# Structure of the Complex between Pyridoxal 5′-Phosphate and the Tyrosine 225 to Phenylalanine Mutant of *Escherichia coli* Aspartate Aminotransferase Determined by Isotope-Edited Classical Raman Difference Spectroscopy†

Jonathan M. Goldberg,‡,§ Jie Zheng,‖ Hua Deng,‖ Yong Q. Chen,‖ Robert Callender,‖ and Jack F. Kirsch*,‡

*Department of Molecular and Cell Biology, University of California, Berkeley, California 94720, and Physics Department, City College of the City University of New York, New York, New York 10031*

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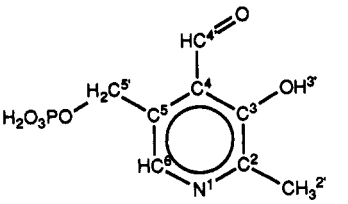
ABSTRACT: The azomethine (Schiff base) linkage between the ϵ-amino group of active-site lysine 258 and the carbonyl moiety of enzyme-bound pyridoxal 5′-phosphate (PLP) normally exhibits absorbance maxima at *ca.* 360 (high-pH form) or *ca.* 430 nm (low-pH form). However, the absorbance maximum is shifted from 358 to 386 nm, a value which is similar to that of free PLP ($\lambda_{max}$ = 388 nm), in a mutant form of *Escherichia coli* aspartate aminotransferase (AATase) in which tyrosine 225, which normally donates a hydrogen bond to the phenolate function of PLP, has been replaced with phenylalanine (Y225F). This spectral shift suggested that PLP binds to Y225F as the free aldehyde. The following evidence from isotope-edited classical Raman spectroscopy proves conclusively that the near-UV spectrum is anomalous and that PLP is bound to Y225F as a Schiff base: (1) A strong cofactor peak at 1630 $cm^{-1}$ in the holoenzyme-minus-apoenzyme difference spectrum of the unprotonated form of Y225F is red-shifted by 18 $cm^{-1}$ in enzyme labeled with $^{15}N$ at lysine 258 and other positions. (2) This isotope-induced red shift is similar to that observed in the unprotonated form of the model Schiff base, PLP–valine. (3) The Raman spectrum of Y225F is unchanged in $H_2{}^{18}O$, while peaks at *ca.* 1670 $cm^{-1}$ in the spectrum of free PLP or in that of a mutant of AATase in which Lys-258 is replaced with Ala, are red-shifted by *ca.* 30 $cm^{-1}$ in $H_2{}^{18}O$. A molecular orbital explanation for the anomalous red shift in the near-UV spectrum of the Y225F–PLP complex is proposed. In addition, the O3′ atom of PLP is found to simultaneously accept hydrogen bonds from Tyr-225 and Lys-258 in the protonated internal aldimine form of AATase.

Aspartate aminotransferase (AATase)[1] catalyzes the reaction (Jansonius & Vincent, 1987; Braunstein, 1973)

$$\text{L-Asp} + \alpha\text{-ketoglutarate} \rightleftharpoons \text{L-Glu} + \text{oxalacetate} \quad (1)$$

Pyridoxal 5′-phosphate (PLP) (Chart I) is an essential cofactor in this and in a large number of other enzyme-catalyzed reactions involved in amino acid metabolism (Hayashi et al., 1990). The cofactor binds covalently via an azomethine, or Schiff base linkage, to an active-site lysine of PLP-dependent enzymes (Lys-258 in the case of AATase, Scheme IA,B) in a complex often referred to as the internal aldimine. The oxygen at the C4′ position of PLP is exchanged for an imine nitrogen, and solvent contacts are replaced by specific interactions with the host protein upon formation of the internal aldimine from free PLP and apoenzyme, and these chemical and environmental changes are reflected in the UV–visible absorbance spectrum of the cofactor. The absorbance maximum of free PLP is 388 nm (Harris et al., 1976), while that of enzyme-bound PLP depends on the protonation state of the imine nitrogen, with the protonated and unprotonated forms displaying maxima at 430 and 360 nm, respectively (Scheme IA,B) (Jenkins & Sizer, 1957; Jenkins et al., 1959). The $pK_a$ value of this transition occurs between pH 6 and 7 in transaminases (Jenkins et al., 1959; Braunstein, 1973; Goldberg et al., 1991). Exceptions to the generalization regarding the $\lambda_{max}$ values of naturally occurring transaminases have not been observed (Kallen et al., 1985; Johnson & Metzler, 1970; Fasella, 1967).

Chart I: Atom Numbering Convention for Pyridoxal 5′-Phosphate (PLP)




The Y225F mutant of AATase, in which a hydrogen bond donated by Tyr-225 to the charged O3′ group of PLP is removed, provides a striking exception to the pattern described above. While the absorbance maximum of the protonated complex between PLP and Y225F (Scheme IC) is similar to those of other PLP-dependent enzymes, the unprotonated form displays an absorbance maximum of 386 nm. Thus, the spectrum of PLP bound to Y225F resembles that of the free aldehyde form of the cofactor more closely than that of the complex between PLP and wild-type AATase, suggesting that the unprotonated form of the Schiff base is hydrolyzed in the mutant active site (Scheme IE).

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‡ University of California.

§ Present address: Department of Biochemistry, Stanford University School of Medicine, Stanford, CA 94305.

‖ City College of the City University of New York.

[1] Abbreviations: AATase, aspartate aminotransferase; PLP, pyridoxal 5′-phosphate; PLP–Val, *N*-PLP–L-valine; PMP, pyridoxamine 5′-phosphate; Y225F, mutant *E. coli* AATase with Tyr-225 replaced by Phe; $^{15}N$ Y225F, Y225F metabolically labeled with $^{15}N$ at the peptide and side-chain nitrogen atoms of Asp, Glu, Arg, Lys, Met, Cys, Asn, Gln, Gly, Ala, Ser, and Thr; K258A, mutant *E. coli* AATase with Lys-258 replaced by Ala; HARD, holoenzyme-minus-apoenzyme Raman difference; CAPS, 3-(cyclohexylamino)-1-propanesulfonic acid; CHES, 2-(cyclohexylamino)ethanesulfonic acid; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*-2-ethanesulfonic acid.

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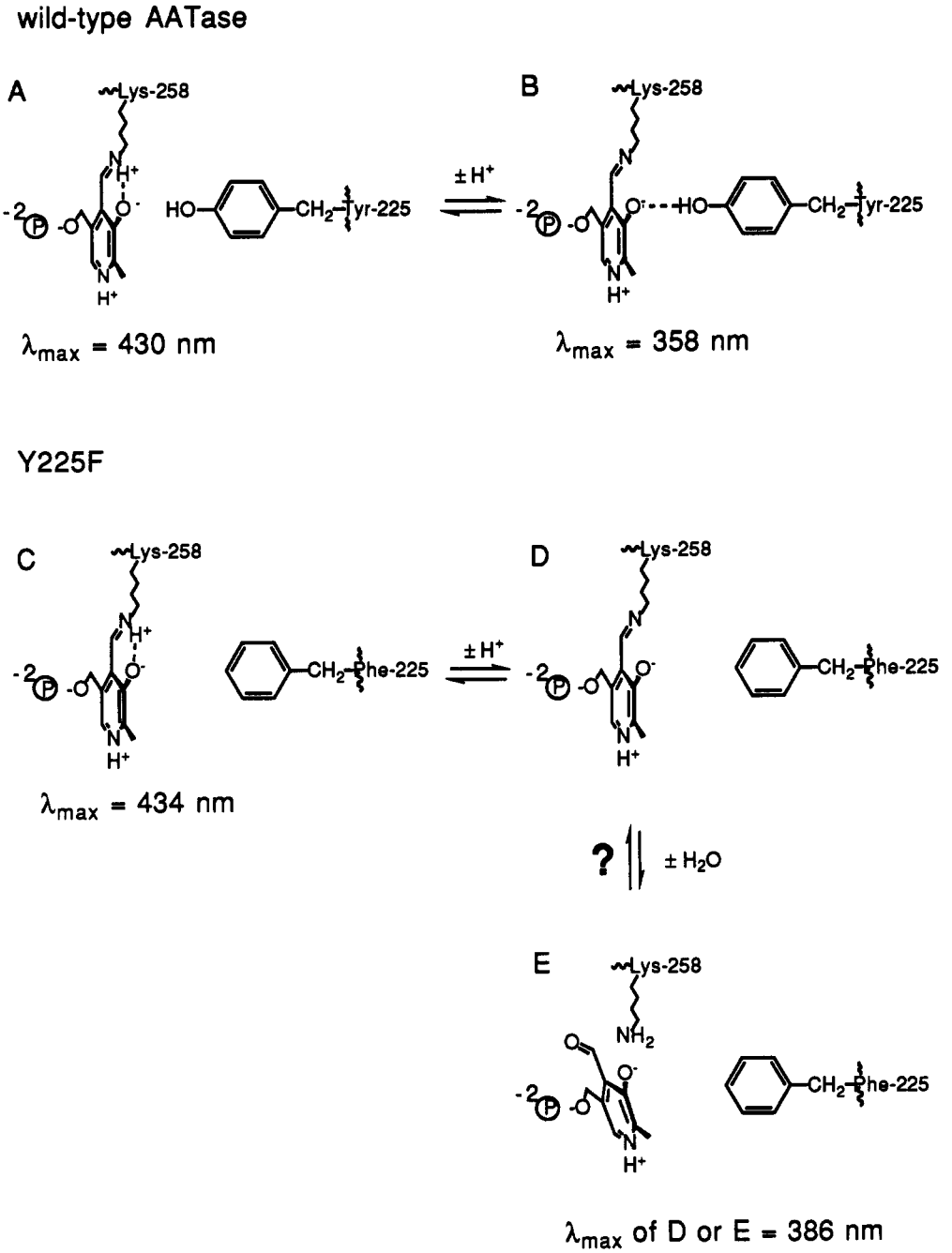
Scheme I: Schematic Representations of Pyridoxal 5′-Phosphate (PLP) and Selected Aspartate Aminotransferase (AATase) Active-Site Residues[a]




[a] (A) The protonated and (B) unprotonated forms of the azomethine (internal aldimine) in wild-type AATase. (C) The protonated form of the internal aldimine of Y225F and (D) the potential unprotonated internal aldimine or (E) free aldehyde forms of the complex of the cofactor with the mutant enzyme. The relative positions of the cofactor and Tyr-225 or Phe-225 are based on crystallographic studies on the wild-type and mutant *E. coli* enzymes (C. Schumacher and D. Ringe, unpublished data).

Current X-ray crystallographic data indicate that the structure of the protonated form of Y225F is similar to that of wild-type AATase (Inoue et al., 1991; C. Schumacher and D. Ringe, unpublished data), but the structure of the unprotonated form of the mutant enzyme has not yet been determined. The nature of the bond in the 386-nm-absorbing complex of PLP and Y225F was probed by acid-catalyzed hydrolysis of the $NaBH_4$-reduced form of the mutant enzyme. This experiment yielded $N^{\epsilon}$-pyridoxyl-L-lysine, the reduction and hydrolysis product of the Schiff base, rather than pyridoxine, the product expected from the reaction of the aldehyde (Goldberg et al., 1991). This finding suggests that PLP binds to Y225F through a Schiff base linkage; however, the chemical reduction experiment could have given a false representation of the structure of the complex. For example, if 95% of the complex were in the free-aldehyde form and 5% were in the azomethine form, more than 95% of the cofactor could still be trapped as $N^{\epsilon}$-pyridoxyl 5′-phosphate–Lys-258 if the ratio of the respective rate constants for $NaBH_4$ reduction of the imine and the aldehyde were greater than 450. This consideration motivated the spectroscopic studies described here.

Raman spectroscopy has the potential to resolve the structural ambiguity surrounding the cofactor–enzyme linkage in Y225F. Spiro and co-workers obtained the resonance Raman spectrum of the protonated form of pig cytosolic AATase and assigned many of the resonances by comparison to the spectra of model compounds (Benecky et al., 1985a,b). However,

they found that resonance Raman spectroscopy cannot be used to determine the structure of the unprotonated form of the cofactor–enzyme complex in AATase, since the fluorescence of this complex obscures the Raman spectrum. Fluorescence interference is avoided in this study by employing classical Raman difference spectroscopy, where the exciting wavelength may be set to values substantially above the cofactor absorbance (Yue et al., 1984; Deng et al., 1989). Raman spectroscopic studies may be enhanced by the use of isotopically enriched model compounds, solvents (Benecky et al., 1985a), and proteins (Lewis et al., 1978; Argade et al., 1981; Manor et al., 1991). We report an investigation of the isotope-edited classical Raman difference spectra of wild-type, Y225F, and K258A AATase. This study resolves the structural ambiguity of the Y225F–PLP linkage in favor of the azomethine and probes other structural attributes of the interaction between PLP and Tyr-225.

## EXPERIMENTAL PROCEDURES

*Preparation of Aspartate Aminotransferases.* Plasmids encoding wild-type, Y225F, and K258A AATase were constructed as described previously (Malcolm & Kirsch, 1985) except that a high expression phagemid vector was used for wild-type and Y225F AATase (J. J. Onuffer, unpublished methods). Site-directed mutagenesis and enzyme expression in *E. coli* strain MG204 were carried out as reported by Danishefsky et al. (1991) except that 0.02% pyridoxine was added to the growth medium. The enzymes were purified as described by Cronin and Kirsch (1988) except that Sepharose G100 was substituted for ACA 44 resin, and absorbance at 430 nm was used to monitor enzyme concentration in column fractions.

Y225F was substituted with $^{15}N$ on the amide and side-chain nitrogen atoms of Asp, Glu, Arg, Lys, Met, Cys, Asn, Gln, Gly, Ala, Ser, and Thr by growth of *E. coli* strain MG204 in a medium containing 0.8% glucose; 0.2 g/L magnesium sulfate heptahydrate; 2 g/L citric acid monohydrate; 10 g/L anhydrous dibasic potassium phosphate; 1 g/L [$^{15}N$]ammonium chloride (IBN Radiochemicals); 12.5 μg/mL tetracycline; 50 μg/mL kanamycin; 100 μg/mL ampicillin; 50 μg/mL each of Tyr, Trp, Phe, Ile, Leu, Val, Pro, and His; 2 mM αKG; and 0.02% pyridoxine. Supplementation of the growth medium with pyridoxine ensured that the bound cofactor was not $^{15}N$-enriched at the N1 position. A liter of culture grown for 60 h at 37 °C yielded 5.8 g of cell paste from which 48 mg of ≥95% pure $^{15}N$ Y225F was obtained.

*Enzyme Resolution.* Wild-type and Y225F AATases were resolved from coenzyme as described by Wada and Snell (1962), with the following variations: A 2-fold molar excess of cysteine sulfinate in 10 mM potassium phosphate buffer, adjusted to pH 7.5 with KOH, was added to a solution of 100–500 μM enzyme. After a few minutes, protein was precipitated by addition of 3 volumes of a saturated ammonium sulfate solution in 10 mM sodium acetate adjusted to pH 4.9 with acetic acid (buffer 1). The supernatants were checked for PMP spectrophotometrically after 1 min of gentle mixing. The precipitates were iteratively dissolved in buffer 1 and reprecipitated until the supernatants were free of PMP. Typically, two repetitions were sufficient. Apoenzyme was dissolved in 10 mM Tris buffer adjusted to pH 7.5 with KOH or HCl, 1 mM $K_2EDTA$, and 0.25 mM DTT such that the final enzyme concentrations were ≤5 mg/mL, and the solutions were dialyzed against the same buffer. Undissolved material was removed by centrifugation or filtration through 0.2-μm filters. Enzyme solutions were exchanged into Raman spectroscopy buffers in a Centricon ultrafiltration device. Temperatures were maintained between 0 and 4 °C throughout. Yields from resolution and reconstitution by addition of a 1.1-fold molar excess of PLP were typically 80% as determined from activity measurements (Goldberg et al., 1991). K258A was resolved similarly except that the precipitation buffer was adjusted to pH 3.5. Prior to Raman spectroscopy, holo-K258A was resolved and reconstituted by addition of a 1.1-fold molar excess of PLP to eliminate PMP which accumulates on the enzyme during preparation and storage at 4 °C (M. D. Toney and J. F. Kirsch, unpublished data).

*Raman and Near-UV–Visible Spectroscopy.* Raman difference spectra were recorded by alternatively scanning apoenzyme and holoenzyme in a split cuvette at 4 °C as described previously (Deng et al., 1989, 1991; Yue et al., 1986). Enzyme concentrations were from 2 to 3 mM; 5 mM PLP and 0.5 M L-valine were combined in 0.1 M potassium phosphate buffer adjusted to pH 13.0 with KOH to obtain Raman spectra of PLP–Val. The pH-dependent formation constant for the Schiff base at pH 13 is approximately 10 (Metzler et al., 1980); thus, above 80% of the total PLP is expected to be in the Schiff base form. Free PLP and valine spectra are subtracted to obtain the pure PLP Schiff base spectrum. AATase solutions were buffered in the following KOH-adjusted solutions: wild-type AATase and Y225F, pH or pD 5.5, 0.2 M sodium acetate; wild-type AATase, pH 9.5, 0.1 M CHES; Y225F, pH 10.5, 0.1 M CAPS; K258A, pH 7.5, 0.1 M HEPES. PLP was buffered in 0.2 M sodium acetate at pH 5.

The excitation wavelength for samples containing wild-type AATase, Y225F, or PLP–Val was 514.5 nm from an argon laser; that for samples containing K258A or PLP was 568.2 nm from a krypton laser. PLP is nearly transparent at these excitation wavelengths; thus, the previously described fluorescence and photoreaction of the cofactor (Benecky et al., 1985b) were minimized. Excitation powers were typically 100 mW. The unsmoothed spectra were calibrated against that of the toluene spectrum; absolute band positions are accurate to within ±3 $cm^{-1}$ and relative band positions to ±1.5 $cm^{-1}$. The slits were set to achieve resolutions of 6–7 $cm^{-1}$. The near-UV–visible spectra were acquired with a Kontron Uvikon 860 spectrophotometer. Stored enzyme was diluted directly into buffered solutions such that the final concentrations were from 20 to 50 μM. The concentration of PLP in the absence of enzyme was 100 μM.

## RESULTS AND DISCUSSION

*Near-UV–Visible Spectroscopy.* The spectra of free PLP, wild-type AATase, Y225F, and K258A are shown in Figure 1, curves b, a, d, and c, respectively. The spectrum of the unprotonated form of wild-type AATase (Scheme IB; Figure 1, curve a) has a $\lambda_{max}$ of 358 nm, typical of PLP-containing enzymes (Kallen et al., 1985; Johnson & Metzler, 1970; Fasella, 1967). The spectrum of the unprotonated form of Y225F (Figure 1, curve d) exhibits an absorbance maximum of 386 nm, similar to 388 nm, the absorbance maximum of free PLP (Harris et al., 1976; Figure 1, curve b), and 398 nm, the absorbance maximum of K258A (Figure 1, curve c). K258A is a mutant of AATase which must bind PLP as an aldehyde since the Schiff-base-forming Lys-258 is replaced by Ala (Toney & Kirsch, 1989). The spectra in Figure 1 are consistent with the hypothesis that Y225F binds PLP as the free aldehyde.

*Raman Spectroscopy.* The nonresonance Raman spectrum of AATase (data not shown) is dominated by protein bands.

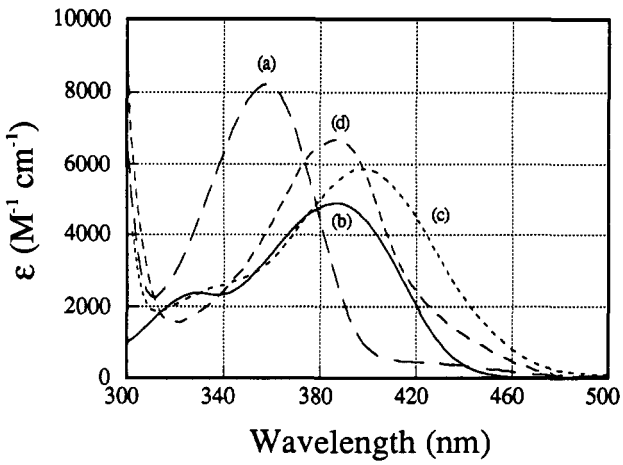



FIGURE 1: Near-UV–visible spectra of free pyridoxal 5′-phosphate and wild-type and mutant aspartate aminotransferases (AATases): (a) wild-type AATase, pH 9.0, 0.2 M CHES; (b) pyridoxal 5′-phosphate, pH 8, 10 mM HEPES; (c) K258A, pH 7.5, 0.2 M HEPES, 0.1 M KCl; (d) Y225F, pH 10.4, 0.2 M CAPS.

Table I: Selected Raman Peak Frequencies of Free Pyridoxal 5′-Phosphate (PLP) and PLP Bound to Aspartate Aminotransferase or to L-Valine: $^{15}N$, $^{18}O$, and $^{2}H$ Isotope Effects at 4 °C

| species | pH or pD | $\nu_{C4'=(N\text{ or }O)}$ ($cm^{-1}$) | | | | $\nu_{C3-O3'}$ ($cm^{-1}$) | |
|---|---|---|---|---|---|---|---|
| | | $^{14}N$ | $^{15}N$ | $^{1}H_2{}^{18}O$ | $^{2}H_2{}^{16}O$ | $^{1}H_2{}^{16}O$ | $^{2}H_2{}^{16}O$ |
| WT[a] | 5.5 | 1650 | | | 1637 | 1355 | 1354 |
| WT[a] | 9.5 | 1639 | | 1639 | | *d* | *d* |
| Y225F[a] | 5.5 | 1650 | | | 1638 | 1348 | 1350 |
| Y225F[a] | 10.5 | 1630 | 1612 | 1630 | | *d* | *d* |
| PLP–Val[b] | 13.0 | 1631 | 1614 | 1631 | | | |
| K258A[a] | 7.5 | 1679 | | 1649 | | | |
| PLP[c] | 5.5 | 1673 | | 1645 | | | |

[a] Peak frequencies from holoenzyme-minus-apoenzyme Raman difference spectra for 2–3 mM wild-type, Y225F, $^{15}N$-enriched Y225F, and K258A aspartate aminotransferases (AATases). Concentrated enzyme solutions were diluted into buffered $H_2{}^{18}O$ or $^{2}H_2O$ for spectral measurements in isotopically enriched solvents. Wild-type AATase, Y225F, and K258A were buffered as follows: pH 5.5 or pD 5.5, 0.2 M sodium acetate; pH 9.5, 0.1 M CHES; pH 10.5, 0.1 M CAPS; pH 7.5, 0.1 M HEPES. Samples were excited at 514 nm from an argon laser (wild-type AATase and Y225F) or at 568.2 nm with a krypton laser (K258A). [b] Peak Raman frequencies from spectra of ~60 mM *N*-PLP–L-valine and $^{15}N^{\alpha}$-enriched *N*-PLP–L-valine, excited as described for wild-type AATase and Y225F, buffered in 0.1 M potassium phosphate. [c] 0.1 M PLP dissolved in $H_2O$ or $H_2{}^{18}O$, excited as described for K258A, and buffered in 0.2 M sodium acetate, pH 5. [d] The C3–O3′ peak frequency for AATase at high pH has not been assigned.[2]

Preresonance effects give rise to cofactor bands in the 1348–1355- and 1612–1650-$cm^{-1}$ regions with amplitudes of *ca.* 100% and 25%, respectively, of the major protein amide I band at 1655 $cm^{-1}$. Our Raman difference spectrometer is currently capable of measurement of differences as small as 0.1% (Yue et al., 1989); thus, extremely good Raman signals of bound cofactor are obtained. At the same time, background fluorescence signals are minimized because the Raman exciting laser light used in this study lies far to the red of any of the protein complexes absorbances.

Holoenzyme-minus-apoenzyme Raman difference (HARD) spectra of AATase were calculated as described previously for alcohol and lactate dehydrogenases (Chen et al., 1987; Deng et al., 1989). The HARD spectrum of wild-type AATase at pH 5.5 (data not shown) is essentially that of the resonance Raman spectrum of pig cytosolic AATase (Benecky et al., 1985b); thus, the assignments of that reference are used in interpreting the present results. Table I collects selected Raman peak frequencies from the spectra of wild-type, Y225F, and K258A AATases, PLP, and PLP–Val. The most intense

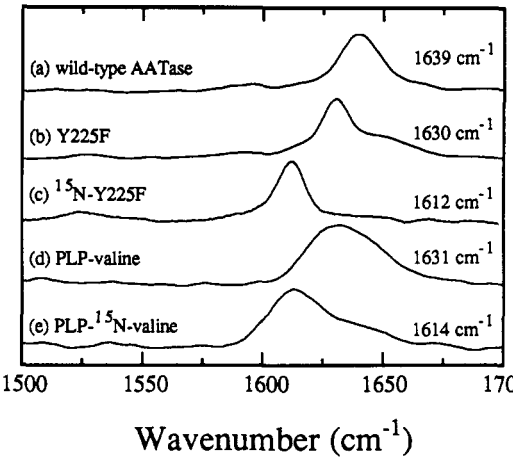



FIGURE 2: Holoenzyme-minus-apoenzyme Raman difference (HARD) spectra of 2–3 mM solutions of wild-type and Y225F aspartate aminotransferases (AATases) at 4 °C, and Raman spectra of *N*-pyridoxal 5′-phosphate–L-valine (PLP–Val). Samples were excited at 514.5 nm with an argon laser. HARD spectra: (a) wild-type AATase, pH 9.5, 0.1 M CHES; (b) Y225F, pH 10.5, 0.1 M CAPS; (c) Y225F labeled with $^{15}N$ on the amide and side-chain nitrogen atoms of Asp, Glu, Arg, Lys, Met, Cys, Asn, Gln, Gly, Ala, Thr, and Ser prepared as described under the Experimental Procedures and measured under the same conditions as (b). The labeling procedure results in quantitative $^{15}N$ enrichment at the $\epsilon$-amino position of active-site Lys-258. Raman spectra: (d) 5 mM PLP–Val, pH 13, 0.1 M potassium phosphate; (e) $\alpha$-amino $^{15}N$-substituted PLP–Val measured under the same conditions as (d).

bands in the HARD spectrum of wild-type AATase occur at 1355 and 1650 $cm^{-1}$ (Table I, row 1). The band at 1355 $cm^{-1}$ is assigned to a ring mode with a large contribution from the C3—O3′ stretch, and the 1650-$cm^{-1}$ peak is assigned to the C=N stretch mode (Benecky et al., 1985b). The HARD spectrum of Y225F (Table I, row 3) at pH 5.5 is similar to that of wild-type enzyme except that the C3—O3′ resonance at 1355 $cm^{-1}$ is shifted to 1348 $cm^{-1}$. The peaks occurring at 1650 $cm^{-1}$ in the spectra of both wild-type and Y225F AATase are red-shifted by about 12 $cm^{-1}$ in $^{2}H_2O$, pD 5.5 (Table I, column 6), consistent with deuteration of the Schiff base nitrogen, while there is little effect of solvent deuteration on the positions of the C3—O3′ stretch frequencies in either enzyme (Table I, column 8).

*PLP Is Bound to Y225F as a Schiff Base.* The Raman spectra covering 1500–1700 $cm^{-1}$ for the unprotonated forms of wild-type AATase, Y225F, and PLP–Val are shown in Figure 2, and the positions of the peak frequencies are indicated in Table I. The intense peak assigned to the C3–O3′ stretch mode (1348–1355 $cm^{-1}$) in the HARD spectra of wild-type and Y225F AATase at pH 5.5 is lost at high pH.[2] The bands located at 1650 $cm^{-1}$ in the spectra of wild-type and Y225F AATase at pH 5.5 (Table I) are shifted to 1639 $cm^{-1}$ for wild-type AATase at pH 9 (Figure 2, curve a; Table I, row 2) and to 1630 $cm^{-1}$ for Y225F at pH 10.5 (Figure 2, curve b; Table I, row 4). The peak at 1630 $cm^{-1}$ in the spectrum of the unprotonated form of Y225F is shifted 18 $cm^{-1}$ to 1612 $cm^{-1}$ in a preparation which is $^{15}N$-enriched at the peptide and side-chain nitrogen atoms of Asp, Glu, Arg, Lys, Met, Cys, Asn, Gln, Gly, Ala, Ser, and Thr (Figure 2, curve c;

[2] Benecky et al. (1985a) assigned the C3–O3′ stretch to a prominent band observed at 1302 $cm^{-1}$ in ultraviolet resonance Raman experiments on PLP–Val complexes at pH 13. The most prominent midrange band in the nonresonance Raman spectrum of this complex lies at 1247 $cm^{-1}$, and no band is observed at 1302 $cm^{-1}$ (data not shown). Thus, the assignment of the C3–O3′ stretch in high-pH conditions must await future study using appropriately isotopically labeled cofactors.

Table I, column 4). Isotopic changes in nitrogen atoms not involved in azomethine linkage are not expected to have large effects on the Schiff base stretch frequency, and Lewis et al. (1978) have shown that $^{15}N$ enrichment of positions other than that of the $\epsilon$-nitrogen atom of the lysine involved in Schiff base formation do not affect the resonance Raman spectrum of the retinal–bacteriorhodopsin azomethine. Thus, it may be inferred that the spectroscopically significant isotopic enrichment in Y225F is that of the $\epsilon$-nitrogen of Lys-258.

The Raman spectra of natural abundance and $^{15}N^{\alpha}$-PLP–Val at pH 13 are shown in Figure 2, curves d and e, and the peak frequencies are indicated in Table I, row 5. The Raman spectrum of PLP–Val is similar to the resonance Raman spectrum of PLP–Val in this range (Benecky et al., 1985a). $\alpha$-Nitrogen $^{15}N$ enrichment of PLP–Val causes a $-19$-cm$^{-1}$ shift in the position of the C=N stretch mode from 1631 to 1614 cm$^{-1}$ (Figure 2, curve e; Table I, column 4). The existence of an $^{15}N$ isotope effect on the HARD spectrum of Y225F, taken together with the similar magnitude of the effect observed for PLP–Val, provides conclusive evidence that the complex between Y225F and PLP is a Schiff base and not a free aldehyde at high pH.

In contrast with the $^{15}N$ isotopic shift observed in peak frequencies in the bands under consideration, there is no effect induced by substituting $H_2{}^{18}O$ for $H_2{}^{16}O$ in wild-type and Y225F AATase (Table I, column 5). A mutant of AATase that lacks the essential lysine at position 258 (K258A), and thus cannot form an active-site Schiff base, has been described (Malcolm & Kirsch, 1985; Toney & Kirsch, 1989). The HARD spectrum of K258A has an intense band at 1679 cm$^{-1}$ (Table I), which is red-shifted by 30 cm$^{-1}$ in $H_2{}^{18}O$ (Table I, row 6). A similar $^{18}O$ isotope effect is observed in the Raman spectrum of free PLP (Table I, row 7). Thus, the peak at 1679 cm$^{-1}$ in the spectrum of K258A may be assigned to the PLP C4′=O bond. The absence of an $^{18}O$ isotope effect on the 1630-cm$^{-1}$ peak of Y225F provides further evidence that its cofactor is not bound as a free aldehyde.

*Models for the Red Shift in the Absorbance Maximum of the Y225F–PLP Complex and Its Decreased C=N Stretch Frequency.* Why is the near-UV absorbance maximum of the internal aldimine shifted 26 nm to the red in Y225F? For the red shift in $\lambda_{max}$, there must be a decrease in the difference between the free-energy levels of the ground ($S_0$) and the first excited singlet state ($S_1$) of the mutant-bound cofactor relative to wild-type enzyme. In addition, any explanation of the red shift must also account for the 9-cm$^{-1}$ decrease in the Schiff base stretch frequency of the mutant relative to the wild-type protein. The shift in stretch frequency is a manifestation of a decrease in bond order of the C4′=N bond and is solely a ground-state effect. A number of explanations are possible, including indirect ones. For example, the C4–C4′ torsion angle may be reduced in Y225F relative to wild-type enzyme, even though the only direct interaction between Tyr-225 and the unprotonated Schiff base form of the cofactor is hydrogen bond donation to O3′ (Jansonius & Vincent, 1987; C. Schumacher and D. Ringe, unpublished data). This torsion angle is 75° in chicken mitochondrial AATase (Jansonius & Vincent, 1987). A decreased angle would allow greater conjugation of the azomethine bond of Y225F with the $\pi$ system of the pyridine ring, which would yield a red shift in $\lambda_{max}$ and a decrease in the Schiff base bond order.

However, our favored explanation is that the spectroscopic results arise from a specific destabilization of the C3–O3′–ground state relative to the excited state through direct electrostatic interactions. Molecular orbital calculations on the dipolar forms of pyridoxal oxime and pyridoxyl-L-valine indicate that the amount of negative charge borne by O3′ in the ground state decreases substantially in $S_1$ while that on the exocyclic nitrogen atom increases (Bazhulina et al., 1974; Kallen et al., 1985). These same calculations also suggest that the C4′=N bond order decreases in $S_1$ relative to $S_0$ as electron density in the C4′=N bond is transferred to the nitrogen atom. Since Tyr-225 stabilizes a negative charge on O3′ by hydrogen bond donation (Scheme IB), removal of this hydrogen bond by site-directed mutagenesis would selectively destabilize the ground state relative to the excited state and hence give rise to a red-shifted $\lambda_{max}$ and the observed decrease of the Schiff base bond order. This explanation is consistent with the observation that the spectra of other forms of the cofactor bound to Y225F are also red-shifted, albeit to a lesser extent than that of the unprotonated Schiff base form. For example, the absorbance maxima of the protonated internal aldimine form, the PMP form, and the $NaBH_4$-reduced form of Y225F are red-shifted by 5, 5, and 9 nm, respectively[3] (Goldberg et al., 1991). Furthermore, the Asn-194 to Leu mutation of AATase, which also results in the loss a hydrogen bond to the O3′ atom, displays a 6-nm red shift with respect to that of wild-type AATase (E. Neymark and J. F. Kirsch, unpublished data).

*The O3′ Atom of PLP Simultaneously Accepts Hydrogen Bonds from Tyr-225 and Lys-258 in the Protonated Internal Aldimine Form of AATase.* It has been proposed that protonation of the $\epsilon$-nitrogen atom of Lys-258 in the internal aldimine breaks the hydrogen bond between Tyr-225 and the O3′ group of PLP (Kirsch et al., 1984). However, the Y225F mutation induces a $-7$-cm$^{-1}$ shift in the position of the C3–O3′ stretch at pH 5.5 (1355 cm$^{-1}$ in wild-type AATase *vs* 1348 cm$^{-1}$ in Y225F; Table I, column 7), suggesting that Tyr-225 retains a hydrogen bond to the exocyclic oxygen atom of the protonated external aldimine.

[3] The smaller shifts in the $\lambda_{max}$ values observed for the protonated Schiff base or amine forms of the Y225F–cofactor complex relative to the shift seen for the unprotonated Schiff base form are probably due to the strong hydrogen bond interaction between the proton associated with the positively charged nitrogen atom and the negatively charged O3′ group. The local charge of C=NH$^+$···O3′ is hence near zero so that the addition or removal of another hydrogen bond would have a much smaller effect.

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