# A Hydrogen-Bonding Network Modulating Enzyme Function: Asparagine-194 and Tyrosine-225 of *Escherichia coli* Aspartate Aminotransferase†

Takato Yano, Takeo Mizuno, and Hiroyuki Kagamiyama*

*Department of Medical Chemistry, Osaka Medical College, Takatsuki, Osaka 569, Japan*

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ABSTRACT: The electron distribution within the coenzyme or coenzyme–substrate conjugate needs to be properly regulated during the catalytic process of aspartate aminotransferase (AspAT). Asn194 and Tyr225 may function in regulating the electron distribution through hydrogen-bonding to O(3′) of the coenzyme, pyridoxal 5′-phosphate (PLP) or pyridoxamine 5′-phosphate (PMP). The roles of Tyr225 have already been explored by site-directed mutagenesis (Inoue et al., 1991; Goldberg et al., 1991). In the present studies, the mutant enzymes Asn194→Ala and Asn194→Ala + Tyr225→Phe were analyzed kinetically and spectroscopically and were compared with the wild-type and Tyr225→Phe enzymes. The kinetic studies showed that Asn194 is not essential for AspAT catalysis, although the $K_d$ values for the substrates were increased by 10- to 50-fold upon the replacement of Asn194. The measurements of the absorption and fluorescence excitation spectra revealed that the ratio of an enolimine to a ketoenamine form was considerably increased as a tautomeric form of the protonated PLP in the active site of the double mutant enzyme. The pH–p$K_d$ relationship for the binding of maleate to AspAT could be explained by a simple thermodynamic cycle where only one ionizing group (the imine nitrogen of the internal aldimine bond) affects the binding of maleate. The analyses of the pH–p$K_d$ curves for the wild-type and mutant enzymes showed that (i) the hydrogen bond between O(3′) of PLP and Asn194 is weakened by the binding of maleate to AspAT, while the hydrogen bond between O(3′) and Tyr225 is not changed, and that (ii) the replacement of Asn194 causes some effect hampering the binding of maleate. Asn194 is also situated within hydrogen-bonding distance to the α-carboxylate group of the substrate and Arg386 which is known to function in substrate binding through the interaction with the substrate α-carboxylate. The above findings were discussed in terms of the hydrogen-bonding network: Tyr225 → O(3′) of the coenzyme ← Asn194 ← Arg386 ← carboxylate group of the substrate (analogue). We propose the idea that Asn194 lowers the p$K_a$ of the imine nitrogen of the internal aldimine bond to facilitate a transaldimination step and that Asn194 functions in substrate binding through hydrogen-bonding to the substrate carboxylate and/or holding the side chain of Arg386 in a position suitable for substrate binding. After formation of the coenzyme–substrate conjugate (an external aldimine intermediate), Tyr225 would be the main residue that regulates the electron distribution through hydrogen-bonding to O(3′) of the coenzyme.

In order to understand the reaction mechanism of an enzyme, it is necessary to eludicate the movement of electrons in each elementary reaction step. A number of studies by site-directed mutagenesis have been made on various enzymes to infer the movement of electrons during the catalytic process. Since aspartate aminotransferase (AspAT)[1] is one of the best characterized enzymes, it is a good material for this purpose.

AspAT catalyzes a reversible transamination reaction between the dicarboxylic α-amino and α-keto acids by a ping-pong bi bi mechanism. On the basis of the X-ray crystallographic studies, a detailed reaction mechanism has been proposed (Ivanov & Karpeisky, 1969; Kirsch et al., 1984; Arnone et al., 1985). The reaction consists of many elementary steps in which the electron distribution within the coenzyme or coenzyme–substrate conjugate must be changed. The amino acid residues surrounding the coenzyme molecule in the active site of AspAT have been supposed to function not only in binding the coenzyme but also in regulating the electron distribution (Kirsch et al., 1984; Arnone et al., 1985). Particularly, amino acid residues interacting with N(1) and O(3′) of the coenzyme are thought to participate effectively in the regulation of the electron distribution which is essential for the efficient catalysis of AspAT. The crystallographic studies showed that N(1) of the coenzyme forms an electrostatic interaction with and a hydrogen bond to the carboxylate group of Asp222[2] and that O(3′) is hydrogen-bonded to the phenolic hydroxyl group of Tyr225. From the studies by site-directed mutagenesis, a negative charge at position 222 was found to be essential for the reaction of the PLP form of AspAT with amino acid substrates (Yano et al., 1992). The negative charge would stabilize the positive charge at N(1) of PLP, which enhances the electron-withdrawing capacity of the coenzyme to facilitate abstraction of the α-proton of amino acid substrates, a prerequisite step of the transamination reaction. The mutation Tyr225→Phe gave more complicated results in that the rate constants were decreased for all the substrates but the affinity for all the substrates was considerably increased (Inoue et al., 1991; Goldberg et al., 1991). From the spectrophotometric titration of the mutant enzyme Y225F, it was supposed that

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* To whom correspondence should be addressed.

[1] Abbreviations: AspAT, aspartate aminotransferase; N194A, AspAT in which Asn194 is replaced by Ala; Y225F, AspAT in which Tyr225 is replaced by Phe; N194A/Y225F, AspAT in which Asn194 and Tyr225 are replaced by Ala and Phe, respectively; HEPES, *N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethanesulfonic acid; MES, 2-(*N*-morpholino)ethanesulfonic acid; PLP, pyridoxal 5′-phosphate; PMP, pyridoxamine 5′-phosphate.

[2] Amino acid residues are numbered according to the sequence of cytosolic AspAT from pig (Ovchinnikov et al., 1973).

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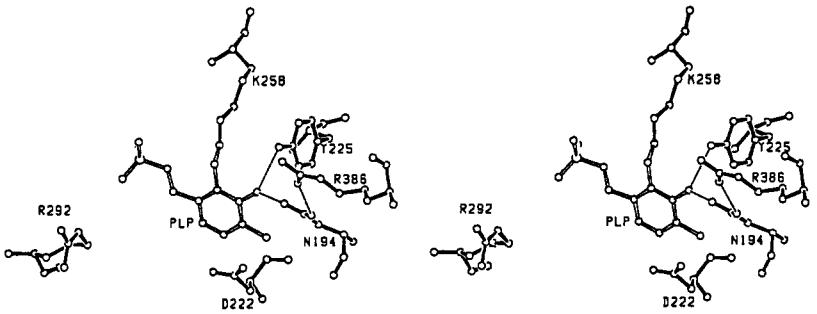



FIGURE 1: Stereo drawing of the active site of wild-type *E. coli* aspartate aminotransferase [modified from Kamitori et al. (1990)]. The hydrogen bonds discussed in this paper are indicated by thin lines.

a hydrogen bond between O(3′) of PLP and Tyr225 lowers the $pK_a$ value of the internal aldimine bond by 1.7 pH units and stabilizes the active unprotonated form of the aldimine bond in the physiological pH region.

The crystallographic studies also showed that the side-chain amide $NH_2$ of Asn194 is situated within hydrogen-bonding distance to O(3′) of the coenzyme and the α-carboxylate group of the substrate and its CO to the guanidinium group of Arg386 (Arnone et al., 1985; Kamitori et al., 1990; see Figure 1). In this study, the roles of Asn194 in the catalytic process were explored. This residue is invariant in all known sequences of AspATs from higher animals to microorganisms and is thus supposed to play an important role in the enzyme function. Asn194 of *Escherichia coli* AspAT has been replaced by Ala, and the double mutant N194A/Y225F has also been constructed. On the basis of the spectroscopic and kinetic analyses of the mutant enzymes, we propose that the active-site residues, Asn194, Tyr225, and Arg386, function in facilitating the transamination reaction through the hydrogen-bonding network formed among them.

## EXPERIMENTAL PROCEDURES

### *Materials*

*Chemicals.* Enzymes for DNA manipulations were obtained from Takara Shuzo Co., Kyoto, except for the "oligonucleotide-directed in vitro mutagenesis system" (Amersham International plc). MES and HEPES were from Dojin Laboratories, Kumamoto; NADH was from Oriental Yeast Co., Tokyo; porcine mitochondrial malate dehydrogenase was from Boehringer Mannheim. 2-Hydroxyglutarate dehydrogenase from *Peptococcus aerogenes* was generously donated by Dr. T. Yagi of Kochi University.

*Mutant AspATs.* Site-directed mutagenesis was performed on the single-stranded M13 phage DNA, into which the *aspC* gene (Kuramitsu et al., 1985) was subcloned, by the method of Nakamaye and Eckstein (1986). The following mixture of primers was used to direct the mutation Asn194→X: GC-TGC-TGC-CAT-N*N*|G*C|-CCA-ACC-GGT-A (asterisks indicate the mismatches). Mutant phages were identified by examining the nucleotide sequences, and the mutant Asn194 (AAC)→Ala(GCG) was picked up. N194A was employed for analyses because the mutation to Ala was the best choice for a nondisruptive mutation. The double mutant N194A/Y225F was constructed by introducing the mutation Tyr225→Phe into the mutant N194A gene. The primer for the mutagenesis of Tyr225→Phe has been published (Inoue et al., 1991). The nucleotide sequences of the entire region of the mutant *aspC* genes were verified by "Dye Deoxy" method using an Applied Biosystems 373A DNA sequencer. The mutant *aspC* genes were subcloned into pUC19 to generate the expression plasmid pKDHE19 (Kamitori et al., 1987). AspAT-deficient *E. coli* strain TY103 (Yano et al., 1991) carrying this plasmid overproduced the mutant enzyme in the pyridoxine-supplemented culture medium (Yano et al., 1992). The enzymes were purified as described previously (Inoue et al., 1989).

### *Methods*

*Spectroscopic Measurements.* Absorption and corrected excitation spectra of AspAT were recorded with a Hitachi spectrophotometer, Model 557, and spectrofluorometer, Model 850, respectively, at 25 °C. Spectrophotometric titrations of AspATs with maleate were performed under the same conditions as those in the measurements of the absorption spectra (Yano et al., 1991). The pH was measured before and after each spectroscopic measurement.

*Protein Concentrations.* The protein concentrations of AspATs were determined spectrophotometrically using the following molar extinction coefficients at 280 nm (Kuramitsu et al., 1990): $4.7 \times 10^4$ $M^{-1}\cdot cm^{-1}$ for the PLP form of the wild-type and N194A enzymes; $4.6 \times 10^4$ $M^{-1}\cdot cm^{-1}$ for the PLP form of Y225F and N194A/Y225F.

*Measurement of AspAT Activity.* The overall transamination reaction of AspAT

$$\text{L-aspartate} + \text{2-oxoglutarate} \rightleftharpoons \text{oxalacetate} + \text{L-glutamate} \quad (1)$$

consists of the following two half-reactions (Kuramitsu et al., 1990):

$$\text{EL} + \text{Asp} \rightleftharpoons \text{EL·Asp} \rightleftharpoons \text{EM·OA} \rightleftharpoons \text{EM} + \text{OA}$$
$$\text{EL} + \text{Glu} \rightleftharpoons \text{EL·Glu} \rightleftharpoons \text{EM·OG} \rightleftharpoons \text{EM} + \text{OG} \quad (2)$$

where EL and EM are the PLP and PMP form of AspAT, respectively; Asp is L-aspartate; OA is oxalacetate; Glu is L-glutamate; and OG is 2-oxoglutarate. The activity of N194A or N194A/Y225F for the four substrates was examined by measuring the pre-steady-state half-transamination reactions at pH 8.0, 25 °C. The half-reactions were followed by monitoring the absorption changes resulting from the conversion of the PLP form into the PMP form or the PMP form into the PLP form (Inoue et al., 1989; Kuramitsu et al., 1990). The reactions were followed by a Union Giken stopped-flow apparatus, RA-401. Since the reactions of the PLP form of N194A/Y225F with the amino acid substrates were slow, they were followed by a spectrophotometer. The data processing unit of the stopped-flow apparatus was used for the nonlinear regression curve fitting. The experiments were performed in a buffer solution of 50 mM HEPES, 0.1 M KCl, and 10 μM EDTA, pH 8.0.

As the reactions of the PLP form of N194A/Y225F with the amino acid substrates were found to suffer from a large contribution of the reverse reactions, the reactions were performed in the presence of the product-removing system (Yano et al., 1992): NADH–malate dehydrogenase for the reactions with aspartate; NADH–2-hydroxyglutarate dehy-

Table I: Kinetic Parameters of AspATs at pH 8.0 and 25 °C[a]

| | $k_{max}$ ($s^{-1}$) | | | | $K_d$ (mM) | | | |
|---|---|---|---|---|---|---|---|---|
| AspAT | Asp | Glu | OA | OG | Asp | Glu | OA | OG |
| wild-type | 550 | 700 | 800 | 600 | 4.5 | 38 | 0.035 | 1.3 |
| N194A | 200 | 140 | 1000 | 310 | 63 | 330[c] | 2.0 | 50 |
| Y225F[b] | 2.6 | 15 | 6.4 | 51 | 0.083 | 12 | 0.0062 | 0.53 |
| N194A/ Y225F | 0.069 | 0.22 | 3.4 | 42 | 58 | 140[c] | 0.69 | 44 |

[a] Abbreviations for the substrates: Asp, aspartate; Glu, glutamate; OA, oxalacetate; OG, 2-oxoglutarate. [b] Data from Inoue et al. (1991). [c] The substrate concentrations were changed in the range covering the $K_d$ values, except for the reactions of the PLP form of N194A and N194A/Y225F with glutamate where the concentrations were below the $K_d$ values (glutamate concentrations were <100 mM to avoid significant changes in the ionic strength of the reaction mixture).

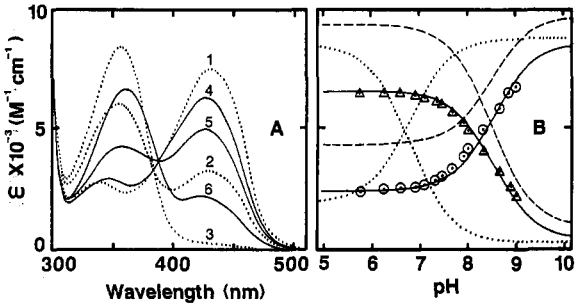



FIGURE 2: pH-dependent spectral changes of the PLP form of wild-type, N194A, and Y225F AspATs at 25 °C. (A) Absorption spectra of wild-type (dotted lines) at pH 5.4 (curve 1), 7.1 (2), and 9.0 (3) and of N194A (solid lines) at pH 7.1 (curve 4), 8.0 (5), and 9.0 (6). The spectra for Y225F (Inoue et al., 1991; Goldberg et al., 1991) are omitted. (B) Titration curves of the apparent molar extinction coefficients at 362 (○) and 426 nm (△) for N194A, at 358 and 430 nm for wild-type, and at 390 and 430 nm for Y225F. The lines are the theoretical curves (see text): solid lines for N194A; broken lines for Y225F; dotted lines for wild-type. For the wild-type and Y225F enzymes, only the theoretical curves are shown. The experiments were performed in a buffer solution of 50 mM MES, HEPES, or borate containing 0.1 M KCl and 10 μM EDTA at a protein concentration of 10–20 μM in a 1-cm cell.

drogenase for those with glutamate. The reactions of the PMP form of N194A/Y225F with the keto acid substrates were performed in the presence of a saturating amount (100 μM) of PMP, because the affinity for PMP was decreased by the double mutation.

## RESULTS

*Catalytic Activity of AspATs.* The kinetic parameters of AspATs for the four substrates are shown in Table I. The replacement of Asn194 by Ala caused only a small reduction (<5-fold) in the $k_{max}$ values but a 10- to 50-fold increase in the $K_d$ values. Y225F has been reported to show a decrease in the rate constants and, more characteristically, a significant decrease in the $K_d$ values for all the substrates (Inoue et al., 1991; Goldberg et al., 1991). The kinetic parameters of the double mutant N194A/Y225F could not be simply interpreted as the sum of the effects of the two single mutations. The $k_{max}$ values of N194A/Y225F for aspartate and glutamate were further decreased by 40- and 70-fold, respectively, compared with those of Y225F, whereas those for the keto acid substrates were similar to the corresponding values of Y225F. The $K_d$ values of N194A/Y225F for the four substrates were similar to those of N194A.

*Spectroscopic Measurements of AspATs.* The absorption spectra of wild-type AspAT at pH 5.4, 7.1, and 9.0 are shown in Figure 2A (curves 1–3). N194A gave pH-dependent spectral changes (curves 4–6) similar to those of the wild-type

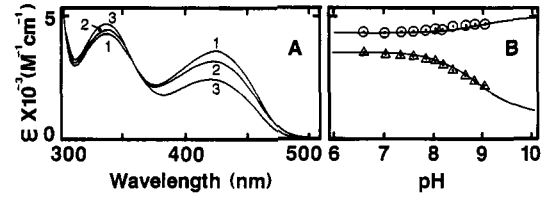



FIGURE 3: pH-dependent spectral changes of the PLP form of N194A/Y225F AspAT at 25 °C. (A) Absorption spectra at pH 6.6 (curve 1), 8.0 (2), and 8.9 (3). (B) Plot of the apparent molar extinction coefficients at 338 (○) and 425 nm (△) as a function of pH. The solid lines indicate the theoretical curves (see text).

enzyme: absorption maxima at 426 nm in an acidic pH region and at 362 nm in an alkaline pH region with an isosbestic point at 388 nm. Y225F gave spectral properties essentially similar to those of N194A: absorption maxima at 430 and 390 nm and an isosbestic point at 403 nm (Inoue et al., 1991; Goldberg et al., 1991), although the reason for the red shift of the 360-nm band to 390 nm is not yet known. These pH-dependent spectral changes have been known to reflect the ionization state of the imine nitrogen of the internal aldimine bond formed between Lys258 and PLP (Jenkins & Sizer, 1959). Figure 2B shows the plot of the apparent molar extinction coefficient of each spectral band as a function of pH. The data for N194A were in good agreement with the theoretical titration curves of p$K_a$ 8.55 (Figure 2B, solid lines), although the data could not be obtained in the region pH > 9.3 where N194A was unstable. In Figure 2, only the theoretical curves are shown for the wild-type (p$K_a$ 6.80) and Y225F (p$K_a$ 8.50) enzymes (Inoue et al., 1991; Goldberg et al., 1991). The p$K_a$ value of the internal almidine bond was increased by 1.75 or 1.70 units upon the mutation of Asn194→Ala or Tyr225→Phe, respectively. N194A/Y225F showed some anomalous spectral properties: one absorption band around 425 nm was titratable, giving a p$K_a$ value of 8.90, whereas the other around 338 nm showed only small pH-dependent changes and was still dominant in the acidic pH region (Figure 3). The theoretical titration curve of p$K_a$ 8.90 was overlaid tentatively on the experimental data for the 338-nm band (Figure 3B). N194A/Y225F was less stable in the alkaline pH region, and thus the data could not be obtained at pH > 9.0.

The corrected excitation spectra (emission at 525 nm) of the wild-type and mutant enzymes were obtained at various pHs (data not shown). Two spectral bands around 340 and 430 nm were observed for all the enzymes. The ratio of the fluorescence intensity of the two bands was different among the enzymes but did not change with pH. The ratio of the peak heights of 340- and 430-nm bands was about 1:5 for the wild-type enzyme, 1:2 for N194A, 1:3 for Y225F, and 1:1 for N194A/Y225F.

An unprotonated form of the internal aldimine, species A in Scheme I, has an absorption band centered around 360 nm. The protonated form of the internal aldimine is thought to exist in two tautomeric species, B and C, as shown in Scheme I (Metzler et al., 1991); the molecular species B, a ketoenamine form, has an absorption band around 430 nm, and C, an enolimine form, around 340 nm (Kallen et al., 1985). It is known that species B and C are fluorescent, whereas A is not (Shaltiel & Cortijo, 1970). Since B and C are the tautomers, the ratio of the peak heights of the two fluorescence bands did not change with pH. The pH-dependent spectral changes, depicted in Figures 2 and 3, reflect the interconversion between A and [B+C]. Figure 2 shows that a major tautomeric form of the protonated internal aldimine should be species B in the wild-type, Y225F, and N194A AspATs. The spectroscopic measurements could show that the molar ratio of the species

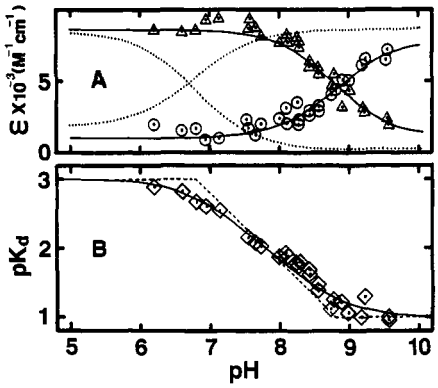
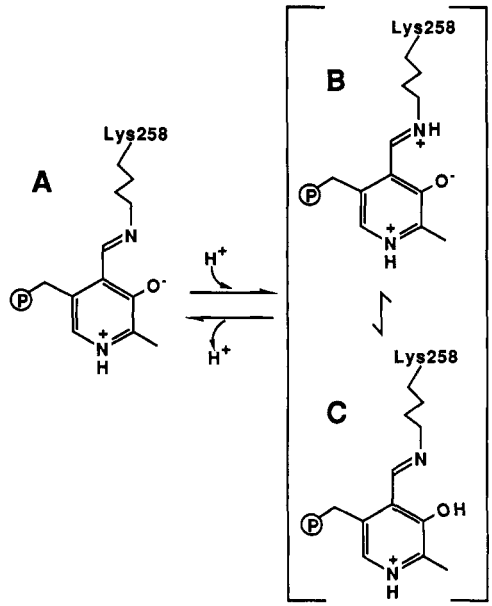



FIGURE 4: Binding of maleate to wild-type AspAT at various pHs at 25 °C. (A) The apparent molar extinction coefficients at 358 (○) and 430 nm (△) in the presence of a saturating amount of maleate were extrapolated and plotted against pH. The data fit to the theoretical curves (solid lines) of p$K_a$ 8.80. The dotted lines are the titration curves of p$K_a$ 6.80 in the absence of maleate shown in Figure 2B. (B) Plot relating the apparent dissociation constant ($K_d$) for maleate to pH. The solid line is a theoretical curve constructed according to Scheme II (see text for details). The dotted line is the additional line indicating each parameter. The experiments were performed in the buffer solutions as described in the legend under Figure 2, at a protein concentration of 10–20 μM.

Scheme I




C to B is increased upon the mutations, and is particularly large upon the double mutation, although the exact molar ratio of C to B could not be determined by this method.

*Binding of Maleate at Various pHs.* Maleate binds noncovalently to the active site of AspAT, simulating the substrate-binding step which precedes a transaldimination step. Upon binding of maleate to the PLP form of AspAT, the following spectral changes are observed: the 430-nm absorption band increases and the 360-nm band decreases. These spectral changes have been known to result from an increase in the p$K_a$ of the imine nitrogen of the internal aldimine bond. The dissociation constant, $K_d$ value, for maleate was determined by monitoring the spectral changes (Fasella et al., 1966; Fonda & Johnson, 1970).

The binding of maleate was examined for the wild-type enzyme in the pH range 6–10 (Figure 4). The apparent molar extinction coefficients of the two spectral bands in the fully maleate-bound enzyme were obtained by extrapolation to a saturating amount of maleate and plotted against pH (Figure 4A). The data fit the theoretical titration curves of p$K_a$ 8.80,

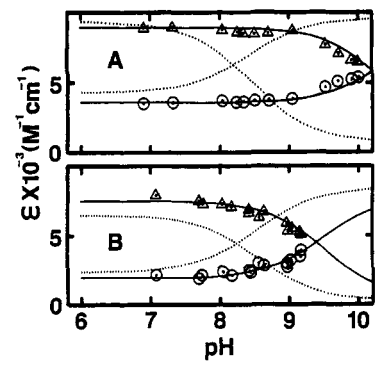



FIGURE 5: Titration curves of the apparent molar extinction coefficients at 390 (○) and 430 nm (△) for Y225F (A), and at 362 (○) and 426 nm (△) for N194A (B) in the presence of a saturating amount of maleate. The dotted and solid lines are the theoretical curves (see text) in the absence and presence of maleate, respectively.

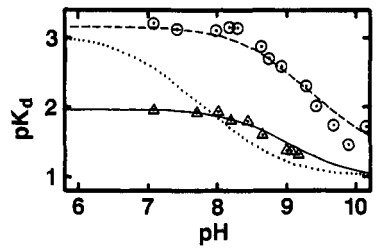
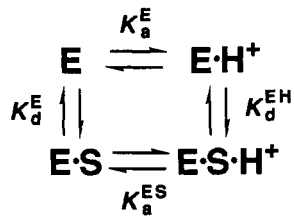



FIGURE 6: Plot of p$K_d$ for maleate against pH for Y225F (○) and N194A (△). The broken and solid lines are the theoretical curves (see text) for Y225F and N194A, respectively, and the dotted line is that for the wild-type enzyme.

Scheme II




showing that the p$K_a$ of the internal aldimine bond of the wild-type enzyme increases from 6.80 to 8.80 upon binding maleate. The plot of the p$K_d$ (–log $K_d$) value for maleate against pH (Figure 4B) implies that the protonation of some ionizing group(s) favors the binding of maleate. Experimental data were analyzed using the equation:

$$pK_d = -\log K_d = -\log \{([H^+] + K_a^E)/([H^+] + K_a^{ES})\} - \log K_d^{EH} \quad (3)$$

This equation is constructed according to a simple thermodynamic cycle shown in Scheme II where only one ionizing group of the enzyme affects the binding of maleate. $K_a^E$ or $K_a^{ES}$ is the acid dissociation constant for the free or maleate-bound enzyme, respectively, and $K_d^E$ or $K_d^{EH}$ is the dissociation constant for maleate of the unprotonated or protonated form of the enzyme, respectively. The experimental data in Figure 4B were in good agreement with theoretical curve constructed using p$K_a^E$ = 6.80, p$K_a^{ES}$ = 8.80, and p$K_d^{EH}$ = 3.0.

The same analyses were performed on the mutant enzymes, and the results are shown in Figures 5 and 6. The p$K_a$ of the internal aldimine bond of Y225F increased from 8.50 to 10.50 upon binding of maleate (Figure 5A), whereas that of N194A increased from 8.55 to 9.55 (Figure 5B). The pH–p$K_d$ plot gave a best fit to the theoretical curve constructed using the following parameters (Figure 6): p$K_a^E$ = 8.50, p$K_a^{ES}$ = 10.50, and p$K_d^{EH}$ = 3.0 for Y225F; p$K_a^E$ = 8.55, p$K_a^{ES}$ = 9.55, and

$pK_d^{EH}$ = 2.0 for N194A. The perturbations in the $pK_a$ and $pK_d$ values for N194A were half of those observed in the wild-type and Y225F enzymes. It should also be noted that the pH–$pK_d$ curve of N194A shifted not only to the right side but also "down" from that of the wild-type enzyme. The double mutant, N194A/Y225F, was also examined (data not shown), but reliable parameters could not be obtained mainly because N194A/Y225F was unstable in the region pH > 9.0. The data obtained below pH 9.0 were not useful for curve fitting to give the value for $K_a^{ES}$ and $K_d^E$. The only reliable parameter obtained was $K_d^{EH} = 3.0 \times 10^{-2}$ M, which was slightly larger than that of N194A.

## DISCUSSION

N194A and Y225F gave pH-dependent spectral changes essentially similar to those of the wild-type enzyme (Figure 2). These spectral changes are attributable to the ionization state of the internal aldimine bond. The pH titration of the absorption spectra showed that the mutation Asn194→Ala or Tyr225→Phe increased the $pK_a$ of the internal aldimine bond by 1.75 or 1.70 units, respectively. These perturbations of the $pK_a$ values would support the idea that hydrogen bonds are actually formed between O(3′) of PLP and Asn194 or Tyr225. When the hydrogen bond to 3′-$O^-$ of PLP from Asn194 or Tyr225 is deleted by the mutation, electrons surrounding the oxygen atom would flow into the $\pi$-electron system which extends from the pyridine ring of PLP to the internal aldimine bond. This would result in the higher electron density around the imine nitrogen of the internal aldimine bond, facilitating its protonation. The finding that the increases in the $pK_a$ value were similar between the two mutations implies that Asn194 and Tyr225 affect the electron distribution within the PLP molecule to the same extent. In other words, the strength of the two hydrogen bonds is nearly equal.

In contrast, the absorption spectra of N194A/Y225F showed some anomalous spectral properties (Figure 3), which could be explained through measurement of the fluorescence excitation spectra at various pHs. The 338-nm absorption band persisting in the acidic pH region could be attributed to an enolimine form (species C in Scheme I), and the enolimine form is a predominant tautomeric form of the protonated PLP in N194A/Y225F. This situation would be expected when both the two hydrogen-bonding groups to 3′-$O^-$ of PLP are removed by the double mutation. The apparent molar extinction coefficient at 338 nm changed only slighly with pH, probably because, in the alkaline pH region, the increase of species A is compensated by the decrease of species C. According to these speculations, the absorption changes at 338 nm should also fit the titration curve of $pK_a$ 8.90, which could not be confirmed because the changes were too small to be examined by curve fitting (Figure 3B). The 430-nm band of Y225F and the 425-nm band of N194/Y225F did not approach zero at high pH (Figures 2B and 3B), which cannot be explained by Scheme I. The reason for this finding is not yet known.

In contrast to Y225F, in which both the $k_{max}$ and $K_d$ values were decreased, the $k_{max}$ values were not largely affected but the $K_d$ values were considerably increased for all the substrates by the mutation Asn194→Ala. The double mutation led to a pronounced decrease in the $k_{max}$ values for the reactions of the PLP form of the enzyme with the amino acid substrates, while the influence was much smaller on the reactions of the PMP form of the enzyme with the keto acid substrates (Table I). The reaction of N194A with [2-$^2$H]aspartate showed a similar deuterium isotope effect to that for the wild-type enzyme ($k^H/k^D$ = 2.2; Kuramitsu et al., 1990). This indicates that the abstraction of the $\alpha$-proton from the amino-acid substrate, which is partially rate-determining in the reaction of the wild-type enzyme, is also rate-determining in the reaction of N194A. On the other hand, it has been reported that the abstraction of the $\alpha$-proton is not rate-determining in the reaction of Y225F (Kirsch et al., 1990). When the rate-determining step is different among the reactions catalyzed by the wild-type and mutant enzymes, kinetic properties of each mutant AspAT cannot be discussed in detail by a simple comparison of the parameters shown in Table I.

Crystallographic studies show that the side-chain amide $NH_2$ of Asn194 can form a hydrogen bond to the $\alpha$-carboxylate group of the substrate and that the side-chain amide CO of this residue is the sole group which can form a hydrogen bond to the side chain of Arg386 except for some water molecules (K. Hirotsu, personal communication). The substrates bind to AspAT through the electrostatic interactions of the two carboxylate groups with the two arginine residues, Arg292 and Arg386. Thus, the pronounced increase in the $K_d$ values of N194A for the substrates (Table I) might be due to loss of the hydrogen bonds between Asn194 and the substrate and/or Asn194 and Arg 386. The reaction of AspAT with maleate is known to stop at the formation of a Michaelis complex and thus would comprise only the substrate-binding step of AspAT. A similar type of analogue, succinate, gave results similar to those of maleate in the experiments with the wild-type enzyme (not shown). pH dependency of maleate-binding could be explained by Scheme II, and the results shown in Figures 4–6 clearly indicate that an ionizing group affecting the binding of maleate is the imine nitrogen of the internal aldimine bond. The binding of maleate to wild-type or Y225F AspAT increased the $pK_a$ of the internal aldimine bond by 2.0 pH units, whereas it increased by 1.0 unit in N194A. The reasons for these perturbations of the $pK_a$ upon binding of maleate are not fully known, but the following situations would participate to some extent: (i) compensation of the positive charges of the two arginine residues, Arg292 and Arg386, by the two carboxylate groups of maleate; and (ii) changes in the electrostatic environments around the active site caused by the conformational changes of AspAT that are induced by the binding of maleate.

The pH–$pK_d$ curve for Y225F shifted only to the right side from that of the wild-type enzyme (Figure 6, broken line), implying that the mutation Tyr225→Phe affects the binding of maleate only through perturbation of the $pK_a$ of the internal aldimine bond without causing any significant conformational changes in the active site. It was suggested previously (Goldberg et al., 1991) and confirmed by X-ray crystallography (Inoue et al., 1991) that the active-site conformation is almost identical between wild-type and Y225F AspATs. In contrast, the pH–$pK_d$ curve for N194A (Figure 6, solid line) showed that the affinity for maleate of the fully protonated form of this mutant enzyme is considerably low as compared with wild-type and Y225F AspATs. This indicates that some factors other than the perturbation of the $pK_a$ of the internal aldimine bond hamper the binding of maleate to N194A. One possible factor is the loss of the hydrogen bond between Asn194 and the ligand carboxylate. The other is some conformational change resulting from the loss of the hydrogen bond between Asn194 and Arg386: Asn194 might function in holding the side chain of Arg386 in a proper position for the binding of the carboxylate group of the substrate. To confirm this hypothesis, X-ray crystallographic studies are now in progress on the mutant enzymes, N194A and N194A/Y225F.

The proper regulation of the electron distribution within the coenzyme molecule during the catalytic process must be essential for the efficient catalysis of AspAT. We propose how Asn194 and Tyr225 contribute to the regulation of the electron distribution through hydrogen-bonding to O(3′) of the coenzyme. The mutations Tyr225→Phe and Asn194→Ala increased the p$K_a$ of the internal aldimine bond to a similar extent, by 1.70 and 1.75 units (6.80 → 8.50 and 6.80 → 8.55), respectively, in the unliganded enzyme. Thus, these two residues participate together in lowering the p$K_a$ of the internal aldimine bond, stabilizing the unprotonated active form of the aldimine bond in the physiological pH region. When maleate binds to the enzyme, however, the perturbations of the p$K_a$ were 1.70 and 0.75 units (8.80 → 10.50 and 8.80 → 9.55) for the mutations Tyr225→Phe and Asn194→Ala, respectively. These results imply that, in the liganded enzyme, the effect of the mutation on the electron distribution within the PLP molecule was smaller for Asn194→Ala than for Tyr225→Phe. In other words, the binding of maleate weakens the hydrogen bond between Asn194 and O(3′) of PLP. This may be the reason for the finding that the catalytic activity was not greatly damaged by the mutation Asn194→Ala. This smaller effect of Asn194 on the electron distribution in the liganded enzyme would be explained by the following assumption: The electrophilicity of the side-chain amide $NH_2$ of Asn194 is intensified by hydrogen-bonding of its CO to the positively charged guanidinium group of Arg386 in the unliganded enzyme. When maleate binds to AspAT, the positive charge of Arg386 would be canceled by the interaction with the carboxylate group of maleate, resulting in the reduction of the electron-withdrawing ability of Asn194 and its influence on the electron distribution within the PLP molecule.

Further support of this idea was obtained by constructing the mutant enzyme Arg386→Leu. The p$K_a$ of the internal aldimine bond was 7.38. The corresponding p$K_a$ of the mutant enzyme Arg292→Leu was 6.95 (Hayashi et al., 1989). Arg386 and Arg292 are positioned at an almost equal distance (about 8 Å) from the imine nitrogen of the internal aldimine bond. Therefore, Arg386 influences the electron distribution within PLP through some mechanism(s) other than the "direct" electrostatic interaction. This finding would support the idea that Arg386 decreases the p$K_a$ through the hydrogen-bonding network to O(3′) of PLP via Asn194.

*Roles of Asn194.* Asn194 would function in two ways. First, Asn194 lowers the p$K_a$ of the imine nitrogen of the internal aldimine bond. This may facilitate a transaldimination step, a reaction step following Michaelis complex formation, because the unprotonated aldimine bond would be favorable for this reaction step (Arnone et al., 1985; Kirsch et al., 1984). Second, Asn194 functions in substrate binding through (i) hydrogen-bonding to the substrate carboxylate and/or (ii) holding the side chain of Arg386 in a position suitable for the binding of the substrate. The function of Asn194 to lower the p$K_a$ of the internal aldimine bond might be intensified by Arg386 through the hydrogen-bonding network and weakened upon binding of the substrate and the concomitant compensation of the positive charge of Arg386. After formation of the coenzyme–substrate conjugate, the regulation of the catalytic process via O(3′) of the coenzyme would be mainly made by Tyr225.

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