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^{13}C NMR Studies of Porphobilinogen Synthase: Observation of Intermediates Bound to a 280 000-Dalton Protein†

Eileen K. Jaffe*,‡ and George D. Markham§

Department of Biochemistry, University of Pennsylvania School of Dental Medicine, Philadelphia, Pennsylvania 19104-6002, and Institute for Cancer Research, Fox Chase Cancer Center, Philadelphia, Pennsylvania 19111

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ABSTRACT: ^{13}C NMR has been used to observe the equilibrium complex of [4-^{13}C]-5-aminolevulinate ([4-^{13}C]ALA) bound to porphobilinogen (PBG) synthase (5-aminolevulinate dehydratase), a 280 000-dalton protein. [4-^{13}C]ALA (chemical shift = 205.9 ppm) forms [3,5-^{13}C]PBG (chemical shifts = 121.0 and 123.0 ppm). PBG prepared from a mixture of [4-^{13}C]ALA and [^{15}N]ALA was used to assign the 121.0 and 123.0 ppm resonances to C_5 and C_3, respectively. For the enzyme-bound equilibrium complex formed from holoenzyme and [4-^{13}C]ALA, two peaks of equal area with chemical shifts of 121.5 and 127.2 ppm are observed (line widths ~ 50 Hz), indicating that the predominant species is probably a distorted form of PBG. When excess free PBG is present, it is in slow exchange with bound PBG, indicating an exchange rate of $<10\ s^{-1}$, which is consistent with the turnover rate of the enzyme. For the complex formed from [4-^{13}C]ALA and methyl methanethiosulfonate (MMTS) modified PBG synthase, which does not catalyze PBG formation, the predominant species is a Schiff base adduct (chemical shift = 166.5 ppm, line width ~ 50 Hz). Free ALA is in slow exchange with the Schiff base. Activation of the MMTS-modified enzyme–Schiff base complex with ^{113}Cd and 2-mercaptoethanol results in the loss of the Schiff base signal and the appearance of bound PBG with the same chemical shifts as for the bound equilibrium complex with Zn(II) enzyme. Neither splitting nor broadening from ^{113}Cd–^{13}C coupling was observed.

Nuclear magnetic resonance (NMR)[1] has long been recognized as a potential probe of the active site chemistry of enzyme-catalyzed reactions. More than 10 years have passed since Mildred Cohn and co-workers first used ^{31}P NMR to investigate the equilibrium complex between substrates bound to an enzyme, arginine kinase (Rao et al., 1976). Although carbon chemistry far exceeds phosphorus chemistry in applicability to enzyme-catalyzed reactions, ^{13}C NMR as a probe of enzyme active sites suffers from the natural 1.1% isotopic abundance of ^{13}C. The low natural abundance dictates synthesis of isotopically enriched substrate molecules, while the high natural abundance contributes substantial resonance intensity (background) from the carbons of the protein. Nevertheless, Mackenzie et al. (1984) in a recent review predict a promising future for the use of ^{13}C NMR to study enzyme–substrate complexes below 50 000 daltons, using cryosolvents to prolong the lifetime of enzyme-bound intermediates.

Porphobilinogen (PBG) synthase (a.k.a. δ-aminolevulinate dehydratase, EC 4.2.1.24) catalyzes the asymmetric condensation of two molecules of 5-aminolevulinate (ALA) to form the pyrrole PBG. One ALA molecule forms the P (propionyl) side of PBG with its amino nitrogen being incorporated into the pyrrole ring, whereas the other ALA molecule forms the A (acetyl) side of PBG, retaining a free amino group (see Figure 1). This reaction is on the pathway for the biosynthesis of porphyrin, chlorophyll, vitamin B_{12}, and a wide spectrum of tetrapyrrole pigments. PBG synthase from bovine liver is an octameric protein of 280 000 daltons and thus might not be considered a promising candidate for investigation of enzyme–substrate complexes by ^{13}C NMR (Mackenzie et al., 1984). However, in a bisubstrate reaction with identical substrates, there are few potentially more powerful techniques for elucidating the chemistry of enzyme-bound reaction intermediates.

Current knowledge of the PBG synthase reaction mechanism is summarized below. The reaction proceeds via a Schiff base intermediate formed between one ALA and an active site lysine residue (Nandi & Shemin, 1968). The Schiff base forms to the C_4 of the ALA, which becomes the P side of PBG

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‡ University of Pennsylvania School of Dental Medicine.
§ Fox Chase Cancer Center.

[1] Abbreviations: ALA, 5-aminolevulinate; NMR, nuclear magnetic resonance; MMTS, methyl methanethiosulfonate; PBG, porphobilinogen; EDTA, ethylenediaminetetraacetic acid.

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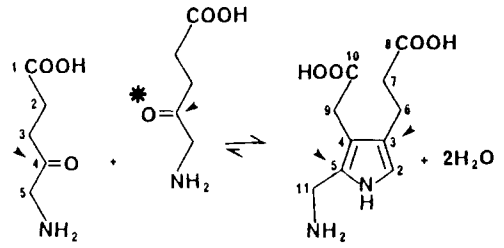

FIGURE 1: Formation of [3,5-^{13}C]porphobilinogen from [4-^{13}C]-5-aminolevulinate. Arrows denote ^{13}C-labeled positions. (*) Denotes the site of P-side Schiff base formation.

[(*) in Figure 1] (Jordan & Seehra, 1980). The C_5 proton of P-side ALA is extracted from the *pro-R* position (Chaudhry & Jordan, 1976). Octameric enzyme requires four Zn(II) for full catalytic activity (Bevan et al., 1980; Jaffe et al., 1984). Schiff base trapping studies and equilibrium binding studies[2] indicate four active sites/octamer or eight sites with strong negative cooperativity (Shemin, 1976; Jaffe & Hanes, 1986), though others have presented data to support eight active sites/octamer (Jordan & Seehra, 1980). Modification of PBG synthase with the small reversible sulfhydryl reagent methyl methanethiosulfonate (MMTS), introduced by Smith et al. (1975), forms a protein that is completely inactive toward PBG production, is free of Zn(II), is highly stable, and can be fully reactivated by the addition of 2-mercaptoethanol and Zn(II) or Cd(II) (Jaffe et al., 1984). MMTS-modified enzyme forms the Schiff base intermediate (Jaffe & Hanes, 1986) but cannot bind A-side ALA.[2]

This study has investigated the interaction of PBG synthase with 5-aminolevulinic acid enriched with ^{13}C at the 4-position. The carbonyl groups at the 4-position of ALA become the aromatic carbons at C_3 and C_5 of PBG (Figure 1). During this transmogrification, the carbon must undergo large changes in bonding leading to large changes in ^{13}C chemical shifts at the various stages of the reaction. From a ^{13}C NMR perspective, the 4-position has several advantageous features. The resonance of the carbonyl group of ALA occurs in a region devoid of protein signals, and the C_3 and C_5 signals from PBG fall in a relatively uncluttered region of the spectrum. Therefore, bound substrate and product are anticipated to be readily distinguished from the protein background. Additionally, the Schiff base intermediate would also be expected to occur in a window in the ^{13}C NMR spectrum. The enriched carbons of both substrate and product are not protonated, eliminating dipolar interactions with directly attached protons as a relaxation mechanism, thus leading to the prediction of relatively narrow lines (<50 Hz) even for a totally immobilized carbon bound to this large protein (Allerhand, 1979). Furthermore, PBG synthase is especially well suited to the ^{13}C NMR approach because the slow turnover rate at 37 °C (0.6 s^{-1}) gives the potential for well resolved signals from free and bound species in chemical exchange. Spectral acquisition at 37 °C provides a mechanism for reducing the sample viscosity and the rotational correlation time of this 280 000-dalton protein.

Experimental Procedures

Materials. KH_2PO_4, MMTS, and D_2O (99.8%) were purchased from Sigma Chemical Co. [4-^{13}C]-5-Aminolevulinate hydrochloride (90% enriched) was purchased from Cambridge Isotope Laboratories and stored frozen at 0.1 M in D_2O. ^{113}CdO was a gift from Professor Mildred Cohn. $^{113}Cd(NO_3)_2$ was prepared by dissolving 30 mg of ^{113}CdO in 0.1 mL of constant-boiling HNO_3 followed by dilution to 1 mL.

Enzyme Preparations and Assays. Purification of bovine liver PBG synthase and preparation and characterization of MMTS-modified apo-PBG synthase were carried out as previously described (Jaffe et al., 1984). The initial specific activity of holo-PBG synthase was ~20 μmol h^{-1} mg^{-1} at 37 °C. MMTS-modified apo-PBG synthase could be reactivated with Zn(II) and 2-mercaptoethanol to a specific activity of 18 μmol h^{-1} mg^{-1}. Activation of MMTS-modified apoenzyme with four Cd(II) per octamer results in specific activity at least equal to that of the Zn(II) enzyme (Jaffe et al., 1984). Following data acquisition, all enzyme samples were found to retain 85–95% the original enzymic activity.

Reconstitution of MMTS-Modified Enzyme–Schiff Base Complex with ^{113}Cd. The MMTS-modified enzyme–Schiff base complex was reconstituted with 2.4 μmol of ^{113}Cd in the presence of 1.8 μmol of [4-^{13}C]ALA and 4.2 μmol of 2-mercaptoethanol.

TCA Extract of Bound Equilibrium Complex. A 200-μL sample of the holoenzyme-bound equilibrium complex was quenched into 200 μL of 0.1 M KP_i, 10% TCA. After 10 min on ice, the precipitated protein was removed by centrifugation, and the supernatant was extracted 3 times with 400 μL of ether. The resulting extract was diluted to 2 mL with 0.1 M KP_i, pH 7, 10% D_2O, and a ^{13}C NMR spectrum was obtained (2000 transients).

Recovery of Enzyme from ^{13}C NMR Samples. An attempt was made to remove the ^{13}C-labeled compound(s) from a bound equilibrium complex sample. The 1.5-mL sample was 3 times diluted and reconcentrated from 10 mL of 0.1 M KP_i, 10 mM 2-mercaptoethanol with the hope that the resulting enzyme might be suitable for additional ^{13}C NMR experiments. Unfortunately, the resulting spectrum revealed signals at 121.5 and 127.2 ppm due to the bound PBG (see Results). Enzyme was finally recovered by 100-fold dilution followed by precipitation with 50% ammonium sulfate. Enzyme recovered in this fashion, like freshly purified enzyme, was shown to contain tightly bound PBG at approximately 30% of the active sites.[3]

Preparation of [^{13}C,^{15}N]PBG Mixture. A preliminary spectrum was obtained on a mixture containing 2 μmol each of [4-^{13}C]ALA and [5-^{15}N]ALA prepared in 2 mL of KP_i, pH 6.8, 10 mM 2-mercaptoethanol, 3 μM $ZnCl_2$, and 25% D_2O. A total of 2.3 units of PBG synthase was added, followed by a 1-h incubation at 37 °C. The probe temperature was reduced to 21 °C, and 12 000 transients were collected to characterize the resultant equimolar mixture of the four isotopically labeled porphobilinogens illustrated in Figure 2.

^{13}C NMR Data Acquisition. Spectra were obtained at 75.45 MHz on a Nicolet NT-300 wide-bore spectrometer with 10-mm GN series (bottom entry) probes. All spectra used a 45° (8 μs) pulse angle, 4-s recycle time, and low-power (0.2 W) broad-band ^{1}H decoupling, with temperature regulation at 37 °C. The spectral width was ±10 000 Hz from the carrier with quadrature phase detection. The chemical shift reference was external dioxane set to 67.4 ppm. Protein spectra (32K data points) were processed with a 30-Hz Gaussian line-broadening function except those presented in Figure 4. Reported line widths are corrected for line broadening.

^{113}Cd NMR Spectra. ^{113}Cd NMR spectra were obtained on the same NMR spectrometer operating at 66.57 MHz. A

[2] E. K. Jaffe, unpublished results.

[3] E. K. Jaffe and P. A. Michini, unpublished results.

Table I: ^{13}C NMR Chemical Shifts for 5-Aminolevulinate and Porphobilinogen

sample description	carbon	chemical shift (ppm)
[4-^{13}C]-5-aminolevulinate (ALA)[a]	C_4	205.9
[3,5-^{13}C]porphobilinogen (PBG)[a]	C_3, C_5	123.0, 121.0
enzyme-bound [3,5-^{13}C]PBG	C_3, C_5[b]	121.5, 127.2
enzyme-bound [3,5-^{13}C]PBG with PBG in excess over active sites	C_3, C_5,[b] bound	121.5, 127.2
	C_3, C_5, free	123.0, 121.0
enzyme-bound P-side Schiff base and free [4-^{13}C]ALA	C_4	166.5, 205.9
natural abundance ALA[c]	C_1, C_2, C_3, C_4, C_5	177.5, 28.6, 35.5, 204.1, 48.2
natural abundance PBG[d,e]	C_8, C_{10}[b]	181.1, 182.9
	C_2, C_3, C_4, C_5	116.4, 122.6, 117.5, 120.8
	C_6, C_7, C_9, C_{11}[b]	38.2, 34.7, 32.8, 21.5

[a] In 0.1 M KP_i, pH 7.0, D_2O, 37 °C. [b] Not necessarily respectively. [c] ALA-HCl in D_2O, ambient temperature (Battersby et al., 1973b). [d] In 0.1 M KP_i, pH 6.5, D_iO, 37 °C. [e] Chemical shifts for C_2 and C_{11} of 115.0 and 36.4 ppm were reported for the dimethyl ester of [2,11-^{13}C]PBG in chloroform (Battersby et al., 1973a).

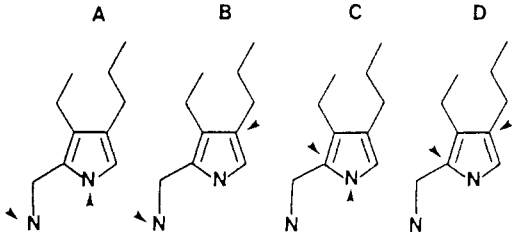

FIGURE 2: Four different isotopically labeled porphobilinogens enzymatically synthesized from an equimolar mixture of [4-^{13}C]ALA and [^{15}N]ALA. Arrows denote ^{15}N- or ^{13}C-labeled positions. Carboxyl oxygens and amino hydrogens are omitted for simplicity.

total of 32K data points were used to cover a 66666-Hz spectral width encompassing the chemical shift range −200 to 800 ppm from 0.1 M $Cd(ClO_4)_2$. A 33° pulse (6 μs) was used. Proton decoupling was not employed. Two data sets were collected: in one experiment 100 000 transients were collected with a 1-s repetition rate; in a second experiment 20 000 transients were collected with a 3.2-s repetition rate. Processing the data with a variety of filter functions revealed no detectable signals.

Results and Discussion

Formation of [3,5-^{13}C]PBG from [4-^{13}C]ALA. ^{13}C NMR (Table I) was used to monitor the formation of [3,5-^{13}C]PBG from [4-^{13}C]ALA. The reaction is illustrated in Figure 1. Prior to addition of enzyme, the only signal observed was a sharp singlet at 205.9 ppm, attributed to the C_4 ketonic carbon of ALA. Apparently ALA is <10% hydrated (predicted chemical shift ~ 100 ppm) under these conditions. After addition of PBG synthase (in the presence of 10 mM 2-mercaptoethanol and 10 μM $ZnCl_2$), there arose two resonances of equal intensity in the aromatic spectral region at 121.0 and 123.0 ppm that are attributed to C_3 and C_5 of PBG. To assign these resonances, PBG was prepared from an equimolar mixture of [4-^{13}C]ALA and [^{15}N]ALA. The mixture of resultant isotopically labeled porphobilinogens is presented in Figure 2: PBG A contains no ^{13}C label; PBG B contains $^{13}C_3$ as a singlet; PBG C contains $^{13}C_5$ coupled to ^{15}N, with an expected J_{CN} ~ 13 Hz (Bundgaard et al., 1975), and PBG D contains both $^{13}C_3$ and $^{13}C_5$ with a small two-bond coupling, J_{CC} = 3.5 Hz (observed in spectra of PBG formed from [4-^{13}C]ALA alone). Analysis of the resulting spectrum allowed us to assign the signals at 121.0 and 123.0 ppm to C_5 and C_3, respectively. Natural abundance spectra of 15 mM PBG in 0.1 M KP_i, pH 6.5, 21 °C, show resonances for the carboxyl groups at 181.1 and 182.9 ppm, the aromatic carbon resonances corresponding to C_3, C_5, C_4, and C_2 at 122.6, 120.8, 117.5, and 116.4 ppm, respectively, and resonances for the remaining aliphatic carbons at 38.2, 34.7, 32.8, and 21.5 ppm. Comparison of proton-coupled and -decoupled spectra allowed assignment of C_4 and C_2 but did not aid in assignment of the aliphatic resonances.

Natural Abundance ^{13}C NMR Spectrum of PBG Synthase. The natural abundance ^{13}C NMR spectrum of PBG synthase is presented in Figure 3B. Although PBG synthase is a relatively large octameric protein of 280 000 daltons, spectra obtained at 37 °C provided sufficiently sharp signals to allow observation of the enzyme-bound equilibrium complex. The line width of the conglomerate signals from the protein carboxylate resonances (182 ppm) and the combined resonances from the C_5 carbon of arginine and the C_ζ carbon of tyrosine (158 ppm) are both approximately 35 Hz. From a Stokes law calculation, the rotational correlation time for the protein is estimated to be 110 ns at 37 °C; with this correlation time, at a magnetic field of 7.05 T the line width for a nonprotonated carbon relaxing by a chemical shift anisotropy mechanism is calculated to be ~25 Hz for a shift anisotropy comparable to a carbonyl group (200 ppm for acetone) (Norton et al., 1978). When spectra were obtained at 4 °C, the line width of the peak at 158 ppm increased substantially with a corresponding ~2-fold decrease in signal to noise.

[4-^{13}C]ALA Bound to PBG Synthase. In our hands, homooctameric PBG synthase contains four functional active sites (Jaffe & Hanes, 1986). The sample used for the experiment illustrated in Figure 3 contains 310 mg of protein, or 4.43 μmol of active sites, each of which can bind two ALA molecules to form one PBG. We have determined that purified PBG synthase contains tightly bound PBG or precursor at approximately 30% of the active sites.[3] Thus, in order to have the substrate fully enzyme bound, addition of [4-^{13}C]ALA to holoenzyme was calculated on the basis of 3.1 μmol of unoccupied active sites and a minimal binding affinity of 0.2 mM.[2]

Parts A and B of Figure 3 show the ^{13}C NMR spectrum of [4-^{13}C]ALA and of holo-PBG synthase. Parts C–F of Figure 3 show spectra obtained after consecutive additions of 1.35 μmol of [4-^{13}C]ALA (90% enriched) to the holoenzyme sample. Two peaks with approximately equal intensity appear at 121.5 and 127.2 ppm with the first addition and increase in intensity with each subsequent addition. The peak at 121.5 ppm lies between the two peaks of free PBG. The peak at 127.2 ppm, although still in the aromatic region, is substantially shifted from the free PBG spectra. These two peaks may be due to C_3 and C_5 of a distorted form of enzyme-bound PBG, or they may arise from the two ^{13}C-labeled carbons of a PBG precursor. Less likely is the possibility that the peaks at 121.5 and 127.2 ppm are exchange-broadened resonances, the former due to both bound PBG carbons and the later due to the two carbons of a PBG precursor. Since the peak at 121.5 ppm is too narrow to cover the 121–123 ppm chemical shift region, if 121.5 ppm were due to both carbons of bound PBG, the

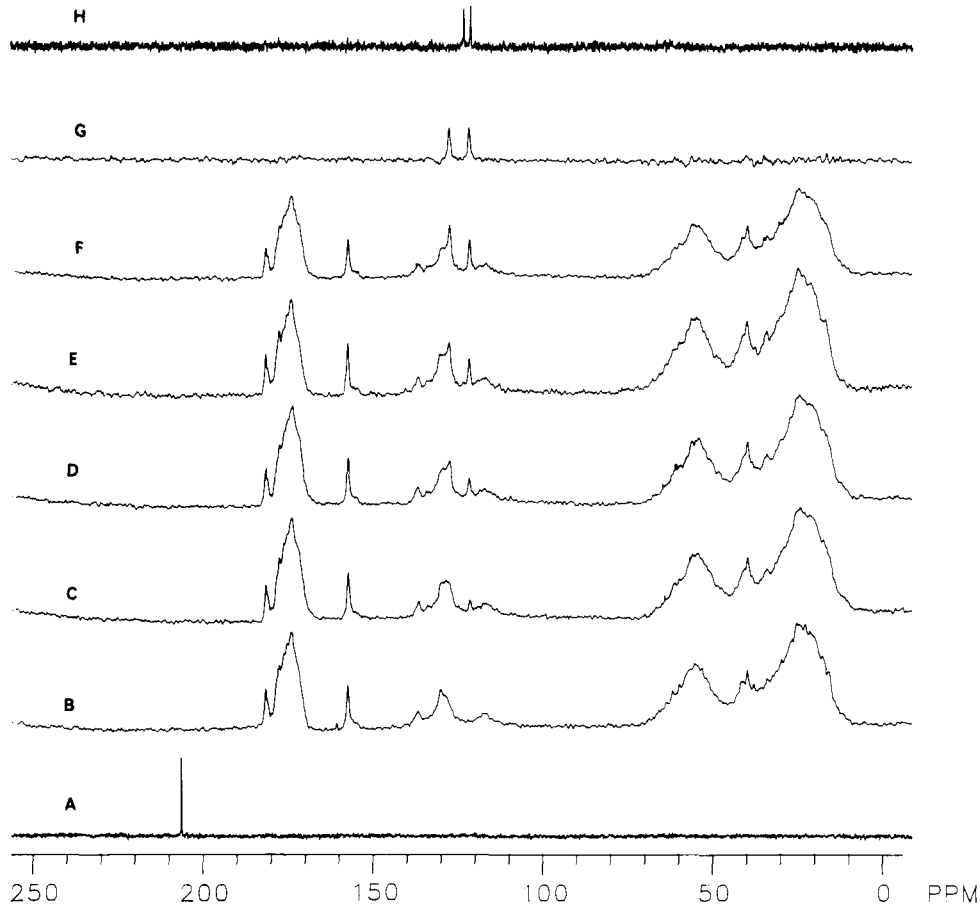

FIGURE 3: ^{13}C NMR spectra of ALA, PBG, and PBG synthase complexes. (A) [4-^{13}C]ALA (4 mM, 352 transients) and (B) holo-PBG synthase. The sample contained 1.8 mL of 172 mg/mL enzyme, 5% D_2O, 10 mM 2-mercaptoethanol, and 10 μM $ZnCl_2$. (C–F) Holoenzyme with successive additions of 15 μL of 90 mM [4-^{13}C]ALA. (G) Free enzyme (spectrum B) was substracted from the spectrum of the bound equilibrium complex (spectrum F) to give the difference spectrum. (H) [3,5-^{13}C]PBG (2 mM, 160 transients). All protein spectra were 12000 transients.

chemical exchange rate (which requires total conversion of ALA to PBG and back) would need to be $\gg$150 s^{-1} in order to obtain exchange narrowing. Although not impossible, this seems improbable as the turnover number for PBG synthase under these conditions is 0.6 s^{-1}. Since an *aromatic* addition product of two ALA molecules that is chemically different from PBG and is a precursor to PBG is unlikely, we conclude that the perturbation causing the chemical shift differences between bound PBG (121.5 and 127.2 ppm for C_3 and C_5, not necessarily respectively) and free PBG (121.0 and 123.0 ppm for C_5 and C_3, respectively) results from the effects of the PBG binding site on the chemical environment of the pyrrole. For instance, a change in pK_a resulting in protonation of the pyrrole, ring strain leading to nonplanarity, or ring current effects from neighboring aromatic amino acid side chains might be responsible for the changes in ^{13}C chemical shift.

Spectrum G of Figure 3 is a difference spectrum between free enzyme (spectrum 3B) and enzyme after the addition of 5 equiv of [4-^{13}C]ALA per octamer (spectrum 3F). The outstanding features are the two resonances at 121.5 and 127.2 ppm, which are notably different from the two resonances of [3,5-^{13}C]PBG in spectrum 3H. There is no detectable intensity at 166.5 ppm, attributable to the P-side Schiff base (see below). The upfield aliphatic region shows many minor alterations in protein resonances at about twice the noise level, which are perhaps attributable to a conformational change but are uninterpretable at this time. There is circumstantial evidence for a conformational change upon formation of the enzyme–substrate complex: (1) the slow activation of MMTS-modified enzyme with Zn(II) and 2-mercaptoethanol (Jaffe et al., 1984) and (2) the observation that PBG synthase crystals shatter upon addition of ALA.[3]

TCA Extract of the Bound Equilibrium Complex. In order to determine if the two major signals of the bound equilibrium complex were due to PBG or some other compound, 10% of the sample was quenched with TCA, and the supernatant was analyzed by ^{13}C NMR (see Experimental Procedures). The observed sharp signals (line width $\sim$ 4 Hz) at 121.0 and 123.0 ppm are attributable to PBG. At a signal to noise of 4 for the PBG resonances, no other signals attributable to quaternary carbons were detected. Thus, other soluble components were less than 25% of the level of PBG. Reaction intermediates covalently bound to the enzyme through an acid-stable bond would not have been detected.

The Bound Equilibrium Complex with Excess PBG. The aromatic region of a difference spectrum between the enzyme-bound equilibrium complex and enzyme is illustrated in Figure 4A. The line widths of the resonances at 127.2 and 121.5 ppm are 51 and 48 Hz ($\pm\sim$5 Hz), respectively. Another holoenzyme sample was prepared that contained the equivalent of nine ALA/octamer. In this case, total ALA is in excess of the active sites, and signals are observed both for free and for bound PBG. Spectrum 4B illustrates the difference spectrum between holoenzyme and the bound equilibrium complex with excess PBG. The intensities from free and bound PBG are in agreement with binding affinities of PBG to holoenzyme.[2] The difference spectrum (spectrum 4B) can be interpreted as two signals (16-Hz line width) at 121.0 and 123.0 ppm, attributable to free PBG, superimposed on the two broader signals (line width $\sim$ 46 Hz) of the bound species, 121.5 and 127.2 ppm. Since free PBG is clearly observed in addition to the bound signals, free and bound PBG are in slow

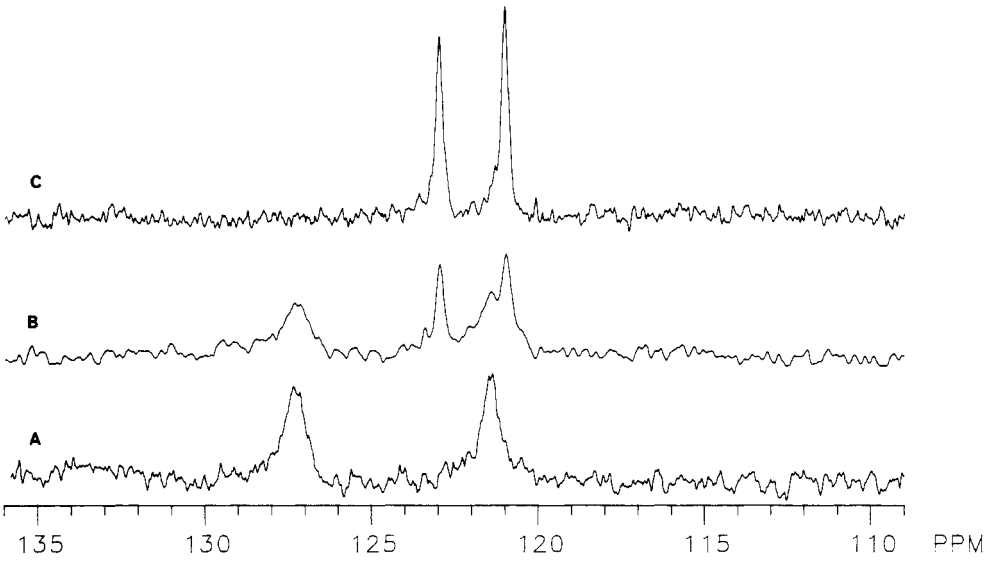

FIGURE 4: Aromatic region of ^{13}C NMR spectra of PBG and PBG synthase complexes. (A) Difference spectrum of six ALA/octamer of holoenzyme, revealing the enzyme-bound PBG spectrum. (B) Difference spectrum of nine ALA/octamer of holoenzyme, showing both free and enzyme-bound PBG. (C) [3,5-^{13}C]PBG prepared from [4-^{13}C]ALA with catalytic amounts of PBG synthase. Spectra were processed with a 5-Hz line-broadening function.

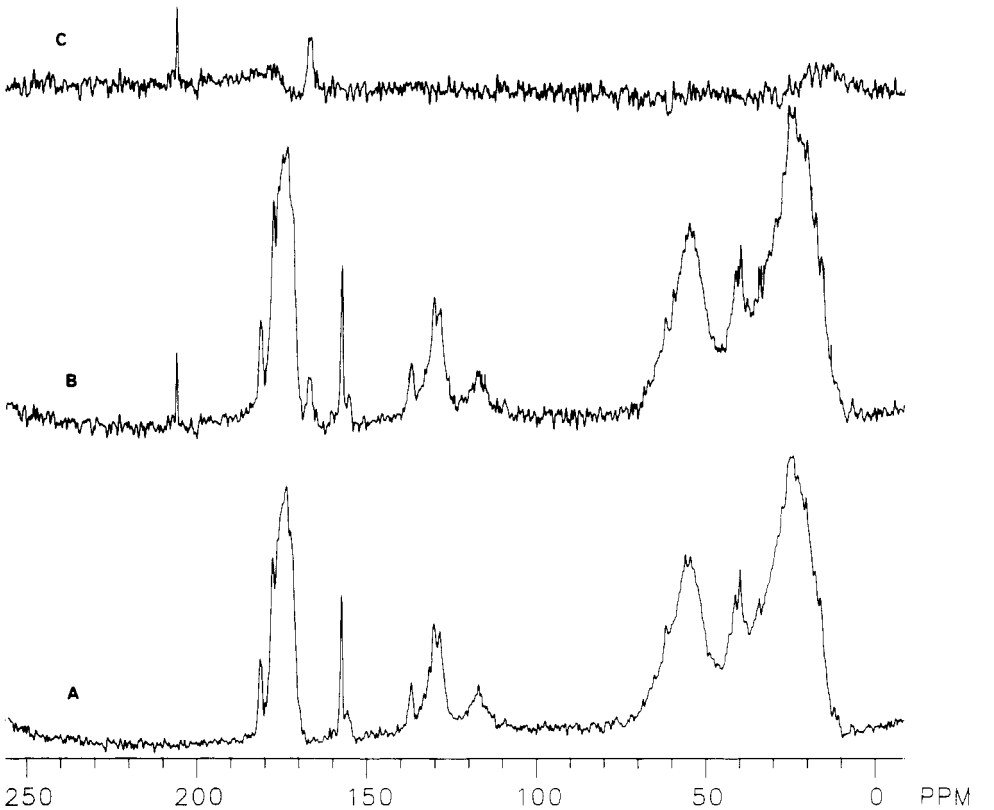

FIGURE 5: ^{13}C NMR spectra of MMTS-modified PBG synthase complexes. (A) MMTS-modified apo-PBG synthase, 2.3 mL of 87 mg/mL, 5% D_2O, 24000 transients. (B) Sample of (A) after the addition of 2.25 μmol of [4-^{13}C]ALA (12000 transients). (C) Difference spectrum, (B) − (A).

exchange. From the line width of free PBG, the upper limit on the exchange rate with the bound species is 10 s^{-1}. The limit on the exchange rate is consistent with the turnover number of 0.6 s^{-1}.

^{13}C NMR of [4-^{13}C]ALA Bound to MMTS-Modified Apo-PBG Synthase. Figure 5A shows the natural abundance ^{13}C NMR spectrum of MMTS-modified apo-PBG synthase. There is no significant difference between this spectrum and the one obtained from holoenzyme (Figure 3A). The enzyme substrate complex of MMTS-modified apo-PBG synthase with [4-^{13}C]ALA is illustrated in Figure 5B. The difference spectrum, Figure 5C, has two notable resonances at 205.9 and 166.5 ppm. The signal at 205.9 ppm is interpreted as free ALA, and the intensity is in agreement with the measured dissociation constant of 0.15 mM for [^{14}C]ALA binding to MMTS-modified enzyme.[2] Although the chemical shift of 205.9 ppm is identical with that of free ALA, the line width of 14 Hz is ~9 Hz broader than the line width for free ALA, consistent with broadening due to exchange with bound ALA or the P-side Schiff base. We can estimate the dissociation rate as less than 9 s^{-1}, and probably less than 5 s^{-1} assuming that some proportion of the line broadening is due to the

sample viscosity. With this dissociation rate and the measured dissociation constant of 0.15 mM, we calculate a maximum rate of binding as 6×10^4 M^{-1} s^{-1}, which is much slower than the diffusion limit, suggesting either a conformational change or a multistep process connecting free ALA with P-side Schiff base. There is no indication of either significant amounts of bound ALA or carbinolamine precursor to the Schiff base, which would be expected to have a chemical shift near 80 ppm (Ray & Harper, 1983).

The signal at 166.5 ppm in the MMTS-modified enzyme–ALA complex is interpreted as the P-side Schiff base for the following reasons: (1) the reaction of ALA with MMTS-modified enzyme is known to proceed at least as far as the P-side Schiff base (Jaffe & Hanes, 1986); (2) imine resonances occur in the region 155–177 ppm (Levy et al., 1980); (3) it is easier to quantitatively trap the P-side Schiff base on MMTS-modified enzyme than on holoenzyme, leading to the suggestion that more of the bound ALA exists as the Schiff base on the modified enzyme than on the holoenzyme (Jaffe & Hanes, 1986). The relative ease of Schiff base trapping was attributed to an increased mole fraction of enzyme containing the Schiff base because modified enzyme is blocked at a step prior to PBG formation. This hypothesis is strongly supported by the ^{13}C NMR data.

Reconstitution of MMTS-Modified Enzyme–Schiff Base Complex with ^{113}Cd. The MMTS-modified enzyme–Schiff base complex illustrated in Figure 5B was reconstituted with 4 molar equiv of ^{113}Cd per octamer in the presence of ca. three [4-^{13}C]ALA per octamer and excess 2-mercaptoethanol. The resulting spectrum was indistinguishable from the holoenzyme equilibrium complex presented in Figure 3F with the exception that additional signals were present at 121.0 and 123.0 ppm, indicative of free PBG as in Figure 4B. From this we conclude that free ALA (205.9 ppm) and P-side Schiff base (166.5 ppm) were converted into bound (121.5 and 127.2 ppm) and free (121.0 and 123.0 ppm) PBG upon reconstitution of the MMTS-modified enzyme-Schiff base complex. The ^{13}C NMR spectrum did not indicate ^{113}Cd–^{13}C spin–spin coupling. This does not exclude direct bonding of the required divalent metal ion to either substrate or product for the PBG synthase catalyzed reaction.

Attempts were made to observe the ^{113}Cd NMR spectra of PBG synthase complexes with this sample. Neither ^{113}Cd NMR experiment described under Experimental Procedures resulted in ^{113}Cd NMR signals for the reconstituted enzyme-bound equilibrium complex. These spectra, acquired at 37 °C, mark our second failure at obtaining a ^{113}Cd NMR spectrum for ^{113}Cd–PBG synthase. Sample preparation for our previous attempt has been described in detail (Jaffe et al., 1984), and those spectra were run at 4 °C. A past study by Sommer and Beyersmann (1984) reported a PBG synthase bound ^{113}Cd NMR signal as a sharp signal at 78.9 ppm. This spectrum was later reported as being attributable to a ^{113}Cd–EDTA complex resulting from an EDTA contaminant in the enzyme sample (Hasnain et al., 1985). Ellis (1983) describes numerous precedents and potential explanations for failure to detect ^{113}Cd bound to a protein.

Conclusions

From this work we can draw a number of conclusions relevant both to PBG synthase and to the general use of ^{13}C NMR for studying enzyme mechanisms. We have demonstrated that it is possible to use ^{13}C NMR to observe ligand signals for the equilibrium complex of the natural substrate bound to a 280 000-dalton protein at 37 °C. The molecular weight of the complex is 5-fold greater than the predicted upper limit for this type of study and has avoided the potential perturbations of cryosolvents (Mackenzie et al., 1984). The thermal stability of the PBG synthase (retention of 85–95% enzymic activity after up to 96 h of spectral accumulations) enables use of elevated temperatures (37 °C), which reduces line-width contributions due to long rotational correlation times. The enzymatic properties of PBG synthase that make it well suited for mechanistic studies by ^{13}C NMR are not unique: quaternary carbons whose bonding changes during the reaction and a slow turnover rate which leads to sharp signals and slow exchange on the NMR time scale for species in chemical exchange (e.g., free and bound). Although large quantities of protein are required for these studies, modern molecular biological methods are reducing the magnitude of this problem. Therefore, we urge others to consider this type of an approach to dissect the chemistry of enzyme-catalyzed reactions.

For PBG synthase in particular, these ^{13}C NMR studies have provided an additional insight into the reaction mechanism. Our previous studies concluded that the MMTS-modified enzyme could form the P-side Schiff base intermediate (Jaffe & Hanes, 1986); equilibrium binding data suggest that MMTS-modified enzyme cannot bind A-side ALA.[2] The NMR studies confirm that the MMTS-modified enzyme can form the Schiff base intermediate. Previous studies also suggested that both the inactive MMTS-modified enzyme and the active Zn(II) and Cd(II) metalloenzymes contained four functional active sites per 280 000 daltons, confirmed here by the observation that free ALA or free PBG for MMTS-modified enzyme and holoenzyme, respectively, is observed after saturation of four active sites. This stoichiometry is in agreement with our binding studies, with the measured dissociation constants, and with the catalytically observed apparent K_m. The NMR results with holoenzyme indicate that $\geq$75% of the species bound to the enzyme under turnover conditions are PBG. This is in agreement with acid-quench studies, which indicate that the mole fraction of PBG in the enzyme-bound complexes at equilibrium is between 0.6 and 0.8, which is markedly different from the solution equilibrium of ALA and PBG, which is 4×10^6 M^{-1} expressed as ($[ALA]^2/[PBG]$).[2]

One question that remains unanswered is the tautomeric structure of the P-side Schiff base intermediate. The chemical shift of 166.5 ppm for the enzyme-bound Schiff base cannot discriminate between a structure containing a double bond between C_4 and the ϵ-amino group of the lysine and a structure containing the double bond between C_4 and C_5 of ALA. Model compounds containing these structural components all have chemical shifts between 150 and 180 ppm (Levy et al., 1980). Future studies with ^{13}C labels in other substrate positions will resolve this ambiguity and will aid in deciphering between the several possible mechanisms for PBG formation from P-side Schiff base and A-side ALA.

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Registry No. PBG A, 108561-51-9; PBG B, 108561-52-0; PBG C, 108561-53-1; PBG D, 108561-54-2; PBG synthase, 9036-37-7; Cd, 7440-43-9.

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Purification and Partial Characterization of the (H^+,K^+)-Transporting Adenosinetriphosphatase from Fundic Mucosa†

Joytirmoy Nandi,‡ Zhou Meng-Ai, and Tushar K. Ray*

Department of Surgery, The State University of New York Health Science Center, Syracuse, New York 13210

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ABSTRACT: The microsomal (H^+,K^+)-ATPase systems from dog and pig fundic mucosa were purified to homogeneity and partially characterized. The method involves sodium dodecyl sulfate (SDS) (0.033% w/v) extraction of the microsomal non-ATPase proteins under appropriate conditions followed by sucrose density gradient centrifugation. Two distinct membrane bands of low (buoyant density = 1.08 g/mL) and high (buoyant density = 1.114 g/mL) densities having distinct enzymatic and chemical composition were harvested. The low-density membrane was highly enriched in Mg^{2+}- or Ca^{2+}-stimulated ATPase and 5′-nucleotidase activities but totally devoid of (H^+,K^+)-ATPase and K^+-*p*-nitrophenylphosphatase activities. The latter two activities were found exclusively in the high-density membrane. SDS–polyacrylamide gel electrophoresis revealed the high-density membranes to consist primarily of a major 100-kilodalton (kDa) protein and a minor 85-kDa glycoprotein, the former being the catalytic subunit of the (H^+,K^+)-ATPase. The amino acid composition of the pure dog (H^+,K^+)-ATPase revealed close similarities with that from pig. The N-terminal amino acid was identified to be lysine as the sole residue. Similar to the high-density membrane-associated pure (H^+,K^+)-ATPase, the low-density membranes containing high Mg^{2+}-ATPase activity also contained a 100-kDa peptide and a 85-kDa glycopeptide in addition to numerous low molecular weight peptides. Also, similar to the pure (H^+,K^+)-ATPase, the Mg^{2+}-ATPase-rich fraction produced an E∼P unstable to hydroxylamine and partially (about 25%) sensitive to K^+ but having a slow turnover. The levels of E∼P produced by the pure (H^+,K^+)-ATPase- and Mg^{2+}-ATPase-rich fractions were 1400 and 178 pmol/mg of protein, respectively. The possibility of the low-density membrane-associated Mg^{2+}-ATPase to be a modified form of the (H^+,K^+)-ATPase has been discussed.

The ouabain-insensitive, membrane-bound (H^+,K^+)-ATPase activity, depending on Mg^{2+} as the sole divalent cation, transports protons across the secretory membrane of the parietal cells in the fundic mucosae (Forte et al., 1976; Ray & Fromm, 1981; Faller et al., 1982). Although much progress has been made in recent years regarding the mechanism of action and regulation of the enzyme system (Forte et al., 1976; Ray & Fromm, 1981; Faller et al., 1982), the precise mechanisms at the molecular level are unknown. One of the approaches for obtaining such molecular insight would be to purify the enzyme to homogeneity in a highly active form and perform appropriate experiments. For instance, studies on the

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* Address correspondence to this author.
‡ Present address: Department of Medicine, SUNY–Health Science Center, Syracuse, NY 13210.

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