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^{15}N and ^{13}C NMR Studies of Ligands Bound to the 280 000-Dalton Protein Porphobilinogen Synthase Elucidate the Structures of Enzyme-Bound Product and a Schiff Base Intermediate†

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ABSTRACT: Porphobilinogen synthase (PBGS) catalyzes the asymmetric condensation of two molecules of 5-aminolevulinic acid (ALA). Despite the 280 000-dalton size of PBGS, much can be learned about the reaction mechanism through ^{13}C and ^{15}N NMR. To our knowledge, these studies represent the largest protein complex for which individual nuclei have been characterized by ^{13}C or ^{15}N NMR. Here we extend our ^{13}C NMR studies to PBGS complexes with [3,3-$^{2}H_2$,3-^{13}C]ALA and report ^{15}N NMR studies of [^{15}N]ALA bound to PBGS. As in our previous ^{13}C NMR studies, observation of enzyme-bound ^{15}N-labeled species was facilitated by deuteration at nitrogens that are attached to slowly exchanging hydrogens. For holo-PBGS at neutral pH, the NMR spectra reflect the structure of the enzyme-bound product porphobilinogen (PBG), whose chemical shifts are uniformly consistent with deprotonation of the amino group whose solution pK_a is 11. Despite this local environment, the protons of the amino group are in rapid exchange with solvent ($k_{exchange} > 10^2$ s^{-1}). For methyl methanethiosulfonate (MMTS) modified PBGS, the NMR spectra reflect the chemistry of an enzyme-bound Schiff base intermediate that is formed between C_4 of ALA and an active-site lysine. The ^{13}C chemical shift of [3,3-$^{2}H_2$,3-^{13}C]ALA confirms that the Schiff base is an imine of *E* stereochemistry. By comparison to model imines formed between [^{15}N]ALA and hydrazine or hydroxylamine, the ^{15}N chemical shift of the enzyme-bound Schiff base suggests that the free amino group is an environment resembling partial deprotonation; again the protons are in rapid exchange with solvent. Deprotonation of the amino group would facilitate formation of a Schiff base between the amino group of the enzyme-bound Schiff base and C_4 of the second ALA substrate. This is the first evidence supporting carbon–nitrogen bond formation as the initial site of interaction between the two substrate molecules.

Porphobilinogen synthase (PBGS)[1] catalyzes the first *common* step in tetrapyrrole biosynthesis. As illustrated in Figure 1, one molecule of 5-aminolevulinate (ALA) forms the propionyl (P) side of PBG, with its amino nitrogen being incorporated into the pyrrole ring (P-side ALA). A second substrate molecule forms the acetyl (A) side of the PBG, retaining its amino group (A-side ALA) (Shemin & Russell, 1953). Zinc is required for enzyme activity, and four Zn(II) per octamer produce full activation (Bevan et al., 1980; Jaffe et al., 1984). The PBGS reaction proceeds via a mechanism where P-side ALA binds first and forms a Schiff base between C_4 and an active-site lysine (Nandi & Shemin, 1968; Jordan & Seehra, 1980). We have shown that Zn(II) and/or sulfhydryl groups are not required for P-side Schiff base formation (Jaffe & Hanes, 1986) but are required for A-side ALA binding (Jaffe & Markham, 1987). Although various reaction mechanisms have been proposed (Nandi & Shemin, 1968; Jordan & Seehra, 1980), there are no concrete data regarding the mechanisms of ALA–ALA bond formation (P-side C_4–A-side C_3 and P-side N–A-side C_4) or which of the bonds forms first.

We have recently demonstrated that ^{13}C NMR can be used to observe nonprotonated carbons or deuterated carbons as ligands bound to PBGS, a 280 000-dalton protein (Jaffe & Markham, 1987, 1988). Our past NMR studies of PBGS utilized ^{13}C label on a quaternary carbon (C_4 of ALA, which becomes C_3 and C_5 of PBG; see Figure 1) and a methylene carbon (C_5 of ALA, which becomes C_2 and C_{11} of PBG, methine and methylene carbons, respectively). In these experiments it was found to be essential to acquire data at elevated temperature (37 °C) to ensure narrow line widths. It was also necessary to deuterate the methylene and methine carbons in order to avoid dipolar broadening from directly attached protons.

The predominant species in the enzyme-bound equilibrium mixture formed from ALA and PBGS at neutral pH was found to be a distorted form of PBG whose chemical shifts closely resembled those of free PBG at pH 12 (Evans et al., 1985). The predominant species in the enzyme-bound complex formed from ALA and methyl methanethiosulfonate (MMTS; Smith et al., 1975) modified PBGS was found to be a Schiff base, which we deduced to be the imine tautomer of *E* stereochemistry. MMTS-modified PBGS had previously been characterized as Zn-free and capable of binding only one of the two substrate molecules; all four of the ALA molecules that bind per octamer can be covalently attached upon incu-

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

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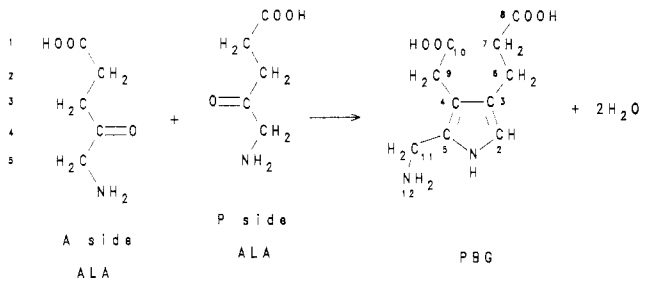

FIGURE 1: PBGS-catalyzed reaction.

bation with $NaBH_4$ (Jaffe et al., 1984, Jaffe & Hanes, 1986). Reaction of MMTS-modified PBGS with Zn and 2-mercaptoethanol results in full recovery of enzymic activity (Jaffe et al., 1984).

To complete the investigation of the carbons whose hybridization changes in the production of PBG from ALA, it is also necessary to characterize the interaction of PBGS and MMTS-modified PBGS by using ALA labeled at C_3. C_3 of ALA is a methylene group that becomes C_4 and C_6 of PBG, a nonprotonated carbon and a methylene carbon, respectively (see Figure 1). Practical considerations for ^{13}C NMR studies of these species are identical with those of [5-^{13}C]ALA (Jaffe & Markham, 1988): the studies require [3,3-$^2H_2$,3-^{13}C]ALA, which can be prepared through enolization of [3-^{13}C]ALA in D_2O.

Additional questions involve the protonation states of the substrate-derived nitrogens in the enzyme-bound product. A direct probe of these nitrogens is ^{15}N NMR. However, a formidable experimental consideration is the low gyromagnetic ratio of ^{15}N, 0.4 times that of ^{13}C, which makes ^{15}N 16% as sensitive as ^{13}C on a given spectrometer. With equivalent relaxation parameters, it would then take 72 h to get an ^{15}N spectra with signal to noise equivalent to our 12-h ^{13}C spectra, which were acquired on a 7.05-T spectrometer. High-quality 50-MHz ^{15}N NMR spectra of [U-^{15}N]proteins as large as 114 kDa have been reported by Smith et al. (1987), and their measurements of ^{15}N relaxation times in proteins have guided our ^{15}N NMR studies, which were performed at 60.8 MHz (14.1 T). In the case of PBGS, there are 670 protein amide bonds per active site; however, the natural abundance of ^{15}N is 0.37% and the ^{15}N chemical shifts of the ligands are separate from the protein-derived resonances. Thus, there is no need to use difference spectra between the enzyme–substrate complex and free enzyme to characterize the enzyme-bound ligands, which greatly reduces instrumental demands. The results of our studies demonstrate that even single ^{15}N nuclei can be observed when bound to large proteins.

Experimental Procedures

Materials. KH_2PO_4, MMTS, and D_2O (99.8%) were purchased from Sigma Chemical Co. [3-^{13}C]ALA (99%) and [^{15}N]ALA (95%) were purchased from Cambridge Isotope Laboratories and used without further purification. All other chemicals were reagent grade or better. All protein NMR samples were prepared in 0.1 M potassium phosphate buffer, pH 6.6 (meter reading) in 97% D_2O or pH 7.0 in 10% D_2O.

Enzyme Preparations and Assays. Purification of bovine liver PBGS, preparation of MMTS-modified PBGS, and activity assays were carried out as previously described (Jaffe et al., 1984). The turnover number for PBGS is 0.6 s^{-1} in aqueous phosphate buffer and 0.25 s^{-1} in D_2O buffer. The enzyme was exchanged into D_2O buffer by repeated 10-fold concentration using either a pressure cell or Amicon Centriprep-10 concentrators.

For spectral acquisition on the enzyme-bound Schiff base complex, PBGS was prepared as the MMTS-modified form. From the same protein sample, holoenzyme was then regenerated through the addition of 2-mercaptoethanol (to 10 mM) and Zn (to 4 equiv/octamer). The PBGS sample used for the complexes with [3,3-$^2H_2$,3-^{13}C]ALA contained 2.9 μmol of active sites in 1.6 mL (97% D_2O). The PBGS sample used for the complexes with protonated [^{15}N]ALA contained 3.2 μmol of active sites in 1.6 mL; the PBGS sample used for deuterated [^{15}N]ALA contained 2.0 μmol of active sites in 1.6 mL.

Deuteration of [3-^{13}C]ALA and [^{15}N]ALA. [3-^{13}C]ALA (40 mM by weight) was deuterated at C_3 by incubation at 37 °C in 0.1 M KP_i buffer, 99.8% D_2O, pH 6.6 (meter reading), for 20 h. Proton-decoupled ^{13}C NMR analysis of [3,3-$^2H_2$,3-^{13}C]ALA showed a 1:2:3:2:1 quintet for the CD_2 species (36.0 ppm, J_{CD} = 19.8 Hz) superimposed on a 1:1:1 triplet for the CHD species (36.3 ppm, J_{CD} = 19.5 Hz) and a singlet for the CH_2 species (36.6 ppm). On the basis of peak heights, the ratio of the species CD_2:CHD:CH_2 was determined to be 75:19:6; this material was used for further studies. The lengthy incubation of ALA in neutral phosphate buffer also facilitates total exchange of the protons at C_5 and N, and results in formation of some autocondensation products (Franck & Stratmann, 1981). The final solution was pale yellow and contained 32 mM ALA by enzymic analysis.

Deuteration of [^{15}N]ALA was achieved by dissolution of [^{15}N]ALA·HCl in D_2O.

NMR Data Acquisition. ^{13}C NMR spectra were obtained at 75.45 MHz on a Bruker AM300 spectrometer by using acquisition parameters identical with those reported previously for our studies with [5,5-$^2H_2$,5-^{13}C]ALA (Jaffe & Markham, 1988). Spectra were acquired with a 45° pulse and a 2.5-s repetition rate and were digitized at a resolution of 1.2 Hz/point. All spectra were obtained at 37 °C. The T_1 of C_3 of deuterated ALA was determined to be 3 s. Protein spectra each included 25 000 scans and were processed with a 30-Hz Lorentzian line-broadening function. Proton decoupling was achieved by using the Waltz-16 pulse sequence.

^{15}N NMR spectra of enzyme-bound complexes were obtained at 60.8 MHz on a Bruker AM600 spectrometer, using a 45° pulse angle and 3-s recycle time, with temperature regulation at 37 °C. The spectral width was 22 kHz, and the internal reference was NH_4^+ (a ubiquitous contaminant in [^{15}N]ALA), which was assigned a chemical shift of −354.0 ppm at neutral pH in H_2O. The T_1 of [^{15}N]ALA was determined to be about 12 s in H_2O and about 100 s in D_2O solution. Protein spectra (32K data points) were processed with 10- or 20-Hz Lorentzian line broadening. Proton decoupling was not employed in the protein experiments to avoid the possibility of signal loss due to a negative nuclear Overhauser effect (Smith et al., 1987). Each of the shown spectra of a ^{15}N protein complex required 24–36 h of data acquisition (see Figures 2 and 3). Attempts to observe resonances of enzyme-bound [^{15}N]PBG in the same samples at 30.4 MHz using the same spectral parameters (except a 2-fold-reduced spectral width) were unsuccessful even after 120 h of data acquisition; this was at least partially due to unavoidable pulse breakthrough, which overloaded the preamplifier for several dwell times. The pH dependence of the chemical shifts of [^{15}N]ALA and [^{15}N]PBG were determined at 30.4 MHz, as were the chemical shifts of the complexes of [^{15}N]ALA with both hydroxylamine and hydrazine.

Studies of Model Schiff Base Complexes. The Schiff base adduct of ALA and hydroxylamine was prepared by mixing

Table I: Chemical Shift Changes (ppm) of Enzyme-Bound PBG Relative to Free PBG in Aqueous Solution at pH 7

atom	bound PBG from free PBG	PBG at pH 12 from pH 7
N_1	−2.0	−3.0
C_2	−2.8	−2.4
C_3	−1.5	−0.4
C_4	−3.4	−3.8
C_5	+6.2	+9.3
C_6	0 ± 1	+1.3
C_{11}	+2.6	+1.0
N_{12}	−12.3	−10.1

equal volumes of 0.2 M NH_2OH and 40 mM [^{15}N]ALA, each of which had been prepared in 0.1 M KP_i buffer, pH 7, 10% D_2O. The Schiff base adduct of ALA and hydrazine contained 39 mM ALA (36% [^{15}N]ALA, 64% [4-^{13}C]ALA) and 79 mM hydrazine in 0.1 M KP_i buffer, pH 7, 10% D_2O. In both cases, the pH was adjusted with KOH for spectral acquisition at higher pH. The basic pH values for which chemical shifts are reported were limited by the chemical stability of the complexes.

Results

Studies with [3-^{13}C]ALA. Catalytic amounts of PBGS converted [3-^{13}C]ALA (C_3 δ = 36.6, J_{CH} = 128 Hz) to [4,6-$^{13}C_2$]PBG (C_4 δ = 118.2, C_6 δ = 22.1, J_{CH} = 125 Hz). When titrated to pH 12, the chemical shifts for C_4 and C_6 of PBG are 114.3 and 22.4 ppm, respectively. The deuterated species, [3,3-$^2H_2$,3-^{13}C]ALA (C_3 δ = 36.0, J_{CD} = 19.8 Hz), was converted to [6,6-$^2H_2$,4,6-$^{13}C_2$]PBG (C_4 δ = 118.3, C_6 δ = 21.4, J_{CD} = 19.2 Hz).

For the complex of MMTS-modified PBGS and [3,3-$^2H_2$,3-^{13}C]ALA, there are two resonances that arise upon addition of the substrate. The resonances are at 36.3 ppm (line width 35 Hz, including contributions from J_{CD}) and 23.4 ppm [line width 60 Hz, presumably effectively ^2H decoupled due to rapid ^2H relaxation (Pople, 1958)] and are assigned to free substrate and the enzyme-bound Schiff base on the basis of chemical shift and line width. As with all other ^{13}C-labeled positions that we have examined, free and enzyme-bound compounds are in slow exchange on the chemical shift time scale, consistent with the slow turnover rate (0.25 s^{-1} in D_2O) of the enzyme. The observed line width of the bound species is comparable to that expected for a totally immobilized CD_2 group [50 Hz, cf. Jaffe and Markham (1988)].

For the complex of [6,6-$^2H_2$,4,6-$^{13}C_2$]PBG and PBGS, there are two substrate-derived signals in the aromatic region of the spectrum, one at 118.2 ppm and the other at 115.9 ppm. These resonances are assigned respectively to the C_4 of free and enzyme-bound PBG on the basis of chemical shifts. The bound peak was ca. 30 Hz broader than that of free PBG, a broadening comparable to that previously observed for the nonprotonated C_3 and C_5 positions and consistent with total immobilization on the enzyme surface. The signals derived from the added substrate are visualized through difference spectra between the enzyme–substrate complex and substrate-free enzyme. In the aliphatic region of the difference spectrum there is a broad peak (ca. 190 Hz) centered at 21.9 ppm, attributable to C_6 of free and bound PBG. Because all previously observed chemical shifts of enzyme-bound PBG resemble those of PBG at pH 12, we would not expect to see a shift larger than 1.3 ppm for this carbon (cf. Table I). Therefore, it is not surprising that there is no significant chemical shift difference for C_6 between free and bound PBG.

^{15}N NMR Studies. ^{15}N NMR studies of free [^{15}N]ALA, [1,12-$^{15}N_2$]PBG, and model Schiff base complexes were carried out at 30.4 MHz. [^{15}N]ALA resonates at −351.0 ppm at neutral pH in H_2O and upon deuteration shifts to −351.8 ppm. In aqueous solution, titration to pH 2 causes an 0.1 ppm downfield shift, while titration to pH 11.6 causes a 13.4 ppm upfield shift (cf. Table II). Formation of a Schiff base adduct with hydroxylamine at pH 7 causes a 2.8 ppm downfield shift in the ^{15}N signal of ALA. However, at pH 9.7 the ^{15}N signal of the ALA–hydroxylamine complex is 7.2 ppm upfield from that of [^{15}N]ALA at neutral pH. Similarly, formation of the Schiff base adduct of ALA and hydrazine at pH 7 causes a 2.1 ppm downfield shift in the ^{15}N signal of ALA. However, at pH 8.5, the ^{15}N signal of the ALA–hydrazine complex is 5.2 ppm upfield from the resonance position of [^{15}N]ALA at neutral pH. Therefore we conclude that, relative to ALA at neutral pH, Schiff base formation to the lysine residue of the enzyme will lead to a downfield shift when the amino group of ALA is protonated but an upfield shift when the amino group is deprotonated, as in the case of ALA itself.

Table II: Effects of pH and Schiff Base Complex Formation Relative to the ^{15}N Chemical Shift of ALA in Neutral Phosphate Buffer

condition	amino N of ALA
enzyme-bound Schiff base	−1.3
ALA at pH 13	−13.4
ALA at pH 2	+0.2
ALA–hydrazine Schiff base, pH 7	+2.1
ALA–hydrazine Schiff base, pH 8.5	−5.2
ALA–hydroxylamine Schiff base, pH 7	+2.8
ALA–hydroxylamine Schiff base, pH 9.7	−7.2

The chemical shifts of [1,12-$^{15}N_2$]PBG are −225.0 and −336.1 ppm, respectively, for the protonated forms of the heteroaromatic and amino nitrogens; the respective chemical shifts are −226.9 and −339.4 ppm for the deuterated forms. In aqueous solution, the heteroaromatic nitrogen resonance shifts 1.8 ppm downfield upon titration to pH 1.8 (protonation of C_8 and C_{10} carboxylates) and shifts 3.0 ppm upfield upon titration to pH 12 (deprotonation of the amino group). The ^{15}N resonance of the amino nitrogen of PBG shifts 0.15 ppm upfield upon titration to pH 1.8 and 10.1 ppm upfield upon titration to pH 12. The one-bond ^1H–^{15}N coupling of the slowly exchanging pyrrole proton was determined to be 98 Hz by ^1H NMR in H_2O.

^{15}N NMR studies of enzyme-bound complexes were carried out at 14.1 T (60.8 MHz) on samples in H_2O and in D_2O in order to assess the possible contributions of dipolar broadening of the signals by directly attached hydrogens. The resulting spectra from the enzyme-bound Schiff base complexes are illustrated in Figure 2 for the protonated and deuterated species. In both cases, there are two sharp ^{15}N resonances, which are assigned to NH_4^+ and ALA (−354.0 and −349.9 ppm in H_2O; −356.1 and −351.9 in D_2O). In addition to these resonances, in both spectra there is a third, broader resonance (−351.1 and −353.4 ppm in H_2O and D_2O, respectively), which is assigned to the amino group of the enzyme-bound Schiff base complex. In both solutions the bound resonance was 2.2–2.5 ppm upfield from free ALA, which is intermediate between the chemical shifts of the protonated and deprotonated signals of the model compounds. In the protonated sample, the line width of ALA was 8 Hz, while the line width of the enzyme-bound species is estimated to be at least 70 Hz. In the sample in D_2O, the ALA signal line width remained 8 Hz, while the enzyme-bound species has a relatively sharp line width of 29 Hz.

The ^{15}N NMR spectra of the enzyme–product complex are illustrated in Figure 3 for the protonated and deuterated species. In this case, presentation of a wide spectral width

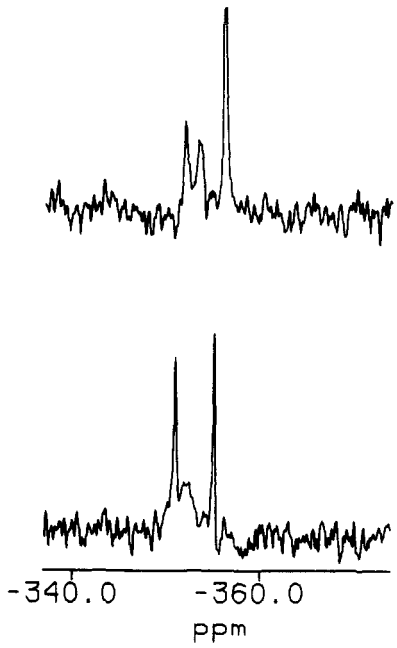

FIGURE 2: ^{15}N NMR spectra of the enzyme-bound Schiff base complex of [^{15}N]ALA and MMTS-modified PBGS. The bottom spectrum represents a sample containing 2.0 mM active sites in buffered H_2O; 30 000 scans were acquired and the data were processed with a 10-Hz Lorentzian line-broadening function. The top spectrum represents a sample of 1.3 mM active sites in buffered D_2O; 43 000 scans were acquired and the data were processed with a 10-Hz Lorentzian line-broadening function. In both cases the farthest upfield resonance is attributed to the ammonium ion, the center resonance derives from the enzyme-bound Schiff base intermediate, and the downfield signal is from free ALA.

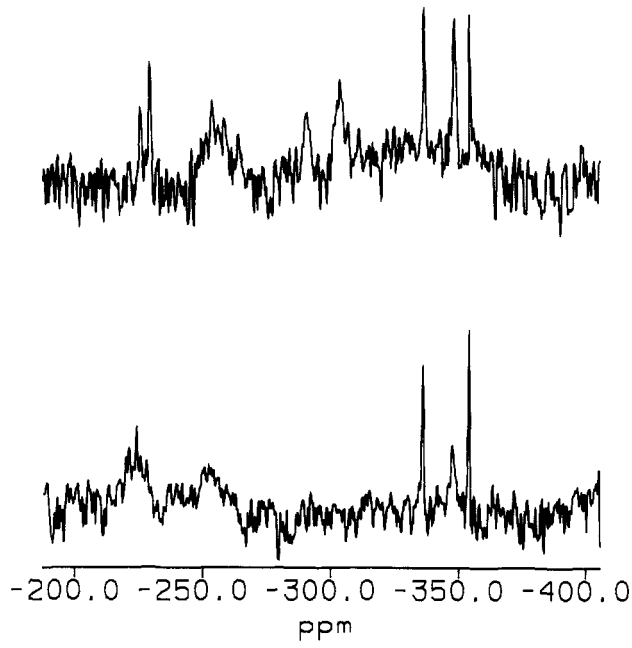

FIGURE 3: ^{15}N NMR spectra of the enzyme-bound product complex of [1,12-$^{15}N_2$]PBG and PBGS. The sample used for the bottom spectrum contained 2 mM active sites in buffered H_2O; 25 000 scans were accumulated and the data were processed with a 20-Hz Lorentzian line-broadening function. The sample used for the top spectrum contained 1.3 mM active sites in buffered D_2O; 40 000 scans were accumulated and the data were processed with a 20-Hz Lorentzian line-broadening function. Moving in the downfield direction, both spectra show the ammonium ion and the N_{12} of enzyme-bound PBG and free PBG; the top spectrum also shows clear signals from the natural abundance ^{15}N of the protein (−310 to −240 ppm). The N_1 of enzyme-bound and free PBG (−230.8 and −227.1 ppm, respectively) are only resolved in the top spectrum.

allows observation of the natural abundance ^{15}N resonances arising from the protein backbone and side chains (−240 to −310 ppm), particularly in the deuterated sample. As in the spectra of the enzyme-bound Schiff base complexes, the amino region of each spectrum contains two sharp signals that correspond to the chemical shifts of NH_4^+ and the amino group of free PBG. There is a third broader signal, at −347.7 and −350.1 ppm in H_2O and D_2O, respectively, which is assigned to the amino group of enzyme-bound PBG. The 2.4 ppm chemical shift change upon deuteration is slightly less than the 3.3 ppm difference observed for the amino group of free PBG. In the protonated sample, the line width for free PBG is 17 Hz, and the line width of the enzyme-bound species is at least 78 Hz. In the sample in D_2O, the PBG amino resonance has approximately the same line width as in H_2O (15 Hz). The enzyme-bound amino species in D_2O is sharper than the corresponding signal in H_2O and exhibits a line width of 56 Hz. In the case of the Schiff base complex, the lack of resolved ^1H–^{15}N splitting indicates fast exchange of the protons with a solvent pool. However, the complexities of exchanging species have deterred a detailed analysis of the line widths.

In contrast to the relatively subtle differences in the amino region of the two spectra illustrated in Figure 3, there is a dramatic difference in the region of the heteroaromatic nitrogen (−226 ppm for free PBG). For the sample in H_2O, there is a very broad signal in the range of −226 ppm with an approximate line width of 400 Hz. There is no obvious signal from the pyrrole nitrogen of free PBG, which is expected to be a doublet due to the coupling with a slowly exchanging proton. In contrast, the aromatic region of the enzyme-bound sample in D_2O shows two well-resolved resonances: one corresponds to free PBG (−227.1 ppm, line width 47 Hz, including contributions from unresolved coupling to deuterium, J_{ND} = 15 Hz), and the other we assign to enzyme-bound PBG (−230.8 ppm, line width 33 Hz). In the case of the aromatic nitrogen there is a substantial advantage to deuteration. Based on the chemical shift anisotropy of the NH group of the imidazole group of histidine hydrochloride (198 ppm, Harbison et al., 1981) and a rotational correlation time of 245 ns, the predicted line widths for the pyrrole nitrogen are 42 Hz for the ND species and an unresolved doublet with the line width of each component of about 110 Hz for the NH species (J_{NH} = 98 Hz). The calculated line widths are in good agreement with the observed line widths, which is a further indication that of the pyrrole moiety of PBG is immobilized at the active site of the enzyme.

Discussion

^{13}C and ^{15}N NMR Probe the Structure of ALA Bound to MMTS-Modified PBGS. PBGS modified with MMTS binds only P-side ALA. The chemical shift of [4-^{13}C]ALA bound to MMTS-modified PBGS confirmed that MMTS-modified PBGS is blocked at the point of the P-side Schiff base intermediate but revealed little about the structure of the intermediate. Three possible structures for the P-side Schiff base are the imine, the C_4–C_5 enamine, C_3–C_4 enamine. Each of these structures exists as two stereoisomers, *E* or *Z*. Based upon model compounds (Breitmaier & Voelter, 1987), the expected chemical shift changes for C_3, C_4, and C_5 of ALA upon formation of the Schiff base are listed in Table III for the various Schiff base structures. The observed chemical shifts are uniformly consistent with the structure illustrated in Figure 4, an imine tautomer with *E* stereochemistry (C_5 trans to the lysine methylene).

Although the chemical shifts of ^{13}C-labeled ALA define the tautomeric structure and stereochemistry of the P-side Schiff

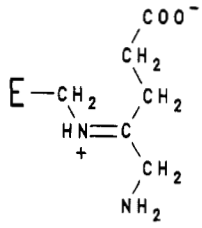

FIGURE 4: Structure of the P-side ALA Schiff base intermediate.

Table III: Expected Chemical Shift Changes upon Formation of the P-Side Schiff Base for a Variety of Schiff Base Structures

substrate	enamine C_3–C_4	enamine C_4–C_5	imine (Z)	imine (E)	obsd shifts
[4-^{13}C]ALA	−40 ± 10	−40 ± 10	−40 ± 10	−40 ± 10	−39.4
[5-^{13}C]ALA	6 ± 4	100 ± 20	−12	≤−3	−2.7
[3-^{13}C]ALA	100 ± 20	6 ± 4	≤−3	−12	−12.5

base, as illustrated in Figure 4, they do not address the protonation states of the imine and amine nitrogens. The chemical shift of [^{15}N]ALA bound to MMTS-modified PBGS shows a 1.6 ppm *upfield* shift for the C_5 amino group of the P-side Schiff base relative to free ALA. To facilitate interpretation of this chemical shift change, we characterized imine formation between [^{15}N]ALA and unlabeled hydoxylamine and hydrazine (Table II). In both cases, formation of a Schiff base to C_4 of ALA causes a 2–3 ppm *downfield* shift for the C_5 amino group. Partial deprotonation of the ALA-derived amino groups of each of these model Schiff base adducts causes the chemical shift to be 5–7 ppm *upfield* of ALA (at neutral pH). Furthermore, deprotonation of the free amino groups of ALA and PBG respectively cause 13.4 and 10.1 ppm *upfield* shifts in the ^{15}N signal of the amino groups. The chemical shift of the enzyme-bound Schiff base is intermediate between the protonated and deprotonated signals of the model compounds. Since the protons of the amino group are in fast exchange with solvent, as shown by lack of resolved ^{1}H–^{15}N coupling, the results could support either the existence of two exchanging populations of enzyme–Schiff base, which differ in the protonation states of the amino group, or the presence of the amino group in an environment that mimics partial deprotonation, perhaps due to hydrogen bonds to protein residues. Deprotonation of the amino moiety of the P-side Schiff base intermediate implies protonation of the imine nitrogen, as illustrated in Figure 4 [cf. Jaffe and Markham (1988)]. Furthermore, deprotonation of the amino group has novel implications with regard to the enzyme-catalyzed formation of PBG. Deprotonation of the C_5 amino group of the P-side Schiff base could promote formation of a second Schiff base to the C_4 of A-side ALA, thus providing the first evidence relating to the mechanism of intersubstrate bond formation.

The ^{15}N resonance from the deuterated enzyme-bound Schiff base is sharper than that of the protonated species (29 vs 70 Hz), but the observed line widths do not correlate well with those calculated for an immobilized amino group on a 280 000-dalton protein (rotational correlation time = 245 ns, chemical shift anisotropy = 0) where the predicted line width for RNH_3^+ is 205 Hz and for RND_3^+ is 12 Hz. The relatively narrow line width for the protonated species indicates that the amino group of the Schiff base intermediate is not completely immobilized. The smaller-than-expected difference between the protonated and deuterated species suggests that the chemical shift anisotropy is not zero, consistent with our interpretation that the substrate-derived nitrogen of the enzyme bound Schiff base is NH_2, an asymmetric group expected to have a nonzero chemical shift anisotropy.

^{13}C and ^{15}N NMR Probe the Structure of Enzyme-Bound PBG. Stoichiometric addition of ALA to PBGS at a protein concentration of >1.5 mM active sites results in the formation of the enzyme-bound equilibrium complex (K_d = 0.15 mM for PBG). Acid quench studies revealed that about 85% of the substrate exists as enzyme-bound PBG. The remainder is distributed between species that are invisible in the NMR spectra of the enzyme-bound equilibrium complex. These species may each be below 5% of the total, or there may be signals arising from species that are in intermediate chemical exchange and thus broadened beyond observation. Consequently, ^{13}C and ^{15}N NMR of isotopically labeled ALA bound to PBGS has not yielded information on the structures of reaction intermediates but has given information on the structure of enzyme-bound PBG, which is in slow exchange with free PBG.

To date, we have observed enzyme-bound product derived from [3-^{13}C]ALA, [4-^{13}C]ALA, [5-^{13}C]ALA, and [^{15}N]ALA, yielding information on positions 1–6, 11, and 12 of PBG (see Figure 1). The observed enzyme-bound signals can be interpreted with respect to line widths and chemical shifts. With the exception of the amino group, the observed line widths suggest that enzyme-bound product is highly immobilized (e.g., the line widths are consistent with the rotational correlation time of a spherical 280 000-dalton entity). The narrow line width of the amino group of PBG in H_2O suggests a rotational correlation time of maximally ca. 100 ns (which would account for the observed line width if the sole relaxation mechanism were dipolar interaction with three attached protons) and indicates that the amino group is more mobile than the pyrrole ring. For each position, the chemical shift of the enzyme-bound species is distinct from "free" PBG. The chemical shift differences between enzyme-bound PBG and free PBG are summarized in Table I. To facilitate structural interpretation of the chemical shift differences between enzyme-bound PBG and PBG in aqueous solution at neutral pH, we obtained the spectra of PBG in aqueous media at pH values between 0 and 13 and in the organic solvents methanol, acetone, acetonitrile, and dimethyl sulfoxide [cf. Jaffe and Markham (1988)]. Chemical shift changes between free and enzyme-bound product are not uniformly consistent with any solvation change. Rather, they are consistent with an effect of enzyme binding similar to deprotonation of the N_{12} amino group, which has a solution pK_a of 11. The changes in chemical shifts of PBG upon going from pH 7 to 12 are included in Table I for comparison.

Although there is reasonable agreement between the chemical shifts of enzyme-bound PBG and PBG at pH 12, it is difficult to rationalize why the enzyme effects a ≥5 pH unit alteration in the pK_a of an amino group that is not obviously involved in the formation of product from substrate. Perhaps deprotonation of the A-side ALA amino group is necessary to stabilize an enzyme-bound reaction intermediate that has yet to be identified. A somewhat related possibility is that the N_{12} amino group of PBG, which derives from the amino group of A-side ALA, is deprotonated in order to facilitate ligand formation to the active-site Zn(II). For instance, ligation to Zn(II) would fix the geometry of A-side ALA and ensure formation of the proper stereochemistry in the proposed Schiff base between C_4 of A-side ALA and N of P-side ALA. In support of an interaction between the A-side amino group and Zn(II), we have demonstrated that either sulfhydryl groups or Zn(II) are required for A-side ALA binding. Furthermore, we have observed that Zn(II) is required for PBG binding.[2]

[2] Unpublished results.

An interesting corollary to the proposed ligation of the A-side amino group and Zn is the increased mobility of the amino group of PBG with respect to the pyrrole ring, as apparent from the ^{15}N line widths. This suggests rapid ligand exchange and might account for our difficulties in observing ^{113}Cd NMR spectra of ^{113}Cd bound to PBGS (Jaffe et al., 1984; Jaffe & Markham, 1987). On the other hand, a counterargument to the *necessity* of the A-side amino group for PBG formation is the report that a mixture of PBGS, ALA, and levulinic acid can form a pyrrole in which levulinic acid must mimic A-side ALA (Nandi & Shemin, 1968); however, no evidence was reported that formation of the mixed pyrrole is enzyme catalyzed. Finally, it is possible that the observed chemical shifts for enzyme-bound product are not indicative of deprotonation at N_{12} but rather of an unusual environment at the enzyme active site that cannot be modeled in solution studies of PBG.

^{15}N NMR Studies of a 280 000-Dalton Protein. This is the first report of ^{15}N studies that examine individual nuclei on a protein larger than 114 kDa. For PBGS, spectral acquisition for a 1–2 mM enzyme-bound ^{15}N species required 24–36 h of spectral acquisition on a 14.1-T spectrometer. As in ^{13}C studies of large proteins, ^{15}N NMR studies are facilitated by deuteration at the nitrogen positions that do not rapidly exchange their directly attached protons with water. For both ^{13}C and ^{15}N, 20–90-Hz line widths were observed for the enzyme-bound species. Thus, in addition to what we have learned about the structure of ligands bound to PBGS, these studies help to establish conditions for observation of ligands bound to a large protein.

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References

Bevan, D. R., Bodlaender, P., & Shemin, D. (1980) *J. Biol. Chem. 256*, 2030–2035.

Breitmaier, E., & Voelter, W. (1987) *Carbon-13 NMR Spectroscopy*, VCH, New York.

Evans, J. N. S., Fagerness, P. E., Mackenzie, N. E., & Scott, A. I. (1985) *Magn. Reson. Chem. 23*, 939–944.

Franck, B., & Stratmann, H. (1981) *Heterocycles 15*, 919–923.

Harbison, G., Herzfeld, J., & Griffin, R. G. (1981) *J. Am. Chem. Soc. 103*, 4752–4754.

Jaffe, E. K., & Hanes, D. (1986) *J. Biol. Chem. 261*, 9348–9353.

Jaffe, E. K., & Markham, G. D. (1987) *Biochemistry 26*, 4258–4264.

Jaffe, E. K., & Markham, G. D. (1988) *Biochemistry 27*, 4475–4481.

Jaffe, E. K., Salowe, S. P., Chen, N. T., & Dehaven, P. (1984) *J. Biol. Chem. 259*, 5032–5036.

Jordan, P. M., & Seehra, J. S. (1980) *J. Chem. Soc., Chem. Commun.* 240–242.

Nandi, D. L., & Shemin, D. (1968) *J. Biol. Chem. 243*, 1236–1242.

Pople, J. A. (1958) *Mol. Phys. 1*, 168–174.

Shemin, D., & Russell, C. S. (1953) *J. Am. Chem. Soc. 75*, 4873.

Smith, D. J., Maggio, E. T., & Kenyon, G. L. (1975) *Biochemistry 14*, 766–771.

Smith, G. M., Yu, L. P., & Domingues, D. J. (1987) *Biochemistry 26*, 2202–2207.