Use of $^1H-^{15}N$ Heteronuclear Multiple-Quantum Coherence NMR Spectroscopy To Study the Active Site of Aspartate Aminotransferase†

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ABSTRACT: Aspartate aminotransferase from *Escherichia coli*, an 88 kDa enzyme, was uniformly and selectively enriched with ^{15}N and was studied by heteronuclear multiple-quantum coherence NMR spectroscopy in H_2O. Good resolution was obtained for the downfield region (above 9.5 ppm chemical shift in the ^1H dimension) for NH protons in the amide, indole, imidazole, and guanidinium group regions and several resonances were tentatively assigned. Two downfield resonances, at 12.6 and 11.36 ppm, appear to belong to oxygen- or sulfur-bound protons. The most downfield amide resonance at 11.78 ppm was assigned to the active site cysteine 192 whose peptide proton is 2.9 Å away from the negatively charged carboxyl group of aspartate 199. Large downfield shifts (up to 1.15 ppm) of the indole NH resonance of the active site tryptophan 140 were observed upon binding of dicarboxylic inhibitors to the pyridoxal 5′-phosphate (PLP) form and of inorganic dianions to the pyridoxamine 5′-phosphate (PMP) form of the enzyme. We discuss these striking differences in the light of the available crystallographic data. Active sites of proteins, as well as specific inhibitory molecules, often contain negatively charged groups. These may be able to form hydrogen-bonds to NH groups and to shift the NH resonances downfield into a less crowded and therefore more readily observable region for many large proteins. Our approach, which makes use of both HMQC spectroscopy and NOE observations, should be widely applicable.

Aspartate aminotransferases (AspAT)[1] are among the most extensively studied pyridoxal 5′-phosphate (PLP)-containing enzymes. A wealth of crystallographic structural information is available for both the PLP and pyridoxamine 5′-phosphate (PMP) forms of the holoenzyme and for their complexes with *quasi*-substrates and inhibitors. Because of the size of the enzymes (∼90 kDa) and the resulting large line widths of the resonance peaks only limited use of NMR spectroscopy has been possible. Nevertheless, one- and two-dimensional ^1H-NMR spectra in H_2O have been recorded for both pig cytosolic AspAT and for the very similar enzyme from *Escherichia coli* (Kintanar *et al*., 1991; Metzler, C. M., *et al*., 1991; Scott *et al*., 1991; Metzler, D. E., *et al*., 1994a−c). They contain a number of downfield resonances whose chemical shift values are remarkably sensitive to the state of protonation of the active site and which are also affected by the binding of substrates or inhibitors. The proton on the ring nitrogen of the coenzyme PLP or PMP, labeled H_a in Figure 1, and the nearby proton H_b on N^{ϵ} of the histidine 143 ring give rise to clearly resolved peaks which are labeled A and B, respectively on Figure 2a. Peak D arises from proton H_d on N^{ϵ} of the histidine 189 ring. Peak D′ in the spectrum of the bacterial enzyme was tentatively assigned to the C192 amide proton and another peak to $HN^{\epsilon 1}$ of the W140 indole ring. A component of peak H was assigned to HN^{ϵ} of the H193 ring and some NOE peaks in the 6−8.5 ppm range to CH protons on the imidazole rings of H143 and H189. These assignments were made by comparing spectra of the apo- and holoenzyme forms and of mutant enzymes recorded under different conditions, by analyzing NOE and NOESY data, and by comparing observed and predicted ^1H chemical shifts (Kintanar *et al*., 1991; Metzler, D. E., *et al*., 1994b,c). Peaks A and B of the PLP form shift by 1.86 and 0.88 ppm, respectively, in opposite directions as the pH is changed around a pK_a of 6.5. This pK_a is attributed largely to the proton H_s on the Schiff base linkage of the coenzyme to the enzyme (see Figure 6). These peaks also shift with changes in the form of the enzyme (apoenzyme, PLP, or PMP form) and with binding of inhibitors or substrates. Some of the other assigned resonances undergo smaller shifts.

Because ^1H chemical shifts are sensitive to any changes in electron density, resonances of active site protons can serve as reporters of the events in the active site. However, to utilize this approach effectively we need to assign as many resonances as possible to the active site protons. It is hard to do this in the 9.5−11 ppm region because of heavy overlap of the peaks (Figure 2). We have therefore turned to two-dimensional $^1H-^{15}N$ heteronuclear multiple-quantum coherence (HMQC) spectroscopy on uniformly and selectively enriched *E. coli* enzyme. This technique correlates the ^1H

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[1] Abbreviations: AspAT, aspartate aminotransferase; PLP, pyridoxal 5′-phosphate; PMP, pyridoxamine phosphate; HMQC, heteronuclear multiple-quantum coherence; NOE, nuclear Overhauser effect; NOESY, nuclear Overhauser enhancement spectroscopy; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; DSS, 2,2-dimethyl-2-silapentane-5-sulfonate.

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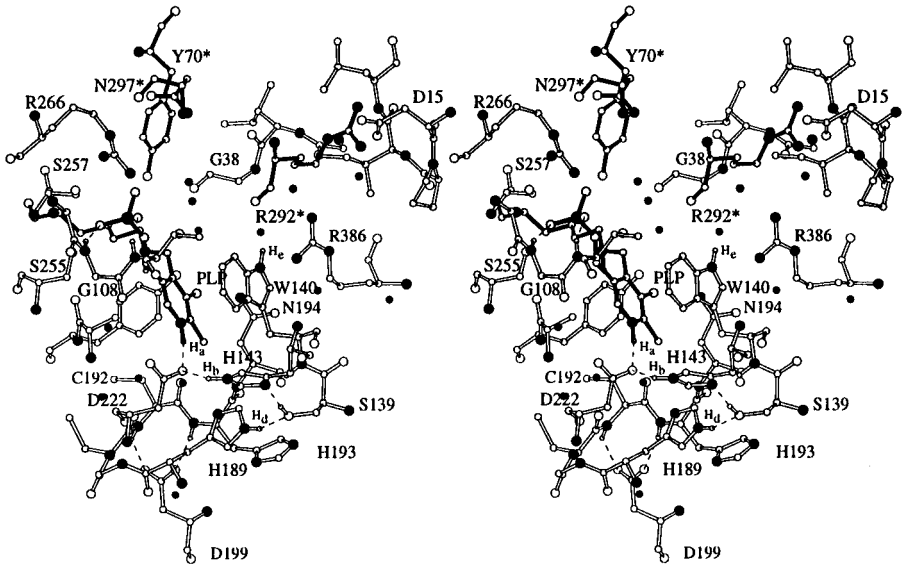

FIGURE 1: Stereoscopic view into the active site of the PLP form of AspAT from *E. coli*. Some residues are labeled; Y70* and R292* are from the second subunit in the dimer. Several active site hydrogen atoms discussed in the text are included in the drawing. These are H_a, H_b, H_d, H_e, and the peptide NH hydrogens of G108, T109, W140, C192, and H193. The small black dots mark the positions of the oxygen atoms of crystallographic water molecules. Coordinates are from Okamoto *et al*. (1994).

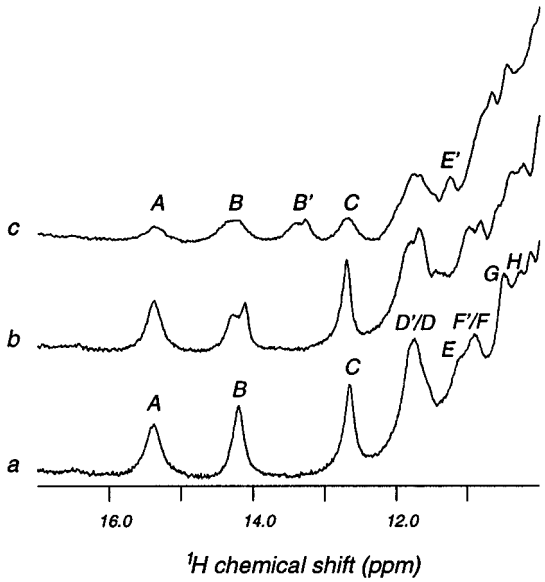

FIGURE 2: One-dimensional ^{1}H NMR spectra of AspAT from *E. coli*, recorded at 21 °C. (a) Spectrum of the unlabeled enzyme at pH 8.0 in 100 mM potassium phosphate buffer. (b) Spectrum of the uniformly ^{15}N-enriched enzyme at pH 8.5 in 20 mM potassium phosphate buffer. Most peaks are split by the 90 Hz coupling to ^{15}N. However, peak A is not split because the coenzyme does not contain appreciable ^{15}N. (c) Spectrum of the uniformly ^{15}N-enriched enzyme at pH 6.5 in 100 mM KCl solution. Peaks B and B′ belong to $HN^{\epsilon 2}$ of H143 in the low- and high-pH forms of the enzyme, respectively, which are in slow exchange in KCl (Metzler, D. E., *et al*., 1994c). Neither peak C nor peak E′ is split, an indication that they must arise from OH or SH protons.

and ^{15}N chemical shifts of directly bonded ^{1}H−^{15}N pairs by selecting for the large ^{1}H−^{15}N scalar couplings of approximately 90 Hz (Bax *et al*., 1990; Norwood *et al*., 1990). It simplifies the proton NMR spectrum of the enzyme yielding only resonances from ^{15}N-labeled protons and adds the dispersion of the indirectly detected ^{15}N dimension. Each resonance in the HMQC spectrum corresponds to an ^{1}H−^{15}N pair in the protein and its coordinates in the spectrum give the proton and nitrogen chemical shifts. In addition to providing improved resolution in the crowded region of the proton spectrum below 12 ppm, the HMQC experiment permits the assignment of peaks by nitrogen type. The ^{15}N resonances of proteins occur in several well-defined and isolated groups (Gust *et al*., 1975). For *E. coli* AspAT these include peptide and side chain amide NH (103−142 ppm in ^{15}N chemical shift), indole NH (130−145 ppm), imidazole NH (165−180 ppm), guanidinium $N^{\eta}H$ (69−77 ppm) and $N^{\epsilon}H$ (81−92 ppm), and primary amines (31−37 ppm).

The active site region of AspAT contains three imidazole rings, the indole ring of tryptophan 140, three guanidinium groups, the side chain amide of asparagine 194, and many peptide NH protons. The indole ring NH of W140 is of special interest. As can be seen in Figure 1, this ring lies in close contact with the coenzyme ring and, as viewed in this figure, in front of it and beneath the substrate-binding site. Numerous crystallographic studies have shown that substrates and inhibitors bind with their carboxylate groups forming hydrogen-bonded salt bridges to the guanidinium groups of arginine 386 and 292* (R292* is located on the second subunit of the symmetric dimer), and with an additional hydrogen-bond from one carboxyl oxygen to $HN^{\epsilon 1}$ of the W140 indole ring (Arnone *et al*., 1985; McPhalen *et al*., 1992; Jäger *et al*., 1994; Miyahara *et al*., 1994; Okamoto *et al*., 1994; Malashkevich *et al*., 1995b). Hydrogen bonding of a negatively charged carboxylate oxygen to this tryptophan should cause a downfield shift of the indole HN. Study of a mutant protein with W140 replaced by phenylalanine (W140F) suggested that a peak in the ^{1}H NMR spectrum that moved downfield from an overlapping region to 11.4 ppm upon binding of succinate was from the W140 indole NH proton (Metzler, D. E., *et al*., 1994c). In this report we confirm that assignment and describe the behavior of this and some other indole, imidazole, and amide resonances in more detail. We also describe effects on the W140 indole NH resonance of the binding of inorganic dianions and dicarboxylic inhibitors into the active site. A preliminary report has been published (Metzler, D. E., *et al*., 1994a).

MATERIALS AND METHODS

Materials. $^{15}NH_4Cl$ and [^{15}N]glycine were obtained from Cambridge Isotope Laboratories. Pyridoxal and pyridoxamine 5′-phosphates, amino acids, and succinic, maleic, and

meso-tartaric acids were purchased from Sigma Chemical Co. Hydroxylapatite was prepared as described by Tiselius *et al.* (1956).

Protein Expression and Purification. Wild type *E. coli* AspAT was produced as described previously (Inoue *et al*., 1989). ^{15}N was uniformly incorporated into the enzyme by growing *E. coli* TY103 cells transformed with the plasmid pKDHE19(*aspC*) (Kamitori *et al*., 1987) in the minimal medium M9 (Sambrook *et al*., 1989) containing $^{15}NH_4Cl$ and supplemented with 6 mg/L thiamin, 3 mg/L pyridoxine hydrochloride, and 50 mg/L ampicillin. All nitrogen atoms in the protein except for the one in the coenzyme are expected to contain >90% ^{15}N. Uniformly ^{15}N-enriched enzyme containing non-labeled tryptophan was obtained by growing cells in a medium identical with that described above but supplemented with 200 mg/L tryptophan. Enzyme containing ^{15}N-enriched glycine, cysteine, serine, and tryptophan was produced by growing cells in an M9 medium supplemented with thiamin, pyridoxine hydrochloride, ampicillin, 125 mg/L [^{15}N]glycine, and 200 mg/L of all standard amino acids except for glycine, cysteine, serine, and tryptophan. Enzyme ^{15}N-enriched in glycine only was produced following the same procedure except that cysteine, serine, and tryptophan were also added to the growth medium.

Both ^{15}N -enriched and unlabeled enzymes were purified at 4 °C following the procedure described by Inoue *et al*. (1989). The cells were collected by centrifugation. Cell paste (30 g) was resuspended in 60 mL of buffer A (10 mM phosphate buffer, pH 6.0, containing 10 mM acetate, 10 mM succinate, and 5 mM 2-mercaptoethanol). The cells were disrupted in a French press (1800 psi) and 2×10^{-5} M PLP was added. Cell debris was removed by centrifugation (27000*g*, 20 min, 4 °C), and the supernatant was collected, diluted in an equal volume of distilled water, and loaded onto a DEAE-Toyopearl 650M column (2.2 × 52 cm) equilibrated in buffer A. AspAT was eluted with a linear NaCl gradient (2 × 500 mL, 0−300 mM NaCl in buffer A) at a flow rate of 1.15 mL/min; fractions were collected and analyzed spectrophotometrically. The AspAT-containing fractions were pooled and concentrated to 50 mL in an Amicon ultrafiltration apparatus. 200 mL of 10 mM potassium succinate, pH 7.0, containing 50 mM α-oxoglutarate was added. The solution was concentrated to 50 mL and loaded on a hydroxylapatite column (2 × 50 cm) equilibrated in buffer B (2 mM potassium phosphate buffer, pH 7.0, containing 10 mM succinate and 5 mM 2-mercaptoethanol). The enzyme was eluted with a phosphate gradient (2 × 500 mL, 2−100 mM phosphate in buffer B) at a flow rate of 0.52 mL/min. The fractions were analyzed spectrophotometrically and on SDS−PAGE. The high-purity fractions were pooled, concentrated to 50 mL, and loaded onto a Sephacryl S-200 column (2 × 50 cm), equilibrated in buffer C (10 mM potassium phosphate buffer containing 10 mM succinate, 0.1 M KCl, and 5 mM 2-mercaptoethanol). The enzyme was eluted with buffer C (flow rate 0.95 mL/min) and the fractions were analyzed as described. The high-purity fractions were pooled, 2×10^{-5} M PLP was added, and the solution was concentrated to ∼16 mg of protein per mL. The yield of enriched protein was typically 35 mg per liter M9 medium. The activity of the purified enzyme was checked by the method of Velick and Vavra (1962) monitoring spectrophotometrically the production of oxalacetate. For k_{cat} of the forward reaction we observed values of 215 ± 12 s^{-1} for the unlabeled enzyme and 223 ± 9 s^{-1} for the labeled enzyme, at 25 °C.

Preparation of Apo-AspAT. Apoenzyme was prepared by treating the PLP-form of AspAT with sodium glutamate (1 M, pH 8.3), which was added to the enzyme in a volume ratio of 2:1 (enzyme:glutamate). The solution was concentrated in a Centricon microconcentrator to the initial enzyme volume; this procedure was repeated twice, after which 2.5 M sodium phosphate, pH 4.8, was added to a final concentration of 1 M. After incubation at 30 °C for 30 min, the solution was dialyzed against 1 M sodium phosphate at room temperature for 6 h with a change of dialysis buffer every 2 h. Finally the sample was dialyzed overnight at 4 °C against the desired buffer for NMR spectroscopy. The enzyme obtained by this procedure contained 98% apoenzyme (estimated spectrophotometrically and by NMR).

Sample Preparation. The NMR samples were prepared as described in previous papers (Kintanar *et al*., 1991; Metzler, D. E., *et al.*, 1994b). They typically contained 40−45 mg (∼2 mM in subunits) of enzyme in 400 μL of buffered H_2O with 50 μL of D_2O added to provide field frequency lock. Most spectra were recorded on samples in 20−100 mM phosphate buffer or 20−100 mM KCl.

NMR Spectroscopy. All the NMR spectra were recorded on a Varian Unity-500 spectrometer equipped with a triple-resonance ^1H [^{15}N, ^{13}C] probe. The ^1H carrier frequency was set on the water resonance and the ^1H spectral widths were 7000 Hz (backbone amide and arginine side chain region) and 10000 Hz (imidazole region). The ^{15}N carrier frequencies were set at 120 ppm (backbone amides), 80 ppm (arginine side chains), and 180 ppm (imidazole side chains), with ^{15}N spectral widths of 4000, 1500, and 5000 Hz, respectively. A total of 80−128 t_1 increments of 2048 complex data points were collected with a total of 96−128 transients per t_1 increment. Enough points were collected to ensure that all resonances had decayed fully. Values of t_1^{max} of 25−30 ms appeared to be optimal for AspAT and in some cases we used fewer t_1 points than collected when processing the data in F_1. A standard HMQC pulse sequence (Summers *et al*., 1986) was used with the delay to build up anti-phase coherence set to 4.54 ms. Water suppression was achieved with either presaturation or a jump-and-return read pulse (Bax *et al*., 1990). Quadrature detection in t_1 was obtained using the method of time-proportional phase incrementation (Marion & Wüthrich, 1983) in the former case and the hypercomplex method (States *et al*., 1982) in the latter. The ^1H-frequency scale was referenced to DSS through the water resonance, and temperature correction was applied. The ^{15}N-frequency scale was referenced to liquid ammonia. The experiments usually required 8−12 h.

The data were processed on a SUN SPARCstation using Varian software or on a Silicon Graphics workstation using FELIX, version 2.3 (Biosym, Inc.). Prior to Fourier transformation, the data sets were zero-filled in order to produce 2K × 1K data matrices. The spectral resolution was enhanced by apodization with a Lorentz−Gauss function in t_2 and a sine bell function shifted by 70° in t_1.

RESULTS

^1H NMR Spectra of the Uniformly ^{15}N-Enriched AspAT. The incorporation of the ^{15}N isotope results in a splitting of the resonances of all ^{15}N-bound protons in the protein due to the scalar coupling of the proton to the ^{15}N nucleus. As

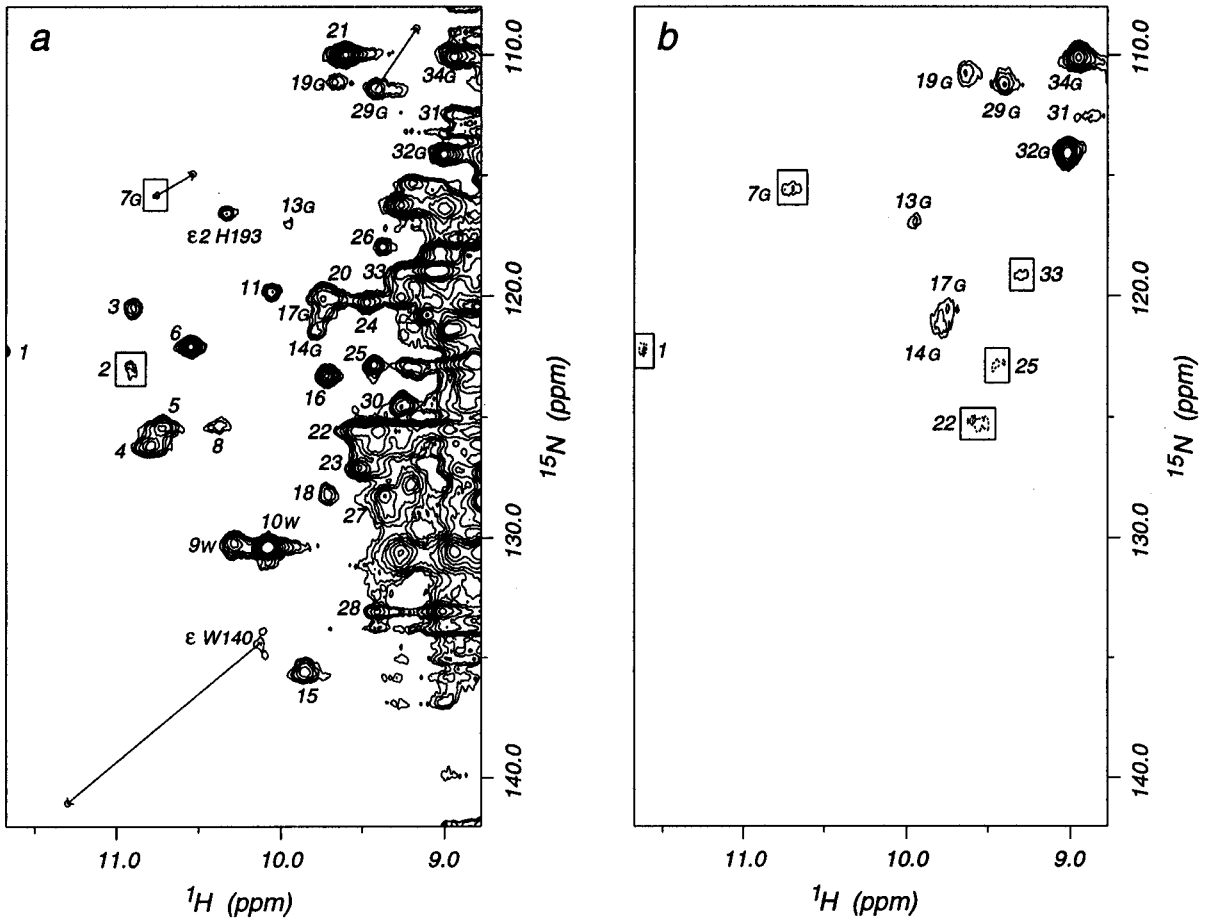

FIGURE 3: $^{1}H-^{15}N$ HMQC spectra of AspAT in 20 mM potassium phosphate buffer, at pH 6, 35 °C. Only the downfield portion of the amide region of the spectrum is shown. The peaks enclosed in boxes are drawn at a lower contour level than is used for the remainder of the spectrum. The arrows point to the positions of the corresponding resonances after addition of 20 mM succinate. The letters after the numbers designate the type of the amino acid residue. (a) Uniformly ^{15}N-enriched AspAT. Peak $\epsilon 2$ H193 is folded into the amide region from 166.2 ppm (^{15}N). (b) [^{15}N]Glycine-enriched enzyme. Peaks shown in dashed lines are from the spectrum of the enzyme containing ^{15}N-enriched glycine, serine, cysteine, and tryptophan.

a consequence, all NH resonances in the one-dimensional ^{1}H NMR spectrum of the enzyme appear as doublets with the two peaks of the doublet ~90 Hz apart (~0.18 ppm at 500 MHz) while protons attached to carbon, oxygen, or sulfur atoms are represented by unsplit resonances.

One-dimensional ^{1}H NMR spectra of the unlabeled and the uniformly ^{15}N-enriched PLP form of AspAT are shown in Figure 2. In all three spectra peak A, representing the nitrogen-bound proton on the PLP ring, is a singlet. This was expected because the pyridoxine supplied in the growth medium does not contain ^{15}N. Peak B, which arises from $HN^{\epsilon 2}$ of H143, does display the characteristic 90 Hz splitting in the labeled enzyme. Most of the other peaks are overlapped but split. However, peak C is a singlet. This downfield peak (12.6 ppm ^{1}H chemical shift), whose position does not change under a variety of conditions, probably belongs to an oxygen- or sulfur-bound proton held in a strong hydrogen-bond. Another peak, which also is not split by ^{15}N, appears when spectra are recorded in KCl instead of phosphate buffer. This is a resonance seen at 11.36 ppm and previously designated E′ (Metzler, D. E., *et al*., 1994c). It is seen in Figure 2c for the enriched enzyme.

HMQC Experiments. We acquired $^{1}H-^{15}N$ HMQC spectra of the enzyme with the ^{15}N carrier frequency positioned at the center of the amide region, histidine region or arginine region using spectral widths in the F_1 (^{15}N) dimension that would allow the observation of the $^{1}H-^{15}N$ correlations of interest without interference from folded resonances. Spectra were collected for apoenzyme and for PLP and PMP forms of the holoenzyme at several pH values and various temperatures, buffer compositions, and ligand concentrations.

Amide Region. Figure 3a shows the downfield portion (>8.8 ppm ^{1}H chemical shift) of the amide region of the HMQC spectrum. The resonances with ^{1}H chemical shift greater than 9.5 ppm are labeled with numbers. Peaks 9w, 10w, and W140 belong to tryptophan *indole* NH protons (see next section). There are 380 possible backbone amide NH resonances in the HMQC spectrum (396 amino acid residues per subunit minus 15 proline residues minus the N-terminal residue). Of these, 30 are expected to arise from glycine amide protons. To identify the glycine resonances we acquired spectra of the free and succinate-complexed PLP form of the [^{15}N]glycine-enriched AspAT. This enzyme was isolated from cells grown on an [^{15}N]glycine-containing medium supplemented with all of the other 19 unenriched amino acids (Experimental section). Although the supplementation suppressed the synthesis of ^{15}N-containing serine, cysteine, and tryptophan, there was some residual labeling of these amino acid residues, particularly of serine (Hoffman & Spicer, 1991), a fact that accounts for the presence of more than 30 peaks in the amide region of the spectrum. For enzyme isolated from cells grown on [^{15}N]glycine and supplemented with all amino acids except glycine, serine, cysteine, and tryptophan, labeling of a number of additional peaks was obtained (Figure 3b). This allowed us to identify the 30 peaks that are most likely to correspond to the 30

Table 1: Observed ^{1}H Chemical Shifts of Selected Resonances upon Binding of Dicarboxylates[a]

	^{1}H chemical shift (ppm)							
	PMP form, pH 7.0, 100 mM phosphate				PLP form, pH 6.0,[b] 20 mM phosphate			
peak	free[c]	succinate	maleate[d]	*m*-tartrate	free	succinate	maleate	*m*-tartrate
1 (D′)[e]	11.65	11.64	**10.76**	11.71	11.73	11.82	**11.84**	11.79
2 (E)	nd [f]	nd	nd	nd	10.93	**11.03**	**11.08**	**11.19**
4 (F)	10.79	10.80	10.83	10.78	10.88	10.79	10.80	10.80
7G (G′)	10.55	**10.43**		10.52	10.70	**10.56**	10.70	10.67
	10.50		10.51					
11 (I)	10.26	10.20	10.20	10.19	10.24	**10.02**	**10.01**	**10.04**
13G (J)	10.07	9.98	9.99	10.02	10.06	9.99	9.98	9.97
19G	9.93	**9.78**		**9.70**	9.96	nd	nd	nd
	9.99		**9.61**					
29G	9.46	**9.34**	**9.26**	**9.31**	9.63	**9.27**	**9.29**	**9.25**
ϵ W140	11.50	**11.14**		**10.48**	10.16	**11.31**	**11.00**	**10.93**
	11.30		**10.81**					

[a] Spectra of the complexes of both enzymes were recorded at saturating concentration of the ligands (20 mM). Chemical shifts for resonances shifting more than 0.1 ppm are shown in bold type. [b] Spectra of the PLP form were recorded at pH 6.0 because dicarboxylic inhibitors bind to this form more strongly at lower pH. [c] A second value, corresponding to the peak position in 20 mM phosphate buffer, is shown for the resonances that shift significantly upon changes in phosphate concentration. [d] Spectra recorded in 20 mM phosphate buffer. [e] The letters in parentheses represent the peak labels used in the one-dimensional proton NMR spectra in Figure 1 and in previous work (Metzler, D. E., *et al*., 1994c). [f] Not detected.

glycines in the amino acid sequence. In Figure 3 the downfield glycine NH resonances are designated by an additional letter G after the number. Peaks 1, 22, 25, 31, and 33 also received some label from [^{15}N]glycine and are probably serine, cysteine, or tryptophan amide resonances.

While most of the amide resonances are in a more upfield region, there are at least 30 with ^{1}H chemical shifts greater than 9.5 ppm. We have focused our attention on these resonances. Over half of them have chemical shift values under the various conditions used that are constant within our limit of reliable detection of chemical shift changes, i.e., less than the half line width (~0.05 ppm in the ^{1}H dimension and 0.5 ppm in the ^{15}N dimension). However, resonances 5, 8, 11, 13, 15, 20−23, and 27 display small but detectable chemical shift changes (<0.1 ppm), and resonances 1, 2, 4, 7G, 11, 14G, 17G, 19G, 26, 28, and 29G show more significant changes. The amide resonances with the most significant chemical shift changes upon ligation are listed in Table 1.

Peak 1, previously called D′ (Metzler, D. E., *et al*., 1994c) and labeled as such in the one-dimensional proton spectrum of Figure 2, is the most downfield amide resonance observed. At pH 8, its position in the apoenzyme and PMP forms is 11.68 ppm. It is found at nearly the same position in the PLP form at pH 5.5 but moves downfield about 0.12 ppm when the pH is raised to 9.0. Binding of an inhibitor to either the low pH form of the PLP enzyme or to the PMP form causes a further downfield shift of about 0.06 ppm. This peak is present in the spectrum of AspAT containing ^{15}N-enriched glycine, serine, cysteine, and tryptophan and in the spectrum of the enzyme containing unlabeled tryptophan but is much weaker in the spectrum of enzyme containing only [^{15}N]glycine. This limits the possible amino acid residues that could give rise to peak 1 to serine or cysteine.

Both peaks 2 and 7G appear to be absent in the apoenzyme spectrum and in the spectrum of the PLP form in KCl solution. Peak 2 is the second-most downfield amide resonance. It is detected only in the spectra of the PLP form of AspAT in phosphate buffer. This resonance exhibits a significant downfield shift upon binding of inhibitors (Table 1) or upon increase of pH (0.28 ppm downfield shift in the pH range 5.5−8.5). Peak 7G is one of the most sensitive amide resonances toward changes in pH, coenzyme form, or ligation. It is seen in the spectrum of the PMP form at 10.55 ppm. It shifts 0.31 ppm further downfield upon conversion of the PMP to the PLP form at pH 7.0. It shifts 0.38 ppm downfield when the pH is raised from 5.5 to 8.5 around an apparent pK_a of ~7. Peak 7G also moves 0.16 ppm upfield upon binding of succinate at pH 6.0 but is virtually unperturbed by the binding of *meso*-tartrate and maleate. It is shifted downfield by sulfate or phosphate anions (Table 2).

Table 2: Changes in the ^{1}H Chemical Shifts in the Presence of Inorganic Anions[a]

	PMP form			PLP form		
	δ(ppm)	$\Delta\delta$(ppm)		δ(ppm)	$\Delta\delta$(ppm)	
peak	free	sulfate[b]	phosphate[c]	free	sulfate	phosphate
1	11.68	0.01	−0.01	11.79	−0.06	−0.06
2	nd	nd	nd	nd	nd	nd
7G	10.39	0.15	0.11	nd	nd	nd
ϵ W140	10.35	0.33	0.95	10.05	nd	0.11

[a] The chemical shifts δ in the free enzyme are measured in 20 mM KCl. Positive values of $\Delta\delta$ correspond to downfield shifts. [b] $\Delta\delta$ for sulfate represents the change in chemical shift upon addition of 20 mM Na_2SO_4 to the enzyme in 20 mM KCl at pH 6.4. [c] $\Delta\delta$ for phosphate represents the difference in chemical shift for the positions of the corresponding peaks in 20 mM phosphate buffer and in 20 mM KCl.

Tryptophan Side Chain Region. Because of the intrinsic downfield shifts of the indole nitrogens and their protons (Wüthrich, 1986), the indole ^{1}H−^{15}N correlations fall in the lower left corner of the amide NH correlation map (Figure 3a) and are often separated from those of most amides. For AspAT this "tryptophan corner" extends from 130 ppm to 141 ppm in the ^{15}N dimension and to greater than 10 ppm in the ^{1}H dimension. We were able to identify the indole resonances by comparing spectra of uniformly ^{15}N-enriched enzyme with those of enzyme samples that were ^{15}N-enriched in all amino acid residues except for tryptophan.

AspAT from *E. coli* contains five tryptophan residues, but only three indole resonances could be seen easily in the spectrum of the holoenzyme. These are labeled 9W, 10W, and ϵ W140 in Figure 3a. Resonances 9W and 10W are strong. Except for a small downfield shift of 0.05 ppm for peak 10W in the PMP form compared to the PLP form of the enzyme, they show no detectable changes in intensity,

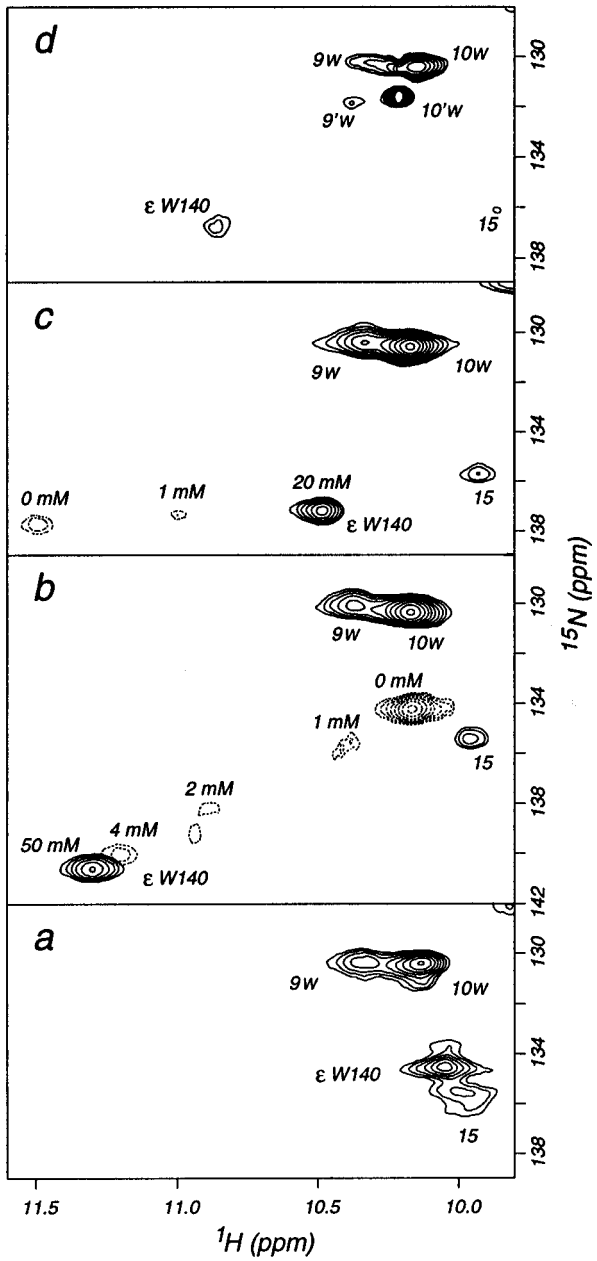

FIGURE 4: The "tryptophan corner" of the HMQC spectrum of AspAT; all spectra are recorded at 21 °C. (a) PLP form in 100 mM KCl, pH 6.5. (b) Titration of the PLP form with succinate in 20 mM phosphate buffer, pH 6.0. The peaks corresponding to 1 and 2 mM succinate concentration are drawn at a contour level which is approximately one-half and one-third that used for the rest of the spectrum, respectively. (c) Titration of the PMP form with *meso*-tartrate in 100 mM potassium phosphate buffer, pH 7.0. The peak at 1 mM *meso*-tartrate concentration is drawn at a contour level which is approximately one-half that used for the rest of the spectrum. (d) Apoenzyme complex with succinate in 20 mM KCl and 150 mM potassium phosphate, pH 6.83.

line width, or spectral position for a variety of experimental conditions (Figure 4). For comparison, we examined the titration behavior of the indole NH proton of free tryptophan in 20 mM potassium phosphate buffer by one-dimensional ^{1}H NMR in the pH range 5−10.8. At pH 8.5 this indole NH resonance is significantly broadened compared to that at low pH, probably as a result of phosphate-catalyzed exchange with the solvent. Peaks at about 10.33 and 10.1 ppm are among the few downfield (>10 ppm) proton resonances that remain more than a few minutes in D_2O (Metzler, D. E., *et al*., 1994c). These peaks were still present in the spectrum after 26 days. The HMQC spectrum of AspAT in 99% D_2O at an apparent pH of 6.5, collected 1 week after addition of D_2O, contained resonances 9W and 10W at virtually unaltered intensity. However, other intense downfield peaks such as 4, 5, 6, 16, 21, 26, 32G, and 34G were completely absent.

The third tryptophan peak in the holoenzyme spectrum, which is assigned to HN$^{\epsilon 1}$ of W140, is very sensitive to both pH and ligation. In the PLP form of the enzyme in KCl it is seen at 10.05 ppm in the ^{1}H dimension (Figure 4a, Table 1). In 20 mM phosphate buffer, it is found at 10.16 ppm (^{1}H)/134.1 ppm (^{15}N); it is strong but broadens as the pH of the sample is raised from 5.5 to 8.5. The peak is barely seen at pH 7 in phosphate buffer and is completely absent from the spectrum at pH 8.5. The rate of exchange with the solvent must be faster than that exhibited by free tryptophan in phosphate buffer. The position of the ϵ W140 peak in 20 mM phosphate remains constant in the pH range where the peak is detected. With increasing concentration of sulfate anions it exhibits a small additional downfield shift (0.14 ppm at 2 mM sulfate concentration) and a significant broadening. It is not detectable at 20 mM sulfate concentration. The ϵ W140 resonance undergoes significant changes in its chemical shift upon binding of dianions (Tables 1 and 2). Binding of succinate into the active site of the PLP form causes a downfield shift of 1.15 ppm (^{1}H)/6 ppm (^{15}N) as shown in Figure 4b [see also Metzler, D. E., *et al*. (1994a)]. Less drastic changes are observed upon binding of *meso*-tartrate or maleate.

In the PMP form of the enzyme in 20 mM KCl the ϵ W140 resonance is 0.30 ppm downfield relative to its position in the free PLP form. Both sulfate and phosphate anions cause additional downfield shifts (Table 2), while binding of dicarboxylates causes large upfield shifts (Table 1 and Figure 4c). Sulfate anions do not broaden the resonance significantly. The changes in the chemical shift of the ϵ W140 resonance in the *apoenzyme* with changes in pH and ligation resemble those of the PMP form in direction and magnitude. However, in the pH range of 6−7 the peak is strong and sharp in the spectra of the PMP form and weak, broad, and sometimes not detectable in the apoenzyme spectra.

We were surprised to find that in the spectrum of the apoenzyme all five tryptophan indole NH$^{\epsilon 1}$ resonances are visible (Figure 4d). The two new peaks 9′W and 10′W were named after the resonances with the closest lower ^{1}H chemical shift. They are absent from the spectrum of the apoenzyme containing unlabeled tryptophan, acquired under the same experimental conditions. They gradually broaden when the pH is raised, are not seen at pH 8.6 in phosphate buffer, and are absent or very weak in spectra of the PMP form.

Peak 9′W is weak. It moves downfield from its position at 10.31 ppm (^{1}H)/131.1 ppm (^{15}N) in KCl solution to 10.40 ppm (^{1}H)/131.9 ppm (^{15}N) when 150 mM potassium phosphate is added but is not affected by succinate binding. Under the same conditions peak 10′W remains constant at 10.2 ppm ^{1}H chemical shift, the same as reported for the indole NH resonance in random coil polypeptide chains (Wüthrich, 1986). This resonance is strong and unusually sharp. Its line width of 19.8 Hz in the ^{1}H dimension at 21 °C is almost an order of magnitude narrower than is expected for a molecule with 130 ns rotational correlation time (Churchich, 1967). For comparison, the sharpest of the peaks observed in the HMQC spectrum have line widths of ∼60

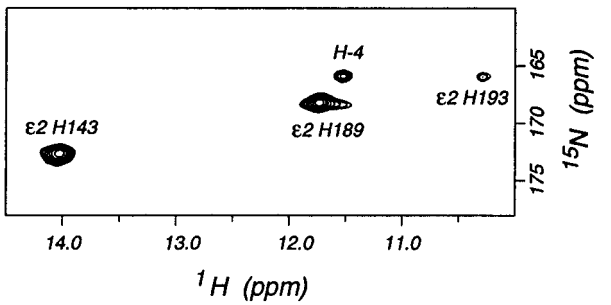

FIGURE 5: Histidine region of the HMQC spectrum of the PMP form in 100 mM potassium phosphate buffer, pH 7.0. H-4 represents a fourth unidentified histidine (H167, H297, or H301).

Table 3: Observed Chemical Shifts for the Imidazole Resonances in the PLP Form at pH 7.0, 35 °C, in 20 mM Phosphate Buffer

peak	chemical shift	
	^{1}H (ppm)	^{15}N (ppm)
H143	14.12	174.0
H189	11.82	167.9
H193	10.41	166.2
H-4 (unassigned)	11.56	166.2

Hz at 21 °C, while the line width we measured for the indole NH resonance of a free tryptophan in phosphate buffer was 20.1 Hz at the same temperature.

Histidine Region. AspAT from *E. coli* contains six histidine residues, but we were able to identify only four imidazole NH resonances in the HMQC spectrum. These are shown in Figure 5, and the measured chemical shifts are presented in Table 3. More peaks are present in the histidine region of the spectrum upfield from peak HN$^{\epsilon 1}$ of H193, but a significant shape distortion and inconsistency in their appearance preclude a definite identification of the fifth and sixth histidine resonances. Peaks B and D (Figure 1) have been assigned previously to the HN$^{\epsilon 2}$ protons of H143 and H189, respectively, and a component of peak H was tentatively assigned to the HN$^{\epsilon 2}$ proton of H193 (Kintanar *et al*., 1991; Metzler, D. E., *et al*., 1994c). The present study confirms that this resonance arises from a histidine side chain NH proton and supports our assignment. The fourth imidazole resonance, labeled H-4 in Figure 5, was previously designated E″ in the one-dimensional ^{1}H NMR spectra.

In the PLP form the H143 resonance moves 0.9 ppm downfield when the pH is raised from 5 to 9 (Metzler, D. E., *et al*., 1994c). The spectral position of the other three histidine peaks in both dimensions of the HMQC spectrum as well as the ^{15}N chemical shift of H143 show only small changes in the pH range of 5.5−8.5. At pH 5.5 all the histidine peaks are significantly broadened. Peak H-4 is weak and has a constant position. Binding of succinate to the PLP form of the enzyme at pH 6.0 shifts peak H143 by 0.12 ppm in the ^{1}H dimension. Peaks H189 and H193 are less affected. Their ^{1}H chemical shift changes are −0.04 and −0.06 ppm, respectively. In the *meso*-tartrate complex of the PMP form peak H143 moves downfield by 0.26 ppm. The corresponding shifts of H189 and H193 are −0.09 ppm and 0.07 ppm.

Arginine N$^{\epsilon}$ Region. We were able to observe 17 out of 22 possible HN$^{\epsilon}$ arginine resonances some of which showed distinct shifts upon binding of dicarboxylates. We have not yet been able to assign any of these resonances.

DISCUSSION

Assignment of Resonances. Although it is difficult to unambiguously assign resonances in such large proteins, we have been able to make a number of tentative identifications on the basis of the following assumptions: (1) Changes in the peak position in response to binding of dicarboxylates or inorganic anions in the active site of the enzyme imply that a proton is probably in the active site; (2) A proton whose resonance undergoes large downfield shifts from the position observed in small peptides probably participates in a strong hydrogen-bond to an adjacent negatively charged atom.

The ∼18 ppm resonance of the H87 imidazole NH in the active sites of serine proteases such as chymotrypsin and trypsin (Robillard & Schulman, 1972; Markley, 1978) is associated with a strong hydrogen-bond to the adjacent buried D102 carboxylate (Frey *et al.*, 1994). An amide proton in ribonuclease A moves 2.3 ppm downfield when an adjacent aspartic acid side chain is deprotonated and forms a hydrogen-bond to the NH (Baker & Kintanar, 1996). Similar downfield shifts of amide NH protons have been reported for troponin C (Krudy *et al*., 1992) and for staphylococcal nuclease (Torchia *et al*., 1989; Wang *et al*., 1990). In our previous studies we have associated downfield NH protons of both imidazole and indole NH groups with hydrogen-bonds to carboxylate oxygen atoms (Kintanar *et al*., 1991; Firsov *et al.*, 1994; Metzler, D. E., *et al.*, 1994b,c).

Amide NH Protons. In attempting to assign amide resonances we took into account all amino acid residues that participate in the ligand binding as well as those involved in the accompanying structural transition from open to closed conformation making use of the information from the high-resolution X-ray crystal structures of free AspAT and its complexes (Jäger *et al*., 1994; Miyahara *et al*., 1994; Okamoto *et al*., 1994). AspAT is a dimer of two identical subunits, each containing a small and a large domain. For the *E. coli* enzyme the transition from open (unliganded) to closed (liganded) conformation involves a 5° rotation of the small domain relative to the large one. Several active site residues, and also residues P16, P30, I37, A346, S361, and G391, which are located in or near the domain interface, change their conformation significantly. Of the six hydrogen-bonds in the domain interface of the open form two are broken upon closure and one new one is formed. All of these alterations in structure and bonding could cause ^{1}H and ^{15}N chemical shift changes and were taken into account when the NMR data were analyzed.

We have carefully examined the changes in ^{15}N chemical shifts for all downfield amide resonances. With the exception of resonances 2, 11, 17G, 23, 28, and 29G whose $\Delta\delta$-(^{15}N) > 0.5 ppm, they all display insignificant ^{15}N chemical shift changes upon ligand binding. Upon transition from the PLP to PMP form, when the major alterations in the conformation of the coenzyme and W140 rings occur, only resonances 1, 7G, 14G, 23, and 29G show significant (>1 ppm) ^{15}N chemical shift changes which might indicate proximity to the active site.

From the downfield position of peak 1 and its sensitivity to pH change, and from comparison with spectra of porcine cAspAT, it was concluded previously that peak 1 could represent the C192 amide NH proton which is hydrogen-bonded to the D199 carboxylate (Metzler, D. E., *et al*., 1994c). The present work indicates that peak 1 belongs to

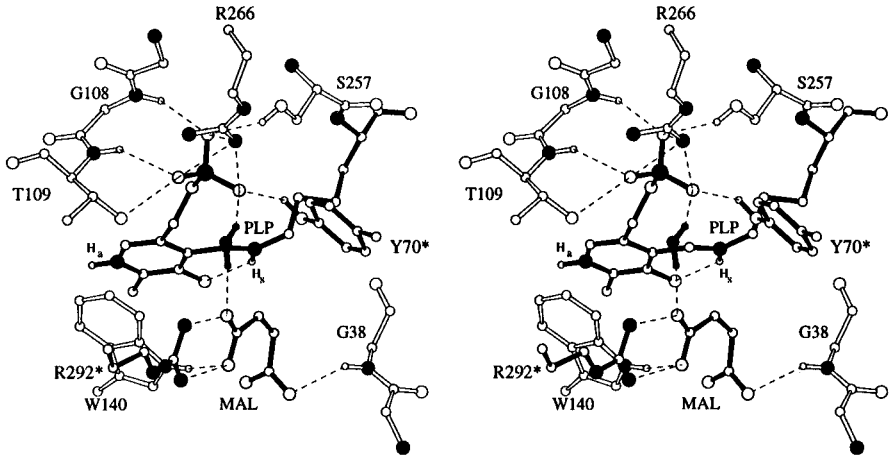

FIGURE 6: Stereoscopic view into the active site of the complex of the PLP form with maleate. This view emphasizes the hydrogen-bonds (dashed lines) from the coenzyme phosphate to the peptide NH groups and also the crystallographically observed conformation of the bound ligand. Notice the hydrogen-bond from the carboxylate of the substrate to the W140 indole NH. Coordinates are from Jäger *et al*. (1994).

a serine or cysteine residue. We made a list of all residues in AspAT whose amide protons are involved in short (<3.5 Å) hydrogen-bonds to negatively charged atoms. Of the 29 protons in the list none belongs to a serine residue and only one (C192 NH) belongs to a cysteine residue. The crystal structure of the enzyme (Okamoto *et al*., 1994) shows that both the C192 and H193 amide nitrogens form hydrogen-bonds, which are 2.9 and 2.8 Å in length, respectively, with the carboxylate oxygens of D199. A strong NOE cross peak to D′ at ~9.6 ppm (Metzler, D. E., *et al*., 1994c) could arise from the H193 amide proton and be assigned to one of the peaks 23−26. The tentative assignment of peak 1 to the C192 NH is strengthened by the present study.

Previous NMR studies of phosphate group binding sites in proteins have shown that amide resonances involved in the coordination of the phosphate groups also exhibit significant downfield shifts (Redfield & Papastavros, 1990; Lowry *et al*., 1992). The peptide NH hydrogens of G108 and T109 of AspAT both form 2.8 Å hydrogen-bonds to oxygen atoms of the phosphate group of the coenzyme (Figure 6). Of the glycine amide NH protons that we have observed, peak 7G has the largest ^{1}H chemical shift. It is the most sensitive to changes in pH and to binding of succinate. The G108 proton is a good candidate for this peak. Peak 2 may arise from the T109 NH proton. Because the phosphate oxygen forming the hydrogen-bond to T109 is also hydrogen-bonded to a guanidinium NH of the R266 side chain it is difficult to predict the relative magnitudes of the negative charge on each of the three phosphate oxygen atoms or the relative chemical shift values of the peptide NH groups of G108 and T109. The third oxygen atom on the phosphate group is hydrogen-bonded to the hydroxyl group of the Y70* side chain and also to a water molecule. At low pH the nearby Schiff base nitrogen is protonated (its pK_a is 6.5 in 10 mM phosphate buffer) (Metzler, D. E., *et al*., 1994c). However, the proton may be resident part of the time on a phosphate oxygen or on the coordinated H_2O molecule as has been suggested by ^{31}P NMR measurements and by analysis of electronic absorption spectra for the cytosolic and mitochondrial enzymes (Kallen *et al*., 1985; Metzler, C. M., & Metzler, 1987; Miura *et al*., 1989). This partial protonation will have the effect of withdrawing negative charge from the other two oxygen atoms, weakening the hydrogen-bonds to the peptide NH groups and causing a more upfield position for their resonances. Raising the pH will result in tightening of these hydrogen-bonds and in the observed downfield shifts in peaks 7G and 2. It is more difficult to explain the additional shift, downfield for peak 2 and upfield for peak 7G, upon binding of succinate. Thus, the assignment of peaks 2 and 7G remains extremely tentative.

Peaks 3−6 lie in the ^{1}H chemical shift range 10.6−10.9 ppm, indicating possible proximity to a carboxylate oxygen. Of the 29 residues in our list with amide hydrogens close to negatively charged atoms, only four, I17, L18, C192, and H193, are near the active site. The assignment of the resonances of the last two amino acid residues was discussed in the preceding paragraphs. I17 and L18 reside on the interface between the small domain and the large domain of the second subunit, and their amide NHs participate in hydrogen-bonds to the carboxyl group of D15, 3.06 and 3.1 Å long, respectively. Upon binding of substrates or inhibitors these two residues undergo large conformational change (Okamoto *et al*., 1994) that could affect the spectral position of their resonances. It is possible that peak 4, which shows a 0.08 ppm upfield shift in the complexes with inhibitors, represents one of these amide hydrogens.

Tryptophan 140 and Its Interaction with Dicarboxylates. Aspartate aminotransferases are known to bind dicarboxylate ions tightly (Jenkins, 1964; Jenkins & D'Ari, 1966; Michuda & Martinez-Carrion, 1969; Cheng *et al*., 1971; Ivanov *et al*., 1973; Bonsib *et al*., 1975; Jenkins & Fonda, 1985; Metzler, C. M., & Metzler, 1987). In a previous ^{1}H NMR study of AspAT from *E. coli* (Metzler, D. E., *et al*., 1994c) a sharp peak was seen to move from a position above 11 ppm downfield to 11.26 ppm upon titration of the enzyme with succinate. This peak was absent from the spectrum of the W140F mutant protein, and we suggested that it might arise from the W140 indole NH. Our HMQC spectra confirm this assignment definitively. There was some confusion in the previous work because another resonance, which, like that of the indole NH was designated E′, was present at 11.36 ppm at pH 9 and was also present and very intense at lower pH values in the absence of phosphate buffer. Our present results show that this latter peak, which we designate E′ in Figure 2c does not arise from W140. It is not split by coupling to an ^{15}N nucleus and probably represents a proton bound to an oxygen or sulfur atom.

In bacterial (Miyahara *et al*., 1994; Okamoto *et al*., 1994), cytosolic (Arnone *et al*., 1985), and mitochondrial (Malash-

kevich *et al*., 1995b) aspartate aminotransferases the side chain of W140 lies within the active site with its indole ring almost parallel to the coenzyme ring. The angle of inclination between the two rings is 20° in the PLP form and 4° in the PMP form of *E. coli* AspAT (Miyahara *et al*., 1994; Okamoto *et al*., 1994). The $HN^{\epsilon 1}$ proton (H_e in Figure 1) points toward the substrate binding site and in complexes with inhibitors forms a hydrogen-bond with the ω-carboxyl group of the ligand (Figure 6). In the absence of a ligand the enzyme is in its open form and the active site is accessible to solvent molecules and buffer ions. In liganded forms the small structural domain of the molecule has moved to cover the active site. In the resulting closed form an additional peptide NH group, that of G38, has assumed a position that allows hydrogen bonding with the α-carboxylate of the substrate or inhibitor (Okamoto *et al*., 1994). In all known crystal structures of the complexes of AspAT with inhibitors and *quasi*-substrates a hydrogen-bond exists between $N^{\epsilon 1}$ of W140 and one of the carboxyl oxygens of the ligand (Figure 6). The distance between the two heavy atoms involved in the bond varies from 3.0 to 3.6 Å depending on the ligand and on the subunit. Crystal structures are available for maleate complexes of both the PLP and PMP form of AspAT. The length of the hydrogen-bond between maleate and W140 is 3.0 Å for one of the subunits of the PLP form (Okamoto *et al*., 1994), and 3.1 Å for the PMP form (Miyahara *et al*., 1994).

The observed 1.15 ppm downfield shift of the W140 indole resonance upon binding of succinate suggests that a definite hydrogen-bond is also formed upon binding of dicarboxylates in solution. Judging by the changes in chemical shift (Table 1), the bond may be strongest in the succinate complex. However, this resonance behaves very differently in the PLP and the PMP forms. It is already quite far downfield in the PMP form in the presence of inorganic phosphate and moves upfield upon addition of a dicarboxylate. We interpret this as a result of the breaking of a strong hydrogen-bond between the indole nitrogen and phosphate and its replacement by a weaker one with the dicarboxylate. In the PMP form the $HN^{\epsilon 1}$ of W140 apparently interacts more strongly with the buffer anions than with the carboxyl group of the dicarboxylate.

For the succinate, maleate, and *meso*-tartrate complexes the W140 indole NH resonance is further downfield in the PLP form than in the PMP form by 0.17, 0.19, and 0.45 ppm, respectively. These differences could arise either from differences in the length of the hydrogen-bond or from changes in the effects of the ring current of the adjacent coenzyme ring and/or in magnetic anisotropies as a result of the altered tilts of the two aromatic rings. To assess the latter possibility we calculated the predicted difference in the chemical shift of $HN^{\epsilon 1}$ of W140 between the maleate complexes of the PMP and the PLP forms of the enzyme using the program SHIFTS (Ösapay & Case, 1991; Metzler, D. E., *et al*., 1994b). The computed value of −0.16 ppm suggests a shift of the ϵ W140 resonance in a direction opposite to the experimentally determined. The predicted shift is mainly an effect of the change in the relative positions of the coenzyme and W140 rings that arise from the tilting of the two rings. This result implies that the differences in the chemical shift of the ϵ W140 resonance in the presence of an inhibitor are in fact greater than observed.

Another factor contributing to the difference between the PLP and PMP forms may be that the inhibitors spend some time in the active site of the enzyme in an alternative binding conformation in which the hydrogen-bond to W140 has a less favorable geometry than in the conformation observed crystallographically. A fast exchange between these conformations would average their effect on the W140 peak shifting it to an intermediate position. There is a different distribution of charges in the active sites of the PLP and PMP forms of the enzyme. In the low-pH species of the PLP form, to which dicarboxylates bind tightly, the positive charge on the Schiff base nitrogen interacts strongly via a hydrogen-bond with the phenolate oxygen of the coenzyme. In the PMP form the rings are tilted toward the substrate-binding site and the positive charge is thought to be shared between the PMP and K258 amino groups which are hydrogen-bonded to each other. This positive charge is carried by atoms that are exposed in the substrate site which now has three positive charges. This allows for alternative binding modes of a dicarboxylate in the active site. Modeling using program CHARMM showed that modes of binding in which the ligand carboxylates interact with the positive charge of the PMP/K258 amino groups are possible and have favorable interaction energies.

Binding of Small Anions. Within the cell, aspartate aminotransferase exists in a highly ionic environment. Similarly, most *in vitro* studies of the enzyme are carried out at relatively high ionic strength. AspAT is known to bind small monoanions and dianions such as Cl^-, formate, acetate, sulfate, and phosphate (Jenkins & D'Ari, 1966; Bergami *et al*., 1968; Metzler, C. M., & Metzler, 1987) as well as dicarboxylates. Anions act as competitive inhibitors and are thought to participate in product displacement reactions (Jenkins & D'Ari, 1966; Cheng & Martinez-Carrion, 1972). Yet, little is known about anion binding sites in AspAT.

We measured a −0.30 ppm shift of the ϵ W140 resonance in the PLP form relative to the PMP form in KCl (Table 2). Most of this shift (−0.17 ppm, as calculated with SHIFTS) is accounted for by the change in the relative positions of the coenzyme and W140 rings. Apparently, if the binding of Cl^- ions into the active site affects the W140 resonance, the effect is small and almost the same for the PLP and PMP forms. However, sulfate (SO_4^{2-}) and phosphate (HPO_4^{2-}) both have strong effects on the chemical shift of $HN^{\epsilon 1}$ of W140. Because of their structural similarities to a carboxylate group they may be expected to occupy the binding sites for the carboxyl groups of the substrate and affect the W140 resonance. In the PLP form in 20 mM phosphate buffer the W140 indole NH proton exchanges faster than does that of free tryptophan. Since there is no base in its vicinity that could catalyze the exchange, it is likely that a loosely bound phosphate in the active site (Iriarte *et al*., 1985) is the catalyst. The small downfield shift produced by the phosphate anions together with the accelerated exchange with the solvent suggest that the inorganic dianions may be bound to the active site of the PLP form but without forming a strong hydrogen-bond to the indole proton. Although sulfate anions cause a somewhat larger downfield shift than do phosphate anions, they also broaden the W140 resonance and most likely interact similarly with the $HN^{\epsilon 1}$ proton of W140. In contrast, both phosphate and sulfate cause large downfield shifts of the indole resonance of the PMP form and suppress the exchange of the $HN^{\epsilon 1}$ proton with the solvent. This suggests the formation of a specific hydrogen-bond between the dianion and W140. The bond with phosphate appears

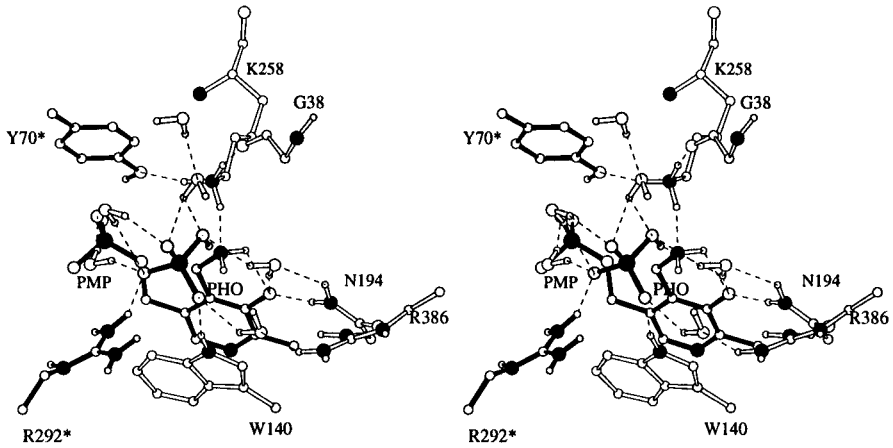

FIGURE 7: Stereoscopic view of a possible model of the complex of the PMP form with phosphate (PHO). The hydrogen-bonds are shown with dashed lines. Several water molecules are also shown. The positive charge was placed on the amino group of K258 while that of the coenzyme was left uncharged.

to be stronger than that with sulfate since the peak shifts almost three times further downfield in phosphate buffer (Table 2).

Why do phosphate and sulfate interact so differently with the PLP and the PMP forms of the enzyme? A clue comes from the observation of two distinct modes of binding of sulfate ions in crystals of the bacterial AspAT. In the structure of the PLP form in the open (unliganded) conformation (Jäger *et al.*, 1994) sulfate binds in the binding site of the α-carboxyl group of the substrate, forming an ion pair with R386 and forming additional hydrogen-bonds with the side chain amide nitrogen of N194 and with $HN^{\epsilon 1}$ of W140. However, in the crystal structure of the PMP form of a K258A mutant (Smith *et al.*, 1989) and a K258H mutant (Malashkevich *et al.*, 1995a) of *E. coli* AspAT the sulfate is in a slightly different position and nearer to the PMP amino group. The sulfate ion pairs with R292* as does the ω-carboxyl group of the substrate. It also hydrogen-bonds with N194, W140, and the PMP amino group. It was suggested that the positive charge on the PMP amino group attracts the sulfate anion toward the cofactor (Malashkevich *et al.*, 1995a). The results of our modeling support this proposal. Whether we place the positive charge on the PMP or the K258 amino group, an HPO_4^{2-} ion placed in the active site cavity moves to form hydrogen-bonds with R292, W140, and PMP groups as shown in Figure 7.

Other Tryptophans. Two out of five tryptophan residues in the amino acid sequence of AspAT give rise to resonances that are always present in the HMQC spectrum and remain unaltered under a variety of conditions. Unlike the indole NH resonances of free tryptophan and W140 of AspAT, peaks 9w and 10w are not broadened at high pH. They are present in the spectra in D_2O for long periods of time. These observations suggest that the two indole NH protons associated with peaks 9w and 10w do not exchange readily with the solvent. Calculation of the solvent accessible area of AspAT with QUANTA (Molecular Simulations, Inc.) using a 1.4 Å probe indicated two tryptophan residues whose $N^{\epsilon 1}$-bound protons are isolated from the solvent. W319 is entirely buried in the protein interior and fully protected from contacts with water. Its indole nitrogen forms a short (2.8 Å) hydrogen-bond with the carbonyl oxygen of N259. The proton involved in this bond may give rise to resonance 10w, which is the strongest tryptophan peak in the spectrum. The second buried indole NH proton belongs to W134 and could be responsible for resonance 9w. This proton participates in a 3.2 Å hydrogen-bond to the carboxyl group of D184 which could explain the ∼0.2 ppm downfield shift of peak 9w relative to 10w.

The remaining two tryptophan residues W205 and W217 have significant solvent accessible areas around their indole nitrogens and are the best candidates for resonances 9′w and 10′w. The high degree of motional freedom of the tryptophan side chain giving rise to peak 10′w in the apoenzyme spectrum suggests an increased mobility of the portion of the polypeptide chain where this tryptophan residue resides. This could be a result of the absence of interactions between the coenzyme and the amino acid residues of the large domain in the apo form.

Protonation State of the Active Site Histidines. The ^{15}N chemical shifts of the four observed imidazole NH nitrogens are all in the range 165−173 ppm characteristic of uncharged rings (Bachovchin, 1986). In the PLP form of the enzyme at pH 7.0 peaks H189, H193, and H-4 have ^{15}N chemical shift values (Table 3) that are very close to 167.5 ppm, the "standard" expected shift for uncharged imidazole ring. The most downfield peak, representing H143, has an ^{15}N chemical shift of 174.0 ppm, 6.5 ppm downfield from the standard value. In model compounds the ^{15}N chemical shifts of imidazole NH nitrogens acting as hydrogen-bond donors shift downfield as much as 10 ppm relative to standard values (Shuster & Roberts, 1979; Roberts *et al.*, 1982). The downfield position of the H143 resonance reflects its involvement in a strong hydrogen-bond to the D222 carboxylate group. Protonation to form an imidazolium ring would increase the ^{15}N chemical shift by ∼9 ppm. The ^{15}N chemical shift changes that we have observed for the histidine NH resonances in the pH range 5.5−8.5 are less than 2 ppm, indicating that all three active site histidines and the unidentified fourth one remain unprotonated at pH 5.5 and above and, therefore, have microscopic pK_a values lower than 5.5. This is in agreement with the results of the ^1H NMR titration studies of porcine cytosolic AspAT (S. Tanase, personal communication) and previous observations that V_{max} of the enzyme does not change in this pH range (Velick & Vavra, 1962; Kiick & Cook, 1983).

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