Mechanism of Action of 5-Aminolevulinic Acid Dehydratase: Stepwise Order of Addition of the Two Molecules of 5-Aminolevulinic Acid in the Enzymic Synthesis of Porphobilinogen

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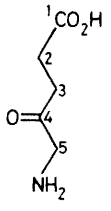
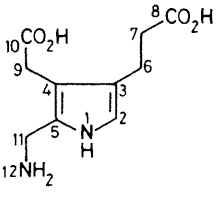
Summary Incorporation of [5-^{14}C]-5-aminolevulinic acid into porphobilinogen in single-turnover enzymic experiments with mammalian 5-aminoleuvlinic acid dehydratase has revealed that of the two molecules of 5-aminolevulinic acid which are utilised for the formation of porphobilinogen it is the one contributing to the propionic acid side [atoms 1, 2, 3, 6, 7, and 8 in (**2**)] which is initially bound to the enzyme.

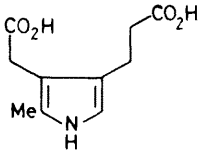
5-AMINOLEVULINIC ACID DEHYDRATASE (porphobilinogen synthetase, E.C. 4.2.1.24) catalyses the formation of the tetrapyrrole precursor porphobilinogen (**2**) from 2 molecules of 5-aminolevulinic acid (**1**).[1]



(1)



(2)



(3)

Extensive studies on the enzyme from *Rhodopseudomonas spheroides* have revealed both the existence of a covalent linkage between the enzyme and substrate and the ability of the enzyme to catalyse the formation of porphobilinogen analogues of the type (**3**) from 1 molecule of levulinic acid and 1 molecule of 5-aminolevulinic acid.[2] As a result of these studies, a mechanism was proposed in which the molecule of 5-aminolevulinic acid which gives rise to the acetic acid side of the product [atoms 4, 5, 9, 10, 11, and 12 in (**2**)] binds initially to the enzyme through a Schiff base linkage and the resulting enimine or its equivalent species then reacts with the second molecule of 5-aminolevulinic acid, the latter coming from solution and giving rise to the propionic acid side of the product [atoms 1, 2, 3, 6, 7, and 8 in (**2**)].[3] Dehydration and loss of a proton then furnishes the product porphobilinogen. With respect to the *R. spheroides* enzyme this mechanism has remained largely unchallenged apart from a recent modification.[4]

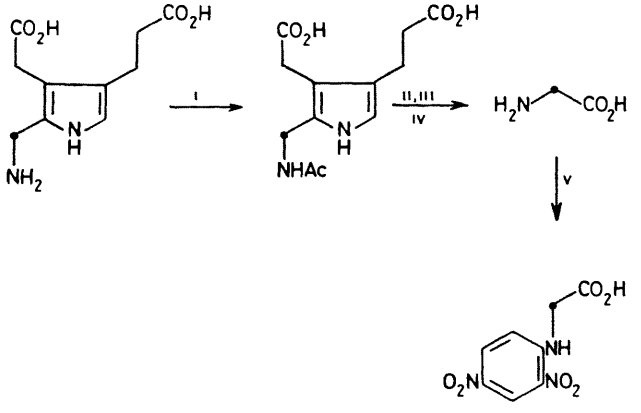
Many of the properties of the mammalian enzyme have been studied particularly with respect to the metal ion requirements[5] and the role played by sulphydryl groups.[6,7] Although the bovine liver enzyme also appears to bind the substrate through a Schiff base,[8] attempts to demonstrate the formation of pyrroles of the type (**3**) have been singularly disappointing[9] and with the exception of the establishment of the stereochemical events which occur at C-2[10] in (**2**) little is known about the mechanistic features of the enzyme reaction.

Paramount to an understanding of the catalytic events which occur at the active site is a knowledge of the order in which the two substrate molecules bind to the enzyme and are subsequently condensed together to form porphobilinogen. The relatively unusual type of reaction catalysed by the dehydratase enzyme, involving two molecules of the *same* substrate, precludes a conventional kinetic evaluation. We thus approached the problem by means of a ^{14}C single-turnover technique† in which a small amount of [5-^{14}C]-5-aminolevulinic acid was initially added to the enzyme followed by a large excess of unlabelled substrate. As long as the affinity of the enzyme for one of the two molecules of substrate is different from that of the other and the concentration of the [5-^{14}C]-5-aminolevulinic acid is equal to or less than that of the enzyme, one or other of the two binding sites will be preferentially occupied by ^{14}C-label. Subsequent addition of an excess of non-labelled substrate will

† This type of approach has been successfully used to determine the order of binding of porphobilinogen to porphobilinogen deaminase (ref. 11) during the enzymic biosynthesis of uroporphyrinogen.

carry the initially bound ^{14}C-substrate through to the product thus completing a single enzyme turnover with respect to the ^{14}C-substrate

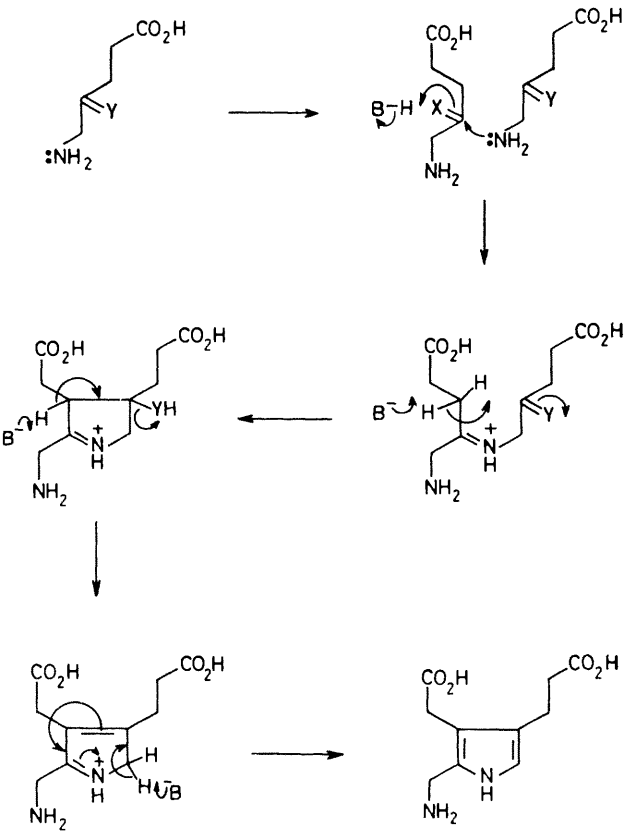
Accordingly, highly purified 5-aminolevulinic acid dehydratase from bovine liver[12] (71 4 nmol) was admixed with [5-^{14}C]-5-aminolevulinic acid (7 1—571 nmol, specific activity 1·1 × 10^5 d p m /nmol) using a rapid mixing device such that enzyme and ^{14}C substrate interacted for 100 ms before the mixture was 'chased' with an excess of unlabelled 5-aminolevulinic acid (60 μmol) The product porphobilinogen was separated from 5-aminolevulinic acid by ion exchange chromatography and was further purified by t l c The ^{14}C label in C-2 and C-11 in the porphobilinogen was determined by degradation to 2,4-dinitrophenylglycine as shown in Scheme 1



SCHEME 1 I, Ac_2O, $NaHCO_3$ (aq), II, O_3–HCO_2H, III, catalase, IV, 6N HCl, 16 h, V, 2,4-dinitro-1-fluorobenzene

The results from the degradation of porphobilinogen enzymically synthesised in single-turnover experiments with 0·1, 1, 2, and 8 mol of [5-^{14}C]-5-aminolevulinic acid per mole of enzyme (experiments 1, 2, 3, and 4 respectively) are summarised in the Table The percentage of radioactivity in the 2,4-dinitrophenylglycine ranged from 4·2 to 13 9% showing that the major proportion of the label was located at C-2 of the porphobilinogen The results suggest that the molecule of 5-aminolevulinic acid initially bound to the enzyme is the one which gives rise to the propionic acid side of porphobilinogen (atoms 1, 2, 3, 6, 7, and 8) If the molecule of 5-aminolevulinic acid first bound to the enzyme was the one contributing to the acetic acid side of the porphobilinogen (atoms 4, 5, 9, 10, 11, and 12) the majority of the label would have been at C-11 As expected, the radioactivity in the porphobilinogen biosynthesised from an excess of [5-^{14}C]-5-aminolevulinic acid was equally distributed between carbon atoms 2 and 11 (experiments 5 and 6)

In addition to demonstrating that a stepwise order of binding of the two 5-aminolevulinic acid molecules to the enzyme occurs, these results also draw attention to several other interesting features about the mechanism of porphobilinogen formation From a kinetic standpoint the



SCHEME 2 X = O or $^+$NH–ENZ, Y = $^+$NH-ENZ

TABLE ^{14}C-Radioactivity in C-2 and C-11 of porphobilinogen enzymically synthesised from [5-^{14}C]-5-aminolevulinic acid [a]

Expt	Number of mols of [5-^{14}C]-5-amino levulinic acid used per mol of enzyme[b]	Specific activity[e] of porphobilinogen /10^3 d p m μmol^{-1}	Specific activity[f] of 2,4-dinitrophenyl-glycine/10^3 d p m μmol^{-1}	% Radioactivity at C-11 of porphobilinogen	% Radioactivity at C-2 of porphobilinogen[g]
1	0·1[c]	41·4	1·75	4 2	95·8
2	1·0[c]	343	27·0	7·9	92·1
3	2·0[c]	770	71·4	9·3	90·7
4	8·0[c]	129	17·6	13·7	86·3
5	Excess[d]	200	102 0	51·0	49·0
6	Excess[d]	173	88·0	50 8	49·2

[a] 5-Aminolevulinic acid dehydratase (20 mg, sp act 34 2 μmol porphobilinogen/mg protein/h) in potassium phosphate buffer (0·1 M, pH 6 8) was rapidly mixed at 20 °C with the required amount of [5-^{14}C] 5-aminolevulinic acid (7 1, 71 4, 143, or 571 nmol, sp act 1 1 × 10^5 d p m /nmol) in 1 ml of potassium phosphate buffer (0 1 M pH 6 8) The porphobilinogen was degraded as shown in Scheme 1 [b] Molecular weight of the enzyme = 280,000 consisting of 8 identical subunits (molecular weight = 35 000) [c] Quantities refer to number of moles of substrate per mol of enzyme based on a molecular weight of 280,000 [d] [5 ^{14}C]-5-Aminolevulinic acid concentration was 160 times that of the enzyme [e] Determined by the reaction of porphobilinogen with *p*-dimethylaminobenzaldehyde in $HClO_4$ (2M), λ_{max} 555 nm, ϵ = 6·02 × 10^4 cm^{-1} (see ref 13) [f] Determined in methanol, λ_{max} 348 nm, ϵ = 1 36 × 10^4 [g] Determined by difference

apparent K_M of the 5-aminolevulinic acid dehydratase from bovine liver ($1{\cdot}5 \times 10^{-4}$ M)[12] almost certainly reflects the affinity of the less tightly bound substrate molecule [*i.e.* the one giving rise to atoms 4, 5, 9, 10, 11, and 12 in (**2**)] as is evident from the preferential incorporation of label into C-2 of porphobilinogen in experiments 1, 2, 3, and 4.

Furthermore, the high level of incorporation of ^{14}C into C-2 in experiment 4, where 8 mol of [5-^{14}C]-5-aminolevulinic acid were utilised per mol of octameric enzyme is consistent with the participation of 8 functional catalytic sites per octamer. More importantly, the results suggest a mechanism for the mammalian enzyme (as shown in Scheme 2) which differs significantly from that proposed for the *R. spheroides* enzyme in which the first molecule of aminolevulinic acid bound to the enzyme is the one which binds second in our mechanism.

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[1] D. Shemin, 'The Enzymes,' 3rd edn., ed. P. Boyer, Academic Press, New York, 1972, vol 7, p 323
[2] D. L. Nandi and D. Shemin, *J. Biol. Chem.*, 1968, **243**, 1231.
[3] D. L. Nandi and D. Shemin, *J. Biol. Chem.*, 1968, **243**, 1236.
[4] M. M. Abboud and M. Akhtar, *J.C.S. Chem. Comm.*, 1976, 1007.
[5] A. Cheh and J. B. Nielands, *Struct. Bonding (Berlin)*, 1976, **29**, 123.
[6] A. G. Chaudhry, M. G. Gore, and P. M. Jordan, *Biochem. Soc. Trans.*, 1976, **4**, 301.
[7] M. G. Gore, P. M. Jordan, and A. G. Chaudhry, *Biochem. Soc. Trans.*, 1976, **4**, 762.
[8] P. E. Gurba, R. E. Sennett, and R. D. Kobes, *Arch. Biochem. Biophys.*, 1972, **150**, 130.
[9] J. S. Seehra and P. M. Jordan (unpublished results).
[10] A. G. Chaudhry and P. M. Jordan, *Biochem. Soc. Trans.*, 1976, **4**, 760.
[11] P. M. Jordan and J. S. Seehra, *FEBS Lett*, 1979, **104**, 364.
[12] J. S. Seehra and P. M. Jordan (in preparation).
[13] D. Mauzerall and S. Granick, *J. Biol. Chem.*, 1956, **219**, 435.