Mechanism of action of 5-aminolaevulinate dehydratase from human erythrocytes

Peter M. JORDAN and Philip N. B. GIBBS

Department of Biochemistry, University of Southampton, Bassett Crescent East, Southampton SO9 3TU, U.K.

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1. Purified 5-aminolaevulinate dehydratase (porphobilinogen synthase, EC 4.2.1.24) from human erythrocytes was incubated initially with limiting amounts of 5-amino[5-¹⁴C]laevulinate in a rapid-mixing apparatus. 2. The single-turnover reaction with respect to the bound labelled 5-aminolaevulinate was completed by the addition of unlabelled 5-aminolaevulinate and the resulting radioactive porphobilinogen was isolated and degraded. 3. The ¹⁴C label was found to be located predominantly at C-2 of the product, demonstrating that, of the two substrate molecules participating in the reaction, the 5-aminolaevulinate molecule initially bound to the enzyme provides the propionic acid 'side' of the porphobilinogen. 4. The same enzyme-[¹⁴C]substrate species that yields regiospecific porphobilinogen may be trapped by reaction with NaBH₄, showing that the substrate molecule initially bound to the enzyme does so in the form of a Schiff base. 5. A conventional incubation with 5-amino[5-¹⁴C]laevulinate yielded porphobilinogen with an equal distribution of the label between C-2 and C-11. 6. The reaction mechanism of the human erythrocyte 5-aminolaevulinate dehydratase thus follows the same course as that of other dehydratases studied in our laboratory by using single-turnover techniques.

5-Aminolaevulinate dehydratase (porphobilinogen synthase, EC 4.2.1.24) catalyses the self-condensation between two molecules of the amino ketone 5-aminolaevulinate to form the pyrrole, porphobilinogen (Shemin, 1972, and references therein). Porphobilinogen is the key monopyrrolic building block that is utilised for the biosynthesis of all porphyrins, chlorophylls and corrins (see Akhtar & Jordan, 1978, for a review).

5-Aminolaevulinate dehydratase has been purified from a wide variety of sources, including bovine liver (Gurba *et al.*, 1972; Tsukamoto *et al.*, 1975), photosynthetic bacteria (van Heyningen & Shemin, 1971) and human erythrocytes (Anderson & Desnick, 1979; Gibbs *et al.*, 1985b). Mammalian 5-aminolaevulinate dehydratase appear to be octameric enzymes with molecular weights of 250 000 to 285 000 (Shemin, 1976; Anderson & Desnick, 1979; Gibbs *et al.*, 1985b).

The mammalian enzyme has a requirement for Zn²⁺ for maximal activity (Tsukamoto *et al.*, 1979; Bevan *et al.*, 1980), and binding studies with ⁶⁵Zn²⁺ have established that eight atoms of Zn²⁺

are bound per molecule of native human erythrocyte enzyme (Gibbs & Jordan, 1981).

The dehydratases from all sources studied to date require the presence of an exogenous thiol such as 2-mercaptoethanol or dithiothreitol for maximum catalytic activity (Shemin, 1972). In the absence of the activating thiol, oxygen causes reversible inactivation of the enzyme; however, full activity can be recovered quantitatively on reincubation of the oxidized enzyme with thiol.

The mechanism by which 5-aminolaevulinate dehydratase catalyses the reaction between two molecules of 5-aminolaevulinate has been investigated comprehensively using the enzyme from *Rhodospseudomonas sphaeroides* (Nandi & Shemin, 1968a,b). These studies revealed the involvement of a Schiff-base linkage between the enzyme and substrate. More recent work with the bovine liver enzyme has shown that the mammalian enzyme also forms a covalent Schiff-base linkage with one of the substrate molecules (Nandi, 1978; Jordan & Seehra, 1980a).

Employing a single-turnover enzyme-reaction technique, investigations in our laboratory using the bovine liver enzyme (Jordan & Seehra,

Abbreviation used: DNP, dinitrophenyl.

1980a,b) have established the order of binding of the two 5-aminolaevulinate molecules to the enzyme active site during the catalytic cycle. On the basis of these studies, a mechanism has been proposed for the enzyme reaction in which the first molecule of 5-aminolaevulinate which is bound to the bovine liver enzyme becomes incorporated into the side of the pyrrole that contains the propionic acid side chain (atoms 1, 2, 3, 8, 9 and 10). However, no mechanistic studies have been carried out on the enzyme isolated from human sources.

The present paper reports studies on the mechanism of action of 5-aminolaevulinate dehydratase from human erythrocytes by using a single-enzyme-turnover reaction technique.

Experimental

Materials

Dithioerythritol, 5-aminolaevulinate hydrochloride and Dowex 1 (X8; 200–400 mesh) were obtained from Sigma. 5-Amino[5- 14 C]laevulinate was from New England Nuclear Corp., and 5-amino[4- 14 C]laevulinate was from Amersham International. Silica-gel 60 t.l.c. plates were obtained from Merck. 4-Dimethylaminobenzaldehyde and all other laboratory reagents were obtained from BDH.

Methods

Purification of 5-aminolaevulinate dehydratase from human erythrocytes. Homogeneous 5-aminolaevulinate dehydratase was isolated from human erythrocytes by the method of Gibbs *et al.* (1985b). The purified enzyme has a specific activity of 24.0 units/mg when assayed in the presence of 0.1 mM- Zn^{2+} and was stored as an $(\text{NH}_4)_2\text{SO}_4$ precipitate at 4°C under N_2 in a screw-top plastic tube. Under these conditions the enzyme activity was unchanged for at least 1 month. When required, portions of the $(\text{NH}_4)_2\text{SO}_4$ precipitate were dissolved in 0.1M-potassium phosphate buffer, pH 6.8, containing 10mM-dithioerythritol and 0.1mM- ZnCl_2 , activated at 37°C for 15min and dialysed before use [see Gibbs *et al.* (1985a) for details].

The enzyme was assayed by the method of Gibbs *et al.* (1985b). One unit of activity is defined as that required to produce 1 μmol of porphobilinogen/h at 37°C.

Single-turnover experiments. Human erythrocyte 5-aminolaevulinate dehydratase (8.04–12.85 nmol) was dissolved in 500 μl of 0.1M-potassium phosphate buffer, pH 6.8, containing 10mM-dithioerythritol and 0.1 mM- Zn^{2+} , and rapidly mixed with the required amount of 5-amino[5- 14 C]laevulinate

(20.6–804 nmol) in 500 μl of the same buffer. The mixing of enzyme and substrate was achieved by using a rapid-mixing device (Jordan & Seehra, 1980a) with two syringe inputs that allowed the enzyme and labelled substrate to interact for a short time ($\approx 100\text{ms}$) before 'chasing' with an excess of unlabelled 5-aminolaevulinate (60 μmol) in the same buffer (1 ml). The resultant reaction mixture was incubated for 5 min at 37°C, carrier porphobilinogen (4mg) was added, and the porphobilinogen was purified as described in Jordan & Seehra (1980a). The specific activity of the porphobilinogen was determined by allowing a known volume of the purified material to react with modified Ehrlich's reagent. The radioactivity in the sample was determined as described below.

NaBH_4 reduction of the enzyme-[14 C]substrate complex was achieved by mixing the enzyme and [14 C]substrate in the rapid-mixing device for 100 ms as described above, followed by quenching the reaction with 1M- NaBH_4 (50 μl) at 0°C. The reduction was allowed to proceed for 10 min at 0°C and the pH was maintained at 6.8 throughout this period by the addition of 1M-acetic acid. The protein was precipitated with $\sim 60\%$ -satd. $(\text{NH}_4)_2\text{SO}_4$ [$\sim 390\text{mg}$ of $(\text{NH}_4)_2\text{SO}_4/\text{ml}$ of solution], purified by chromatography on Sephadex G-100 and counted for radioactivity in Tritoscint (see below).

Conversion of porphobilinogen into DNP-glycine to determine regioselectivity of label

The porphobilinogen samples (3.04–4.44 mg) from the single-turnover experiments were converted into DNP-glycine by the method of Jordan & Seehra (1980a). The concentration of the DNP-glycine was determined spectrophotometrically ($\lambda_{\text{max.}} = 348\text{nm}$; ϵ 13600 litre $\cdot$ mol $^{-1}$ $\cdot$ cm $^{-1}$ in methanol). The radioactivity associated with the DNP-glycine was determined as described below. The specific radioactivity of the DNP-glycine could then be calculated.

Counting of radioactive samples

All determinations were made with a Phillips P4700 scintillation spectrophotometer programmed to give a quench correction to d.p.m. Non-aqueous and aqueous samples up to 200 μl in volume were counted for radioactivity in 10 ml of toluene/methanol (3:1, v/v) containing butyl PBD [5-(4-biphenyl)-2-(4-t-butylphenyl)-1-oxa-3,4-diazole] (6g/litre), whereas aqueous samples with volumes up to 2 ml were counted for radioactivity in the scintillation fluid Tritoscint, composed of xylene (2 litres), synperionic NXP (1 litre), PPO (2,5-diphenyloxazole) and POPOP [1,4-bis-(5-phenyloxazol-2-yl)benzene] (1.5g).

Results and discussion

A prerequisite for the formulation of the mechanism of an enzyme catalysing a bisubstrate reaction is a knowledge of the sequence of the binding of the two substrates. Steady-state kinetics (Cleland, 1970) can often provide this information and can also provide evidence for the participation of covalent intermediates. In the case of 5-aminolaevulinate dehydratase, steady-state kinetics cannot be employed, since the two molecules of substrate are identical and therefore in kinetic terms cannot be evaluated independently. In these present studies we therefore utilized an alternative strategy involving a single-turnover enzyme reaction approach in which the two substrate molecules which participate in the reaction were distinguished from one another by isotopic labelling.

In this method, stoichiometric equivalents of 5-amino[5- ^{14}C]laevulinate and homogeneous human 5-aminolaevulinate dehydratase were initially mixed together in a rapid-reaction device for 100 ms (Jordan & Seehra 1980a), the reasoning being that if one of the two 5-aminolaevulinate-binding sites had a higher affinity for the substrate than the other, it would be occupied preferentially by the labelled substrate. Subsequent addition of a large excess of unlabelled 5-aminolaevulinate was used to force this enzyme-bound labelled substrate through into the product, porphobilinogen, thus completing a single turnover, at least with respect to the *labelled substrate*.

As long as one of the two substrate-binding sites has an affinity at least an order of magnitude higher than that of the other site, such a single-turnover reaction would be expected to label the porphobilinogen regiospecifically either in C-2 or C-11, the positions that arise from the C-5 of 5-aminolaevulinate (see Scheme 1) (Shemin & Russell, 1953; Neuberger & Scott, 1953).

Two broad mechanisms for the human 5-aminolaevulinate dehydratase reaction may be considered. In mechanism 1 (Scheme 1) the substrate molecule initially bound to the enzyme of a Schiff base is the one that gives rise to carbon atoms 4, 5, 6, 7 and 11 in porphobilinogen and which occupies the 'acetic acid' side of the active site. A single-turnover reaction of the type described above would lead to the label being incorporated largely into C-11 of porphobilinogen. In mechanism 2, on the other hand, the initially bound substrate ultimately gives rise to carbon atoms 2, 3, 8, 9 and 10 in porphobilinogen and occupies the 'propionic acid' side of the active site (Scheme 1). If this mechanism was operative, a single-turnover reaction would result in the incorporation of label preferentially into C-2 of the product.

These two possibilities may be resolved by determining the percentage of radioactivity present at C-2 and C-11 in the porphobilinogen derived from single-turnover experiments by degradation of this product into glycine. This was accomplished by a sequence involving ozonolysis and hydrolysis in which the glycine thus obtained contained, at its α -position, the carbon atom originally at C-11 in porphobilinogen (see Jordan & Seehra, 1980a, for details).

In order to maximize the chances of obtaining regiospecific labelling in porphobilinogen, various molar-equivalent ratios were used (1.6, 8 and 16 mol of 5-amino[5- ^{14}C]laevulinate/mol of octameric enzyme) and the results from these single-turnover experiments are shown in Table 1(a).

The data clearly show that the distribution of ^{14}C label between C-2 and C-11 of porphobilinogen in all cases favours the labelling of C-2. In experiments where excess 5-amino[5- ^{14}C]laevulinate was mixed with enzyme (Table 1a) and the resulting porphobilinogen was degraded by the same procedure, there was, as expected, an essentially equal distribution of radioactivity between C-11 and C-2.

From these cumulative results, it may be concluded that the first molecule of 5-aminolaevulinate to bind to *human* 5-aminolaevulinate dehydratase is the one that gives rise to the 'propionic acid' side of porphobilinogen (carbon atoms 2, 3, 8, 9 and 10). These findings thus support mechanism 2 (Scheme 1) and are in close agreement with the conclusions of Jordan & Seehra (1980a,b) and Seehra (1980) obtained from the use of this single-turnover-reaction approach with the 5-aminolaevulinate dehydratase enzymes from bovine liver and *Rhodopseudomonas sphaeroides*. A comparison of these latter results with those from the human enzyme are shown in Table 1(b).

Previous work on several 5-aminolaevulinate dehydratases has established the existence of a Schiff-base linkage between the enzyme and one of the substrate molecules (Nandi & Shemin, 1968a,b). Treatment of the enzyme with NaBH_4 in the presence of 5-aminolaevulinate leads to inactivation of the enzyme, and if ^{14}C -labelled substrate is used, the inactivation is accompanied by the incorporation of label into the enzyme protein (Nandi & Shemin, 1968a,b; Seehra, 1980). In order to determine whether the enzyme-[^{14}C]substrate complex responsible for the production of C-2 regiospecifically labelled porphobilinogen in the single-turnover experiments was the same Schiff-base species amenable to trapping with NaBH_4 , it was important to demonstrate the existence of this enzyme-substrate complex both under the conditions and within the time course of the rapid-mixing experiments.

Scheme 1. *Alternative fates of 5-amino[5-¹⁴C]aevulinic acid (ALA) in the enzymic synthesis of porphobilinogen using a single-turnover enzyme-reaction technique*

The symbol ● represents the location of the ¹⁴C label; X = oxygen or enzyme-NH.

Several experiments were therefore carried out in which, after the rapid mixing of labelled 5-aminolaevulinate with the human dehydratase, the resulting mixture was allowed to react with NaBH₄, rather than with excess substrate, under the same conditions used for the single-turnover-reaction experiments. When 8 molar equivalents of

labelled 5-amino[4-¹⁴C]aevulinate were used per mol of enzyme octamer, after quenching the mixture in NaBH₄, 2.8–3.2 mol of 5-aminolaevulinate were incorporated per mol of the octameric enzyme, equivalent to 0.35–0.40 mol of 5-aminolaevulinate per mol of enzyme subunit. This represents an occupancy of 30–40% of available

Table 1. (a) ^{14}C radioactivity in C-11 and C-2 of porphobilinogen enzymically synthesized from 5-amino[5- ^{14}C]laevulinate by means of a single-turnover reaction using human erythrocyte 5-aminolaevulinate dehydratase and (b) comparison of ^{14}C radioactivity incorporated into C-11 and C-2 of porphobilinogen enzymically synthesized from 5-amino[5- ^{14}C]laevulinate by means of a single-turnover reaction using 5-aminolaevulinate dehydratase isolated from different sources

Stoichiometric quantities of human erythrocyte 5-aminolaevulinate dehydratase (8.04–12.85 nmol) and 5-amino[5- ^{14}C]laevulinate (20.5–804 nmol) were mixed in a rapid-mixing device so that the enzyme and [^{14}C]substrate interacted for approx. 100 ms, before the mixture was 'chased' with a large excess of unlabelled 5-aminolaevulinate (60 μmol). After purification by ion-exchange chromatography and t.l.c., the regioselectively labelled porphobilinogen was converted into DNP-glycine (Jordan & Seehra, 1980a) in order to determine the distribution of the label between C-11 and C-2. Notes: a specific radioactivity of amino[5- ^{14}C]laevulinate = $381 \times 10^2 \text{ d.p.m./nmol}$ (expts. 1 and 3) and $63.5 \times 10^2 \text{ d.p.m./nmol}$ (expt. 4); b expressed per mol of the octameric enzyme (M_r 285 000); the amount of enzyme used = 12.85 nmol (expt. 1) and 8.04 nmol (expts. 2, 3 and 4); c specific radioactivity of human erythrocyte 5-aminolaevulinate dehydratase = 21.7 units/mg when assayed in the presence of 0.1 mM-Zn^{2+} ; d determined by reaction of porphobilinogen with p -dimethylaminobenzaldehyde in 2M-HClO_4 (λ_{max} = 555 nm; ϵ 60 200 $\text{litre} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$); e after purification, the yield of porphobilinogen from 5-amino[5- ^{14}C]laevulinate was approx. 30% (expts. 1, 2 and 3); f determined in methanol (λ_{max} = 348 nm; ϵ 13 600 $\text{litre} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$); g determined by comparing the specific radioactivities of the DNP-glycine and the original porphobilinogen; h quantities refer to the number of mol of labelled 5-aminolaevulinate added in the single-turnover experiments based on an M_r of 280 000–285 000; i 5-aminolaevulinate dehydratase purified from human erythrocytes (Gibbs *et al.*, 1985b); j data from Jordan & Seehra (1980a) and Seehra (1980); k 5-aminolaevulinate dehydratase from *R. sphaeroides* exists as a hexameric protein of subunit M_r 47 500. Abbreviation used: ALA, 5-aminolaevulinate.

Expt. no.	No. of mol of [5- ^{14}C]ALA ^a used/mol of enzyme ^{b,c}	Porphobilinogen			DNP-glycine			Radioactivity (%) at:	
		Yield ^{d,e} (μmol)	$10^{-3} \times \text{Total radioactivity}$ (d.p.m.)	$10^{-3} \times \text{Specific radioactivity}$ (d.p.m./ μmol)	Yield ^f (μmol)	$10^{-3} \times \text{Total radioactivity}$ (d.p.m.)	$10^{-3} \times \text{Specific radioactivity}$ (d.p.m./ μmol)	C-11 of porphobilinogen	C-2 of porphobilinogen
1	1.6	19.28	118.7	6.16	3.25	1.35	0.42	6.8	93.2
2	8.0	13.32	444.4	33.37	2.13	12.08	5.67	17.0	83.0
3	16.0	15.60	1651.3	105.85	2.57	77.53	30.17	28.5	71.5
4	Excess (100.0)	14.75	3765.2	255.27	2.65	324.79	123.56	48.0	52.0

Source of enzyme	No. of mol of [5- ^{14}C]ALA used/mol of enzyme ^b	$10^{-3} \times \text{Specific radioactivity}$ (d.p.m./ μmol) of:		Radioactivity (%) at:	
		Porphobilinogen	DNP-glycine	C-11 of porphobilinogen	C-2 of porphobilinogen
Human ⁱ	8	33.4	5.7	17	83
Bovine ^j	8	129	17.6	14	86
<i>R. sphaeroides</i> ^j	6 ^k	896	360	40	60
Control	Excess	255.3	123.6	48.0	52.0

catalytic sites (assuming eight per octamer), a value that correlates well with the observation, in single-turnover experiments, that approx. 30% of the labelled substrate was carried through into porphobilinogen. These results suggest that the enzyme-substrate Schiff base is a catalytically viable intermediate which, in the presence of further substrate, can yield porphobilinogen, but which, when treated with NaBH_4 , is trapped as an inactive reduced complex. The results further demonstrate most importantly that it is the *same* molecule of substrate bound initially to the enzyme which forms a Schiff-base linkage with the dehydratase enzyme.

On the basis of these results a mechanism for the formation of porphobilinogen by the human dehydratase enzyme may be proposed in which the first molecule of 5-aminolaevulinate binds to the 'propionic acid' side of the catalytic site forming a Schiff base with a reactive amino group. Subsequent addition of the second substrate molecule to the 'acetic acid' side of the catalytic site eventually leads to the formation of the product porphobilinogen. This mechanism, first proposed by Jordan & Seehra (1980*a,b*) differs substantially from the mechanism proposed by Nandi & Shemin (1968*b*), in which the converse order of addition of the two molecules of 5-aminolaevulinate to the enzyme was favoured. The approach by these latter workers involved the use of the pseudosubstrate, laevulinate, which together with 5-aminolaevulinate yielded a 'mixed' pyrrole with the dehydratase from *Rhodopseudomonas sphaeroides*. Since laevulinate was also shown to form a Schiff base with the enzyme, it was suggested that laevulinate interacted only at the 'acetic acid' site and it was argued that the first substrate was also bound at this site.

This reasoning, however, may be suspect, since it takes no account of the fact that laevulinate could interact with *both* substrate binding sites: acting as a *substrate* at the acetic acid site (A in Scheme 1) but, since it has no amino group like the true substrate, acting only as an *inhibitor* at the 'propionic acid' site (B in Scheme 1). Our findings that the first 5-aminolaevulinate molecule binds to the enzyme in the form of a Schiff base leads us to suggest, by analogy, that the enzyme inactivation which occurs when the enzyme is allowed to react with NaBH_4 in the presence of laevulinate (Nandi & Shemin, 1968*b*) is as a result of the binding of the laevulinate to the same site, the P-site (Scheme 1).

The single-turnover approach described above is a powerful technique for the study of homopolymerization reactions and highlights the importance of using the physiological substrate rather

than inhibitors or pseudosubstrates. This technique has also been used successfully in our laboratory to elucidate mechanistic features of other enzymes catalysing homopolymerization reactions, including β -oxoacyl-CoA thiolase (Jordan & Gibbs, 1983) and porphobilinogen deaminase (Jordan & Seehra, 1979; Seehra & Jordan, 1980).

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