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Evidence for a dipyrromethane cofactor at the catalytic site of *E. coli* porphobilinogen deaminase

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Porphobilinogen deaminase isolated from *Escherichia coli* is shown to contain a dipyrromethane cofactor (DPMC) linked covalently to the enzyme. The structure of the cofactor is proposed on the basis of its reaction with Ehrlich's reagent and from its chemical properties. The cofactor is involved in the binding of intermediates during the catalytic reaction but is not incorporated into the product preuroporphyrinogen, *E. coli* strains containing the cloned porphobilinogen deaminase gene *(hemC)* when grown on 5-amino[^{14}C]-levulinic acid incorporate ^{14}C radioactivity specifically into the dipyrromethane cofactor of porphobilinogen deaminase.

Porphobilinogen deaminase; Dipyrromethane cofactor; Enzyme intermediate complex; (E. coli)

1. INTRODUCTION

Porphobilinogen deaminase (hydroxymethylbilane synthase, EC 4.3.1.8) catalyses the tetrapolymerization of the pyrrole porphobilinogen (**1**) to yield the unstable hydroxymethylbilane, preuroporphyrinogen (**2**) [1,2]. Uroporphyrinogen III synthase subsequently catalyses the isomerization and cyclization of the preuroporphyrinogen to uroporphyrinogen III (**3**) [3], the key precursor for heme, chlorophyll and all other tetrapyrroles. In the absence of the synthase, preuroporphyrinogen cyclizes chemically to give uroporphyrinogen I (**4**) [3,4].

The deaminases from a wide variety of sources have been purified to homogeneity and all appear to be monomeric enzymes with an M_r ranging from 34000 to 44000. More recently the cloning and sequencing of the gene coding for *E. coli* porphobilinogen deaminase (*hemC*) has been reported [5]. The application of molecular cloning has also resulted in the generation of *E. coli* strains which produce almost 100 times the wild type deaminase levels [6].

One of the most striking properties of the porphobilinogen deaminases is their ability to form stable enzyme intermediate complexes with one, two, three or four pyrrole units bound to the catalytic site [7,8]. Single turnover experiments with labelled substrate have shown that the first porphobilinogen molecule to bind at the active site is destined to become ring a in the preuroporphyrinogen (**2**) [9,10] with rings b, c and d being added in sequence.

Studies with the enzyme from *Rhodopseudomonas sphaeroides* have established that a covalent bond is formed between the enzyme and the first bound substrate [11]. The nature of the enzymic group involved in the covalent linkage with ring a has been a matter of great interest. Lysine has been favoured by Battersby et al. [12] on the basis of ^{13}C NMR experiments and binding studies with pyridoxal phosphate [13]. It has also been suggested that the SH group of cysteine is involved with the binding of the substrate [14].

In this paper we present preliminary evidence

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that a completely unsuspected and novel enzyme bound cofactor, in the form of a dipyrromethane, is responsible for the binding of the substrate and for directing the synthesis of the tetrapyrrole at the catalytic site.

2. EXPERIMENTAL

2.1. *Materials*

Porphobilinogen and [3,5-$^{14}C_2$]porphobilinogen were prepared as in [11]. 5-Amino-[5-^{14}C]levulinic acid was purchased from New England Nuclear (Southampton, England). All other chemicals were from BDH Chemicals (Poole, Dorset).

Porphobilinogen deaminase was purified from *E. coli* ST1048 as described in [6]. This strain contains the plasmid pST48, a pBR322 derivative harbouring the *hemC* gene and produces almost 100 times the deaminase compared to the wild type. Purified enzyme was stored at −20°C. Enzyme activity and protein were determined as described [8]. The purified enzyme had a specific activity of 43 μmol/h per mg.

Porphobilinogen deaminase, in which the dipyrromethane cofactor was labelled with ^{14}C, was prepared from bacteria which had been grown in the presence of 5-amino[5-^{14}C]levulinic acid (20 μCi/l).

2.2. *Spectroscopic measurements and reagents*

Colorimetric and spectroscopic measurements were carried out at room temperature using a Pye Unicam SP8-400 UV/VIS spectrophotometer. Ehrlich's reagent was prepared as in [15].

2.3. *Formation and isolation of porphobilinogen deaminase intermediate complexes*

Enzyme (1 mg, 30 nmol) and porphobilinogen (60 nmol) were mixed in a rapid mixing device consisting of two 1-ml syringes linked by a Y-shaped tube. The enzyme and enzyme intermediate complexes were isolated either by electrophoresis as described in [8] or by high-resolution ion-exchange chromatography using a Pharmacia FPLC system. Application of the enzyme to a MonoQ HR 5/5 column in 10 mM Tris-HCl buffer, pH 7.5, followed by a linear gradient of NaCl permitted separation of the enzyme and enzyme intermediate complexes which eluted in well separated peaks in the order E, ES, ES_2 and ES_3 between 0.2 M and 0.3 M salt.

3. RESULTS AND DISCUSSION

3.1. *Removal of the bound substrate and isolation of the 'holoenzyme'*

It is now well established that the addition of the substrate porphobilinogen to the deaminase enzyme leads to the formation of stable enzyme intermediate complexes with one (ES), two (ES_2) and three (ES_3) substrate molecules bound covalently at the active site [8]. The bound substrates can be removed readily with hydroxylamine (0.2 M) which regenerates free enzyme [11]. This process can also be performed by heating the enzyme to 60°C for 5 min.

The effectiveness of these treatments for the removal of bound substrate was confirmed by first incubating the enzyme with [3,5-$^{14}C_2$]porphobilinogen (spec. act. 1 μCi/90 nmol) to yield ^{14}C-labelled ES, ES_2 and ES_3 intermediate complexes. Treatment of any of these complexes with either hydroxylamine or by heating released all the bound ^{14}C-label and regenerated the fully active enzyme. A typical experiment in which [^{14}C]ES_2 (1540×10^3 dpm/mg) was treated with hydroxylamine yielded active enzyme with less than 0.1% of the radioactivity remaining (1.03×10^3 dpm/mg). Treatment with hydroxylamine also released the ^{14}C-label from ^{14}C-labelled ES and ES_3 complexes. The enzyme generated from ES, ES_2 or ES_3 exhibited the same specific activity (43 μmol/h per mg) as the purified enzyme. The enzyme thus free of substrates is termed the holoenzyme.

3.2. *Spectral properties of the enzyme*

The purified holoenzyme is faint light orange-yellow in colour with weak absorption bands of 410 nm and 500 nm (fig.1a) at pH 8.

Acidification of the holoenzyme to pH 3.5 leads to a dramatic colour change, the solution turning to an intense pink-red colour. The deaminase is irreversibly inactivated by this process. The absorption spectrum of the deaminase at pH 3.5 is very different with a large absorption maximum at 490 nm (fig.1b).

Similar observations have been made with crystals of the enzyme: crystals grown in acid buf-

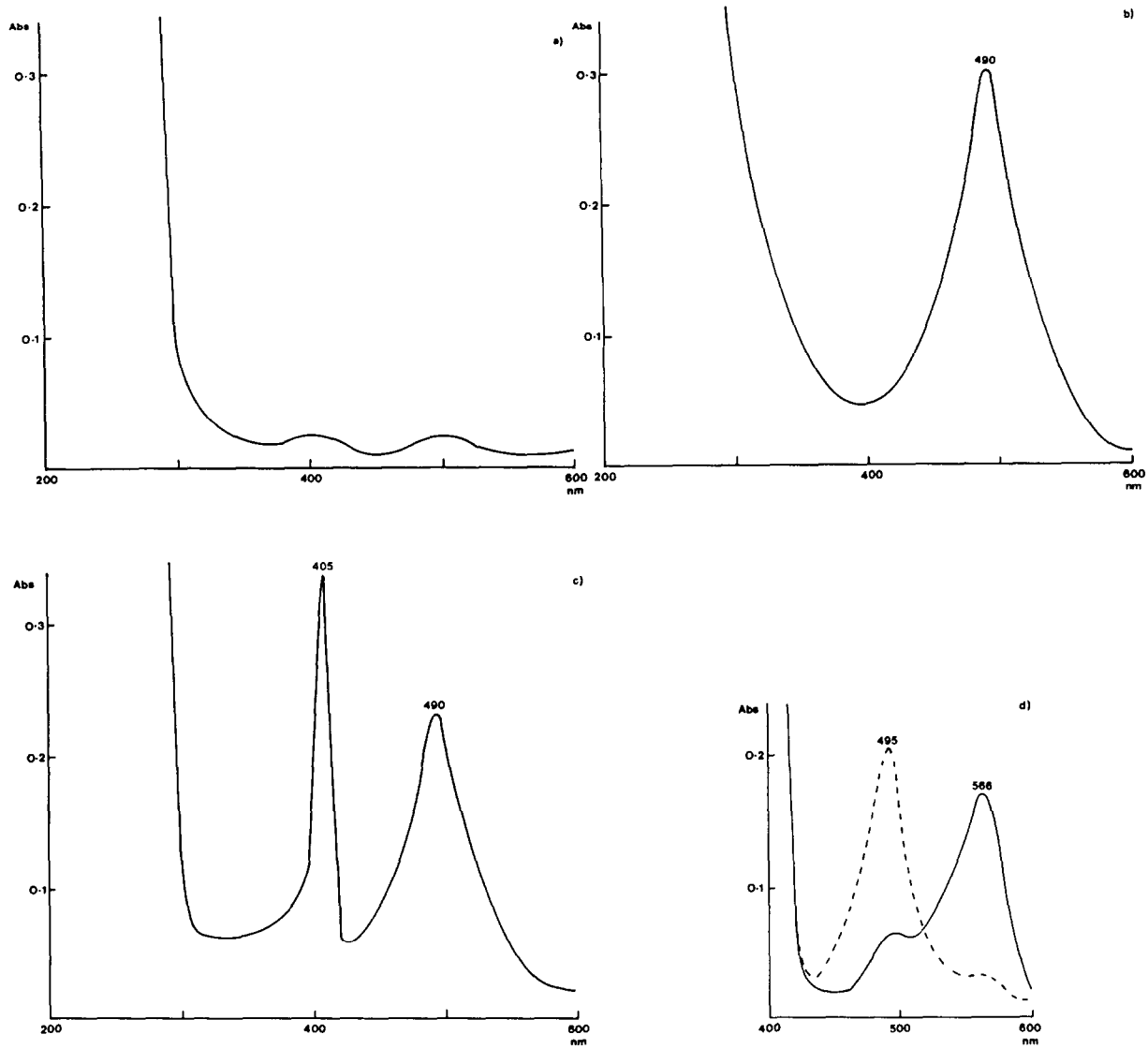


Fig.1. Spectra of porphobilinogen deaminase holoenzyme at (a) pH 8.0 in Tris-HCl buffer, (b) at pH 3.5 in acetate buffer, (c) after treatment with formic acid (10%, v/v), (d) after reaction with Ehrlich's reagent for 1 min (——) and 15 min (– – –).

fers are pink in colour, whereas those grown at higher pH are a pale straw colour. These findings suggested the presence of a prosthetic group which is protonated to give a pink chromophore under acid conditions but which at neutral pH is almost colourless (5).

When the holoenzyme was subjected to treatment with acid (10% HCOOH, v/v), over a period of an hour, a strong absorption maximum developed at 405 nm characteristic of a porphyrin and the peak at 490 nm declined (fig.1c). Under long wavelength ultraviolet light, this solution exhibited a strong pink fluorescence also indicative of the presence of a porphyrin. The nature of the porphyrin was unambiguously determined by HPLC using a reverse-phase C18 column developed with sodium phosphate buffer, pH 7.5, containing 4% (v/v) acetonitrile [16]. Comparison with authentic standards established that the porphyrin formed was uroporphyrin I (4). The

uroporphyrin I was not associated with the enzyme protein since after precipitation with trichloroacetic acid (10%, w/v) followed by centrifugation it remained in the supernatant.

It is well established that, under acid conditions, porphyrins are formed from pyrromethanes, first by their polymerization to porphyrinogens which are then rapidly oxidized to their respective porphyrins. Our observations are thus consistent with the presence of a dipyrromethane bound to the porphobilinogen deaminase. Furthermore, the ability to form uroporphyrinogen I establishes that the dipyrromethane is made up of pyrrole units similar in structure to that present in porphobilinogens (**1**).

3.3. *Properties of the bound prosthetic group*

The likelihood that we were observing a dipyrromethane prompted us to treat the holoenzyme with Ehrlich's reagent, which gives a characteristic colour reaction with such compounds [17]. The initial reaction of the enzyme with Ehrlich's reagent yielded an intense purple colour over a period of 30 s, with an absorption maximum at 566 nm. This colour quickly faded over a period of 15 min to an orange-yellow colour with a new absorption maximum at 495 nm. Such spectroscopic behaviour is also very strong evidence for the presence of a dipyrromethane [17]. The reaction with Ehrlich's reagent also demonstrates that the dipyrrole has a free α-position, the significance of which will be discussed below. From the foregoing information it is clear that the dipyrromethane is tightly bound to the porphobilinogen deaminase under both weakly basic or weakly acidic conditions. Stronger acid treatment leads to the liberation of the pyrromethane and its rapid polymerization.

In an attempt to isolate the dipyrromethane, the porphobilinogen deaminase was exhaustively digested with pronase. The digest was then separated by TLC under acidic conditions (butanol/acetic acid/water, 5 : 1 : 1, v/v). The compound migrated as a faint pink band (R_f 0.70) which reacted rapidly with Ehrlich's reagent to give an immediate purple colour changing to yellow-orange after 5 min. The behaviour of the isolated material with Ehrlich's reagent was therefore similar to that of the enzyme bound species.

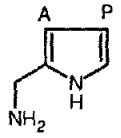
The oxidation state of the prosthetic group is likely to be that of a dipyrromethane rather than a dipyrromethene since prior reduction of the holoenzyme with I_2 not only inhibited the Ehrlich's reaction but also diminished the yield of porphyrin on treatment with formic acid.

3.4. *Mechanistic significance of the dipyrromethane*

The evidence described above points to the structure of the prosthetic group as being a tightly bound dipyrromethane (**5**) formed from two molecules of the substrate. In the presence of acid, we suggest that the enzyme bound dipyrromethane is cleaved and rapidly converted into uroporphyrinogen I (**4**). We believe that this dipyrrole represents part of the catalytic site of the porphobilinogen deaminase and that the tetrapyrrole product is assembled whilst bound covalently to the free α-position of the bound dipyrromethane through the sequence (**5**) $\longrightarrow$ (**6**) $\longrightarrow$ (**7**) $\longrightarrow$ (**8**) $\longrightarrow$ (**9**) $\longrightarrow$ (**2**).

A crucial test for this hypothesis is that the ES_2 complex (**7**) should be shown to contain a tetrapyrrole structure composed of the dipyrromethane from the holoenzyme linked to the dipyrromethane generated from the binding of two substrate molecules (rings a and b). Accordingly, the ES_2 complex was generated, purified by FPLC and treated with Ehrlich's reagent. A very rapid reaction occurred characteristic of a tetrapyrromethane [18]. This is similar to the profile shown in fig.1d except that the transition from 566 nm to 495 nm occurs about 10-times faster.

The existence of a bound tetrapyrrole was further substantiated by formic acid treatment of the ES_2 intermediate complex which gave twice the yield of uroporphyrin I and at a far more rapid rate than did the holoenzyme under the same conditions. We conclude therefore that treatment of the ES_2 complex with formic acid also releases pyrromethanes, which react spontaneously to yield uroporphyrinogen I. This lends further support to the suggestion above that the prosthetic group actually represents an active site dipyrromethane cofactor responsible for binding covalently the growing pyrrole chain. It is important to recognize that the dipyrromethane of the holoenzyme is not exchangeable with substrate under normal conditions of pH and that it is not released with hydrox-



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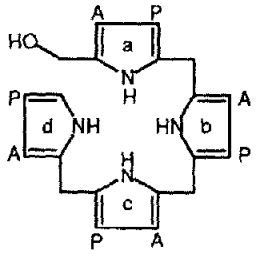

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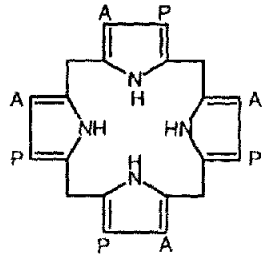

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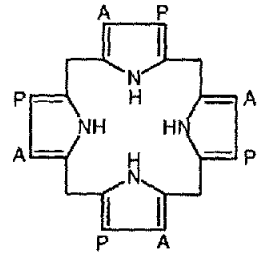

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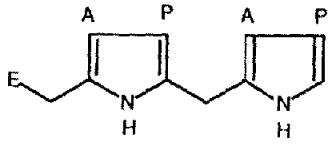

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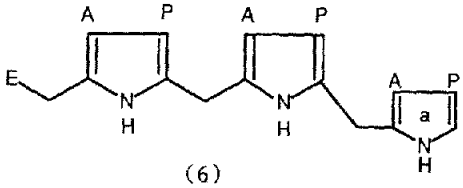

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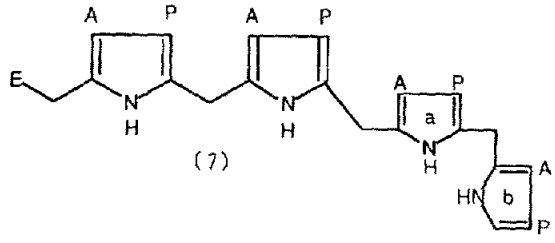

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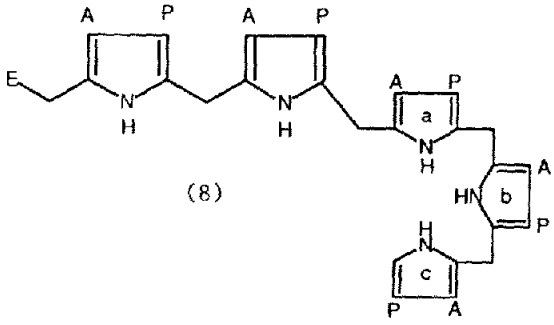

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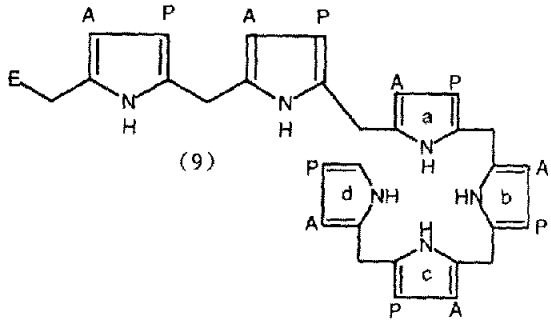

(9)

Scheme 1.

ylamine in sharp contrast to the bound substrates (rings a and b in structure **7**).

One as yet unanswered question relates to the mechanism by which the dipyrromethane cofactor is synthesised and whether this is catalysed by the deaminase molecule to which it is ultimately bound. A partial answer to this question has come from preliminary experiments in which bacteria were grown in the presence of 5-amino-[5-^{14}C]levulinic acid. This led to the exclusive labelling of the porphobilinogen deaminase with ^{14}C radioactivity, suggesting that the dipyrromethane cofactor is incorporated into the enzyme during the biosynthesis of the proteins. The ^{14}C-labelled enzyme does not release radioactivity when catalysing its reaction, however on treatment with formic acid as described above there is a stoichiometric release of ^{14}C radioactivity, accompanied by the formation of [^{14}C]uroporphyrin I.

It is hoped that the precise role of the dipyrromethane cofactor will be revealed when the X-ray structure of the enzyme is solved, nevertheless it is tempting to postulate that an active site dipyrromethane could serve as a 'moving arm' on which the growing tetrapyrrole can be covalently held as the sequential condensations with successive substrate molecules occur. The existence of such 'moving arms' is well substantiated in enzymes such as fatty acid synthase [19] and pyruvate dehydrogenase [20]. However, this is the first evidence to suggest a dipyrromethane cofactor as a means for manipulating the bound substrate at the active site of an enzyme.

ACKNOWLEDGEMENTS

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