Biosynthesis of the Natural Porphyrins: Proof that Hydroxymethylbilane Synthase (Porphobilinogen Deaminase) uses a Novel Binding Group in its Catalytic Action

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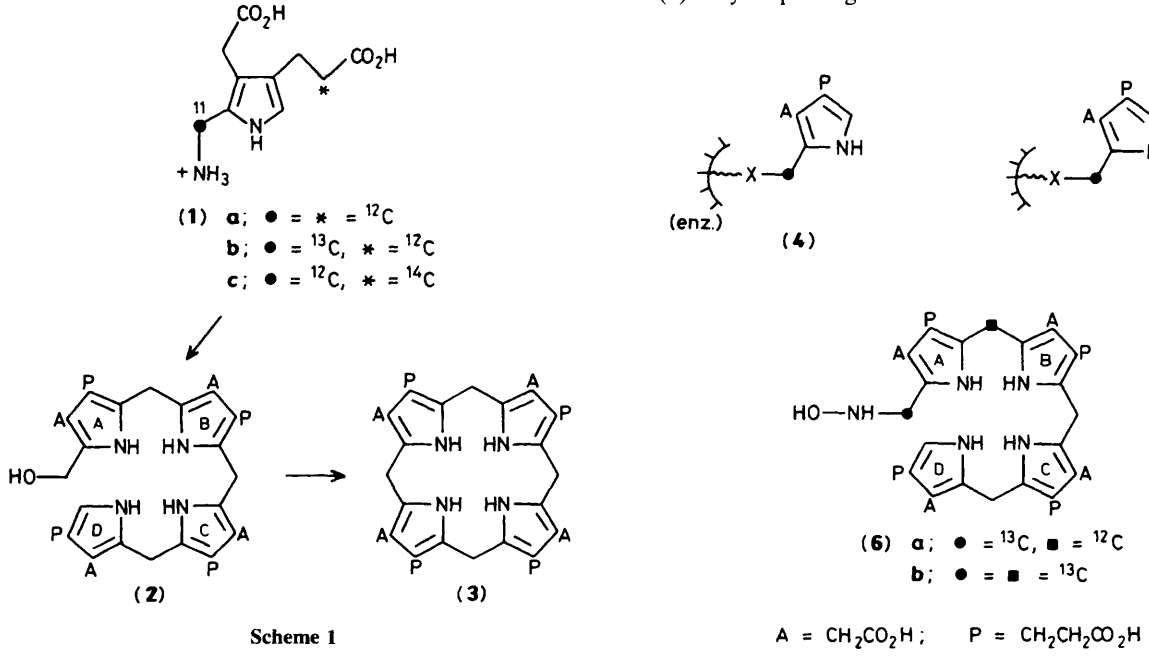
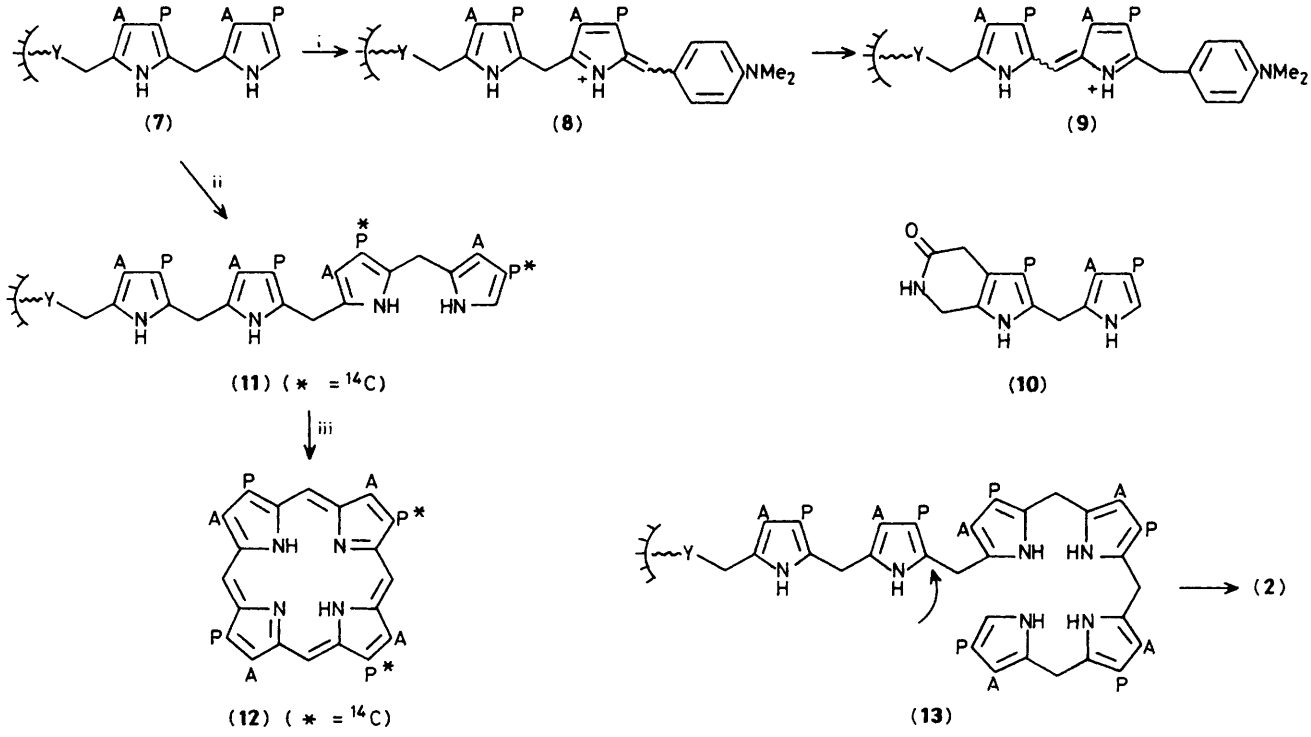
Hydroxymethylbilane synthase builds a bilane by assembling 4 monopyrrolic units, the first of these being bound covalently to the enzyme through a group X; it is proved that X represents a unique enzymic cofactor based on a pyrromethane system.

Uroporphyrinogen III (uro'gen III) (**3**), the biosynthetic parent of all the naturally occurring porphyrins, chlorins and corrins, is derived from hydroxymethylbilane (**2**) in a rearrangement step catalysed by uro'gen III synthase (Scheme 1).[1] The bilane (**2**) is built from four molecules of porphobilinogen (PBG) (**1a**) by the enzyme hydroxymethylbilane synthase (HMBS),[1] and the growing oligopyrrolic chain is known to be covalently attached[2,3] to the enzyme through some group X [see (**4**) and (**5**)]. This paper outlines our proof that the X group of HMBS is an entirely novel one.

The work was made possible by (i) the development of a strain of *Escherichia coli* which overproduces HMBS by *ca.* 200 fold[4] and (ii) knowledge of conditions which stabilise X–CH_2–pyrrole systems.[5,6] Incubation of a limited amount of 90 atom % [11-^{13}C]PBG (**1b**) with *E. coli* HMBS[7] followed by ion-exchange fast protein liquid chromatography (f.p.l.c.) allowed approximately equal amounts of enzyme–^{13}C-PBG_1, mono-complex (**4**) and enzyme–^{13}C-PBG_2, di-complex (**5**), to be isolated. The binding ratios were established using a small amount of ^{14}C-PBG (**1c**) of known specific activity in admixture with the ^{13}C-PBG. The two isolated ^{13}C-labelled complexes were prepared for proton-decoupled ^{13}C n.m.r. studies and all spectra were recorded under the stabilising conditions given in Fig. 1a which shows the well-resolved signals observed from the enzyme–^{13}C-PBG_1 mono-complex (**4**). By repeating the above work but with unlabelled



Scheme 1



Scheme 2. *Reagents:* i, *p*-Dimethylaminobenzaldehyde, HCO_2H/2 M-HCl (70 : 30); ii, ^{14}C-PBG; iii, 50% aqueous HCO_2H, air oxidation.

^{12}C-PBG (**1a**), a natural abundance ^{13}C n.m.r. spectrum was obtained from the enzyme–^{12}C-PBG_1 mono-complex that was almost identical to Figure 1a, only lacking the strong signal at δ 24.6. The difference spectrum from the enzyme–^{13}C-PBG_1 minus enzyme–^{12}C-PBG_1 spectra showed a single strong signal at δ 24.6, Figure 1b, characteristic of a pyrrole–$^{13}CH_2$–pyrrole group.[5]

The ^{1}H-decoupled ^{13}C spectrum from the enzyme–^{13}C-PBG_2 di-complex (**5**) was also as shown in Figure 1a, but with the signal at δ 24.6 far larger; the difference spectrum from the enzyme–^{13}C-PBG_2 minus enzyme–^{12}C-PBG_1 spectra, showed one strong signal at δ 24.6, and the ratio of intensity of this signal to the difference signal in Figure 1b was 1.7 ± 0.2 : 1 (theoretically 2 : 1). Since a ratio of two difference values was involved, high accuracy was not expected. The ^{13}C-signal at δ 24.6 from the enzyme–^{13}C-PBG_1 complex was proved to arise from a $>CH_2$ group by the appropriate spin-echo experiments.

All these results strongly suggested that the PBG unit added first in the building process binds to a tightly bound pyrrole unit already present in the enzyme.

N.m.r. analysis of the enzyme–^{13}C-PBG_1 mono-complex (**4**) at pH 8.5 (not pH 12 as above) showed no discrete signals, a result presumably due to relaxation problems (buried ^{13}C-pyrrole residues and/or tumbling time). These problems were overcome by adjustment to pH 12, when the characteristic δ 24.6 signal could be accumulated as rapidly as the other signals in the spectrum. The pH of this sample was adjusted back to 8.5 and hydroxylamine and ^{12}C-PBG (**1a**) were added so that if the ^{13}C-PBG residue was still bound to the normal site, turnover would occur to yield tetrapyrrole which from previous knowledge[12] would be trapped as hydroxyaminomethylbilane (**6a**) for study by ^{13}C n.m.r. spectroscopy at pH >12. The characteristic signal[2] at δ 51.0 for HONH–$^{13}CH_2$-pyrrole was observed. Even more striking, when the enzyme–^{13}C-PBG_2 di-complex (**5**) was carried through this turnover and trapping sequence, two peaks at δ 51.0 and 24.6 were seen of integral ratio 1.04 : 1.0, indicating the formation of doubly-labelled hydroxyaminomethylbilane (**6b**).

These experiments prove that the enzyme–^{13}C-PBG_1 mono-complex (**4**) and enzyme–^{13}C-PBG_2 di-complex (**5**) observed by n.m.r. spectroscopy are active complexes at pH 8.5 and when turned over, the first ^{13}C–PBG unit which had been bound to the enzyme becomes ring *A* of the bilane (**6a**), in agreement with earlier experiments on order of assembly of the bilane system.[2,8] In confirmation, turnover of 12 mol ^{14}C-PBG (**1c**) per mol HMBS, followed by reisolation of the protein by f.p.l.c., gave unlabelled enzyme still carrying the normal amount of pyrrole determined as outlined below. So the tightly bound (enzymic) pyrrole system is present on the enzyme in addition to the PBG units which are turned over during the assembly process.

A surprising observation now fell in place; it was that treatment of HMBS with 70% aqueous formic acid rapidly yielded uroporphyrins shown by reverse phase f.p.l.c. to be type I : type III/IV : type II in the ratio *ca.* 57 : 40 : 3. The porphyrins arise by aerial oxidation of the initially formed uro'gens. The combined amount of uroporphyrins released corresponded in different experiments to 0.8—1.2 mol bound pyrrole per mol enzyme. But it was very likely that considerably more pyrrole than this estimate was in fact present on the enzyme since non-enzymic cyclisations of pyrromethanes[9] and bilanes[10] to uroporphyrins give *ca.* 40—50% yields. The formation of uro'gen isomers (leading to isomeric uroporphyrins) is expected under acidic conditions.[11]

To gain information about the nature of the bound pyrrole

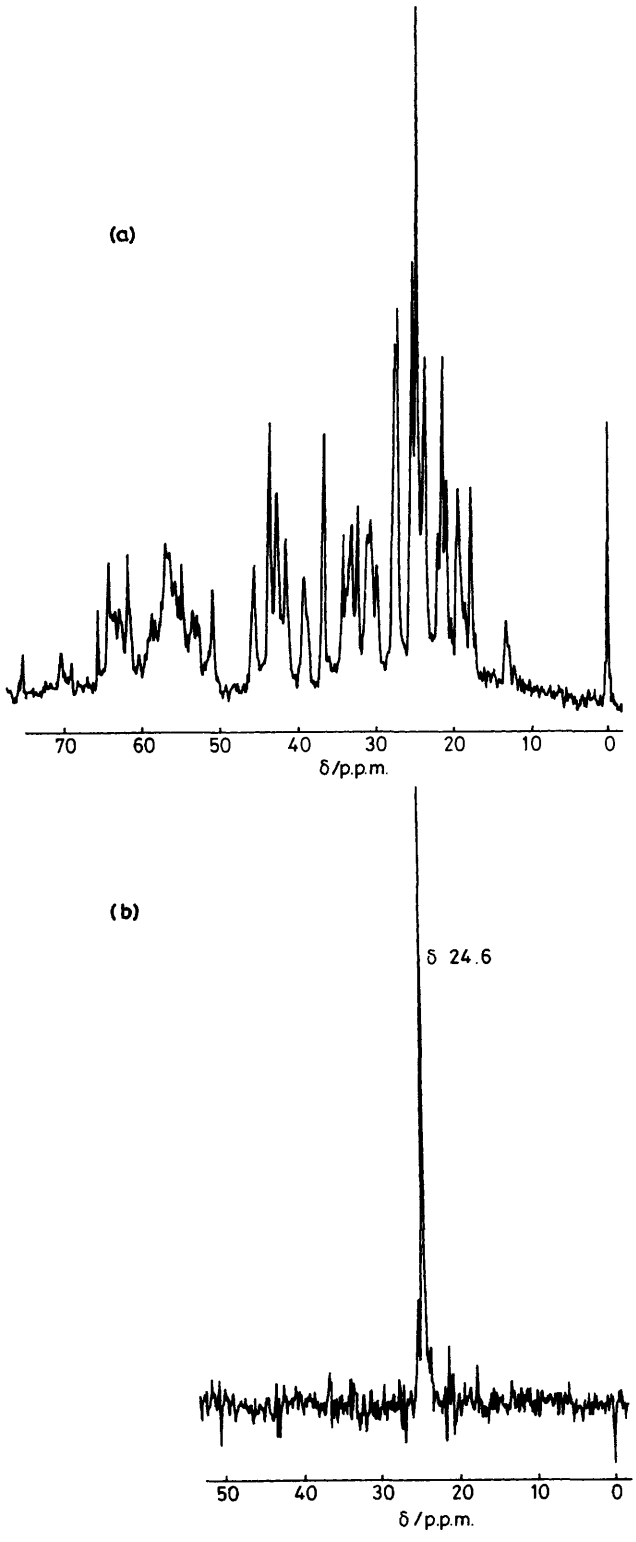


Figure 1. (a) Proton-decoupled ^{13}C n.m.r. spectrum of the enzyme–^{13}C-PBG_1 mono-complex (**4**) run at 100.6 MHz, 10 °C and pH 12; the data were processed by exponential multiplication and 10 Hz line broadening. The mono-complex had been concentrated by ultrafiltration and freed from Tris buffer by dialysis at 0 °C against nitrogen-saturated phosphate buffer, pH 12.

(b) Difference spectrum from spectrum (a) minus the natural abundance proton-decoupled ^{13}C spectrum of the enzyme–^{12}C-PBG_1 mono-complex; the complex was prepared for spectroscopy and the spectrum was acquired as for (a).

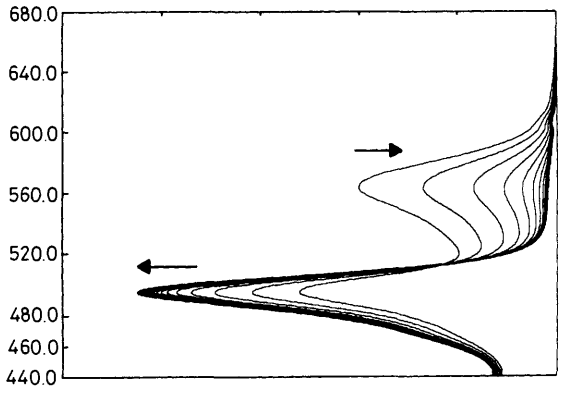


Figure 2. Absorption spectra of products formed by treating hydroxymethylbilane synthase with *p*-dimethylaminobenzaldehyde (Ehrlich's reagent) under acidic conditions. Sequential plots were run every 2 min and the absorption at 564 nm was gradually replaced by that at 495 nm.

system, HMBS was treated with acidic *p*-dimethylaminobenzaldehyde (Ehrlich's reagent). This caused the remarkable spectroscopic changes shown in Figure 2 which we interpret as being due to the enzymic pyrrolic system being a pyrromethane (**7**) covalently bonded to the protein through a Y group.† The initially formed Ehrlich pigment (**8**) (λ_{max} 564 nm), could undergo tautomerisation to the pyrromethene (**9**) (λ_{max} 495 nm), which was shown to be covalently bound to the enzyme. This interpretation was shown to be correct by reacting the synthetic pyrromethane (**10**) under the same conditions with Ehrlich's reagent. The behaviour shown in Figure 2 was reproduced almost exactly, the two λ_{max} values being 562 and 488 nm.

Finally, HMBS was converted using ^{14}C-PBG (**1c**), into the enzyme–^{14}C-PBG_2 di-complex and the entire bound oligopyrrole, which on the above basis will be species (**11**), was cleaved from Y with acid. The resulting uroporphyrins were separated to yield uroporphyrin I (**12**), 72% of total, and uroporphyrin III/IV, 28% of total. The illustrated labelling of uroporphyrin I (**12**) shows that its specific molar activity should (since two unlabelled pyrroles have been cleaved from the enzyme) be only 50% that of uroporphyrin I produced by the normal action of HMBS on the same sample of PBG (when all four pyrrole rings are derived from substrate PBG). The value found was 49.6%.

All the foregoing evidence interlocks to support the view that the terminus of the X-group of HMBS which binds PBG is a pyrromethane (**7**) bound to the enzyme through a group Y; the nature of Y is being studied.‡ This result contrasts with the report[12] that the X-group of HMBS is sulphur or, less likely, nitrogen. On the basis of our findings, the building of the tetrapyrrole system leading to release of hydroxymethylbilane (**2**) generates a hexapyrrole (**13**). Specific protonation of this system at the arrowed carbon would lead to release of the assembled tetrapyrrole ready for a new cycle of building.

† Hydroxymethylbilane synthase isolated from the alga *Euglena gracilis*[2] shows the same behaviour with Ehrlich's reagent.

‡ It is possible, in principle, that the complete X-group is a linear tripyrrole or even a linear tetrapyrrole, in which case the group Y in (**7**) will be, respectively, a mono- or di-pyrrolic unit. These possibilities are being examined by current experiments though the amount of tightly bound pyrrole (not turned over) detected on the enzyme (see text), points to a pyrromethane (**7**) as being the whole of the X-group.

To our knowledge, the covalently bound pyrromethane (**7**) of hydroxymethylbilane synthase represents a unique enzymic cofactor.

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