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MECHANISM-BASED INACTIVATION OF PORPHOBILINOGEN SYNTHASE: SUBSTITUTION OF KETONE FUNCTIONALITY IN THE SUBSTRATE WITH A THIOESTER

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Abstract: The thioester (**5**) was found to inactivate the enzyme porphobilinogen synthase. Replacement of the ketone functionality in aminolevulinic acid (ALA), shown to be involved in Schiff-base linkage with an active site lysine residue, by a thioester moiety was found to result in loss of enzyme activity. A plausible mode of inactivation is proposed.

Porphobilinogen synthase (PBGS, ALA-dehydratase) catalyzes the condensation of two molecules of 5-aminolevulinic acid (ALA,**1**) to give porphobilinogen (PBG,**2**), the heterocyclic precursor of all naturally-occurring porphyrin and corrin cofactors.[1] The native enzyme in *E.coli* is an octamer of molecular weight 280 kDa (subunit:35 kDa) and requires Zn(II) for catalysis.[2] The two molecules of ALA come to reside in the final product in an asymmetric fashion, one giving rise to the acetate (A) side chain and the other forming the propionate (P) side chain of porphobilinogen (Fig. 1).

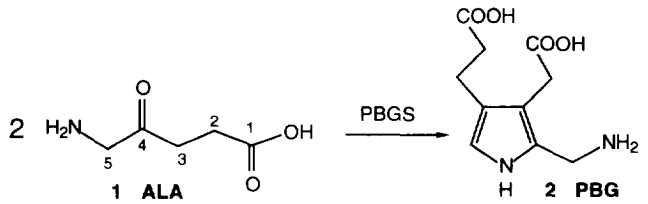

Fig. 1

The proposed enzymatic mechanism involves formation of a Schiff-base intermediate between an active site lysine[3] and the C-4 carbonyl group of one of the ALA molecules denoted as P-side ALA since the propionate side chain of the product is derived from it. Subsequently the free amino group of the ALA-Schiff base intermediate undergoes Schiff-base formation with the second ALA molecule, denoted as the A-side ALA. Subsequent reactions lead to the formation of PBG (Fig. 2).

5-chlorolevulinic acid was introduced as an inactivator of the enzyme by Seehra and Jordan.[4] Further work carried out by Jaffe and co-workers showed that the α-chloroketone functionality labelled an active site cysteine believed to be a ligand for the catalytic zinc.[5] Heterogeneous modification of the enzyme was observed as evidenced by the fact that at least two different cysteines were labelled. We now report the mechanism-based inactivation of the enzyme by using a modified substrate analog approach. Heteroatom modification of the C-3 methylene group of the substrate to produce the ester **4** and thioester **5** results in the generation of a potential scissile C-X (X=O,S) bond while minimising structural deviation. If the active site lysine involved in Schiff-base

formation carried out nucleophilic attack at the ester or thioester functionality this could result in inactivation of the enzyme as depicted in Fig. 3.

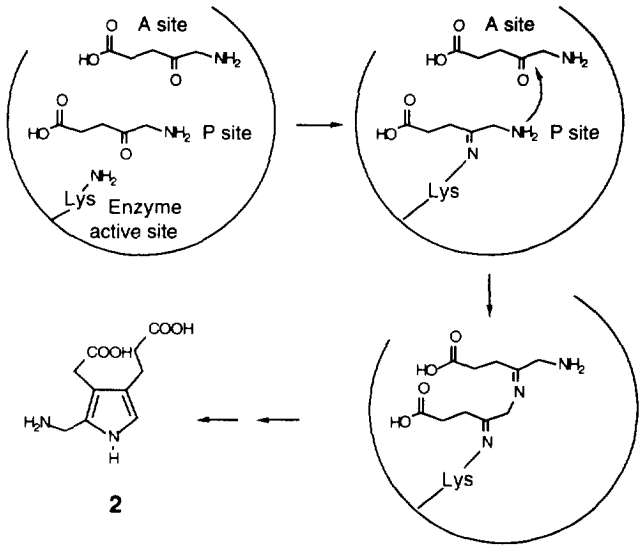

Fig. 2: Proposed sequence of events occurring at the enzyme active-site leading to the formation of PBG **2**

The ε-amino group of lysine in proteins has been shown to be involved in protein crosslinking by way of nucleophilic attack at a thioester moiety, in a reaction catalyzed by γ-glutamyl-ε-lysine amino transferases.[6] We reasoned therefore that the active site lysine in PBGS might conceivably be glycinated by the inhibitors **4** or **5**.

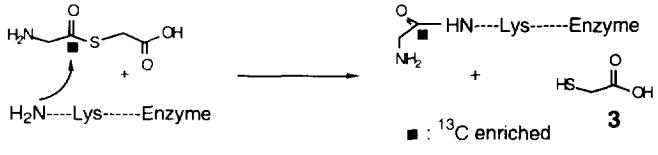

Fig. 3: Possible mode of enzyme inactivation

Ester **4** and thioester **5** were synthesized from glycine as shown in Fig. 4. The deprotected products were characterized by ^{1}H NMR and FAB-MS. No loss of enzyme activity was observed when PBGS was pre-incubated with ester **4** (5 mM) for 30 minutes. However the enzyme exhibited time-dependent loss of activity in the presence of ≈150 μM thioester **5**, losing 80% of its activity over 30 minutes. In contrast, when ALA was included in the incubation mixture at a concentration of 0.3 mM the enzyme retained over 80% of its activity, indicating substrate protection of the active site. The thioester also undergoes spontaneous hydrolysis at pH=7, leading to glycine and thioglycolic acid **3**.[7]

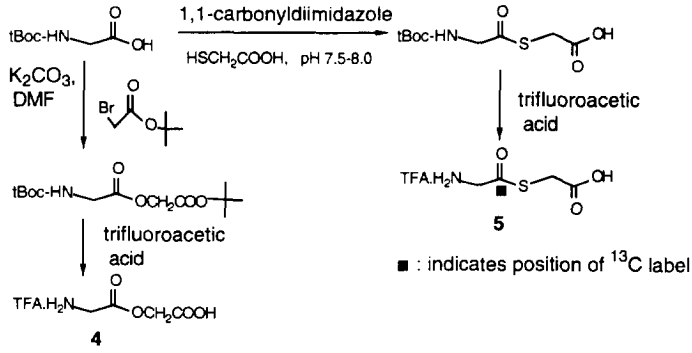

Fig. 4: Synthesis of ester **4** and thioester **5** from glycine

The thioester was next synthesized using glycine enriched with ^{13}C at the carboxylic position. If the mechanism of action of the inhibitor is as proposed in Fig. 3 then inhibition should result in the glycination of the ε-amino group of the active site lysine. A 1mM solution of the enzyme was incubated with the thioester (≈5mM, pH 6.6) for 2h at 25 °C. The enzyme solution was then extensively dialyzed in 10mM phosphate buffer (pH 6.8), concentrated and analyzed by 125 MHz ^{13}C NMR. As a control experiment the enzyme was also incubated with inhibitor lacking the ^{13}C label and analyzed. The sample incubated with ^{13}C labelled inhibitor showed new features in the carbonyl region (160-180 ppm) but background signals from the protein amide backbone made analysis difficult (data not shown). In order to improve resolution and remove any ^{13}C label-derived, non-covalently bound species, 7M urea was added to denature the protein and the solution dialyzed in 10mM phosphate buffer (pH 6.6, 2 x 1000 mL). The denatured protein was then concentrated and analyzed by ^{13}C NMR. The NMR spectrum derived from the enzyme sample treated with the ^{13}C labelled inhibitor (Fig 5A) clearly shows the presence of a new peak at 175 ppm that is absent in the sample treated with unlabelled inhibitor (Fig 5B).

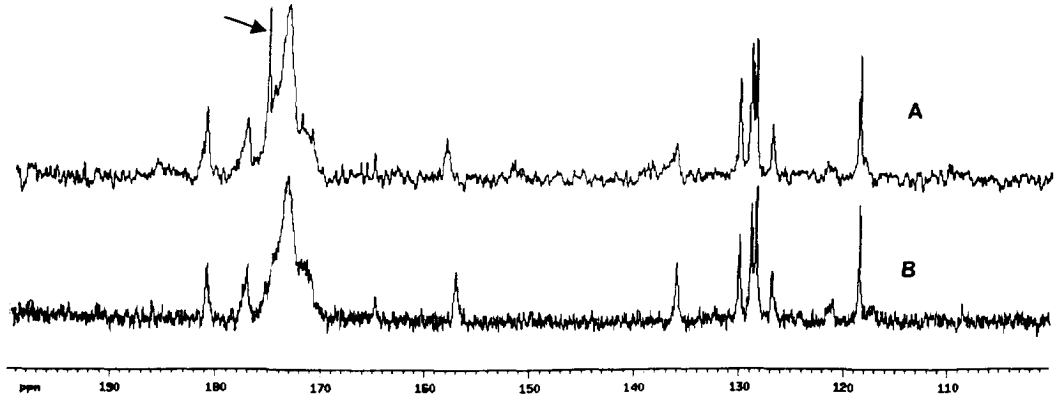

Fig. 5: Partial ^{13}C NMR of denatured PBGS (pH 12) after incubation with the thioester **5**

The peak at 175 ppm exhibited no decomposition at pH 12-13 over 8 hours making it unlikely that it arose

from an ester species, thus excluding the possibility that modification of a serine or threonine residue could have occurred. Likewise, the chemical shift lies upfield from that expected for the carboxylic acid group in glycine (≈ 181 ppm), the decomposition product resulting from hydrolysis of the substrate. Also the chemical shift of the thioester moiety in **5** is 193 ppm. The ^{13}C chemical shift of the carboxamide moiety in glycine model compounds of the form NH_2CH_2CONHR, where R is alkyl, was shown to be in the region of 173-175.5 ppm at pH 12.

Earlier work by Jaffe and co-workers has demonstrated the utility of ^{13}C NMR as a probe for gleaning mechanistic details of the enzyme.[8a,b] It will be of interest to study whether the free amino group of the glycinyl moiety that is presumably appended to the active site lysine (Fig. 3) is capable of forming a Schiff base with ALA bound at the A site. This would offer the possibility of studying partial reactions catalyzed by the enzyme. ^{13}C and ^{15}N labelled inhibitors show promise of throwing further light on the enzymatic mechanism.

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References and Notes:

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2. Spencer, P.; Jordan, P. M. In *The Biosynthesis of the Tetrapyrrole Pigments*; Chadwick, D. J.; Ackrill, K., Eds.; John Wiley: Chichester, 1994; p 50. The enzyme was isolated from a recombinant strain of *E.coli* containing a plasmid bearing the *hemB* gene: Roessner, C. A.; Spencer, J. B.; Ozaki, S.; Min, C.; Atshaves, B. P.; Nayar, P.; Anousis, N.; Stolowich, N. J.; Holderman, M. T.; Scott, A. I. *Protein Expression Purif.* **1995**, *6*, 155.
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7. This unforeseen hydrolytic instability of the thioester **5** is reminiscent of the decomposition of succinyl CoA due to internal (anchimeric) assistance from the C-1 carboxylic acid group. The thioester is stable at pH< 5. Samples of thioester **5** always contained glycine and thioglycolic acid due to decomposition of the product during preparation, and thus we were unable to determine k_i. The contaminants could not be removed by purification. However tests were carried out to ensure that enzyme inhibition was not due to the presence of these contaminants.
8. (a) Jaffe, E. K.; Volin, M.; Myers, C. B.; Abrams, W. R. *Biochemistry* **1994**, *33*, 11554.
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