

PREUROPORPHYRINOGEN, A UNIVERSAL INTERMEDIATE IN THE BIOSYNTHESIS OF UROPORPHYRINOGEN III

P. M. JORDAN and A. BERRY

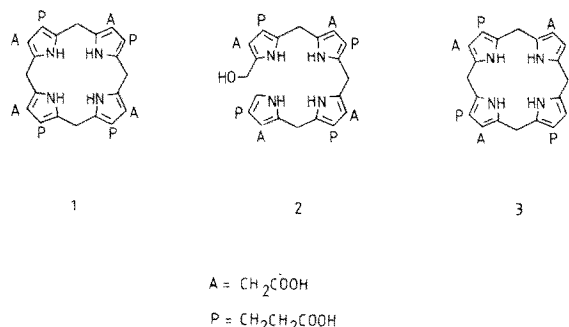
Department of Biochemistry, University of Southampton, Southampton, SO9 3TU, England

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1. Introduction

Since their initial recognition over 20 years ago [1–3] the two enzymes which catalyse the formation of uroporphyrinogen III (1), namely porphobilinogen deaminase and uroporphyrinogen III cosynthetase[†], have been isolated from a variety of sources (reviewed [4]). Numerous attempts have been made to unravel the mechanism by which the enzymes catalyse the formation of uroporphyrinogen III, the key intermediate in the biosynthesis of heme, chlorophylls and corrins. Until recently, the two enzymes had been widely considered to function in a complex with the catalytic events between porphobilinogen and uroporphyrinogen III occurring through enzyme bound intermediates. This has been arrived at by surmise rather than from direct experimental observations (reviewed [5]).

Our concept of the mechanism by which the tetrapyrrole ring is biosynthesised has been totally altered by reports that in the photosynthetic bacterium



Rhodospseudomonas spheroides the enzyme porphobilinogen deaminase catalyses the formation of an enzyme free tetrapyrrole intermediate [6] preuroporphyrinogen* (2) which acts as the substrate for uroporphyrinogen III cosynthetase [7], leading to the formation of uroporphyrinogen III in almost quantitative yield. In the absence of the cosynthetase the preuroporphyrinogen is rapidly and non-enzymically transformed into uroporphyrinogen I (3), scheme 1.

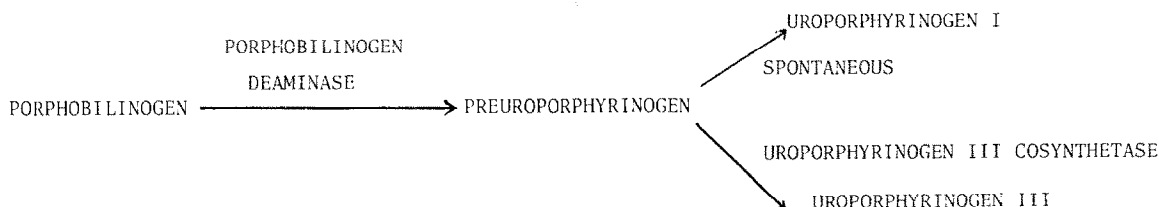
To establish whether the intermediacy of preuroporphyrinogen in the biosynthesis of uroporphyrinogen III is a universal phenomenon and also occurs in eukaryotes we have investigated the conversion of preuroporphyrinogen into uroporphyrinogen III by the cosynthetases from several other living systems.

2. Materials and methods

Porphobilinogen was prepared enzymically from 5-aminolevulinic acid using purified 5-aminolevulinic acid dehydratase [8]. Porphobilinogen deaminase, free of uroporphyrinogen III cosynthetase, was isolated from *Rps. spheroides* (NCIB 8253) by the method in

[†] In view of the findings reported here and in [7] the name uroporphyrinogen I synthetase is no longer tenable since the enzyme does not catalyse the formation of uroporphyrinogen I, the latter spontaneously arising from preuroporphyrinogen in a chemical reaction. Porphobilinogen deaminase or preuroporphyrinogen synthetase should therefore be used. In terms of the second enzyme, uroporphyrinogen III cosynthetase is also no longer meaningful since this enzyme acts independently on preuroporphyrinogen. Uroporphyrinogen III synthetase is thus preferable

* The structure of preuroporphyrinogen (2) has been discussed elsewhere and several possibilities have been considered, the chemical shift data from ¹³C NMR experiments [6] favouring the hydroxymethylbilane (2)



Scheme 1

[9] except that the hydroxyl apatite stage was omitted and Biogel A.5 was substituted for Sephadex G-100. The bacterium was grown semi-anaerobically in the light as in [10].

Porphobilinogen deaminase was assayed either by following the disappearance of porphobilinogen or the formation of uroporphyrin [9]. One unit of porphobilinogen deaminase consumes 1 μ mol porphobilinogen/h.

Uroporphyrinogens were oxidised to uroporphyrins and after conversion to their methyl esters [11] the ratio of isomers I and III was determined by HPLC using a Waters Assoc. liquid chromatography system fitted with a μ porasil column (60 cm) and a model 440 detector (405 nm filter) [7]. The developing solvent of *n*-heptane:glacial acetic acid:acetone:hexane:water (540:300:200:60:0.1, by vol.) was run at 1 ml/min. Uroporphyrinogen III cosynthetases were obtained in semi-purified form and stored at pH 8.2 in 0.2 M Tris-HCl. Enzymes from *Rps. spheroides* [12], yeast [13], mouse spleen [14], wheat [15],

human reticulocytes [16], and chicken erythrocytes [17] were employed in this study.

The conversion of porphobilinogen into preuroporphyrinogen and the formation of uroporphyrinogen III with various cosynthetases is described in table 1. After incubations, porphyrinogens were oxidised with benzoquinone and the samples were lyophilised. Esterification was carried out with $\text{BF}_3\text{:MeOH}$ (Sigma) after which the methyl esters were extracted into chloroform and purified by TLC on silica gel (benzene:ethyl acetate:ethanol, 75:23:2, by vol.). The purified esters were then analysed by HPLC for isomers I and III as above and the quantity of each isomer was obtained by integration. Protein was determined by the method in [18].

3. Results and discussion

The sequential nature of the formation of uroporphyrinogen III from porphobilinogen, namely porpho-

Table 1

Source of cosynthetase	Cosynthetase spec. act. (munits/mg) (pH 8.2)	Uroporphyrinogen III formed (nmol)	% Conversion preuroporphyrinogen into uroporphyrinogen III
<i>Rps. spheroides</i>	5.2	2.95	53.8
Wheat leaves	20.8	0.55	10.0
Mouse spleen	36.9	3.98	72.6
Yeast	2.3	3.26	59.5
Human reticulocytes	1.0	5.48	99.6
Chicken erythrocytes	0.2	0.07	1.3

Porphobilinogen (200 nmol) was incubated with 14 units porphobilinogen deaminase in 0.2 M Tris-HCl buffer (pH 8.2) containing 6.7 μ mol NaBH_4 under anaerobic conditions in 1.0 ml final vol. The porphobilinogen was consumed at 37°C in 1.5 min after which 150 μ l aliquots of preuroporphyrinogen (5.50 nmol) were incubated with 500 μ l cosynthetase samples in the same buffer for 2.25 min. The samples were then oxidised by the addition of 200 μ l 10 mM benzoquinone and were processed as described in section 2 [9,11]. Controls contained no preuroporphyrinogen and the values obtained were subtracted to correct for the small amount of endogenous uroporphyrinogen present. All values are corrected for the uroporphyrinogen formed (20%) during the initial incubation with deaminase alone. One unit of cosynthetase forms 1 μ mol uroporphyrinogen III from preuroporphyrinogen per hour [19]

bilinogen→preuroporphyrinogen, catalysed by porphobilinogen deaminase, and preuroporphyrinogen→uroporphyrinogen III by uroporphyrinogen III cosynthetase in *Rps. spheroides* enables preuroporphyrinogen to be generated enzymically by porphobilinogen deaminase (scheme 1).

Because of the inherent instability of preuroporphyrinogen at physiological pH ($t_{1/2} = 4$ min at 37°C, pH 8.5) the incubation in which the porphobilinogen was consumed was carried out rapidly to ensure that the amount of uroporphyrinogen I formed chemically from preuroporphyrinogen was minimal. As the formation of uroporphyrinogen I lags considerably behind porphobilinogen consumption preuroporphyrinogen could be obtained in yields >80% under our experimental conditions. Incubation of cosynthetases from various sources with the preuroporphyrinogen thus generated, resulted in the formation of uroporphyrinogen III in all systems investigated (table 1). Since the porphobilinogen was completely consumed during the initial incubation with porphobilinogen deaminase, there was no possibility of uroporphyrinogen III arising directly from porphobilinogen rather than from preuroporphyrinogen. Excellent yields of uroporphyrinogen III were obtained from preuroporphyrinogen with *Rps. spheroides*, mouse spleen, yeast and human reticulocyte cosynthetases. The conversion with the human cosynthetase was virtually quantitative. Wheat leaf cosynthetase gave a lower but significant incorporation probably due to the small number of units of enzyme from this source. The only cosynthetase giving a very low conversion was that from chicken erythrocytes however, because of the necessity of carrying out incubations at pH 8.2 due to the instability of preuroporphyrinogen, we were not able to incubate the chicken enzyme at its pH optimum of 7.4. The very low incorporation in this case is likely to be explained on this basis.

Our results lead one to the unequivocal conclusion that preuroporphyrinogen plays a crucial role as a substrate for the cosynthetases⁺ in all the systems investigated in this study and firmly establishes preuroporphyrinogen as a universal intermediate in tetrapyrrole biosynthesis in all the major classes of living organisms.

⁺ The generation of preuroporphyrinogen by deaminase and its subsequent utilization as a substrate for cosynthetase represents the first direct assay method for cosynthetase further details of which are to be published [19]

Acknowledgement

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Note added in Proof

Since the first reports [6,7] on the structure and role of preuroporphyrinogen (2) the Cambridge group has repeated several of our key experiments using our approach but with enzyme preparations from *Euglena gracilis* [20] and has subsequently come to similar conclusions to those put forward in our original hypothesis.

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