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REVIEW

Biosynthesis of the Pigments of Life: Structure and Mode of Action of a Novel Enzymatic Cofactor

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Biosynthesis of the organic nuclei of hemes, chlorophylls, cytochromes, and vitamin B_{12} involves, as a first stage, the building of a linear tetrapyrrole by the enzyme hydroxymethylbilane synthase. This enzyme has been found to use a novel dipyrrolic cofactor whose structure has been established. The function of this cofactor in the building process and the way in which the cofactor is bound to the enzyme have both been determined.

The pigments of life such as protoheme, chlorophyll, and vitamin B_{12} are all organometallic complexes and the organic ligand is one of a family of related tetrapyrrolic macrocycles, see, e.g., protoheme **8**. Remarkably, the different organic macrocycles which are used by living systems to allow a wide variety of functions to be performed are all derived biosynthetically from a single intermediate, uroporphyrinogen III (uro'gen III)[1] having structure **7**. Examples of the range of biological functions are oxygen or electron transport (protoheme), photosynthesis (chlorophyll), and molecular rearrangements (vitamin B_{12}).

The key importance of uro'gen III for the biosynthesis of the pigments of life led to a powerful effort to gain an understanding of its biosynthesis. By 1979, the pathway had been largely elucidated (*1*) from the starting pyrrolic precursor, porphobilinogen, PBG, **1a** leading forward to uro'gen III **7**. The roles of the two essential enzymes had also been defined. One is called hydroxymethylbilane synthase (HMBS), also called PBG deaminase, and the other is uroporphyrinogen III synthase, also called cosynthetase.

HMBS was shown to be the assembly enzyme (*2*) which joins four PBG units **1a** head to tail to produce the hydroxymethylbilane **5**. As illustrated in Fig. 1, the first PBG unit is bound covalently to the enzyme through some group X. In this way,

[1] Abbreviations used: PBG, porphobilinogen; HMBS, hydroxymethylbilane synthase; uro'gen III, uroporphyrinogen III; FPLC, fast protein liquid chromatography.

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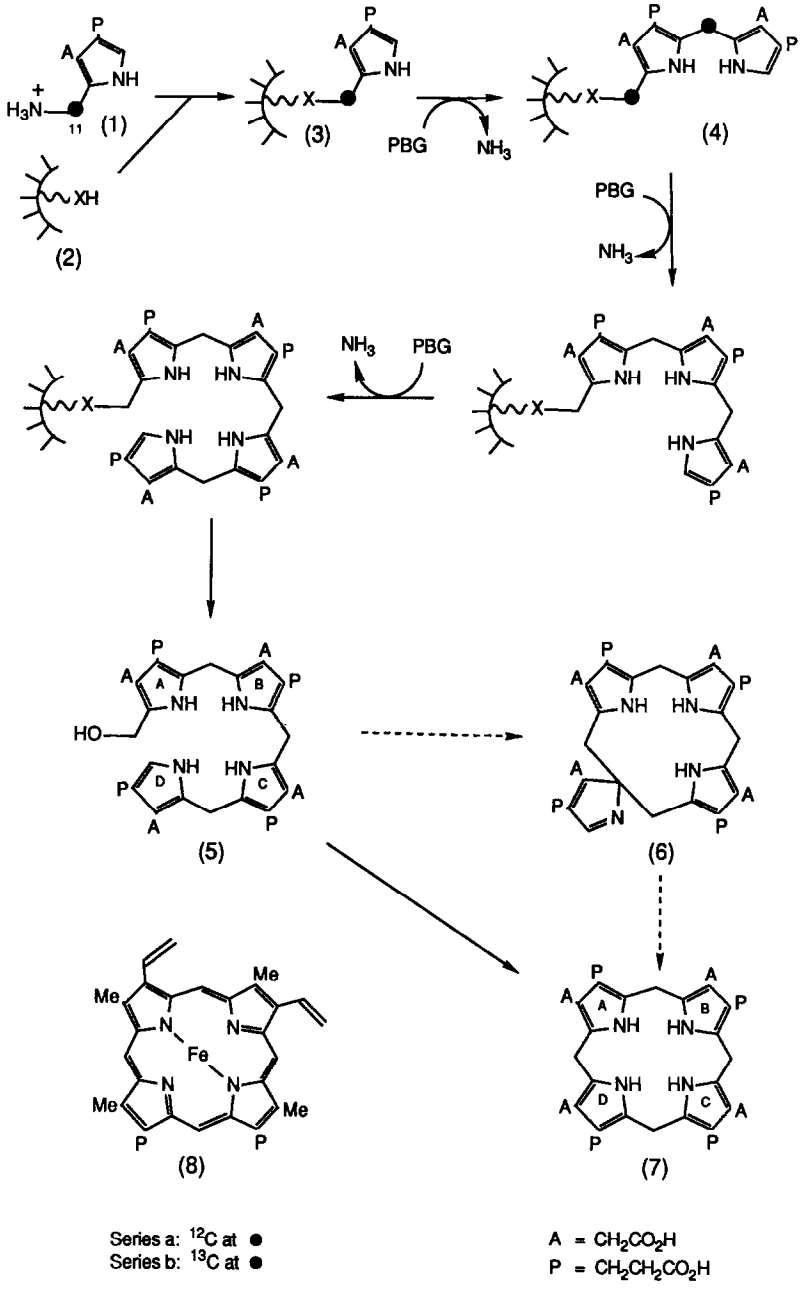


FIG. 1

the first pyrrolic unit is firmly anchored while the second, third, and fourth PBG residues are sequentially attached. Finally, the bilane **5** is released and this is the substrate for the second enzyme, cosynthetase, which brings about cyclization, with intramolecular rearrangement (*3*) of ring-D, to generate uro'gen III **7**. There

is strong evidence (*4*) that the spiro-system **6** is an intermediate in this rearrangement process.

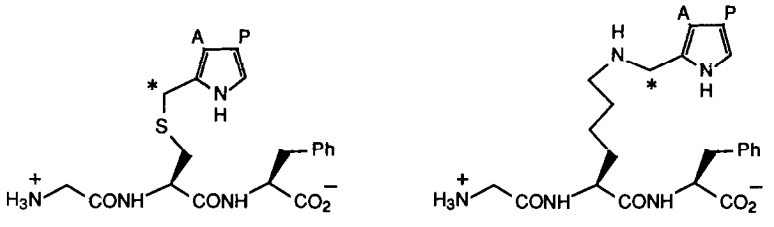
Much of our recent research in this area has been aimed at a full understanding of the mode of action of the enzyme HMBS; initially, the emphasis was on the nature of the X-group (see Fig. 1). These efforts combined in a powerful way the techniques of enzymology, molecular biology, synthetic organic chemistry, and NMR spectroscopy. The solution to the X-group problem which emerged was a great surprise and the key steps were as outlined in the sequel.

PRODUCTION OF HMBS

Most of the earlier work (*1*) in Cambridge on HMBS had been carried out with small amounts of enzyme isolated from *Euglena gracilis*. By cloning the gene for HMBS from *Escherichia coli* and overexpressing it, a strain was developed (*5*) which produced ca. 200 times the wild-type yield of HMBS leading to 60 mg of enzyme being isolable from one overnight fermenter run. This advance opened the door to enzymic experiments on a scale which allows study by ^{13}C NMR spectroscopy.

SYNTHESIS OF STANDARD SYSTEMS

We expected the X-group of HMBS to be a hetero atom of one of the protein amino acids, with the N of lysine or the S of cysteine being likely candidates. Earlier experience indicated that such $-X-CH_2$-pyrrole systems would be labile and therefore the model tripeptide Gly · Cys(S-PBG) · Phe **9** and the analogous Gly · Lys(NH–PBG) · Phe **10** were synthesized (*6*) (Fig. 2). It was then possible (a) to find conditions (high pH) which stabilized these molecules and (b) to determine the ^{13}C NMR chemical shifts for the asterisked methylene groups under these stabilizing conditions.



(9) (10)

FIG. 2

NATURE OF THE X-GROUP

It was first established by several experiments based on ion-exchange fast protein liquid chromatography (FPLC) that the isolated form of HMBS normally carries no bound substrate, i.e., the enzyme is unloaded, **2.** Incubation of this form (to be called *holo*enzyme) with a limited quantity of 90 atom% [11-^{13}C]PBG **1b** followed by FPLC gave roughly equal amounts of enzyme–[^{13}C]PBG_1 mono-complex **3b** and enzyme–[^{13}C]PBG_2 di-complex **4b** which were clearly distinguished chromatographically from each other and from the unloaded *holo*enzyme **2.** A small amount of [^{14}C]PBG was used in admixture with the [^{13}C]PBG to allow the above binding radios to be deduced. Figure 3a shows the sharp ^{13}C NMR signals (proton decoupled) from the mono-complex **3b** run under the stabilizing high pH conditions established above (*6*). An equivalent ^{13}C spectrum at natural abundance was obtained from unlabeled enzyme–[^{12}C]PBG_1 mono-complex prepared analogously and the difference spectrum (enzyme–[^{13}C]PBG_1 minus enzyme–[^{12}C]PBG_1) showed a single strong signal at δ24.6 from the ^{13}C-enriched center (*7*) (Fig. 3b). Surprisingly, this chemical shift did not correspond to group X being S or N or even O; however, we knew this shift of δ24.6 to be characteristic of a pyrrole–$^{13}CH_2$–pyrrole system (*2*).

These results proved that the PBG unit added first in the building process binds to a tightly bound pyrrole residue already present in the enzyme (*7*). Similar NMR studies on the enzyme–[^{13}C]PBG_2 di-complex **4b** fully supported this conclusion. Also clear evidence was gained by turnover and trapping experiments using the mono-complex **3b** that the first PBG unit which had been bound to the enzyme appeared as ring-A of the bilane **5** (*7*). A final important experiment established that turnover of 12 mol [^{14}C]PBG/mol HMBS followed by reisolation of the *holo*enzyme **2** by FPLC gave *unlabeled* enzyme still carrying the normal amount of bound pyrrole determined as outlined later. Thus the tightly bound (enzymatic) pyrrole is present on the enzyme in addition to the PBG units which are turned over during the assembly process (*7*).

STRUCTURE OF THE TIGHTLY BOUND PYRROLIC SYSTEM

The foregoing results now interlocked with an earlier observation that acidification of a solution of HMBS caused a pink coloration. The color was shown to be due mainly to uroporphyrin I **14** arising by aerial oxidation of the initially formed uro'gen I **13** (Fig. 4). The production of uro'gen I proved that the tightly bound pyrrolic system carries acetic and propionic side chains together with a one-carbon residue able to provide the interpyrrolic bridges of uro'gen I **13.** It was thus likely that the tightly bound pyrrolic system is derived from PBG **1a.**

The amount of porphyrin formed from HMBS as compared with yields from acid-catalyzed cyclizations of standard pyrromethanes (*8*) indicated that the enzyme carries a pyrromethane system **11** (Fig. 4). This was supported by treating HMBS with *p*-dimethylaminobenzaldehyde (Ehrlich's reagent) when striking spectroscopic changes occurred. The initially generated Ehrlich pigment (λ_{max},

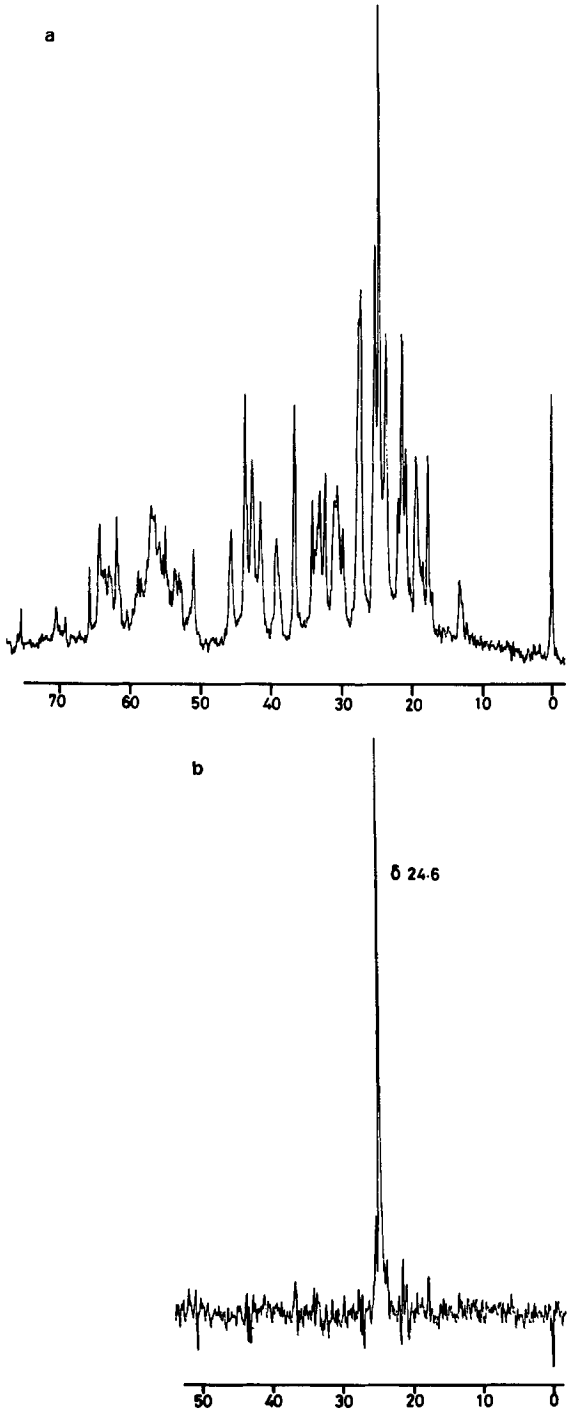


FIG. 3. (a) Proton-decoupled ^{13}C NMR spectrum of the enzyme–[^{13}C]PBG_1 mono-complex **3b**, Fig. 1, run at 100.6 MHz, 10°C, and pH 12. (b) Difference spectrum generated by subtraction of the natural abundance proton-decoupled ^{13}C spectrum of enzyme–[^{12}C]PBG_1 mono-complex from the spectrum (a).

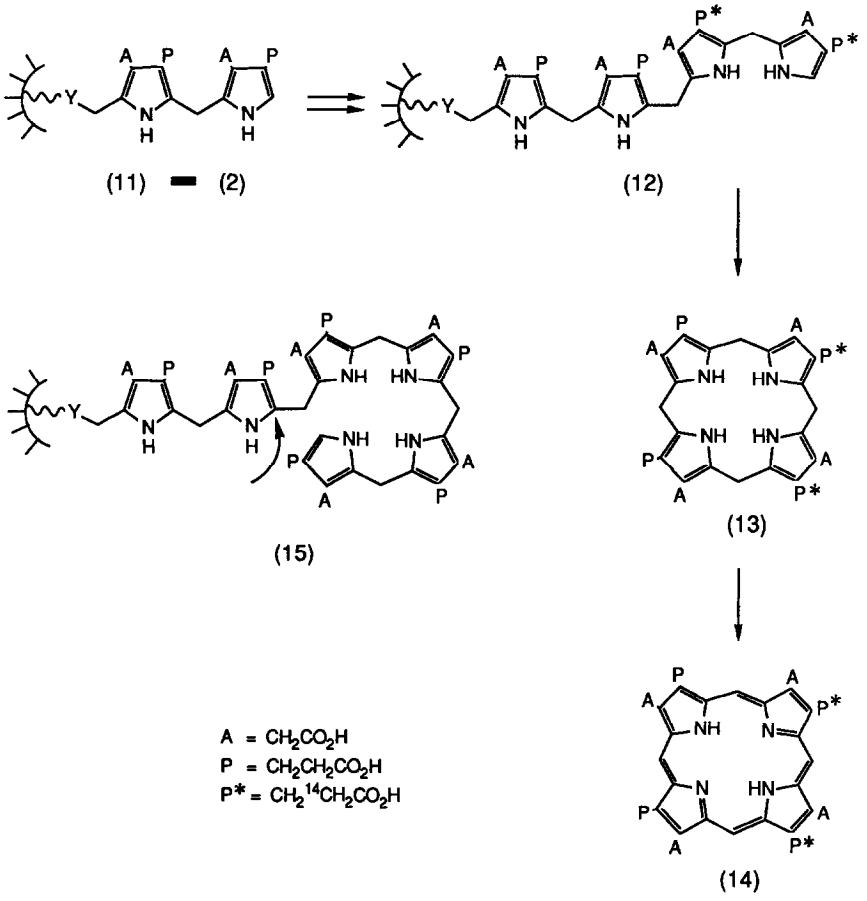


FIG. 4

564 nm) changed during 15–20 min to a different pigment (λ_{max}, 495 nm). This change was closely matched by that which occurred when a synthetic pyrromethane, equivalent to structure **11,** was treated with Ehrlich's reagent; the change is due to the initially formed Ehrlich pigment undergoing tautomerism to a pyrromethene (*7*). The clear conclusion could now be drawn that the X-group of HMBS is a pyrromethane **11** bound to the enzyme through a group Y.

Final confirmation came from preparing from HMBS **11** and [^{14}C]PBG the enzyme–[^{14}C]PBG_2 di-complex, which on the above basis will be the tetrapyrrolic species **12.** Cleavage of the Y–CH_2-link with acid gave, after oxidation of the generated uro'gens (see **13**), largely uroporphyrin I **14.** The illustrated labeling shows that its molar specific activity should (since two unlabeled pyrroles have been derived 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 labeled). This was indeed found (result 49.6%).

It was subsequently reported independently (*9*) that HMBS carries a pyrromethane.

NATURE OF THE Y-GROUP

The problem of the Y-group was tackled in two complementary ways. The first depended on being able to cleave the tightly bound pyrromethane system from the Y-group to uncover Y-H **16** with minimum damage to the protein structure. Conditions were eventually found to achieve this step and the resultant protein (*apo*-enzyme) had lost essentially all its catalytic activity. However, when the *apo*enzyme **16** was incubated with PBG **1a**, 50–60% of the initial enzymatic activity was restored (*10*). It follows that no additional enzyme is needed to build the pyrromethane system onto the Y-group of *apo*enzyme **16** to produce HMBS **11**; the *apo*enzyme itself possesses all the necessary catalytic activity.

By incubating *apo*enzyme **16** with [11-^{13}C]PBG to regenerate the pyrromethane cofactor, a ^{13}C atom was bound directly to the Y-group (see **17**) (Fig. 5). ^{13}C NMR studies on this labeled HMBS as earlier but, under even more strongly alkaline conditions to increase the mobility of the pyrromethane system, showed the expected signal at δ24.6 for the interpyrrolic $^{13}CH_2$ group (which confirms the pyrromethane structure), but also a new signal at δ29.5. This corresponded exactly to the position of the methylene signal from the –S–CH_2–pyrrole group of the synthetic peptide **9.** It was clear that the Y-group is sulfur and so it is cysteine in HMBS which carries the pyrromethane cofactor.

The complementary approach involved direct incorporation of 5-[^{13}C]aminolevulinic acid into HMBS by adding this known precursor of PBG **1** to the medium

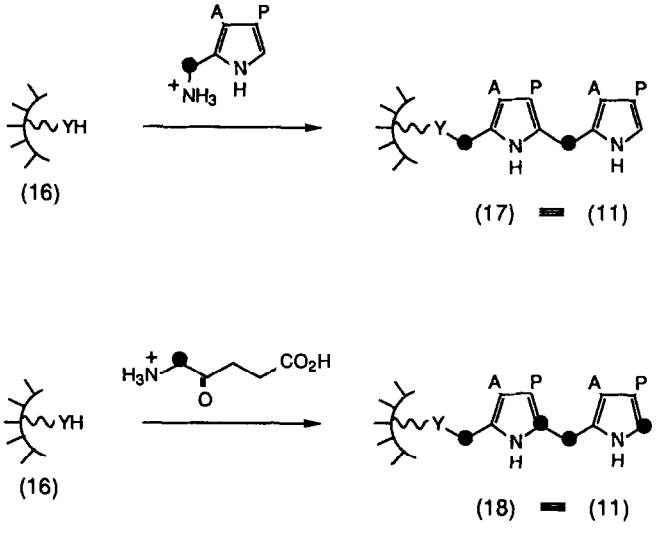


FIG. 5

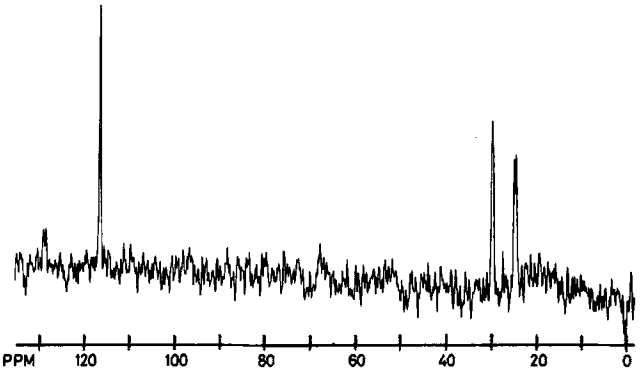


FIG. 6. Difference spectrum generated by subtraction of the natural abundance proton-decoupled ^{13}C spectrum of *holo*enzyme **11** from the equivalent ^{13}C spectrum of *holo*enzyme **18**, Fig. 5, which had been ^{13}C-labeled from 5-[^{13}C]aminolevulinic acid.

in which the overproducing strain of *E. coli* was grown. The *holo*enzyme **18** was isolated by FPLC and examined by proton decoupled ^{13}C NMR spectroscopy under the highly alkaline conditions used above. Three strong signals and a weaker one were observed in the difference spectrum, Fig. 6, which established (*11*) the labeling pattern illustrated on structure **18.** One at δ29.5 proved that the Y-group in naturally formed HMBS is sulfur, in confirmation of the foregoing results using reconstituted enzyme. Now the signal from the interpyrrolic methylene group at δ24.5 was a doublet ($J = 45 \pm 5$ Hz) proving its direct attachment to another ^{13}C atom. The signal for the latter carbon was weaker than the others as expected for a quaternary center; it appeared at δ128.3 in agreement with the sp^2-hybridization of the carbon and again the splitting ($J = 45 \pm 5$ Hz) was quite clear. Finally, a signal at δ116.2 matched exactly the expected position from a ^{13}C α-free carbon atom of the terminal pyrrole nucleus.

These interlocking experiments on the ^{13}C-labeled cofactor of the naturally formed **18** and reconstituted **17** HMBS made it certain that the structure of the pyrromethane cofactor of the enzyme is as illustrated and that it is attached to the sulfur of cysteine (*11*).

PRESENT KNOWLEDGE AND PROSPECTS

The knowledge of the mode of action of HMBS which has emerged from the research outlined above was totally unexpected. Indeed, the bound pyrromethane of HMBS **11** represents a unique enzymatic cofactor which the *apo*enzyme **16** can self-assemble from PBG **1a** onto a cysteine residue. We know of no other enzyme using a bound cofactor which is essential for the catalytic process (but not turning over) yet is built from the *substrate* for that enzyme. The biosynthesis of hydroxymethylbilane **5** involves four PBG units being added sequentially to the bound pyrromethane **11.** This generates a hexapyrrole **15** covalently attached to the

enzyme and specific protonation of this intermediate at the arrowed site would allow release of the constructed tetrapyrrole, leaving the enzyme ready for a new assembly cycle.

In the future, we aim to discover which of the enzyme's cysteine residues carries the pyrromethane cofactor. We also want to understand the molecular recognition process which controls the interaction of PBG **1a** with the enzyme. We believe that the combination of site-directed mutagenesis with complementary synthetic modification of the pyrrolic building blocks will lead to exciting advances.

ACKNOWLEDGMENTS

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