^{13}C NMR Studies of Methylene and Methine Carbons of Substrate Bound to a 280 000-Dalton Protein, Porphobilinogen Synthase†

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Received December 23, 1987; Revised Manuscript Received February 12, 1988

ABSTRACT: ^{13}C NMR has been used to observe the equilibrium complex of [5,5-^{2}H,5-^{13}C]-5-aminolevulinate ([5,5-^{2}H,5-^{13}C]ALA) bound to porphobilinogen (PBG) synthase (5-aminolevulinate dehydratase), a 280 000-dalton protein. [5,5-^{2}H,5-^{13}C]ALA (chemical shift 46.9 ppm in D_2O) was prepared from [5-^{13}C]ALA through enolization in deuteriated neutral potassium phosphate buffer. In the PBG synthase reaction [5,5-^{2}H,5-^{13}C]ALA forms [2,11,11-^{2}H,2,11-^{13}C]PBG (chemical shifts 116.2 ppm for C_2 and 34.2 ppm for C_{11} in D_2O). For the complex formed between [5,5-^{2}H,5-^{13}C]ALA and methyl methanethiosulfonate (MMTS) modified PBG synthase, which does not catalyze PBG formation but can form a Schiff base adduct, the chemical shift of 44.2 ppm (line width 92 Hz) identifies an imine structure as the predominant tautomeric form of the Schiff base. By comparison to model compounds, the stereochemistry of the imine has been deduced; however, the protonation state of the imine nitrogen remains unresolved. Reconstitution of the MMTS-modified enzyme–Schiff base complex with Zn(II) and 2-mercaptoethanol results in the holoenzyme-bound equilibrium complex; this complex contains predominantly enzyme-bound PBG, and spectra reveal two peaks from bound PBG and two from free PBG. For bound PBG, C_2 is −2.8 ppm from the free signal and C_{11} is +2.6 ppm from the free signal; the line widths of the bound signals are 55 and 75 Hz, respectively. To aid in interpretation of these shifts, and those previously observed with [4-^{13}C]ALA as substrate (which forms [3,5-^{13}C]PBG) [Jaffe, E. K., & Markham, G. D. (1987) *Biochemistry 26*, 4258–4264], the ^{13}C NMR chemical shifts of PBG were investigated as functions of pH and a variety of organic solvents. The observed shifts of bound PBG are not consistent with simple protonation/deprotonation of PBG nor with changes that can be duplicated by solvation by simple organic solvents.

We have recently demonstrated that ^{13}C NMR can be utilized to observe single nonprotonated carbons of substrate bound to a protein as large as 280 000 daltons (Jaffe & Markham, 1987). The protein under study is porphobilinogen (PBG)[1] synthase [5-aminolevulinate (ALA) dehydratase; EC 4.2.1.24], which catalyzes the conversion of two molecules of ALA to PBG (Shemin & Russell, 1953). This unique bisubstrate addition reaction occurs in the biosynthetic pathway toward porphyrin, chlorophyll, vitamin B_{12}, and a broad spectrum of tetrapyrrole pigments.

Our past work utilized a ^{13}C label on a nonprotonated carbon (C_4 of ALA, which forms the C_3 and C_5 of PBG; see Figure 1). In these experiments, it was found that the predominant species in the enzyme-bound equilibrium mixture was a distorted PBG whose ^{13}C resonances were distinct from the free PBG with which it was in slow exchange. Furthermore, using a modified form of PBG synthase, it was possible to observe a complex with ALA bound as a Schiff base, believed to be an intermediate in the formation of PBG. However, in order to fully characterize the chemistry of substrate and product bound to PBG synthase, we must also investigate the chemical shifts of the methylene carbons that change hybridization during the reaction (e.g., C_3 and C_5 of ALA). Unfortunately, the protons directly attached to methylene carbons lead to dipolar interactions which are predicted to result in several hundred hertz wide lines for compounds bound to large proteins (Wilbur et al., 1976; Allerhand, 1979). One possible mechanism for reducing the dipolar relaxation problem is to replace the directly attached protons with deuterons, which have a smaller magnetic moment. Replacement of each hydrogen by deuterium should lead to an ~16-fold reduction in the efficiency of dipolar relaxation. Although in small molecules deuteriation leads to well-resolved splittings of ^{13}C NMR spectra due to ^{2}H–^{13}C coupling, rapid ^{2}H relaxation in macromolecular complexes is anticipated to effectively decouple ^{13}C from ^{2}H (Pople, 1958), obviating the need to use deuterium decoupling in studies of large molecules.

This study investigates the interaction of PBG synthase with [5,5-^{2}H,5-^{13}C]ALA, which becomes [2,11,11-^{2}H,2,11-^{13}C]-PBG (see Figure 1; ^{13}C atoms are marked by arrows). In the PBG synthase catalyzed reaction, a Schiff base intermediate forms between one ALA and a lysine residue on the enzyme (Nandi & Shemin, 1968). The site of Schiff base formation is to the ALA which becomes the P (propionyl side chain) side of PBG, first demonstrated by Seehra and Jordan (1980). The C_2 of PBG arises from a carbon α to the sp^2 carbon of the Schiff base, and in the conversion from the C_5 methylene group of ALA to the aromatic C_2 carbon of PBG the *pro-R* proton is lost (Chaudry & Jordan, 1976). The C_{11} position of PBG lies on the A (acetyl side chain) side, and this carbon does not change hybridization during the reaction. The data presented here indicate that deuteriation at ^{13}C results in reasonably

† Supported in part by grants from the National Institutes of Health, ES-03654 (E.K.J.), CA-06927, and RR-05539, and an appropriation from the Commonwealth of Pennsylvania.

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

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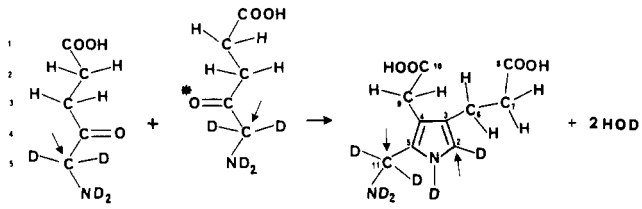

FIGURE 1: Formation of [2,11,11-^{2}H,2,11-^{13}C]porphobilinogen (PBG) from [5,5-^{2}H,5-^{13}C]-5-aminolevulinate (ALA) in D_2O. The carbons of ALA and PBG are numbered; ^{13}C labeling is depicted by arrows; (*) denotes the site of enzyme-bound Schiff base formation.

sharp signals for enzyme-bound species, allowing further insight into the structures of enzyme–substrate complexes.

Experimental Procedures

Materials. KH_2PO_4, methyl methanethiosulfonate (MMTS), and D_2O (99.8% and 99.96%) were purchased from Sigma Chemical Co. [5-^{13}C]-5-Aminolevulinate hydrochloride (99% enriched) was purchased from Merck Sharp & Dohme Isotopes. All NMR samples were prepared in 0.1 M potassium phosphate, pH 6.6 (meter reading), in 97% D_2O.

Enzyme Preparations and Assays. Purification of bovine liver PBG synthase and the preparation and characterization of MMTS-modified PBG synthase were carried out as previously described (Jaffe et al., 1984). MMTS is a small, reversible sulfhydryl modification reagent first introduced by Kenyon and co-workers (Smith et al., 1975). MMTS modification of PBG synthase results in a Zn(II)-free enzyme that cannot catalyze PBG formation (Jaffe et al., 1984) but can form the P-side Schiff base intermediate as shown by BH_4^- trapping studies (Jaffe & Hanes, 1986). The specific activity of MMTS-modified PBG synthase, after reactivation with Zn(II) and 2-mercaptoethanol, was 21 μmol h^{-1} mg^{-1} in 100 mM KP_i, pH 6.8, 10 mM 2-mercaptoethanol, and 10 μM Zn. A unit is defined as 1 μmol h^{-1} at 37 °C. Following data acquisition, which required about 5 days at 37 °C, the enzyme samples were shown to retain 75% of the original enzymic activity. Like many enzymes, PBG synthase is stabilized by high protein concentration and the presence of substrate. Some loss of activity was shown to be due to trace protease activity in the enzyme sample.

Determination of Enzyme Activity under Conditions of Deuteriations. Deuteriation of both enzyme and substrate can affect the PBG synthase catalyzed reaction either through changes in protein structure, through solvent isotope effects, and/or through primary deuterium isotope effects from the four protons that are lost during the reaction (both C_3 protons from A-side ALA and one C_5 and one nitrogen proton from P-side ALA). The combined deuterium isotope effects on V_{max} from solvent and protein were measured by comparing enzymic activity of deuteriated PBG synthase (exchanged for 48 h at 4 °C in 90% D_2O) with that of protonated PBG synthase with protonated ALA as substrate; under these conditions the amino group of ALA is 90% deuteriated. In this case deuteriation decreased enzymic activity to 70% of the protonated system. The total primary deuterium isotope effect was measured by using [3,3,5,5-^{2}H]ALA as substrate for protonated enzyme in protonated buffer (10% D_2O). [3,3,5,5-^{2}H]ALA was prepared by exhaustive enolization (15 h, 37 °C) in 99.96% D_2O and 0.2 M KP_i, pH 7.0 (see below). Under these conditions, enzymic activity was reduced to 60% of the rate when [3,3,5,5-^{1}H]ALA was used as substrate. Both isotope effect determinations used a 5-min fixed-time assay and 10 mM ALA, which is saturating under normal conditions ($K_M \simeq 0.15$ mM). Thus, the turnover rate of deuteriated enzyme in D_2O with deuteriated ALA as substrate is $\simeq 0.7 \times 0.6 \times 0.6$ s^{-1} = 0.25 s^{-1} (the normal turnover number is 0.6 s^{-1}).

Deuteriation of [5-^{13}C]ALA. A total of 3.6 mg of [5-^{13}C]ALA-HCl was dissolved in 0.5 mL of 99.96% D_2O. This solution was transferred to a tube in which 50 μmol of KP_i, pH 7 (in H_2O), had been lyophilized. The solution was neutralized with 19 μmol of KOD and placed at 37 °C. The exchange of deuterium into the C_5 position, which occurs through buffer-catalyzed enolization (Michini & Jaffe, 1987), was monitored by ^{13}C NMR as illustrated in Figure 2. Following complete exchange, the [5,5-^{2}H,5-^{13}C]ALA was stored at −20 °C between additions to the NMR sample of PBG synthase. Complete exchange of the C_5 protons of ALA through enolization in KP_i/D_2O also results in partial exchange of the C_3 protons since ALA can form two different enolates, C_5–C_4 and C_3–C_4. At pH 6.4 (meter reading in D_2O), 37 °C, the $t_{1/2}$ for deuterium exchange at C_5 and C_3 are approximately 15 min and 3 h, respectively (Michini & Jaffe, 1987). Procedures for deuteriation of ALA specifically at C_3 or C_5 have been published (Lerman & Whitaker, 1980).

pH Titrations of ^{13}C-Labeled PBG. [3,5-^{13}C]PBG was synthesized from 20 μmol of [4-^{13}C]ALA in 2 mL of 10 mM KP_i, 10 mM 2-mercaptoethanol, and 3 μM Zn (final pH 6.8) by using 6.5 units of PBG synthase. After 3 h at 37 °C, the sample was brought to 10% D_2O, the pH was determined to be 6.3, and the ^{13}C NMR spectrum was obtained. ^{13}C NMR spectra (25 °C) were obtained at pH 7.13, 8.82, 9.40, 10.27, 10.72, 11.44, 12.19, and 12.69 after which the solution was neutralized with HCl. The pH was adjusted using 5-μmol aliquots of KOH up to pH ~11, above which 25-μmol aliquots were added. Then, 1 N HCl (or 12 N) was used to decrease the pH, and spectra were obtained at pH 6.33, 4.93, 4.23, 3.84, 3.25, 2.77, 2.29, 1.72, 1.04, and 0.63. A similar procedure was used to obtain the pH titration curve of [2,11-^{13}C]PBG which was synthesized from 20 μmol of [5-^{13}C]ALA. In this case the pH values at which the ^{13}C NMR spectra were obtained were 6.46, 7.17, 7.90, 8.90, 9.46, 10.08, 10.57, 11.40, 12.22, 12.71, 7.22, 6.60, 5.78, 5.52, 4.60, 4.03, 3.76, 2.95, 2.63, 2.21, 1.60, 0.75, and 0.20. Chemical shifts are referenced to an external standard of 0.1 M tetramethylammonium chloride which was assigned as 47.9 ppm.

Effects of Organic Solvents on ^{13}C NMR Chemical Shifts on PBG. [3,5-^{13}C]PBG and [2,11-^{13}C]PBG were each synthesized from 20 μmol of [4-^{13}C]ALA or [5-^{13}C]ALA, respectively, in 10 mM KP_i as described above. When PBG production was complete (determined by analysis with Ehrlich's reagent), the reaction mixtures were adjusted to pH 7 with KOH and the samples were lyophilized. The samples were dissolved in 2 mL of 91% CD_3OD and 9% D_2O, followed by ^{13}C NMR characterization with low-power ^{1}H decoupling. The samples were lyophilized and dissolved in 77% $(CD_3)_2CO$ and 27% D_2O, and the ^{13}C NMR spectra were obtained. Deuteriated solvents were utilized in order to minimize the intensity of the natural abundance ^{13}C NMR signal from solvent. The procedure of lyophilization, dissolution, and spectral acquisition was then repeated with 60% CD_3CN and 40% D_2O and subsequently 91% $(CD_3)_2SO$ and 9% D_2O as solvents.

Effects of Deuteriation on Organic Amines. The organic amines methylamine, glycine, and glycine methyl ester were each prepared at 0.1 M in 10 mM KP_i, final pH 7.0. Two-milliliter aliquots were lyophilized and redissolved in both 10% D_2O and 99.8% D_2O for ^{13}C NMR spectral analysis. ^{13}C NMR characterization of these model compounds allowed quantification of the deuterium isotope shift resulting from

Table I: ^{13}C NMR Parameters of [2,11-^{13}C]PBG Production from [5-^{13}C]ALA Free in Solution and Bound to PBG Synthase

sample description	carbon	chemical shift (ppm)	coupling constants (Hz)
[5-^{13}C]ALA, pH 6.8, in KP_i, H_2O	5	47.8	J_{CH} = 144
[5-^{13}C]ALA, pH 6.4 (meter reading), in KP_i, D_2O	5	47.4	J_{CH} = 144
[5,5-^{2}H,5-^{13}C]ALA, pH 6.4 (meter), in KP_i, D_2O	5	46.9	J_{CD} = 22
enzyme-bound P-side Schiff base and free [5,5-^{2}H,5-^{13}C]ALA	5 (free)	46.9	
	5 (Schiff base)	44.2	
[2,11-^{13}C]PBG, pH 6.8, in KP_i, H_2O	2	116.7	J_{CH} = 184
	11	35.1	J_{CH} = 144
[2,11,11-^{2}H,2,11-^{13}C]PBG, pH 6.4 (meter), in KP_i, D_2O	2	116.2	J_{CH} = 28
	11	34.2	J_{CD} = 20
enzyme-bound PBG and free [2,11,11-^{2}H,2,11-^{13}C]PBG	2 (free)	116.2	
	2 (bound)	113.4	
	11 (free)	34.2	
	11 (bound)	36.8	

deuteriating an amino group adjacent to a methylene carbon analogous to the C_5 of ALA, i.e., $RCH_2NH_3^+$ vs $RCH_2ND_3^+$.

^{13}C NMR Data Acquisition. Spectra were obtained at 75.45 MHz on a Nicolet NT-300 wide-bore spectrometer with GN series (bottom entry) probes. Some spectra of small molecules were obtained on a Bruker AM300 spectrometer. All spectra used a 45° pulse angle and 2-s recycle time, with temperature regulation at 37 °C. Except where specified, proton decoupling was not utilized. The spectral width was ±10 000 Hz from the carrier using quadrature phase detection. The chemical shift reference was external dioxane set to 67.4 ppm. Protein spectra (32K data points) were processed by using a Gaussian line broadening function of 30 or 50 Hz (see figure legends). Most spectra of small molecules were processed by using a Lorenzian line broadening function of 3 or 15 Hz (see figure legends). The T_1 of [5,5-^{2}H,5-^{13}C]ALA was determined by using the 180°–τ–90° sequence to be ~6 s.

Results

Formation of [2,11-^{13}C]PBG from [5-^{13}C]ALA. ^{13}C NMR was used to monitor the formation of [2,11-^{13}C]PBG from [5-^{13}C]ALA. Chemical shifts and coupling constants are summarized in Table I. Prior to addition of enzyme the only signal observed was a sharp singlet at 47.8 ppm (with proton decoupling; the signal was a triplet in proton-coupled spectra). This signal is attributed to the C_5 methylene carbon of ALA. From the observation of only one resonance that is clearly due to a CH_2 group, we conclude that the steady-state concentrations of the C_4–C_5 enol or enolate tautomers of ALA are less than 5% of the total ALA under these conditions (0.1 M KP_i, pH 6.8, 10% D_2O, 37 °C). Data on conjugated systems (e.g., acetylacetone) predict the carbon α to the ketone to be shifted by ~+50 ppm in the enol tautomer (Breitmaier & Voelter, 1987).

Upon addition of PBG synthase, 10 mM 2-mercaptoethanol, and 10 μM $ZnCl_2$ (final concentrations) there arose two resonances of equal intensity at 35.1 and 116.7 ppm.[2] These signals are assigned to C_{11} and C_2, respectively, on the basis of the sp^3 hybridization of C_{11} and the aromatic character of C_2. Proton-coupled spectra showed the C_{11} resonance to be a triplet, J_{CH} = 144 Hz, and the C_2 signal to be a doublet, J_{CH} = 184 Hz. At pH 8 in pyrophosphate buffer and 10% D_2O, the chemical shifts of these signals were reported as 34.3 and 116.1 ppm, respectively (Evans et al., 1985).

Formation of [5,5-^{2}H,5-^{13}C]ALA and Its Conversion to [2,11,11-^{2}H,2,11-^{13}C]PBG. The formation of [5,5-^{2}H,5-^{13}C]ALA from [5-^{13}C]ALA in neutral KP_i (99.96% D_2O) was monitored by ^{13}C NMR as illustrated in Figure 2 (see Experimental Procedures). Figure 2A shows an early time point, when most of the C_5 carbons exist as CH_2 (47.42 ppm), and the deuterium isotope shifted, deuterium-coupled CHD signal is undetected. The insert in Figure 2, which illustrates a spectrum of a slightly later time point with proton coupling, reveals that the CH_2 signal is 1:2:1 triplet (J_{CH} = 143 Hz), while the CHD signal is an isotope-shifted doublet (J_{CH} = 142 Hz) of 1:1:1 triplets (J_{CD} = 22 Hz). Later time points, spectra B, C, and D, show the evolution of the proton-decoupled signal through CHD (a 1:1:1 triplet, centered at 47.16 ppm, where the downfield line of the CHD multiplet falls under the CH_2 singlet) to the CD_2 1:2:3:2:1 quintet (centered at 46.88 ppm, J_{CD} = 22 Hz). The overall deuterium isotope shift is −0.27 ppm per deuteron. Unfortunately, at 75.4 MHz the isotope shift is nearly equal to the carbon deuterium coupling constant, producing an interpretable but complex spectrum at intermediate time points in the exchange (e.g., Figure 2B,C).

The spectra also reveal a solvent isotope effect on the ^{13}C resonance of the C_5 of ALA. All other conditions being identical (pH, temperature, buffer, and ALA concentration),

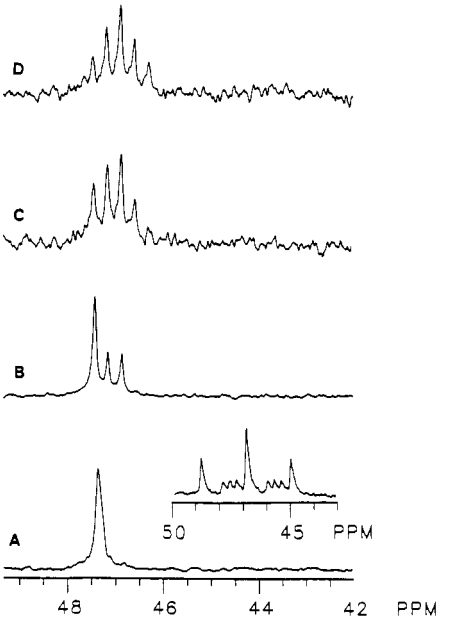

FIGURE 2: Time course for deuterium exchange at C_5 of [5-^{13}C]ALA. Sample components and spectral parameters are described under Experimental Procedures. Spectra A–D depict ^{1}H-decoupled spectra of [5-^{13}C]ALA at progressive times during the deuteriation (processed with 3-Hz line broadening function). The insert is an early time point shown without proton decoupling. The decrease in signal to noise from spectrum A to spectrum D is largely the result of the loss of the nuclear Overhauser effect as protons are lost. See Results for description of signal multiplicities.

[2] In addition to the peaks from ^{13}C-labeled PBG, the spectra showed the natural abundance ^{13}C signals of the added 2-mercaptoethanol at 63.6 and 26.2 ppm.

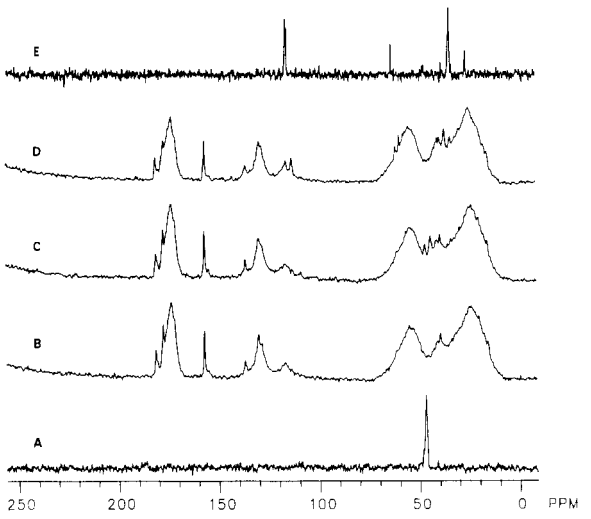

FIGURE 3: ^{13}C NMR spectra of ALA, PBG, and PBG synthase complexes. (A) [5,5-^{2}H,5-^{13}C]ALA; spectrum processed with 15-Hz line broadening. The sample is identical with that of the spectrum presented in Figure 2D which illustrates the ^{13}C–^{2}H coupling. (B) MMTS-modified PBG synthase; 2.0 mL of 123 mg/mL (3.5 μmol of active sites), 97% D_2O, 32 000 transients, 30-Hz line broadening. (C) MMTS-modified enzyme after the addition of 2.34 μmol of [5,5-^{2}H,5-^{13}C]ALA; 36 000 transients, 30-Hz line broadening. (D) Reconstituted equilibrium reaction mixture. To the sample in spectrum C was added 3 μmol of Zn(II), 40 μmol of 2-mercaptoethanol, and 1.53 μmol of [5,5-^{2}H,5-^{13}C]ALA. A total of 44 000 transients were collected, and 30-Hz line broadening was used. (E) [2,11,11-^{2}H,2,11-^{13}C]PBG[2], with proton decoupling; spectrum processed with 15-Hz line broadening (see Results for description of signal multiplicity).

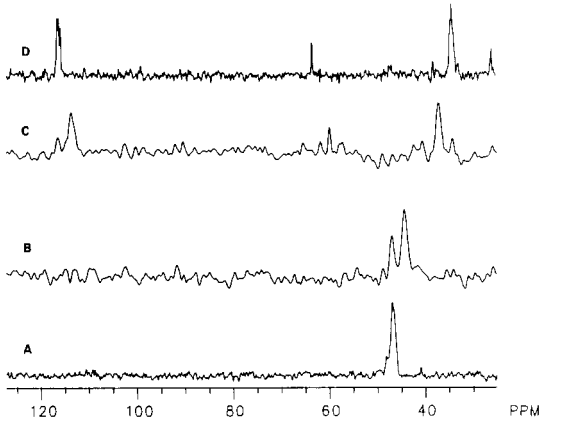

FIGURE 4: Difference spectra between PBG synthase and PBG synthase complexes. The difference spectra presented were processed with 50-Hz line broadening prior to subtraction. (A) [5,5-^{2}H,5-^{13}C]ALA, processed with 15-Hz line broadening. (B) Difference spectrum of enzyme-bound Schiff base complex and MMTS-modified PBG synthase (Figure 3C minus Figure 3B). (C) Difference spectrum of enzyme-bound equilibrium reaction mixture and MMTS-modified PBG synthase (Figure 3D minus Figure 3B).[4] (D) [2,11,11-^{2}H,2,11-^{13}C]PBG, processed with 15-Hz line broadening.[2]

the use of D_2O as solvent produces a –0.4 ppm shift in the C_5 signal from the CH_2 species. On the basis of model compounds, we attribute this chemical shift change to deuteriation of the C_5 amino group (which is expected to exchange rapidly). Deuteriation of the amino groups of methylamine, glycine, and glycine methyl ester causes chemical shift changes in the adjacent methylene ^{13}C signal of –0.33, –0.29, and –0.90 ppm, respectively, which is interpreted as being due to a secondary deuterium isotope shift.

Addition of 35 nmol of PBG synthase to 20 μmol of [5,5-^{2}H,5-^{13}C]ALA (in the presence of 10 mM 2-mercaptoethanol and 10 μM $ZnCl_2$) resulted in the production of [2,11,11-^{2}H,2,11-^{13}C]PBG as illustrated in Figures 3E and 4D. Although the deuterium coupling is not easily observed in these plots, C_2 is a 1:1:1 triplet (116.2 ppm, J_{CD} = 28 Hz) and C_{11} is a quintet (34.2 ppm, J_{CD} = 20 Hz).[2]

Natural Abundance ^{13}C NMR Spectrum of MMTS-Modified PBG Synthase. The conditions under which the natural abundance ^{13}C NMR spectrum of MMTS-modified PBG synthase (and spectra of other enzyme complexes shown in Figure 3) were acquired differ from the previously published conditions (Jaffe & Markham, 1987) in two ways: (1) The spectra in Figure 3 were acquired without proton decoupling, and (2) a recycle time of 2.0 s rather than 4.0 s was used. Both sets of spectra utilized a 45° pulse width. Nevertheless, the spectra are nearly superimposable.

Formation of the Schiff Base Complex between [5,5-^{2}H,5-^{13}C]ALA and MMTS-Modified PBG Synthase. Addition of ALA to MMTS-modified PBG synthase results in formation of the P-side Schiff base intermediate (Jaffe & Hanes, 1986). The MMTS-modified PBG synthase–Schiff base complex shows no evidence of a carbinolamine precursor and appears unable to bind A-side ALA (Jaffe & Markham, 1987). When [5,5-^{2}H,5-^{13}C]ALA is used as substrate, the resulting ^{13}C NMR spectrum, illustrated in Figure 3C, reveals two additional signals at 46.9 and 44.2 ppm (a difference spectrum between spectra B and C of Figure 3 is illustrated in Figure 4B). These peaks are identified as free ALA and enzyme-bound Schiff base, respectively, by the following three criteria: (1) under these conditions, the chemical shift of free ALA is 46.9 ppm (see Table I); (2) a peak area ratio of 30% free and 60% bound, in slow exchange on the chemical shift time scale, is in agreement with our previous results (Jaffe & Markham, 1987); and (3) addition of further [5,5-^{2}H,5-^{13}C]ALA (to 6 equiv per octamer) resulted in an increase in the intensity of the 46.9 ppm signal relative to the 44.2 ppm signal (spectrum not shown).

The line widths (corrected for line broadening) of the signals illustrated in Figure 4B were 80 Hz for 46.9 ppm (free) and 92 Hz for 44.2 ppm (bound). The apparent width at half-height of "free" ALA, which is an unresolved 1:2:3:2:1 quintet due to deuterium coupling (J_{CD} = 22 Hz), can be estimated to be 3 times the coupling constant plus line broadening due to the high sample viscosity, and this estimate is in reasonable agreement with the observed signal width. The finding that free [5,5-^{2}H,5-^{13}C]ALA and the Schiff base which have resonances separated by 2.7 ppm are in slow exchange on the chemical shift time scale is in agreement with the only slight broadening of the resonance of the free [4-^{13}C]ALA observed in the previous study, which suggested an exchange rate of <5 s^{-1} (Jaffe & Markham, 1987).

Reconstitution of the Schiff Base Complex with Zn(II) and 2-Mercaptoethanol To Form the Bound Equilibrium Complex with Excess PBG. The MMTS-modified enzyme–Schiff base complex was reconstituted to give holoenzyme by adding 40 μmol of 2-mercaptoethanol, 3 μmol of Zn(II), and 1.53 μmol of [5,5-^{2}H,5-^{13}C]ALA. The resulting spectrum of the bound equilibrium complex is illustrated in Figure 3D. These conditions were expected to reveal signals only from bound PBG as observed previously when [4-^{13}C]ALA was substrate. However, signals from both free and bound PBG are present.[2] It appears that the dissociation constant for PBG from PBG synthase is sensitive to deuteriation of either the enzyme, the substrate, or both. The chemical shifts of "free" and "bound" (identified by their similarities to "free" PBG in the absence of enzyme and by adding substrate in excess of the active sites) were for C_2 116.2 (free) and 113.4 ppm (bound) and for C_{11} 34.2 (free) and 36.8 ppm (bound). The effects of enzyme

Table II: Comparison of the Effects of pH and Organic Solvents vs Enzyme Binding on the Chemical Shifts of Porphobilinogen

condition	carbon			
	C_3	C_5	C_2	C_{11}
neutral pH to enzyme bound	−1.50	6.20	−2.80	2.60
or				
neutral pH to enzyme bound	4.20	0.50	−2.80	2.60
neutral pH to pH 1.6	−1.17	0.38	+1.14	−0.40
neutral pH to pH 12.7	−0.36	9.25	−2.40	0.97
water to 91% CD_3OD and 9% D_2O	+1.13	−0.08	−0.96	+0.62
water to 77% $(CD_3)_2CO$ and 27% D_2O	+1.07	+0.21	−0.28	+0.62
water to 60% CD_3CN and 40 D_2O	+0.67	−0.04	−0.29	+0.14
water to 91% $(CD_3)_2SO$ and 9% D_2O	+1.51	+1.02	−2.04	+0.35

binding on the chemical shifts of C_2 (−2.8 ppm) and C_{11} (+2.6 ppm) are small and in opposite directions (see Discussion).

The measured line widths for bound PBG are 55 Hz for C_2 and 75 Hz for C_{11}. The origins of the line widths are considered below (Discussion). Here it is simply noted that the width of the C_2 deuteriated methine resonance is not significantly larger than the widths of the quaternary C_3 and C_5 resonances reported in our previous study.

pH Titrations of Porphobilinogen. PBG contains at least three ionizable groups with pK_a values between 2 and 12. These pK_a values have been reported as 3.7, 4.95, and 10.1 (Windholz, 1976). The higher pK_a, attributed to the free amino group at C_{11}, has also been reported to be 11.0 by Evans et al. (1985) using ^{13}C NMR of [3,5-^{13}C]PBG. These investigators demonstrated a >+9 ppm shift in the C_5 signal upon deprotonation of the amino nitrogen. This shift is reminiscent of the +6.2 ppm shift at C_5 upon binding to PBG synthase (Jaffe & Markham, 1987) (see Discussion).

Following the work of Evans et al. (1985), in order to aid in interpretation of the chemical shift changes of PBG on binding to PBG synthase, we have investigated the effect of pH on the ^{13}C chemical shifts of C_2, C_3, C_5, and C_{11} of PBG. The chemical shifts of all four carbon are sensitive to the basic pK_a, which we determine to be 10.5. The magnitudes of the shifts are −2.4, −0.4, +9.3, and +1.0 for C_2, C_3, C_5, and C_{11}, respectively (see Table II). The unexpectedly large magnitude of the shift at C_5 was attributed by Evans et al. (1985) as partially due to breaking an internal salt bridge between the deprotonated C_{10} carboxylic acid group and the protonated C_{11} amino group. If this were the case, then protonation of the C_{10} carboxylic acid should also result in an upfield shift at C_5. Protonation of both carboxylic acid groups, estimated to be complete by pH 1, results in a +0.4 ppm shift at C_5. Thus, if breaking the salt bridge contributes to the large downfield shift at C_5 observed when the amino group is deprotonated (+9.3 ppm), the contribution appears to be small (less than 0.4 ppm).

There are two additional pK_a values for PBG corresponding to protonation of the ring, predicted to occur at C_2 (Chiang & Wipple, 1963), and to deprotonation of the pyrrole nitrogen. The pH titrations provide no evidence for these two pK_a values, suggesting that they are $\ll$1.0 and $\gg$13.0, respectively.

Effects of Organic Solvents on Chemical Shifts of PBS. As an alternative approach to understanding the chemical shift changes upon enzyme binding, we have investigated the effects of a variety of organic solvents on the chemical shifts of PBG. The results, presented in Table II, reveal that dissolution in polar organic solvents does not have a profound effect on the ^{13}C NMR spectra of PBG. Of the four solvents (91% methanol, 77% acetone, 60% acetonitrile, and 91% dimethyl sulfoxide), the largest effect was seen in dimethyl sulfoxide, where the changes in chemical shifts on C_2, C_3, C_5, and C_{11} were −1.5, +1.0, −2.0, and +0.4 ppm, respectively, compared to water.

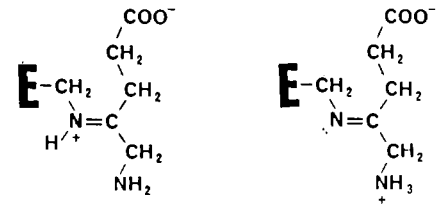

FIGURE 5: Stereochemistry and possible protonation states of the P-side Schiff base intermediate. The C_5 of ALA is trans to the lysine methylene group; only one nitrogen carries a positive charge.

Discussion

Structure of the P-Side Schiff Base Intermediate. Previous spectra of complexes with the ^{13}C label in the ketonic position of ALA revealed an sp^2 carbon consistent with either an imine or enamine structure (Jaffe & Markham, 1987). Possible tautomers are the imine, the C_4–C_5 enamine, and the C_3–C_4 enamine. The C_4–C_5 enamine is an attractive intermediate as it contains a double bond in correct position for PBG formation. The C_3–C_4 enamine appears unlikely since it is chemically irrelevant to the formation of PBG. The imine and C_4–C_5 enamine tautomers differ in hybridization at C_5. The observed chemical shift of 44.2 ppm for the C_5 of the P-side Schiff base intermediate is consistent with sp^3 hybridization. Thus, the predominant form of the Schiff base is concluded to be the imine. If the C_4–C_5 enamine also exists, it is less than 20% of the total bound species. Alternatively, in a reaction mechanism proposed by Jordan and Seeha (1980) a P-side enamine is not involved.

The −2.7 ppm chemical shift difference between C_5 of ALA and of the Schiff base can be interpreted with respect to the stereochemistry of the imine. With simple aliphatic compounds and acetone derivatives as models, the following chemical shift changes at C_5 might be expected upon formation of the enzyme-bound Schiff base: (1) going from ketone to imine would be expected to give a small shift (<−3 ppm) if C_5 is trans to the lysine methylene but a larger shift (~−12 ppm) if cis; (2) if analogous to ALA, deprotonation of the free amino group from ND_3^+ to ND_2 would result in a +3.2 ppm shift in C_5; and (3) protonation of the imine would result in ~−1.5 ppm shift (Breitmaier & Voelter, 1987). Making the assumption that these chemical shift changes are additive, the consistent structures for the P-side Schiff base intermediate are presented in Figure 5: the imine is the predominant tautomer, C_5 is trans with respect to the lysine methylene, and the protonation states of the two nitrogens are opposite (e.g., either the free amino group remains protonated and the imine is not protonated or the amino group is deprotonated and the imine is protonated).[3] These structural predictions can, and will, be tested as follows: (1) [3-^{13}C]ALA is expected to show a larger (~−12 ppm) shift upon formation of the Schiff base because if C_5 is trans to the lysine methylene group, then C_3 must be cis (this observation would also disprove the C_3–C_4 enamine structure), and (2) with [^{15}N]ALA as substrate for the P-side Schiff base formation and ^{15}N NMR, the protonation state of the free amino group will be determined. De-

[3] In ALA there may be an internal hydrogen bond between the free amino group and the ketone oxygen; an analogous hydrogen bond could exist between the free amino group and the imine nitrogen of the Schiff base. Because models for the chemical shift differences between these two structures are elusive, the predicted structure does not consider internal hydrogen bonding.

[4] The additional signal at about 59.7 ppm in the difference spectrum is due to the disulfide product of mercaptoethanol and the methyl mercaptan released from MMTS-modified apoenzyme.

termination of the protonation state of the imine nitrogen requires ^{15}N-labeled protein. This experiment, though not impossible, is not currently within our grasp since the gene for the bovine enzyme has not yet been cloned.

Strucure of Enzyme-Bound PBG. The effect of PBG synthase on the chemical shifts of C_2 and C_{11} of PBG, −2.8 and +2.6 ppm, respectively, is small and is in opposite directions (see Table II). Since the literature on ^{13}C NMR of substituted pyrroles is limited, we have investigated the effects of pH and solvent on the ^{13}C NMR of PBG to serve as models for the effects of enzyme binding.

For the enzyme-bound signals from [3,5-^{13}C]PBG, we have tentatively assigned the peak at 127 ppm to C_5 and the peak at 121.5 ppm to C_3 because the 127 ppm peak is consistently broader than the 121.5 ppm peak (see below). Furthermore, the directions of the chemical shifts at four carbons, C_2, C_3, C_5, and C_{11}, are consistent with the effects of deprotonation at the C_{11} nitrogen; see Table II. For free PBG, deprotonation of the group(s) with pK_a ~10.5 results in chemical shift changes of −2.4, −0.36, +9.25, and +0.97 ppm for the 2, 3, 5, and 11 carbons, respectively. By comparison, enzyme binding results in chemical shift changes of −2.8, −1.5, +6.2, and +2.6 ppm for these same carbons. Although the direction of the chemical shift changes upon enzyme binding are mainly consistent with deprotonation of the group with a pK_a of ~10.5, the magnitudes of the chemical shift changes do not follow accordingly. Therefore, the perturbation due to enzyme binding is not simply equivalent to a local pH change. Of the organic solvents examined, all were necessarily polar due to the highly functionalized nature of PBG. The effect on C_3 ranged from +0.7 to +1.5 ppm, opposite to the effect of enzyme binding (see Table II). The effects of organic solvents on C_5, C_2, and C_{11} were −0.1 to +1.0, −2.0 to −0.3, and +0.1 to +0.6 ppm, respectively, which uniformly do not compare favorably with the effects of the enzyme, which are +6.2, −2.8, and +2.6 ppm, respectively. Table II also considers the alternative ^{13}C assignment for bound [3,5-^{13}C]PBG.

Line Widths of Enzyme-Bound PBG. The relatively narrow line widths of the enzyme-bound C_2 and C_{11} of PBG may appear unexpected. We have estimated the various contributions to the widths of the signals for C_2, C_3, C_5, and C_{11} as described by Wilbur et al. (1976). The previously reported line widths of C_3 and C_5 were 48 and 51 Hz (tentative assignment). Assuming a chemical shift anisotropy for C_3 of 145 ppm [analogous to the C_γ of tryptophan (Cross, 1981)] and dipolar interactions with three protons at two-bond distances of 0.21 nm, from a 48-Hz line width a rotational correlation time of 245 ns is calculated. This is consistent with the rotational correlation time estimated by Stokes' law for a 280 000-dalton protein at 37 °C in a viscous solution containing 123 mg/mL protein. Assuming the same chemical shift anisotropy for C_5, calculations indicate that C_5 is expected to be ca. ~2 Hz broader than C_3 due to additional contributions from dipolar relaxation by the directly attached ^{14}N. The reproducibly greater line width of the 127.2 ppm resonance of bound [3,5-^{13}C]PBG supports assignment of this peak to C_5.

The line width of C_2 of PBG should have significant contributions from the dipolar interaction with the attached deuteron, as well as from the attached ^{14}N and chemical shift anisotropy contributions. By use of a C–D bond length of 1.05 Å, as observed for pyrrole (Cumper, 1958), and a correlation time of 245 ns, the deuterium dipolar contribution to the C_2 line width is calculated to be 21 Hz. The observed width of C_2 is ca. 55 Hz, and the residual line width is presumably primarily due to contributions from the chemical shift anisotropy mechanism. (Since we were unable to find data for a suitable model, we cannot reliably estimate the size of the contribution; however, a 145 ppm chemical shift anisotropy would contribute 31 Hz to the line width.) For a protonated rather than deuteriated C_2 with the C–H distance of pyrrole, the calculated line width is 420 Hz from the proton dipolar interaction alone, which would render observation of the resonance essentially impossible.

The observed 75 Hz of the exocyclic C_{11} is also comparable to the calculated line width of 50 Hz. The calculations assumed the same correlation time as for the ring carbons, dipolar interactions with two directly attached ^2H and ^{14}N (at standard bond lengths of 1.09 and 1.35 Å, respectively), and a chemical shift anisotropy of 57 ppm, analogous to C_1 of ethanol (Mehring, 1983). The additional observed line width may be due to chemical exchange between species containing a protonated vs unprotonated amino group. For ^1H at C_{11} rather than ^2H, at the same correlation time, the line width would be expected to be >650 Hz from the dipolar interaction with ^1H alone.

The line widths observed in these studies are therefore comparable to those predicted for totally immobilized PBG. It should be expected therefore that signals for deuteriated methine and methylene carbons will readily be observed even for totally immobilized groups bound to large proteins. Excessive line widths should not be a serious impediment to the study of structures of enzyme-bound ligands.

ACKNOWLEDGMENTS

We thank Patti Michini for assistance in the purification of bovine liver PBG synthase and Robert Dykstra for his assistance with NMR data acquisition.

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High-Resolution NMR Studies of Fibrinogen-like Peptides in Solution: Resonance Assignments and Conformational Analysis of Residues 1–23 of the Aα Chain of Human Fibrinogen†

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Received October 27, 1987; Revised Manuscript Received February 12, 1988

ABSTRACT: The proton resonances of the following synthetic linear human fibrinogen-like peptides were completely assigned with two-dimensional NMR techniques in solution: Ala(1)-Asp-Ser-Gly-Glu-Gly-Asp(7)-Phe-Leu-Ala-Glu-Gly(12)-Gly(13)-Gly(14)-Val(15)-Arg(16)-Gly-Pro-Arg-Val-Val-Glu-Arg (F10), Ala-Asp-Ser-Gly-Glu-Gly-Asp-Phe-Leu-Ala-Glu-Gly-Gly(13)-Gly(14)-Val-Arg (F11), and Gly-Pro-Arg-Val-Val-Glu-Arg (F12). No predominant structure was found in the chain segment from Ala(1) to Gly(6) for F10 in both H_2O and dimethyl sulfoxide. The previous suggestion that there is a hairpin loop involving residues Gly(12) to Val(15) in the Aα chain of human fibrinogen is supported by the slow backbone NH exchange rates of Gly(14) and Val(15), by an unusually small NH chemical shift of Val(15), and by strong sequential NOE's involving this region in F10. This local chain fold within residues Asp(7) to Val(20) may place the distant Phe residue near the Arg(16)–Gly(17) peptide bond which is cleaved by thrombin.

The specific removal of fibrinopeptides A and B by thrombin exposes complementary polymerization sites near the N-terminus of the Aα and Bβ chains (Laudano & Doolittle, 1980) located in the central domain of the soluble plasma protein fibrinogen (Telford et al., 1980), an event which initiates the spontaneous polymerization of the resultant fibrin monomer into the insoluble fibrin clot [Scheraga (1983, 1986) and references cited therein]. Much has been learned in recent years about the mechanism of the interaction of thrombin with fibrinogen, especially about the cleavage of the Arg–Gly peptide bond in the Aα chain of human fibrinogen [Scheraga (1983, 1986) and references cited therein]. Among other things, by use of an active-site mapping approach, one of the thrombin (catalytic) binding sites has been found to lie within the region from Asp(7) to Val(20) near the N-terminus of the human fibrinogen Aα chain [Meinwald et al., 1980; Marsh et al. (1983) and references cited therein]. In particular, residues Asp(7) and Phe(8), which are located ten and nine residues away, respectively, from the thrombin cleavage site, have been shown to influence the effectiveness of the binding of synthetic peptide substrates to thrombin (Marsh et al., 1982, 1983). These kinetic data help explain the observations that Asp(7) and Phe(8) are strongly conserved in many species (Blombäck, 1967; Henschen et al., 1983) and that mutations of Asp(7) to Asn(7) and Arg(19) to Asn(19) or Ser(19) produce bleeding disorders [Henschen et al., 1983; Menache (1983) and references cited therein].

In contrast to the understanding that has been gained about the influence of residues around the Arg–Gly peptide bond on the kinetics of cleavage of this bond, very little progress has been made to elucidate the detailed mechanism of the interaction of fibrinogen with thrombin, primarily because crystal structures of thrombin and fibrinogen are not available. With use of physicochemical techniques such as NMR[1] spectroscopy, attempts have been made to determine conformational features of small peptide substrates of thrombin in aqueous and in nonaqueous solutions such as DMSO (von Dreele et al., 1978; Rae & Scheraga, 1979). However, these studies were limited to very small peptides, whereas it is now clear that long-range interactions not present in those small

† This work was supported by research grants from the National Institute of General Medical Sciences (GM-24893) and the National Heart, Lung, and Blood Institute (HL-30616 and HL-31048), National Institutes of Health. It was carried out with the GN-500 500-MHz NMR spectrometer at the NIH Regional Research Resource for Multinuclear NMR and Data Processing at Syracuse University (RR-01317). This is paper 16 in a series. Marsh et al. (1985) is paper 15 of this series.
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[1] Abbreviations: NMR, nuclear magnetic resonance; DMSO, dimethyl sulfoxide; NOE, nuclear Overhauser effect; HPLC, high-performance liquid chromatography; RPLC, reverse-phase HPLC; TFA, trifluoroacetic acid; PAM, phenylacetamidomethyl; Tos, tosyl; *t*-Boc, *tert*-butyloxycarbonyl; Bzl, benzyl; DCC, *N,N*-dicyclohexylcarbodiimide; HF, hydrofluoric acid; DMSO-d_6, fully deuteriated DMSO; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; COSY, two-dimensional scalar correlation spectroscopy; DQ-COSY, double quantum filtered COSY; NOESY, two-dimensional nuclear Overhauser and exchange spectroscopy; relayed COSY, two-dimensional relayed *J*-correlation spectroscopy; FpA, fibrinopeptide A; FpAP, phosphorylated fibrinopeptide A; des-A fibrin, fibrin produced after only FpA and FpAP are removed from fibrinogen.

0006-2960/88/0427-4481$01.50/0