5-Aminolaevulinic acid dehydratase: metals, mutants and mechanism

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Abstract

5-Aminolaevulinic acid dehydratase catalyses the formation of porphobilinogen from two molecules of 5-aminolaevulinic acid. The studies described highlight the importance of a bivalent metal ion and two active-site lysine residues for the functioning of 5-aminolaevulinic acid dehydratase. Dehydratases fall into two main categories: zinc-dependent enzymes and magnesium-dependent enzymes. Mutations that introduced zinc-binding ligands into a magnesium-dependent enzyme conferred an absolute requirement for zinc. Mutagenesis of lysine residues 247 and 195 in the Escherichia coli enzyme lead to dramatic effects on enzyme activity, with lysine 247 being absolutely essential. Mutation of either lysine 247 or 195 to cysteine, and treatment of the mutant enzyme with 2-bromethylamine, resulted in the recovery of substantial enzyme activity. The effects of the site-directed alkylating inhibitor, S-chlorolaevulinic acid, and 4,7-dioxosebacic acid, a putative intermediate analogue, were investigated by X-ray crystallography. These inhibitors reacted with both active-site lysine residues. The role of these two lysine residues in the enzyme mechanism is discussed.

Introduction

5-Aminolaevulinic acid dehydratase (porphobilinogen synthase; EC 4.2.1.24) catalyses the dimerization of two molecules of 5-aminolaevulinic acid to give porphobilinogen in a Knorr-type pyrrole synthesis (Scheme 1). Porphobilinogen is the pyrrole precursor utilized by all living systems for the biosynthesis of tetrapyrroles, including haems, chlorophylls and corphins [1]. 5-Aminolaevulinic acid dehydratases have been isolated from a number of sources, and their properties extensively characterized. Two features unify the enzymes; namely, they all utilize covalent catalysis forming a Schiff base with the substrate during catalysis, and most, if not all, appear to be metalloenzymes, requiring a bivalent metal ion for activity. The genes/cDNAs specifying a number of 5-aminolaevulinic acid dehydratases have been isolated and sequenced and, from a comparison of the derived amino acid sequences [2], it is clear that their structure is highly conserved throughout the biosphere.

The X-ray structures of eukaryote (yeast) [3] and prokaryote (Escherichia coli) [4] dehydratases have been determined in our laboratories, confirming the high degree of conservation of the enzyme structure suggested from the amino acid sequences. Thus both enzymes exist as homooligomers with D2 symmetry, made up from four pairs of subunits. The protein fold of each subunit is in the form of a classical (a/β)8-barrel, with the active site located in its hydrophobic centre. Two substrate-binding sites, termed the P-site and the A-site, are evident from the X-ray structures. The ‘P-site’ binds the 5-aminolaevulinic acid substrate molecule that gives rise to the propionic-acid-side-chain side of porphobilinogen, and the ‘A-site’ recognizes the 5-aminolaevulinic acid molecule that forms the side of porphobilinogen carrying the acetic-acid side chain (Scheme 1). In the E. coli enzyme, the P-site is distinguished by the presence of lysine-247, which forms a Schiff base with the P-site substrate. Serine-273 and tyrosine-312 recognize the carboxyl group of the substrate, and phenylalanine-204 forms hydrophobic interactions with the two methylene groups. The A-site is characterized by three cysteine residues, at positions 120, 122 and 130, which form ligands for the zinc ion. The carboxyl group of the A-site substrate is bound by two arginine residues at positions 205 and 216. In addition to these invariant residues, lysine-195, phenylalanine-204, aspartate-118 and serine-165 are located in prominent positions within the A-
Scheme I

The reaction catalysed by 5-aminolaevulinic acid dehydratase

\[
\begin{align*}
\text{H}_2\text{N} & \quad \text{O} \quad \text{CO}_2\text{H} \\
& + \quad \text{O} \quad \text{CO}_2\text{H} \\
\text{5-aminolaevulinic acid} & \xrightarrow{\text{dehydratase}} \\
& \quad \text{NH}_2 \quad \text{H} \\
& \quad \text{porphobilinogen}
\end{align*}
\]

Figure I

The active site of 5-aminolaevulinic acid dehydratase: X-ray structure of the active site of *E. coli* 5-aminolaevulinic acid dehydratase with porphobilinogen bound, showing the key features of the A- and P-sites

The amino acid residues are denoted by single letters.

Results

Mutations at the metal-binding site

Two broad classes of 5-aminolaevulinic acid dehydratases exist, one requiring zinc, found predominantly in animals, fungi and some bacteria, and the other requiring magnesium, representatives of which are present in plants and some bacteria [1,2]. The zinc metal ion in *E. coli* dehydratase is bound by three cysteines (C) in a sequence of 11 amino acids (CXCxC) extending from residues 120–130 [4]. Modification of these cysteine residues with thiophilic reagents, or oxidation in air, leads to a loss of zinc ion and inactivation of the enzyme, although the ability to bind the substrate covalently through a Schiff base at the P-site is not impaired [5,6]. In the magnesium-dependent enzymes, two of the cysteine residues are substituted by aspartic acid (D) residues, and the other by alanine (A) in the sequence AXDX,D [7], lending further support to the suggestion that this region of the enzyme is important in defining the bivalent metal ion specificity.
To provide evidence that this sequence of amino acids is indeed responsible for the metal ion specificity, two types of hybrid enzyme were created by site-directed mutagenesis: first, the magnesium-dependent pea dehydratase was mutated by introducing zinc-binding cysteine residues and, secondly, the zinc-dependent *E. coli* dehydratase was mutated by substituting several of the key residues found in the putative magnesium-binding site. The amino acid sequences and the nature of the modifications made are shown in Table 1 below.

The results of the manipulation of the pea sequence were as anticipated, with the hybrid pea dehydratase now obligatorily requiring zinc for activity. Compared with the native pea dehydratase, the hybrid enzyme was surprisingly active, considering the extensive nature of the modification, and possessed 10% of the native pea dehydratase activity. Interestingly, the hybrid enzyme was activated by magnesium in a way similar to that found with *E. coli* dehydratase [6]. Other workers have obtained results broadly similar to these [8].

The converse experiment, to generate a hybrid *E. coli* enzyme harbouring the putative magnesium-binding sequence, yielded an inactive enzyme, despite strenuous attempts to stimulate activity with a variety of bivalent metal ions. It should, however, be recognized that all the mutations were made without the knowledge of an X-ray structure of a plant dehydratase, and it is therefore remarkable that a hybrid pea enzyme was obtained with as much as 10% of the native activity, given the magnitude of the changes made. The overall conclusions from these experiments strongly suggest that the metal-binding sequence may occupy broadly similar topological positions within the active site of zinc- and magnesium-dependent enzymes.

The precise role of the metal ion in the mechanism is of some debate since, in principle, zinc could act either as a Lewis acid to polarize the carbonyl group of the A-site substrate, facilitating the deprotonation at the 3-position or, alternatively, could function in the form of a zinc hydroxide and participate as the base in deprotonation at the 3-position (or both) (see also Scheme 2). The X-ray structure indicates the likely presence of a water molecule as the fourth ligand to the triple cysteine metal ion, and this is close enough to the A-site substrate to participate as an enzymic base. It would be unusual for a zinc metal ion bound to three cysteine residues to act as a Lewis acid, and a metal hydroxide mechanism is more likely. The fact that cadmium, when exchanged for zinc, also supports catalysis is also consistent with a metal hydroxide mechanism. Whether an analogous reaction occurs with the magnesium-dependent dehydratases requires further investigation, since the X-ray structure of the magnesium-dependent dehydratase from *Pseudomonas aeruginosa* has not revealed a magnesium ion in an analogous location to the zinc ion [9]. The role of the metal ion must not be considered in isolation, since it may act in concert with lysine-195 and other nearby groups.

### Table 1

<table>
<thead>
<tr>
<th>Species</th>
<th>Amino acid sequence</th>
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<tbody>
<tr>
<td>E. coli</td>
<td>VMSDTFCFCEYTSKGGCHSVLCEH</td>
</tr>
<tr>
<td>Human</td>
<td>VACDVCLCPYTSKGGCHSLLSEN</td>
</tr>
<tr>
<td>Yeast</td>
<td>IIICDVCLCEYTSKGGCHSVLYDD</td>
</tr>
<tr>
<td>Spinach</td>
<td>IYTDVALDFYYYGDHDGIVTQH</td>
</tr>
<tr>
<td>Pea</td>
<td>IYTDVALDFYSSGDHDGIVRED</td>
</tr>
<tr>
<td>Pea sequence with Zh site</td>
<td>VACDVCLCPYTSKGGCHSLLSEN</td>
</tr>
<tr>
<td><em>E. coli</em> sequence with Mg site</td>
<td>IMSDTAFDFYTDGDGDGVLS ED</td>
</tr>
</tbody>
</table>

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The importance of the two active-site lysine residues
Treatment of 5-aminolaevulinic acid dehydratase with sodium borohydride, in the presence of the substrate 5-aminolaevulinic acid, has long been known to result in the irreversible inactivation of the enzyme, indicating that a Schiff base is involved [10]. Borohydride reduction of the enzyme in the presence of 5-aminolaevulinic acid, followed by proteolytic digestion of the labelled protein and isolation and sequencing of the labelled peptide, identified lysine-252 in the human enzyme [11] as being the active site residue of importance in the invariant sequence valine-lysine-proline. Single-turnover experiments using either 14C- or 13C-labelled substrate established that the 5-aminolaevulinic acid molecule that binds to this lysine, as a Schiff base, becomes the propionic acid side of porphobilinogen and is the first of the two substrate molecules to be bound to the enzyme [12,13]. Similar experiments with the E. coli enzyme established that lysine-247 is involved in Schiff base formation [14].

As mentioned above, the X-ray structures of the yeast [3] and E. coli [4] enzymes highlighted a second invariant active-site lysine at position-195 in the E. coli enzyme (residue 210 in the yeast enzyme). These two lysine residues are located close to one another and play a co-ordinated role in the mechanism of porphobilinogen synthesis. Their dual role has been investigated by site-directed mutagenesis and inhibitor studies supported by X-ray crystallography.

Site-directed mutagenesis of lysine-247 and lysine-195 in E. coli 5-aminolaevulinic acid dehydratases
Mutations of the hemB gene of E. coli were generated by standard procedures, and the mutant proteins were expressed in a hemB− strain of E. coli that requires haem for growth [15]. Mutations of lysine-247 to alanine or cysteine resulted in recombinant strains that would not grow unless haem was present in the growth medium. Strains harbouring mutations of lysine-247 to glutamine and lysine-195 to alanine or cysteine grew poorly, but significantly, in the absence of added haem. The lysine-247 and lysine-195 mutants were purified and their properties were investigated. Mutation of lysine-247 to either alanine or cysteine yielded proteins that were essentially inactive. Mutations of lysine-195 were less damaging, but resulted in enzymes with only 0.1% of the wild-type activity. The mutation of lysine-195 also had a dramatic effect on the ability of lysine-247 to form a Schiff base with the P-site substrate, and caused the loss of a titratable group with a pKₐ of 7.2. These results suggest that the presence of lysine-195 is essential for lysine-247 to form an enzyme-substrate Schiff base, assisting in lowering the pKₐ of lysine-247 by approximately four orders of magnitude.

The mutation to glutamine-247 was particularly interesting, since this showed only 0.01% of enzyme activity and was clearly unable to form a Schiff base with the P-site substrate. A structural comparison between the lysine (-CH₂CH₂CH₂CH₂NH₂) and glutamine (-CH₂CH₂CONH₂) side chains suggests an explanation for the small amount of enzyme activity of this mutant. The amide side chain -CONH₂ is long enough to interact with 5-aminolaevulinic acid at the P-site, with the amide forming a hydrogen bond with the ketone oxygen (N-H—O═C) to orient the substrate in the correct position for pyrrole formation. In the wild-type, the distance from C2 of lysine 247 to the imine carbon of the bound substrate is 7.1 Å (1 Å = 0.1 nm), close to the distance of 7.3 Å that exists between the C2 of glutamine-247 and the carbonyl carbon of 5-aminolaevulinic acid in the mutant. In the glutamine-247 mutant, the presence of a ketone, rather than an imine, dramatically lowers the electrophilicity of C4, with the result that the rate of pyrrole formation is reduced by four orders of magnitude. The lysine-247 → glutamine mutation may therefore provide an approximate measure of the contribution to the overall rate enhancement afforded by a protonated imine in comparison with a ketone.

Rescue experiments with lysine-247 and lysine-195 → cysteine mutants
Attempts were made to 'rescue' the activity of the lysine-247 and -195 → cysteine mutants by reacting the purified proteins with 2-bromoethylamine. 2-Bromoethylamine reacts with cysteine, leading to the formation of a -CH₂S-CH₂CH₂NH₂ side chain related to that of lysine (-CH₂CH₂CH₂CH₂NH₂). The 2-bromoethylamine reagent had little effect on the native enzyme; however, the modified cysteine-247 mutant was restored to 6% of the wild-type activity and the cysteine-195 mutant recovered 10%. These experiments highlight further the essential nature of lysine residues-247 and -195 for the correct functioning of the enzyme [15].
Figure 2

Inhibitors that modify both active-site lysine residues in 5-aminolaevulinic acid dehydratases

Left-hand panel: X-ray structure of the adduct formed on reaction of E. coli 5-aminolaevulinic acid dehydratase with 5-chlorolaevulinic acid. Lysine-247 forms a Schiff base that promotes alkylation of the lysine-195 by the resulting α-haloimine. Right-hand panel: X-ray structure of the adduct formed between yeast 5-aminolaevulinic acid dehydratase and 4,7-dioxosebacic acid. Both lysine residues appear to have been covalently modified as Schiff bases.
Inhibitor studies with 5-aminolaevulinic acid dehydratase

4-Oxo-acids, such as laevulinic acid (4-oxopentanoic acid), are good inhibitors of the enzyme at the P-site, since the ketone forms a Schiff base with lysine-247 and the carboxyl group interacts with serine-273 and tyrosine-312. A great many inhibitors on the basis of this structure have since been synthesized, one of the most notable being succinylacetone, a metabolite overproduced in type I tyrosinaemia [16]. Such 4-oxo-acids normally act as competitive inhibitors by forming a Schiff base at the P-site, and can be linked irreversibly to the enzyme by reduction with sodium borohydride [1,2,6,10,17].

Several inhibitors have higher affinity for the magnesium-dependent plant enzymes compared with the zinc-dependent enzymes [18,19]. One particularly interesting inhibitor is the homologue of 5-aminolaevulinic acid, 4-amino-3-ketobutyrate, that cannot form a Schiff base at the P-site, but which interacts specifically with the A-site [18], but only in the presence of substrate.

Inhibitors that react with both active-site lysine residues

Chemical modification by the active-site-directed inhibitors, 3-chlorolaevulinic acid and 5-chlorolaevulinic acid [20], showed interesting differences in their reaction with the bovine enzyme, with 3-chlorolaevulinic acid exhibiting half-site reactivity. Curvilinear kinetics suggested that 5-chlorolaevulinic acid was binding to the enzyme reversibly before irreversible reaction takes place. X-ray structure studies with the E. coli enzyme, inhibited by 5-chlorolaevulinic acid, have now shown that the inactivation results from the initial formation of a Schiff base with lysine-247, followed by alkylation of lysine-195 by the resulting reactive chloroimide [15]. The X-ray structure of the E. coli 5-aminolaevulinic acid dehydratase, modified covalently by 5-chlorolaevulinic acid, is shown in Figure 2(left panel). Other detailed studies of inhibitors of the enzyme have been carried out [21,22], and the synthesis and action of a number of inhibitors have been comprehensively reviewed [23].

One particularly interesting inhibitor is 4,7-dioxosebacic acid, a compound resembling a putative intermediate analogue in the reaction to form porphobilinogen. The X-ray structure of the yeast enzyme–inhibitor complex is shown in Figure 2(right panel), and reveals that one of the two oxo-groups of the inhibitor has formed a Schiff base with lysine-263 and the other with lysine-210 [24,25]. This raises the intriguing possibility that lysine residues 263 and 210 in yeast dehydratase (lysine residues 247 and 195 in the E. coli enzyme) may both participate in covalent catalysis. Whether this complex represents a true intermediate analogue of the two natural substrates or is the expected adduct from the reaction of a dioxo-compound with an enzyme active site containing two reactive lysine residues is difficult to assess. Thus the structure may represent a true enzyme intermediate analogue that is too short-lived to be trapped when the natural substrates and intermediates are bound to the A- and P-sites. On the other hand, it may represent an abortive complex that is formed slowly during crystal-

Scheme 2

A possible mechanism for porphobilinogen synthesis catalysed by 5-aminolaevulinic acid dehydratase, involving the participation of both active-site lysine residues as Schiff bases

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lization. A mechanism involving the participation of two Schiff bases is mechanistically plausible, and has already been proposed [12, 23]. Scheme 2 illustrates a possible mechanism in which the C–C bond is formed first, followed by the C–N bond. The mechanism of the enzyme reaction has been debated at length and more detailed discussions are available [1, 23, 26, 27].

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References

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Terminal steps of haem biosynthesis
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Abstract
The terminal three steps in haem biosynthesis are the oxidative decarboxylation of coproporphyrinogen III to protoporphyrinogen IX, followed by the six-electron oxidation of protoporphyrinogen to protoporphyrin IX, and finally the insertion of ferrous iron to form haem. Interestingly, Nature has evolved distinct enzymic machinery to deal with the antepenultimate (coproporphyrinogen oxidase) and penultimate (protoporphyrinogen oxidase) steps for aerobic compared with anaerobic organisms. The terminal step is catalysed by the enzyme ferrochelatase. This enzyme is clearly conserved with regard to a