THE ISOLATION AND CHARACTERIZATION OF CATALYTICALLY COMPETENT PORPHOBILINOGEN DEAMINASE–INTERMEDIATE COMPLEXES

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1. Introduction

Porphobilinogen deaminase catalyses the homopolymerization of 4 molecules of porphobilinogen (1) to give preuroporphyrinogen (2) [1,2]. Preuroporphyrinogen is the natural substrate for uroporphyrinogen III cosynthetase leading to the formation of uroporphyrinogen III (3) [3] in quantitative yield. In the absence of cosynthetase, preuroporphyrinogen spontaneously converts to uroporphyrinogen I (4). The order in which the deaminase catalyses the assembly of the 4 porphobilinogen molecules has been determined in [4–6] and it appears that addition of the 4 pyrrole rings to the enzyme follows the sequence ring *a* followed by ring *b* and *c* and finally ring *d* (see structure (2)). Observations that the addition of stoichiometric amounts of porphobilinogen to deaminase give tightly bound species in which 1,2 or 3 mol substrate (or equivalent) are bound to the enzyme have suggested that discrete enzyme intermediate complexes exist [5]. Elegant experiments [7] involving excess [^{3}H]-porphobilinogen and human deaminase have shown the existence of 4 labelled protein bands with deaminase enzyme activity again suggesting the existence of discrete enzyme intermediate complexes. Related work [8] with the deaminase from *Rhodopseudomonas spheroides* has largely confirmed these latter findings although only 3 discrete labelled protein bands are formed with the bacterial enzyme when incubated with [^{14}C]porphobilinogen. In addition we have established that the enzyme forms a relatively stable covalent link with the bound pyrrole residues [9].

Although the appearance of 3-intermediate complexes in the case of the bacterial deaminase suggests the existence of complexes with 1,2 and 3 pyrrole units linked to the enzyme, the demonstration of 4 complexes in the case of the human deaminase is less easy to explain. It was therefore important to isolate the labelled enzyme intermediate complexes and to convert each into product. Since the order in which the pyrrole rings are incorporated into the tetrapyrrole is known [5] the degradation of the products formed from each labelled complex should reveal the exact location of label and thereby allow the structural characterization of each enzyme intermediate complex. This paper deals with experiments carried out with the deaminase from *Rhodopseudomonas spheroides.*

2. Experimental

Biogel HTP was obtained from BioRad Labs. (St Albans, Herts); DEAE cellulose (DE52) was purchased from Whatman (Maidstone, Kent) and Sephadex G-100 (fine) from Pharmacia (London W5); Acrylamide and methylene *N,N'*-bis-acrylamide were obtained from Koch Light Labs. Porphobilinogen and [3,5-$^{14}C_2$]porphobilinogen were prepared as in [9]. All other chemicals were from BDH Chemicals (Poole, Dorest).

Porphobilinogen deaminase was isolated as in [10] from *Rhodopseudomonas spheroides* (NC1B 8253) obtained from the Torry Research Station (Aberdeen, Scotland) which was grown according to [11,12]. Enzyme activity was assayed as in [9].

2.1. *Formation and isolation of porphobilinogen deaminase–intermediate complexes*

Enzyme and [3,5-$^{14}C_2$]porphobilinogen (spec. act.

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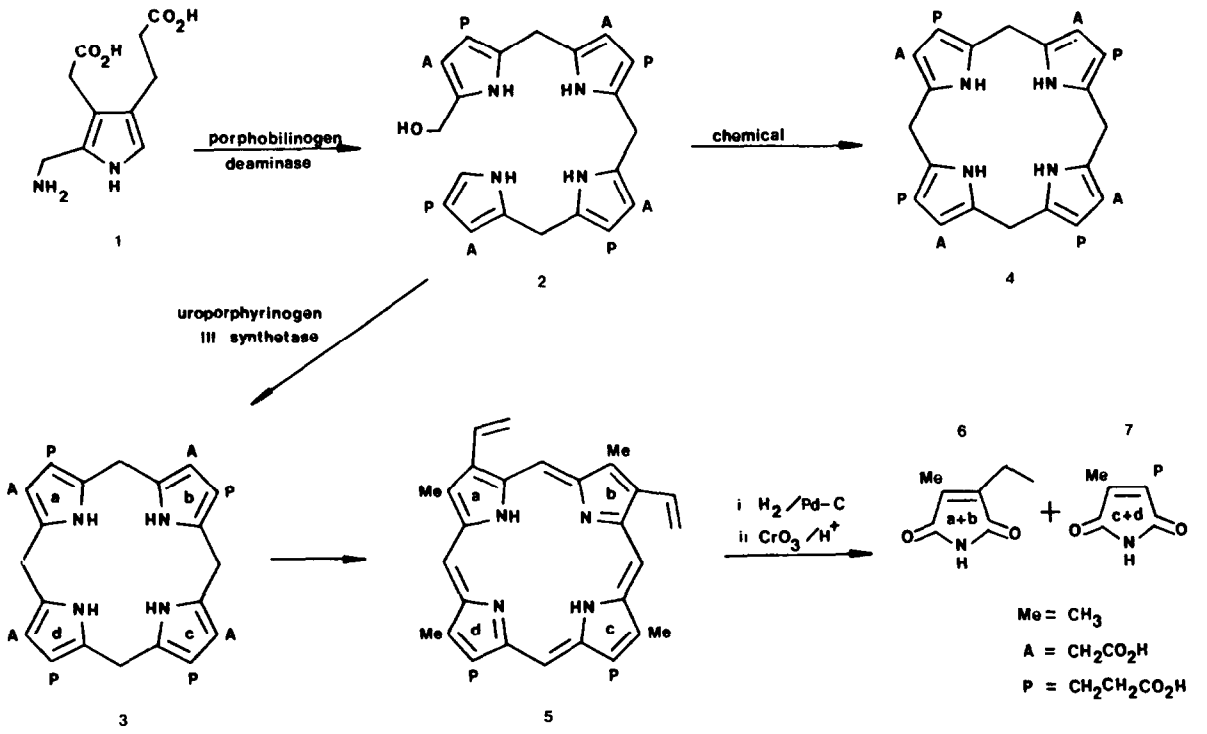


Fig.1.

106 μCi/μmol) were mixed in a rapid mixing apparatus (see table 1 for amounts). The enzyme intermediate complexes were isolated by electrophoresis in a 7.5% polyacrylamide gel slab (11 cm X 24.5 cm X 5 mm) in the long dimension. All manipulations were carried out at 0°C. After electrophoresis the enzyme bands were visualised by incubation of a 1 mm strip from each gel track with 2 mM porphobilinogen at 37°C for 30 min. The strips were then treated with benzoquinone in methanol (1%, w/v) and the fluorescent areas were detected under UV light. The corresponding enzyme intermediate complexes were then recovered from the remainder of the gel track by homogenising the gel in 0.1 M Tris/HCl buffer (pH 8.2) followed by extraction at 4°C for 1.5 h.

The deaminase–intermediate complexes, (table 1) were incubated with excess unlabelled porphobilinogen (3.6 mg) and the resulting preuroporphyrinogen was converted in situ to protoporphyrin IX in a linked enzyme system from *Rhodopseudomonas spheroides* containing uroporphyrinogen III cosynthetase, uroporphyrinogen III decarboxylase and coproporphyrinogen oxidase as in [5].

2.2. *Isolation and degradation of protoporphyrin IX*

The enzyme reaction was terminated by the addition of benzoquinone (6 mg) in methanol resulting in oxidation of any remaining porphyrinogens into porphyrins. Protoporphyrin IX was isolated by extraction as in [5,13]. Protoporphyrin IX [5] was reduced to mesoporphyrin IX and the latter was oxidized by CrO_3:H_2SO_4 to hematinic acid and ethyl methyl maleimide [14]. The maleimides were purified by vacuum sublimation [5].

2.3. *Radioactivity measurements*

All samples were counted in a Philips Liquid Scintillation System (Model PW 4700) in toluene methanol (75:25, v/v) containing 5-(biphenylyl)2-(4-butylphenyl)-1-oxa-4-diazole (6 g/l) as the scintillant. All samples were corrected to dpm using the appropriate quench curves. Specific radioactivities of protoporphyrin, ethylmethyl maleimide and hematinic acid were determined using the following extinction coefficients; protoporphyrin in ether $\epsilon_{408} = 165\,000\ M^{-1} . L^{-1}$; hematinic acid in water $\epsilon_{295} = 475\ M^{-1} . L^{-1}$; ethyl methyl maleimide $\epsilon_{295} = 435\ M^{-1} . L^{-1}$.

Table 1
Properties of porphobilinogen deaminase intermediate complexes

Band	Mobility% tracker dye	Deaminase activity	Total counts isolated intermediate	Total counts isolated protoporphyrin	Specific activity protoporphyrin (dpm/μmol)	Specific activity hematinic acid (dpm/μmol)	Specific activity ethyl methyl-maleimide (dpm/μmol)	Ratio % ethyl methyl-maleimide: hematinic acid
Native deaminase	56	+	–	–	–	–	–	–
A	59	+	23 150	15 200	3389	0	1710	100:0
B	65	+	214 830	57 700	14 310	0	7180	100:0
C	70	+	118 476	30 820	6502	998	2200	68:32

Porphobilinogen deaminase (1 nmol) was admixed with [3,5-$^{14}C_2$]porphobilinogen (1–3 nmol); spec. act. 106 μCi/μmol, and the resulting labelled intermediate complexes A–C were purified as in section 2. Electrophoretic mobilities of the complexes were measured from the fluorescent bands of uroporphyrin I formed on incubation of a strip of the gel with porphobilinogen. Complexes A, B and C (0.1–0.5 nmol) were incubated with unlabelled porphobilinogen (~15 μmol) and the resulting protoporphyrin IX was purified (~3 μmol) and degraded into ethyl methyl-maleimide and hematinic acid (~1.0 μmol of each). Specific activities refer to dpm/μmol

3. Results and discussion

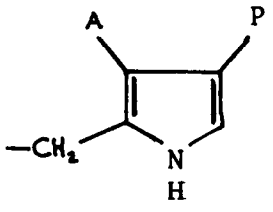
Polyacrylamide electrophoresis of porphobilinogen deaminase admixed with increasing stoichiometric amounts of [3,5,-$^{14}C_2$]porphobilinogen revealed 3 new protein bands with progressively higher electrophoretic mobility (table 1A–C) suggesting an increase in net negative charge compared to that of the native deaminase. All 3 newly formed protein bands exhibited deaminase catalytic activity and were labelled with ^{14}C. The label was quantitatively incorporated into uroporphyrinogen I on incubation with unlabelled prophobilinogen [8].

It has been established [5] that porphobilinogen is incorporated into the linear tetrapyrrole, preuroporphyrinogen, in a sequential reaction with ring *a* being the initial pyrrole residue bound to the deaminase followed by rings *b*, *c* and finally *d*. The possibility thus arises that there may be at least 4 distinct enzyme intermediate complexes E(CH_2pyr)*, E(CH_2pyr)$_2$, E(CH_2pyr)$_3$ and E(CH_2pyr)$_4$. Our finding that in the deaminase from *Rhodopseudomonas spheroides*, only 3 complexes are formed together with the observation [7] that the human deaminase forms 4 labelled complexes highlighted the importance of isolating and characterizing each intermediate complex to determine the number of pyrrole residues bound in each.

Accordingly, porphobilinogen deaminase was incubated with increasing amounts of [3,5-$^{14}C_2$]-porphobilinogen under conditions required to maximise the formation of each intermediate complex (see table 1). The labelled complexes were then purified by preparative polyacrylamide slab gel electrophoresis and each intermediate complex was incubated with an excess of unlabelled porphobilinogen to complete the turnover of the labelled enzyme bound intermediate to form regiospecifically labelled preuroporphyrinogen. Preuroporphyrinogen is highly unstable ($t_{1/2}$ = ~ 4 min at 37°C, pH 8.2) and forms the symmetrical uroporphyrinogen I in a rapid chemical reaction. Since the identification of the position labelled in the tetrapyrrole depends on a regioselective degradation, it was important to convert preuroporphyrinogen into uroporphyrinogen III and thence into protoporphyrin IX (5). The specificity of the enzymes involved allow the utilization of only the assymetric III isomer series, and more importantly, ensures a specific modification of the propionate residues on rings *a* and *b* to vinyl groups thus allowing a distinction between rings *a* + *b* and rings *c* + *d* in the protoporphyrin IX. Hence a comparison of the radioactive label in hematinic acid (6) and ethyl methyl maleimide (7) isolated from protoporphyrin (5) originating from labelled enzyme intermediate complexes will enable the number of pyrrole residues bound to the complexes to be determined. Of 4 possible intermediate complexes only 3 are observed (bands A–C) and since these 3 bands were separated by similar distances on the polyacrylamide gel (table 1) it follows that are almost certainly observing either the sequential intermediates E(CH_2pyr), E(CH_2pyr)$_2$ and E(CH_2pyr)$_3$ or E(CH_2pyr)$_2$, E(CH_2pyr)$_3$ and E(CH_2pyr)$_4$. These two possibilities may be distinguished by a comparison of the ratios of radioactivity between ethyl methyl maleimide and hematinic acid. Should the 3 intermediate complexes represent E(CH_2pyr), E(CH_2pyr)$_2$ and E(CH_2pyr)$_3$ then the ratio of ethyl methyl maleimide to hematinic acid would be 100:0, 100:0 and 67:33, respectively. Should the three bands be due to E(CH_2pyr)$_2$, E(CH_2pyr)$_3$ and E(CH_2pyr)$_4$ then the ratios observed should be 100:0, 67:33 and 50:50.

E = deaminase

(CH_2pyr) =



The results shown in table 1 are very close to those expected for the complexes containing 1,2 and 3 pyrrole rings bound to the deaminase enzyme. Further indirect evidence for this conclusion comes from a close investigation of the electrophoretic mobility of bands A–C compared with the native deaminase. The observed increase in mobility between bands A and B and between bands B and C is about twice that noted for the distance between native deaminase and band A thus suggesting the net increase in negative charge on forming of band A is only ~½ of that compared with the conversion of A–B and B–C. Since the binding of each pyrrole residue would contribute 2 net negative charges (after loss of $-\dot{N}H_3$) it follows that the binding of the first pyrrole residue to the enzyme must be neutralizing a negatively charged enzyme group thus only providing a net increase of one negative

charge. The mobility data together with those obtained from the degradation of the labelled protoporphyrin IX samples thus enable an unambiguous assignment of bands A–C to $E(CH_2pyr)$, $E(CH_2pyr)_2$, $E(CH_2pyr)_3$, respectively. Under no conditions were we able to find a band of higher mobility than C, indicating that under our conditions the isolation of an enzyme bound tetrapyrrole is not possible although such an intermediate must exist. Such a complex would be expected to form free enzyme and preuroporphyrinogen in a very facile manner. The existence of 3 relatively stable covalent enzyme intermediates which are catalytically competent complexes and which can be isolated and characterised is nevertheless a remarkable property of this enzyme.

Acknowledgement

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References

[1] Burton, G., Fagerness, P. E., Hosozawa, S., Jordan, P. M. and Scott, A. I. (1979) J. Chem. Soc. Chem. Commun. 202–204.
[2] Scott, A. I., Burton, G., Jordan, P. M., Matsumoto, H., Fagerness, P. E. and Pryde, L. (1980) J. Chem. Soc. Chem. Commun. 384–387.
[3] Jordan, P. M., Burton, G., Nordlov, H., Schnieder, M. M., Pryde, L. M. and Scott, A. I. (1979) J. Chem. Soc. Chem. Commun. 204–205.
[4] Jordan, P. M. and Seehra, J. S. (1979) FEBS Lett. 104, 364–366.
[5] Seehra, J. S. and Jordan, P. M. (1980) J. Am. Chem. Soc. 102, 6841–6847.
[6] Batersby, A. R., Fookes, C. J. R., Matcham, G. W. J. and McDonald, E. (1979) J. Chem. Soc. Chem. Commun. 539–541.
[7] Anderson, P. M. and Desnick, R. J. (1980) J. Biol. Chem. 255, 1993–1999.
[8] Berry, A. and Jordan, P. M. (1981) Biochem. Soc. Trans. in press.
[9] Jordan, P. M. and Berry, A. (1981) Biochem. J. 195, 177–181.
[10] Jordan, P. M. and Shemin, D. (1973) J. Biol. Chem. 248, 1019–1024.
[11] Lascelles, J. (1956) Biochem. J. 62, 78–93.
[12] Lascelles, J. (1959) Biochem. J. 72, 508–518.
[13] Tait, G. H. Biochem. J. (1972) 128, 1159–1169.
[14] Muir, H. M. and Neuberger, A. (1950) Biochem. J. 47, 97–104.