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^{13}C-N.M.R. STUDIES ON THE PYRROMETHANE COFACTOR OF HYDROXYMETHYLBILANE SYNTHASE

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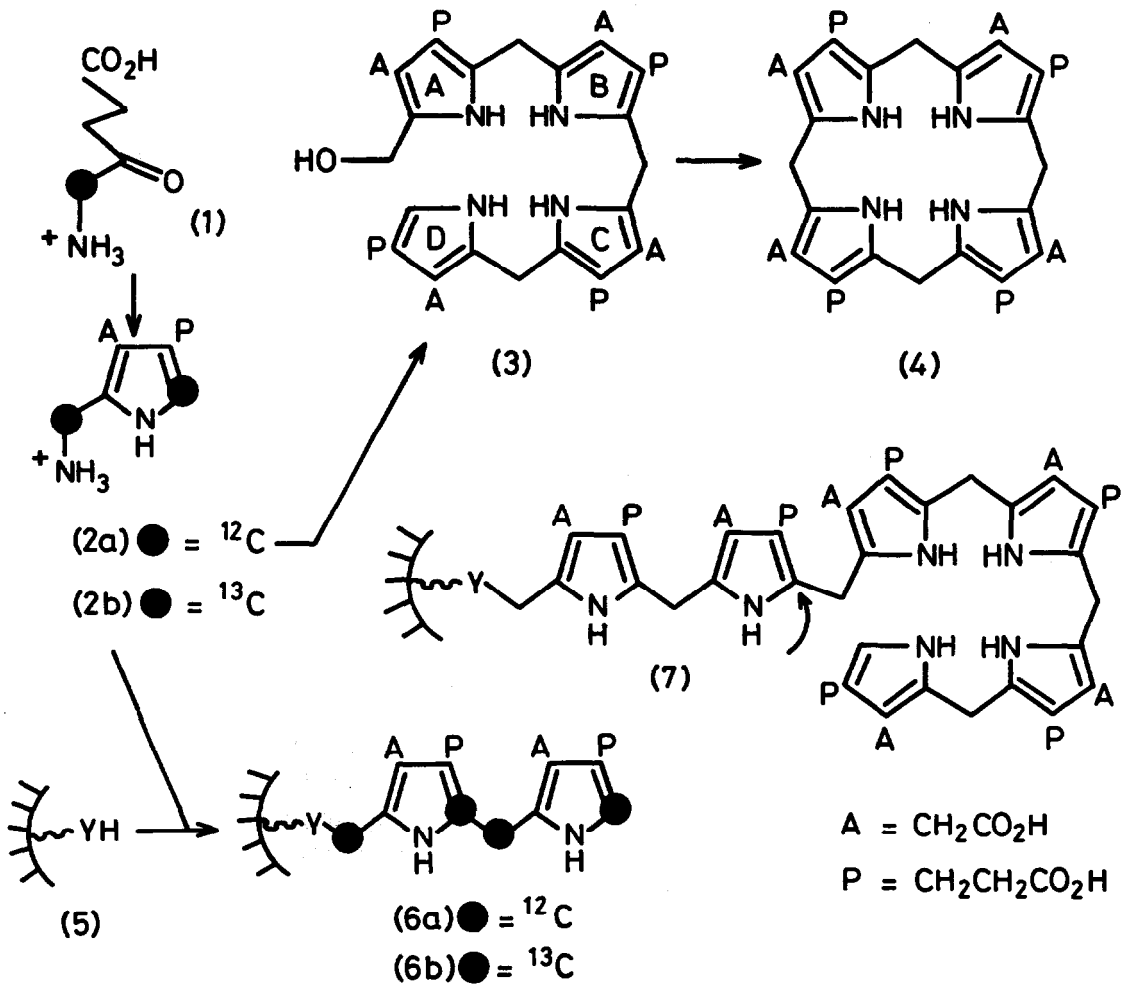
Summary: By growing Escherichia coli in the presence of 5-amino [5-^{13}C]laevulinic acid, the enzyme hydroxymethylbilane synthase is produced carrying ^{13}C-labels in its pyrromethane cofactor. It is then proved by ^{13}C-n.m.r. spectroscopy that the cofactor is bound to the protein via the sulphur atom of a cysteine residue.

Hydroxymethylbilane synthase (HMBS), also called porphobilinogen deaminase, is one of the key enzymes involved in the biosynthesis[1] of uroporphyrinogen III (4), shortened to uro'gen III. This tetrapyrrolic macrocycle is the parent of all the pigments of life such as the haems, chlorophylls and vitamin B_{12}. The role of HMBS is to construct hydroxymethylbilane[2] (3) by assembling four molecules of porphobilinogen (2a), PBG, head-to-tail with loss of four molecules of ammonia. The bilane (3) then acts as the substrate for a second enzyme, uroporphyrinogen III synthase, which cyclises the bilane (3) with concomitant intramolecular rearrangement of the terminal ring-D[3] to generate uro'gen III (4).

It has recently been shown[4] that the PBG unit which eventually forms ring-A of bilane (3) is covalently bound, as a first step, to a unique pyrromethane cofactor present in HMBS having structure (6a). This cofactor[5] is itself covalently bound to the protein of HMBS by way of a group Y. It must be emphasised that though the structure of the cofactor corresponds to the union of two PBG units, this pyrromethane remains firmly bound to HMBS (6a) and is not lost[4,5] during the assembly process which generates the bilane (3). What happens is that a hexapyrrolic system (7) is built from which the tetrapyrrolic bilane (3) is cleaved leaving the pyrromethane cofactor (6a) in place ready for the next cycle of assembly. It is probable that this cleavage is triggered by protonation of the cofactor at the arrowed α-position. We now describe studies which firmly establish the nature of group Y and confirm the pyrromethane structure for the cofactor previously deduced from other evidence.[4]

A strain of Escherichia coli which overproduces HMBS ca. 200 fold[6] was grown in the presence of 5-amino[5-^{13}C]laevulinic acid (1), ALA; the latter was synthesised[7] from [2-^{13}C]glycine. Two molecules of ALA are used for the biosynthesis of PBG[8] so generating the labelling pattern shown in structure



(2b). This labelling was chosen so that as the holoenzyme (6b) is formed from the apo-enzyme (5), a ^{13}C-atom will be directly connected to the Y-group. Also, as will be seen later, this labelling allowed us to probe other structural features of the cofactor in the enzyme.

HMBS (6b) isolated[9] from the above *E. coli* cells was purified by fast protein liquid chromatography[4] and a solution of it (*ca.* 22 mg) at pH 14 was used to determine the proton decoupled ^{13}C-n.m.r. spectrum. Remarkably sharp signals were observed which indicates that the protein and its attached cofactor are unwound and mobile under the conditions used. The natural abundance ^{13}C-spectrum from an unlabelled sample of HMBS (6a) was recorded under exactly the same conditions and the difference spectrum (^{13}C-labelled HMBS minus ^{12}C-HMBS) is shown in Fig. 1. This approach reveals the signals

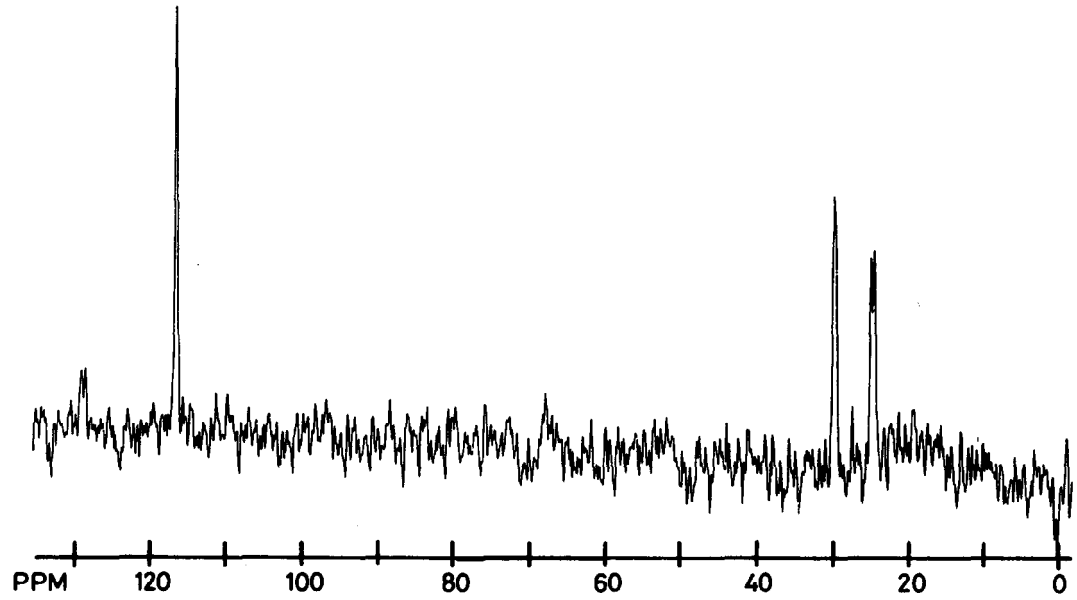


Figure 1

from the ^{13}C-enriched sites in the cofactor of HMBS (6b).

Standard peptide model systems had already been synthesised[10] containing RS-$^{13}CH_2$-pyrrole and RNH-$^{13}CH_2$-pyrrole residues and their clearly distinct ^{13}C-spectra were available. The signal at δ 29.5 in Fig. 1 lies precisely where that from the model RS-$^{13}CH_2$-pyrrole appeared. It is thus established that the cofactor of HMBS produced normally from its natural precursor is bound to the sulphur of a cysteine residue.

The doublet centred at δ 24.5 is at a well-known position[2] corresponding to an interpyrrolic $^{13}CH_2$ group. The fact that this signal is a <u>doublet</u> proves that the interpyrrolic $^{13}CH_2$ is directly bonded to another ^{13}C-atom and the splitting (J = 45±5Hz) is consistent with an sp^3-sp^2 connection (see 6b). The signal from the sp^2-^{13}C atom of this pair was weaker than the others as expected for a quaternary centre when rapid pulsing conditions are used. It appeared at δ 128.3 in agreement with a quaternary α-pyrrolic carbon and again the splitting was quite clear (J = 45±5Hz). Finally, the signal at δ 116.2 corresponds exactly to an unsubstituted α-carbon of a pyrrole (see 6b).

The foregoing results not only establish that the Y-group is sulphur[11] but all the other main structural features of the pyrromethane cofactor are strongly supported. The availability of HMBS specifically labelled in its pyrromethane cofactor opens the way to experiments probing the mechanism of action of HMBS.

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