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NMR SPECTRA OF EXCHANGEABLE PROTONS OF PYRIDOXAL PHOSPHATE-DEPENDENT ENZYMES

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We have recorded ^1H NMR spectra in H_2O for exchangeable protons of four pyridoxal phosphate-dependent enzymes: D-serine dehydratase, aspartate aminotransferase, tryptophan: indole-lyase and glutamate decarboxylase. The molecular masses range from 48 - 250 kDa. In every case there are downfield peaks which are lost when the apoenzyme is formed. In most cases some peaks shift in response to interactions with substrates and inhibitors and with changes in pH. We associate one downfield resonance with the proton on the ring nitrogen of the coenzyme and others with imidazole groups that interact with coenzyme or substrates. The chemical shift for the coenzyme-bound proton differs for free enzyme, substrate Schiff base or quinonoid forms.

Coenzymes are major components of the active sites of many enzymes and imidazole groups are also often present. Recently we observed that nitrogen-bound protons of the coenzyme pyridoxal 5'-phosphate (PLP) as well as those of the imidazole groups of histidine side chains, can be observed readily in the ^1H NMR spectra of cytosolic aspartate aminotransferase (EC 2.6.1.1) in H_2O (1, 2) These resonances are found in the 10-18 ppm range, which also contains peaks from indole NH (3) and probably from some peptide backbone NH groups (4). Even though this aminotransferase is a large 93 kDa dimeric protein, a number of peaks represent single proton resonances.

We are also investigating three other PLP-dependent enzymes isolated from *Escherichia coli*: the 48 kDa monomeric D-serine dehydratase (EC 4.2.1.14), the 180 kDa tetrameric tryptophan:indole-lyase (tryptophanase; EC 4.1.99.1) and glutamate decarboxylase (EC 4.1.1.15), a 250 kDa hexamer.

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METHODS

Enzymes: The fully active alpha subform of cytosolic aspartate aminotransferase was isolated from pig hearts (5,6) Apoenzyme was prepared as described by Furbish *et al.* (6). Tryptophanase was obtained from *E.coli*. B/7t (7) as the holoenzyme by a modification of the method of Behbahani-Nejad *et al.* (8). Apoenzyme was prepared by dialysis against 0.1 M sodium phosphate and 0.1 M L-alanine. (7) Glutamate decarboxylase was isolated from *E. coli* as described by Yang *et al.* (5) with piperazine substituted for pyridine. Apoenzyme was prepared by treatment with 0.1M α-methylglutamate at pH 4.6 (9). The 442-residue D-serine dehydratase was isolated as described previously (10) and apoenzyme was prepared by resolution with 2 M 2-hydroxyethylamine (11) at pH 7.8 and a protein concentration of about 0.3 mg/ml.

NMR Spectroscopy: Most of the ^{1}H NMR spectra were recorded on enzymes in 50 mM phosphate buffers. The samples were brought to the desired initial pH by thorough dialysis against appropriate buffers, and were then concentrated by centrifugal ultrafiltration to about 2 mM. Further pH adjustments were made by addition of small amounts of 50 mM K_2HPO_4 adjusted to pH 12.2 with KOH or of 50 mM KH_2PO_4. The pH was measured with a Radiometer PHM 84 meter before transfer of the sample to the NMR tube and often again immediately after the NMR measurement, always at about 25°C. The pH drift was < 0.1 unit. NMR samples typically contained 17 mg of D-serine dehydratase or 35-40 mg of one of the other proteins in 400 μl of buffered H_2O with 50 μl of D_2O added to provide field frequency lock. Up to an additional 100 μl of concentrated solutions of substrates or inhibitors were added to some samples.

All spectra were collected at 500 MHz on a Varian Instruments Unity 500 NMR spectrometer. The water resonance was suppressed using the 1-1 spin-echo selective excitation pulse sequence described by Sklenar and Bax (12). The sweep width was 18 kHz and 16000 data points were collected. The delay between 90° pulses (τ) was 35 μs and the 90° pulse length was 10 μs. No homospoil pulse was used. Spectra were obtained with from 256-4096 scans, typically 1024 for D-serine dehydratase and the aminotransferase and somewhat more for tryptophanase and the decarboxylase. The recycle time was 2.44 s per scan. The free induction decays were zero-filled to 64K points and apodized with 4Hz lorentzian line broadening prior to fourier transformation.

RESULTS

The spectrum of the aminotransferase in the PLP form at pH 8.3 is shown in Fig 1 together with those of the apoenzyme, of the pyridoxamine 5'-phosphate (PMP) form, and of the DL-α-methylaspartate and L-*erythro*-β-hydroxy-aspartate complexes. Note the peak marked A which is absent in the apoenzyme and which assumes distinctly different positions in the PLP and PMP forms. In the α-methylaspartate complex peak A is partially replaced by the peak marked A_e ("external aldimine" or substrate Schiff base) and B appears to be partially replaced by X. Peaks A and B undergo distinct changes in position with changes in pH (1,2). Peak A moves downfield as the pH is decreased while peak B moves upfield. Both changes are centered around a pK_a of 6.2. At low temperature a new peak, marked A' in Fig 1, appears at 16.8 ppm. It also titrates, moving to 13.6 ppm around a pK_a value of 9.5 (2). With L-*erythro*-β-hydroxyaspartate peak A is replaced by peaks A_1 and A_2 at 16.3 ppm and 15.6 ppm respectively. These may represent ketimine and quinonoid forms. All peaks move 0.1 - 0.3 ppm downfield in going from 6° to 21°C.

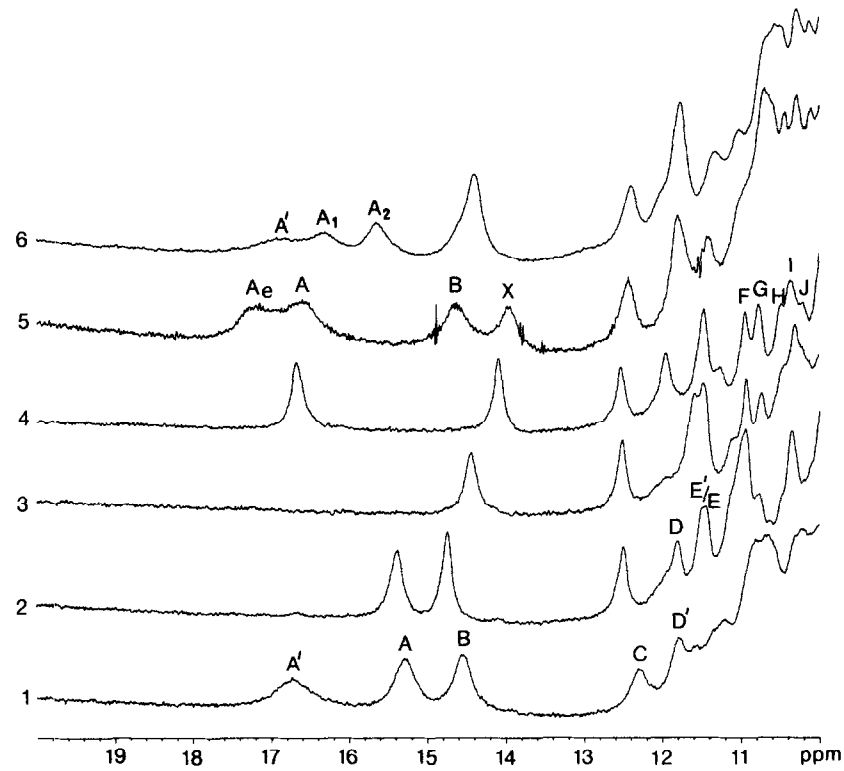

Fig 1. ^{1}H-Nuclear magnetic resonance spectra at 500 MHz for pig cytosolic aspartate aminotransferase in H_2O with 50 mM potassium phosphate buffers. All are at pH 8.3. 1. Holoenzyme, 6°C. 2. same at 35°C. 3. apoenzyme, 35°C. 4. pyridoxamine 5'phosphate form of the enzyme, 35°C. 5. Holoenzyme + 60 mM DL-α-methylaspartate, 21°C. 6. Holoenzyme + 10 mM L-*erythro*-β-hydroxyaspartate, 21°C.

Spectra of tryptophanase are shown in Fig. 2. Four well-resolved peaks, which we have designated A - D, are seen at 26°C and an additional broad peak B' emerges at ~ 16.6 ppm at low temperature. The peaks move very little with pH changes, but there are changes in relative heights which are centered around a pH of about 7.6. This is the midpoint pH for a change in the ultraviolet-visible absorption spectrum by which the 420 nm absorption band seen at low pH is largely replaced by a 324 nm band (7,13). In the tryptophanase apoenzyme peaks A, B, and C are all undetectable. They are restored by readdition of PLP. Addition of L-alanine, which converts a large fraction of the enzyme to a quinonoid form (7,13,14), causes new peaks, marked Q_1 and Q_2 to appear at 16.3 and 14.3 ppm. Addition of L-threonine converts most of the enzyme to an external aldimine, as judged by changes in absorption and circular dichroism in the coenzyme absorption bands (14). A new peak appears at 17.7 ppm in the NMR spectrum (Peak E, Fig. 2). As the new peaks Q or E appear peak B seems to lose intensity relative to C and D but does not disappear.

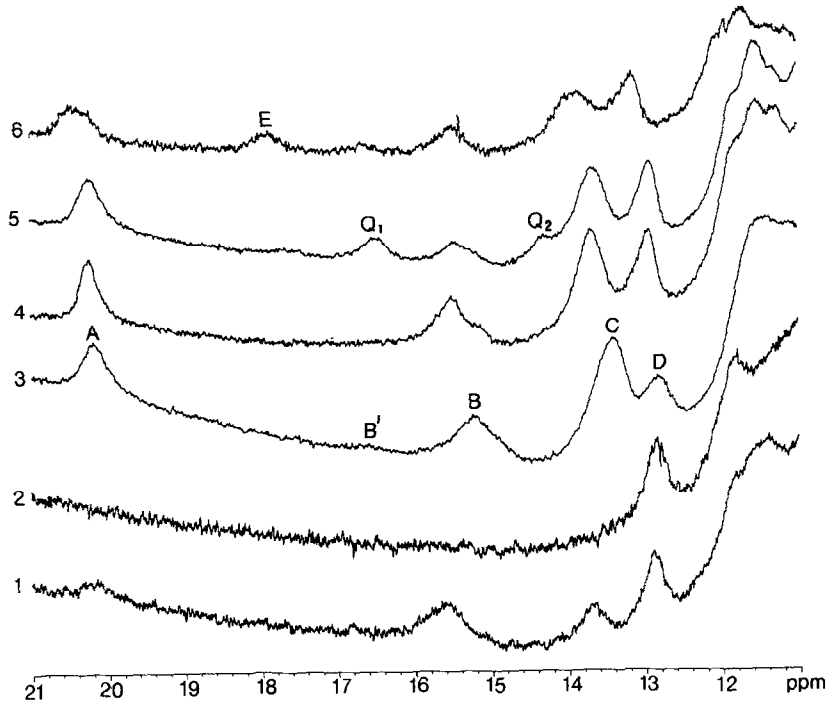

Fig. 2. ^{1}H-NMR spectra of tryptophanase. 1. Holoenzyme pH 6.8, 6°C. 2. Apoenzyme, pH 6.8, 6°C. 3. Holoenzyme, pH 8.5, 6°C. 4. Holoenzyme, pH 8.5, 26°C. 5. Holoenzyme + 40 mM L-alanine, pH 8.5, 26°C. 6. Holoenzyme + 100 mM L-threonine, pH 8.5, 26°C.

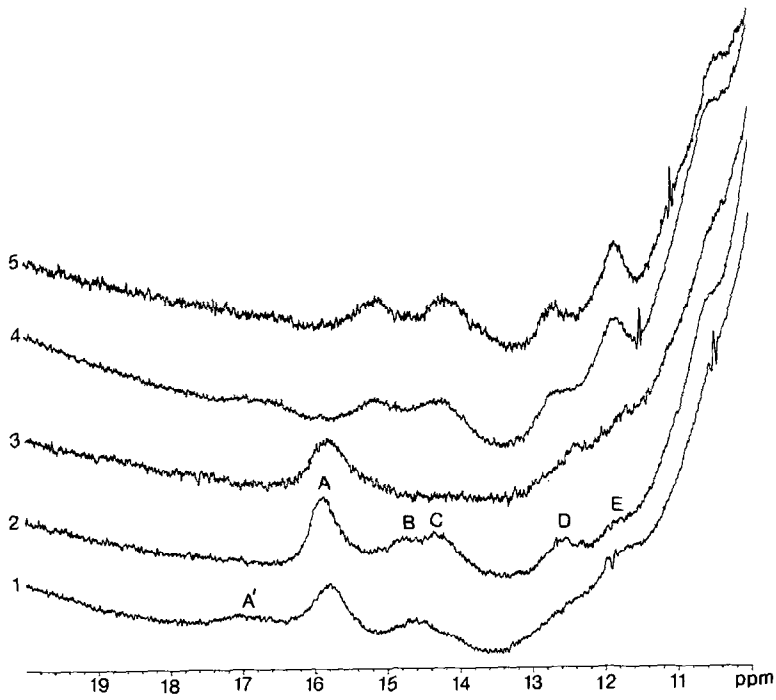

Fig. 3 H^1-NMR spectra of glutamate decarboxylase in 0.1 M piperazine-HCl buffers. 1. Holoenzyme, pH 4.60, 6°C. 2. Same at 21°C. 3. Apoenzyme, pH 4.7, 21°C. 4. Holoenzyme, pH 6.67, 6°C. 5. Holoenzyme, pH 6.67, 21°C.

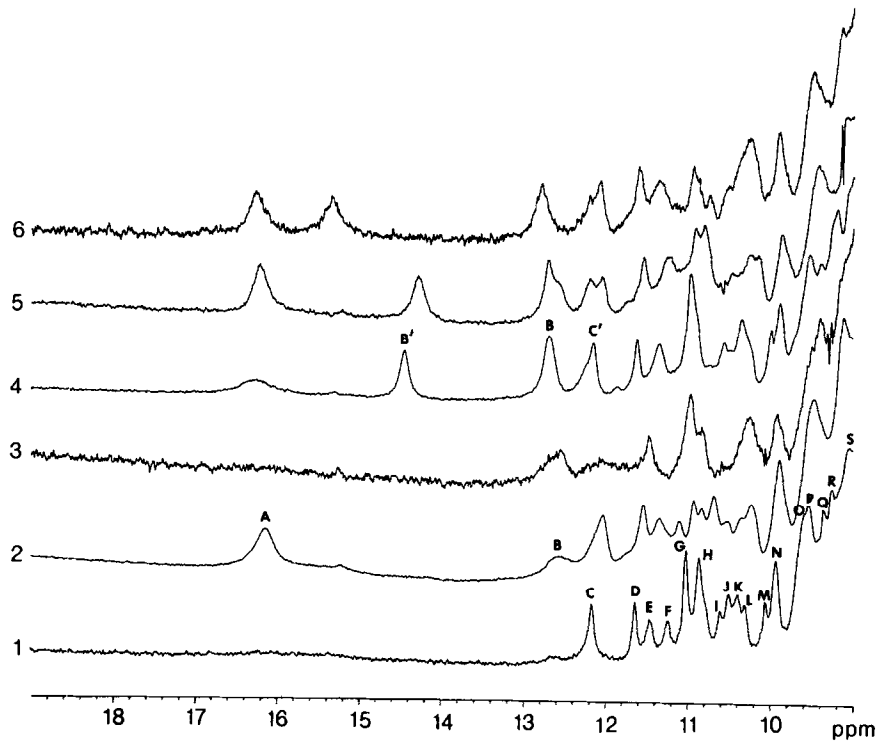

Fig. 4. ^1H NMR spectra of D-serine dehydratase in H_2O at pH 7.8. 1. Holoenzyme at 31°C. 2. Same at 6°C. 3. Apoenzyme, 6°C. 4. Holoenzyme after addition of 100 mM glycine, 6°C. 5. Same at 31°C. 6. Holoenzyme plus 200 mM sodium glycolate at 6°C.

Despite its large size the hexameric glutamate decarboxylase displays several reasonably well-resolved downfield resonances, labeled A-E in Fig. 3. An additional broad peak A' appears at lower temperatures. As with tryptophanase, the relative intensities of the peaks change as the pH is raised past 5.6, which is the midpoint of a distinct spectral transition by which the 420 nm absorption band shifts to 340 nm. This multiple-proton cooperative transition appears to involve a conformational change at the active site (15, 16). Addition of the competitive inhibitor glutarate has no apparent effect on either the NMR or the electronic spectrum.

D-serine dehydratase is the smallest of the proteins studied (10) and because of its more rapid tumbling, it has the sharpest NMR spectrum (Fig. 4). At least 18 distinct resonances are seen in the 9.4 - 17 ppm range. The downfield resonance A broadens as the temperature is raised and becomes undetectable at 26°C. It moves from 16.1 to 16.3 ppm at 6°C and other changes are also seen as the pH is lowered from 7.8 to 6.8. Addition of glycine and subsequent formation of a highly fluorescent PLP:glycine transimination

complex in the active site (10, 11), causes a small 0.1 ppm downfield shift of peak A and appearance of a new peak B' at 14.3 ppm. Peak B is shifted slightly to 12.7 ppm and is greatly intensified. Peak A of the transimination complex broadens and is not detectable when the temperature is raised to 26° but peaks B' and B do not lose intensity. Addition of glycine also causes a new peak C' to appear just downfield of C while peaks E and F merge into a single peak (at 11.3 ppm at 6°C). Other changes can also be seen. These alterations are about 50% completed at glycine concentrations of 4 mM at 6°C and 6 mM at 26°C. L-alanine has a similar effect but peak B' appears at 13.9 ppm. When the competitive inhibitor sodium glycolate was added the new peak B' appeared at 15.3 ppm and peak B was shifted and enhanced as with glycine. However both peaks B' and B were broadened and nearly undetectable at 26°C. Glycolate binds several-fold more tightly at pH 6.8 than at 7.8 judging by the intensity of peak B' at various ligand concentrations.

Peak A appears to be gone in apo-D-serine dehydratase. Several other resonances in the upfield region are broadened, shifted or diminished in the apoenzyme. Readdition of PLP in a pH 7.8 Tris buffer followed by dialysis into potassium phosphate leads to a return to the original holoenzyme spectrum.(not shown) Attempts to demonstrate an NOE between peaks A and C of the holoenzyme or between A and B' or B or between B' and B in the glycine complex were unsuccessful.

DISCUSSION

The 93 kDa aspartate aminotransferase contains one PLP in Schiff base linkage to K258 in each of its two 412-residue subunits. Of the ~17 distinct resonances seen in the 10-18 ppm range, the resonance marked A in Fig. 1 has been identified by its absence in the apoenzyme and by its changes with pH and in the presence of inhibitors or substrates as arising from proton H_a on the ring nitrogen of the PLP (1,2; See Fig. 5). NMR peak B was identified by a nuclear Overhauser effect (NOE) between peaks A and B as the imidazole proton H_b on H143. The chemical shift of peak A is very sensitive to the donation of electrons into the ring from the phenolate oxygen atom. This accounts for the fact that upon dissociation of proton H_s from the Schiff base nitrogen with a pK_a of ~6.2, peak A moves from 17.4 to 15.45 ppm at 21°C. Proton H_b also senses the change and peak B moves in the opposite direction by 1 ppm. Peak A_e of the α-methyl-aspartate complex (Fig. 1) probably represents proton H_a in the external aldimine. Peak X also arises from the external aldimine and may represent either H_b or H_s.

Spectra of the other three enzymes also contain downfield proton resonances. However, we cannot make the kind of simple association of a pK_a value with movement of the ^1H resonance of a proton attached to the coenzyme.

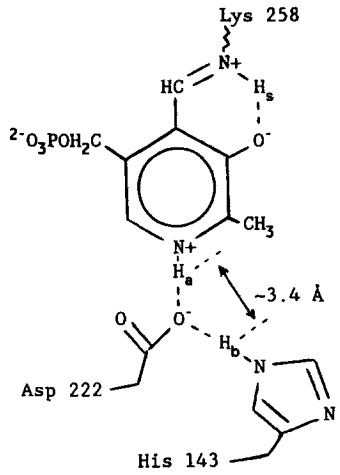

Fig. 5. Partial structure of the active site of aspartate aminotransferase.

Since in each case more than one resonance is lost when the apoenzyme is formed, assignment of a peak to the ring nitrogen of PLP is more difficult. However, the peaks at 17.7 and 16.3 ppm observed for tryptophanase in the presence of threonine and alanine, respectively can reasonably be attributed to this proton in the external aldimine and quinonoid species, respectively. These positions are similar to those seen for the aminotransferase in the α-methylaspartate complex (17.2 ppm) and *the erythro*-β-hydroxyaspartate complex (15.65 or 16.44 ppm), respectively. The apparent loss of intensity in the 15.5 ppm peak (B) suggests a possible location for the ^1H resonance of the protonated PLP ring in free tryptophanase.

The 16.2 ppm peak A of D-serine dehydratase may also arise from this proton and its peak B may represent a proton on an imidazole group that interacts with the coenzyme. We speculate that peak B', which appears in the glycine, alanine and glycolate complexes of this enzyme, may arise from an imidazolium ion that binds the α-carboxylate group of the substrate or of a competitive inhibitor such as glycolate. Weak binding of the glycine dipolar ion could be followed by transfer of a proton from the glycine $-NH_3^+$ to the imidazole with a resultant tighter binding and a readiness for the glycine $-NH_2$ to initiate the transimination sequence. This proton transfer would not occur readily with primary amines lacking the α-carboxylate and which do not undergo rapid transimination (17). The stronger binding of glycolate at low pH is consistent with this interpretation.

Typical peak widths at half-height at 21-26°C are D-serine dehydratase, 30-40 Hz, aspartate aminotransferase, 70-150 Hz, tryptophanase, 170-220 and glutamate decarboxylase 250-300 Hz. These widths increase monotonically with the molecular sizes. The line widths for neither holo nor apo forms of glutamate decarboxylase change in going from pH 4.6 to 6.7. Thus, the

dissociation into dimers that occurs at pH 6 at lower apoenzyme concentrations (18) does not take place at our relatively high concentrations.

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