THE ENZYMATIC INCORPORATION OF A DIPYRRYLMETHANE INTO UROPORPHYRINOGEN III

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1. Introduction

Uroporphyrinogen III 2 is the biosynthetic precursor of heme, chlorophylls and all the natural porphyrins. During the enzymatic conversion of porphobilinogen 1 into uroporphyrinogen III 2 an intramolecular rearrangement takes place (see [1] and references therein for a literature survey). The polymerization of four units of porphobilinogen by the combined action of the enzymes porphobilinogen deaminase and uroporphyrinogen III cosynthetase does not afford uroporphyrinogen I 3 – as could be expected from a repetitive head-to-tail condensation of porphobilinogen – but the isomeric uroporphyrinogen III 2 where an inversion in the order of the β-substituents took place. Since no chemically defined pyrrylmethanes were isolated during the enzymatic reaction that could help explain that inversion, the synthetic 2-aminomethyldipyrrylmethane 4 – resulting from the formal head-to-tail condensation of two units of porphobilinogen – was examined as a first intermediate of the enzymatic system involved in porphobilinogen polymerization [1].

It was found that in the presence of porphobilinogen it was incorporated exclusively into uroporphyrinogen I, and not into uroporphyrinogen III. We then proposed [1] that both isomers originate by different pathways from the start of the polymerization and that the dipyrrylmethane 5 – resulting from the formal head-to-head condensation of two units of porphobilinogen followed by a 2-aminomethyl migration – would be the first intermediate in uroporphyrinogen III biosynthesis**. The incorporation data obtained with dipyrrylmethane-^{12}C 5 and [^{14}C]dipyrrylmethane 5 lends support to that proposal and will be discussed in this report.

2. Materials and methods

Porphobilinogen and [^{14}C]porphobilinogen were prepared by synthesis [3]. Dipyrrylmethane 5 – (2-aminomethyl-3,4′-(β-carboxyethyl)-4,3′-carboxymethyldipyrrylmethane) – and [^{14}C]dipyrrylmethane 5 were prepared according to the described synthetic outline [1]. The label was at C-2 of the acetic acid side chain R_3′. Other chemicals were reagent grade. Porphobilinogen deaminase and uroporphyrinogen III cosynthetase were isolated and purified from wheat germ and from human erythrocytes [4, 5]. When the incorporation of dipyrrylmethane 5 into uroporphyrinogens was measured by the isotope dilution method (table 1), the incubated system contained in a final volume of 100 μl: 10 μmoles of phosphate buffer (pH 7.4), 6 nmoles of [^{14}C]porphobilinogen (4,000 cpm/nmole), 20 μl of enzyme (a porphobilinogen deaminase–uroporphyrinogen III cosynthetase whole system from wheat germ), and the indicated amounts of dipyrrylmethane 5. Incubations

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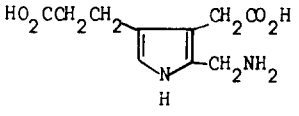
** This suggestion was first advanced by Sir Robert Robinson at the First Weizmann Memorial Lecture [2].



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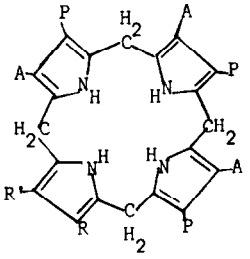


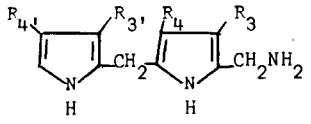
A= CH_2CO_2H

P= $CH_2CH_2CO_2H$

2. R= P; R'= A

3. R= A; R'= P

Fig. 1–3.



4. R_3= CH_2CO_2H; R_4= $CH_2CH_2CO_2H$; $R_{3'}$= CH_2CO_2H; $R_{4'}$= $CH_2CH_2CO_2H$.

5. R_3= $CH_2CH_2CO_2H$; R_4= CH_2CO_2H $R_{3'}$= CH_2CO_2H; $R_{4'}$= $CH_2CH_2CO_2H$.

Fig. 4, 5.

Table 1
Enzymatic incorporation of dipyrrylmethane 5 into uroporphyrinogen III, isotope dilution data.

Enzymatic system	Dipyrrylmethane added	Uroporphyrinogens[a]							
		Isomer I				Isomer III			
	(nmoles)	(nmoles)	(cpm)	Specific activity	Decrease in specific activity	(nmoles)	(cpm)	Specific activity	Decrease in specific activity
Incubated	None	0.165	2,670	16,180	–	0.465	7,335	15,730	–
Control	10	0.195	2,496	12,800	–	0.42	6,000	14,285	3,785
Incubated	10	0.21	2,700	12,850	–	0.30	3,150	10,500	
Control	20	0.198	2,475	12,500	–	0.36	4,563	12,400	3,100
Incubated	20	0.180	2,340	13,000	–	0.24	2,232	9,300	
Control[b]	15	0.195	2,250	13,070	–	0.285	3,705	13,000	4,200
Incubated[b]	15	0.129	1,740	13,490	–	0.135	1,188	8,800	

The incubation conditions were those described in Methods. a) Measured as coproporphyrins (see Methods). b) A recombined system containing purified wheat germ deaminase and purified human erythrocyte cosynthetase was used.

were carried out at 37° during 60 min. Strict anaerobiosis was not needed since no oxidation to pyrrylmethenes took place under the described incubation conditions. The controls were performed by mixing an incubated system as described above with a blank obtained by incubating at 37° and 60 min a mixture containing 10 μmoles of phosphate buffer (pH 7.4), 6 nmoles of [^{14}C]porphobilinogen and the indicated amounts of dipyrrylmethane 5 (table 1).

The reaction was stopped by adding 10 μl of 1% iodine solution. The solution was then evaporated to dryness, the resulting product esterified with a 5% sulfuric acid solution of methanol, and the obtained uroporphyrin octamethyl esters decarboxylated to

Table 2
Enzymatic incorporation of [^{14}C]dipyrrylmethane 5 into uroporphyrinogen III.

System	Dipyrrylmethane added	Uroporphyrinogens*							
		Isomer I				Isomer III			
	(nmoles)	(nmoles)	(cpm)	Specific activity	Δ Specific activity	(nmoles)	(cpm)	Specific activity	Δ Specific activity
Incubated	None	0.24	–	–	–	0.70	–	–	–
Incubated	10	0.32	50	156	–	0.77	410	532	84
Control	10	0.23	30	152	–	0.67	300	448	
Incubated	20	0.24	65	270	–	0.53	545	1028	171
Control	20	0.22	61	277	–	0.72	610	847	

The incubation conditions were the described in Methods.

* Measured as coproporphyrins (see Methods).

the corresponding coproporphyrins by heating at 180° with hydrogen chloride [6a]. Coproporphyrins I, II and III were separated by paper chromatography [6b], located by fluorescence, eluted from the paper with an ammonium hydroxide solution, and estimated in the eluates by spectrophotometric methods using as reference a calibration curve prepared with pure samples. Radioactivity was measured with a gas-flow counter. When the incorporation of [^{14}C]dipyrrylmethane 5 into uroporphyrinogen III was measured (table 2) the incubation mixtures and the controls were prepared as described above, using porphobilinogen-^{12}C (8 nmoles) and [^{14}C]dipyrrylmethane 5 (1200 cpm/nmole). Uroporphyrinogens were transformed into coproporphyrins as described and measured as such.

3. Results

The dipyrrylmethane 5 was not a substrate of either porphobilinogen deaminase, uroporphyrinogen III cosynthetase or the combined enzymatic system. When incubated together with porphobilinogen in the presence of uroporphyrinogen III cosynthetase, no enzymatic uroporphyrinogen formation was detected. When the dipyrrylmethane 5 was heated at 37° under the described incubation conditions it dimerized, affording exclusively uroporphyrinogen II in 10% yield. In the presence of porphobilinogen a small amount (2%) of uroporphyrinogen III was formed due to the chemical condensation of porphobilinogen with the dipyrrylmethane. When the dipyrrylmethane 5 was incubated with the whole deaminase-cosynthetase enzymatic system in the presence of [^{14}C]porphobilinogen, a decrease in the specific activity of uroporphyrinogen III was evident indicating that the dipyrrylmethane was incorporated into the same (table 1). No decrease was observed in the specific activity of uroporphyrinogen I. The control experiments (see Methods) were performed in order to account for the small chemical formation of uroporphyrinogen III (see above) which may be a source of error. The data also indicated that increasing amounts of dipyrrylmethane inhibited the formation of uroporphyrinogen III, without increasing correspondingly the amount of uroporphyrinogen I. When a recombined enzymatic system was used, prepared by mixing purified porphobilinogen deaminase from wheat germ with uroporphyrinogen III cosynthetase from erythrocytes, a higher enzymatic incorporation of dipyrrylmethane was achieved.

The incorporation of dipyrrylmethane 5 into uroporphyrinogen III was confirmed by using [^{14}C]dipyrrylmethane and unlabelled porphobilinogen (table 2). By using this method higher incorporations were detected with increasing concentrations of dipyrrylmethane. The uroporphyrinogen III formed

at expense of the dipyrrylmethane amounted to 10% of the total uroporphyrinogen III formed when 10 nmoles of the dipyrrylmethane were added, and to 26% of the total when 20 nmoles of **5** were added. The amount of label incorporated into uroporphyrinogen III in the control experiments was due to the chemical condensation of porphobilinogen with the dipyrrylmethane and was deducted in each run. The small amount of label that appeared in isomer I was of a non-systematic nature and its origin is not yet clear.

4. Discussion

The obtained results clearly indicated that dipyrrylmethane **5** was specifically incorporated into uroporphyrinogen III. Since the structure of dipyrrylmethane **5** is derived from a formal head-to-head condensation of two units of porphobilinogen followed by an intramolecular migration of the 2-aminomethyl group, this mechanism must then be the origin of the side chain inversion in the biosynthesis of uroporphyrinogen III. These data also agree with the results obtained with dipyrrylmethane **4** which was enzymatically incorporated exclusively into uroporphyrinogen I [1], indicating that both uroporphyrinogens start by different pathways from the beginning of the enzymatic polymerization of porphobilinogen. The specificity of these incorporations was confirmed by the lack of any enzymatic incorporation of "biologically nonsense" 2-aminomethyldipyrrylmethanes, isomeric with **4** and **5** [1].

Even when the dipyrrylmethane **5** was present in a good excess with respect to porphobilinogen its total incorporation was low, as was the case with dipyrrylmethane **4** [1]. Thus, the 2-aminomethyldipyrrylmethanes are not free substrates of the enzymatic system behaving only as intermediates of the process. The overall polymerization of porphobilinogen under normal conditions must take place at all the stages without liberation of soluble pyrrylmethanes.

Acknowledgements

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