Biosynthesis of Porphyrins and Corrins. 2. Isolation, Purification, and NMR Investigations of the Porphobilinogen–Deaminase Covalent Complex†

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ABSTRACT: The procedures for the generation of enzyme–substrate complexes from labeled porphobilinogens ([2,11-^{13}C]PBG and [2,6,11-^{3}H]PBG) with deaminase and the methods employed for their purification are described. Use of ^{13}C NMR failed to detect the substrate bound to the enzyme, suggesting that the line width must be inordinately large. The complex was found to disproportionate with time when stored at 25 °C. However, enzyme-bound uroporphyrinogen I (uro'gen I) was detected, both in the intact protein and in the oligopeptides from tryptic digestion and peptide mapping. The first detection of an enzyme–substrate complex by ^{3}H NMR is described for [^{3}H]PBG and deaminase. The line widths of the observed resonances were found to be extremely large and dependent upon temperature, giving chemical shifts that suggest the involvement of a sulfhydryl group as the nucleophilic enzyme group that binds the substrate. The catalytic competence of this complex was also demonstrated by displacing bound [^{3}H]PBG with unlabeled PBG. During the resultant formation of [^{3}H]uro'gen I, a transient low-intensity signal was detected that has been tentatively assigned to the highly reactive azafulvene species, proposed in several mechanistic schemes for porphyrin biosynthesis.

The enzyme porphobilinogen (PBG)[1] deaminase (EC 4.3.1.8)[2] catalyzes the head-to-tail condensation of 4 mol of the monopyrrole porphobilinogen (PBG, **1**) to preuroporphyrinogen (pre-uro'gen), whose release and stabilization as (hydroxymethyl)bilane (HMB, **7**) has been the subject of extensive investigation [see preceding paper (Evans et al., 1986)]. HMB may cyclize chemically to uro'gen I or serve as the substrate for uro'gen III cosynthase (EC 4.2.1.75) to form uro'gen III (Scheme I).

The mechanism by which PBG deaminase polymerizes PBG and the structure of the true product of the enzyme have been the subject of much speculation [see preceding paper and reviewed in Buckley (1977) and Akhtar & Jordan (1978)] but to date has remained unclear. The order of assembly of the four units was investigated independently by Battersby et al. (1979a,b, 1983a,b), Jordan and Seehra (1979), and Seehra and Jordan (1980). Both groups established that the order of assembly is the expected one: ring A, followed by rings B, C, and D. The discovery by Anderson and Desnick (1980) that human deaminase forms stable covalent complexes bearing up to four condensed PBG units stimulated studies on the enzyme–substrate (ES) complexes from *Rhodopseudomonas spheroides* (Jordan & Berry, 1981; Berry & Jordan, 1981; Berry et al., 1981), *Euglena gracilis* (Williams et al., 1981; Battersby et al., 1983a,b), and rat spleen (Williams, 1984). Studies on the *R. spheroides* ES complex by Jordan and Berry (1981) suggested that the substrate is covalently attached to the enzyme, and further work by Berry and Jordan (1981) and Berry et al. (1981) identified the existence of four bands by gel electrophoresis. Using [^{14}C]PBG, Berry et al. elegantly showed that the bands corresponded to native enzyme and ES complexes bearing one, two, and three bound PBG units. Interestingly, unlike the human enzyme, no complex corresponding to bound tetrapyrrole was detected.

The identification and characterization of such ES complexes and the discovery of their unusual stability[3] suggest that it may be possible to probe the structure of the active site and the nature of binding of the substrate. A suitable technique for this purpose is NMR spectroscopy, and in particular ^{13}C NMR spectroscopy, the power of which has already been established [reviewed in Mackenzie et al. (1984)] in defining the structure and stereochemistry of enzyme–inhibitor and enzyme–substrate complexes with proteases of modest molecular weight (<30 000). In this paper, we describe the use of ^{13}C NMR and tritium (^{3}H) NMR spectroscopy in an attempt to elucidate the structure and mechanism of PBG deaminase.

Experimental Procedures

Materials. All reagents employed were of the highest grade obtainable. [^{14}C]- and [^{13}C]PBG were prepared by the methods outlined in the preceding paper. [^{3}H]PBG was prepared by similar methods from [^{3}H]aminolevulinic acid (ALA) (Evans et al., 1985). Trypsin (TPCK treated), Pronase E, and polyacrylamide gel electrophoresis reagents were obtained from Sigma Chemical Co. All enzyme manipulations were carried out at 4 °C except where stated otherwise.

Purification of PBG Deaminase. Deaminase for enzyme–substrate NMR investigations was prepared from ca. 3 kg wet

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[1] Abbreviations: PBG, porphobilinogen; NMR, nuclear magnetic resonance; pre-uro'gen, preuroporphyrinogen; HMB, (hydroxymethyl)-bilane; uro'gen I, uroporphyrinogen I; uro'gen III, uroporphyrinogen III; ALA, aminolevulinic acid; DEAE, diethylaminoethyl; Tris, tris(hydroxymethyl)aminomethane; FID, free induction decay.

[2] IUB has recently recommended the name hydroxymethylbilane (HMB) synthase, but as is clear from the work presented here, this name no better represents the enzyme's function than does the old systematic name uroporphyrinogen I synthase. However, the name porphobilinogen deaminase is noncommittal as to the nature of the product of the enzyme and is therefore employed in this paper.

[3] The stability of the ES complexes at high protein concentration is a point that we dispute in this paper.

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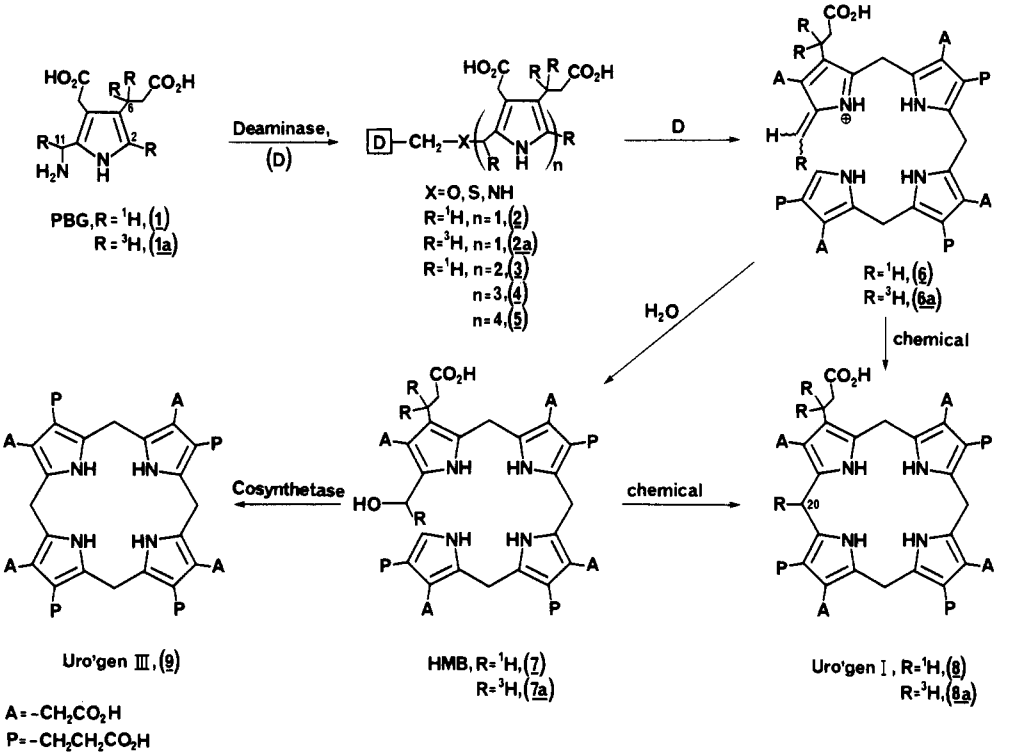
Scheme I



weight of *R. spheroides* (from 500 L of cell culture) divided into eight batches. Typically, *R. spheroides* (350–400 g) was suspended in buffer (700–800 cm^3, 0.05 M potassium phosphate, pH 7.6) and sonicated (using a Heat-System-Ultrasonic Model W200R sonicator) in 100-cm^3 batches for a total time of 9 min in 3-min bursts with 3 min between each burst for cooling. The temperature was maintained by a cooling bath with circulating ethylene glycol–water at −20 °C. In this way, the temperature of the cell suspension was kept below 10 °C.

The homogenate was then centrifuged at 35000*g* (12 min) and the supernatant carefully decanted. This was heated to 60 °C for 15 min in 150-cm^3 batches, before being cooled in ice and then centrifuged at 100000–150000*g* (60 min). The supernatant (ca. 810 cm^3) from this was made 10 mM in β-mercaptoethanol (567 μL) and brought to 40% saturation in ammonium sulfate (183.06 g) with stirring. After 30 min, the mixture was centrifuged (20000*g*, 15 min) and the supernatant then brought to 55% saturation in ammonium sulfate and then stirred for 30 min. After centrifugation (20000*g*, 15 min), the pellet of deaminase was dissolved in 0.01 M Tris–Cl$^-$, pH 8.0, buffer (ca. 20 cm^3) and dialyzed against two changes of buffer (2 L, 0.01 M Tris–Cl$^-$, pH 8.0, plus 5 mM β-mercaptoethanol).

The dialyzate (ca. 50 cm^3) was applied to a freshly equilibrated DEAE-Sephacel column (500-cm^3 bed volume, 14 × 6.5 cm), which had been pretreated with base and acid washes. Elution was carried out with a linear salt gradient of 0.05 M KCl (750 cm^3, in 0.01 M Tris-Cl$^-$, pH 8.0, plus 5 mM β-mercaptoethanol) to 0.6 M KCl (750 cm^3, in 0.01 M Tris-Cl$^-$ pH 8.0, plus 5 mM β-mercaptoethanol) at 30 cm^3 h^{-1}. Fractions containing active enzyme were pooled (ca. 1800 units) and concentrated (Amicon PM-10 membrane) to ca. 30 cm^3.

The whole process was repeated with another batch of cells, and the pooled enzyme from DEAE-Sephacel chromatography combined with the first batch and further concentrated (Amicon PM-10 membrane) to ca. 30 cm^3. This pooled enzyme was applied to a freshly equilibrated Sephadex G-100 column and eluted with 0.01 M Tris–Cl$^-$, pH 8.0, plus 5 mM β-mercaptoethanol at 30 cm^3 h^{-1}. Fractions containing active enzyme were pooled (ca. 3200 units) and concentrated (Amicon PM-10 membrane) to ca. 15 cm^3.

Another six batches of cells were processed in pairs in an identical manner, furnishing after gel filtration four 15-cm^3 samples of the enzyme (total ca. 12000 units), which were pooled and concentrated (Amicon PM-10 membrane) to ca. 20 cm^3. This was then heated at 60 °C for 15 min to ensure complete inactivation of any residual cosynthase. As no precipitation was observed, the enzyme was rapidly cooled in ice and applied to a freshly equilibrated Sephadex G-100 column. Elution was performed with 0.01 M Tris-Cl$^-$, pH 8.0, plus 5 mM β-mercaptoethanol at 30 cm^3 h^{-1}. Fractions containing active enzyme were pooled and concentrated (Amicon PM-10 membrane) to ca. 100 cm^3 (total ca. 8000 units), 20 units mg^{-1}).

Deaminase was routinely assayed by consumption of PBG (Bogorad, 1972). One unit is defined as the amount of enzyme required to consume 1 μmol of PBG/h.

Generation and Purification of PBG–Deaminase Complex by Analytical Gel Electrophoresis. For preliminary studies, the generation of [^{14}C]PBG–deaminase complexes was carried out with a rapid mixing device (Evans, 1984), and the resultant complexes were analyzed by gel electrophoresis. Gels (7.5% polyacrylamide) were electrophoresed on an LKB Multiphor system operating at a running pH of 9.5 (using glycine–Tris electrode buffer, pH 8.9). Gels were stained for protein with Coomassie Brilliant Blue R-250 and for enzymic activity by incubation of the gel with PBG solution (640 μg in 20 cm^3 of 0.01 M Tris, pH 8.0) for 45 min, subsequent treatment with iodine [0.2% (w/v)I_2 in 1 M HCl], and visualization under UV light. For ^{14}C assays, bands were cut out, dried (vacuum desiccator), and then treated with 0.5 cm^3 of 30% H_2O_2 at 80 °C until completely dissolved (ca. 90 min). To this, Aquasol liquid scintillant (New England Nuclear, 4.5 cm^3) was added, and the bands were counted with a Packard PL Prias Tri-carb scintillation counter.

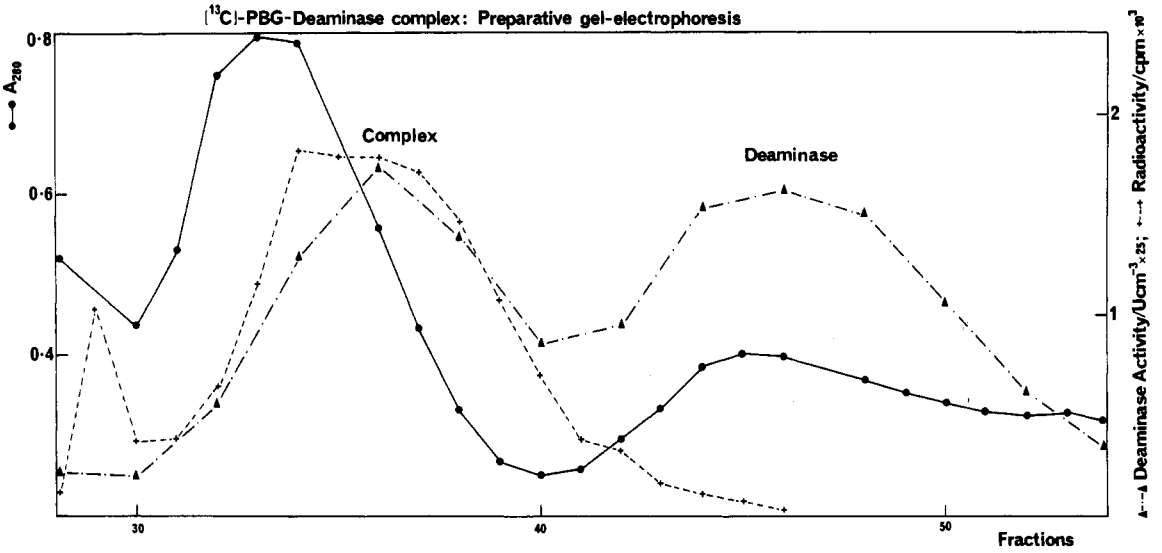


FIGURE 1: Purification of [^{13}C,^{14}C]PBG–deaminase covalent complex and deaminase by preparative gel electrophoresis: (●) A_{280}; (▲) deaminase activity (units cm^{-3} × 25); (+) radioactivity (cpm × 10^3).

Preparative Gel Electrophoresis. In a typical preparation, purified deaminase (8000 units, 20 units mg^{-1} in 50 cm^3 of 0.01 M Tris–Cl$^-$, pH 8.0, buffer) and labeled PBG [750 μg of [^{13}C]PBG plus 37.5 μg of [^{14}C]PBG, 6 789 555 dpm μmol^{-1} [^{13}C]PBG, in 50 cm^3 of 0.01 M Tris-Cl$^-$, pH 8.0, plus 5 mM EDTA plus 25 mM lysine (optional)] were mixed in a rapid mixing device, and the resultant complex was immediately concentrated (Amicon PM-10 membrane) to ca. 2 cm^3. This sample was then subjected to preparative gel electrophoresis on a Buchler Poly-prep 2000 preparative vertical column apparatus in a 210-cm^3 7.5% polyacrylamide gel cooled to 4 °C and with Tris–barbital (pH 7.5) for the upper electrode buffer and Tris buffer (pH 7.5) for the lower electrode and elution buffers. The sample, containing sucrose [5% (w/v)] and bromophenol blue tracking dye [10 μL, 0.25% (w/v) in Tris buffer], was carefully layered onto the gel and concentrated at 30 mA (<100 V) for ca. 15 min, and electrophoresis continued at 50–65 mA (ca. 150 V). Initially, the elution buffer was collected in bulk (150 cm^3), but after 6 h, fractions (10 cm^3) were collected, and electrophoresis was continued for approximately another 17 h. The running pH was ca. 7.9.

The fractions were assayed for radioactivity (liquid scintillation counting), protein (A_{280} used as a rough guide, since barbital interferes at this wavelength), and enzyme activity (see Figure 1). Fractions containing complex (38–45) were concentrated (Amicon PM-10 membrane) to ca. 0.4 cm^3 and then diluted and reconcentrated (twice) with 0.05 M sodium pyrophosphate and 20% D_2O, pH 8.0, buffer. Fractions containing pure (80–90%) deaminase were used for preparing additional, very pure complex (i.e., complex used in ^{3}H NMR studies).

NMR Spectroscopy. All spectra were recorded on a 7.0497-T Bruker WM-300 wide-bore spectrometer; ^{13}C NMR spectroscopy was conducted at 75.473 MHz and ^{3}H NMR at 320.121 MHz, both in 5-mm tubes (0.5 cm^3). ^{13}C NMR spectra were indirectly referenced to *p*-dioxane (δ_C = 66.5 ppm), and ^{3}H NMR spectra were referenced to a Me_4Si ghost signal (Elvidge et al., 1978).

RESULTS

^{13}C NMR Spectroscopy. Preliminary investigations have established, with [3,5-^{14}C]PBG and purified deaminase from *R. spheroides* in a rapid mixing device, that the same complexes as those reported by Berry et al. (1981) were obtained. Furthermore, the proportions of bound mono-, di-, and tripyrroles could be altered by varying the stoichiometry of the PBG and deaminase. Deaminase is a monomer of M_r ≃36 000, and a typical preparation yields 8000 units or 63 mg (1.7 μmol),[4] which could theoretically bind 392 μg of ^{13}C-enriched PBG as the monopyrrole complex; the NMR detectable concentration of enriched PBG is thus equivalent to 35.6 mg at natural abundance (natural abundance of ^{13}C = 1.1%). The substrate, even if it were to bind with a 100% yield, will be present at similar NMR detectable concentrations as the natural abundance protein background.

In a typical preparation, deaminase (8000 units, 34 μM) and [^{13}C]PBG (34 μM) and 5% [^{14}C]PBG were mixed at 4 °C in a rapid mixing device (Evans, 1984). The concentrated complex was then purified by preparative polyacrylamide gel electrophoresis at pH 8.0 as shown in Figure 1. The complex thus obtained was usually ca. 70% pure (determined by analytical gel electrophoresis) and mainly (>90%, by gel electrophoresis) corresponded to mono-PBG complex (see Figure 4) bearing approximately 256 μg of PBG (51% yield).

The ^{13}C NMR investigation of the ES complex employed [2,11-^{13}C]PBG, in which the chemical shift of C-11 would be highly diagnostic of the nature of the binding of the substrate. Accordingly, the NMR spectrum of the ^{13}C complex [see Figure 2 (spectrum ii)] was compared with electrophoretically pure unenriched complex [Figure 2 (spectrum i)], and a small extra peak is visible at 123.93 ppm, with no clear differences in the aliphatic region. In the difference spectrum [Figure 2 (spectrum iii)], the peak at 123.93 ppm is clearly visible and additionally a peak of similar line width at 22.15 ppm. These shifts are consistent with enzyme-bound uro'gen I, which exhibits resonances at 123.1 and 21.8 ppm (see preceding paper). They are also consistent with enzyme-bound di- or tripyrrole, but in this case we would also expect resonances to occur at ca. 112 ppm for the terminal pyrrolic carbon (see preceding paper) and at an unknown shift for the methylene directly attached to the enzyme. Figure 2 (spectrum iii) shows a very low intensity peak at 114.2 ppm, and two other broad peaks at 44.9 and 31.6 ppm, but the poor signal-to-noise ratio does not allow any conclusions to be drawn from these resonances.

[4] Determined by using the value of 128 units mg^{-1} reported by Jordan and Shemin (1973) for the specific activity of the pure *R. spheroides* enzyme.

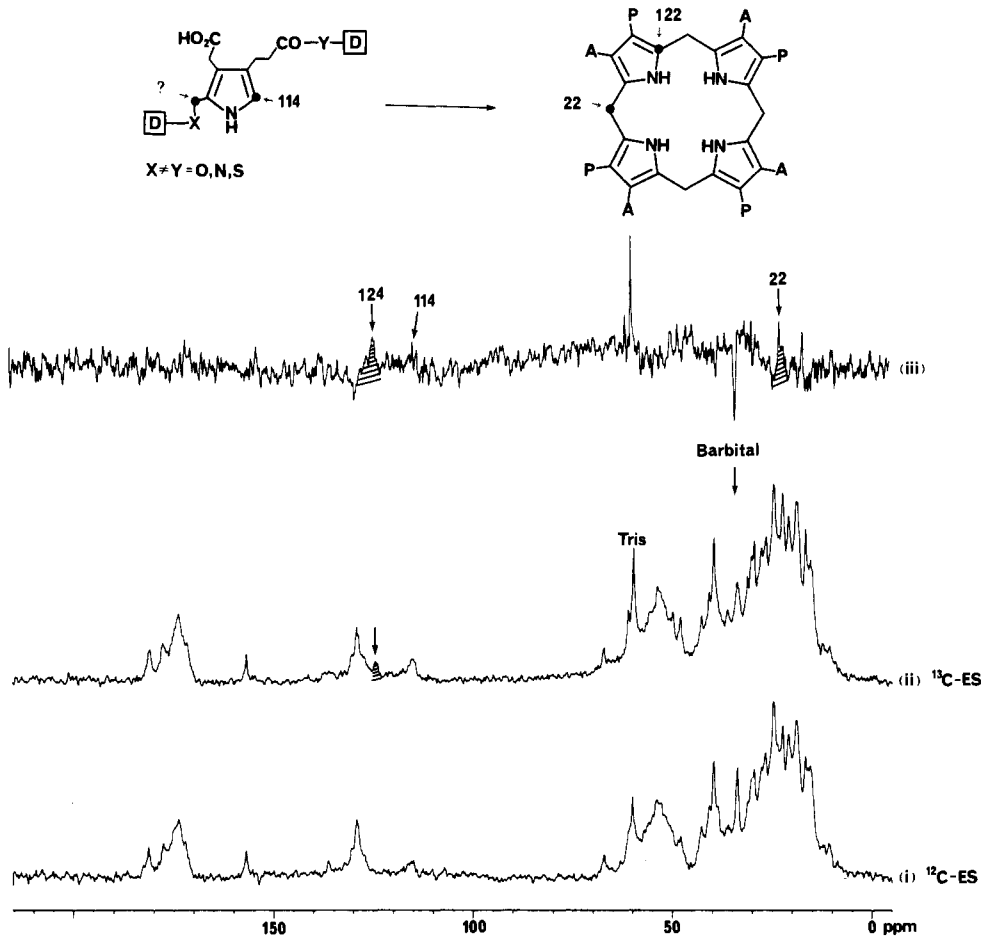


FIGURE 2: ^{1}H-decoupled (0.8 W broad band continuous) 75.5-MHz ^{13}C NMR spectra of (i) electrophoretically pure unenriched complex (ca. 800 units, 63 units mg^{-1} in 0.4 cm^3 of 0.01 M sodium pyrophosphate and 10% D_2O, pH 8.0) with NS (number of scans) = 136 800, (ii) [2,11-^{13}C]PBG–deaminase complex (192 μg bound to 6000 units of deaminase after gel electrophoresis, in 0.4 cm^3 of 0.01 M sodium pyrophosphate and 10% D_2O, pH 8.0) with NS = 79 200, and (iii) the difference (ii – i). Data were recorded at 11.0 °C, with pulse width = 54°, with acquisition time = 0.492 s, with size of data table = 16K, and with the first two data points of the combined FIDs removed, zero-filled to 32 K, exponentially multiplied with a broadening of 20 Hz, and Fourier transformed, and the spectra base line was corrected by a spline fit.

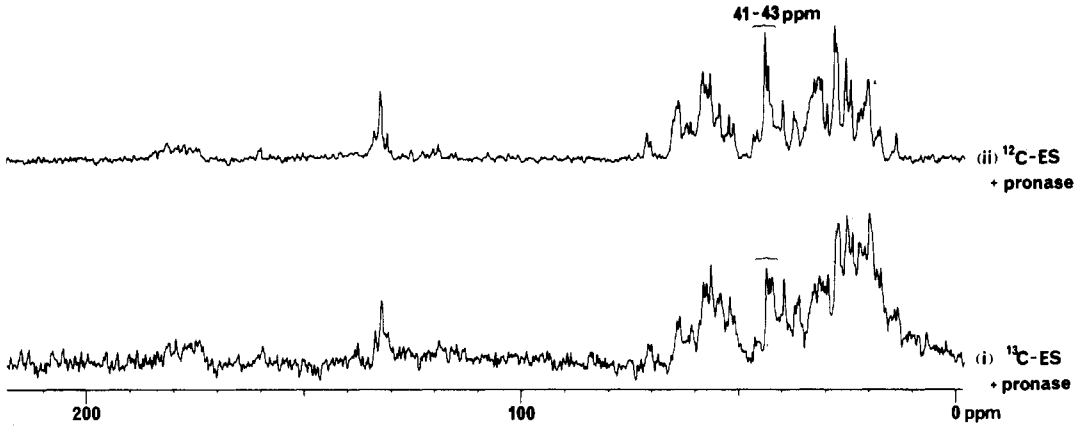


FIGURE 3: ^{1}H-decoupled (0.8 W broad band continuous) 75.5-MHz ^{13}C NMR spectra of (i) [2,11-^{13}C]PBG–deaminase complex (50 μg bound to 3000 units of deaminase after gel electrophoresis, in 0.2 cm^3 of 0.05 M sodium pyrophosphate and 10% D_2O, pH 8.0) with added Pronase E (1.855 mg), NS = 18 000, and (ii) electrophoretically pure unenriched complex (0.2 cm^3 in 0.05 M sodium pyrophosphate and 10% D_2O, pH 8.0) with added Pronase E (0.9788 mg), NS = 37 533; both were after incubation for 12 h at 25 °C and run at 12.5 °C, with pulse width = 90°, acquisition time = 0.983 s, and size of data table = 32K. Data were processed exactly as in Figure 2.

When [$^{13}C/^{14}C$]PBG–deaminase complex was digested with trypsin and chromatographed on Sephadex G-50, the radioactive peptides corresponding to small molecules (M_r <2000) were subjected to peptide mapping. The main radioactive band obtained was analyzed by UV spectroscopy and exhibited a λ_{max} of 395 nm, presumably corresponding to peptide-bound uroporphyrin I. The latter could arise by disproportion of the complex to give uro'gen I, which readily oxidizes, or from a covalently bound, oxidized, oligopyrrole having similar absorption maxima. Attempts to examine the peptides by ^{13}C NMR failed to reveal significantly enriched resonances.

When [2,11-^{13}C]PBG–deaminase complex was digested with Pronase E in the NMR tube [Figure 3 (spectrum i)] and compared with pure unenriched complex treated in an identical manner [Figure 3 (spectrum ii)], after 24 h a large number of peaks appeared, corresponding to free oligopeptides. Se-

lected resonances can be assigned to certain amino acid residues, but none can be easily assigned to ^{13}C-enriched PBG. Thus, no conclusions could be drawn from these spectra, which is in contrast to Battersby et al. (1983a,b), who recently published work along very similar lines. In their work, the oligopeptides resulting from a Pronase incubation with [11-^{13}C]PBG–deaminase complex were examined by ^{13}C NMR. They detected a family of peaks at 42–43 ppm, which were assigned to X–CH_2–pyrrole, and a peak at 24.5 ppm, which was assigned to pyrrole–CH_2–pyrrole. Battersby concluded that the group on the enzyme to which the substrate binds is the ϵ-NH_2 of lysine. The spectra in Figure 3 clearly show a family of peaks at 41–43 ppm in both the ^{13}C-enriched complex *and* the unenriched complex and can be assigned to the ϵ-CH_2 of lysine, the β-carbon of leucine, or the δ-carbon of arginine. Furthermore, a difference spectrum (not shown) exhibited no significant resonances. We chose, therefore, not to draw conclusions from the observation of such peaks.

Further attempts at the detection of bound substrate by ^{13}C NMR spectroscopy involved mild acid denaturation and also the use of [2,11-^{13}C,$^{2}H_3$]PBG in an attempt to remove ^{13}C–^{1}H dipolar relaxation and thereby reduce the carbon line width by a factor of 2 or 3. Both these techniques failed to reveal any significant peak in the aliphatic region. Following the observation (Evans et al., 1985) that C-5 of [3,5-^{13}C]PBG is sensitive to the pK_a of the amino group, titration of the [3,5-^{13}C]PBG–deaminase complex was attempted. Again, despite the fact that the substrate quaternary carbons should exhibit line widths of the order of 100 Hz for a protein of this molecular weight (Mackenzie et al., 1984), the resonances were too weak for definite conclusions to be drawn.

^{3}H NMR Spectroscopy. We sought a general solution to structural problems in covalent enzyme–substrate complexes where the lack of a "window region" in a high natural abundance background precludes rigorous assignment. We now describe [part of this work has been reported as a preliminary paper (Evans et al., 1984)] the first application of tritium, a nucleus of high sensitivity (20% greater than ^{1}H at full enrichment) and low ($<10^{-16}$) natural abundance, as an NMR probe of chemical shift in the environment of a productive covalent enzyme–substrate complex.

The ES complex of [2,6,11-^{3}H]PBG[5] (**1a**) and highly purified (>95% by analytical gel electrophoresis) deaminase (4000 units) was generated in a rapid-mixing device and separated from the small molecules by gel filtration on Sephadex G-50. Lysine (25 mM) was included in the PBG solution in order to provide a large excess of small charged molecules, which might form an ion pair with any uro'gen I produced, without nucleophilically trapping any tetrapyrrole as is the case with ammonia.[6] Analysis of the concentrate by analytical gel electrophoresis (Figure 4) showed it to be >90% of the monopyrrole complex (**2a**; 16 mCi). The remaining 10% is the dipyrrole complex (**3** and Figure 4). The ^{1}H-decoupled ^{3}H NMR spectrum [Figure 5 (spectrum i)] of **2a** exhibits resonances at 6.18 (C-2, C–*T*), 3.28 ± 0.1 (pyrrole–CH*T*–X–Enz), and 2.48 ± 0.1 ppm (pyrrole–CT_2–CH_2CO_2H) at 5.5 °C. At 23 °C [Figure 5 (spectrum ii)], the signals at 6.18 and 3.28 ppm disappear and are replaced by new resonances at 3.58 ± 0.05 [*meso*-methylenes (CH*T*) of uro'gen I (**8a**) and methylenes (CH*T*) of complexes **3–5**; R = ^{3}H], 2.75 ± 0.05 (–C$T_2$$CH_2CO_2H$ of **3–5**; R = ^{3}H), 2.48 ± 0.05 [C$T_2$$CH_2CO_2H$ of uro'gen I (**8a**)], and 4.69 ppm (HO*T*; exchanged from C-2 and present in spectra 5i, 5ii, and 6).

To demonstrate the catalytic competence of the monopyrrole complex (**2**), *unlabeled* PBG (**1**) was added to the ^{3}H complex (**2a**) at 3.5 °C, and the formation of uro'gen I was monitored by ^{3}H NMR. At 3.5 °C, a transient low-intensity signal was observed at 4.76 ppm [Figure 6 (spectrum i)]. On being warmed to 23 °C [Figure 6 (spectrum ii)], sharp resonances for unbound uro'gen I appeared at 3.58 (20-*meso*-CH*T*) and

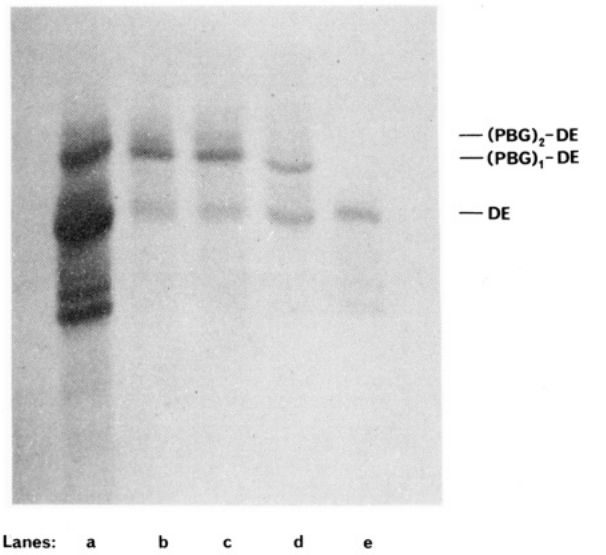


FIGURE 4: Polyacrylamide (7.5%) analytical gel electrophoresis of [^{13}C,^{14}C]PBG–deaminase covalent complex. (Lane a) Complex prior to preparative gel electrophoresis; (lanes b and c) purified (ca. 70%) monopyrrole (ca. 90%) complex [(PBG)$_1$-DE] and the dipyrrole (ca. 10%) complex [(PBG)$_2$-DE]; (lane d) complex in lanes b and c after 24 h at 4 °C, showing increase in deaminase band; (lane e) pure deaminase (DE).

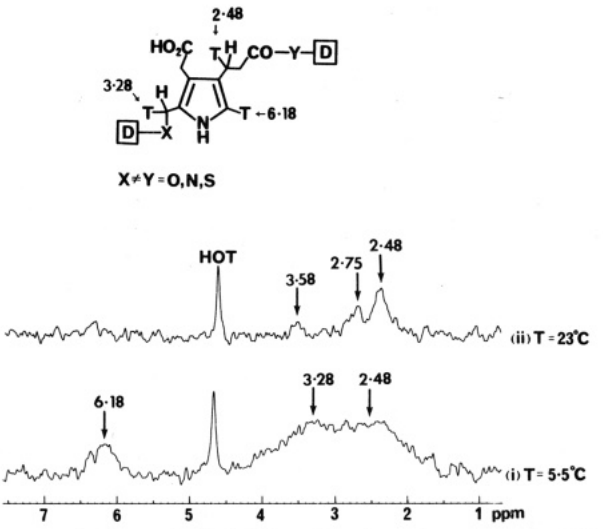


FIGURE 5: ^{1}H-decoupled (0.8 W broad band continuous) 320.13-MHz ^{3}H NMR spectra of [2,6,11-^{3}H]PBG–deaminase complex (16 mCi bound to 4000 units of electrophoretically pure deaminase in 0.4 cm^3 of 0.05 M sodium pyrophosphate and 90% D_2O, pH 8.0): (i) at 5.5 °C with NS = 10 800 and (ii) half of the sample in (i) reequilibrated at 23 °C with NS = 12 600. Data were recorded with pulse width = 90°, with acquisition time = 1.065 s, with size of data table = 8K, and with the first two data points of the FIDs removed, zero-filled to 16 K, exponentially multiplied with a broadening of 10 Hz, and Fourier transformed, and the spectra base line was corrected by a spline fit.

[5] The [^{3}H]PBG employed contains all multiply tritiated species up to the fully tritiated [2,6,6,11,11-$^{3}H_5$]PBG, but consisted mainly of [2,6,6,11-$^{3}H_4$]PBG.

[6] Control experiments with and without lysine confirmed that its presence did not affect the yield or stoichiometry of the complex produced.

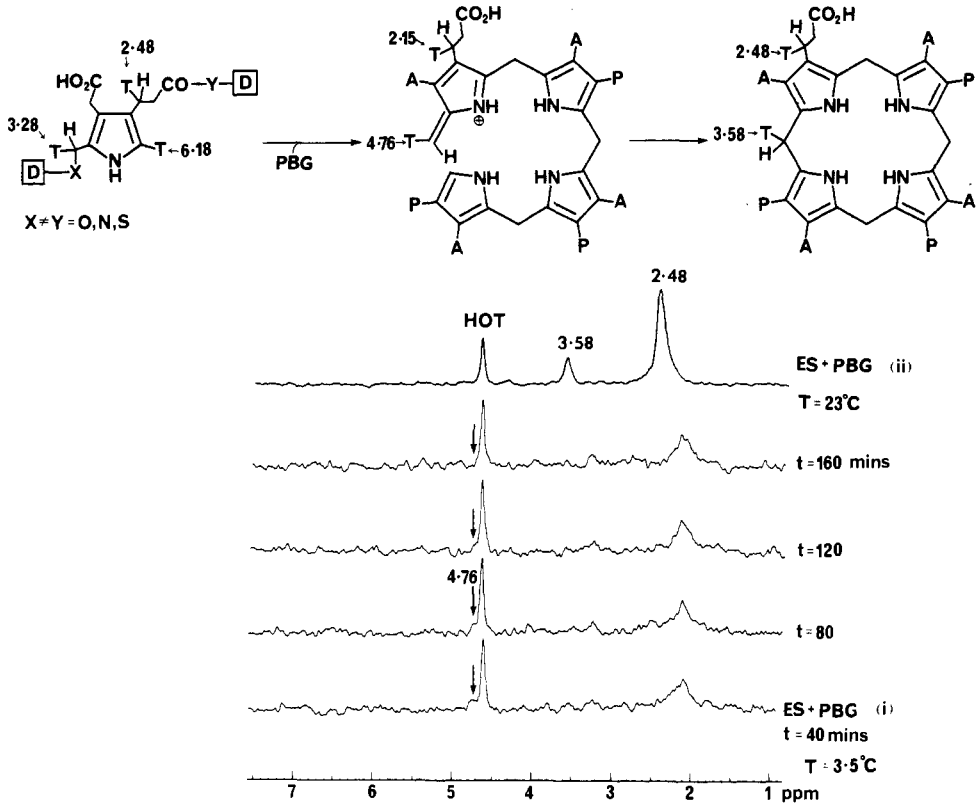


FIGURE 6: ^{1}H-decoupled (0.8 W broad band continuous) 320.13-MHz ^{3}H NMR spectra of half the sample described in Figure 5 with added unlabeled PBG (749 μg in 50 μL of 0.1 M sodium pyrophosphate and 90% D_2O, pH 8.0): (i) at 3.5 °C, 20 min between spectra, NS = 4500 each; (ii) at 23 °C, NS = 1800. Data were recorded with pulse width = 90°, with acquisition time = 0.532 s, with size of data table = 4K, and with the first two data points of the FIDs removed, zero-filled to 16 K, exponentially multiplied with a broadening of 10 Hz, and Fourier transformed.

2.48 ppm (propionate–CH*T*), corresponding to the ^{1}H chemical shifts in an enzyme-free sample of uro'gen I (see preceding paper).

Discussion

The formation of uro'gen I detected by ^{13}C NMR in samples known initially to comprise of monopyrrole complex is ascribed to disproportionation of complex **2a** via **3**–**5** to yield ultimately enzyme-bound uro'gen I (**8**). Since these samples were stored at 4 °C for the duration of the experiment, the stability of the ES complexes (vide supra) is questionable. A similar effect was also seen in the ^{3}H NMR studies where the formation of uro'gen I (**8**) in the complex **2a** at 23 °C was observed in the absence of free PBG. The methylene (CH*T*) and side-chain groups ($-CT_2CH_2CO_2H$) were observed along with those of uro'gen I in the signals at 3.58, 2.75, and 2.48 ppm [Figure 5 (spectrum ii)]. Such a disproportionation also accounts for the disappearance of the signal at 3.28 ppm, which would be expected to lose up to 90% of its original intensity, on the basis of the statistical randomization of ^{3}H label in the reaction

$$E + S \rightleftarrows ES_1 \rightleftarrows ES_2 \rightleftarrows ES_3 \rightleftarrows ES_4 \rightleftarrows E + P$$

whose equilibrium clearly lies far to the right under conditions of high protein concentration and temperatures > 23 °C.[7]

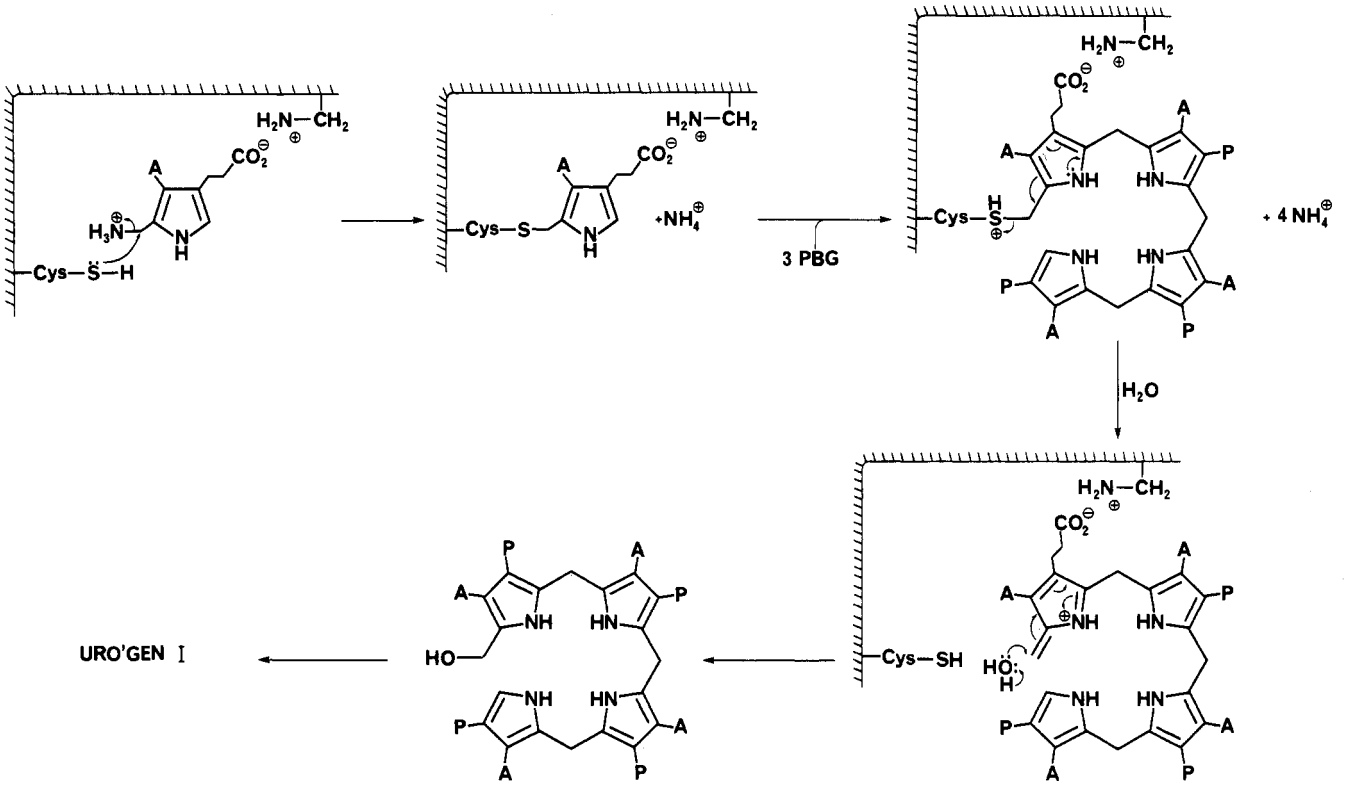
The large ^{3}H NMR line widths (ca. 150–300 Hz) of the spectrum in Figure 5 (spectrum i) reflect an environment in which the active site of the enzyme is buried within the protein. Furthermore, the implications of such line widths for ^{13}C NMR explain the comparative lack of success with that nucleus, since the predicted ^{13}C line width[8] would be ca. 285 Hz and therefore very difficult to detect. That the large ^{3}H NMR line widths might be due to a dynamic equilibrium between two conformers cannot be ruled out, nor can it be easily tested, since increasing the temperature causes disproportionation and decreasing the temperature necessitates the use of cryosolvents. However, a dynamic equilibrium between free and bound pyrrole can be discounted, since the pyrrole is known to bind with loss of ammonia (Akhtar & Jordan, 1978) and thus the only free species would be the monopyrrole azafulvene (cf. **6**). The averaged signal from bound **2** and monopyrrole azafulvene (cf. **6**) would have a chemical shift of ca. 4.2 ppm [averaged between 4.9 ppm for **6** and ca. 3.5 ppm (H. Rexhausen, N. E. Mackenzie, and A. I. Scott, unpublished results)].

The propionate side-chain line width suggests that it is either covalently attached or ionically associated with the protein. This is consistent with reports of PBG analogues in which changes in both the aminomethyl and the propionate side chains gave rise to competitive inhibition of deaminase (Heath & Hoare, 1959a,b; Bogorad, 1957, 1960; Carpenter & Scott, 1961; Frydman & Feinstein, 1974). In addition, Berry et al. (1981) interpreted the increase in the mobility between the mono-PBG complex band and the di-PBG complex band as evidence for the neutralization of a negatively charged enzyme group (after loss of $-NH_3^+$), thus providing only a net increase

[7] Proof of an enzyme-bound uro'gen I species is not possible by gel electrophoresis since uro'gen I dissociates from the protein under the conditions described under Experimental Procedures.

[8] Using the line width $\nu_{1/2} \sim 300$ Hz to calculate a value for T_2, we can calculate an approximate value for the rotational correlation time $\tau_R = 50$ ns using the rigid-rotor nearest-neighbor (RRNN) model and assuming the extreme narrowing condition to hold ($\omega^2\tau_R^2 \ll 1$). The value for τ_R can be used to predict a T_2 for the $^{13}CH_2$ of the ES complex, and hence $\nu_{1/2}$. In reality, this estimated value for T_2 is probably extremely conservative.

Scheme II



of one negative charge. However, the single net negative charge is much more likely to arise from involvement of one of the pyrrole carboxylates in binding, probably the propionate, as well as elimination of $-NH_3^+$. This is further supported by the observation that, under conditions of high protein concentration, uro'gen I is found to associate with the enzyme, presumably through ionic bonding via the propionate carboxylate.

The conspicuous lack of accumulation of any obvious intermediates such as HMB (**7**) in the spectra in Figure 6 (spectrum i) is surprising in the light of the evidence presented in the preceding paper for its involvement in porphyrin biosynthesis. This can be attributed to the high protein concentrations (ca. 1.6 mM) employed in these experiments where intermediates such as HMB, which may represent the nucleophilic trapping of an azafulvene species (**6**) with water, never have an opportunity to accumulate. Rather, the highly reactive azafulvene species cyclizes directly to uro'gen I. The transient peak observed at 4.76 ppm supports this view, since the chemical shift is consistent with the vinyl hydrogen of the azafulvene (**6**), which was demonstrated to be 4.86 ppm in model systems (Falk & Schlederer, 1981).

The ^3H chemical shift (3.28 ± 0.1 ppm) of the methylene directly attached to the enzyme allows conclusions to be drawn as to the nature of the nucleophilic group "X" in Scheme I. That the methylene is bound to the oxygen of a serine residue can be ruled out, since HMB (**7**) and its methyl ether have chemical shifts of 4.4 and 4.2 ppm, respectively [see preceding paper and Evans (1984)]. Model studies (Dauner et al., 1976; Evans, 1984) predict $\delta_H \approx 3.8$ ppm for a methylene attached to a secondary amine, some 0.5 ppm downfield from the observed resonance. Substituted thiomethylpyrroles give a chemical shift for the methylene bridge (–S–CH_2–pyrrole) of $\delta_H = 3.50 \pm 0.1$ ppm (H. Rexhausen, N. E. Mackenzie, and A. I. Scott, unpublished results). That the PBG molecule might be in a position unusually close to an aromatic amino acid residue in the enzyme active site cannot be ruled out. However, the fact that the propionate side-chain resonance shows no ring current upfield shift, nor indeed does the bridgehead methylene of enzyme-bound uro'gen I [Figures (spectrum i)], suggests that the observed value of 3.28 ± 0.1 ppm is *unlikely* to be the result of ring current effects. We therefore suggest that the active site nucleophilic group in deaminase is a cysteine thiol residue or, less probably, the ϵ-amino group of lysine. The former possibility is further supported by the observation (Jordan & Shemin, 1973) that deaminase is reversibly inhibited by sulfhydryl blocking reagents.

Recent kinetic studies using purified deaminase from *Euglena gracilis* (Williams et al., 1981) and rat spleen (Williams, 1984) have established from the pH dependence of V_{max} that two ionizable groups are involved in any one of the ES complexes with pK_a values of 6.1 and 8.9 (*Euglena*). The value of K_m was found to decrease with decreasing pH, and this was interpreted as the absence of a pK_a in the free enzyme or substrate corresponding to the pK_a of 6.1. Thus, if the effect is due to ionization of the substrate part of the ES complex, then the pK_a of the carboxyl functions of the substrate complex must be raised from 3.7 or 4.95 (Granick & Bogorad, 1953) to 6.1. From our NMR results, such a highly perturbed value is consistent with the propionate carboxylate forming an ionic bond with a suitably basic enzyme group such as the ϵ-NH_2 of a lysine residue, the guanidine group of an arginine residue, or the imidazole of a histidine residue. It was also found that the dependence of V_{max}/K_m on pH showed a single ionization (pH 8.2), reflecting ionizations in the free enzyme or its three pyrrolic forms. This might reflect ionizations in the enzyme portion of the ES complex, which is only slightly perturbed (0.7 pH units) by the presence of substrate. If this were true, then the free enzyme pK_a is closer to typical values for a sulfhydryl group (pK_a = 8.3–8.6) than for an ϵ-ammonium group (pK_a = 9.4–10.6) (Cohn & Edsell, 1943).

Thus, a mechanism for deaminase can be proposed on the basis of the evidence acquired to date, as shown in Scheme

II. The first PBG unit alkylates a free thiol group with the elimination of 1 mol equiv of ammonia, and the propionate forms an ion pair with a lysine or arginine residue, as supported by the recent work of Russell et al. (1984) and Hart et al. (1984). Subsequent condensations with PBG result in the bound tetrapyrrole. At this point, the first step in the release of the tetrapyrrole is the regeneration of the free thiol and formation of an *exo*-methylene double bond. The highly reactive azafulvene species, under appropriate conditions, could be intercepted by endogenous water, or any other suitable nucleophile, as has been shown by numerous trapping experiments (Davies & Neuberger, 1973; Radmer & Bogorad, 1972; Battersby et al., 1983a). This species or its trapped derivative (HMB) is presumably released into the medium by breaking the propionate ionic bond, for further cyclization to uro'gen I. The observation by Battersby et al. (1983b) that uro'gen III cosynthase can act upon deaminase-bound tetrapyrrole supports our view that the true product of the enzyme is in fact the azafulvene species and not HMB, as initially thought.

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