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Identification of [2Fe-2S] Clusters in Microbial Ferrochelatases

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The terminal enzyme of heme biosynthesis, ferrochelatase (EC 4.99.1.1), catalyzes the insertion of ferrous iron into protoporphyrin IX to form protoheme. Prior to the present work, [2Fe-2S] clusters have been identified and characterized in animal ferrochelatases but not in plant or prokaryotic ferrochelatases. Herein we present evidence that ferrochelatases from the bacteria Caulobacter crescentus and Mycobacterium tuberculosis possess [2Fe-2S] clusters. The enzyme from C. crescentus is a homodimeric, membrane-associated protein while the enzyme from M. tuberculosis is monomeric and soluble. The clusters of the C. crescentus and M. tuberculosis ferrochelatases are ligated by four cysteines but possess ligand spacings that are unlike those of any previously characterized [2Fe-2S] cluster-containing protein, including the ferrochelatase of the yeast Schizosaccharomyces pombe. Thus, the microbial ferrochelatases represent a new group of [2Fe-2S] cluster-containing proteins.

The enzyme ferrochelatase (EC 4.99.1.1) catalyzes the insertion of ferrous iron into protoporphyrin IX to form protoheme IX (heme) (4, 6). This enzyme is present in essentially all eukaryotes and prokaryotes with the exception of a few obligate pathogens and some anaerobic prokaryotes. Recently a gene sequence that appears by homology to be ferrochelatase was reported for the archaeon, Thermoplasma acidophilum (17). A comparison of all currently known ferrochelatase sequences reveals the existence of three distinct segments or domains (6). The first segment (I) is present in all eukaryotes and is identifiable as an amino-terminal organelle-targeting motif that is proteolytically removed after organelle targeting (12, 16). The second (II) represents the core 330 amino acid residues of the enzyme and is present in all ferrochelatases. The third (III) is a 30- to 50-amino-acid-residue sequence found at the carboxyl terminus of some ferrochelatases. Region III of animal ferrochelatases, which is involved in the dimerization motif for these enzymes (21), contains three of the four cysteinyl ligands for a [2Fe-2S] cluster with the fourth cluster ligand present in region II (3, 5, 19, 21). Region III of plant ferrochelatases is approximately 50 residues in length, and its role is currently unknown (6). Plant ferrochelatases do not possess the cysteinyl residues in region III and do not contain [2Fe-2S] clusters. The ferrochelatase of the yeast Saccharomyces cerevisiae, which has been cloned, purified, and characterized (9, 10, 13), possesses region III, does not contain any cysteinyl residues in this region, and does not possess a [2Fe-2S] cluster. The gene for ferrochelatase of the yeast Schizosaccharomyces pombe has been sequenced (GenBank accession number AL022245) and, interestingly, revealed the presence of four cysteinyl residues in the same sequence position as is found in animal ferrochelatases. Previously, as part of a study on the carboxyl termini of eukaryotic ferrochelatases, it was determined that S. pombe ferrochelatase contains a [2Fe-2S] cluster (14).

All bacterial ferrochelatases previously biochemically characterized possessed only region II (see references 6 and 11). However, with the plethora of genome sequencing projects, there are now several putative ferrochelatases in the bacterial genome databases which also possess a region III in their derived amino acid sequences. Of the region III-possessing bacterial ferrochelatases identified to date, all contain cysteinyl residues, but none have the same cluster-ligating cysteinyl residue motif as is found in animal ferrochelatases or other [2Fe-2S]-containing proteins.

In the present work we have expressed, purified, and characterized ferrochelatases from the bacteria Caulobacter crescentus and Mycobacterium tuberculosis. Both of these possess region III. The C. crescentus enzyme is homodimeric and membrane-associated and contains [2Fe-2S] clusters. However, although the M. tuberculosis ferrochelatase contains region III and a [2Fe-2S] cluster, it is monomeric and soluble.

MATERIALS AND METHODS

Cloning of the ferrochelatase genes from C. crescentus and M. tuberculosis. The DNA sequence coding for the putative C. crescentus ferrochelatase was found in The Institute for Genomic Research database with Drosophila ferrochelatase as the query sequence. C. crescentus genomic DNA was a gift from Michael Laub. The primers used for PCR of the full-length clone from the genomic DNA were sense, 5'-GGTTCTTGAGTACCCAGAAGCTGCGTCG-3' and antisense, 5'-GGTTCTTAAGGTTTCACGGCAAGGGCTTCG-3'. The italicized portion was added to facilitate cloning (2); restriction sites for cloning are in bold; the underlined portion is homologous to the target gene. The gene was amplified with Pfu polymerase (Promega), cleaned with Qiagen (Qiagen), and cloned into pTrcHisA (Invitrogen) by using the Nhel and HindIII cloning sites.

Mutagenesis of C. crescentus ferrochelatase residues H104A, T133C, T133A, and DIII cloning sites. The mutagenesis was accomplished by using Quikchange mutagenesis (Stratagene). The Δ328-347 truncation was accomplished with the same sense primer as for the wild-type expression plasmid and the antisense primer 5'-GGTTCTTAAGGTTTCACGGCAAGGGCTTCG-3'; the coding region was amplified by PCR and cloned into pTrcHisA by using the Nhel and HindIII sites as described above. Following confirmation of ferrochelatase activity, the sequence for C. crescentus ferrochelatase was submitted to GenBank.
RESULTS

Ferrochelatases of *C. crescentus* and *M. tuberculosis* were cloned and expressed as hexahistidine amino-tagged proteins in *E. coli* in the vector pTrcHisA. The *C. crescentus* enzyme was efficiently expressed by *E. coli* and yielded at a rate of approximately 10 mg per liter of culture. The yield of the *M. tuberculosis* preparations were somewhat lower, approximately 2.5 mg per liter of culture. The single Talon matrix column was sufficient to obtain homogeneous protein preparations (not shown). Unlike most animal ferrochelatases, the ferrochelatases of *C. crescentus* and *M. tuberculosis* were relatively stable upon storage at 4°C. The *C. crescentus* enzyme preparations remained in solution and active after over a week of storage at 4°C, and surprisingly, the characteristic spectra of the cluster remained even in the presence of 1% (wt/vol) SDS at room temperature for several minutes. In order to quantitate the iron content of this enzyme it was necessary to include 2% (wt/vol) SDS, ferrozine, and ascorbic acid in the buffer and to heat the sample to 90°C. Under these conditions, a stoichiometry of 1.6 Fe/mole of enzyme was found. The determination of subunit composition for the purified proteins was done by FPLC in the presence of 1.0% (wt/vol) sodium cholate. The *C. crescentus* enzyme was found to be a dimer based upon the predicted monomer molecular weight of 38,491. This observation is consistent with the suggestion based on the structure of human ferrochelatase that dimerization is mediated by carboxyl-terminal extension (21), which this ferrochelatase possesses. The enzyme required detergent for solubilization and partitioned into the detergent phase, rather than the aqueous phase, of the Triton X-114 two-phase systems, thereby demonstrating a hydrophobic nature, which is consistent with membrane association. This observation is consistent with the proposal that membrane association for ferrochelatase is attributable to the presence of a hydrophobic surface largely composed of a 12-amino-acid-residue region that is present as a hydrophobic lip on the active site pocket of human ferrochelatase (6, 21). It is present in both *S. pombe* (T. A. Dailey and H. A. Dailey, unpublished data) and *C. crescentus* ferrochelatases, but it is absent in known soluble ferrochelatases such as *Bacillus subtilis* ferrochelatase (11). The ferrochelatase of *M. tuberculosis* differs from the *C. crescentus* enzyme in that it does not partition into the detergent phase of the Triton X-114 two-phase system, indicating that it is not membrane associated, and by FPLC it was determined to be monomeric based upon its predicted molecular weight of 41,043.

Kinetic parameters were determined for the enzymes. For *C.
**C. crescentus** the apparent $K_m$ s are 6.2 $\mu$M for protoporphyrin and 30 $\mu$M for iron. The $V_{max}$ is 14.5 min$^{-1}$. Interestingly, **C. crescentus** ferrochelatase does not use cobalt as do **S. pombe** (data not shown) and other animal ferrochelatases, but it does catalyze metallization with nickel with an apparent $K_m$ of 13 $\mu$M and a $V_{max}$ of 16.5 min$^{-1}$. Kinetic parameters were not determined for the **M. tuberculosis** enzyme since the activity of that enzyme was very low. Currently we do not have an explanation for this low level of enzyme activity.

The absorbance spectra for ferrochelatases of all three organisms contain features characteristic of [2Fe-2S] cluster-containing proteins (Fig. 1). The amino acid sequence of **S. pombe** ferrochelatase contains cysteines analogous to the [2Fe-2S] cluster-ligating cysteines that are found in animal ferrochelatases. However, **C. crescentus** ferrochelatase does not possess cysteines in these same positions, so a series of site-directed mutants were prepared and examined to determine the cluster ligands (Table 1). Since the spacing of the available cysteine residues of this protein differs dramatically from what has been found for animal ferrochelatases (Fig. 2), five cysteine residues, one histidine residue, and one serine residue were individually mutated. In addition, one triple mutation in the region from 132 to 134 was made and a carboxyl-terminal truncation was engineered. The data obtained with these mutants are consistent with a four-cysteinyl ligation involving C158, C332, C339, and C341.

**C. crescentus** has a four-cysteinyl ligation involving C158, C332, C339, and C341.
DISCUSSION

Prior to the present work it was generally believed that no prokaryotic ferrochelatases possessed [2Fe-2S] clusters (4, 6). The yeast S. cerevisiae (13) and plant ferrochelatase sequences do not contain the cysteine ligands involved in cluster ligation in animal ferrochelatases, and the expression of nonanimal ferrochelatases had revealed no evidence of clusters. The initially published bacterial ferrochelatase sequences did not contain the carboxyl-terminal domain III (see references 6 and 11) which is necessary for cluster formation in animal ferrochelatases (3–6, 8, 19, 21), and there was no spectral evidence of a cluster in expressed bacterial ferrochelatase proteins. Thus, the tacit assumption has been that the [2Fe-2S] cluster arose during evolution in the animal kingdom. Because of this proposition, explanations for the cluster’s role became related to the evolution of multicellular animals (6, 8, 20). The data presented previously (14) and above, however, clearly show that the cluster exists not only in multicellular animals but also in at least one unicellular eukaryote and, most significantly, in some prokaryotes.

Since no [2Fe-2S] cluster has ever been reported for a bacterial ferrochelatase, the discovery of a [2Fe-2S] cluster in C. crescentus ferrochelatase was unexpected. Visual examination of the primary sequence revealed the presence of a carboxyl-terminal extension of a length similar to that of animal ferrochelatases, and this region contains three cysteine residues as are found in animal ferrochelatases (Fig. 2). However, the spacing of these residues (C-X2-C-X-C) is unlike that found in animal and S. pombe ferrochelatases (C-X2-C-X-C) (6–9, 14, 20). More significant, however, was that the most-amino-terminal cluster-ligating cysteine residue of animal (C196 of human ferrochelatase) and S. pombe (C162) ferrochelatases is absent in C. crescentus. The residues surrounding the most-amino-terminal cysteine are also highly conserved between S. pombe and animal ferrochelatases (Y-P-Q-W/Y-S-C-A/S-T-S/T-G). Instead, there is a threonine in this position in C. crescentus (T133). While the possibility for a noncysteinyl cluster ligand exists, mutations of this threonine had no effect upon enzyme activity. A role as a dimerization motif (6, 21) alone is not supported by the discovery that M. tuberculosis ferrochelatase contains both this region and a [2Fe-2S] cluster but is monomeric as isolated. C. crescentus ferrochelatase may provide an important handle in addressing the question of a possible catalytic role, since the noncluster mutants produced in the present study, unlike those made previously for animal ferrochelatases, still possess minimal enzyme activity.

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REFERENCES