2-Amino-3-ketobutyrate−CoA Ligase from Beef Liver Mitochondria: An NMR Spectroscopic Study of Low-Barrier Hydrogen Bonds of a Pyridoxal 5′-Phosphate-Dependent Enzyme†

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ABSTRACT: A study of protons associated with low-barrier hydrogen bonds in 2-amino-3-ketobutyrate−CoA ligase (AKB-ligase, EC 2.3.1.29) by NMR is reported. Three resonances are observed in the range of δ_H = 15−20 ppm when the NMR spectrum of AKB-ligase is recorded at 600 MHz. These low-barrier hydrogen bonds are associated respectively with a side chain proton, the PLP pyridinium ring nitrogen proton, and the PLP Schiff base proton at the active site of the ligase. The pyridinium proton has been assigned a chemical shift of 19.10 ppm and the Schiff base proton 14.90 ppm. The third low-barrier hydrogen bond associated proton resonating at 16.20 ppm is assigned to a proton of a side chain group. All three resonances disappear when pyridoxal phosphate is removed from the ligase. Consistent with NOE coupling, the side chain group proton should be close to the proton of the Schiff base nitrogen of the pyridoxal 5′-phosphate. The effects of temperature, pH, substrate, and NOE on the three resonances are also studied, in order to assign the protons. The three low-barrier hydrogen bonds described in this report may serve to anchor the cofactor in the active site of 2-amino-3-ketobutyrate−CoA ligase.

2-Amino-3-ketobutyrate−CoA ligase (AKB-ligase)[1] functions in a coupled system with L-threonine dehydrogenase (TDH, EC 1.1.1.103), to catalyze interconversion between L-threonine and glycine in both eukaryotic and prokaryotic cells (Mcgilvray & Morris, 1969; Bell & Turner, 1976a,b; Dale, 1978; Bird & Nunn, 1979; Komatsubara et al., 1978; Bird et al., 1984; Boylan & Dekker, 1981). Studies showed that threonine in biological systems is degraded mainly through this metabolic pathway (Bird & Nunn, 1983; Ravnikar & Somerville, 1987; Aoyama & Motokawa, 1981).

2-Amino-3-ketobutyrate−CoA ligase has been purified from beef liver mitochondria (Tong & Davis, 1994). Pyridoxal 5′-phosphate (PLP) is an essential coenzyme for the ligase. Similar to most PLP-requiring enzymes, the cofactor is covalently bonded to an active site lysine and the holoenzyme has an absorption maximum at 430 nm. The spectrum does not change significatly over the pH range 5.1−9.4. Spectral changes that might occur in the presence of CoA and acetyl CoA are obscured by the absorption of these substrates. Therefore, the UV−visible changes that serve as probes for enzyme substrate species of other PLP-requiring enzyme cannot be used to obtain information about AKB-ligase. However, the use of high field NMR has proven to be a useful probe for enzyme substrate species of the AKB-ligase. Sklenar and Bax (1987) developed a spin-echo water suppression method that we have used to obtain NMR spectra of protons at the active site of AKB-ligase. The AKB-ligase is a 43 000 dalton monomer, making it ideal for NMR studies of its PLP-binding site. The most unique signals in the spectrum of AKB-ligase appear to arise from very strong hydrogen bonded protons. The signals in the spectrum of holo-AKB-ligase in the 15−20 ppm region are characteristic of low-barrier hydrogen bonded protons. Studies of the ^{1}H resonances of AKB-ligase in this region when the Schiff base linkage is reduced, at different pH values, and in the presence of substrates or inhibitors have enabled us to assign the protons associated with specific resonances.

EXPERIMENTAL PROCEDURES

The Schiff base double bonds in AKB-ligase were reduced by adding solid sodium borohydride to a sample solution. The excess reagent was removed with a Centricon-30 microconcentrator (Amicon), when necessary. Apoenzyme was obtained by reacting the ligase with hydroxylamine and subsequently removing the cofactor by dialysis against 50 mM phosphate buffer, pH 7.0, containing 5 mM hydroxylamine for 6 h. The hydroxylamine was removed by dialysis for 6 h against three changes of 50 mM phosphate buffer, pH 7.0. Some denaturation occurred, and precipitated protein was removed by centrifugation. The undenatured protein was concentrated using a Centricon-30 microconcentrator (Amicon).

Samples of AKB-ligase for NMR experiments typically contained 10−14 mg/mL enzyme (~0.5 mM) in 0.4 mL of 50 mM potassium phosphate buffer, at pH 7.0, plus 8−10% deuterium oxide. Substrate was added to the sample in 5−10% of the original volume of the sample. The pH titration was carried out with two samples initially at pH 7.0. The pH was adjusted by adding either a small amount of 1.0 M potassium phosphate buffer, pH 2.0, to make the solution more acidic, or 1.0 M potassium phosphate buffer, pH 12, in a small amount for more basic conditions.

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[1] Abbreviations: PLP, pyridoxal 5′-phosphate; AKB, 2-amino-3-ketobutyrate; AKB-ligase, 2-amino-3-ketobutyrate−CoA ligase; AAT, aspartate aminotransferase; TDH, threonine dehydrogenase; NMR, nuclear magnetic resonance; BSA, bovine serum albumin; NOE, nuclear Overhauser effect.

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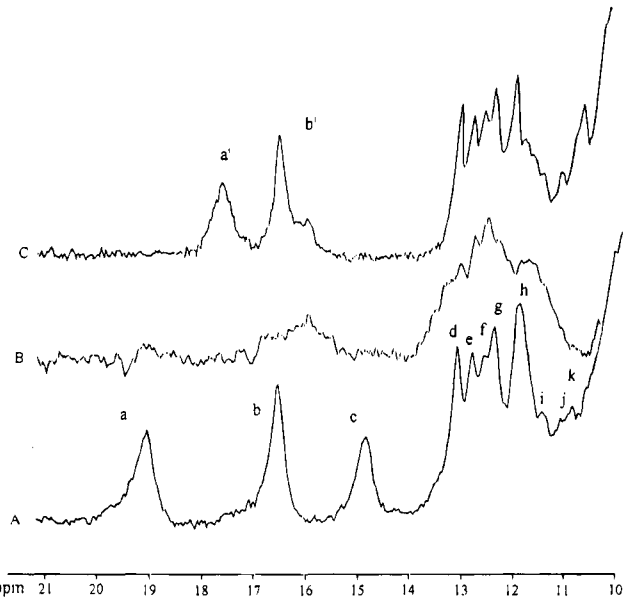

FIGURE 1: 600 MHz ^{1}H NMR spectra: (A) 2-amino-3-ketobutyrate–CoA ligase, pH 7.0, 295 K; (B) 2-amino-3-ketobutyrate–CoA ligase, after the removal of the PLP from the enzyme, pH 7.0, 295 K; and (C) reduced 2-amino-3-ketobutyrate–CoA ligase, pH 7.0, 295 K.

Proton NMR spectra were obtained using a Bruker 600 MHz AMX spectrometer. All spectra were recorded using a modified 1−1 spin-echo water suppression method, as described by Sklenar and Bax (1987), Kintanar et al. (1991), and Metzler et al. (1994a,b). The acquisition parameters included the following: D19, the delay between 90° pulse, was 25 μs; D20, the short spin-echo delay, was 100 μs; and P1, 90° transmitter high power pulse, was 7.7 μs. The sweep width was 25 kHz, and 16K data points were collected. The spectra of exchangeable protons were typically obtained with 256−512 scans.

RESULTS

The spectrum of the ligase in the PLP form at pH 7.0 was recorded at 295 K and is shown in Figure 1A. Approximately eleven peaks labeled "A", "B", "C", etc., from left to right were observed between 11 and 20 ppm. Well isolated peaks A, B, and C are considered to represent resonances of single low-barrier hydrogen bonded protons. Figure 1C shows the spectrum of fully reduced enzyme at pH 7.0: the peak labeled C is absent in borohydride reduced ligase and peak A is replaced by a peak labeled A′. A′ is 1.5 ppm upfield to peak A. Peak B is unchanged. Peaks D through K appear to be combined peaks. All peaks shifted slightly upfield at 275 K relative to 295 K (not shown) and the line width of peaks A, B, and C increased. The broadening at the lower temperature could be due to slower tumbling of the protein in the solution. Peaks from D to K merged at the lower temperature. Figure 1B shows the spectrum of apoenzyme. The three resonances in the 15–20 ppm region disappear when apoenzyme is formed.

Changes in both chemical shift and area of some peaks were observed when the pH of solutions of the ligase was adjusted to more acidic or basic values (Figure 2). A downfield shift of approximately 0.1 ppm of peak B occurred when the pH is incrementally changed from 7.0 to 5.1. Peaks A and C undergo significant changes in intensity as a function of pH. The signal associated with peak A diminishes in area as a function of incrementally changing the pH from 7.0 to 5.1 as shown in Figure 3A. The pH-dependent change in the area of peak A is centered around an apparent pK_a of 6.2. Peak C diminishes in area in a similar fashion centered around an apparent pK_a of 8.74 as shown in Figure 3B.

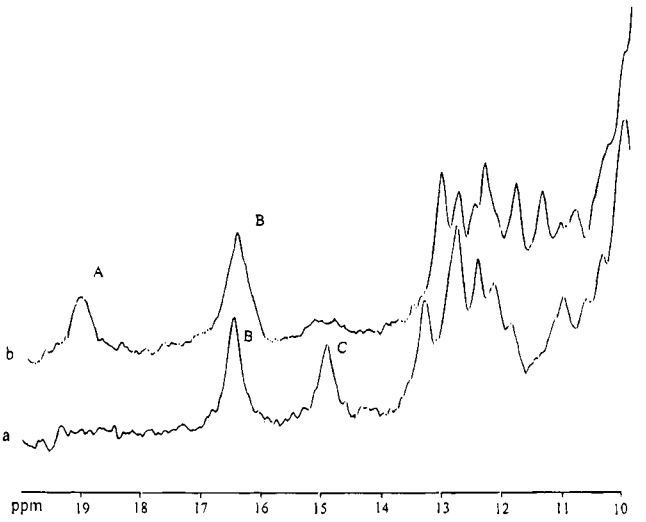

FIGURE 2: 600 MHz ^{1}H NMR spectra of 2-amino-3-ketobutyrate–CoA ligase at (a) pH 5.1 and (b) pH 9.4, 295 K.

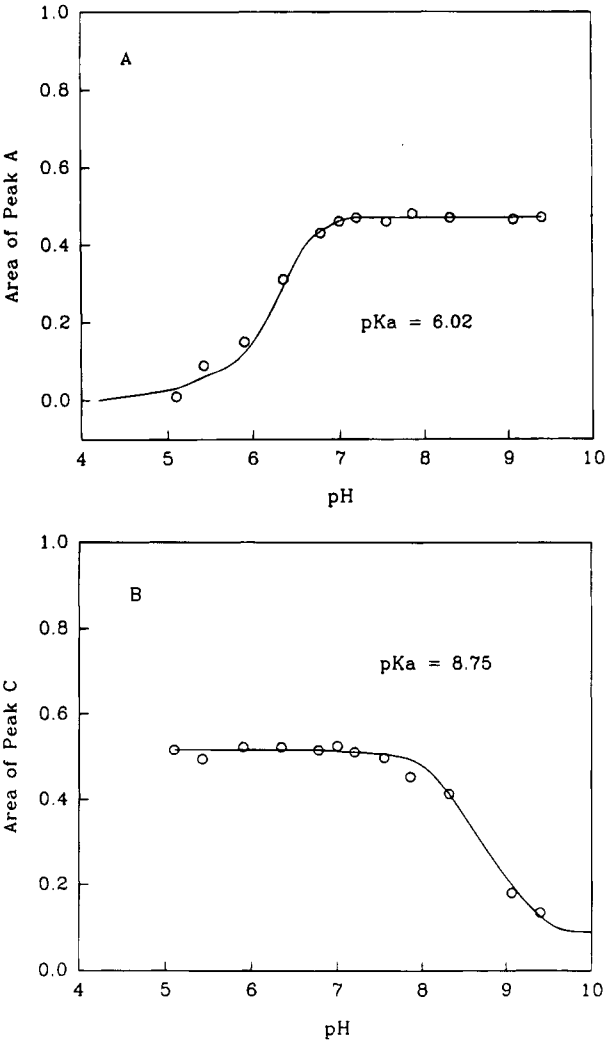

FIGURE 3: Titration curves for (A) peak A and (B) peak B.

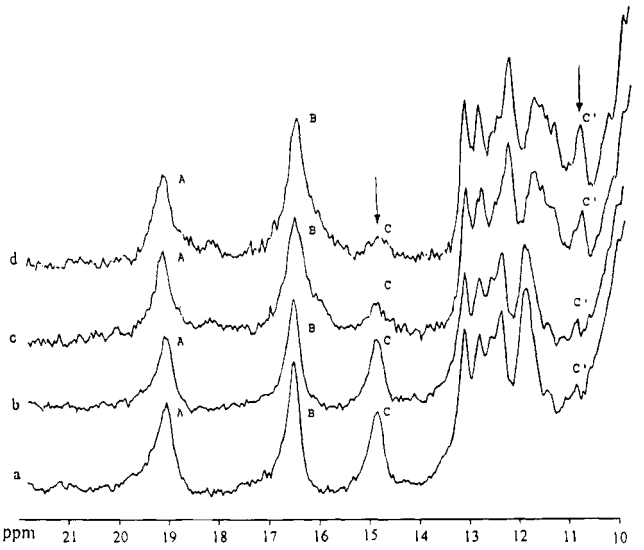

FIGURE 4: 600 MHz ^{1}H NMR spectra of 2-amino-3-ketobutyrate–CoA ligase in the presence of different concentrations of glycine: (a) without glycine; or with (b) 5 mM glycine, (c) 100 mM glycine, or (d) 200 mM glycine.

AKB-ligase reacts with glycine to form an external aldimine (Fubara et al., 1986). A small visible spectrum shift from 430 to 426 nm accompanies external aldimine formation. However, major shifts in peak C occur in the NMR spectrum of the ligase when an external aldimine is formed between glycine and the ligase. With increasing concentration of glycine in a ligase sample, the signal at 14.90 ppm (peak C) shifts to a limiting value of 11 ppm (Figure 4). At the same time, peak A (19.10 ppm) undergoes a shift of 0.4 ppm. Additional shifts occur when the cosubstrate acetyl CoA is added to the mixture. Peak A shifts to a limiting value of 18.62 from 19.10 ppm, and a new resonance appears in the 16.0 ppm region at 16.82 ppm (Figure 5b). Aminomalonate will also form an external aldimime with AKB-ligase (Fubara et al., 1986). The effect of aminomalonate on the NMR spectrum of AKB-ligase is shown in Figure 5c. Peak C is diminished, and peaks A and B are shifted to 18.80 and 16.34 ppm, respectively. When acetyl CoA is added to this sample, a spectrum emerges that is very similar to the spectrum observed with glycine and acetyl CoA as substrates.

The ^{1}H NMR spectrum of the ligase is also affected by addition of saturating amounts of acetyl CoA as the sole substrate. In the presence of acetyl CoA three signals are observed at 19.22, 16.15, and 14.50 ppm (Figure 5d) compared to signals at 19.10, 16.60, and 14.90 ppm in holoenzyme in the absence of acetyl CoA. Peak B at 16.60 ppm in holoenzyme is significantly shifted to 16.15 ppm in the presence of acetyl CoA.

L-Cysteine reacts with pyridoxal 5′-phosphate to form a thiazolidine (Schirch & Mason, 1963), which has a UV absorption maximum at 335 nm, and can be used to prepare apoenzyme with many PLP-dependent enzymes. Cysteine is, however, unable to remove the pyridoxal 5′-phosphate from AKB-ligase, but does form a 335 nm absorbing species, and the changes in UV absorption are cysteine dependent (Fubara et al., 1986). The NMR spectrum of AKB-ligase is also altered in the presence of cysteine as shown in Figure 5e. Peaks A and C disappeared, and a broad peak appeared in the 16 ppm region.

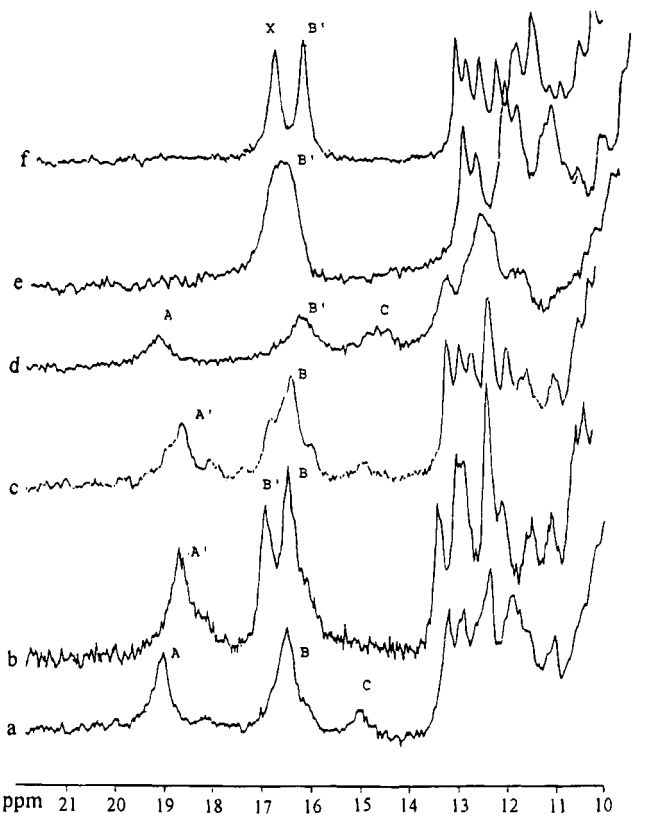

FIGURE 5: 600 MHz ^{1}H NMR spectra of 2-amino-3-ketobutyrate–CoA ligase in the presence of substrates: (a) 100 mM glycine; (b) 200 mM glycine and 0.06 mM acetyl CoA; (c) 120 mM aminomalonate; (d) 0.06 mM acetyl CoA; (e) 10 mM cysteine; and (f) 10 mM cysteine and 0.06 mM CoA.

NOE experiments were carried out with a pulse sequence consisting of a 200-ms presatuation pulse followed by the 1–1 spin-echo sequence. A 50-dB attenuator was added to the low power decoupler channel of the spectrometer to obtain a sufficiently soft presatuation pulse. Typically, 512 scans were collected at each frequency offset. The difference NOE spectrum of the holoenzyme at pH 7.0, 298 K, is shown in Figure 6. When peak B is irradiated, a difference NOE spectrum is observed with peaks at 14.90 and 12.80 ppm. The 14.90 ppm peak coincides with the resonances associated with peak C, and the 12.80 ppm resonance coincides with the resonance labeled peak D in the spectrum of the holoenzyme. However, no NOE was observed when peaks A, C, or D were irradiated.

DISCUSSION

2-Amino-3-ketobutyrate–CoA ligase is a member of the PLP family of enzymes that exhibit maximum absorption at 430 nm and which do not undergo significant spectral changes as a function of pH or external aldimine formation (Morino & Nagashima, 1984). However, like many other PLP-requiring enzymes, the Schiff base can be reduced with borohydride to form a secondary amine with a maximum absorption at 340 nm (Tong & Davis, 1994). The ligase also reacts with L-cysteine to form an enzyme species absorbing at 335 nm (Fubara et al., 1986; Tong & Davis, 1994; Ulevitch & Kallen, 1977). However, cysteine binding is reversible and does not result in cofactor dissociation. Because the UV–visible spectrum does not change in ϵ or wavelength as a function of pH or when the enzyme forms

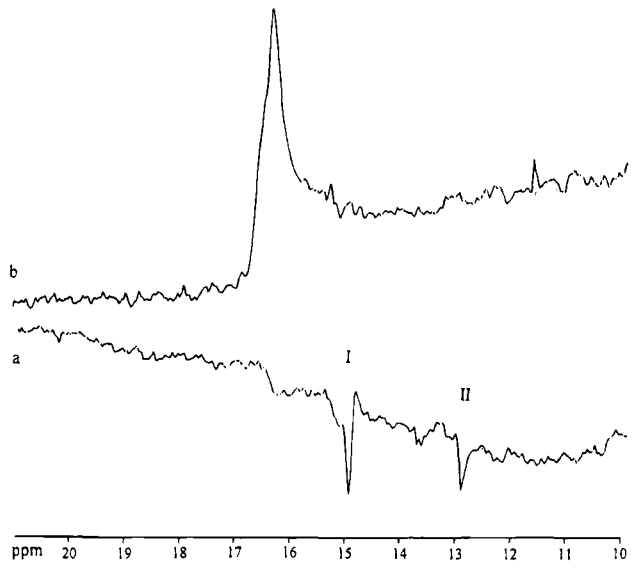

FIGURE 6: Different NOE spectra of the hololigase at pH 7.0, 295 K. (a) NOE were observed at 14.9 (peak I) and 12.8 ppm (peak II) when peak B was irradiated. (b) No NOE was found when peak C and peak D were irradiated.

an external aldimine with glycine or aminomalonate, high field NMR has been used to probe the state of protonation of the PLP in the ligase and the mechanistic implications associated with substrate binding. The enzyme is well suited for NMR studies, having a molecular weight of 43 000 daltons, being a monomer, and containing a single PLP per monomer. The ^{1}H NMR spectrum of AKB-ligase contains three well resolved signals in the 15−20 ppm region (Figure 1). The three peaks represent single proton resonances associated with low-barrier hydrogen bonds.

The ^{1}H NMR low field signals of PLP-requiring enzymes in the 15−20 ppm range may represent protons associated with low-barrier hydrogen bonds, since many of the signals are too far removed from the range (9−12 ppm) where nitrogen bonded protons normally exhibit chemical shifts. Current theory (Frey et al., 1994; Cleland & Kreevoy, 1994) suggests that NMR signals of PLP-requiring enzymes in the downfield region may arise from low-barrier hydrogen bonded protons. Such bonds are characterized by high stabilization energies of up to 20 kcal mol^{-1}, short distances between donor−acceptor atoms, and ^{1}H NMR signals in the downfield area of the spectrum (δ_H = 16−20 ppm). The chemical shift range is one of the most unambiguous parameters for characterizing low-barrier hydrogen bonds (Hibbert & Emsley, 1990). The 600 MHz NMR spectrum of AKB-ligase can be used to establish the identity of groups at its active site and, as well, can be used to monitor changes occurring when enzyme−substrate complexes are formed.

Experiments have been conducted to assign specific protons with the observed low field signals in the 15−20 ppm region. Specifically, the protons of the Schiff base and ring nitrogen of the PLP have been assigned. The assignment was facilitated by the study of isolated apoenzyme. All three low field signals in the 15−20 ppm region are absent in the apoenzyme.

A strong case can also be made for assigning peak C to the Schiff base proton on the basis of changes that occur in the NMR spectrum of holoenzyme when the Schiff base double bond is reduced. On the basis of the disappearance of the signal at 14.90 ppm upon reduction of the enzyme, this signal has been assigned to a low-barrier hydrogen bonded proton of the Schiff base nitrogen in a donor−acceptor pairing. However, the nature of the acceptor cannot be inferred from the position of the resonance. The proton associated with this hydrogen bond is labeled Hc (Figure 1) and is presumably hydrogen bonded to the phenolic oxygen of the PLP. Since the ligase has an absorption maximum at 430 nm that does not change over the pH range 5.1−9.4, we believe the Schiff base proton does not dissociate. The fact that the Schiff base proton does not dissociate over this wide range implies that some unique structural feature contributes to its stability. On the other hand, the melting of the 14.90 ppm resonance centered around a pK_a of 8.74 differs from the observed spectroscopic determined Schiff base pK_a of 6.1 for the aminotransferase (Kintanar et al., 1991) but is closer to the normal pK_a of 10.9 for the Schiff base proton in model systems (Metzler et al., 1980). The melting of the 14.90 ppm peak centered around a pK_a of 8.74 is, however, not consistent with the lack of spectral changes with pH. Therefore, the pH-dependent intensity changes in peak C may not be associated directly with dissociation of the Schiff base proton. It may be associated with dissociation of a nearby hydrogen bonded group. However, at this time it is not clear why dissociation of a nearby group would cause the observed melting of peak C.

In addition to the spectral changes of peak C observed on reduction of the holoenzyme, additional evidence for its assignment arises from NMR spectral changes that occur when the ligase is saturated with glycine or aminomalonate. The signal at 14.90 ppm undergoes a major shift when an external aldimine is formed between the ligase and glycine or aminomalonate. Increasing glycine shifts the signal at 14.9 ppm to a limiting value of 11 ppm as shown in Figure 3. This shift of the low-barrier hydrogen bonded proton to a range normally associated with non-hydrogen-bonded protons associated with a Schiff base nitrogen would suggest that the Schiff base proton is not hydrogen bonded or weakly hydrogen bonded in the external aldimine. Peak C also disappears in the presence of aminomalonate. On the other hand, thiazolidine formation between PLP and cysteine results in the disappearance of both peaks A and C. The signal at 14.90 ppm disappearing in the presence of cysteine provides additional evidence in the assignment of the Schiff base proton signal.

In all these experiments with amino acid substrate or inhibitor binding to the PLP of the ligase, the most sensitive resonance is the one at 14.90 ppm and the Schiff base proton is the one most likely to be affected by reactions at the 4′ formyl position or reduction of the Schiff base double bond of the enzyme.

The assignment of protons hydrogen bonded by donor−acceptor heteroatoms to specific low-barrier hydrogen bonded protons with signals at 19.10 and 16.20 ppm is much more problematic. Of the two signals, the 19.10 ppm peak is more sensitive to pH changes and substrates than the 16.20 ppm peak. The 19.10 ppm signal is labeled peak A in Figure 1. In reduced AKB-ligase, peak A shifts upfield by 0.2 ppm whereas peak C disappears from the spectrum. It would appear that the hydrogen bonded proton associated with the signal at 19.10 ppm is, thus, only slightly influenced by reducing the Schiff base linkage. The small upfield shift observed could result from the oxygen anion, if previously hydrogen bonded to the proton of the Schiff base nitrogen,

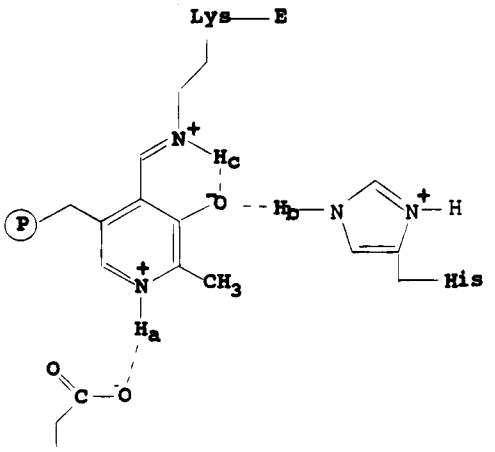

FIGURE 7: A proposed intermediate complex of glycine, acetyl CoA, and PLP at the active site of the enzyme.

now being able to donate better its electrons to the ring (Scott et al., 1985; Metzler & Snell, 1955; Kinatanar et al., 1991; Metzler et al., 1994a,b). This change in chemical shift of peak A could, therefore, be the result of a change in the pK_a of the ring nitrogen proton. Such a change could effect the donor–acceptor pK_a pairing and therefore the strength of the low-barrier hydrogen bond.

The broadening of peak A and its eventual disappearance is centered around an apparent pK_a of 6.2. Peak A is also shifted upfield in the presence of glycine and aminomalonate. However, its most dramatic shift occurs when both glycine and acetyl CoA are reacted with the ligase. In the presence of glycine a small shift in peak A occurs, however, a shift to 18.62 from 19.10 ppm occurs when both glycine and acetyl CoA are added to the ligase. A similar shift of peak A occurs in the presence of aminomalonate and acetyl CoA. Peak A is little influenced by the addition of acetyl CoA alone. Peak A undergoes a major shift with thiazolidine formation. Peak A either disappears or is shifted to the 16 ppm region in the presence of cysteine as shown in Figure 5. These observations support assigning peak A to the pyridinium proton. The fact that the signal (peak A) is that of a low-barrier hydrogen bonded proton requires that the proton is involved in a donor–acceptor system. Likely acceptors are the heteroatoms of the imidazoles of a histidine or an oxygen of aspartate ω-carboxylic acid side chains as in the case of the aminotransferase (Kintanar et al., 1991).

While peak B is found to be inert to pH changes and reduction of the ligase, the presence of substrates result in significant resonance shifts. The largest shift occurs in peak B when both glycine and acetyl CoA are added to the ligase. Significant changes also occur in peak B with thiazolidine formation. The proton of peak B is most likely associated with a side chain residue at the active site, and candidates are oxygen or nitrogen heteroatoms. Additional experiments are needed to establish the identity of the residue whose bonded proton is involved in the low-barrier hydrogen bond of peak B.

Additional information on the properties of the signal at 14.90 ppm is derived from NOE experiments. Nuclear Overhauser effect (NOE) studies indicate the H_C and H_B are close to each other. Assuming that our assignment of peak C is correct, H_B should be in close proximity to the Schiff base proton at the active site of the ligase in this position (Figure 7). The NOE results eliminate the ring nitrogen proton from being associated with peak B.

Finally, the shifts of the three low-barrier hydrogen bonded protons occurring in the presence of the cosubstrates glycine and acetyl CoA may suggest a structure for its enzyme–

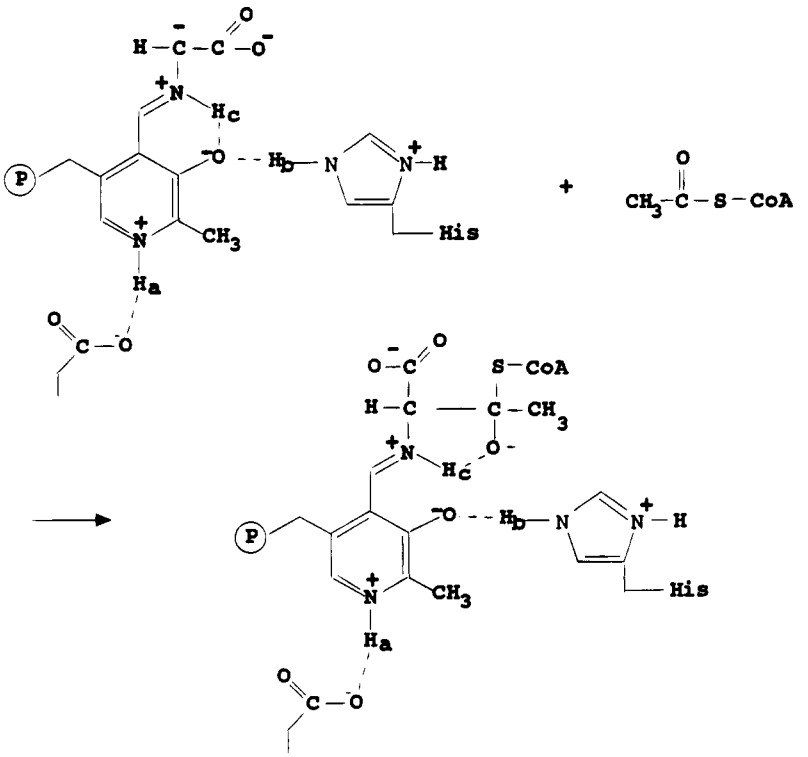

FIGURE 8: Proposed structure of pyridoxal 5′-phosphate at the active site, a possible electronegative atom such as an oxygen atom in a carboxylic group of aspartic acid or glutamic acid, or in a hydroxyl group of tyrosine, as well as a nitrogen atom in histidine. The hydrogen bond formed between COO^- and the ring nitrogen proton may stabilize the system.

substrate complex. When both glycine and acetyl CoA are added to the enzyme, peak A moves upfield and peak C moves downfield. In other words, proton A becomes involved in a weaker hydrogen bond and proton C becomes associated with a stronger hydrogen bond. AKB-ligase is observed to form a stable enzyme−substrate complex when catalyzing the reverse reaction or functioning as an aminoacetone synthetase (Fubara et al., 1986). Therefore, the NMR spectrum of the ligase in the presence of glycine and acetyl CoA with peaks at 18.80, 16.80, and 16.40 ppm may represent the intermediate complex shown in Figure 7. When the carbanion of glycine attacks the carbonyl of acetyl CoA, the carboxyl of the acetyl group becomes (sp^3) hybridized and an anion is generated capable of forming a new hydrogen bond at the active site. Possible hydrogen donors are the side chain proton (peak B) or the Schiff base. A postulated mechanism would have the intermediate stabilized by forming a hydrogen bond with either the Schiff base proton or the putative side chain proton. Since the intensity of the negative charge on this anion would be higher than that on the phenolate anion, any newly formed hydrogen bond should be stronger, which would explain the observed downfield shift of peak C. In the overall process, the phenolate anion appears to donate more electrons into the pyridine ring, the consequence being an upfield shift of peak A.

In summary, it would appear that three low-barrier hydrogen bond associated protons exist at the active site of AKB-ligase. The assignment of both the Schiff base and pyridinium heteroatoms as sites of two of the low-barrier hydrogen bonded protons suggests that functional groups such as a charged ω-carboxylate group and an imidazole may be present at the PLP-binding site. Evidence is presented to place a donor side chain close to the Schiff base, the strongest evidence being the NOE observed between the Schiff base proton and the proton involved in a low-barrier hydrogen bond nearby. On the other hand, evidence is presented suggesting that the proton associated with the pyridinium heteroatom is also involved in a low-barrier hydrogen bonding system. A likely candidate is an ω-carboxylate group. A proposed structure for the PLP at the active site of the ligase is presented in Figure 8. If both assumptions prove to be accurate, then the PLP of AKB-ligase is very tightly bound by these low-barrier hydrogen bonds to its protein, explaining the difficulty experienced in attempting to resolve the holoenzymes of its PLP cofactor.

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