Mechanism of Action of Porphobilinogen Deaminase: Ordered Addition of the Four Porphobilinogen Molecules in the Formation of Preuroporphyrinogen†

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Abstract: The tetrapyrrole preuroporphyrinogen is assembled by porphobilinogen deaminase from four molecules of porphobilinogen (**1**) in a linear ordered sequence. Using single turnover enzyme reactions, we have established that of the four porphobilinogen molecules involved it is the one giving rise to ring a in the tetrapyrrole (**4**) which is bound to the enzyme first, with the other three rings following in the order b, c, and finally d. The location of the ^{14}C label is determined by a combination of enzymatic and chemical degradation methods. The mechanism of action of porphobilinogen deaminase is discussed.

Introduction

The biosynthesis of uroporphyrinogen III, the key tetrapyrrole precursor of heme, chlorophylls, and corrins,[1] is mediated by the action of two enzymes, porphobilinogen deaminase and uroporphyrinogen III synthetase[2] (formerly uroporphyrinogen III cosynthetase). The individual role played by each enzyme in their coordinated action to form uroporphyrinogen III (**4**, Scheme I) has occupied considerable interest, as has the mechanism by which one of the pyrrole rings is rearranged with respect to the unimportant physiological isomer uroporphyrinogen I (**3**).

The view has generally been held that the close association of the enzymes in a complex was an obligatory requirement for the formation of the rearranged III isomer.[3] The first demonstration that porphobilinogen deaminase catalyzes the formation of the unstable enzyme free tetrapyrrolic intermediate, termed preuroporphyrinogen (**2**), from four molecules of porphobilinogen[4,5] in bacteria has completely altered our concept of the nature of urogen III biosynthesis. Preuroporphyrinogen is either nonenzymatically modified to give uroporphyrinogen I or, more importantly, acts as the true physiological substrate for uroporphyrinogen III synthetase, resulting in the formation of uroporphyrinogen III in quantitative yield.[5] Preuroporphyrinogen has since been shown to act as the substrate for uroporphyrinogen III synthetase in mammals and plants[6] and euglena.[7]

The resolution of uroporphyrinogen III biosynthesis into these two clearly defined stages, namely, porphobilinogen → preuroporphyrinogen and preuroporphyrinogen → uroporphyrinogen III, allows the detailed investigation of each enzyme step in isolation. The present study deals with the investigation into the mechanism by which porphoblinogen deaminase binds and constructs the tetrapyrrole structure of preuroporphyrinogen (**3**) from four molecules of the substrate porphobilinogen, with particular emphasis on the order of incorporation of the four pyrrole rings during the assembly process. Preliminary accounts from our laboratory[8] and elsewhere[9] have been published.

Results and Discussion

The order in which substrate molecules bind to an enzyme catalyzing a multisubstrate reaction is traditionally investigated using steady-state kinetics in which the effect of varying the concentration of each substrate and product on the overall rate of product formation is measured.[10] The study of the sequence of events in a reaction, such as that catalyzed by porphobilinogen deaminase in which four *identical* substrate molecules are involved in the formation of the tetrapyrrole preuroporphyrinogen, requires a somewhat different approach, since a conventional kinetic evaluation is not possible.

We therefore adopted a single turnover technique[11] in which the deaminase was first exposed to an equimolar amount of ^{14}C-labeled porphobilinogen, after which the enzyme "turnover" was completed with respect to the [^{14}C]porphobilinogen by the addition of excess unlabeled porphobilinogen. Examination of the product to determine the fate of the ^{14}C label would provide information about the mechanism of assembly of the tetrapyrrole ring from porphoblinogen. In the context of this approach, several broad mechanisms may be considered for the assembly of preuroporphyrinogen by porphobilinogen deaminase.

(1) Firstly, an ordered mechânism where a *single* high-affinity catalytic site was occupied initially by the [^{14}C]porphobilinogen. Subsequent addition of nonlabeled porphobilinogen and completion of the tetrapyrrole would ultimately result in the incorporation of the majority of the label into only one ring of the product, the position of labeling representing the porphobilinogen that is first bound to the enzyme. Extension of this approach to the utilization of 2 or more equiv of [^{14}C]porphobilinogen at the initial stage, followed by a subsequent evaluation of the position of the ^{14}C label in the product, would also provide information about the *order* in which the four porphoblinogen units are incorporated into preuroporphyrinogen. Such a mechanism could theoretically proceed through one of eight ordered sequential routes, namely, a, b, c, d; b, c, d, a; c, d, a, b; d, a, b, c; d, c, b, a; c, b, a, d; b, a, d, c; or a, d, c, b.

(2) Secondly, a mechanism involving several catalytic sites which are "charged" with their substrate in an ordered sequence. This may be expected to yield results similar to mechanism 1.

(3) Thirdly, a sequential mechanism where the binding of the first molecule of porphobilinogen induces an increase of the affinity of the enzyme for the second, third, and fourth molecules of porphobilinogen. Such a mechanism would give rise to ^{14}C labeling in all four pyrrole rings in the product, although the ring bound first may be expected to have a slightly higher ^{14}C labeling.

(4) Fourthly, a random mechanism in which the labeled porphobilinogen molecules interact with several catalytic sites in a nonordered reaction. This mechanism would result in an equal

† Dedicated to Professor David Shemin on the occasion of his 70th birthday.

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(11) This approach has also been adopted for the elucidation of the mechanism of action of 5-aminolevulinic acid dehydratase: see Jordan, P. M.; Seehra, J. S. *J. Chem. Soc., Chem. Commun.* **1980**, 240-242.

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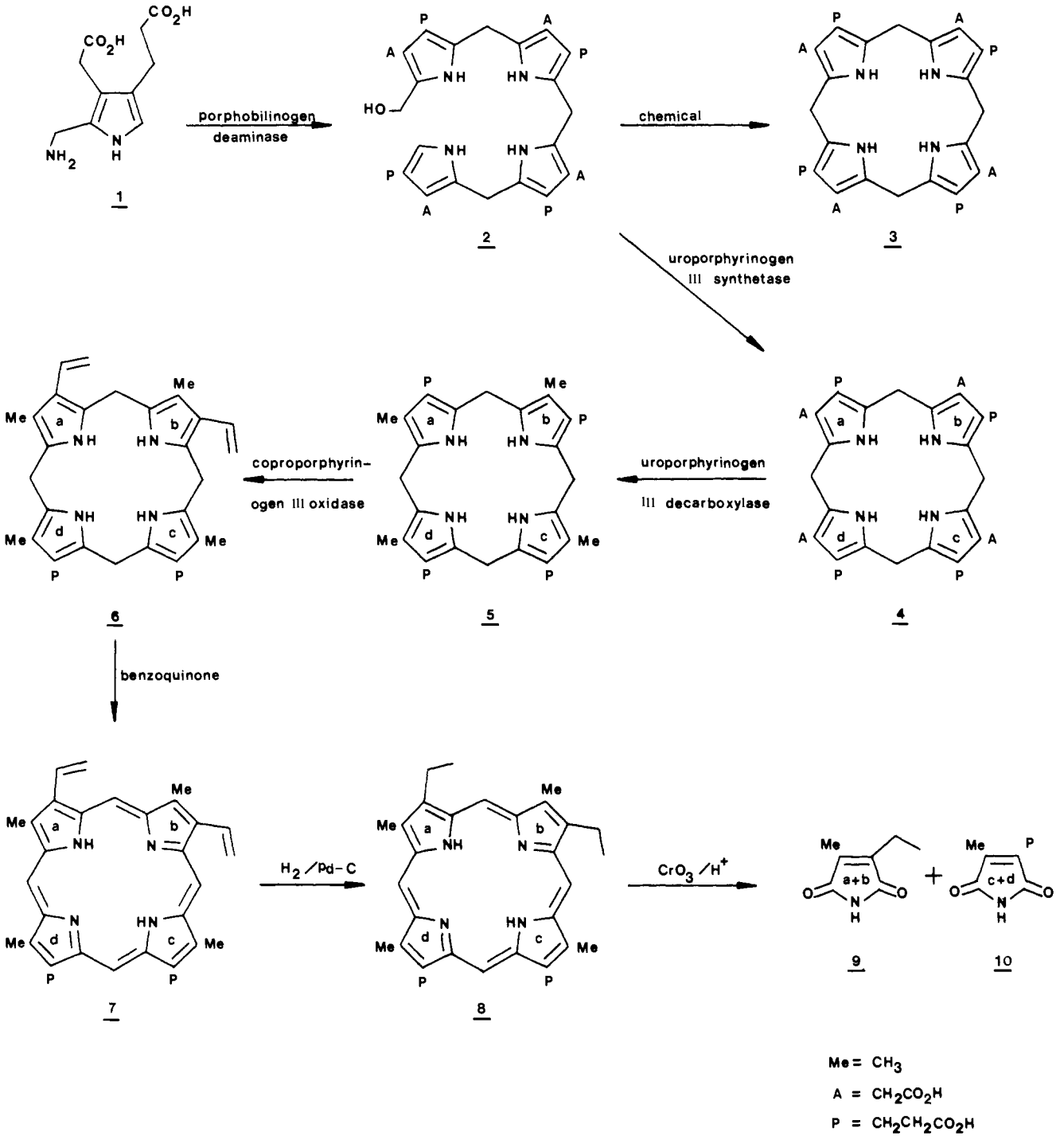
Scheme I. Biosynthesis of Protorporphyrin IX and Degredation to Ethylmethylmaleimide and Hematinic Acid



labeling of all four pyrrole rings in the product.

The asymmetric product of porphobilinogen deaminase, preuroporphyrinogen, is highly unstable at physiological pH ($t_{1/2}$ = 4 min at 37 °C and pH 8.2) and forms uroporphyrinogen I in a rapid chemical reaction. If this occurred the symmetrical nature of the latter would preclude a regiospecific evaluation for the ^{14}C label, as all four rings of uroporphyrinogen I are chemically equivalent. Accordingly, preuroporphyrinogen was rapidly converted into the asymmetric uroporphyrinogen III isomer by uroporphyrinogen III cosynthetase. Although asymmetric, the four pyrrole rings in uroporphyrinogen III all have the same β substituents, and, like those in uroporphyrinogen I, are indistinguishable after chemical degradation. The enzymatic conversion of uroporphyrinogen III to protoporphyrinogen IX by uroporphyrinogen III decarboxylase and coproporphyrinogen III oxidase in a linked enzyme system results, however, in the selective modification of rings a and b (in **4**), which can then be distinguished from rings c and d by chemical degradation.[12] In addition, the specificity of the enzymes ensure that only the asymmetric uroporphyrinogen III is converted via coproporphyrinogen III (**5**) into protoporphyrinogen IX (**6**) (Scheme I).

A series of experiments (experiments 1–3; Table I) were carried out with the linked enzyme system to which 1, 2, or 3 mol of [2,11-$^{14}C_2$]porphobilinogen was initially mixed with 1 mol equiv of deaminase. An excess of nonlabeled porphobilinogen was then added to complete the turnover of the [^{14}C]porphobilinogen bound to the deaminase, and the preuroporphyrinogen thus formed was converted into protoporphyrin IX (**7**) as described in the Experimental Section. A control using 60 mol (excess) equiv of

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Table I. Distribution of Radioactivity in Protoporphyrin IX, Ethylmethylmaleimide and Hematinic Acid Derived from Regiospecifically Labeled Samples of Uroporphyrinogen III[a]

no. of moles of [2,11-$^{14}C_2$]PBG[b] used		yield, mg	total radioact, 10^4 dpm	sp act, 10^3 dpm/μmol	% radioactive label in maleimides	sequence eliminated
1	protoporphyrin IX[c]	25.0	390	87.7		c, d, a, b
	mesoporphyrin IX	21.3	330	87.5		d, a, b, c
	ethylmethylmaleimide	4.02	123.5	42.7	99	d, c, a, b
	hematinic acid	3.63	1.1	0.57	1	c, b, a, d
2	protoporphyrin IX	14.3	210	82.5		b, c, d, a; c, d, a, b
	mesoporphyrin IX	12.9	188	82.3		d, a, b, c; d, c, b, a
	ethylmethylmaleimide	2.6	63	33.6	81.6	c, b, a, d
	hematinic acid	2.03	8.4	7.57	18.4	a, d, c, b
3	protoporphyrin IX	13.3	190	80.2		b, c, d, a
	mesoporphyrin IX	12.1	171	80.0		c, d, a, b
	ethylmethylmaleimide	1.8	30.2	23.3	58.2	d, c, b, a
	hematinic acid	1.6	14.6	16.7	41.8	a, d, c, b
excess	protoporphyrin IX	14.0	220	88.3		
	mesoporphyrin IX	13.1	203	87.3		
	ethylmethylmaleimide	2.1	33.0	21.85	50.1	
	hematinic acid	1.8	21.4	21.75	49.9	

[a] Experiment 1: [2,11-$^{14}C_2$]Porphobilinogen (0.43 μmol, sp act. 3.5×10^7 dpm/μmol) was intially added to porphobilinogen deaminase (0.43 μmol) and the enzyme-bound, labeled porphobilinogen was carried through to the product by the addition of an excess on unlabeled porphobilinogen (18 μmol). Experiments 2 and 3: [2,11-$^{14}C_2$]Porphobilinogen (0.77 μmol, sp act. 3×10^6 dpm/μmol; 1.1 μmol, sp act. 2×10^6 dpm/μmol, respectively) was initially added to porphobilinogen deaminase (0.37 μmol) followed by unlabeled porphobilinogen (18 μmol). Experiment 4: [2,11-$^{14}C_2$]Porphobilinogen (20 μmol, sp act. 1.2×10^5 dpm/μmol) was added to porphobilinogen deaminase (0.37 μmol). The preuroporphyrinogen was converted to protoporphyrin IX and degraded to ethylmethylmaleimide and hematinic acid as outlined under Experimental Section. [b] Porphobilinogen. [c] Total yield of protoporphyrin IX in experiment 1 was 86 mg. The remainder of the protoporphyrin IX was degraded using osmium tetroxide.

labeled porphobilinogen (experiment 4; Table I) was carried out simultaneously.

The results (Table I) show that the distribution of radioactivity in ethylmethylmaleimide (**9**) and hematinic acid (**10**) derived from the degradation of labeled samples of protoporphyrin IX biosynthesized from uroporphyrinogen III labeled with 1, 2, and 3 mol of [^{14}C]porphobilinogen in the single turnover experiments are consistent with an ordered sequential mechanism (mechanism 1 or 2) and eliminate the mechanism involving positive cooperativity (mechanism 3) and also the random-binding mechanism (mechanism 4). A comparison between the results obtained and the theoretical labeling expected from a sequential binding mechanism shows that of the eight ordered sequences only a, b, c, d and b, a, d, c are consistent with the results (Table II).

To distinguish between these two sequences, a degradation of protoporphyrin IX (**7**) was required in which rings a and b of protoporphyrin IX (**6**) (and hence uroporphyrinogen) could be resolved. This was accomplished by oxidation of protoporphyrin IX dimethyl ester (**11**) with *1* mol of OsO_4[13] to yield a statistical mixture of the two *monoglycol* derivatives (**12** and **13**), together with some bisglycol (**14**) and unreacted protoporphyrin IX ester. The two monoglycols were separated using preparative thin-layer chromatography and were unambiguously characterized.[13] Chromic oxidation of each monoglycol derivative, after reduction of the remaining vinyl group using H_2/Pd through intermediates **15** and **16**, gave ethylmethylmaleimide derived solely from ring a or ring b of protoporphyrin IX (Scheme II). A single turnover experiment involving 1 mol of [^{14}C]porphobilinogen equivalent to deaminase (as in experiment 1; Table I) would result in the label being incorporated preferentially into ring a if sequence a, b, c, d is operative or into ring b for sequence b, a, d, c. The results of the degradation (Table III) show that in a single turnover experiment using 1 mol of [^{14}C]porphobilinogen 89% of the label is in ring a and 11% is in ring b. These results establish that the *first* molecule of porphobilinogen bound to the deaminase enzyme is the one which gives rise to ring a of the tetrapyrrole preuroporphyrinogen (**3**) and, hence, ring a in uroporphyrinogen III (**4**). Porphobilinogen deaminase thus catalyzes the assembly of the tetrapyrrole molecule from four molecules of porphobilinogen in the specific unidirectional sequence a, b, c, d.

Table II. Radioactivity in Ethylmethylmaleimide and Hematinic Acid Predicted for an Ordered Mechanism (See Text) from Single Turnover Experiments[a]

possible sequence	expected labeling pattern, % radioactivity[b]							
	expt 1		expt 2		expt 3		expt 4	
	EM	HA	EM	HA	EM	HA	EM	HA
a, b, c, d	100		100		67	33	50	50
b, c, d, a	100		50	50	33	67	50	50
c, d, a, b		100		100	33	67	50	50
d, a, b, c		100	50	50	67	33	50	50
d, c, b, a		100		100	33	67	50	50
c, b, a, d		100	50	50	67	33	50	50
b, a, d, c	100		100		67	33	50	50
a, d, c, b	100		50	50	33	67	50	50
obsd	99	1	82	18	58	42	50	50

[a] Where 1, 2, or 3 mol equiv or an excess of [^{14}C]porphobilinogen was added to porphyrinogen deaminase. [b] EM = ethylmethylmaleimide (9). HA = hematinic acid (10).

In addition to showing the sequence in which the four porphobilinogen molecules are incorporated into the tetrapyrrole preuroporphyrinogen, several important features of the catalytic mechanism are highlighted. The high proportion of ^{14}C label incorporated into ring a (89% in experiment 1) suggests that once bound to the deaminase the substrate does not readily dissociate from the enzyme. This point is also reinforced by the observation of the rapid disappearance of the ^{13}C signal for [11-^{13}C]porphobilinogen when exposed to an equimolar quantity of deaminase in the NMR tube, pointing to the immobilization of the ^{13}C-labeled substrate at the activity site.[14] The results from experiments 2 and 3 (Table I) show that there is a strong binding of the second and third porphobilinogen molecules, suggesting that the intermediate di- and tripyromethanes also cannot dissociate from the enzyme. The apparent lack of liberation of intermediates into solution when the enzyme is functioning under normal conditions is also consistent with this observation. The participation of a covalent enzyme–substrate intermediate linkage in the catalytic

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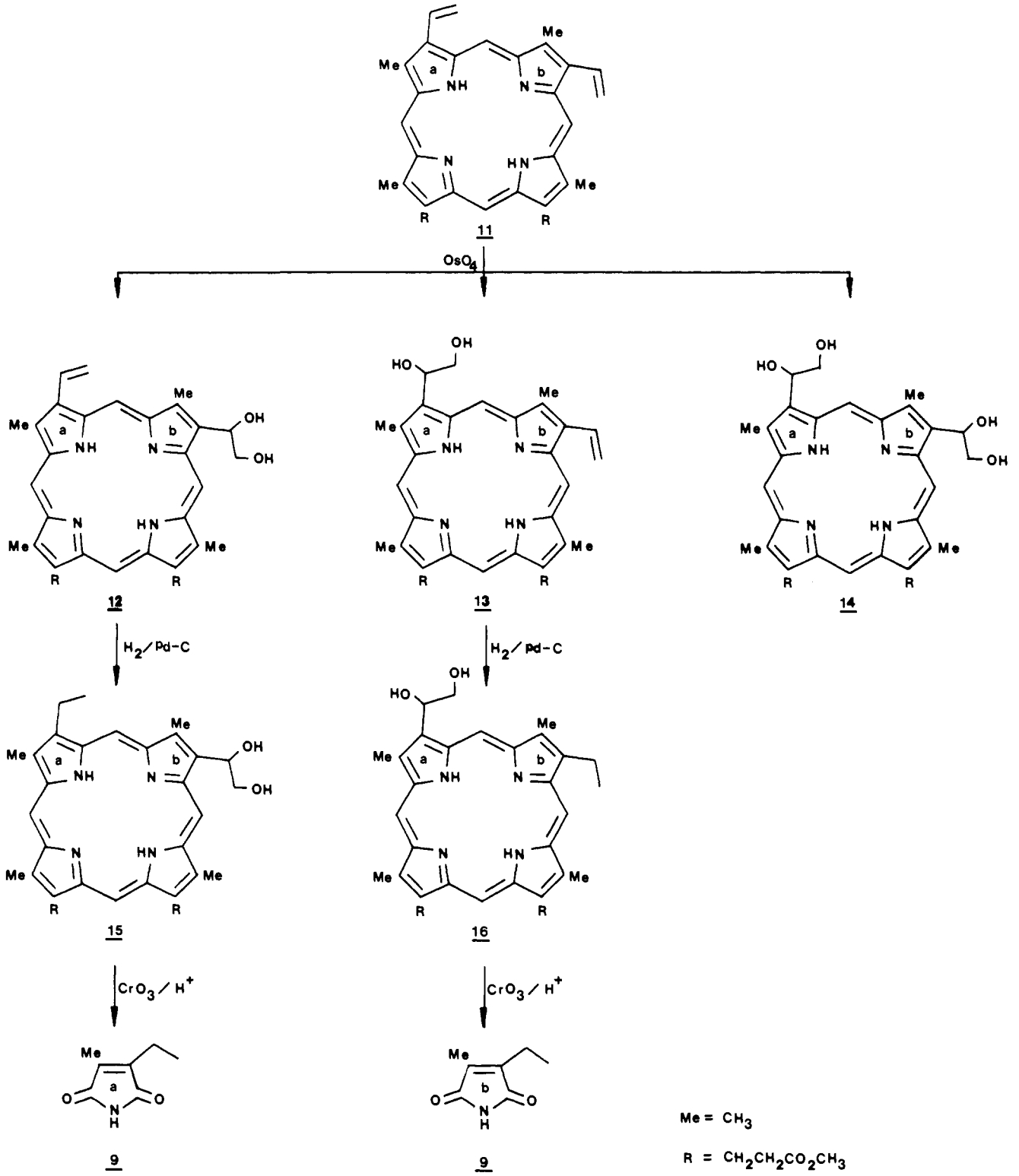
(14) Jordan, P. M., unpublished observations.

Scheme II. Selective Degradation of Protorporphyrin IX Dimethyl Ester to Ethylmethylmaleimide (Ring a or b)



mechanism is clearly attractive,[15] but as yet no direct evidence exists (see Note Added in Proof), since the strong binding observed does not necessarily imply the involvement of a covalent bond. In fact, conformational changes in the enzyme on interaction with the substrate could equally well account for a slow "off" rate for the substrate. Whatever the reason for the observed results, it would appear that the deaminase prefers to biosynthesize preuroporphyrinogen from porphobilinogen units rather than from synthetic dipyromethane or tripyromethanes.[16] This may be due to the fact that each condensation is obligatorily linked to a translocation stage in which the growing polypyrrole is manipulated on the enzyme surface before the next porphobilinogen ring can bind. This may be expected to occur if a single catalytic site was involved. Although our results do not distinguish between a mechanism involving a single catalytic site which is used several times (mechanism 1) or multiple catalytic sites used once in

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Table III. Distribution of the ^{14}C Label in Ethylmethylmaleimide Derived from Ring a or Ring b of Protoporphyrin IX Dimethyl Ester Arising from Uroporphyrinogen III (4) Regiospecifically Labeled as in Experiment 1

	yield, mg	total radioact, 10^5 dpm	sp act., 10^4 dpm/μmol	% radioact in maleimide
protoporphyrin IX	60	93.6	8.77	
protoporphyrin IX dimethyl ester	58	86.2	8.77	
8-(1,2-dihydroxyethyl)-3-vinyldeuteroporphyrin IX dimethyl ester	20.5	28.8	8.77	
8-(1,2-dihydroxyethyl)-3-ethyldeuteroporphyrin IX dimethyl ester	12.8	17.9	8.74	
ethylmethylmaleimide (ring a)	2.7	7.54	3.80	89
3-(1,2-dihydroxyethyl)-8-vinyldeuteroporphyrin IX dimethyl ester	20	28.1	8.75	
3-(1,2-dihydroxyethyl)-8-ethyldeuteroporphyrin IX dimethyl ester	12.3	17.1	8.70	
ethylmethylmaleimide (ring b)	2.0	0.799	0.44	11

sequence (mechanism 2), the small size of the deaminase protein (M_r 35 000)[17] in comparison to the size of the tetrapyrrole product makes it unlikely on intuitive grounds that several catalytic sites could be present. Since the binding of the porphobilinogen molecules is very tight, it is interesting to speculate on the mechanism by which the tetrapyrrole product preuroporphyrinogen is ultimately able to leave the enzyme. If the conformation adopted by the bound tetrapyrrole is similar to that in uroporphyrinogen III, then the ring (a) may well obstruct the addition of a "fifth" porphobilinogen, allowing the attack of OH^- either by a direct displacement of NH_3 or by reacting with a previously formed covalent enzyme–tetrapyrromethane bond. In this context it is interesting to note that aminomethylbilane[18] is the only polypyrrole which is accepted by deaminase and converted into preuroporphyrinogen, a reaction not requiring translocation.[14] Clearly, much future work is required to unravel the intricacies of the mechanism of this remarkable enzyme.

Experimental Section

Chemicals. Porphobilinogen was enzymatically synthesized from 5-aminolevulinic acid, using purified bovine liver 5-aminolevulinic acid dehydratase[19] [EC 4.2.1.24]. [2,11-$^{14}C_2$]Porphobilinogen was prepared from 5-amino[5-^{14}C]levulinic acid as above. Protoporphyrin IX and osmium tetroxide were obtained from Sigma Chemical Co., London. Al_2O_3 (Merck) for column chromatography and silica gel type 60 for TLC (Merck) were obtained from BDH, Poole, Dorset. All other laboratory reagents were obtained from BDH.

Growth of Bacteria. *Rhodopseudomonas spheroides* [NCIB 8253] was grown semianaerobically in the light, using Lascelles' medium S[20] supplemented with yeast extract (0.2% w/v) and iron(III) citrate (2.5 mg/L). The cells were grown at 30 °C and were harvested when in the log phase by using a Sharples ultracentrifuge at 65 000 rpm. The cells were washed with potassium phosphate buffer (0.1 M; pH 7.9) (saturated with nitrogen) and used immediately for the isolation of enzymes.

Purification of Enzymes. Porphobilinogen deaminase[17] and uroporphyrinogen III cosynthetase[21] were purified by using the initial stages of methods already described elsewhere. A partially purified system containing the enzymes, uroporphyrinogen decarboxylase and coproporphyrinogen III oxidase, was prepared using freshly harvested *R. spheroides*.[19]

Incubations. Incubations were carried out anaerobically in the dark, using a linked enzyme system containing porphobilinogen deaminase, uroporphyrinogen III cosynthetase, uroporphyrinogen III decarboxylase, and coproporphyrinogen III oxidase. The incubation mixture contained, in a final volume of 200 mL, porphobilinogen deaminase (~2000 units), uroporphyrinogen III cosynthetase (~1000 units), uroporphyrinogen III decarboxylase and coproporphyrinogen III oxidase (~2.5 units), $MgSO_4$ (2 mmol), ATP (0.8 mmol), L-methionine (0.2 mmol), NADH (0.6 mmol), $NADP^+$ (0.4 mmol), and potassium phosphate buffer, pH 7.9 (20 mmol). All buffers were saturated with N_2 prior to the addition of the enzymes. Incubations were carried out anaerobically under an atmosphere of nitrogen. The activity of the deaminase system was measured by the rate of consumption of porphobilinogen, the latter being estimated spectrophotometrically after reaction with Ehrlich's reagent.[22]

[2,11-$^{14}C_2$]Porphobilinogen (quantities described in the legend to Table I) was added to the linked enzyme systems at 0 °C. The temperature of incubation was raised to 10 °C, unlabeled porphobilinogen (5 mg) was added, and the mixture was incubated at 37 °C anaerobically in the dark. After 4 h, KCN (2 mmol) was added and the incubation was continued for a further 2 h aerobically. The combination of aerobic and anaerobic activities of coproporphyrinogen III oxidase[23] gave protoporphyrin IX in approximately 90% yield (radiochemical yield from porphobilinogen).

Extraction of Protoporphyrin IX. The reaction was stopped by the addition of benzoquinone (6 mg) in methanol (1 mL) so that any remaining porphyrinogens were oxidized to porphyrins. Protoporphyrin IX (90 mg)[23] was then added and the porphyrins were extracted using a modified method of Tait[23] as follows. Ethyl acetate–acetic acid (3:1, v/v; 500 mL) was added to the above solution, and after vigorous stirring the mixture was centrifuged. The upper layer was removed and the lower layer (which also contained the protein pellet) was reextracted twice. Diethyl ether (500 mL) was added to the combined extracts, and the resulting solution was washed with water (4 × 300 mL). The volume of the ethereal extract, which contained all the porphyrins, was reduced to 200 mL in vacuo. The porphyrins were then extracted from the ether into 8 M hydrochloric acid (100 mL) and the acid solution was diluted to 250 mL with water. Diethyl ether (100 mL) was added to the acid solution, and sodium acetate (solid) was added to the mixture (with stirring) until all the porphyrins were extracted into the ether. The etheral layer was extracted with 0.01 M HCl to extract the uroporphyrins, followed by 0.05 M HCl and 0.15 M HCl to extract the coproporphyrins. The etheral layer which contained protoporphyrin IX was washed with water (2 × 250 mL) and dried over anhydrous sodium sulfate, and the solvent was removed in vacuo.

A sample of protoporphyrin IX (~100 μg) was esterified with BF_3/MeOH (1.0 mL) at 60 °C for 10 min. Water (2 mL) was added and the protoporphyrin IX dimethyl ester was extracted into chloroform (0.5 mL). The chloroform extract was washed with 2 M sodium carbonate (1 mL), washed with water, and dried over anhydrous sodium sulfate. The purity of the protoporphyrin IX methyl ester was determined by TLC on silica plates developed in benzene–ethyl acetate–methanol (80:18:2, v/v). The bands were located by fluorescence of porphyrins under UV. Samples of protoporphyrin IX dimethyl ester were also analyzed by high-performance LC to verify their purity.[24]

Large-Scale Preparation of Protoporphyrin IX Dimethyl Ester.[25] Protoporphyrin IX (250 mg) was added to dry methanol (100 mL) containing 5% H_2SO_4. The mixture was stirred in the dark for 36 h at 4 °C. Diethyl ether (200 mL) was added and the organic phase was washed three times with aqueous Na_2CO_3 (10% w/v) and then with water. The organic phase was dried over anhydrous Na_2SO_4 and the solvent was removed in vacuo. The protoporphyrin IX dimethyl ester (**11**) was dissolved in a small volume of dry benzene (a few drops of $CHCl_3$ being necessary for complete solution) and was applied to a column of grade IV Al_2O_3 (3.5 × 20 cm) prepared with dry benzene, and the protoporphyrin IX dimethyl ester was eluted with benzene–chloroform (95:5, v/v). A small quantity of the free acids remained strongly bound to the column. The solvent was removed in vacuo: UV λ_{max} 408 nm (ϵ 165 000), 507 (13 700), 542 (11 600), 575 (6600), 600 (2000), 630 (5300).

Preparation of 3-(1,2-Dihydroxyethyl)-8-vinyldeuteroporphyrin IX Dimethyl Ester and 8-(1,2-Dihydroxyethyl)-3-vinyldeuteroporphyrin IX Dimethyl Ester.[13] A solution of osmium tetroxide (25 mg) in diethyl ether (2.5 mL) containing pyridine (50 μL) was added to a solution of

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protoporphyrin IX dimethyl ester (60 mg) and the mixture was stirred in the dark for 48 h. A solution of Na_2SO_3 (250 mg) in water (2.5 mL) was added to the black suspension and refluxed for 75 min. The suspension was filtered, and the filterate was added to methylene chloride (50 mL) and extracted with water (3 × 25 mL). The organic phase was dried over anhydrous Na_2SO_4 and evaporated to dryness, and the residue was chromatographed on alumina (grade V), elution being with chloroform–acetone (95:5, v/v). The first mobile band contained unreacted protoporphyrin IX dimethyl ester, and the second mobile band contained the required isomeric mixture of the two glycols. This bisglycol (**14**) remained bound to the column and was eluted with chloroform–acetone (50:50, v/v). The isomeric mixture of 3-(1,2-dihydroxyethyl)-8-vinyldeuteroporphyrin IX dimethyl ester (**13**) and 8-(1,2-dihydroxyethyl)-3-vinyldeuteroporphyrin IX dimethyl ester (**12**) was separated by preparative TLC on silica gel (type 60) with the use of 1-mm thick plates. The plates were developed twice in 6% THF (distilled) in CH_2Cl_2. The more mobile band afforded the 3-(1,2-dihydroxyethyl)-8-vinyldeuteroporphyrin IX dimethyl ester and the less mobile band gave the 8-(1,2-dihydroxyethyl)-3-vinyldeuteroporphyrin IX dimethyl ester. The physical properties of the two isomers were the same as those described in literature.[13] 3-(1,2-Dihydroxyethyl)-8-vinyldeuteroporphyrin IX dimethyl ester: UV λ_{max} 405 nm (ϵ 170 000), 505 (13 500), 540 (12 500), 575 (7500), 600 (1500), 627 (4000). 8-(1,2-Dihydroxyethyl)-3-vinyldeuteroporphyrin IX dimethyl ester: UV λ_{max} 405 nm (ϵ 160 000), 505 (13 000), 540 (10 700), 575 (6500), 600 (1500), 627 (3500).

Reduction of Protoporphyrin IX, 3-(1,2-Dihydroxyethyl)-8-vinyldeuteroporphyrin IX Dimethyl Ester, and 8-(1,2-Dihydroxyethyl)-3-vinyldeuteroporphyrin IX Dimethyl Ester. Protoporphyrin IX (20 mg) was dissolved in formic acid (98–100%; 3.1 mL) and methyl methacrylate (40 mg) was added. The solution was stirred for 1 h at room temperature in the dark to dissolve the methyl methacrylate, the mixture was cooled in ice, and 10% Pd/C (22 mg) was added. Hydrogen gas was passed over the mixture held at 48 °C for 3 h, after which dry diethyl ether (200 mL) was added and the precipitate of methyl methacrylate was removed by filteration. Removal of the solvent in vacuo afforded mesoporphyrin IX: UV λ_{max} 400 nm (ϵ 16 500), 495 (13 000), 535 (9500), 565 (6000), 595 (1000), 620 (4500).

The reduction of the vinyl group in 3-(1,2-dihydroxyethyl)-8-vinyldeuteroporphyrin IX dimethyl ester and 8-(1,2-dihydroxyethyl)-3-vinyldeuteroporphyrin IX dimethyl ester was carried out as described above for protoporphyrin IX.

3-(1,2-Dihydroxyethyl)-8-ethyldeuteroporphyrin IX dimethyl ester (**16**): UV λ_{max} 400 nm (ϵ 16 000), 500 (12 500), 530 (10 000), 570 (6500), 600 (1500) and 620 (5000). 8-(1,2-Dihydroxyethyl)-3-ethyldeuteroporphyrin IX dimethyl ester (15): UV λ_{max} 400 nm (ϵ 16 000), 500 (12 500), 530 (9800), 570 (6500), 600 (1400), 620 nm (4800).

Chromic Acid Oxidation.[12] Mesoporphyrin IX (~30 mg) was dissolved in H_2SO_4 (3.5 mL; 20%, v/v, in H_2O), and chromium trioxide (55 mg) in water (0.1 mL) was added over a period of 30 min. The mixture was left stirring in the dark for 18 h at 20 °C. Ethylmethylmaleimide and hematinic acid derived from mesoporphyrin IX were extracted into ether (continuous extraction for 24 h). The hematinic acid was removed from the ethyl maleimide by washing the etheral extract with 5% aqueous $NaHCO_3$ (5.0 mL) and then twice with water (5 mL). The ethylmethylmaleimide remained in the etheral layer. The combined bicarbonate and aqueous washings were acidified to pH 2.0 with sulfuric acid (2 M). The hematinic acid was extracted with chloroform (3 × 5 mL). The maleimides were purified by vacuum sublimation. Ethylmethylmaleimide sublimed at 80–90 °C (0.05 mmHg) and hematinic acid at 160–170 °C (0.05 mmHg). Ethylmethylmaleimide and hematinic acid were estimated by measuring the UV spectra, both maleimides having a broad absorption at 295 nm in water. Ethylmethylmaleimide: UV λ_{max} 295 nm (ϵ 435). Hematinic acid: UV λ_{max} 295 nm (ϵ 472) in water.[12]

Radioactive Counting. Radioactive samples were counted in toluene–methanol (75:25, v/v) containing 5-(4-biphenylyl)-2-(4-butylphenyl)-1-oxa-4-diazole (6 g/L), in an Intertechnique SL30 programmed for correction to disintegrations per minute.

Note Added in Proof: Current investigations on the mode of interaction of the deaminase with the substrate have provided strong evidence for the participation of a covalent linkage.

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