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Mechanism of action of porphobilinogen deaminase

The participation of stable enzyme substrate covalent intermediates between porphobilinogen and the porphobilinogen deaminase from *Rhodopseudomonas spheroides*

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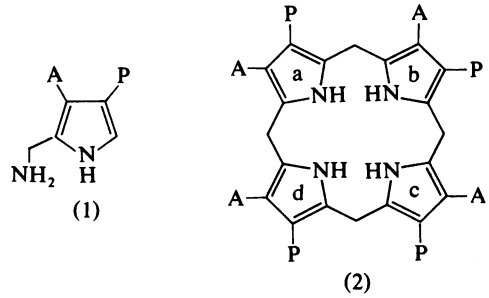
Highly stable labelled complexes are formed between porphobilinogen deaminase and stoicheiometric amounts of [^{14}C]porphobilinogen. On completion of the catalytic cycle by the addition of excess of substrate, the complexes yield labelled product and display all the properties expected from covalently bound enzyme intermediates involved in the deaminase catalytic sequence.

Porphobilinogen deaminase and uroporphyrinogen III co-synthetase catalyse the transformation of porphobilinogen (1) into uroporphyrinogen III (2) (Bogorad, 1958*a*,*b*,*c*). Uroporphyrinogen III is the precursor for all the major tetrapyrroles found in biological systems (Akhtar & Jordan, 1979).

The biosynthesis of uroporphyrinogen III has been shown to proceed via the unstable linear tetrapyrrolic intermediate pre-uroporphyrinogen (3), which arises from porphobilinogen under the influence of porphobilinogen deaminase (Burton *et al.*, 1979). Pre-uroporphyrinogen (3) subsequently acts as the physiological substrate for uroporphyrinogen III co-synthetase, leading to the formation of uroporphyrinogen III in high yield (Jordan *et al.*, 1979).

The mechanism by which porphobilinogen deaminase catalyses the formation of the tetrapyrrole from four molecules of porphobilinogen has been investigated by single-turnover experiments with labelled porphobilinogen (Jordan & Seehra, 1979; Battersby *et al.*, 1979; Seehra & Jordan, 1980), and it has been established that the order in which the four porphobilinogen rings are added to the deaminase is ring a followed by b, c and finally ring d (see structures 1 and 2). Under normal conditions no intermediates between porphobilinogen and pre-uroporphyrinogen are liberated into solution, the deaminase preferring to synthesize the tetrapyrrole from porphobilinogen by a mechanism involving only enzyme-bound intermediate species (Frydman *et al.*, 1976).

The present paper describes the isolation and characterization of covalent enzyme–intermediate complexes formed by the interaction of [^{14}C]-porphobilinogen with porphobilinogen deaminase and their significance in the catalytic mechanism of the enzyme.



Abbreviation used: SDS, sodium dodecyl sulphate.

Materials and methods

Methods

Bio-Gel HTP was obtained from Bio-Rad Labora-

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tories (St. Albans, Herts., U.K.); DEAE-cellulose (DE-52) was supplied by Whatman (Maidstone, Kent, U.K.); Sephadex G-100 (fine grade) and G-50 (fine grade) were purchased from Pharmacia (London W.5, U.K.). Dithioerythritol was obtained from Sigma Chemical Co. (Poole, Dorset, U.K.). Opsopyrroledicarboxylic acid (4) [3(4)-carboxymethylpyrrole-4(3)-β-propionic acid] was generously given by Dr. S. F. MacDonald (Division of Pure Chemistry, National Research Council of Canada, Ottawa, Ont., Canada). 5-Amino[4-^{14}C]laevulinic acid hydrochloride was obtained from The Radiochemical Centre (Amersham, Bucks., U.K.). Porphobilinogen was prepared enzymically from 1g of 5-aminolaevulinic acid (6mmol) by using purified 5-aminolaevulinate dehydratase (Seehra & Jordan, 1980). [3,5-$^{14}C_2$]Porphobilinogen was prepared by a similar procedure from 5-amino[4-^{14}C]-laevulinic acid (42mCi/mmol) (1μmol). *Rhodopseudomonas spheroides* (N.C.I.B. 8253) was from the Torry Research Station (Aberdeen, Scotland, U.K.), and was grown as described by Lascelles (1956, 1959). All other chemicals were purchased from BDH Chemicals (Poole, Dorset, U.K.).

Porphobilinogen deaminase

Porphobilinogen deaminase was isolated from *Rhodopseudomonas spheroides* as previously described (Jordan & Shemin, 1973). The enzyme was assayed by incubation with 100nmol of porphobilinogen in a final volume of 1.3ml of 0.1M-Tris/HCl buffer, pH 8.2. The reaction was stopped after 6min by the addition of 250μl of 5M-HCl. The porphyrinogens formed were oxidized to porphyrins with benzoquinone and the absorbance was measured at 405nm.

Isolation of enzyme–substrate intermediates

Deaminase (1nmol) was added to [3,5-$^{14}C_2$]-porphobilinogen (84mCi/mmol; 1–5nmol) in 0.1M-Tris/HCl buffer, pH 8.2, in a rapid-mixing apparatus (Jordan & Seehra, 1980). The resulting mixture (400μl) was chromatographed on a Sephadex G-50 (fine grade) column (29cm × 1cm) equilibrated in the same buffer. Fractions (1.4ml) were collected and analysed for enzyme activity, prophyrin, porphobilinogen and radioactivity. This procedure separates enzyme, porphyrin and porphobilinogen from one another. All manipulations were conducted at 4°C.

Radioactivity measurements

Water was added to the samples (200–800μl) to give a final volume of 1ml. Then 10ml of a detergent-based scintillant (Wood *et al.*, 1975) was added to obtain a clear solution. The radioactivities of the samples were then counted in a Philips liquid-scintillation spectrometer programmed to d.p.m. quench correction.

Results and discussion

Isolation of enzyme–porphobilinogen complexes

Single-turnover experiments performed previously in our laboratory (Seehra & Jordan, 1980) suggested that 1, 2, 3 or 4 molar equivalents of porphobilinogen become bound to the deaminase when stoicheiometrically related amounts of enzyme and substrate are mixed. Elegant experiments by Anderson & Desnick (1980) also suggest the existence of stable complexes between porphobilinogen and deaminase, although they have not yet established their significance in the overall catalytic reaction. These findings have been extended in order to isolate and characterize deaminase enzyme–substrate complexes and establish their role in the catalytic cycle of the enzyme.

Porphobilinogen deaminase (1nmol) was added to 1nmol of [3,5-$^{14}C_2$]porphobilinogen as described in the Materials and methods section and the mixture was analysed on a column of Sephadex G-50. Fig. 1 shows that most of the ^{14}C label was associated with

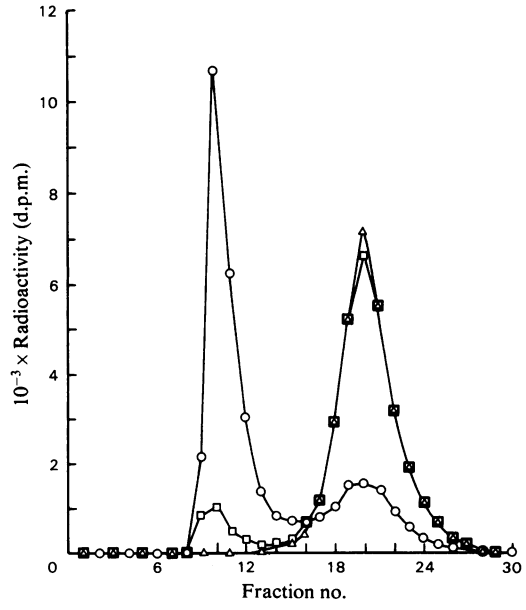

Fig. 1. *Isolation of a complex formed from porphobilinogen deaminase and [^{14}C]porphobilinogen*
The Figure shows the chromatography elution profile from Sephadex G-50 column (29cm × 1cm diam.) after rapid mixing of 1nmol of [3,5-$^{14}C_2$]porphobilinogen (84mCi/μmol) with 1nmol of native deaminase (○), 1nmol of boiled enzyme (△) or 1nmol of native deaminase after pretreatment with 1mM-opsopyrroledicarboxylic acid (□).

the column fraction corresponding to the enzyme, indicating that a stable complex between the active enzyme and the substrate had been formed. The small peak of 'unbound' radioactivity is attributable to porphobilinogen oxidation products rather than to free porphobilinogen. Controls (Fig. 1), with boiled enzyme and [^{14}C]porphobilinogen or [^{14}C]porphobilinogen alone, resulted in the elution of all the ^{14}C radioactivity in the small-molecules fraction. Pretreatment of the enzyme with the competitive inhibitor opsopyrroledicarboxylic acid (4) before the addition of porphobilinogen prevented the binding of porphobilinogen to the deaminase. Exposure of the isolated enzyme–substrate complex to freezing and thawing, incubation for 2h at 37°C, storage for 1 week at 4°C or at pH 6.5, 7.8 and 9.0 all failed to liberate any significant label from the enzyme–porphobilinogen complex.

Similar experiments in which increasing molar equivalents of substrate were mixed with enzyme gave a corresponding increase in label associated with the protein. However, when more than 4 molar equivalents of porphobilinogen were added to deaminase, uroporphyrinogen was formed at the expense of the enzyme-bound species. The above findings suggest that the interaction of the enzyme with the substrate leads to highly stable enzyme–substrate complexes.

Properties of the enzyme–porphobilinogen complex

A requirement of an enzyme–substrate complex is that completion of the enzyme turnover by the addition of further substrate should lead to all bound substrate being carried into the product. Accordingly, excess of unlabelled porphobilinogen was added to the complex formed from 1nmol of deaminase and 1nmol of [^{14}C]porphobilinogen. Re-chromatography on Sephadex G-50 revealed that all the ^{14}C radioactivity was now associated with uroporphyrin, as judged by absorbance at 405nm (Fig. 2*a*). There was little evidence of ^{14}C radioactivity associated with either the protein or the porphobilinogen peaks. It appears that the enzyme–[^{14}C]porphobilinogen complex is thus a catalytically viable species and is able to participate in the formation of the tetrapyrrole.

The formation of any physiologically significant deaminase–porphobilinogen complex should be affected by competitive inhibitors such as opsopyrroledicarboxylic acid (4) (Bogorad, 1957). When opsopyrroledicarboxylic acid was used to pretreat the enzyme before the addition of [^{14}C]porphobilinogen, then no label was bound to the enzyme (see Fig. 1). However, when opsopyrroledicarboxylic acid was added to the enzyme–[^{14}C]porphobilinogen complex, it failed to displace the label (Fig. 2*b*). Opsopyrroledicarboxylic acid (4,), though having the acetate and propionate side chains and the aromatic ring for recognition by the deaminase active site, has no $-CH_2-NH_2$ function, and is not likely to be able to displace bound substrate from the deaminase, particularly if the latter is covalently bound.

Previous studies (Davies & Neuberger, 1973; Radmer & Bogorad, 1972) have suggested that intermediates normally bound to the enzyme may be displaced by NH_3, NH_2OH or NH_2O-CH_3. The complex between enzyme and porphobilinogen would also be expected to be sensitive to the attack of NH_3 or NH_2OH. This was found to be the case, since when the enzyme–[^{14}C]porphobilinogen complex was treated with NH_2OH a large proportion of the ^{14}C label was found to have been displaced from the complex and was found to be associated with the small-molecules fraction (Fig. 2*c*).

Evidence for a covalent linkage between enzyme and porphobilinogen

Paramount to our understanding of the mechanism of the enzyme reaction is a knowledge of the type of interaction between enzyme and substrate. Whether the binding of a substrate with the enzyme is by covalent bonding or by strong physical interaction may be resolved by denaturing the enzyme–[^{14}C]porphobilinogen complex in a detergent such as SDS. A covalent bond would survive such treatment, whereas physical interaction would be destroyed by this treatment. Fig. 2(*d*) shows the effect of the addition of SDS (1%, w/v) on the effectiveness of the deaminase to bind porphobilinogen. No displacement of the ^{14}C label into solution was noted, the radioactivity remaining tightly associated with the protein. However, when porphobilinogen was added to the deaminase after the latter had been denatured by SDS there was no formation of a protein–substrate complex, indicating the importance of an active enzyme for the formation of the enzyme–[^{14}C]porphobilinogen complex (results not shown). These findings thus provide the first direct evidence for the participation of a covalent linkage after reaction between the deaminase and the porphobilinogen. It is noteworthy that NH_2OH is also able to liberate ^{14}C label from the complex that had previously been treated with SDS, suggesting that the action of the inhibitory base is almost certainly a chemical displacement involving cleavage of an enzyme $X-CH_2$ pyrrole bond, where X is either an O or N function. The conclusion from this and previous work (Seehra & Jordan, 1980) may be used to propose a mechanism for the action of porphobilinogen deaminase in which the first porphobilinogen unit, ($^{+}NH_3-\overset{*}{C}H_2$-pyr), which becomes ring a in the product (Jordan & Seehra, 1979), is initially bound to the enzyme (Enz) through a covalent bond concomitant with the displacement of NH_3 (eqn. 1).

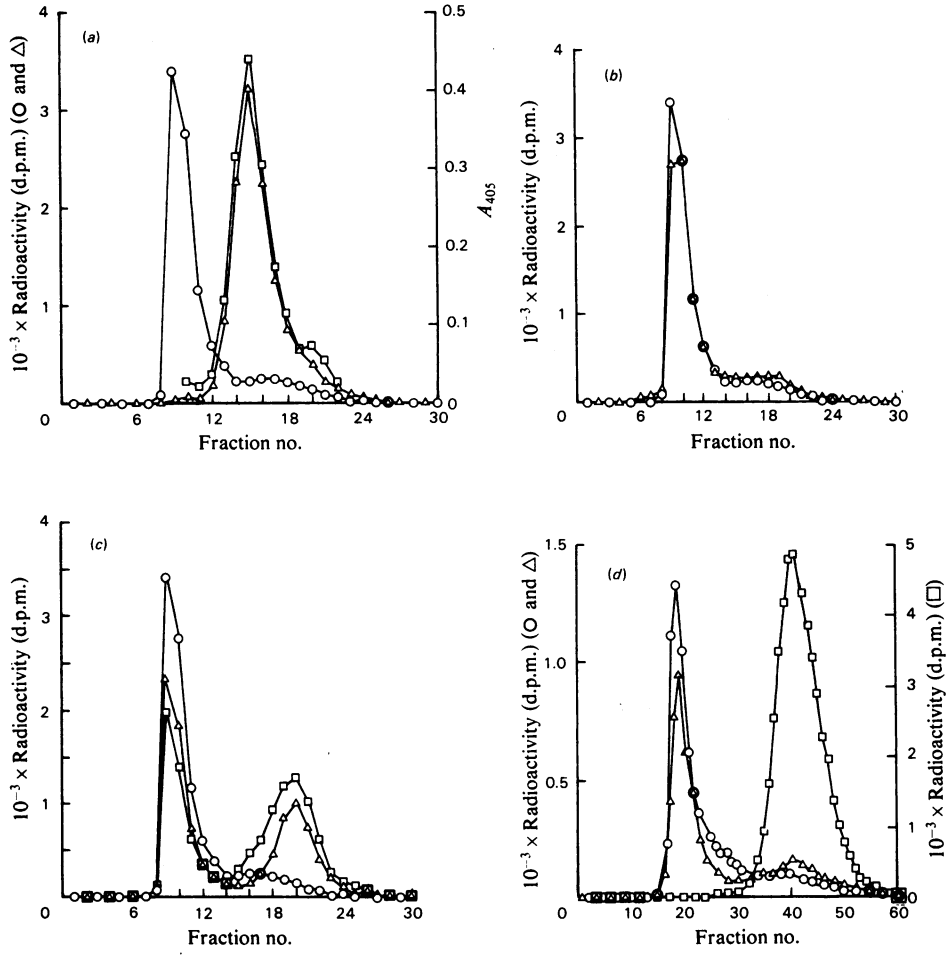

Fig. 2. *Effects of porphobilinogen, opsopyrroledicarboxylic acid, hydroxylamine and SDS on the deaminase–[^{14}C]porphobilinogen complex*

Enzyme–[^{14}C]porphobilinogen complex was isolated as described in Fig. 1. The resulting complex was treated with porphobilinogen, opsopyrroledicarboxylic acid, NH_2OH or SDS as follows. (*a*) Porphobilinogen: O, 125pmol of enzyme–[^{14}C]porphobilinogen complex chromatographed on a Sephadex G-50 column after incubation for 10min at 37°C; △, after incubation at 37°C for 10min with excess (100nmol) of unlabelled porphobilinogen to complete the enzyme turnover; □, porphyrinogens formed in this latter experiment oxidized to porphyrins and determined at 405nm in 1M-HCl. (*b*) Opsopyrroledicarboxylic acid: O, 125pmol of enzyme–[^{14}C]porphobilinogen complex re-chromatographed on a Sephadex G-50 column; △, 100pmol of enzyme–[^{14}C]porphobilinogen complex after treatment with opsopyrroledicarboxylic acid (final concn. 1mM) and chromatography on Sephadex G-50. (*c*) Hydroxylamine: O, 125pmol of enzyme–[^{14}C]porphobilinogen complex re-chromatographed on a Sephadex G-50 column after incubation at room temperature for 10min; △, after treatment with NH_2OH (0.2M) for 10min at room temperature; □, after treatment with NH_2OH (0.5M) for 10min at room temperature. (*d*) SDS: O, 60pmol of enzyme–[^{14}C]porphobilinogen complex re-chromatographed on a Sephadex G-50 column equilibrated in 1% SDS (w/v) in 0.1M-Tris/HCl buffer, pH8.2, after denaturation with 1% SDS (w/v) at room temperature for 5min; △, as above but incubation with SDS for 2h; □, [^{14}C]porphobilinogen incubated for 2h in the absence of deaminase.

The term enzyme-porphobilinogen complex used throughout this paper is thus strictly an enzyme–intermediate complex (enzyme–$\overset{*}{C}H_2$–opsopyrroledicarboxylic acid) formed by displacement of NH_3 from porphobilinogen and *C*-alkylation of the enzyme.

Succeeding porphobilinogen units are then added sequentially while the initially bound pyrrole residue

remains covalently linked to the enzyme (eqn. 2). Subsequent liberation of bound intermediate occurs at the tetrapyrrole stage by cleavage of the covalent enzyme intermediate linkage (eqn. 3):

$$\text{EnzX} + {}^{+}\text{NH}_3\text{–}\overset{*}{\text{C}}\text{H}_2\text{–pyr} \rightarrow \text{EnzX–}\overset{*}{\text{C}}\text{H}_2\text{–pyr} + \text{NH}_3 \quad (1)$$

$$\text{EnzX–}\overset{*}{\text{C}}\text{H}_2\text{–pyr} + 3\ {}^{+}\text{NH}_3\text{–CH}_2\text{–pyr} \rightarrow\rightarrow\rightarrow \text{EnzX–}\overset{*}{\text{C}}\text{H}_2\text{–pyr–CH}_2\text{–pyr–CH}_2\text{–pyr–CH}_2\text{–pyr} + 3\text{NH}_3 \quad (2)$$

$$\text{EnzX–}\overset{*}{\text{C}}\text{H}_2\text{–pyr–CH}_2\text{–pyr–CH}_2\text{–pyr–CH}_2\text{–pyr} \rightarrow\rightarrow \text{EnzX} + \text{HO}\overset{*}{\text{C}}\text{H}_2\text{–pyr–CH}_2\text{–pyr–CH}_2\text{–pyr–CH}_2\text{–pyr} \quad (3)$$

This mechanism differs from that proposed by Anderson & Desnick (1980) in that the amino group of the porphobilinogen giving rise to ring a is displaced on initial binding to the enzyme by virtue of the formation of the covalent bond.

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