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DEUTERIUM ISOTOPE EFFECTS ON PORPHOBILINOGEN SYNTHESIS CATALYSED BY 5-AMINOLAEVULINIC ACID DEHYDRATASE

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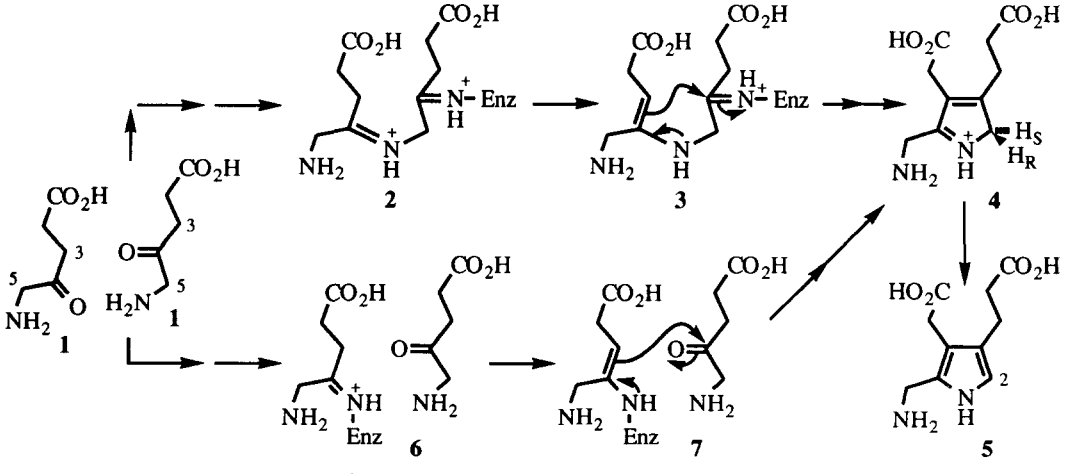
Abstract. Deuteriation of 5-aminolaevulinic acid (ALA) at C-5 has no effect on the rate of porphobilinogen synthesis by ALA dehydratase from *Bacillus subtilis* but deuteriation at C-3 gave isotope effects on k_{cat} and k_{cat}/K_M of 3.4 and 2.3 respectively. Reisolated ALA after 50% reaction shows no significant loss of deuterium at C-3, indicating that it is probably the first deprotonation at this carbon which is rate-determining.

In tetrapyrrole biosynthesis, the dimerisation of 5-aminolaevulinic acid (ALA) **1** to give porphobilinogen (PBG) **5** is catalysed by ALA dehydratase (PBG synthase, EC 4.2.1.24).[1] The enzymes from bovine liver[2,3] and more recently *E. coli*[4,5] have been much studied and purification and characterisation of dehydratases from many other sources have also been reported. We have overproduced and purified ALA dehydratase from *Bacillus subtilis*[6] and we have recently reported the effects of some inhibitors on the enzyme from this source.[7]

It has been found that ALA dehydratases from all sources form an imine linkage between ALA and a lysine residue in the active site. It has been suggested that the imine is formed in the P-site,[2,8] *i.e.* with the ALA molecule that provides the propionate side-chain of PBG, and may proceed *via* intermediates such as **2** and **3** (Scheme 1). A plausible alternative, however, is formation of the imine in the A-site (see **6**), which would then involve attack of the enamine in the A-site on the ketone in the P-site (see **7**).[9] For either mechanism, the last

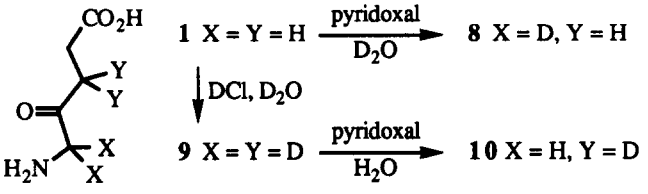


Scheme 1. Alternative mechanisms for ALA dehydratase

intermediate is likely to be the protonated 2*H*-pyrrole **4** and it has been established that it is H_R which is lost from the carbon which ends up as C-2 of PBG **5**.[10]

Three hydrogen atoms are lost during the course of the reaction, two from C-3 of the ALA molecule in the A-site and one from C-5 of the ALA molecule in the P-site and yet, surprisingly, there has been no report of a study of isotope effects. In order to start to dissect the mechanism and obtain some information about the kinetics of individual steps, we were interested to find out whether the overall enzymic reaction shows an isotope effect.

Very convenient procedures for the specific deuteriation of ALA at either C-3 or C-5 have been published.[11] Thus treatment of ALA with pyridoxal and pyridine in D_2O results in specific exchange next to the amino group at C-5 to give [5,5-$^2H_2$]ALA **8**. On the other hand, reaction with DCl in D_2O results in exchange at both C-3 and C-5 to give [3,3,5,5-$^2H_4$]ALA **9** and then washing out the deuterium on C-5 using pyridoxal and pyridine in H_2O gives [3,3-$^2H_2$]ALA **10**. In our preparations, the level of deuteriation in **8** was *ca.* 80% and in **9** and **10** was *ca.* 95% by ^1H NMR spectroscopy.



Scheme 2. Deuteriation of ALA

The rates of reaction catalysed by ALA dehydratase from *Bacillus subtilis* were measured spectrophotometrically, after reaction of the PBG produced with modified Ehrlich's reagent.[12] Various concentrations of the substrates, undeuteriated ALA **1** and the three deuteriated forms **8**–**10**, were used (see Fig. 1). Fitting of the Michaelis-Menten equation to the data gave k_{cat} and K_M values for the four substrates (see Table). For [5,5-$^2H_2$]ALA **8** the rates were essentially identical to those for unlabelled ALA **1** but both [3,3,5,5-$^2H_4$]ALA **9** and [3,3-$^2H_2$]ALA **10** showed substantial isotope effects on k_{cat} and on k_{cat}/K_M.

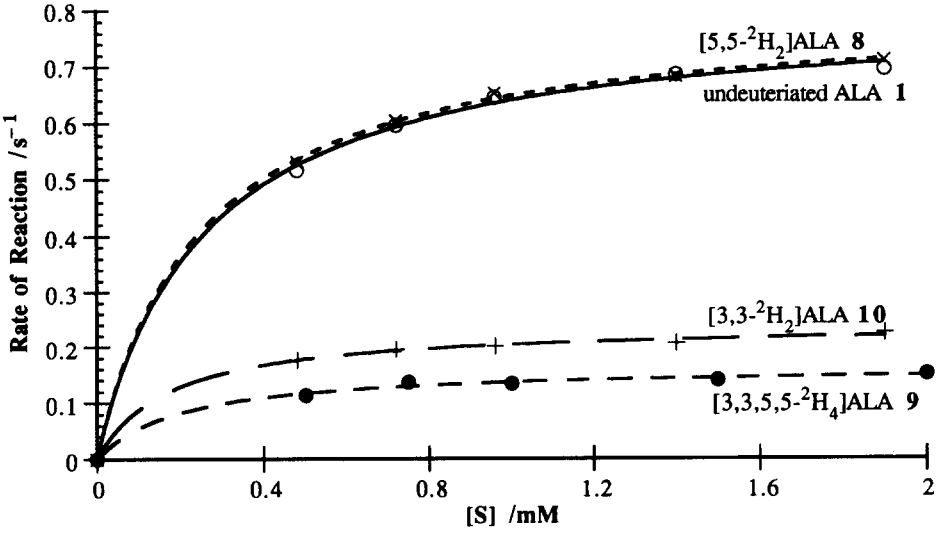


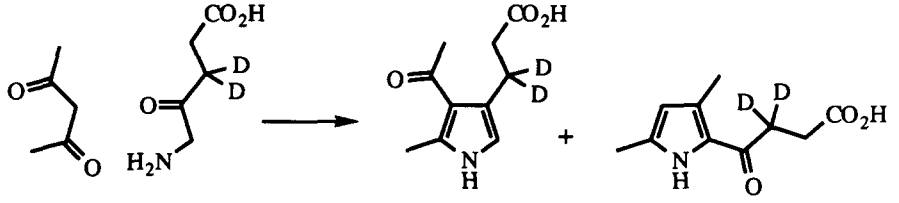
Figure 1. Rates of reaction of the deuteriated forms of ALA at different concentrations.

Table. Kinetic parameters[13] and isotope effects measured for the deuteriated forms of ALA.

Substrate	k_{cat}/s^{-1}	$K_M/\mu M$	Isotope Effect on k_{cat}	Isotope Effect on k_{cat}/K_M
ALA **1**	0.803 ± 0.015	253 ± 23	–	–
[5,5-$^2H_2$]ALA **8**	0.805 ± 0.009	241 ± 13	1.00 ± 0.02	0.95 ± 0.10
[3,3,5,5-$^2H_4$]ALA **9**	0.163 ± 0.007	195 ± 46	4.93 ± 0.23	3.80 ± 0.98
[3,3-$^2H_2$]ALA **10**	0.240 ± 0.009	172 ± 27	3.35 ± 0.14	2.27 ± 0.42

The implication of the deuterium isotope effect on k_{cat} using [3,3-$^2H_2$]ALA **10** is that one of the two steps involving deprotonation at C-3 must be rate-determining. Furthermore, the fact that the isotope effect on k_{cat}/K_M is also quite large implies that there is not a great deal of commitment to catalysis by this stage,[14] *i.e.* the steps leading up to this rate-determining step must, in part, be reversible. The magnitude of the isotope effects observed for [3,3,5,5-$^2H_4$]ALA **9** support this conclusion.[15]

If the rate-determining step just identified were the second of the two deprotonation steps at C-3, then molecules which reach this stage but then return to starting materials rather than proceeding to product would be very likely to regain protium rather than deuterium. The result would be gradual exchange of the deuterium in the starting ALA as the reaction progressed. To test for this, three parallel incubations of [3,3-$^2H_2$]ALA **10** (10 mg) were set up with different amounts of ALA dehydratase. After 6 h the assay for PBG showed the three reactions had reached 16, 46 and 57% completion, respectively. The remaining ALA was then reisolated by reaction with acetylacetone **11** to give the Knorr pyrrole **12**, along with a minor amount (ratio 1:7) of the Fischer-Fink pyrrole **13**.[12,16] The ^1H NMR spectrum of the mixture of pyrroles from each enzymic incubation showed little if any (< 10%) exchange of deuterium for protium at the relevant position of **12** when compared with **12** produced from [3,3-$^2H_2$]ALA **10** which had been incubated under the same conditions but without enzyme.



11 **10** **12** **13**

Scheme 3. Derivatisation of ALA

We conclude that the enzyme does not catalyse any significant hydrogen exchange at C-3 of ALA and, therefore, the rate-determining step is probably the first deprotonation at this site, *i.e.* **2** → **3** or **6** → **7**. The alternative is that the rate-determining step is the second deprotonation but that the deuterium atom taken off in the first deprotonation step does not exchange with the medium. This does not seem very likely for two reasons: firstly, all the steps up to the rate-determining deprotonation have to be reversible and this would have to include the C–C bond forming step if it were the second deprotonation; secondly, a likely mechanism would use the same basic group in the active site to effect both the deprotonations at C-3 and if this is correct then the base would have to lose the first proton before it could effect the second deprotonation step.

In summary, the deuterium isotope effects reported here have, for the first time, defined the rate-determining step (or one of the rate-determining steps) of the ALA dehydratase reaction as one of the two deprotonations (probably the first one) occurring at C-3 of ALA in the A-site. The steps leading up to this deprotonation step must be reversible. The deprotonation at C-5 in the P-site, thought to be the final step (**4** → **5**), does not appear to be rate-determining. The finding that the deprotonation at C-3 is a kinetically significant step should open the possibility of further experiments to help define the elusive mechanism of this enzymic reaction. In particular, we hope in the future to use ALA stereospecifically deuteriated at C-3 to probe the stereochemistry of the deprotonation step.

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