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Reconstitution of apo-porphobilinogen deaminase: structural changes induced by cofactor binding

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Expression of porphobilinogen deaminase in a *hemB*$^-$ strain of *E. coli* has permitted the isolation of the apoenzyme, i.e. deaminase lacking the porphobilinogen-derived dipyrromethane cofactor. Incubation of purified apoenzyme with porphobilinogen resulted in reconstitution of the covalently attached dipyrromethane cofactor, indicating no additional cofactors or enzymes are required for biosynthesis of holoenzyme. Electrophoretic and ^{13}C-NMR spectroscopic analyses demonstrate that the apoenzyme exists in a conformationally unstable form which is converted to a highly stable tertiary structure on covalent attachment of the dipyrromethane cofactor.

Porphobilinogen deaminase; NMR spectroscopy, ^{13}C-; Dipyrromethane cofactor; Enzyme conformation; Reconstitution; (*E. coli*)

1. INTRODUCTION

Previous work from this laboratory has demonstrated that porphobilinogen deaminase (EC 4.3.1.8) catalyzes the polymerization of PBG (**1**) (fig.1) to preuroporphyrinogen (hydroxymethylbilane, HMB) (**2**) [1,2] which is rearranged and cyclized to the unsymmetrical uro'gen III (**3**) by the subsequent enzyme of tetrapyrrole biosynthesis, uro'gen III synthase (EC 4.2.1.75). In the absence of the latter enzyme, preuro'gen cyclizes chemically to afford uro'gen I (**4**) [3]. We have previously reported the construction of a plasmid bearing the *hemC* gene of *E. coli* which led to the over-expression of deaminase in *E. coli* [4]. The acquisition of substantial quantities of pure enzyme has allowed detailed NMR and genetic studies of the active site [5,6] which consists of a novel dipyrromethane cofactor, constituted from head to tail condensation of two molecules of the substrate PBG covalently bound at cysteine 242 [6]. The cofactor's α-free pyrrolic center ($\longrightarrow$) is the point of attachment of four succeeding substrate molecules ($ES_1 \longrightarrow ES_4$) during the synthesis of hydroxymethylbilane (**2**) (fig.1). We have also previously demonstrated that the apoenzyme of deaminase was expressed in a *hemB*$^-$ strain of *E. coli*. Since this mutant is unable to synthesize PBG, the expressed protein lacked the essential dipyrromethane cofactor.

In this paper we present evidence that the apoenzyme can be reconstituted to afford a holoenzyme with about 50% of the expected catalytic activity simply by incubation with PBG at pH 8.0, thereby proving that no additional cofactors or enzymes are required for attachment of the dipyrromethane cofactor to the apoenzyme during biosynthesis of the holoenzyme. It has also been possible to study the conformational states of apo- and holoenzyme

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Abbreviations: PBG, porphobilinogen; ALA, 5-aminolevulinic acid; uro'gen, uroporphyrinogen; PE buffer, phosphate-EDTA buffer (100 mM KH_2PO_4, 2 mM EDTA adjusted to pH 8.0 with NaOH); PAGE, polyacrylamide gel electrophoresis; ES_1, ES_2, ES_3, and ES_4, enzyme-substrate complexes in which PBG deaminase with the covalently attached dipyrromethane cofactor (E) has reacted with 1, 2, 3, or 4 mol PBG

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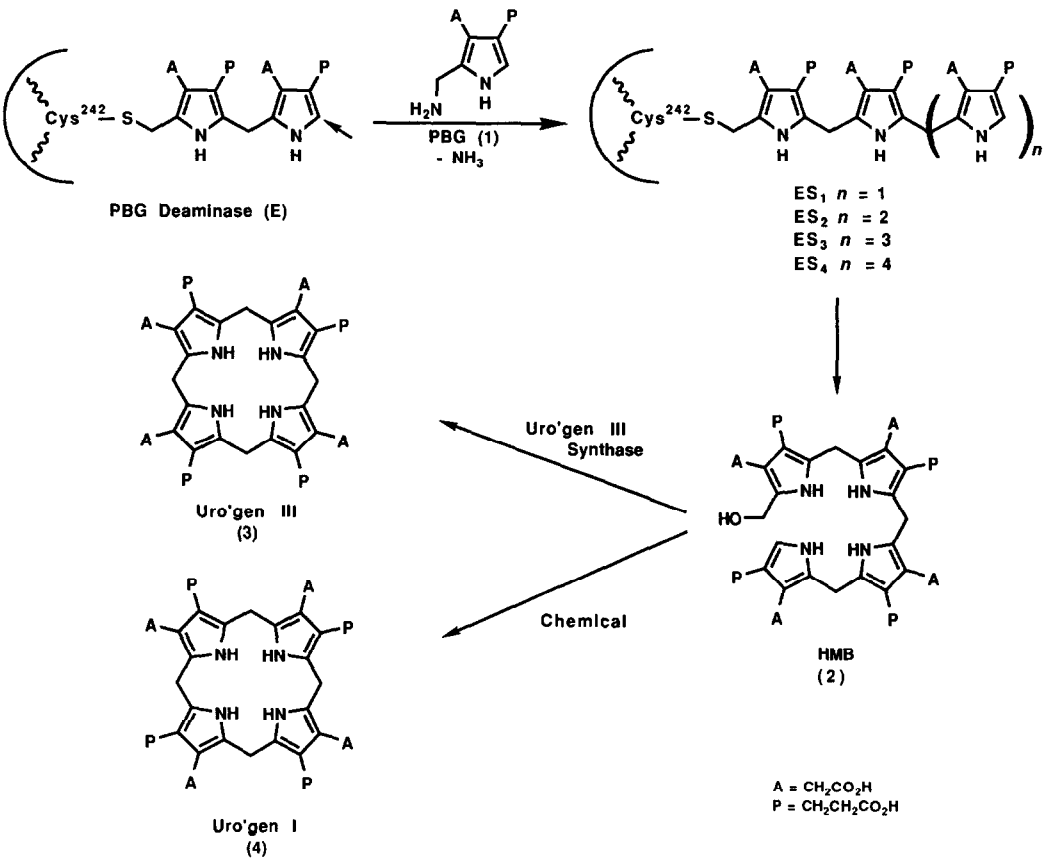

Fig.1. Biosynthesis of uroporphyrinogens catalyzed by PBG deaminase and uro'gen III synthase.

by biochemical techniques and by ^{13}C-NMR spectroscopy.

2. EXPERIMENTAL

2.1. *Expression and purification of holo- and apoenzyme*

Bacteria were grown on LB medium or LB agar plates containing 10 g of tryptone, 5 g of yeast extract and 5 g of sodium chloride per liter of water. Ampicillin, when required, was added at 50 μg/ml. All DNA manipulations were carried out by standard techniques [7]. TB1 is an rK$^-$ mK$^+$ derivative of JM83 used as a host for pUC plasmids [8]. RP523 is a *hemB*$^-$ (ALA dehydratase minus) derivative of *E. coli* C600 [9] that grows well on hemin and was provided by S. Cosloy. Plasmid pBG101 was constructed by ligation of a 1.6 kb *Bam*HI-*Sal*I restriction fragment from pLC41-04 [10] containing the *hemC* gene [11] into the *Bam*HI-*Sal*I restriction sites of pUC18. Colonies with the plasmid-containing insert were selected as white colonies on LB plates containing 100 μg/ml ampicillin and 40 μg/ml X-gal.

The holoenzyme was purified from TB1 transformed with pBG101 and the apoenzyme was purified from RP523 transformed with pBG101. The appropriate strain was inoculated (1.0 ml of a mid-log culture per liter) into 4 l of LB-ampicillin and incubated in a rotary shaker 16–18 h at 37°C. Cultures of RP523(pBG101) also contained hemin at 10 μg/ml. The cells were collected by centrifugation and suspended in 80 ml of PE buffer. The cells were lysed by sonication, heated to 60°C for 15 min, and the heat-treated lysate centrifuged at 10000 × *g* for 10 min. (The heat step was omitted in the purification of apoenzyme.) The supernatant was then fractionated with ammonium sulfate. The 35–55% ammonium sulfate fraction was dialyzed against PE buffer and applied to a DEAE-Sephacel column equilibrated with PE buffer. PBG deaminase was retained on this column, but on further washing with PE buffer, eventually eluted as a broad peak of >90% pure enzyme which was concentrated by ultrafiltration on a PM10 membrane and stored at 4°C in PE buffer containing 0.2 M NaCl. The enzyme was stable for several weeks when stored at concentrations of <1 mg/ml but was rapidly degraded when at concentrations of >10 mg/ml. For some purposes the proteolytic activity was removed by FPLC. The enzyme was dialyzed against 20 mM Tris-HCl, pH 8.0, and loaded onto a MonoQ column (Pharmacia). The protein was eluted from the column with a gradient of 0–0.4 M NaCl in 20 mM Tris-HCl, pH 8.0.

2.2. *PBG deaminase assay*

PBG deaminase activity was determined by following the consumption of PBG with Ehrlich's reagent (1 g of dimethylaminobenzaldehyde dissolved in 42 ml concentrated acetic acid, 8 ml of 70% perchloric acid, and 50 ml water) as previously described [12]. One unit of activity is defined as the amount of enzyme required for the consumption of 1.0 μmol PBG in 1 h. PBG deaminase activity in situ in native polyacrylamide gels was determined by incubating the gel for 20 min at 37°C in PE buffer (100 mM KH_2PO_4, 2 mM EDTA adjusted to pH 8.0 with NaOH), containing 0.2 mg/ml PBG followed by oxidation in 0.01% iodine in 1 N HCl and observation of the resulting fluorescent uroporphyrins on an ultraviolet transilluminator at 300 nm. Protein concentrations were determined with Bradford's reagent [13].

2.3. *Reconstitution of apoenzyme*

Apoenzyme was reconstituted by incubation of the purified enzyme (0.6 mg/ml) with a three-fold molar excess (0.05 mM) of PBG in 1.5 ml PE buffer at 4°C for a minimum of 4 h.

2.4. *Polyacrylamide gel electrophoresis*

Denaturing SDS-PAGE was performed as described [14]. Native (non-denaturing) PAGE was performed as for SDS-PAGE except the SDS was omitted and the samples were not treated with SDS, 2-mercaptoethanol or heat before loading. Urea-PAGE was performed as for native PAGE except the sample and gel contained 6 M urea. The gels were stained with a solution of 50% trichloroacetic acid containing 0.12% Coomassie brilliant blue R250 and destained with 7% acetic acid.

2.5. *Syntheses*

2-Aminomethylpyrrole (**5**) was synthesized from pyrrole-2-carboxaldehyde by formation of the corresponding oxime followed by hydrogenation [15]. Aminomethyldipyrromethane (**6**) was synthesized by coupling of benzyl-5-acetoxymethyl-3-(2-methoxycarbonylethyl)-4-methoxycarbonylmethylpyrrole-2-carboxylate (prepared by lead tetraacetate acetoxylation of the corresponding methyl pyrrole [16]) with porphobilinogen lactam methyl ester in boiling methanol containing toluene-*p*-sulphonic acid as a catalyst [17]. Hydrogenolysis of the product of the above reaction followed by decarboxylation and basic hydrolysis afforded the required aminomethyldipyrromethane.

2.6. *NMR spectroscopy*

^{13}C-NMR spectra were recorded at 125.76 MHz on a Bruker AM-500 spectrometer equipped with 5 mm dual probehead and Aspect 3000 computer. Spectra were recorded at ambient temperature (18–20°C) using a 60° pulse width, a repetition time of 0.5 s and an acquisition time of 0.3 s. Low power (1/4 W) proton decoupling was accomplished using the WALTZ-16 sequence available in the Bruker software. Exponential line broadening of 15 Hz was applied to the 16K data point FID prior to Fourier transformation. NMR samples were prepared by concentrating 10–15 mg aliquots (≈1 mg/ml in PE buffer, pH 8) of the DEAE purified apoenzyme to a volume of 0.3 ml (Amicon PM-10) at 5°C. D_2O (0.1 ml) was added and the solution microfuged for 1 min to remove fine particles before being transferred to the NMR tube. The apoenzyme which had been reconstituted as described above was prepared for NMR in a similar fashion.

3. RESULTS AND DISCUSSION

The PBG deaminase expression plasmid pBG101 was transformed into *E. coli* RP523, a strain that cannot synthesize PBG since it lacks the enzyme ALA dehydratase [9]. Deaminase expressed in this strain would, therefore, be expected to lack the PBG-derived dipyrromethane cofactor. The protein was purified to >90% by a slight modification of the procedure for the purification of the holoenzyme. The purified protein displayed no PBG deaminase activity when assayed for consumption of PBG or production of uroporphyrin. The purified protein also did not form the typical chromophore normally observed when the dipyrromethane cofactor of the holoenzyme reacts with Ehrlich's reagent [18], thus demonstrating the absence of the cofactor. That the purified protein was, indeed, the apoenzyme of PBG deaminase was demonstrated when it was reconstituted to a specific activity of about 20 units/mg (compared to a specific activity of about 40 units/mg typically seen with purified holoenzyme) by incubation of the purified protein with 3 molar equivalents of PBG at 4°C for 6 h. A similar treatment of crude cell lysates containing apoenzyme failed to reconstitute activity. Treatment of the apoenzyme with 6 M urea followed by slow removal of the urea prior to incubation with PBG had no effect on the efficiency of reconstitution nor did incubation with higher concentrations of PBG (80 molar equivalents).

As previously reported [4], the active holoenzyme, purified as described in section 2, displayed a single band of 33 kDa when analyzed by SDS-PAGE, but had multiple active bands when analyzed under native (non-denaturing) gel conditions (fig.2, lane 1). Incubation of the active enzyme with PBG was shown to result in the formation of a stable ES_2 complex consisting of holoenzyme containing two additional molecules of substrate [4]. The purified apoenzyme displayed a mobility equal to that of the holoenzyme when analyzed by SDS-PAGE. However, when analyzed under native gel conditions (fig.2, lane 2), the apoenzyme displayed no active bands at the position corresponding to the holoenzyme and had, in addition to a major protein band migrating slightly

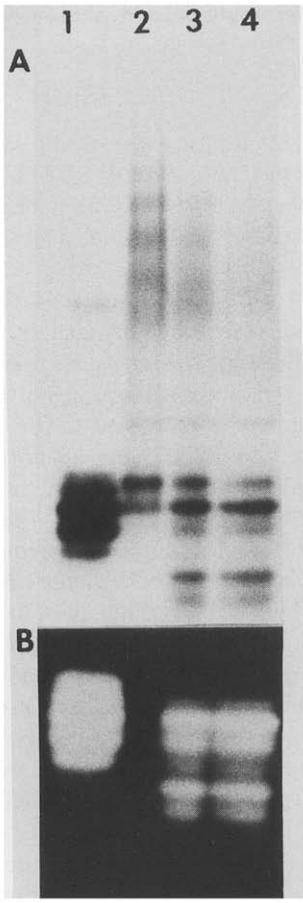

Fig.2. Native PAGE analysis of the reconstitution of PBG deaminase. Purified holoenzyme (lane 1), apoenzyme (lane 2), or apoenzyme that had been incubated for 6 h at 4°C with 3 molar equivalents of PBG (lanes 3 and 4, two different enzyme preparations) were analyzed on a native gel which was stained with Coomassie blue (A) after in situ detection of enzymatic activity (B). Each sample contained 24 μg of protein.

behind the holoenzyme, a ladder of bands of decreasing electrophoretic mobility that was not seen with the holoenzyme. Incubation of the purified apoenzyme with three molar equivalents of PBG at 4°C led to a reduction in the amount and number of these bands with the concomitant appearance of active bands at the positions corresponding to holoenzyme and ES_2 (fig.2, lane 3). When the ladder of bands observed on native gels of apoenzyme was excised from the gel and re-electrophoresed under denaturing conditions, the protein contained in each band comigrated with the purified apoenzyme demonstrating that the protein contained in these bands was deaminase. Analysis of the apoenzyme by gel-filtration chromatography (FPLC with a Superose 12 column) revealed a single peak with a retention time equal to that of the holoenzyme, thus indicating that the multiple bands are not due to aggregation of the protein into multimeric forms. The properties of apo- and holoenzyme were further compared by examining the effect of urea on their electrophoretic mobility. When examined by urea-PAGE, the average relative mobility of the bands seen with holoenzyme or reconstituted apoenzyme was the same as the average relative mobility of the bands seen with holoenzyme or reconstituted apoenzyme when electrophoresed under native conditions (M_r = 0.70). However, the ladder of bands that was observed on electrophoresis of the apoenzyme on native gels was not seen when the apoenzyme was examined by urea-PAGE. Instead, only three closely spaced bands of low relative mobility (average M_r = 0.14) were seen, presumably corresponding to apoenzyme completely unfolded by the presence of urea. These results suggest that the holoenzyme exists in a highly stable conformation since it is resistant to denaturation by 6 M urea. This high stability apparently is achieved on the addition of the dipyrromethane cofactor since the apoenzyme appears to exist in an unstable conformational state or states sensitive to denaturation by 6 M urea.

Differences in the conformational states of apo- and holoenzyme were further substantiated by NMR spectroscopy. ^{13}C-NMR analysis of the apoenzyme at pH 8 (fig.3A) revealed a spectrum containing sharp peaks reminiscent of the spectrum observed with the holoenzyme at pH 12 [5,6] indicating that the apoenzyme exists in a conformationally mobile state similar to the denatured state at pH 12. Apoenzyme reconstituted with PBG displayed, however, a ^{13}C-NMR spectrum (fig.3B) containing the broad peaks characteristic of native holoenzyme at pH 8.0.

In order to examine the specificity of the reconstitution process, the PBG analog, aminomethylpyrrole (**5**) (fig.4), and the cofactor analog, aminomethyldipyrromethane (**6**) (fig.4), were incubated with apoenzyme. Incubation of either analog with the purified protein under the same conditions used for reconstitution with PBG had no detectable effect on the banding pattern

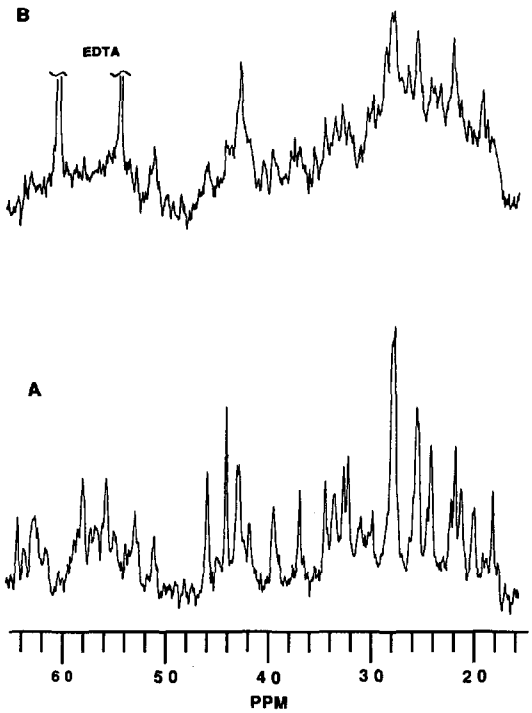

Fig.3. Aliphatic region of ^{13}C-NMR spectra of purified apoenzyme (A) and purified apoenzyme following reconstitution with PBG (B).

displayed by the apoenzyme on native PAGE. Enzyme activity, when assayed in situ for production of porphyrins, was reconstituted to less than 5% of that seen with PBG. These results indicate (i) the importance of the acetate and/or propionate side chain in the recognition of PBG and, (ii) that the cofactor is added to the enzyme by binding PBG one molecule at a time rather than forming the dipyrromethane prior to covalent attachment at Cys-242.

We have presented evidence that PBG deaminase exists in a conformationally unstable state after ribosomal synthesis but, in the presence of PBG, assumes the native tertiary structure, concomitant with formation of the dipyrromethane-thiol linkage at Cys-242, as is evidenced by the increase in its stability (resistance to thermal and urea denaturation) and by NMR spectroscopy (line width analysis). Furthermore, the conversion of apoenzyme to holoenzyme requires no additional cofactors or enzymes for deaminative attachment of 2 PBG units, head to tail, to the Cys-242 nucleophilic center at the active site. We further

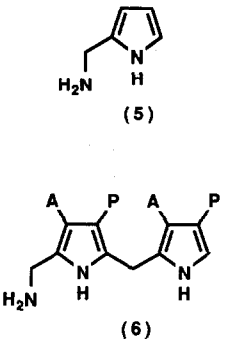

Fig.4. The structure of aminomethylpyrrole (5) and aminomethyldipyrromethane (6).

propose that the initial attachment of the first PBG unit to Cys-242 is a kinetically slow step, presumably guided by recognition of PBG's acetate and propionate side chains to the correct cysteine in a conformationally mobile active site. The relatively slow time course of reconstitution may, therefore, be a result of the apoenzyme existing in an equilibrium mixture of several conformational variants, only one of which is capable of binding PBG effectively enough to begin the reconstitution process. In the presence of excess

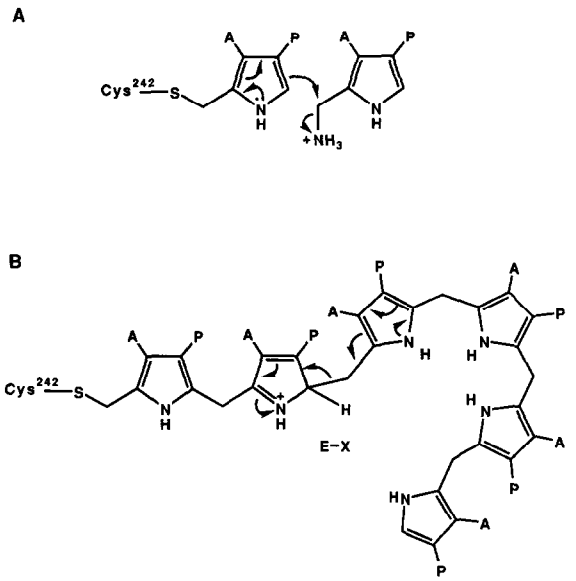

Fig.5. Proposed mechanisms for the formation of the dipyrromethane cofactor of PBG deaminase (A) and detachment of the tetrapyrrole following polymerization of 4 PBG units (B).

PBG, the binding of the second and subsequent PBGs occurs at a much faster rate, giving rise to the mixture of E, ES_1, and ES_2 complexes observed. We suggest that, under physiological conditions, these complexes are present at steady-state concentration but are decomposed on purification of the holoenzyme.

Indeed, the mechanism of the formation of the dipyrromethane cofactor (fig.5a) corresponds closely to the synthetic machinery employed by the holoenzyme to polymerize the succeeding 4 PBG units, after which an editing mechanism detaches the newly added tetrapyrrole as hydroxymethylbilane (**2**). This process is then repeated without transfer of either of the holoenzyme's cofactor pyrrole units to the discharged tetrapyrrole species. This remarkable mechanism for the detachment of the tetrapyrrole must depend on the regiospecific protonation of the second pyrrole of the cofactor (fig.5b). The fact that the enzyme always edits itself back to the dipyrromethane and not a monopyrrole suggests that this protonation is assisted by an active site residue which is adjacent to the second pyrrole unit, but out of hydrogen bonding distance to the other pyrrole units.

Reconstitution experiments with preparations of deaminase from which the dipyrromethane cofactor had been removed by strong acid treatment (1 M HCl) have recently shown [19] that ~40% of the activity could be restored by addition of PBG followed by isolation of the holoenzyme from the mixture of proteins.

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