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OBSERVATION OF ENZYME BOUND INTERMEDIATES IN THE BIOSYNTHESIS OF PREUROPORPHYRINOGEN BY PBG DEAMINASE

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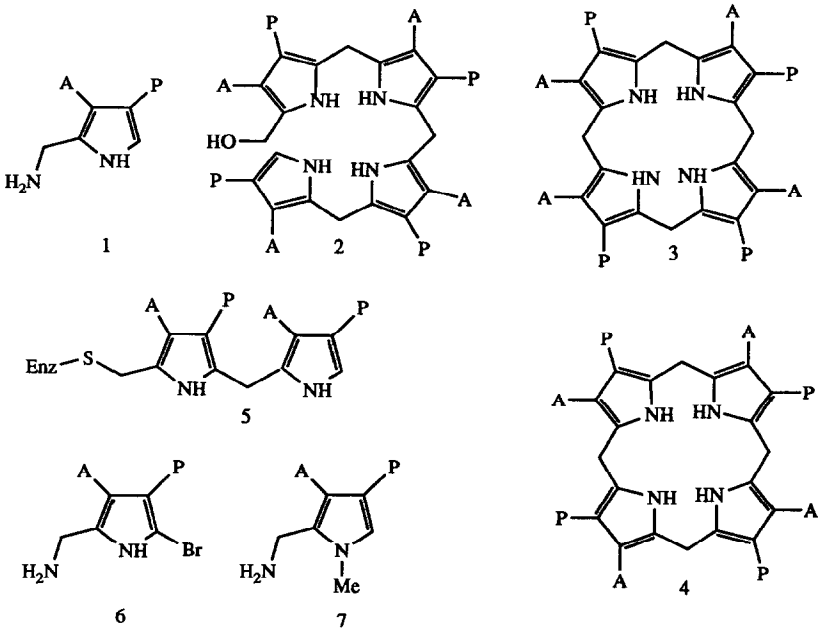
Abstract: Electrospray mass spectrometry was used to observe covalently bound enzyme-intermediate complexes during the catalytic assembly of the linear tetrapyrrole, preuroporphyrinogen, by the enzyme porphobilinogen deaminase.

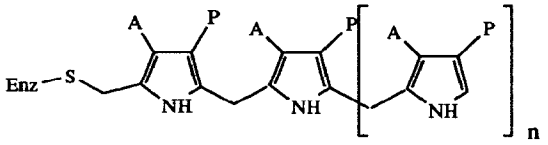
A key step in the biosynthesis of the porphyrins and modified tetrapyrroles is the assembly of four molecules of porphobilinogen (PBG, 1), catalysed by PBG deaminase (E.C. 4.3.1.8, hydroxymethylbilane synthase), to give preuroporphyrinogen (preuro'gen, 2), a linear hydroxymethylbilane.



Preuro'gen (2) is subsequently converted, by the action of urogen III synthase (cosynthetase), the suceeding enzyme in the pathway, into the cyclic tetrapyrrole, uroporphyrinogen III (3), the progenitor of haems, chlorophylls, correnoids, and related porphyrinoids. In the absence of the cosynthetase, HMB (2) cyclises chemically to uro'gen I (4).

PBG deaminase has been isolated from several sources and studies on the limited amounts of material available have demonstrated that a covalent bond is formed between substrate and the enzyme.[3] The assembly of the linear tetrapyrrole product occurs in an ordered stepwise sequence,[3c,4] involving isolable stable enzyme-substrate complexes (ES_1 - ES_4). The cloning of the gene (*hem*C) encoding for the *Escherichia coli* PBG deaminase[2] has enabled large amounts of the recombinant protein to be isolated and has permitted more extensive mechanistic investigations, which have resulted in the discovery that PBG deaminase contains an unusual dipyrromethane cofactor (5), derived from two PBG (1) molecules.[5] The cofactor acts as the attachment point for the growing oligopyrrolic chain within the active site cleft of the enzyme. Furher investigations, using n.m.r. revealed that the cofactor is attached to the enzyme through a sulphydryl linkage (cys-242) and that the enzyme itself is responsible for the attachment and synthesis of its own cofactor.[5c]

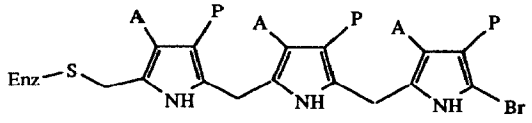
The most recent mechanism for the enzyme (E),[1c,6] postulates that there are two pyrrole binding sites. One for the incoming substrate molecule and the other for either the cofactor or preceding pyrrole. The binding of the first substrate (S) to the free α-position of the cofactor with concomitant loss of NH_3 forms the ES_1 complex. This process is repeated until the ES_4 complex is formed. With the *E. coli* enzyme, however, the ES_4 complex is not normally observed as it quickly dissociates, to preuro'gen (2) and free enzyme.



n = 1, ES_1
n = 2, ES_2
n = 3, ES_3
n = 4, ES_4

Electrospray mass spectrometry (ESMS) has recently been developed as a convenient and accurate technique for the determination of the molecular mass of proteins[7] and has been used to analyse both covalently inhibited enzymes and enzyme-substrate complexes.[8] Herein, we report the direct observation, by ESMS, of complexes formed with PBG deaminase and it's substrate and inhibitors.

The holo enzyme,[9] containing dipyrromethane cofactor, was studied initially. The analyses were carried out in methanol:water (1:1) containing 4% formic acid. The data revealed that the major species (>80%) had a molecular mass of 34271 (±3), in good accord with the predicted value for holo-PBG deaminase (*M* 34270*, Table 1). The experimentally determined molecular mass of the apo-protein[9] (*M* 33852) was also in agreement with the calculated value (*M* 33852).



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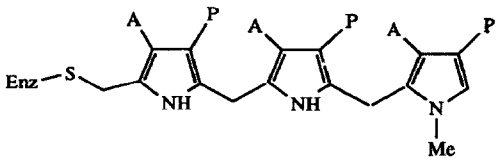

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We next examined the complexes formed between PBG deaminase and two irreversible inhibitors, 2-bromo-PBG (6)[5c] and N-methyl-PBG (7).[5d] The enzyme was incubated with the inhibitor (5equiv.) in 20mM TRIS.HCl (pH 8-8.5) for a period of up to thirty minutes and then then quenched with methanol:water (1:1) containing 4% formic acid to an apparent pH of ca 4-5. Electrospray analysis was carried out as before and in the case of (6) the formation of the inhibitor complex was essentially complete (>90%) after 30minutes. The data obtained were consistent with the irreversible formation of a 1:1 covalent

complex (8) between enzyme and inhibitor (6) (calculated for PBG deaminase + (6) - NH_3 = *M* 34558, found: *M* 34556±2). For inhibitor (7) it was possible to observe an adduct of *M* 34494±9, as a low abundance series (calculated for PBG deaminase + (7) - NH_3 = 34493), after incubation for 10 minutes followed by quenching with methanol:water (1:1) containing 4% formic acid, consistent with the formation of complex (9).

Incubation of PBG deaminase with it's substrate, PBG (1) gave very informative results. Thus, reaction of the enzyme (in 20mM TRIS.HCl, pH 8) with 5 equiv. of PBG for 10minutes at 4C, followed by quenching with methanol: water (1:1) containing 2% acetic acid (final concentration PBG deaminase: 25pmol/µl) and analysis by ESMS gave the results shown in Table 1 (Figure 1). It was possible to identify peaks corresponding to the enzyme-substrate complexes (ES_1 to ES_4), in addition to the 'resting' enzyme. After 30minutes the peak corresponding to ES_2 was the major species, fully consistent with prior reports that the latter is the most kinetically stable of the ES complexes[5c].

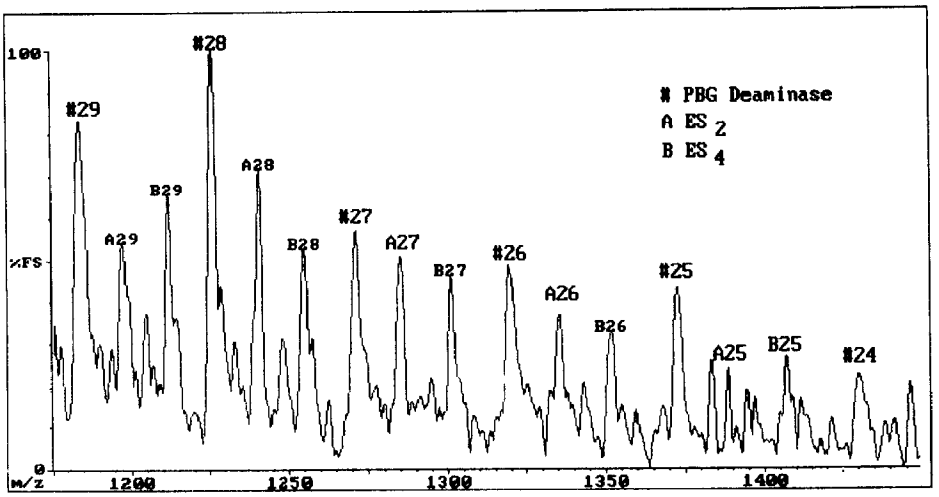

Figure1: Part of the electrospray mass spectrum of PBG deaminase after incubation with PBG (1). Details as for Table 1; # = PBG deaminase; A = ES_2; B = ES_4. Numbers refer to the charge state.

	Calculated*	Found	Approximate Relative Intensities
E	34270	34271	100
ES_1	34479	34477	27
ES_2	34688	34689	62
ES_3	34898	34902	31
ES_4	35107	35104	69

Table 1 ESMS analysis of an incubation of PBG deaminase with PBG (1) (5equiv.) at pH 8 for 10min, followed by quenching with methanol:water (1:1) containing 2% acetic acid. *Molecular weights were calculated using the average isotopic masses weighted by abundance, i.e. C = 12.011, H = 1.008, N = 14.007 etc. This corresponds to the centroid of the molecular ion distribution. The electrospray mass spectra were measured on a VG BIO Q triple quadrupole mass spectrometer equipped with an electrospray interface.

These experiments allow us to make several important conclusions: (i) the change in molecular weight from apo to holo enzyme, is consistent with the insertion of a dipyrromethane molecule; (ii) the inhibitors 2-bromo-PBG (6) and N-methyl-PBG (7) form covalent attachments to the enzyme; (iii) the enzymic reaction with the substrate PBG (1) proceeds via enzyme-intermediate complexes, containing one, two, three, or four pyrrole units.

This is the first demonstration of the ES4 complex with the *E. coli* wildtype enzyme. The stability of this species under the ESMS conditions is possibly due to the lowering of pH after initial reaction with substrate, since analysis was performed at higher pH failed to unequivocally identify an ES4 peak. A time course analysis (reaction at pH 8-8.5, then acid quench) indicated that the ES4 complex disappeared as the substrate was utilised. This study also provides further evidence as to the power of ESMS to investigate the mechanism of enzymic reactions which involve covalent intermediates.

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