROSOPHILA MELANOGASTER*

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The terminal enzyme in heme biosynthesis, ferrochelatase (E.C. 4.99.1.1), catalyzes the insertion of iron into protoporphyrin IX. Nuclear-encoded and produced in the cytoplasm, ferrochelatase is proteolytically processed upon translocation into the mitochondrion. In eukaryotes, the mature-length 42,000 Da protein is associated with the inner mitochondrial membrane, with the active site facing the mitochondrial matrix (1). The proposed catalytic mechanism (2) initially involves a metal-dependent, enzyme-mediated distortion of the bound porphyrin ring allowing rapid insertion of Fe^{2+} into the bent porphyrin (3). Distinct from the enzyme's catalytic activity, a labile [2Fe-2S]^{2+} cluster has been identified in several animal ferrochelatases including human (4), mouse (5), chicken, and frog (6). Although similar to the [2Fe-2S]^{2+} centers found in plant ferredoxins, the ferrochelatase iron-sulfur cluster is more labile with enhanced sensitivity to degradation by nitric oxide (7).

That the cluster plays no direct role part in catalysis is evidenced by the observation that bacterial, plant, and yeast ferrochelatases do not contain the metal center, and by studies showing that the redox state of the cluster is inconsequential to enzyme activity (4). However, when the cluster is disassembled, enzyme activity is lost (4, 7) and the protein readily precipitates. Hence the cluster appears to play a crucial role in maintaining protein structure in animal ferrochelatases and coupled with the sensitivity to nitric oxide, this has lead us to postulate that the cluster may serve as a regulatory NO-sensor as part of an immune response (7).

Site-directed mutagenesis of the five conserved cysteines closest to the COOH terminus of recombinant human ferrochelatase, identified Cys-403, Cys-406, and Cys-411 as ligands to the [2Fe-2S] cluster (8). Additionally, at this time it was proposed that certain spectroscopic anomalies and the unusual lability of the cluster may result from one noncysteinyl oxygenic ligand. The objective of the present investigation was to identify the fourth cluster ligand via additional mutagenesis experiments and by cloning, expression, and characterization of cluster-containing ferrochelatases from more distantly related organisms. We report here the first characterization of ferrochelatase from Drosophila melanogaster and, together with the new mutagenesis results with the human enzyme, these new data support the assignment of the cysteine located at position 196 in the human ferrochelatase as the fourth cluster ligand. Tracing the biological evolution of the iron-sulfur cluster in ferrochelatase also provides further insight concerning the specific role of this metal center.

EXPERIMENTAL PROCEDURES

Strains and Cell Culture—Escherichia coli strain JM109 was used to express recombinant human ferrochelatase, mutant human ferrochelatase, and recombinant Drosophila ferrochelatase as described elsewhere (9). For the mutagenesis procedure described below, E. coli strain BHI mut-s was used to amplify the mutant plasmid. E. coli strain DW35 3fo(labcD) and ΔsdhC::kan was used for whole cell EPR as described previously (8). This strain of E. coli has been altered so that any interference from the metal centers in fumarate reductase and

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1 The abbreviations used are: NO, nitric oxide; MOPS, 4-morpholinepropanesulfonic acid.
succinate dehydrogenase is eliminated during whole cell EPR studies (10). *E. coli* *hem* H, a ferrochelatase-deficient strain (11), which requires exogenous heme for survival, was used in the cloning of *Drosophila* ferrochelatase (12).

**Construction of Human Ferrochelatase C196S Mutation—**Site-directed mutagenesis of recombinant wild-type human ferrochelatase pHDTP20 (9) was performed using a modification of the published procedure of Deng and Nickoloff (13). The C196S mutation was produced using an antisense selection primer, which converts a *Spy*HI site to an *Nhe*I site in the noncoding region of the vector, and an antisense mutagenic primer containing a single base change to create the Cys to Ser mutation. The procedure was modified by performing the extension or synthesis reaction at a high temperature to improve primer specificity using high temperature Vent DNA polymerase and *Taq* DNA ligase (New England Biolabs, Boston, MA) at the *Tm* of the mutagenic primer. To purify the resulting DNA, the mixture was phenol/chloroform-extracted, then chloroform-extracted, and ethanol-purified. Then, following digestion with *Spy*HI, the mixture was used to transform *E. coli* JM109. The resulting colony was used to transform *E. coli* strain JD109. Colonies were screened for the presence of the new *Nhe*I site by digestion. Positives were confirmed by double-stranded sequencing at the Molecular Genetics Instrumentation Facility, University of Georgia, Athens, GA. Recombinant C196S mutant human ferrochelatase was purified as described previously (9).

**Cloning and Expression of Drosophila melanogaster Ferrochelatase—**Drosophila melanogaster ferrochelatase cDNA was isolated using complementation of *E. coli* *hem* H, a ferrochelatase-deficient strain (11), with a λ cDNA library. The λ ZAP cDNA library (Stratagene Inc.) was rescued in the pBluescript phagemid according to the in vivo mass excision protocol of Stratagene, using the Exassist/SOLR system. The pBluescript phagemid was then electroporated into *E. coli* *hem* H. Recovered clones, which complemented the ferrochelatase-deficient cells, were assayed for ferrochelatase activity. Following the isolation of plasmid DNA, the DNA sequence was obtained by the Molecular Genetics Instrumentation Facility. For high levels of expression and simple purification, an expression vector containing a six-histidine NH2-terminal leader was constructed (14). Polymerase chain reaction was used to incorporate the His6 tag upstream from the processed length of the protein’s NH2 terminus and to incorporate appropriate restriction sites. The sense primer, 5′-GAT-GCT-CCATTGA-CAC-CAT-CAT-CAT-CAC-GGT-CTG-GCA-GGA-GTG-C-3′, was used to create an *Nco*I site (underlined) and 6 histidine codons. The antisense primer, 5′-GCG-CCG-CGA-TTA-GTT-GCA-GCA-CTG-GCG-3′, corresponds to the 3′ coding region containing the stop codon and was used to incorporate a *Bgl*II site (underlined). The polymerase chain reaction contained 50 ng of recovered *Drosophila* ferrochelatase DNA as a template, 1 μM of each primer, 10 μl of polymerase buffer, 20 μM dNTPs, 2 mM MgCl2, and 5 units of *Taq* DNA polymerase in a total volume of 100 μl. The polymerase chain reaction product was isolated and cloned into the *Nco*I site of *pTrcHisC* (Invitrogen, Carlsbad, CA). The resulting *Drosophila* ferrochelatase expression vector, named *pTDFc*, was used to transform electrocompetent *E. coli* strain JD109.

**Purification of Drosophila Ferrochelatase—**One-liter cultures of *C. elegans* (BIO 101, Inc.) supplemented with 100 ng/ml ampicillin were inoculated with *pTDFc* in *E. coli* JD109. Cultures were grown 18–20 h at 37 °C with shaking. Cells were harvested by centrifugation at 9,000 × g for 15 min at 4 °C, suspended in solubilization buffer (50 mM Tris·HCl, pH 8.1, that contained excess sodium dithionite for 15 min at 4 °C, suspended in solubilization buffer (50 mM Tris-MOPS, pH 8.0, 0.1 M KCl, 1% sodium cholate). The cells were then sonicated on ice three times for 30 s and centrifuged at 100,000 × g for 30 min at 4 °C. The supernatant was loaded onto a TALON matrix (CLONTECH Palo Alto, CA) column that was previously equilibrated with sodium cholate buffer. The column was washed with 30 ml of 50 mM Tris-MOPS, pH 8.0, 0.1 M KCl, 0.2% sodium cholate. Recombinant *Drosophila* ferrochelatase was eluted in 50 mM Tris-MOPS, pH 8.0, 0.1 M KCl, 0.2% sodium cholate, and 200 mM imidazole. Protein purity was assessed by SDS-polyacrylamide gel electrophoresis (15).

**Procedures and Sources—**Ferrochelatase activity was determined using the pyridine hemochromagen assay (16). Ferrous iron and protoporphyrin (Porphyrin Products, Logan, UT) were the substrates used in the assay. The UV absorption at 278 nm, ε = 46,900 M cm−1, was used to determine protein concentration. UV-visible spectra were recorded using a Cary-G1 spectrophotometer. X-band EPR spectra were obtained with a Bruker ESP 300E EPR spectrometer fitted with an Oxford Instruments ESR-9 liquid helium flow cryostat. Whole cell EPR samples were spun down at low rpm, to avoid cell lysis, and then transferred into an anaerobic chamber. The cell pellet was suspended in a solution of 500 mM Tris-HCl, pH 8.1, that contained excess sodium dithionite and then transferred to an EPR tube. A small amount of chloroform was stirred into each EPR tube immediately prior to freezing. Oligonucleotides for mutagenesis, cloning, and sequencing were synthesized with an Applied Biosystems model 391 DNA synthesizer.

**RESULTS**

**Characterization of the C196S Mutant of Human Ferrochelatase—**Several new mutant forms of human ferrochelatase were constructed and characterized in this work. Each involved point mutations leading to substitution for conserved residues that were considered potential candidates for cluster ligation, i.e. C196S, S387C, S396C, S402C, S416C, and S420C. As evidenced by UV-visible absorption, EPR, and activity studies of purified enzymes, the [2Fe-2S] cluster was assigned in each of the serine mutants with no discernible affect on the spectroscopic properties of the cluster or the enzymatic activity (data not shown).

In contrast, the UV-visible spectrum of purified C196S ferrochelatase (Fig. 1) shows no sign of the Fe-S cluster. The band at 330 nm and a broad absorption feature ranging from ~400 to 600 nm that are characteristic of the [2Fe-2S]2+ center (4, 5, 7), are absent in the C196S spectrum. Moreover, neither cell extracts nor the purified protein exhibited any ferrochelatase activity. To investigate the possibility that a cluster is assembled but lost during enzyme purification, whole cell EPR was used to assess the presence of a reduced cluster in intact cells expressing C196S human ferrochelatase (8). No EPR resonance was discernible above background for the C196S mutant expressed in *E. coli* DW35 3fdrABCDD and ΔsdhC::kan (data not shown). As for the point mutants involving the cysteines at positions 403, 406, and 411 (8), this result implies that the metal center is not assembled in cells expressing the C196S mutant of human ferrochelatase.

**Expression and Purification of Drosophila Ferrochelatase—**The 1.4-kilobase *Drosophila melanogaster* ferrochelatase cDNA obtained by complementation contains 1,152 nucleotides
(Fig. 2) that encode a 384-amino acid protein. As isolated, the 5′-untranslated region contains only 6 bases, a property which may have enabled rather successful complementation. The 3′-untranslated region contains 170 bases with the putative pol-yadenylation site underlined at position 1,295 (Fig. 2). From the amino acid sequence of the processed length ferrochelatase, the molecular weight is approximately 41 kDa. Although homologous to the previously known animal ferrochelatases, *Drosophila* ferrochelatase contains a higher percentage of charged amino acids (Fig. 3). Also, the mitochondrial-targeting leader sequence at the amino terminus is shorter than the leader sequences of the other eukaryotic ferrochelatases that have been investigated. Additionally, *Drosophila* ferrochelatase contains only four of the conserved cysteines, verifying that Cys-196, Cys-403, Cys-406, and Cys-411 are cluster ligands. Other published sequences of animal ferrochelatases contain two additional conserved cysteines, Cys-236 and Cys-323, but these residues are not conserved in *Drosophila* ferrochelatase (Fig. 3) and so were not subjects for site-directed mutagenesis.

The construction of the *Drosophila* ferrochelatase expression vector utilizes the addition of a His_{6} tag upstream from the amino terminus of the processed length protein. After expression in *E. coli* JM109, the protein is rapidly purified using metal chelate chromatography (Fig. 4). One liter of bacterial culture yields approximately 20 mg of purified *Drosophila* ferrochelatase. Purified *Drosophila* ferrochelatase is like animal ferrochelatases in general (4), requiring detergent to solubilize the protein and to keep it in solution.

**Characterization of Drosophila Ferrochelatase**—The UV-visible absorption spectrum of *Drosophila* ferrochelatase (Fig. 1) clearly demonstrates the presence of the iron-sulfur cluster. The spectrum is indistinguishable from that of other [2Fe-2S]^{2+} cluster-containing ferrochelatases (4, 5, 7), with the band at 410 nm resulting from residually bound porphyrin, which can be removed by further purification if necessary (17). Further evidence that the cluster is identical to that found in animal ferrochelatases comes from a comparison of the EPR spectra for samples reduced with dithionite. Fig. 5 shows a comparison of the EPR spectra of dithionite-reduced samples of recombinant chicken, *Drosophila* and human ferrochelatase. In each case, the resonances, *g* = 2.00, 1.94, 1.90–1.91, exhibit relaxation properties indicative of a [2Fe-2S]^{1+} cluster, i.e. readily saturated at microwave powers >0.2 mW at 10 K and observable without significant broadening up to 100 K. The only minor difference in the three spectra is a small increase in *g*-value anisotropy in order human, *Drosophila*, chicken, which results in a more resolved high-field component for chicken ferrochelatase. In addition, the [2Fe-2S] cluster in *Drosophila* ferrochelatase displays virtually the same sensitivity to nitric oxide as that of human ferrochelatase (7). Under identical conditions, the timescales for NO-induced cluster degradation in *Drosophila* and human ferrochelatase, using the S-
nitrosyl-N-acetylpenicillamine as the NO donor (18), were the same within experimental error (data not shown).

DISCUSSION

Previously we have presented data supporting a role for Cys-403, Cys-406, and Cys-411 as ligands for the \([2\text{Fe}-2\text{S}]^2\) cluster of animal ferrochelatase (8). In addition, anomalous spectral data were interpreted as possible support for involvement of an oxygen as the fourth cluster ligand. In the current work Drosophila ferrochelatase has been cloned, sequenced, expressed, and the isolated protein characterized. It was shown to contain a \([2\text{Fe}-2\text{S}]^2\) cluster with properties analogous to those found in animal ferrochelatases. The observation that only four cysteines are conserved between Drosophila and animal ferrochelatases, coupled with the mutagenesis results presented herein and in our previous mutagenesis studies (8) provides strong evidence that Cys-196, Cys-403, Cys-406, and Cys-411 ligate the cluster in human ferrochelatase. In addition results from site-directed mutagenesis experiments described above demonstrate that the potential oxygen ligands of Ser-387, Ser-396, Ser-402, Ser-416, and Ser-420 are not ligands. Unpublished data from this laboratory also rule out Asp-340, Glu-343, Glu-347, Tyr-123, Tyr-165, Tyr-191, and Tyr-276 because alteration of these residues does not alter the cluster, although some have a significant impact on enzyme kinetics. Data from others suggest that Ser-102 and Ser-174 (19) along with His-157, His-263, His-341, and His-388 (20) would probably not be involved in cluster ligation. Examination of the available ferrochelatase sequences show that Cys-196 is found only in animal and two bacterial ferrochelatases, and this position is occupied by serine in three bacterial enzymes, thus arguing against a general disruption of protein structure by the C196S alteration as an explanation for cluster loss. Inspection of the crystal structure for Bacillus subtilis ferrochelatase (21), the only known enzyme structure but one that lacks a cluster, shows that serine residues at positions 195, 261, and 303 par-
Cluster Ligands of Ferrochelatase

The key role of Fe-S clusters in determining protein structure makes them important regulatory targets for controlling enzyme activity or gene expression in response to external stimuli such as intracellular Fe, NO, O2, or O2 concentrations. Regulatory control can be accomplished via cluster degradation/assembly (e.g., the [4Fe-4S] clusters in the mammalian iron-regulatory protein-1 (24, 25) and \textit{B. subtilis} glutamine phosphoribosylpyrophosphate amidotransferase (26)), cluster interconversions (e.g., [4Fe-4S] + [2Fe-2S] conversions in \textit{E. coli} fumarate nitrate reductase regulatory protein (27, 28) and possibly the emerging class of S-adenosylmethionine-dependent Fe-S enzymes (29)), or redox processes (e.g., the [2Fe-2S]^{2+} cluster in \textit{E. coli} SoxR (30, 31)). Our current working hypothesis for ferrochelatase is that NO-induced degradation of the [2Fe-2S] cluster is part of a local immune response that prevents bacteria from using heme synthesized by the host organism (7) and that the requirement for iron to assemble the cluster in ferrochelatase may, in itself, present an additional iron regulatory step in heme biosynthesis during erythropoiesis.

The arrangement of cluster ligands in ferrochelatase also explains the absence of the cluster in several exon deletion mutants. A genetic defect in human ferrochelatase causes the disease erythropoietic protoporphyria. Exon deletions are common causes of this disease (32–36). A set of mutants containing individual deletions of exons 3 through 11 was previously constructed to study the underlying biochemical nature of erythropoietic protoporphyria (37). These exon deletion mutants possessed no enzyme activity, and in each case the Fe-S cluster failed to assemble. With cluster ligands spanning the mature-length protein, the excision of entire exons must affect the ability of the protein to properly assume its native conformation with an intact iron-sulfur cluster. If the cluster itself is responsible for maintaining the protein structure, failure to generate the cluster may result in a protein that never reaches its proper conformation. Distant spacing of cluster ligands may be crucial for regulation of global as opposed to local structure.

The identification of complete cysteinylation coordination for the ferrochelatase Fe-S cluster also has important consequences for the interpretation of resonance Raman and variable temperature magnetic circular dichroism spectra of [2Fe-2S] centers. On the basis of spectral comparisons with mutant ferredoxins with one noncysteinyl ligand, it was argued that the Fe-S stretching frequencies of the oxidized [2Fe-2S]^{2+} center and the pattern of S → Fe(III) charge transfer bands in the variable temperature magnetic circular dichroism spectrum of the reduced [2Fe-2S]^{+} center could be interpreted in terms of one oxygenic ligand at the nonreducible Fe site of the [2Fe-2S] cluster in human ferrochelatase (8). It now seems likely that the overall simplification of the S → Fe(III) charge transfer region in the variable temperature magnetic circular dichroism spectrum of reduced human ferrochelatase is a consequence of heterogeneity resulting in poor spectral resolution, rather than partial noncysteinyl ligation, and that there is greater overlap in the ranges of ligand-sensitive Fe-S stretching modes than previously established. For example, with the inclusion of ferrochelatase as a cluster with complete cysteinylation coordination, the lowest energy Fe-S stretching mode, which involves out-of-phase combination of the symmetric Fe-S(Cys) stretching, has now been observed in the range 281–295 cm^{-1} for [2Fe-2S]^{+} clusters with complete cysteinylation coordination and in the range 289–302 cm^{-1} for [2Fe-2S]^{+} clusters with one oxygenic ligand. The present study serves to demonstrate that although these techniques are useful in suggesting the possibility of anomalous cluster coordination, they clearly require confirmation in the form of structural or mutagenesis evidence.

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