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Accelerated Publications

Site-Directed Mutagenesis and High-Resolution NMR Spectroscopy of the Active Site of Porphobilinogen Deaminase†

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ABSTRACT: The active site of porphobilinogen (PBG)[1] deaminase (EC 4.3.1.8) from *Escherichia coli* has been found to contain an unusual dipyrromethane derived from four molecules of 5-aminolevulinic acid (ALA) covalently linked to Cys-242, one of the two cysteine residues conserved in *E. coli* and human deaminase. By use of a *hemA*⁻ strain of *E. coli* the enzyme was enriched from [5-^{13}C]ALA and examined by ^{1}H-detected multiple quantum coherence spectroscopy, which revealed all of the salient features of a dipyrromethane composed of two PBG units linked head to tail and terminating in a CH_2–S bond to a cysteine residue. Site-specific mutagenesis of Cys-99 and Cys-242, respectively, has shown that substitution of Ser for Cys-99 does not affect the enzymatic activity, whereas substitution of Ser for Cys-242 removes essentially all of the catalytic activity as measured by the conversion of the substrate PBG to uro'gen I. The NMR spectrum of the covalent complex of deaminase with the suicide inhibitor 2-bromo-[2,11-$^{13}C_2$]PBG reveals that the aminomethyl terminus of the inhibitor reacts with the enzyme's cofactor at the α-free pyrrole. NMR spectroscopy of the ES_2 complex confirmed a PBG-derived head-to-tail dipyrromethane attached to the α-free pyrrole position of the enzyme. A mechanistic rationale for deaminase is presented.

Previous work from this laboratory (Scott et al., 1988a,b) and elsewhere (Jordan & Warren, 1987; Hart et al., 1987) has defined the active site of PBG[1] deaminase to contain a dipyrromethane (DPM) cofactor linked covalently to the enzyme. It was further shown by ^{13}C NMR spectroscopy of deaminase biosynthetically enriched from [5-^{13}C]ALA that

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[1] Abbreviations: PBG, porphobilinogen; NMR, nuclear magnetic resonance; ALA, 5-aminolevulinic acid; HMB, (hydroxymethyl)bilane; DPM, dipyrromethane; LB, Luria broth; X-Gal, 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside; SDS, sodium dodecyl sulfate; PE, phosphate–EDTA; EDTA, ethylenediaminetetraacetic acid.

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the ^{13}C chemical shifts of the enriched enzyme were in full accord with those expected for partial structure **1**, where the DPM is covalently linked to a cysteinyl sulfhydryl group. Rigorous confirmation of the structure of the cofactor, its location on the enzyme, and the catalytic function of its unoccupied α-pyrrolic terminus are described in this paper. First it is shown that the ^1H NMR spectrum of ^{13}C-enriched deaminase edited by multiple quantum coherence spectroscopy reveals the chemical shifts of the key protons in the cofactor. Site-specific mutagenesis defines the site of covalent attachment of DPM as Cys-242. The suicide inhibitor 2-bromo-PBG reacts at the α-free pyrrole position of **1** (→) to give a covalent inhibitor complex whose ^{13}C NMR spectrum reveals the site of attachment of the growing oligopyrrolic chain leading to uroporphyrinogen I. The chain elongation process can be observed pari passu by ^{13}C NMR spectroscopy of the catalytically competent ES_2 complex.

Experimental Procedures

Strains and Construction of pBG101. All DNA manipulations were carried out by standard techniques (Maniatis et al., 1982). The bacterial strain *Escherichia coli* TB1 is an rK^- mK^+ derivative of JM83 used as a host for pUC plasmids (Baldwin et al., 1984). *E. coli* SASX41B is a *hemA*$^-$ mutant requiring ALA for growth and was provided by B. Bachman (Yale University). *E. coli* strains CJ 236 (*dut*$^-$, *ung*$^-$) and MV1190 (*dut*$^+$, *ung*$^+$) along with the vector pTZ18U were purchased from Bio-Rad. Plasmid pBG101 was constructed as previously described (Scott et al., 1988b) by ligation of a 1.6-kb *Bam*HI–*Sal*I restriction fragment from pLC41-04 (Clarke & Carbon, 1976) containing the *hemC* gene (Jordan et al., 1986) into the *Bam*HI–*Sal*I restriction sites of pUC18. Bacteria were grown at 37 °C on LB medium or LB agar plates containing 10 g of tryptone, 5 g of yeast extract, and 5 g of NaCl per liter of water. Additional supplements were added as noted. Ampicillin, when required, was added at 100 μg/mL.

Biochemical Methods. PBG deaminase activity was determined by following the consumption of PBG (Sigma) with Ehrlich's reagent [1 g of (dimethylamino)benzaldehyde dissolved in 42 mL of concentrated acetic acid, 8 mL of 70% perchloric acid, and 50 mL of water] as previously described (Mauzerall & Granick, 1956). PBG deaminase activity in situ in native polyacrylamide gels was determined by incubation of the gel for 20 min at 37 °C in PE buffer (100 mM KH_2PO_4 and 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 (Bradford, 1976). Sodium dodecyl sulfate and nondenaturing polyacrylamide gel electrophoresis were performed as previously described (Scott et al., 1988b).

Expression and Purification of PBG Deaminase. TB1 containing pBG101 was inoculated (10 mL of a mid-log culture/L) into 4 L of LB–ampicillin and incubated in a rotary shaker for 16–18 h at 37 °C. The cells were collected by centrifugation and suspended in 80 mL of PE buffer. The cells were lysed by sonication and heated to 60 °C for 15 min, and the heat-treated lysate was centrifuged at 10000*g* for 10 min. The supernatant was then fractionated with ammonium sulfate. The 35–55% ammonium sulfate pellet 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 and was stored at 4 °C in PE 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 0–0.3 M NaCl in 20 mM Tris-HCl, pH 8.0. Isotopically labeled enzyme was prepared by addition of either 5-amino-[5-^{13}C]levulinic acid (ALA, 100 mg/L) or [4-^{14}C]-ALA (30 mCi/100 mL, specific activity 50 mCi/mmol; Amersham) to the growth medium and purification of the enzyme as described above.

Site-Directed Mutagenesis. Site-directed mutagenesis was performed by the method of Kunkel et al. (1987) using a kit purchased from Bio-Rad. The *hemC* gene from pBG101 was subcloned into the vector pTZ18U to constuct pTZ18U:*hemC*, which was transformed into strain CJ236 (*dut*$^-$, *ung*$^-$) for the production of single-stranded phagemid DNA containing uracil. A 40-base oligonucleotide primer was synthesized which was designed to change the codon for amino acid 99 from TGT(Cys) to TCT(Ser) and to change the codon for amino acid 105 from CGC to CGG, eliminating one of the two *NruI* retriction enzyme sites found in the gene while conserving the amino acid (Arg). A 30-base primer was synthesized which was designed to change the codon for amino acid 242 from TGT(Cys) to TCT(Ser) and to change the codon for amino acid 244 from GTG to GTC, eliminating a *Ban*I restriction enzyme site in the gene while conserving the amino acid (Val). Isolation of single-stranded DNA, annealing of the primers, complementary strand synthesis, and transformation of the double-stranded DNA into strain MV1190 (*dut*$^+$, *ung*$^+$) were performed according to the directions provided by Bio-Rad. Plasmid DNA from transformed cells was screened by restriction analysis. Plasmids in which the *Nru*I or *Ban*I restriction enzyme sites had been eliminated were sequenced through the region of the primer to confirm the described base changes. *E. coli* strain MV1190 bearing pTZ18U:*hemC* or the mutagenized plasmids were grown for 18–24 h in 50 mL of LB medium containing ampicillin at 50 μg/mL. The cells were collected by centrifugation, suspended in 2.0 mL of PE buffer, and lysed by sonication. PBG deaminase activity in the lysates was assayed by measuring the amount of PBG consumed in 10 min as determined with Ehrlich's reagent. One unit is defined as the consumption of 1.0 μmol of PBG in 1 h.

Preparation of Porphobilinogen Deaminase Samples for NMR Spectroscopy. The sample of ^{13}C-labeled holoenzyme for the multiple quantum experiment was prepared from *E. coli* SASX41B (pBG101) grown on LB containing [5-^{13}C]-ALA (100 mg/L) as described previously (Scott et al., 1988a). The deaminase-containing DEAE-Sephacel fractions from the purification scheme described above were dialyzed extensively against 0.5 M NaCl in 0.1 M phosphate buffer, initially at pH 8 and then subsequently at pH 12, to remove the EDTA used throughout the initial purification. The ^{13}C-labeled enzyme was finally dialyzed against 2 mM NaCl/2 mM phosphate buffer (pH 12) and the sample lyophilized. The residue was repeatedly dissolved in D_2O and lyophilized to remove residual H_2O. The final NMR sample was prepared under argon in 0.3 mL D_2O (99.99 D atom %) to a final protein concentration of 2 mM.

Samples of deaminase for ^{13}C NMR spectroscopy of enzyme substrate–inhibitor complexes were prepared as follows from enzyme isolated from *E. coli* TB1 (pBG101) as described

above. Deaminase–ES_2 complexes were prepared by rapid mixing at 5 °C of a volume (20 mg of deaminase) of the DEAE-Sephacel stock with 2.2 equiv of [2,11-$^{13}C_2$]PBG dissolved in an equal volume of PE buffer (pH 8). The deaminase–PBG complex was allowed to stir for 30 min at 5 °C and then concentrated to a volume of 0.3 mL (PM10 membrane; Amicon). 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. After measurement of the pH 8 data, the pH of the sample was adjusted by brief dialysis against 0.1 M phosphate/NaOH buffer (pH 12) at 5 °C. In a similar fashion the ES_2 complex of ^{13}C-labeled deaminase (from the *hemA*$^-$ mutant described above) was prepared by using *unlabeled* PBG. The NMR sample of the bromo-PBG-inhibited enzyme complex was prepared in a similar manner. A solution of deaminase (20 mg) was reacted with a 5-fold excess of 2-bromo-[2,11-$^{13}C_2$]PBG for 30 min and then concentrated as described above. In this case the pH was adjusted to pH 12 by careful addition of 1 M NaOH directly to the NMR sample. The final deaminase concentration for ^{13}C NMR in all cases was approximately 1.5 mM.

Preparation of ^{13}C-Labeled Compounds. [5-^{13}C]ALA and [2,11-$^{13}C_2$]PBG were prepared as previously described (Evans et al., 1986). 2-Bromo-[2,11-$^{13}C_2$]PBG was prepared by bromination of [2,11-$^{13}C_2$]PBG according to the method suggested by Dr. G. Müller, Stuttgart (private communication).

NMR Spectroscopy. ^{13}C NMR spectra were recorded on either Bruker WM-300 wide-bore or AM-500 spectrometers, both equipped with 5-mm dual probe beads and Aspect 2000A computers. Spectra obtained at 125.76 MHz (AM-500) were recorded at ambient room temperature (18–20 °C) while the 75.46-MHz (WM-300) data were measured at approximately 10 °C, the temperature maintained by the VT-100 temperature controller. The actual sample temperature was estimated by previously determining the effect of decoupler heating with similiar samples and conditions and then adjusting the temperature controller correspondingly lower. Low-power ($^1/_4$ W) proton decoupling was accomplished by using the WALTZ-16 sequence available in the Bruker software. Approximately 60° pulse widths were employed with 0.3- and 0.5-S recycle delays for data recorded at pH 8 and pH 12, respectively. In general, 40 000–80 000 acquisitions were obtained with 15-Hz exponential line broadening applied to the 16K data point FID prior to Fourier transformation.

The proton-detected zero and double quantum coherence experiment was recorded on the WM-300 spectrometer operating at 300.13 MHz by using the pulse sequence suggested by Müller (1979) with the modified phase cycling suggested by Cavanaugh and Keller (1988). This pulse sequence was chosen over others because it contains the fewest delays and thus minimizes the loss of magnetization due to relaxation during the pulse sequence. The spectrometer was configured in the normal proton mode with ^{13}C pulses supplied via a Bruker BSV-3 selective power amplifier gated through the decoupler channel. A PTS-160 synthesizer operating at 75.46 MHz was used as the frequency source. A home-built 5-mm "inverse" probe head optimized for proton detection was employed with 90° pulse widths of 12.6 and 16.6 μs for the proton and carbon coils, respectively.

RESULTS

Proton-Detected Multiple Quantum Coherence Spectroscopy of PBG Deaminase Enriched from [5-^{13}C]ALA. Prophobilinogen (PBG) deaminase from *E. coli* is a monomeric enzyme of M_r 33 000. The relevant sections of the proton

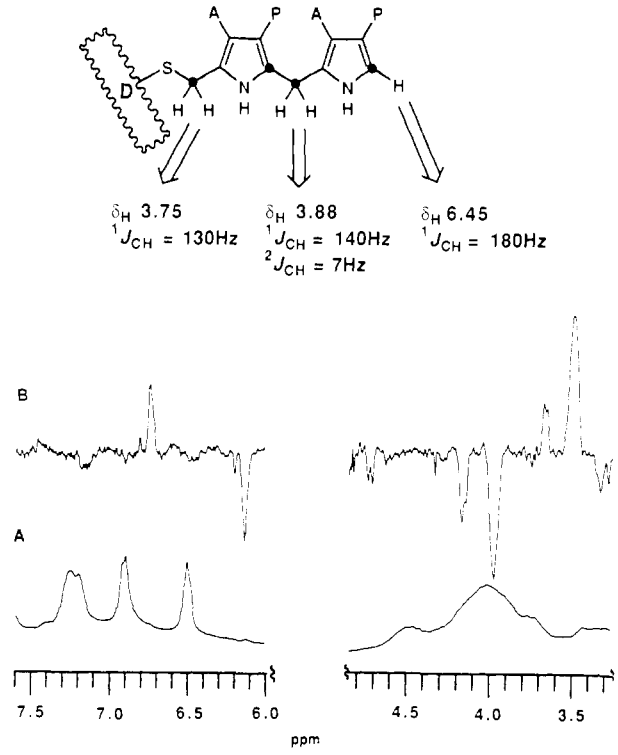
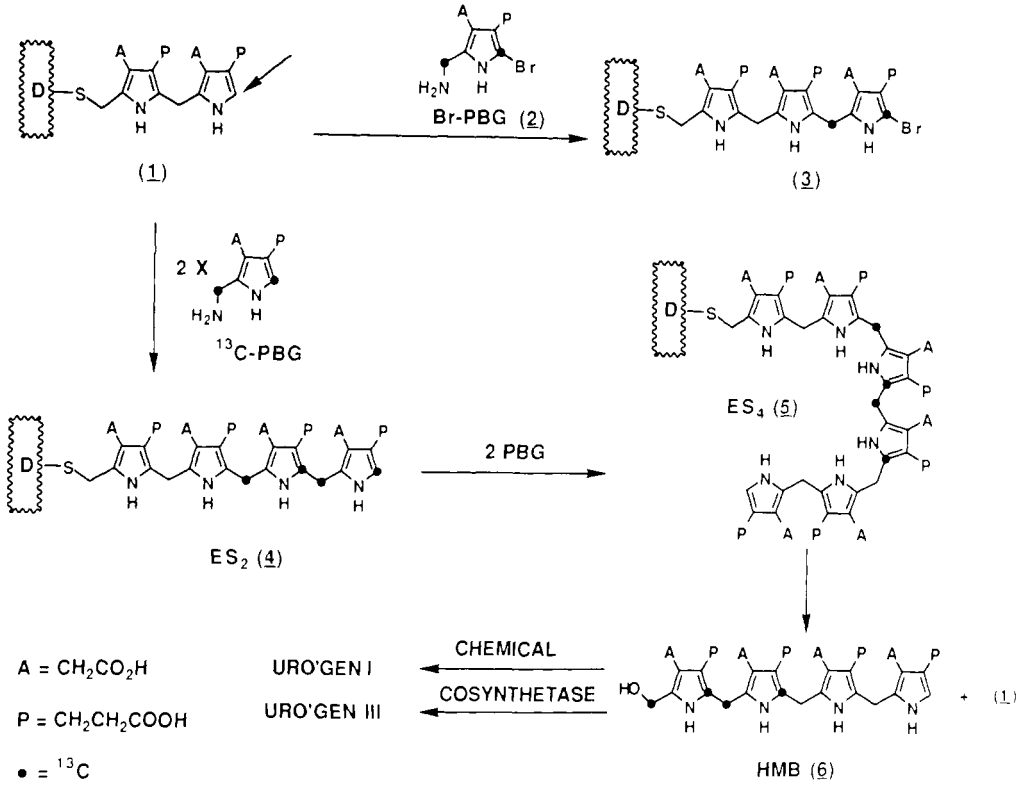


FIGURE 1: 300-MHz ^1H NMR spectra of PBG deaminase enriched from [5-^{13}C]ALA showing the relevant regions of (A) the normal proton spectrum obtained with presaturation of the residual water resonance referenced to δ 4.8 and (B) the proton-detected zero and double quantum coherence spectrum of same region containing the three proton signals originating from the biosynthetically enriched dipyrromethane cofactor. Conditions: 2.0 mM deaminase in 0.1 M phosphate buffer, pH 12, 20 °C Number of scans: (A) 384, (B) 6440.

NMR spectrum of ^{13}C-enriched PBG deaminase are shown in Figure 1A. As expected, on the basis of the DNA sequence (Thomas & Jordan, 1986), the spectrum is composed of broad, overlapping resonances resulting from ca. 1000 protons in the molecule conveying no clear structural features associated with ^{13}C enrichment. A two-dimensional NMR experiment of deaminase would be expected to sort the spectrum into distinct groupings of amino acids, but with substantial overlap in each grouping. The heteronuclear multiple quantum coherence experiment, as first suggested by Müller (1979) and applied to proteins by Wilde et al. (1986), offers the possibility of "editing out" the bulk proton signal, thereby allowing the observation of only those protons directly bound to a ^{13}C nucleus. Application of this technique to a sample of deaminase biosynthetically enriched with ^{13}C in its dipyrromethane cofactor resulted in the spectrum shown in Figure 1B. The edited spectrum in the region of interest now consists of pairs of antiphase doublets originating from the one-bond coupling ($^1J_{CH}$) to the directly attached ^{13}C nucleus. On the basis of the observed chemical shifts of a model dipyrromethane attached to sulfur (Scott et al., 1988a), the individual protons attached to the ^{13}C-enriched sites of the dipyrromethane cofactor can be readily assigned (Figure 1B). Thus the two pairs of antiphase doublets centered at d 3.75 ($^1J_{CH}$ = 130 Hz) and d 3.85 ($^1J_{CH}$ = 140 Hz) are assigned to the thio methylene (py-CH_2-SR) and the bridging methylene (py-CH_2-py), respectively, the latter confirmed by the observed long-range ($^3J_{CH}$ = 7 Hz) coupling to the neighboring ^{13}C (see Figure 1B). The terminal pyrrolic proton, the initial site of substrate binding, was observed at d 6.45 ($^1J_{CH}$ = 180 Hz).

^{13}C NMR Spectroscopy of PBG Deaminase Covalent Complexes. PBG deaminase catalyzes the stepwise polymerization of PBG, resulting in the formation of the linear

Scheme I



tetrapyrrole (hydroxymethyl)bilane (HMB). Previous work by this group (Scott et al., 1988a,b) and by Warren and Jordan (private communication) has indicated that the addition of two PBG units to deaminase (denoted as ES_2 complex) appears to be kinetically more favorable than the formation of ES_1, ES_3, or ES_4 complexes. Accordingly, the formation of the ES_2 complex with [2,11-$^{13}C_2$]PBG was chosen for NMR studies. The addition of 2.2 equiv of labeled PBG to deaminase at 5 °C resulted in the formation of the stable ES_2 complex, as demonstrated by ^{13}C NMR spectroscopy. The resulting difference spectrum, obtained via subtraction of the spectrum of an unlabeled deaminase sample prepared under identical conditions, is shown in Figure 2. At 10 °C, three signals are clearly evident both at pH 8 (Figure 2A) and at pH 12 (Figure 2B). At pH 8, the three resonances can be assigned to the α-substituted pyrrole (δ 127.8) and α-free pyrrole (δ 118.2) while the nearly equivalent methylene carbons (py-CH_2-py) are coalesced at 25.1 ppm. In addition to the characteristic line broadening displayed, the spectrum is devoid of signals originating from free PBG (δ NH_2-CH_2-py 35 ppm), indicating complete formation of the covalent complex **4** as shown in Scheme I.

Upon increasing the pH to 12, considerable sharpening of the resonances is evident (Figure 2B) and can be attributed to the unfolding of the protein at high pH. Both α-pyrrole carbons (α-substituted, δ 126.7 ppm; α-free, δ 116.0 ppm) as well as two superimposed methylene carbon resonances (δ 24.5) all have shifted slightly upfield. In addition, partial resolution of the methylenes is suggested by the upfield shoulder, which may be attributed to the methylene of the terminal PBG unit of ES_2 (**4**, Scheme I). This resonance would be expected to be broader and lower in intensity due to C–C coupling to the α-substituted pyrrole carbon at 126.7 ppm, which is similarly broadened.

^{13}C NMR Spectroscopy of Bromo-PBG-Inhibited Deaminase. The reaction of deaminase with the inhibitor 2-bromo-PBG has been shown to result in the formation of a relatively stable monocovalent enzyme–inhibitor complex (Scott et al., 1988a,b). Accordingly, a sample of deaminase was inhibited with 2-bromo-[2,11-$^{13}C_2$]PBG, immediately adjusted to pH 12 as described under Experimental Procedures, and subjected to NMR analysis. The difference spectrum recorded at 20 °C (Figure 3) displays two relatively sharp signals at 97.5 ppm (α-Br pyrrole) and 24.6 ppm [py-CH_2-py(Br)]. The complete conversion of the aminomethyl

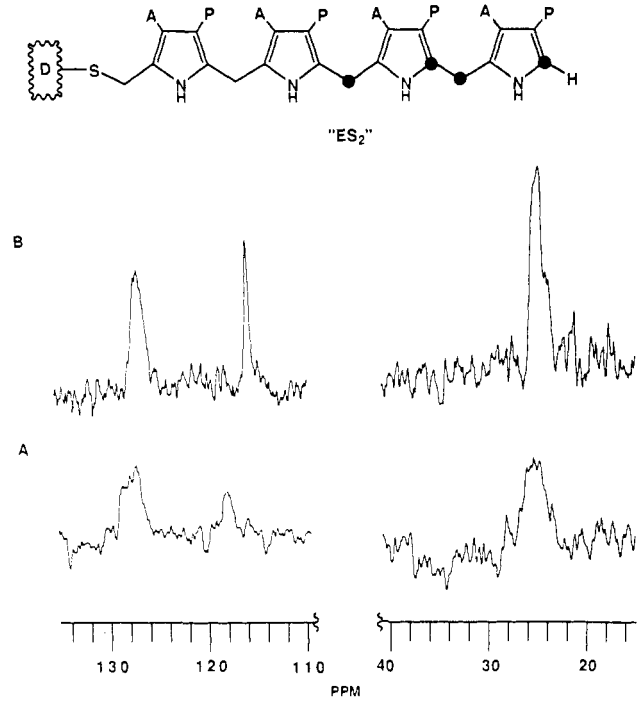


FIGURE 2: ^{1}H-decoupled 75-MHz ^{13}C NMR difference spectra of the PBG deaminase–ES_2 complex derived from incubation of 2.2 equiv of [2,11-$^{13}C_2$]PBG and deaminase. Conditions: 1.5 mM deaminase in 0.1 M phosphate buffer containing 2 mM EDTA, 10 °C; (A) pH 8; (B) pH 12.

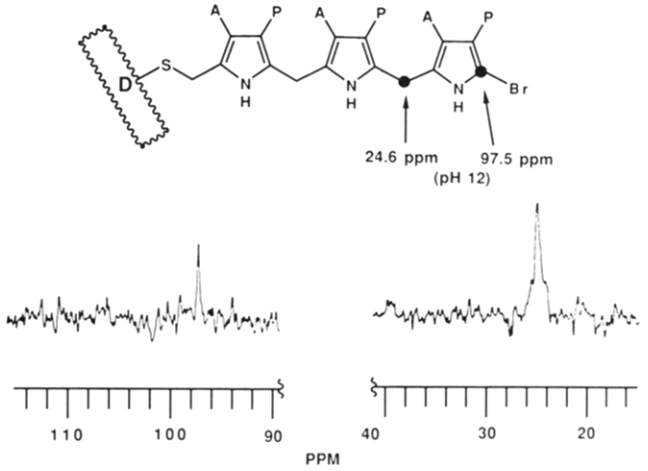


FIGURE 3: ^{1}H-decoupled 125-MHz ^{13}C NMR difference spectrum of deaminase inhibited with 2-bromo-[2,11-$^{13}C_2$]PBG. Conditions: 1.5 mM deaminase in 0.1 M phosphate buffer containing 2 mM EDTA, pH 12, 20 °C.

group of the inhibitor (δ 35 ppm) to a bridging methylene carbon characterized by a chemical shift of 24.6 ppm confirms covalent attachment, consonant with structure **3** (Scheme I).

^{13}C NMR of PBG Deaminase under Catalytic Conditions. Convincing evidence has been presented above for the dipyrromethane cofactor as the site of substrate attachment. However, direct spectroscopic evidence is lacking for the conversion of the α-free pyrrole carbon of the dipyrromethane cofactor to an α-substituted carbon on covalent attachment of PBG with loss of ammonia. A sample of ^{13}C-labeled deaminase was therefore prepared as described under Experimental Procedures and examined by ^{13}C NMR at 10 °C and pH 8. The difference spectrum (not shown) confirms the enrichment of the dipyrromethane cofactor as assigned (Scott et al., 1988a). Upon preparation and examination of the labeled ES_2 complex with *unlabeled* PBG at 10 °C, the signal at 118.3 ppm previously attributed to the α-free pyrrole carbon is no longer observed. Moreover, the signal at 129.7 ppm has now doubled in intensity, indicating that a second α-substituted pyrrole carbon has been formed. The disproportionation of the substrate and hence liberation of free enzyme were then effected simply by increasing the temperature of the sample directly in the NMR tube. Upon heating, the time-dependent return of the signal at 118 ppm was observed with concomitant production of uroporphyrinogen.

Site-Directed Mutagenesis of Residues Cys-99 and Cys-242. *E. coli* PBG deaminase contains four cysteine residues (Thomas & Jordan, 1986), two of which, Cys-99 and Cys-242, are conserved in the human enzyme (Raich et al., 1986). With the foregoing identification of an active site sulfhydryl group involved with attachment of the dipyrromethane cofactor, residues Cys-99 and Cys-242 were therefore targeted for site-directed mutagenesis. Accordingly, the *hemC* gene was subcloned into the vector pTZ18U and overexpressed from *E. coli* MV1190. The level of deaminase overexpression was 50–100-fold, which was 2–4-fold less than that previously reported for the expression of the *hemC* gene cloned into the vector pUC18 (Scott et al., 1988b).

Site-directed mutagenesis of the *hemC* gene which led to the alteration of amino acid residue Cys-99 to Ser-99 had little effect on the specific activity of lysates of *E. coli* MV1190 bearing the mutagenized plasmid (Table I). However, a similar alteration of amino acid Cys-242 to Ser-242 reduced the activity of the lysates to levels undetectable under the conditions of the assay. Further convincing evidence toward

Table I: Specific Activity of PBG Deaminase in Lysates of *E. coli* MV1190 Bearing Wild-Type pTZ18U:*hemC* or Mutagenized Plasmids

plasmid	sp act. (units/mg)
pTZ18U:*hemC* (Cys-99, Cys-242 = wild type)	3.8
pTZ18U:*hemC* (Ser-99)	3.4
pTZ18U:*hemC* (Ser-242)	0.0[a]

[a] No activity detectable with Ehrlich's reagent after a 10-min incubation with ten times more protein than used for determining the specific activity of the wild-type and Ser-99 lysates.

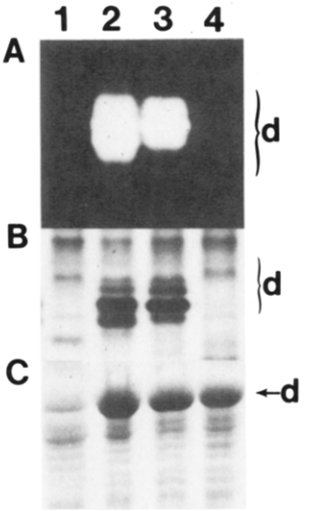
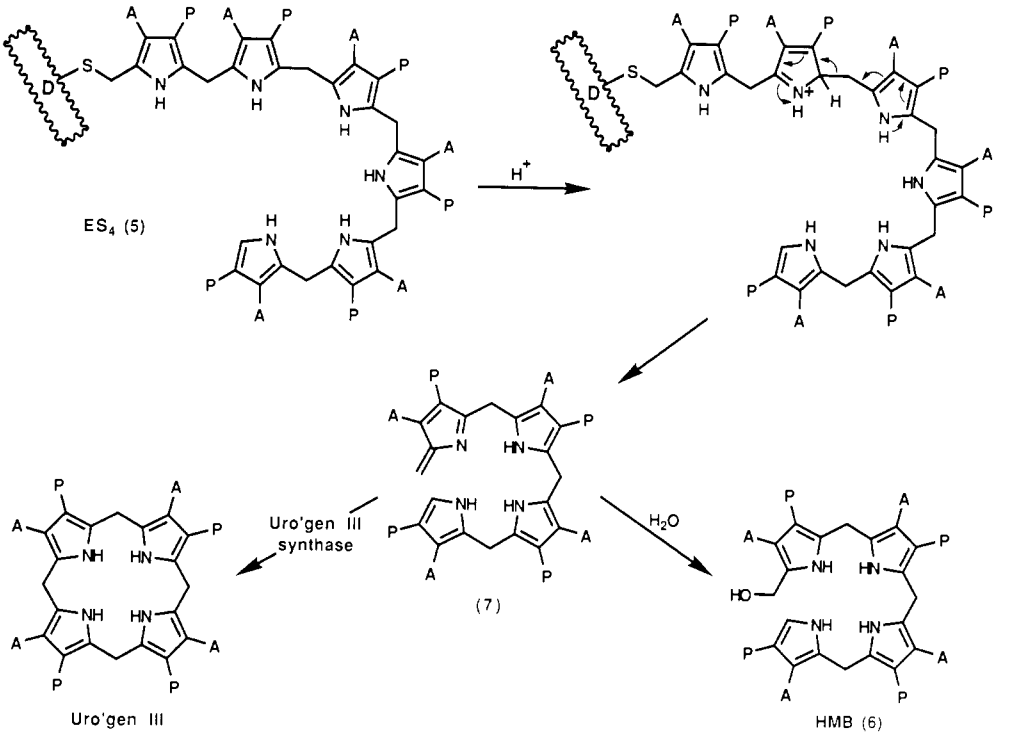


FIGURE 4: Polyacrylamide gel electrophoretic analysis of lysates of *E. coli* MV1190 containing the cloning vector (pTZ18U, lane 1), the vector with the wild-type *hemC* gene (pTZ18U:*hemC* Cys-99, Cys-242, lane 2), and the vector with mutant *hemC* genes (pTZ18U:*hemC* Ser-99, lane 3, or pTZ18U:*hemC* Ser-242, lane 4). The lysates (100 μg of total protein) were electrophoresed under nondenaturing conditions (A and B) or denaturing conditions (C) and stained for PBG deaminase activity (A) or with Coomassie Blue (B and C). PBG deaminase bands are marked with a d.

the role of Cys-242 in catalysis was obtained via a comparison of the three enzymes on polyacrylamide gel electrophoresis.

As previously reported (Scott et al., 1988b), when subjected to nondenaturing polyacrylamide gel electrophoresis, purified deaminase displays multiple forms, all of which are catalytically competent when assayed for fluorescent uroporphyrins. Analysis of the above MV1190 lysates under nondenaturing conditions followed by I_2 staining for the production of uroporphyrins (Figure 4A) or Coomassie Blue staining (Figure 4B) confirmed the lack of detectable PBG deaminase activity in the Ser-242 lysates (lane 4, Figure 4A). Furthermore, both the wild-type and Ser-99 lysates displayed multiple forms of the enzyme (lanes 2 and 3, Figure 4B) while no obvious protein bands corresponding to PBG deaminase were seen in the Ser-242 lysates (lane 4, Figure 4B). However, analysis of the lysates by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (Figure 4C) demonstrated in all three lysates approximately equal amounts of protein in the band associated with PBG deaminase. A similar loss of the multiple banding pattern displayed by deaminase has previously been observed by us with PBG deaminase synthesized in a *hemB*$^{-}$ strain of *E. coli* that does not synthesize PBG and, therefore, produces deaminase lacking the dipyrromethane cofactor (Scott et al., 1988b). Final confirmation of Cys-242 being responsible for the binding of the dipyrromethane cofactor came from radioisotopic feeding experiments. Gel electrophoresis of the above *E. coli* MV1190 lysates grown in the presence of [4-^{14}C]ALA indicated that both the wild-type and Ser-99 forms

Scheme II



of deaminase equally incorporated radioactivity into the multiple bands of protein displayed under nondenaturing conditions (data not shown). The Ser-242 lysate however, in addition to not displaying the multiple banding pattern, was clearly devoid of radioactivity. These results suggest that the loss of activity in the Ser-242 mutant is not due to reduced expression of the protein but rather due to the inability of the protein to incorporate the dipyrromethane cofactor required for activity. On the basis of these results, we predict the cysteine at amino acid residue 242 of PBG deaminase to be the position of attachment of the dipyrromethane cofactor bound in the active site of the enzyme.

Discussion

Previous NMR studies of the active site of PBG deaminase were hindered by line-broadening problems associated with measurment of the spectra of the enzyme substrate complexes corresponding to succesive addition of up to 3 mol of PBG to a nucleophilic center in the enzyme. Thus, while a Cambridge group favored a lysine residue as the nucleophile, on the basis of ^{13}C NMR data (Battersby et al., 1979; Hart et al., 1984), a study using ^{3}H NMR (Evans et al. 1986) suggested a cysteinyl sulfhydryl as the site of attachment of the growing oligopyrrolic chain of PBG units to form the ES_1–ES_4 covalent complexes. Recent work with biosynthetically enriched enzyme using gentically engineered *E. coli* TBI (pBG101) to overproduce deaminase (Scott et al., 1988a,b; Jordan et al., 1988) has allowed the acquisition of sufficient quantities of deaminase to address both the structural and mechanistic problems posed by this enzyme, whose function is to assemble the linear tetrapyrrole HMB, which in turn becomes the substrate for uro'gen III synthase (Scheme I) [for a review, see Scott (1987)]. Thus, examination of the ^{13}C NMR difference spectrum of deaminase enriched in its active site from [5-^{13}C]ALA led to the proposal (Scott et al., 1988a,b; Scheme I) that a dipyrromethane formed from two PBG molecules constituted a unique cofactor which is linked to a cysteine residue of deaminase and which conserves an α-free pyrrolic position (→; Scheme I) as the nucleophilic center for attachment of each incoming PBG molecule with loss of ammonia during the buildup of the enzyme substrate complexes $ES_1 \rightarrow ES_4$.

In this paper we have extended the structural analysis to include ^{1}H NMR spectroscopy of the active site and ^{13}C NMR spectroscopy of the catalytic cycle, interrupted both at the stage of the ES_2 complex and in the complex with the suicide inhibitor 2-bromo-PBG. It has also been possible to define the location of the cysteine residue to which the cofactor is attached by site-specific mutagenesis.

The technique of proton-detected multiple quantrum coherence spectroscopy in which only those protons attached to the biosynthetically enriched carbons of **1** are observed not only has confirmed the head-to-tail arrangement in the dipyrromethane cofactor **1** (see Figure 1 for labeling pattern) but also conclusively shows, on the basis of a chemical shift of δ 3.75, that the CH_2 of the dipyrromethane is attached to sulfur, since the corresponding signal (CH_2–S) in a number of synthetic model pyrroles (Scott et al., 1988a) falls in the range 3.72–3.78 ppm. The power of this method is clearly revealed by comparing the normal ^{1}H spectrum (Figure 1A) with the edited spectrum (Figure 1B), which shows only those protons attached to ^{13}C-enriched carbons. The application of this edited ^{1}H NMR pulse sequence to ^{13}C-enriched proteins would appear to have general utility (Wilde et al., 1986), especially where unusual active site residues are present.

The property of the *E. coli* deaminase to form stable, catalytically competent adducts with up to 3 molar equiv of substrate covalently bound to the active site was exploited in order to examine the structure of the most stable of these complexes, viz., the ES_2 species **4**. As can be seen from Figure 2, four resonances can be observed in the ^{13}C NMR spectrum of the ES_2 complex prepared from 2 equiv of [2,11-$^{13}C_2$]PBG. The sp^2 centers in the complex can be assigned to substituted (δ 126.7) and α-free pyrrolic (δ 116) carbons. The chemical shifts of the two methylene signals overlapping at δ 24.5 ppm are attributed to py-CH_2-py, which confirms the point of attachment of the growing oligopyrrolic chain at the α-position (→) in **1**. Strong supporting evidence for the above structural

proposals is provided by two further sets of ^{13}C NMR experiments. In the first of these, a suicide inhibitor of deaminase, 2-bromo-PBG, was synthesized as the 2,11-$^{13}C_2$ isotopomer, which retains the aminomethyl terminus necessary for covalent attachment to position (→) in **1** (Scheme I) and has the α-pyrrolic position substituted with bromine, thereby rendering the "ES_1" complex incapable of further elaboration to di-, tri-, etc. pyrrolic chains. The site of attachment of the aminomethyl group via loss of ammonia is revealed by the familiar chemical shift of δ 24.5 ppm in the inhibitor complex **3** (Figure 3).

As final proof of the reactivity of the α-pyrrolic carbon of the dipyrromethane cofactor, a sample of ^{13}C-enriched deaminase labeled as in Figure 1 was converted to the ES_2 adduct **4** with *unlabeled* PBG (2.2 equiv) and examined by ^{13}C NMR difference spectroscopy. The signal for the original α-pyrrole center (δ 118.3) has been replaced by a new resonance at δ 129.7 ppm characteristic of a CH_2-substituted quaternary pyrrolic carbon overlapping the signal at 129 ppm already present in the labeled active site, which is assigned to the α-substitued carbon of the first pyrrole unit (see labeling pattern in Figure 1). The previous assignment of sulfhydryl for the nucleophilic center (Evans et al., 1986) must therefore have been incorrectly assigned due to considerable line broadening of the ^3H spectrum. Similarly, the attribution since withdrawn (Hart et al., 1987) of ϵ-NH_2 from a lysine residue as the nucleophile (Battersby et al., 1979; Hart et al., 1984) probably reflects a poor ^{13}C NMR spectrum of an impure specimen of the ES_1 complex and its products of proteolytic digestion.

With these structural features firmly established, it remained for us to identify which of the two cysteine residues (Cys-99 or Cys-242) conserved in *E. coli* (Thomas & Jordan, 1986) and human (Raich et al., 1986) deaminase is attached to the dipyrromethane cofactor. As discussed under Results, substitution of serine for each of these cysteine residues in turn followed by enzymatic assay of the lysates from the transformed *E. coli* strains revealed that alteration of cysteine-242 abolished the enzymatic activity, while the same substitution of serine for Cys-99 left the activity unaltered, within experimental error. Furthermore, the loss of activity associated with the Ser-242 mutant is a direct consequence of the failure to incorporate the dipyrromethane cofactor into the active site.

The NMR data discussed above contain the necessary information to prove beyond doubt the structure of the dipyrromethane at the active site and also to propose a mechanism of tetrapyrrole assembly, in which the nucleophilicity of the α-pyrrolic position in the cofactor, and in the succeeding $ES_1 \rightarrow ES_3$ complexes, is used for each elongation step. We suggest that, on reaching the ES_4 complex (Scheme II), the tetrapyrrolic fragment is cleaved regiospecifically by a reversal of the mechanism that mediates the chain propagation to release preuro'gen (HMB; **6**) with regeneration of the enzyme (**1**). Although HMB (**6**) is used efficiently as a chemical substrate for uro'gen I sythesis and as a biochemical substrate for uro'gen III synthetase (cosynthetase), the actual species discharged from the enzyme would appear most likely, on mechanistic grounds, to be the azafulvene **7** formed by the mechanism shown in Scheme II; i.e., the true substrate for cosynthetase is the unstable species **7**.[2]

[2] For these reasons and those recently adumbrated (Rosê et al., 1988), we suggest that the name recommended by IUB [(hydroxymethyl)bilane synthase] for the enzyme be restored to the original name, porphobilinogen deaminase, at least until the true biochemical substrate for uro'gen III is firmly established.

Added in Proof

Since submitting this manuscript, the two remaining cysteine residues, Cys-134 and Cys-205, have separately been altered to serine without measurable loss of deaminase activity, confirming the site of cofactor attachment at Cys-242.

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Registry No. PBG deaminase, 9074-91-3; L-Cys, 52-90-4.

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