Mechanism-based inhibition of 5-aminolaevulinic acid dehydratase from *Bacillus subtilis* by the 3-thia analogue of the substrate

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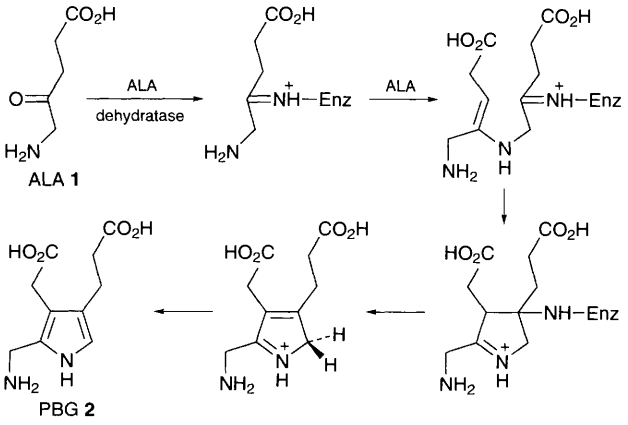
The interaction of various substrate analogues with 5-aminolaevulinic acid dehydratase (porphobilinogen synthase) from *Bacillus subtilis* is studied kinetically and by electrospray mass spectrometry; 5-chlorolaevulinic acid is shown to be a non-specific alkylating agent but 5-amino-3-thialaevulinic acid is a potent mechanism-based inactivator.

In tetrapyrrole biosynthesis, the conversion of the first committed precursor common to all organisms, 5-aminolaevulinic acid (ALA) **1**, into the monopyrrole porphobilinogen (PBG) **2** is catalysed by the enzyme ALA dehydratase (PBG synthase, EC 4.2.1.24). Previous studies on the enzymes from bovine liver[1–3] and *Escherichia coli*[4,5] have shown that ALA forms an imine linkage (Schiff's base) with a lysine residue. Two molecules of ALA are combined in an unsymmetrical fashion during the reaction and the mechanism commonly proposed involves imine formation at the site that provides the propionate side-chain of PBG, see Scheme 1, but an equally plausible mechanism involves imine formation at the alternative acetate site.[6] Here we present studies of the interaction of ALA dehydratase from *Bacillus subtilis* with some inhibitors, which have implications for the mechanism and active-site functional groups of the enzyme from this and other organisms.

We have overexpressed in *E. coli* the *hemB* gene from the *hemAXCDBL* operon[7] in *B. subtilis* and purified and characterised the resulting ALA dehydratase (K_M for ALA, 0.17 mmol dm^{-3}; k_{cat}, 0.95 s^{-1}).[8] Electrospray mass spectrometry (ESMS) of the enzyme gave a peak† at 36 082 ± 3 Da [Fig. 1(*a*)] in close agreement with the predicted weight of 36 078.5 Da after removal of the terminal methionine. Reaction with ALA and $NaBH_4$ gave a new peak, 36 195 ± 3† [Fig. 1(*b*)], due to reduction of the imine linkage between the enzyme and ALA. As in a recent study on the *E. coli* enzyme,[4c] there was no evidence of the half-of-sites reactivity (four sites per octamer) observed with the bovine enzyme.

5-Chlorolaevulinic acid (CLA) **3** is a known competitive inhibitor and inactivator of ALA dehydratase from other sources.[9–11] With the enzyme from *B. subtilis* CLA is a competitive inhibitor with a K_i value of 1.7 mmol dm^{-3} but no inactivation was observed at this concentration: a concentration of 100 mmol dm^{-3} was required to obtain 59% inactivation after 15 min.‡ Furthermore, the rate of inactivation increased with the concentration of CLA with no apparent saturation. This suggests that, although CLA does bind in the active site (hence the competitive inhibition), the alkylation reaction that causes inactivation does not occur at that site and may not involve prior non-covalent binding of CLA to the enzyme at all. The nature of the inactivation reaction was investigated by ESMS after reaction of the enzyme with six different concentrations of CLA. Aliquots were also assayed for enzymic activity. At the lowest concentration (2 mmol dm^{-3}) relatively clean monoalkylation was observed [Fig. 1(*c*)] but virtually no inactivation. With CLA at 50 mmol dm^{-3}, a series of peaks corresponding to mono- to tetra-alkylation [Fig. 1(*d*)] was observed but the inactivation was modest (45%). At 100 mmol dm^{-3} the peaks attributed to native enzyme and its monoalkylation product decreased further and the major species were tri- and tetra-alkylated but still the level of inactivation was only 59%. Comparison of the peak heights with the residual activity indicated that even the trialkylated enzyme must retain substantial activity.



Scheme 1 One proposed mechanism for ALA dehydratase

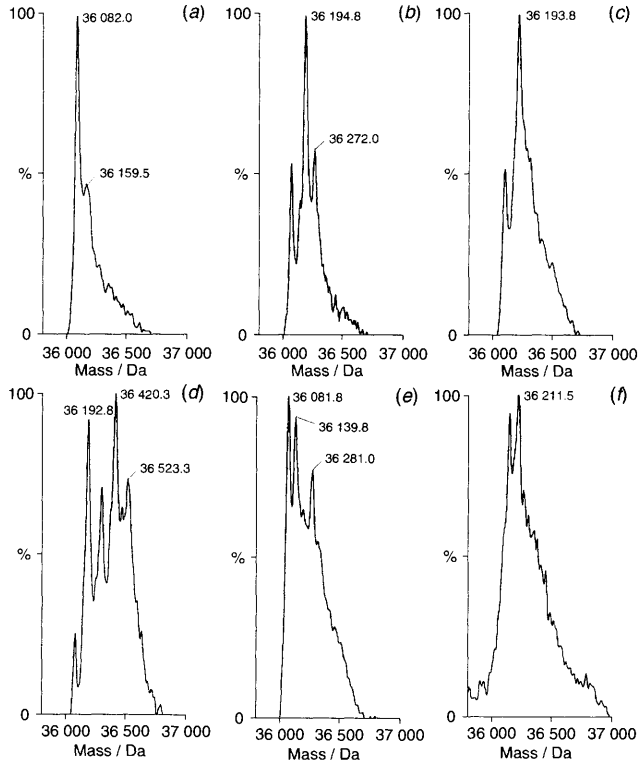
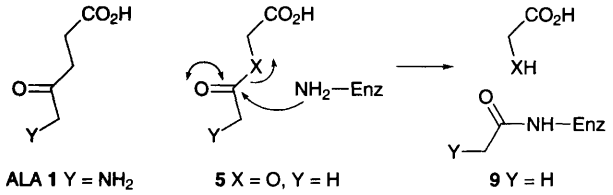


Fig. 1 Electrospray mass spectra of ALA dehydratase: (*a*) native enzyme; (*b*)–(*f*) after inactivation for 15 min at 37 °C with (*b*) ALA **1** and $NaBH_4$, (*c*) CLA **3** (2 mmol dm^{-3}), (*d*) CLA (50 mmol dm^{-3}), (*e*) 3-ThiaALA **8** (0.5 mmol dm^{-3}), (*f*) 3-ThiaALA (1.5 mmol dm^{-3})

These experiments show that CLA is not an active site-directed inhibitor of ALA dehydratase from *B. subtilis* but a non-specific alkylating agent, most likely alkylating cysteine residues. There are only 4 cysteines in the sequence,[7] which matches the maximum number of alkylations observed per subunit. The similarity between this ALA dehydratase and those from *E. coli* (48% identity) and bovine liver suggest that the three conserved cysteines are involved in binding Zn^{2+}.[12] It is unlikely that Zn^{2+} would still bind after alkylation of at least two of its ligands and so we conclude that this zinc ion is not essential for activity. It has been proposed, however, that these enzymes contain a second zinc binding site, which is the one essential for catalysis.[12]

In order to find a true active site-directed inhibitor, we aimed to use the known attack of a lysine residue on the ketone group of ALA. If this were replaced by an ester or thioester (as in compounds **5–8**), then attack of the amino group would be followed by expulsion of the leaving group to give an amide (**9** or **10**, Scheme 2). Each of these four compounds§ (and laevulinic acid **4**) was tested as a competitive inhibitor and inactivator of ALA dehydratase, Table 1. The K_i values for competitive inhibition show that the thioesters bind better than the esters and also compounds having the 5-amino group bind much more strongly (**4** binds much more weakly than ALA **1** whereas for the bovine and *E. coli* enzymes the K_i for **4** is similar to the K_M for ALA). Compounds **6**, **7** and **8** all cause appreciable inactivation of the enzyme at concentrations similar to or less than that required for competitive inhibition.

The specificity of the reaction between ALA dehydratase and the thioester inactivators was investigated by ESMS. In both cases the expected mass increases were observed corresponding to acetyl-enzyme **9** from thioester **7** and glycyl-enzyme **10** from **8**. In Fig. 1(*e*) the two major peaks correspond to native enzyme and monoacylated enzyme **10**. With a higher concentration of **8**



ALA **1** Y = NH_2
CLA **3** Y = Cl
LA **4** Y = H

5 X = O, Y = H
6 X = O, Y = NH_2
7 X = S, Y = H
8 X = S, Y = NH_2

9 Y = H
10 Y = NH_2

Scheme 2 Mechanism for the inactivation of ALA dehydratase by inhibitors **6–8**

Table 1 Inhibition of ALA dehydratase[a] by compounds **3–8**

Compound	K_i (competitive)[b] /mmol dm^{-3}	% Inactivation[c]	Conc.[c] /mmol dm^{-3}
3	1.7	59	100
4	21	—	—
5	>200	0	222
6	26	18	12
7	116	54	15
8	1.1	86	1.5

[a] Enzyme was preincubated in bis-tris propane buffer (12 mmol dm^{-3}; pH 9.0) containing DTT (5.55 mmol dm^{-3}), NaCl (140 mmol dm^{-3}), $ZnSO_4$ (50 μmol dm^{-3}) and $MgSO_4$ (500 μmol dm^{-3}) for 10 min at 37 °C and then assayed in the same buffer (100 mmol dm^{-3}) with ALA (0.5–2 mmol dm^{-3}) for 10 min at 37 °C. PBG formed was measured by reaction with Ehrlich's reagent.[1–6] [b] The competitive inhibition experiments were performed with a preparation of enzyme which gave a K_M value for ALA of 0.29 mmol dm^{-3}. The measurements of the K_i values for **6**, **7** and **8** may be affected by inactivation of the enzyme occurring during the assays. [c] Inactivation experiments involved incubation of the enzyme with the indicated concentration of inactivator for 15 min at 37 °C followed by dilution and assay in the normal way.

[Fig. 1(*f*)] a peak appeared which may in part correspond to diacylated enzyme.† The remaining enzymic activity in these samples (53 and 14% respectively) correlates well with the amount of native enzyme, indicating that, 3-thiaALA **8** is a specific active site- directed inhibitor of ALA dehydratase from *B. subtilis*.

The rate of inactivation of ALA dehydratase was measured at several different concentrations of 3-thiaALA **8** and to our surprise the rate of inactivation is approximately proportional to the square of the concentration of 8 up to 1.7 mmol dm^{-3} (at higher concentrations the rate of inactivation became too fast to measure using our standard procedures). This indicates that two molecules of inhibitor bind reversibly before the inactivation reaction occurs. By analogy it is likely that two molecules of ALA bind before imine formation occurs. This is significant because the proposal that the imine is formed in the propionate side of the active site, as in Scheme 1, is based on the belief that, for the bovine enzyme, the second molecule of ALA binds after the first molecule has formed the imine.[2]

The strategy demonstrated here for the inactivation of an imine-forming enzyme (replacement of a methylene group of the substrate by a sulphur atom so as to convert the ketone into a thioester) may be valuable for the design of inhibitors of other imine-forming enzymes such as aldolases and decarboxylases.

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Footnotes

† A shadow peak was often observed at *ca.* 78 mass units higher [*e.g.* 36 159.5 in Fig. 1(*a*) and 36 311 in (*b*)]; the nature of this adduct is unknown but a similar effect has previously been observed for the enzyme from *E. coli*.[4c] The peak at 36 211.5 in Fig. 1(*f*) may in part be due to the same effect. The peak at 36281 in Fig. 1(*e*) is thought to be due to the adduct of glycyl-enzyme with dithiothreitol (DTT) (or enzyme with glycyl-DTT).

‡ The enzyme was first dialysed to remove DTT which reacts with the alkylating agent. Little activity was lost due to oxidation if the buffers were well deoxygenated. The results are all expressed as a percentage of the activity measured in the control which lacked inhibitor.

§ Acetoxyacetic acid **5** was from Aldrich; **6** was made by *O*-alkylation of *N*-Boc-glycine with *tert*-butyl chloroacetate followed by deprotection using TFA; **7** was made by acetylation of mercaptoacetic acid using Ac_2O; **8** was made by activation of *N*-Boc-glycine with EtOCOCl and Et_3N followed by reaction with mercaptoacetic acid and deprotection using TFA.

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