addition, the transition between native and denatured DAC-α-chymotrypsin occurs at a lower temperature than measured on the protein as a whole (Privalov, 1974). This transition occurs at an even lower temperature than with the DAB-α-chymotrypsin, which is 52 °C at pH 4.0 (Argade et al., 1983). The region around the active site appears to denature earlier with the acyl enzyme than with the protein as a whole and even depends on the nature or size of the acyl group. These observations, coupled with the lack of a single isosbestic point, are consistent with there being more than two spectrally distinct species along the denaturation pathway. Whether these species are local or more generalized intermediates might be of interest to investigate. We were not able to obtain Raman spectra of partially denatured acyl enzyme at high temperatures.

Added in Proof

An alternative explanation for the experimental results given here is that of Warshel and Russell (1984), who propose that the reduced bond order of the acyl carbonyl in the native acyl enzyme compared to that in the denatured enzyme could be due to a positive charge in the protein located close to the carbonyl oxygen. [See Figure 42 of Warshel and Russell (1984).] The negative charge on the carbonyl oxygen could also perturb the sp^2 hybridization and give rise to a nonplanar conformation of the ester linkage proposed above.

Registry No. DACHO, 6203-18-5; DACA, 1552-96-1; DAC-Im, 59708-13-3; DAC-O-Me, 7560-48-7; triethylamine, 121-44-8; imidazole, 288-32-4; α-chymotrypsin, 9004-07-3.

References

Argade, P. V., Gerke, G. K., Weber, J. P., & Peticolas, W. L. (1984) *Biochemistry 23*, 299–304.

Bernhard, S. A., & Lau, S. J. (1971) *Cold Spring Harbor Symp. Quant. Biol. 36*, 75–83.

Bernhard, S. A., & Malhotra, O. P. (1974) *Isr. J. Chem.* 12, 471–481.

Bernhard, S. A., Lau, J., & Noller, H. (1965) *Biochemistry 4*, 1108.

Caplow, M., & Jencks, W. P. (1962) *Biochemistry 1*, 883–893.

Henderson, R. (1970) *J. Mol. Biol. 54*, 341–354.

Komiyama, M., & Bender, M. L. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 557–560.

MacClement, B. A. E., Carrier, R. G., Phelps, D. J., & Carey, P. R. (1981) *Biochemistry 20*, 3438–3447.

Mishimura, Y., & Tsuboi, M. (1980) *Science (Washington, D.C.) 210*, 1358–1360.

Peticolas, W. L. (1982) *Raman Spectroscopy, Linear and Nonlinear* (Lascombe, J., & Huong, P. F., Eds.) pp 694–702, Wiley, New York.

Warshel, A., & Russell, S. T. (1984) *Q. Rev. Biophys. 17*, 283–422.

Identification of Coenzyme Aldimine Proton in ^1H NMR Spectra of Pyridoxal 5′-Phosphate Dependent Enzymes: Aspartate Aminotransferase Isoenzymes†

Yoshimasa Morino,* Fujio Nagashima, Sumio Tanase, Masaki Yamasaki, and Tsuyoshi Higaki

Department of Biochemistry, Kumamoto University Medical School, 2-2-1 Honjo, Kumamoto 860, Japan

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ABSTRACT: The pyridoxal form of the α subform of cytosolic aspartate aminotransferase (EC 2.6.1.1) is fully active and binds pyridoxal 5′-phosphate via an aldimine formation with Lys-258 whereas the γ subform is virtually inactive and lacks the aldimine linkage. Comparison of ^1H NMR spectra between the α and γ subforms suggested that peak 1 of the α subform at 8.89 ppm contains a resonance assignable to the internal aldimine 4′-H. Reaction with a reagent that cleaves or modifies the internal aldimine bond [(aminooxy)acetate, L-cysteinesulfinate, NH_2OH, $NaBH_4$, or $NaCNBH_3$] caused the disappearance of a resonance line at 8.89 ppm that possessed a broad line width and corresponded in intensity to a single proton. These reagents were also used successfully for the identification of the aldimine 4′-H resonance in the mitochondrial isoenzyme. In contrast to the cytosolic isoenzyme whose resonance for the 4′-H did not show any detectable change in chemical shift with pH, the corresponding resonance in the mitochondrial isoenzyme exhibited pH-dependent chemical shift change (8.84 ppm at pH 5 and 8.67 ppm at pH 8) with a pK value of 6.3, reflecting the interisozymic difference in the microenvironment provided for the internal aldimine. Validity of the signal assignment was further shown by the two findings: (a) the resonance assigned to the 4′-H emerged upon conversion of the pyridoxamine into the pyridoxal form, and (b) the resonance appeared upon reconstitution of the apoenzyme with [4′-^{1}H]pyridoxal phosphate but not with [4′-^{2}H]pyridoxal phosphate.

The importance of imine bond formation is apparent in many enzymic reactions catalyzing the transformation of carbonyl compounds and amines. Occurrence of such bonds seems to be universal in pyridoxal-P[1]-dependent enzymes, in which the formyl group at position 4 of the coenzyme forms an aldimine bond with the ε-amino group of a specific lysyl residue (Snell & Di Mari, 1970). Since this binding mode of pyridoxal-P was shown for glycogen phosphorylase (Fischer et al., 1958)

† This paper is dedicated to Professor Esmond E. Snell on the occasion of his 70th birthday. This work was supported by a Grant-in-Aid for scientific research from the Ministry of Education, Science and Culture. Preliminary reports on this work have been presented (Morino et al., 1982; Morino, 1984).

[1] Abbreviations: pyridoxal-P, pyridoxal 5′-phosphate; pyridoxamine-P, pyridoxamine 5′-phosphate; NMR, nuclear magnetic resonance; cAspAT, cytosolic aspartate aminotransferase; mAspAT, mitochondrial aspartate aminotransferase; EDTA, ethylenediaminetetraacetate; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

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and aspartate aminotransferase (Hughes et al., 1962), all pyridoxal-P-dependent enzymes so far examined (Morino & Nagashima, 1984) have been demonstrated to bind the coenzyme via aldimine linkage to a specific lysyl residue. The high reactivity of the internal aldimine (Jencks & Cordes, 1963) is obviously essential for transimination, which leads to the formation of an external aldimine between the the enzyme-bound pyridoxal-P and a substrate, a prerequisite step to subsequent catalytic events. A unique microenvironment probably entails high reactivity of the internal aldimine. Although properties of the internal aldimine in many pyridoxal-P-dependent enzymes have been extensively studied by using mostly absorption spectroscopy, there has so far been no study dealing with the observation of this important internal aldimine by ^{1}H NMR spectroscopy, which should be expected to provide further information on its microenvironment.

The purpose of this study is to identify the hydrogen atom (4′-H) attached to the internal aldimine carbon in ^{1}H NMR spectra of aspartate aminotransferase (EC 2.6.1.1) from pig heart. This enzyme exists in two isozymic forms in mammalian tissues; one is localized in mitochondria and the other in cytosol (Boyd, 1961; Katunuma et al., 1962; Morino & Wada, 1963). Both isoenzymes are dimeric proteins (M_r 90 000) composed of two identical subunits, each of which binds one molecule of pyridoxal-P. In spite of only 48% sequence identity between the two isoenzymes (Ovchinnikov et al., 1973; Kagamiyama et al., 1977), the three-dimensional structures are very similar to each other (Ford et al., 1980; Arnone et al., 1982). Recent studies using ^{1}H NMR spectroscopy have revealed substantial differences between the two isoenzymes (Morino et al., 1984). To elucidate details of structural similarity and dissimilarity between the two isoenzymes, it should be important to observe the internal aldimine 4′-H in ^{1}H NMR spectra as a feature common to both isoenzymes, on one hand, and to compare its microenvironment between them, on the other. One of the experimental approaches to this aim is to compare the ^{1}H NMR spectrum of the enzymically fully active α subform of the cytosolic isoenzyme with that of the catalytically inactive γ subform, which is believed to lack the internal aldimine bond. The second approach is to observe the effect of internal aldimine breaking reagents on the spectra of both isoenzymes. The third approach is to observe the effect of pyridoxal-P on the spectra of apoenzymes. The latter two approaches should also be applicable to the identification of the internal aldimine 4′-H in other pyridoxal-P-dependent enzymes.

Experimental Procedures

Materials. Mitochondrial aspartate aminotransferase (mAspAT) and three subforms of the cytosolic isoenzyme (cAspAT) were purified from pig heart as described (Martinez-Carrion et al., 1967; Morino et al., 1977). Spectral and catalytic properties of the enzyme preparations were as follows: α subform of cAspAT, A_{430nm}/A_{340nm}(pH 5) = 3.6–3.7, specific activity = 290 μmol min mg^{-1} at 25 °C; β subform of cAspAT, A_{430nm}/A_{340nm} = 1.2, specific activity = 120 μmol min^{-1} mg^{-1}; γ subform of cAspAT, A_{430nm}/A_{340nm} = 0.06, specific activity = 7 μmol min^{-1} mg^{-1}; mAspAT, A_{430nm}/A_{340nm} = 2.6, specific activity = 200 μmol min^{-1} mg^{-1}. The enzyme activity was determined by measuring the absorbance at 280 nm for the formation of oxalacetate in the reaction mixture containing 10 mM each L-aspartate and 2-oxoglutarate in 1.0 mL of 0.1 M Tris-HCl buffer (pH 8.0). (Aminooxy)acetic acid and L-cysteinesulfinic acid were obtained from Sigma; ^2H_2O (99.7%), from Merck. Other reagents were of the best grade available from commercial sources.

Preparation of Holoenzyme Samples for NMR Measurement. The concentrated enzyme solutions (50–100 mg/mL) were dialyzed overnight at 4 °C against 50 mM sodium phosphate buffer (pH 6.8) and then overnight against distilled water containing 0.5 mM EDTA. The dialyzed samples were lyophilized and dissolved in ^2H_2O. Solution pHs were adjusted by using appropriate buffers as described for the individual experiments. Ionic strength was adjusted to 0.1 by adding an appropriate amount of 1 M NaCl solution in ^2H_2O. pH values of the solutions are expressed as pH*, the direct pH meter readings uncorrected for the isotope effect on the glass electrode. A pHM 84 research pH meter (Radiometer, Copenhagen) was used. (Aminooxy)acetate, cysteinesulfinate, and other substrate ligands were dissolved in ^2H_2O at 0.2–0.5 M each and neutralized by adding NaO^{2}H solution. Each ligand solution in microliter amounts were placed on the tip of a thin glass rod, dried under gentle air, and then mixed into the enzyme solution contained in a 5-mm NMR tube. the concentration of enzyme samples was 0.8–0.9 mM with respect to the monomeric unit of the enzyme.

Preparation of Apoenzyme Samples. Lyophilization of apoenzymes both of cAspAT and mAspAT resulted in the formation of considerable amounts of insoluble materials. Hence, the concentration and exchange for a desired medium were carried out by using centrifugation of sample solutions in Centriflo ultrafiltration membrane cones (type CF25, Amicon). All buffer solutions used were prepared in ^2H_2O. A stock solution of the pyridoxal form of cAspAT was concentrated to approximately 200 mg/mL and then diluted with a 10-fold volume of 0.1 M sodium succinate buffer (pH 6.0), followed by concentration. After another repetition of this step, the sample was taken up in 5 mL of the same succinate buffer and was warmed at 65 °C for 30 min to enhance the exchange of polypeptide amide protons for deuterons. Small amounts of denatured proteins were removed by centrifugation. The resulting solution was again concentrated in the ultrafiltration membrane cone as described above. The sample was twice washed by ^2H_2O. Then, 5 mL of 50 mM L-cysteinesulfinate in 20 mM sodium phosphate buffer (pH 8.0) was added to convert the pyridoxal form of the enzyme into the pyridoxamine form, followed by centrifugation. This step was repeated twice. A portion of the resulting sample was withdrawn and stored as the pyridoxamine form of the enzyme for NMR measurements. To the remaining portion was added 5 mL of 0.5 M sodium phosphate buffer (pH* 4.8), followed by centrifugation. This procedure was repeated again to ensure the dissociation of pyridoxamine-P from the enzyme. The sample was then washed 3 times with 20 mM sodium phosphate buffer (pH* 7.0) by means of ultrafiltration. The resulting apoenzyme of cAspAT showed a A_{280}/A_{330} value greater than 90 and a specific activity higher than 85% that of the original holoenzyme upon reconstitution with pyridoxal-P. This procedure for preparation of the pyridoxamine form and the apoenzyme is based on that described previously (Jenkins & D'Ari, 1966a; Scardi et al., 1963). However, apoenzyme samples of mAspAT similarly prepared showed only 30–40% of the original activity upon reconstitution with pyridoxal-P. Further decrease in the activity was observed during NMR measurements at room temperature. Thus we have not been able to obtain an apoenzyme preparation of mAspAT suitable for proton NMR experiments.

Preparation of [4′-^{2}H]Pyridoxal-P. Pyridoxamine dideuterated at position 4′ was prepared by incubating pyridoxamine (20 μmol) and pyridoxal (70 μmol) for several weeks at 30 °C in 2 mL of ^2H_2O at pH* 4.5 (Dunathan et al., 1968),

followed by chromatography on an Amberlite CG-50 column (Peterson & Sober, 1984). The proton NMR spectrum of the purified sample showed only a residual (7%) signal at 4.29 ppm (4′-N-CH_2). The dideuterated pyridoxamine was phosphorylated by the method using phosphorus oxychloride (Heyl et al., 1951), followed by purification on an Amberlite CG-50 column. The resulting dideuterated pyridoxamine-P was converted to pyridoxal-P by incubating for 30 min at 30 °C in the presence of 20 mM glyoxylate and 1 mM $CuSO_4$ (Metzler et al., 1954). [4′-^{2}H]Pyridoxal-P was isolated by chromatography on a Dowex 50 column equilibrated with 10 mM ammonium formate (pH 3.25) (Tiselius, 1972). Fractions containing pyridoxal-P were combined and lyophilized. The sample thus obtained showed an absorption spectrum identical with that of an authentic sample of pyridoxal-P. Its proton NMR spectrum (pH* 2.0) showed 3 protons as a singlet at 2.51 ppm (2-CH_3), 2 protons as a singlet at 5.14 ppm (5-C-H_2–), 1 proton as a singlet at 7.82 ppm (6-H), and a residual (about 0.1-proton) singlet signal at 10.47 ppm (4′-H). The deuterated pyridoxal-P-reconstituted cAspAT showed absorption spectra indistinguishable from that of the native pyridoxal form of cAspAT.

^{1}H NMR Spectroscopy. ^{1}H NMR spectra were obtained at 199.5 MHz by using Fourier transform FX-NMR-200 spectrometer (Jeol, Tokyo). A spectral width of 4000 or 5000 Hz was used. Free induction decays were multiplied by an exponential function to yield a line broadening of 1.2 Hz. Convolution difference spectra were obtained as described (Campbell et al., 1973). Chemical shift was expressed in parts per million (ppm) downfield from 3-(trimethylsilyl)[2,2,3,3-^{2}H]propionic acid as an external reference. The variable temperature controller was calibrated by the temperature-dependent chemical shift change of OH of 1,3-propanediol.

Results

Comparison of ^{1}H NMR Spectra of α, β, and γ Subforms of cAspAT. Three naturally occurring subforms of pig heart cAspAT are distinguished from one another both catalytically and spectrally (Martinez-Carrion et al., 1967). In principle, the α subform is fully active, the β subform is half-active, and the γ subform is totally inactive. In accordance with this difference in the enzymic activity, the α subform shows a predominant absorption band at 430 nm in an acidic solution (pH 6). In contrast, the γ subform is lacking in the 430-nm band and instead absorbs predominantly at 340 nm. The β subform possesses a spectral feature just intermediate between the α and γ subforms. Such spectral differences are considered to result from a different mode of combination of the coenzyme, pyridoxal-P, with the apoprotein. A catalytically active mode of combination must be via an aldimine linkage to the ε-amino group of a lysyl residue.

It is speculated that in the γ subform the coenzyme may exist in both subunits as a substituted aldimine derivative. Hence, the β subform may represent a heterodimer in which one subunit combines with the coenzyme as an aldimine and the other as a substituted aldimine (hence, half-active). Recent crystallographic studies have shown that a substrate-like molecule is present in one of the active sites of the β subform (Arnone et al., 1982). Thus a comparison of ^{1}H NMR spectra between the α and γ subforms should provide a logical, straightforward means for the spectral indentification of 4′-H of pyridoxal-P in the aldimine. However, no apparent difference in the ^{1}H NMR spectra was observed between the three subforms at the 8–9 ppm region where an aldimine proton would resonate (Figure 1). Taking advantage of a fairly high thermal stability of cAspAT, NMR spectra were

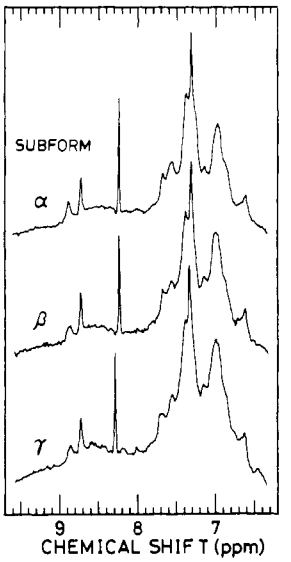


FIGURE 1: Comparison of ^{1}H NMR spectra between subforms of cAspAT. (Upper spectrum) Pyridoxal form of the α subform of cAspAT in 20 mM sodium phosphate buffer, pH* 6.58. Peaks 2, 3, and 6, which titrate with pH (Morino et al., 1984), are all visible. The pH-titratable peak 4 is very broad at this pH range and overlaps peak 3. (Middle spectrum) Pyridoxal form the β subform in 20 mM sodium phosphate buffer, pH* 6.58. (Lower spectrum) The γ subform in 20 mM sodium phosphate buffer, pH* 6.49. The enzyme concentration of the three samples was adjusted to 0.9 mM with respect to the monomeric unit of the enzyme. A total of 1000 transients were accumulated for each spectrum at 28 °C. Spectra are resolution-enhanced by the convolution difference method.

observed at higher temperatures in the hope that a resonance line for the aldimine proton might resolve from other lines. As can be seen in Figure 2, peak 1 of the α subform sharpened increasingly with the rise in temperature while the corresponding signal of the γ subform broadened conversely. It is evident that the nature of peak 1 is quite different between the two subforms. Upon cooling back to room temperature, the two subforms became spectrally indistinguishable again from each other. Thus peak 1 in the α subform seems to represent a composite of at least two resonances, one of which reflects 4′-H of the internal aldimine.[2] In fact, close inspection of the spectra in Figure 1 reveals that peak 1 of the γ subform is a little smaller than that of the α subform and resonates at a chemical shift upfield by 0.03 ppm from the corresponding resonance in the α subform. These findings suggest that the 4′-H of pyridoxal-P in the aldimine should resonate at 8.89 ppm. There were no significant differences between the two subforms in the distribution pattern of other resonances in the 9–6 ppm region. Differences in chemical shift of some resonances between the two samples resulted from the difference in pH values of the samples, since the titration curves for His C-2 protons of the γ subform (unpublished results) were identical with those for the α subform (Morino et al., 1984).

Effect of L-Cysteinesulfinate on ^{1}H NMR Spectra of the Pyridoxal Form of cAspAT. The internal aldimine 4′-H could be identified on ^{1}H NMR spectra by a comparison between the pyridoxal and the pyridoxamine form of each isoenzyme. L-Cysteinesulfinate is an ideal amino acid substrate for this

[2] The complex nature of peak 1 is understood in light of the effect of various internal-aldimine-cleaving reagents on this peak. The data in Figures 3–5 show that after the resonance assigned to the aldimine 4′-H disappeared upon addition of these ragents, a broad resonance P-1′ still remains at the position upfield by 0.03 ppm to resonance P-1 observed prior to reaction with these reagents. Resonance P-1′ is probably identical in nature with peak 1 of the γ subform. Thus resonance P-1 appears to represent a composite of the resonance for 4′-H and the resonance P-1′.

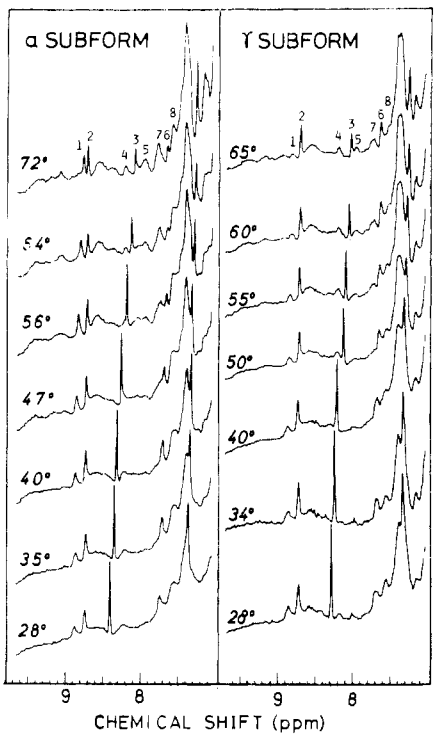


FIGURE 2: Differential effect of increasing temperature upon ^1H NMR spectra of α and γ subforms of cAspAT. (Left-hand panel) α subform (0.9 mM) in pyridoxal form in 0.5 mL of ^2H_2O containing 12 mM sodium glutarate, pH* 6.31, and 0.5 mM EDTA. (Right-hand panel) γ subform (0.8 mM) in 0.5 mL of ^2H_2O containing 12 mM sodium glutarate, pH* 6.58, and 0.5 mM EDTA. In both runs, the spectra were serially obtained at increasing temperatures as indicated on the spectra. Prior to the NMR measurement a period of 10 min was allowed for thermal equilibration of the samples at a desired temperature. Each spectrum was obtained by accumulating 1000 transients. Although the data were not shown, the NMR spectra of both samples obtained upon cooling back to 28 °C were indistinguishable from those obtained at 28 °C before raising the temperature, except for peak 3, which reflects an exposed histidyl C-2 proton (Morino et al., 1984) and, hence, showed a reduced signal intensity due to its ready exchange for medium deuterons. The numbering of resonances corresponds to that described previously (Morino et al., 1984).

purpose. Addition of an equimolar amount of this amino acid to a solution of the pyridoxal form of enzyme in an NMR tube would be sufficient for its stoichiometric conversion to the pyridoxamine form, since the corresponding keto acid formed upon transamination is spontaneously decomposed to pyruvate, which is a very poor keto acid substrate (Jenkins & D'Ari, 1966a), thus rendering the conversion to the pyridoxamine form virtually irreversible and allowing us to observe spectral changes specific for this conversion. The ^1H NMR difference spectrum obtained by subtracting the spectrum prior to the addition of cysteinesulfinate from that after its addition showed a number of positive–negative changes over the entire range of the spectrum (Figure 3). These changes presumably reflect conformational redistribution of side-chain protons, which may accompany a tilt of the coenzyme pyridine ring occurring during the conversion of the pyridoxal to the pyridoxamine form of the enzyme (Ford et al., 1980; Arnone et al., 1982). Close inspection of the spectrum revealed a distinct negative change at 8.89 (Figure 3B). Similar spectral observations at several different pH values showed a negative change at 8.89–8.90 ppm (Figure 4), which uniformly corresponded in its signal intensity to a single proton as compared with the signal P-2 previously assigned to the C-2 proton of His-68 (Morino et al., 1984). This situation was reproducible with several batches of enzyme samples over the range of pH from 5 to 9. Thus, the ionization state of the internal aldimine does

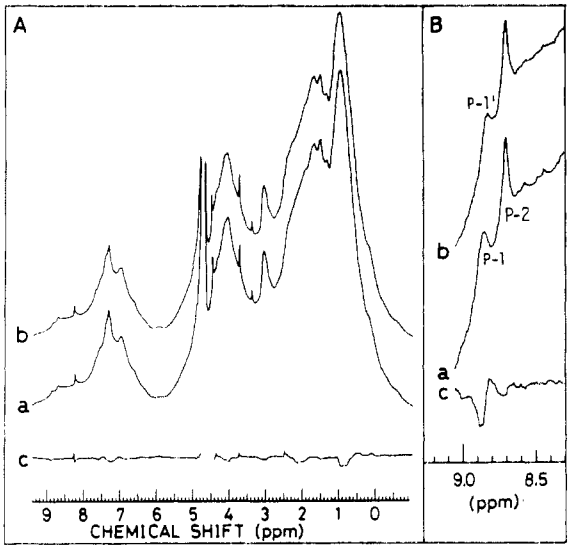


FIGURE 3: Effect of cysteinesulfinate on NMR spectra of cAspAT in the pyridoxal form. The solution contained, in 0.5 mL of ^2H_2O, 0.9 mM pyridoxal form of the α subform of cAspAT, 0.1 mM EDTA, and 20 mM sodium phosphate buffer, pH* 6.58. The spectra were obtained by accumulating 1000 transients at 30 °C. (A) (a) Spectrum before adding 1.2 mM L-cysteinesulfonate; (b) spectrum after the addition; (c) difference between spectra b and a (spectrum b minus spectrum a). The water resonance at the 4.5–4.8 ppm region was deleted. (B) The 8–9-ppm region of the spectra in (A) are 16-fold expanded. The symbols a, b, and c, correspond to those in (A). P-1, P-2, and P-3 denote respectively peaks 1, 2, and 3 as defined previously (Morino et al., 1984). P-1′ denotes peak 1 of the pyridoxamine form.

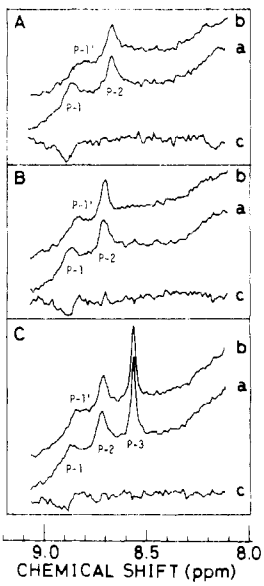


FIGURE 4: Internal aldimine 4′-H in ^1H NMR spectra of cAspAT at various pH values. Conditions for samples and NMR measurement were the same as those for Figure 3, except that buffers used were 20 mM sodium citrate, pH* 8.18 (A), 20 mM sodium phosphate, pH* 6.92 (B), and 20 mM sodium citrate, pH* 5.63 (C). (a) Spectra before the addition of 1.2 mM L-cysteinesulfinate; (b) spectra after the addition; (c) difference (spectrum b minus spectrum a). P-1, P-2, P-3, and P-1′ are as defined for Figure 3.

not seem to affect appreciably the chemical shift of the aldimine 4′-H in cAspAT. It is also noted that after conversion to the pyridoxamine form there still remained a broad resonance (P-1′ in Figure 3 and 4) slightly upfield to the peak 1 in the pyridoxal form of the enzyme.

Effect of (Aminooxy)acetate on ^1H NMR Spectra of the Pyridoxal Form of cAspAT. A variety of reagents are known to disrupt the aldimine bond formed between pyridoxal-P

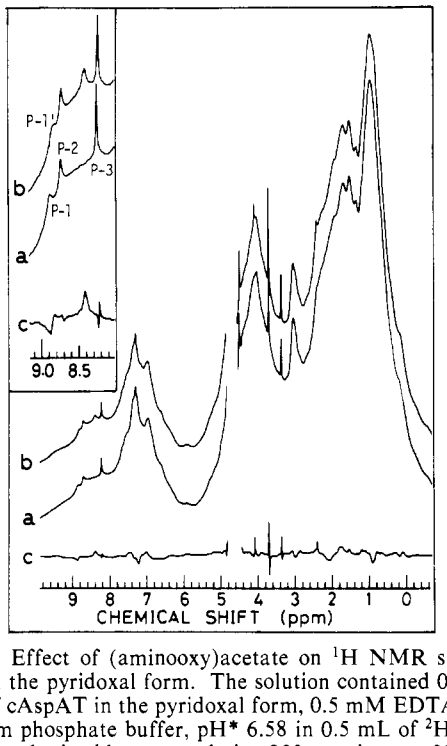


FIGURE 5: Effect of (aminooxy)acetate on ^1H NMR spectra of cAspAT in the pyridoxal form. The solution contained 0.8 mM α subform of cAspAT in the pyridoxal form, 0.5 mM EDTA, and 20 mM sodium phosphate buffer, pH* 6.58 in 0.5 mL of ^2H_2O. The spectra were obtained by accumulating 800 transients at 30 °C. (a) Spectrum before the addition of 1.0 mM (aminooxy)acetate; (b) spectrum after the addition; (c) difference between spectra b and a. The inset shows a 4-fold expansion of the spectra at the 8–9-ppm region. P-1, P-2, and P-3 are as defined for Figure 3. P-1′ denotes the resonance remaining after the enzyme reacted with (aminooxy)acetate. The water resonance at the 4.5–4.8-ppm region was omitted.

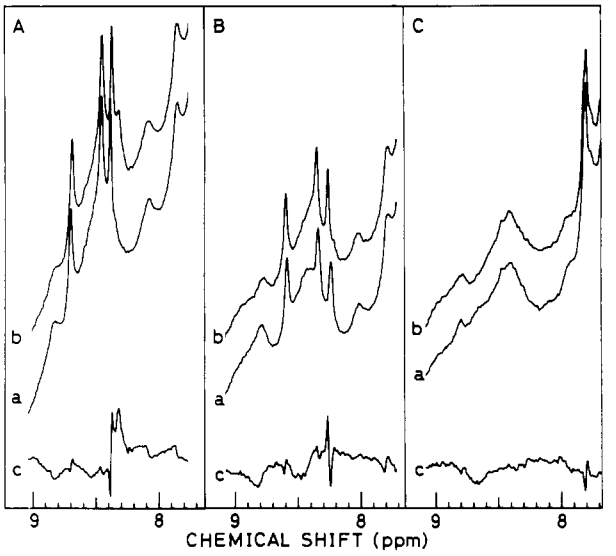


FIGURE 6: Effect of (aminooxy)acetate (A) and cysteinesulfinate (B and C) on ^1H NMR spectra of mAspAT in the pyridoxal form. (A) The sample solution contained 0.8 mM pyridoxal form of mAspAT, 20 mM sodium cacodylate buffer, pH* 5.77, and 0.2 mM EDTA in 0.5 mL of ^2H_2O. (a) Spectrum before the addition of 1.2 mM (aminooxy)acetate; (b) spectrum after the addition; (c) difference (spectrum b minus spectrum a). (B) The sample solution contained 0.9 mM pyridoxal form of mAspAT, 0.2 mM EDTA, and 20 mM sodium cacodylate buffer, pH* 6.01. (C) Same as in (B) except that 20 mM sodium borate buffer, pH* 8.30, was used. In both (B) and (C), spectra a represent those before addition of 1.0 mM L-cysteinesulfinate; spectra b, after the addition; spectra c, difference (spectrum b minus spectrum a). Each spectrum was obtained by accumulating 1000 transients at 30 °C.

4-formyl and Lys ϵ-amino group. (Aminooxy)acetate is one of these reagents and is known to form an imine complex with some pyridoxal-P-dependent enzymes (Torchinsky & Korenova, 1964). At acidic and neutral pH values, the pyridoxal form of either cAspAT or mAspAT was rapidly converted by an equimolar amount of (aminooxy)acetate into an imine complex absorbing at 380 nm (data not shown). By use of this stoichiometric reactivity, ^1H NMR spectra of cAspAT were compared before and after the addition of (aminooxy)-acetate in a slight excess over the amount of enzyme-bound pyridoxal-P (Figure 5). Comparison of both spectra showed the appearance of a new signal at 8.40 ppm and a broad resonance (P-1′ in spectrum b) slightly upfield to peak 1 (P-1 in spectrum a). The difference spectrum clearly showed that, upon the external imine formation, a signal at 8.89 ppm appeared as a negative change and a new signal emerged at 8.40 ppm. These changes corresponded in intensity to 1 proton (Figure 5, inset). In accord with the conclusion derived from the comparison of the spectra between the α and γ subforms as well as from the experiment using cysteinesulfinate, the signal at 8.89 ppm should be assigned to the pyridoxal-P 4′-H of the internal aldimine. The signal at 8.40 ppm must reflect the corresponding proton in the external aldimine. Similar experiments were performed at several pH values to see whether or not the pH affects the chemical shift of these signals. However, variation in pH did not exert any detectable effect on the chemical shift of the internal aldimine proton (data not shown), as described above for the experiment (Figure 4) using cysteinesulfinate. Although a value of pK 6.3[3] was assigned to the protonation and deprotonation of the internal aldimine of cAspAT (Jenkins et al., 1959), the ionization state of the aldimine nitrogen does not seem to affect the chemical shift of the 4′-H in cAspAT.

Internal Aldimine 4′-H in mAspAT. As described for cAspAT, (aminooxy)acetate and cysteinesulfinate were utilized for identification of the internal aldimine 4′-H of mAspAT. Upon the addition of these ligands in a slight molar excess over the enzyme-bound pyridoxal-P, redistribution of many resonances to a relatively limited extent was observed over the entire region of ^1H NMR difference spectra as in the case of cAspAT. Overall patterns were largely similar to each other with both ligands (data not shown). The spectral changes occurring at the 8–9 ppm region upon reaction with (aminooxy)acetate and cysteinesulfinate are shown in Figure 6. A negative change corresponding in signal intensity to 1 proton was observed at a position close to but not identical with the chemical shift (8.80 ppm) for peak 1 (Morino et al., 1984) with both ligands, and an additional positive change at 8.31 ppm with (aminooxy)acetate (Figure 6A) was observed. By analogy with the case of cAspAT, the former could be assigned to the internal aldimine 4′-H while the latter could be assigned to the corresponding proton of an external imine formed with (aminooxy)acetate. In contrast with cAspAT, however, the chemical shift of the negative change in mAspAT was found to vary with pH (Figure 6B,C; also see Figure 7). The function of chemical shift vs. pH revealed a pK value of 6.3 (Figure 7), which coincides with that determined spectrophotometrically for the dissociation of the proton from the internal aldimine nitrogen (Wada & Morino, 1964). Thus, the chemical shift of the internal aldimine 4′-H of mAspAT changes with the ionization state of the imine group. This is in striking contrast to the situation with cAspAT, where the corresponding proton did not sense the variation of the solvent pH.

[3] Under the present conditions, spectrophotometric titration yielded a pK value of 6.3 for ionization of the aldimine in both isoenzymes.

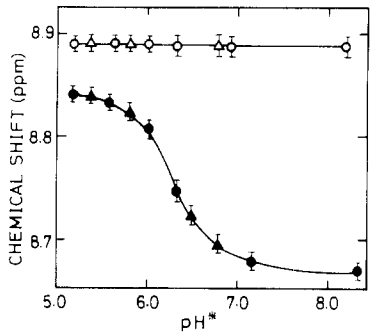


FIGURE 7: Comparison of pH-dependent changes in chemical shift of internal aldimine 4′-H between cAspAT and mAspAT. With each isoenzyme, the resonance for 4′-H was obtained from ^{1}H NMR difference spectra at 30 °C before and after the addition of cysteinesulfinate (○, ●), or (aminooxy)acetate (△, ▲). Open symbols show data for cAspAT; closed symbols, data for mAspAT. The data were obtained from the experiments described for Figure 3–6 and from additional experiments performed at different pH values. Each point represents an average of triplicate experiments. Vertical bars denote ranges of chemical shift values.

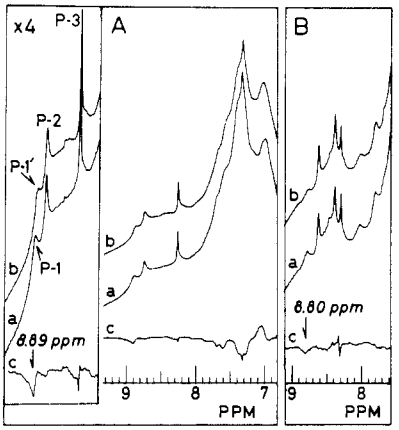


FIGURE 8: Effect of NH_2OH and $NaCNBH_3$ on the ^{1}H NMR spectra of cAspAT (A) and mAspAT (B). (A) The solution contained 0.9 mM pyridoxal form of cAspAT, 20 mM sodium phosphate buffer (pH* 6.56), and 0.2 mM EDTA in 0.5 mL of ^2H_2O. (a) Spectrum before the addition of 2 mM NH_2OH; (b) spectrum after the addition; (c) difference (spectrum b minus spectrum a). The inset shows 4-fold-expanded spectra. P-1, P-2, and P-3 are as defined for Figure 3. P-1′ denotes the resonance remaining after the enzyme reacted with NH_2OH. (B) The solution contained 0.8 mM pyridoxal form of mAspAT, 20 mM sodium cacodylate buffer (pH* 5.95), and 0.2 mM EDTA in 0.5 mL of ^2H_2O. (a) Spectrum before the addition of 2 mM $NaCNBH_3$; (b) spectrum at 30 min after the addition; (c) difference (spectrum b minus spectrum a).

Effect of Other Internal-Aldimine-Destroying Reagents. NH_2OH, $NaBH_4$, and $NaCNBH_3$ reacted with the pyridoxal form of both cAspAT and mAspAT in a stoichiometric fashion. Figure 8A shows typical NMR difference spectra before and after the reaction of NH_2OH with the pyridoxal form of cAspAT. The negative change at 8.89 ppm showed 1-proton intensity as compared with signal P-2 and should represent the internal aldimine 4′-H, as demonstrated above with cysteinesulfinate and (aminooxy)acetate (Figures 3 and 5). The reaction of $NaCNBH_3$ with the pyridoxal form of mAspAT gave rise to a negative change at 8.80 ppm (Figure 8B). This is in accord with the results obtained with other reagents (see Figure 6).

Conversion of the Pyridoxamine Form to the Pyridoxal Form. The ^{1}H NMR spectra of the pyridoxamine form of cAspAT did not appreciably differ from that of the pyridoxal form. A difference was that peak 1 (P-1′ in Figure 9B) resonated upfield by 0.03–0.04 ppm in the pyridoxamine form

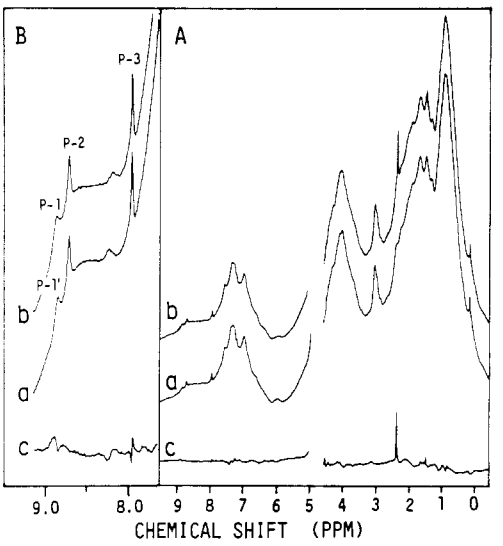


FIGURE 9: Effect of pyruvate on the ^{1}H NMR spectrum of the pyridoxamine form of cAspAT. (A) (a) Spectrum of 0.8 mM pyridoxamine form of cAspAT in 20 mM sodium phosphate buffer (pH* 7.45); (b) spectrum after the addition of 3 mM pyruvate; (c) difference (spectrum b minus spectrum a). (B) 10-fold expanded spectra. The spectra correspond to those in (A). P-1, P-2, P-3, and P-1′ are as defined for Figure 3.

as compared to that (P-1) in the pyridoxal form (Morino et al., 1984). The addition of pyruvate[4] in a 3 times molar excess over the amount of the bound pyridoxamine-P fully converted the pyridoxamine form of the enzyme into the pyridoxal form as judged from the change in absorption spectra (data not shown). Upon the addition of pyruvate, redistribution of many resonances to a relatively limited extent was observed over the entire region of the ^{1}H NMR difference spectrum (Figure 9A), reflecting a slight conformational difference between the two coenzymic forms. It should be noted that a new broad resonance line appeared at 8.89 ppm (Figure 9B), which could be assigned to the internal aldimine 4′-H. By use of the same procedure as described above for cAspAT, it was shown that the addition of pyruvate to the pyridoxamine form of mAspAT at pH* 8.25 induced the appearance of a new resonance at 8.67–8.68 ppm which should be assigned to 4′-H of the internal aldimine (see Figure 7).

Reconstitution of cAspAT Apoenzyme with [4′-^{1}H]-Pyridoxal-P and [4′-^{2}H]Pyridoxal-P. The ^{1}H NMR difference spectrum of the cAspAT apoenzyme before and after the addition of an equimolar (with respect to the monomeric unit of the enzyme) amount of pyridoxal-P showed redistribution of many resonance lines over the entire region of the spectrum, reflecting a limited degree of conformational changes occurring upon reconstitution with pyridoxal-P (Figure 10A,B). Among these spectral changes, a new broad resonance line appeared at 8.89 ppm which corresponds in chemical shift to that for the coenzyme aldimine 4′-H. Comparison of the difference spectra c in panels A and B of Figure 10 showed that the chemical shift for this resonance was identical at two different

[4] Pyruvate is a poor substrate for aspartate aminotransferase but reacts with the pyridoxamine form of either cAspAT or mAspAT at a rate sufficient for the present purpose. This keto acid has also an advantage in that it induced only a small conformational change detectable in the NMR spectra, and thus the major spectral change at the 9-ppm region was restricted to that resulting from the appearance of the resonance for the internal aldimine 4′-H. In contrast, a natural dicarboxylic substrate like 2-oxoglutarate caused a dramatic redistribution of many NMR signals over the entire region of the spectra, which reflects a large conformational change, and, hence, complicated the interpretation of the spectral changes occurring at the 9-ppm region.

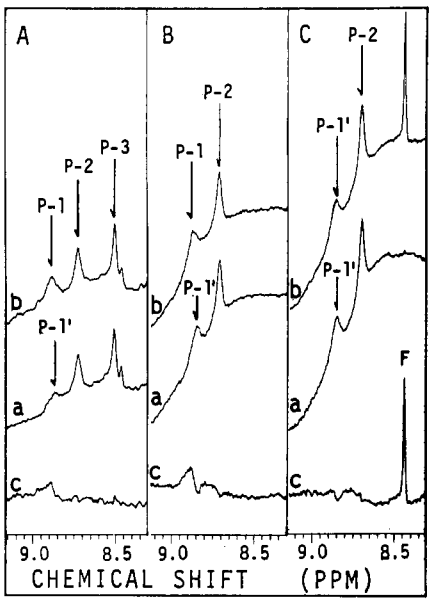


FIGURE 10: Binding of pyridoxal-P and deuterated pyridoxal-P to the cAspAT apoenzyme. (A) (a) Spectrum of 0.8 mM cAspAT apoenzyme in 20 mM sodium cacodylate buffer (pH* 5.91); (b) spectrum at 10–20 min after the addition of 0.8 mM pyridoxal-P to the apoenzyme; (c) difference (spectrum b minus spectrum a). (B) Same as in (A) except that 20 mM sodium phosphate buffer (pH* 7.40) replaced the cacodylate buffer. (C) [4′-^{2}H]Pyridoxal-P (1 mM) was added to 0.9 mM cAspAT apoenzyme in 20 mM sodium phosphate buffer (pH* 7.60). (a) Apoenzyme alone; (b) apoenzyme plus deuterated pyridoxal-P; (c) difference spectrum (spectrum b minus spectrum a). F denotes the signal from contaminating formic acid. P-1, P-2, and P-3 are as defined for Figure 3. P-1′ in the apoenzyme may be identical with P-1′ in the pyridoxamine form and the complex with (aminooxy)acetate of the enzyme.

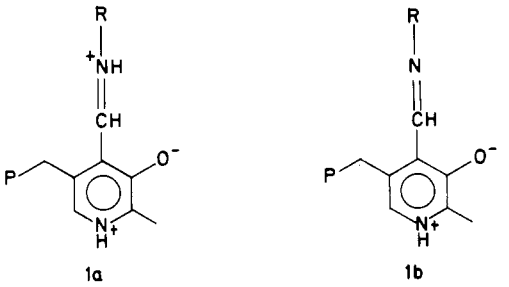
pH values, supporting the result shown in Figure 7. Addition of a slight molar excess of cysteininesulfinate to the reconstituted enzyme resulted in the disappearance of the resonance at 8.89 ppm, in accord with the result shown previously in Figure 3. Similar reconstitution experiments were attempted with mAspAT. However, the apoenzyme of mAspAT was found to be not sufficiently stable for the purpose, as described under Experimental Procedures.

To further confirm the above result, [4′-^{2}H]pyridoxal-P was synthesized, and its effect on the ^{1}H NMR spectrum of the cAspAT apoenzyme was examined. The reconstitution with the deuterated pyridoxal-P produced a difference spectrum that was apparently indistinguishable from that obtained with the nondeuterated pyridoxal-P over the entire spectral region (data not shown). A single distinct difference was observed at the 8.9-ppm region; namely, upon reconstitution with [4′-^{2}H]pyridoxal-P, no distinct resonance emerged at 8.89 ppm (Figure 10C). This must result from the replacement of hydrogen by deuterium at position 4′ of pyridoxal-P. Thus the result provides further evidence that the resonance at 8.89 ppm represents the 4′-H of the internal aldimine in cAspAT.

Discussion

This paper has described three approaches to the identification of the coenzyme aldimine 4′-H in ^{1}H NMR spectra of aspartate aminotransferase isoenzymes. The first is the comparison of ^{1}H NMR spectra between the α and γ subforms of cAspAT. The second is the use of reagents to modify the internal aldimine bond in a specific fashion. This approach can be conveniently applied also to other pyridoxal-P dependent enzymes by using $NaBH_4$, $NaCNBH_3$, or imine-forming reagents such as NH_2OH, (aminooxy)acetate, etc. The third is to observe the effect of pyridoxal-P on the ^{1}H NMR spectrum of an apoenzyme. Although it is a direct way for the present purpose, it is practicable only when the coenzyme is readily dissociable under mild conditions and the resulting apoenzyme is sufficiently stable.

Chart I



The pyridoxal form of cAspAT shows pH-dependent changes in the visible region of absorption spectra that are attributed to protonation of the internal aldimine nitrogen (Jenkins et al., 1959). Then it is readily conceived that protonation on and unprotonation from the nitrogen atom might influence the chemical shift of vicinal 4′-H. In fact, the 4′-H of the Schiff base formed between L-valine and pyridoxal showed on the ^{1}H NMR spectrum a pH-dependent chemical shift change, at 8.80 ppm (30 °C) for the protonated Schiff base **1a** (see Chart I) and 8.76 ppm for the unprotonated form **1b**, with a proteolytic pK around pH* 10.6, which coincides with that determined spectrophotometrically (unpublished result). In accord with this is the pH-dependent variation in chemical shift of the coenzyme aldimine 4′-H in mAspAT. In contrast, the aldimine 4′-H of cAspAT did not show any detectable change in the chemical shift by the variation of pH which obviously caused a pH-dependent shift of absorption bands at 360 and 430 nm with a pK of 6.3 (Jenkins et al., 1959). This may result from a unique immediate environment of the internal aldimine 4′-H in cAspAT, which acts in a manner counteracting a possible electronic influence from the ionization state of the adjacent nitrogen. A similar interisozymic difference in the pH dependency of the chemical shift for the phosphorus of the enzyme-bound pyridoxal-P was reported: chemical shift differences between high- and low-pH forms of mAspAT (Mattingly et al., 1982) and cAspAT (Schnackerz, 1984) are 1.3 and 0.5 ppm, respectively. The pH dependency showed an identical pK value of 6.3, which corresponds exactly to the spectrophotometrically determined pK value for the dissociation of the proton from the internal aldimine group. Thus the ^{31}P nucleus in the two isoenzymes appears to sense the ionization state of the coenzyme aldimine differently. Various possibilities for tautomerism in the enzyme-bound coenzyme molecule are raised (Arnone et al., 1985). Subtle interisozyme differences in the microenvironment provided by protein side chains may influence differently the electronic state of the bound coenzyme.

By a simple comparison of the NMR spectra before and after the addition of an internal-aldimine-cleaving reagent (Figures 3–5), it was hard to detect the resonance for the internal aldimine 4′-H. The difference spectrum revealed this resonance as a negative change corresponding in intensity to a single proton by comparison with the intensity of peak 2 or 3. In spite of the disappearance of the 4′-H resonance, there still remained a broad resonance (P-1′ in spectra b in Figures 3–5) at a position similar but slightly upfield by 0.03 ppm to that for peak 1 of cAspAT prior to reaction with the reagent. This remaining resonance did not change the chemical shift in the range of pH* 5–9 and showed a much broader line width than peaks 2 and 3 which have been shown to titrate with pH

as His C-2 protons (Morino et al., 1984). It is not known at present whether or not this broad resonance may represent His C-2 proton in an abnormal environment. A similar argument may be extended also to the situation of the peak 1 of mAspAT. In this case, however, the resonance assignable to the internal aldimine 4′-H changed in its chemical shift with pH (Figure 7), leaving a broad resonance at 8.80 ppm independently from the change in pH (see Figure 6).

In both isoenzymes, the 4′-H resonance showed a considerable broadening of the line width in going from 20 to 30 Hz. An extensive broadening of signals appears to be common to proteins of high molecular weight and is understood to result from the slow tumbling of the molecule. With both isoenzymes having a large rotational correlation time [4.3×10^{-8} s for cAspAT (Churchich, 1967)], the line width of a proton resonance would become considerably broad (~30 Hz) when a single proton existed within ~2.3 Å in its vicinity (Farrar & Becker, 1971), and such a situation may well be expected for the internal aldimine 4′-H. Candidates for such protons might be the 5-(hydroxymethyl) protons, which should be close enough to 4′-H and restricted in mobility, since it is linked to the phosphate group which interacts strongly with the positively charged group of Arg-266, peptide carbonyl groups belonging to Ser-107 and -106, and the hydroxyl of Ser-255 (Ford et al., 1980; Arnone et al., 1982).

With both isoenzymes, the difference spectra observed upon the binding of ligands showed positive–negative changes in the entire spectral regions, reflecting redistribution of 10–15 protons upon the complex formation (see Figures 3 and 4). This rather small change suggests that the structural perturbation has occurred to the residues in the very vicinity of the bound pyridoxal-P molecule, since the coenzyme pyridine ring is well expected to tilt upon cleavage of the internal aldimine bond, as was demonstrated by crystallographic studies on the complex with 2-methylaspartate (Ford et al., 1980; Arnone et al., 1982).

A group of dicarboxylic ligands such as glutamate, aspartate, and *erythro*-3-hydroxyaspartate could in principle be utilized for the identification of the internal aldimine 4′-H in aspartate aminotransferase. When these reagents were tested, however, ^{1}H NMR difference spectra showed complex changes at the 8.9–9.1-ppm region which were evidently more than the change reflecting the disappearance of the internal aldimine 4′-H. The extent of redistribution of many resonances observed over the entire region of the difference spectra was also far more extensive than that elicited by more specific ligands such as (aminooxy)acetate (unpublished results). When glutamate or aspartate is added in excess over the amount of the pyridoxal form, the equilibrium mixture would contain the amino acid and the corresponding keto acid, both of which should interact with either the pyridoxal or the pyridoxamine form of enzyme (Jenkins & D'Ari, 1966b; Michuda & Martinez-Carrion, 1970), thus producing, on ^{1}H NMR spectra, various changes similar to those elicited upon the interaction with succinate or glutarate, typical dicarboxylic competitive inhibitors for aspartate aminotransferase. Details of NMR studies on the interaction of these dicarboxylic ligands with both isoenzymes of aspartate aminotransferase will be described elsewhere.

Registry No. Pyridoxal-P, 54-47-7; aspartate aminotransferase, 9000-97-9.

REFERENCES

Arnone, A., Bailey, P. D., Rogers, P. H., Hyde, C. C., Metzler, C. M., & Metzler, D. M. (1982) in *Molecular Structure and Biological Activity* (Griffin, J. F., & Duax, W. L., Eds.) pp 57–74, Elsevier/North-Holland, New York.

Arnone, A. Christen, P., Jansonius, J. N., & Metzler, D. E. (1985) in *Transaminases* (Christen, P., & Metzler, D. E., Eds.) pp 326–357, Wiley, New York.

Boyd, J. W. (1961) *Biochem. J. 81*, 434–441.

Campbell, I. D., Dobson, C. M., Williams, R. J. P., & Xavier, A. V. (1973) *J. Magn. Reson. 11*, 172–181.

Churchich, J. E. (1967) *Biochim. Biophys. Acta 147*, 511–517.

Dunathan, H. C., Davis, L., Kury, P. G., & Kaplan, M. (1968) *Biochemistry 7*, 4532–4542.

Farrar, T. C., & Becker, E. D. (1971) *Pulse and Fourier Transform NMR*, Chapter 4, Academic Press, New York.

Fischer, E. H., Kent, A. B., Synder, E. R., & Krebs, E. G. (1958) *J. Am. Chem. Soc. 80*, 2906–2907.

Ford, G. C., Eichele, G., & Jansonius, J. N. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 2559–2563.

Heyl, P., Luz, E., Harris, A. A., & Folkers, K. (1951) *J. Am. Chem. Soc. 73*, 3436–3437.

Hughes, R. C., Jenkins, W. T., & Fischer, E. H. (1962) *Proc. Natl. Acad. Sci. U.S.A. 48*, 1615–1618.

Jencks, W. P., & Cordes, E. (1963) in *Chemical and Biological Aspects of Pyridoxal Catalysis* (Snell, E. E., Fasella, P. M., Braunstein, A. E., & Rossi Fanelli, A., Eds.) pp 57–67, Pergamon Press, Elmsford, NY.

Jenkins, W. T., & D'Ari, L. (1966a) *Biochem. Biophys. Res. Commun. 22*, 376–382.

Jenkins, W. T., & D'Ari, L. (1966b) *J. Biol. Chem. 241*, 5667–5674.

Jenkins, W. T., Yphantis, D. A., & Sizer, I. W. (1959) *J. Biol. Chem. 234*, 51–57.

Kagamiyama, H., Sakakibara, R., Wada, H., Tanase, S., & Morino, Y. (1977) *J. Biochem. (Tokyo) 82*, 291–294.

Katunuma, N., Matsuzawa, T., & Huzino, A. (1962) *J. Vitaminol. 8*, 74–84.

Martinez-Carrion, M., Turano, C., Chiancone, E., Bossa, F., Giartosio, A., Riva, F., & Fasella, P. (1967) *J. Biol. Chem. 242*, 2397–2409.

Mattingly, M. E., Mattingly, J. R., Jr., & Martinez-Carrion, M. (1982) *J. Biol. Chem. 257*, 8872–8878.

Metzler, D. E., Olivard, J., & Snell, E. E. (1954) *J. Am. Chem. Soc. 76*, 644–648.

Michuda, C. M., & Martinez-Carrion, M. (1970) *J. Biol. Chem. 245*, 262–269.

Morino, Y. (1984) in *Chemical and Biological Aspects of Vitamin B_6 Catalysis, Part B* (Evangelopoulos, A. E., Ed.) pp 255–265, Liss, New York.

Morino, Y., & Wada, H. (1963) in *Chemical and Biological Aspects of Pyridoxal Catalysis* (Snell, E. E., Fasella, P. M., Braunstein, A. E., & Rossi Fanelli, A., Eds.) pp 175–184, Pergamon Press, Elmsford, NY.

Morino, Y., & Nagashima, F. (1984) *Methods Enzymol. 106*, 116–137.

Morino, Y., Tanase, S., Watanabe, T., Kagamiyama, H., & Wada, H. (1977) *J. Biochem. (Tokyo) 82*, 847–852.

Morino, Y., Tanase, S., Yamasaki, M., & Nagashima, F. (1982) Abstracts of the 12th International Conference on Magnetic Resonance in Biological Systems, Stanford, CA, Aug 29–Sept 3, 1982, p 124.

Morino, Y., Yamasaki, M., Tanase, S., Nagashima, F., Akasaka, K., Imoto, T., & Miyazawa, T. (1984) *J. Biol. Chem. 259*, 3877–3882.

Ovchinnikov, Yu. A., Egorov, C. A., Aldanova, N. A., Feigina, M. Y., Lipkin, V. M., Abdulaev, N. G., Grishin, E. V., Kiselev, A. P., Modyanov, N. N., Braunstein, A. E., Polyanovsky, O. L., & Nosikov, V. V. (1973) *FEBS Lett. 29*, 31–34.

Peterson, E. A., & Sober, H. A. (1954) *J. Am. Chem. Soc. 76*, 169–175.
Scardi, V., Scotto, P., Iaccarino, M., & Scarano, E. (1963) *Biochem. J. 88*, 172–175.
Schnackerz, K. D. (1984) in *Chemical and Biological Aspects of Vitamin B_6 Catalysis, Part B* (Evangelopoulos, A. E., Ed.) pp 195–208, Liss, New York.
Snell, E. E., & Di Mari, S. J. (1970) *Enzymes (3rd Ed.) 2*, 325–370.
Tiselius, H.-G. (1972) *Clin. Chim. Acta 40*, 319–324.
Torchinsky, Yu. M., & Koreneva, L. G. (1964) *Biochim. Biophys. Acta 79*, 426–429.
Wada, H., & Morino, Y. (1964) *Vitam. Horm. (N.Y.) 22*, 411–444.

Trimethoprim Binding to *Lactobacillus casei* Dihydrofolate Reductase: A ^{13}C NMR Study Using Selectively ^{13}C-Enriched Trimethoprim†

H. T. A. Cheung,‡,§ M. S. Searle,‡ J. Feeney,*,‡ B. Birdsall,‡ G. C. K. Roberts,‡ I. Kompis,∥ and S. J. Hammond‡
Physical Biochemistry Division, National Institute for Medical Research, Mill Hill, London NW7 1AA, U.K., and F. Hoffmann-La Roche and Company, CH-4002 Basle, Switzerland
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ABSTRACT: We have measured the ^{13}C chemical shifts for trimethoprim molecules selectively enriched with ^{13}C at the 2-, 4-, 5-, 6-, and 7-positions and the *p*-OCH_3 position in their complexes with *Lactobacillus casei* dihydrofolate reductase in the presence and absence of coenzyme analogues. The C2 carbon shifts indicate that the pyrimidine ring is protonated at N1 in all the complexes of trimethoprim with the enzyme and coenzymes and in each case the pyrimidine ring is binding in a similar way to that of the corresponding part of methotrexate in the enzyme–methotrexate complex. The C6 carbon of trimethoprim shows a large upfield shift in all complexes (3.51 to 4.70 ppm) but no shift in the complex of 2,4-diaminopyrimidine with the enzyme: these shifts probably arise from steric interactions between the C1′ and C2′ carbons and the H6 proton, which approach van der Waals contact in the folded conformation adopted by trimethoprim when bound to the enzyme. The large shift observed for C6 in all complexes indicates that the basic folded conformation is present in all of them. A comparison of the ^{13}C shifts in the enzyme–trimethoprim–NADPH complex with those in the enzyme–trimethoprim binary complex shows substantial changes even for carbons such as C6 and *p*-OCH_3 (0.46 and −0.36 ppm, respectively), which are remote from the coenzyme: these are caused by ligand-induced conformational changes that may involve displacement of the helix containing residues 42–49. In the ternary complex with $NADP^+$, the two conformational states previously described are further characterized: separate signals are seen for conformations I and II for the C2, C4, C5, C6, and C7 carbons. One set of chemical shifts has values similar to those measured in the binary complex with trimethoprim and also to those in the ternary complex with the methyl β-riboside of 2′-phosphoadenosine 5′-(diphosphoribose); these are assigned to conformation II of the complex. The complex of [*m-methoxy*-^{13}C]brodimoprim [[*3′-methoxy*-^{13}C]- or [*5′-methoxy*-^{13}C]-2,4-diamino-5-(3′,5′-dimethoxy-4′-bromobenzyl)pyrimidine] with the enzyme shows two equal-intensity ^{13}C signals at 274 K, which coalesce to a single absorption when the temperature is raised to 287 K. This two-site exchange between nuclei at the C3′ and C5′ positions has been characterized in terms of "ring flipping", and the rate of this process can be estimated to be 65 ± 8 s^{-1} at 287 K. For the ternary complex with $NADP^+$, the ^{13}C spectrum showed the same coalescence temperature (287 K) as observed for the binary complex: at this temperature the ternary complex is predominantly in form II (75%).

The antibacterial drug trimethoprim (TMP)[1] acts by selectively inhibiting the enzyme dihydrofolate reductase in bacterial cells. Considerable efforts have been made to understand the factors controlling the specificity of trimethoprim binding to the bacterial enzyme, and numerous structure–activity studies using trimethoprim-related inhibitors have been reported (Roth & Cheng 1982). Previously, we have used NMR methods to investigate the protonation state of the pyrimidine ring of bound trimethoprim (Roberts et al., 1981; Bevan et al., 1985), to determine its conformation in complexes with both bacterial and mammalian dihydrofolate reductase (Cayley et al., 1979; Birdsall et al., 1983), and to investigate the presence of multiple conformations in these complexes (Gronenborn et al., 1981a,b; Birdsall et al., 1984). One of the major problems encountered in studying complex NMR spectra is that of assigning the signals to particular nuclei in the protein–ligand complexes. This can be largely overcome by using isotopic labeling to simplify the NMR spectra of the proteins or their bound ligands. Isotopic labeling with ^{13}C

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‡ National Institute for Medical Research.
§ On leave of absence from the Department of Pharmacy, University of Sydney, Sydney, New South Wales, Australia.
∥ F. Hoffmann-La Roche and Co.

[1] Abbreviations: TMP, trimethoprim; DAP, 2,4-diaminopyrimidine; PADPR-OMe, methyl β-riboside of 2′-phosphoadenosine 5′-(diphosphoribose); NIC, nicotinamide; DMF, dimethylformamide.

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