THE BIOSYNTHESIS OF UROPORPHYRINOGEN III: ORDER OF ASSEMBLY OF THE FOUR PORPHOBILINOGEN MOLECULES IN THE FORMATION OF THE TETRAPYRROLE RING

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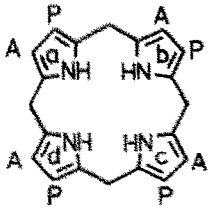
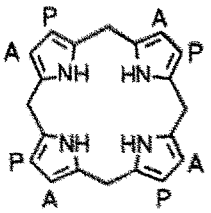
1. Introduction

The elaboration of the macrocyclic ring of uroporphyrinogen III (1) from four molecules of the monopyrrole precursor porphobilinogen (5-(amino-methyl)-4-(carboxymethyl)-pyrrole-3-propionic acid) requires the participation of two enzymes, porphobilinogen deaminase and uroporphyrinogen III cosynthetase (review [1]). The former enzyme catalyses the uptake of porphobilinogen into the tetrapyrrole intermediate preuroporphyrinogen [2] while the role of the cosynthetase is to rearrange this intermediate into uroporphyrinogen III [3]. In the absence of cosynthetase, preuroporphyrinogen is transformed into uroporphyrinogen I (2) in a rapid chemical reaction.



1



2

A = CH_2COOH

P = CH_2CH_2COOH

The assembly of the tetrapyrrole from four identical precursor monopyrrole molecules poses unusual kinetic and mechanistic problems. Of particular interest is the sequence in which the four porphobilinogen molecules are built into uroporphyrinogen III. Using stoichiometric quantities of ^{14}C-labelled porphobilinogen and porphobilinogen deaminase followed by the addition of excess unlabelled porphobilinogen we have shown that the biosynthesis of uroporphyrinogen occurs by a specific unidirectional sequence in which the porphobilinogen molecule giving rise to ring a in structure 1 is the one initially incorporated into the tetrapyrrole.

2. Materials and methods

[2,11-$^{14}C_2$]Porphobilinogen was prepared from 5-amino [5-^{14}C]levulinic acid using purified 5-aminolevulinic acid dehydratase from bovine liver [4].

Porphobilinogen deaminase, uroporphyrinogen III cosynthetase, uroporphyrinogen III decarboxylase and coproporphyrinogen III oxidase were semi-purified [4] from *Rhodopseudomonas sphaeroides* (NCIB 8253) grown semi-anaerobically in the light [5] and were utilised in a linked enzyme system. Porphobilinogen deaminase was estimated by measuring the rate of uptake of porphobilinogen, and the quantity of deaminase present in the experiments was calculated on the basis of a specific activity of 128 units/h/mg and a mol. wt of 36 000 [6].

[2,11-$^{14}C_2$]Porphobilinogen (2.13 μmol; 4.09×10^7 dpm) was added to the linked enzyme system (170 ml) at 0°C, which contained 1.97 μmol of porphobilinogen deaminase. The temperature of the incubation mixture was raised to 10°C, unlabelled porphobilinogen (70 μmol) was added and the mixture was incubated at 37°C anaerobically in the

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dark for 20 min and then for a further 2 h aerobically in the presence of KCN (170 μmol) [7]. At the end of the incubation, the porphyrinogens were oxidized to porphyrins with benzoquinone (3 mg) and after the addition of protoporphyrin IX (20 mg), the porphyrins were extracted into ethyl acetate/acetic acid [7]. Protoporphyrin IX and coproporphyrin III were separated [7] and their purities were determined by TLC of their methyl esters as in [8]. Protoporphyrin IX was reduced to mesoporphyrin IX which was then degraded with CrO_3/H_2SO_4 to hematinic acid and ethyl methyl maleimide [9]. The hematinic acid and ethyl methyl maleimide were separated from one another by extraction and were finally purified by sublimation (ethyl methyl maleimide and hematinic acid sublimed at 80°C and 140°C, respectively, at 0.05 mm Hg). Their purity was confirmed by comparing their ultraviolet spectra with authentic standards.

3. Results and discussion

The order in which the four molecules of the monopyrrole, porphobilinogen are built into uroporphyrinogen III can theoretically proceed by one of eight biosynthetic routes, namely, abcd, bcda, cdab, dabc, dcba, cbad, badc, or adcb (see structure 1) although on intuitive grounds abcd or dcba are the most likely sequences. Recent studies have shown that an amino methyl bilane [10] is accepted as a poor substrate for porphobilinogen deaminase/uroporphyrinogen III cosynthetase and is converted into uroporphyrinogen III thereby providing experimental evidence favouring either the sequences abcd or dcba.

These two possibilities were investigated by means of a single enzyme turnover experiment using ^{14}C-labelled porphobilinogen. If one mole of ^{14}C-labelled porphobilinogen is added to the deaminase enzyme, a high proportion of the enzyme molecules would be charged with a single molecule of porphobilinogen. Subsequent completion of the enzyme turnover by the addition of an excess of non-labelled substrate would result in a ^{14}C-regiospecific synthesis of the first molecule of tetrapyrrole, in which the porphobilinogen unit bound initially to the enzyme would contain the majority of the label. In order to determine the position of the ^{14}C label, the enzyme uroporphyrinogen III cosynthetase was also utilized ensuring that the only uroporphyrinogen formed would be the asymmetric type III isomer (the position of the label in any individual pyrrole ring cannot be determined in the type 1 isomer). Uroporphyrinogen III thus formed was converted via coproporphyrinogen III into protoporphyrinogen IX by the enzymes uroporphyrinogen III decarboxylase and coproporphyrinogen III oxidase, thus providing a convenient enzymic method for selective modification of rings a and b. The protoporphyrinogen IX was oxidized to protoporphyrin IX and was subsequently chemically degraded to ethyl methyl maleimide (rings a and b) and haematinic acid (rings c and d).

Table 1
Degradation of protoporphyrin IX

	Yield (mg)	Total radioactivity/ 10^4 dpm	Specific activity/ 10^3 dpm/μmol	Radiochemical yield (from protoporphyrin IX) (%)
Protoporphyrin IX	20	802	225	–
Mesoporphyrin IX	18.8	750	225	93
Ethyl methyl maleimide	3.70	232	109[a]	29
Hematinic acid	3.54	3.1	1.21	<0.5

[a] As expected the specific activity in ethyl methyl maleimide is half that of protoporphyrin IX since the *meso* carbon atoms are lost as CO_2 in the chromic acid oxidation

The results in table 1 show that negligible radioactivity from protoporphyrin IX is found in the hematinic acid after degradation*. Had the enzymic assembly of the tetrapyrrole from porphobilinogen proceeded via the sequence dcba the hematinic acid would have been heavily labelled. This sequence is thus eliminated. On the other hand the results show almost quantitative (99%) incorporation of ^{14}C label into ethyl methyl maleimide. As this is derived exclusively from rings a and b (previously in uroporphyrinogen III) we therefore conclude that rings a and b are built into the tetrapyrrole initially and that the order of assembly of the four porphobilinogen molecules by the deaminase must proceed in the order abcd.

* In a parallel experiment with a 35-fold molar excess of [2,11-$^{14}C_2$]porphobilinogen over deaminase the ethyl methyl maleimide and hematinic acid arising from the degradation of the protoporphyrin IX had essentially identical specific radioactivities

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