Conformational Change in Aspartate Aminotransferase on Substrate Binding Induces Strain in the Catalytic Group and Enhances Catalysis*

Received for publication, September 9, 2002, and in revised form, November 25, 2002
Published, JBC Papers in Press, December 17, 2002, DOI 10.1074/jbc.M209235200

Hideyuki Hayashi, Hiroyuki Mizuguchi, Ikuko Miyahara, Yoshitaka Nakajima, Ken Hirotsu, and Hiroyuki Kagamiyama

Aspartate aminotransferase has been known to undergo a significant conformational change, in which the small domain approaches the large domain, and the residues at the entrance of the active site pack together, on binding of substrates. Accompanying this conformational change is a two-unit increase in the pKₐ of the pyridoxal 5’-phosphate-Lys²⁵⁸ aldimine, which has been proposed to enhance catalysis. To elucidate how the conformational change is coupled to the shift in the aldimine pKₐ and how these changes are involved in catalysis, we analyzed structurally and kinetically an enzyme in which Val₃⁹ located at both the domain interface and the entrance of the active site was replaced with a bulkier residue, Phe. The V₃⁹F mutant enzyme showed a more open conformation, and the aldimine pKₐ was lowered by 0.7 unit compared with the wild-type enzyme. When Asn¹₉⁴ had been replaced by Ala in advance, the V₃⁹F mutation did not decrease the aldimine pKₐ, showing that the domain rotation controls the aldimine pKₐ via the Arg³⁸⁶-Asn¹₉⁴-pyridoxal 5’-phosphate linkage system. The maleate-bound V₃⁹F enzyme showed the aldimine pKₐ, 0.9 unit lower than that of the maleate-bound wild-type enzyme. However, the positions of maleate, Asn¹₉⁴, and Arg³⁸⁶ were superimposable between the mutant and the wild-type enzymes; therefore, the domain rotation was not the cause of the lowered aldimine pKₐ value. The maleate-bound V₃⁹F enzyme showed an altered side-chain packing pattern in the 37–39 region, and the lack of repulsion between Gly³⁸⁸ carboxyl O and Tyr²²⁵ O₆W seemed to be the cause of the reduced pKₐ value. Kinetic analysis suggested that the repulsion increases the free energy level of the Michaelis complex and promotes the catalytic reaction.

Aspartate aminotransferase (aspartate, 2-oxoglutarate aminotransferase, EC 2.6.1.1; AspAT) is a pyridoxal 5’-phosphate (PLP)-dependent enzyme and catalyzes the reversible transfer of the amino group of aspartate to 2-oxoglutarate by the following Ping Pong Bi Bi mechanism (1), in which E-PLP and E-PMP denote the PLP form (see Equation 1) and the pyridoxamine 5’-phosphate (PMP) form (see Equation 2) of the enzyme, respectively.

\[
\text{aspartate} + \text{E-PLP} \rightleftharpoons \text{oxalacetate} + \text{E-PMP} \quad (\text{Eq. 1})
\]

\[
2\text{-oxoglutarate} + \text{E-PMP} \rightleftharpoons \text{glutamate} + \text{E-PLP} \quad (\text{Eq. 2})
\]

The homodimeric structures have been solved for cytosolic (2), mitochondrial (3–5), and Escherichia coli (6, 7) enzymes. Each subunit is composed of large and small domains, and the active site containing the PLP-Lys²⁵⁸ aldimine is located between the two domains. The ω-carboxylate group of the dicarboxylic substrates binds to Arg³⁸⁶ located at the small domain and the ω-carboxylate group to Arg²⁹² located at the large domain. An asterisk indicates that the residue comes from the neighboring subunit. On the basis of structural, steady-state (1), and transient (8, 9) kinetic studies, the reaction mechanism of AspAT has been proposed (10, 11) and refined (9) (see Scheme I).

The PLP-Lys²⁵⁸ aldimine (internal aldimine) of the unliganded AspAT has an imine pKₐ value of ~8.5, which is strikingly lower than that of the PLP-amide aldimines with protonated pyridine N (~11; see Ref 13). We have shown that the principle factor that decreases the aldimine pKₐ value of AspAT is the imine-pyridine torsion of the PLP-Lys²⁵⁸ aldimine (as represented by the torsion angle C3–C4–C4’–N₆C, expressed as χ, in the panel of Scheme I), rather than the historically accepted electrostatic effect of the neighboring positive charges of Arg²⁹² and Arg³⁸⁶. The torsion of the aldimine, the angle of which spans the range from ~35° (protonated aldimine) (2) to ~90° (unprotonated aldimine) (7), causes a decrease of 3 units in the aldimine pKₐ (14), whereas the positive charges of the arginine residues decreases the pKₐ by only 0.7 unit (15). The catalytic significance of the aldimine torsion is considered to be that it increases the energy level of the protonated form of the aldimine in the unliganded enzyme, thereby decreasing the

* This work was supported in part by Scientific Research on Priority Areas 13125101 (to H. H. and K. H.) and by Grant-in-aid for Scientific Research 13880897 (to H. H.) from the Japan Society for the Promotion of Science. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† To whom correspondence should be sent: Dept. of Biochemistry, Osaka Medical College, 2-7 Daigakumachi, Takatsuki 569-8686, Japan. Tel: 81-72-683-1221 ext. 2645; Fax: 81-72-684-6516; E-mail: med001@art.osaka-med.ac.jp

‡ From the Department of Biochemistry, Osaka Medical College, Takatsuki 569-8686 and †Department of Chemistry, Faculty of Science, Osaka City University, Sumiyoshi-ku, Osaka 558-8585, Japan

§ Department of Biochemistry, Osaka Medical College, Takatsuki 569-8686 and ¶Department of Biochemistry, Osaka City University, Sumiyoshi-ku, Osaka 558-8585, Japan

1 The abbreviations used are: AspAT, aspartate aminotransferase (aspartate, 2-oxoglutarate aminotransferase, EC 2.6.1.1); PLP, pyridoxal 5’-phosphate; PMP, pyridoxamine 5’-phosphate; V₃⁹F AspAT, mutant AspAT in which the residue Val₃⁹ has been replaced with a phenylalanine residue (other mutant AspATs are expressed in the same way); WT, wild-type; MES, 4-morpholinethanesulfonic acid; TAPS, 3-[2-hydroxy-1,1-bis(hydroxymethyl)methyl]amino]-1-propanesulfonic acid.

This paper is available on line at http://www.jbc.org
free energy gap between the starting state (the unliganded enzyme plus the free substrate) and the transition state leading to the first irreversible step, i.e. the release of the product oxaloacetate (see Scheme I). This decrease in the free energy gap is estimated to be 16 kJ mol\(^{-1}\) and increases the \(k_{\text{cat}}/K_m\) value for amino acid substrates by 10\(^{10}\)-fold (14).

The intrinsic \(pK_a\) of the external aldimine, i.e. the aldimine of PLP and the substrate aspartate, has been estimated to be \(>11\) (14). The elevated \(pK_a\) is considered to be caused by the aldimine being fixed to a near planar conformation (\(\chi = -25^\circ\)) (7) and the deprotonated form being destabilized (14). Accordingly, the external aldimine reduces the fraction of the dead end species \(H^+E_1S\). The shift from \(H^+E_1S\) to \(E_2H^+S\) is favorable for the progress of the 1,3-prototropic shift (\(E_2H^+S \rightarrow E_2H^+S\rightarrow K\) in Scheme I), the first rate-determining step of the entire catalytic reaction (16).

In comparison to the case of the external aldimine, less is understood about the mechanism of controlling the aldimine \(pK_a\) and its role in catalysis in the Michaelis complex, where PLP still forms the aldimine with Lys\(^{258}\) (6). The intrinsic \(pK_a\) of the aldimine has been estimated to be 8.8, by spectroscopic analysis of the maleate-bound enzyme, and it has been explained that the increase in the \(pK_a\) is because of the electrostatic effect of the anionic ligand (17, 18). However, taking into account the relatively weak electrostatic effect (0.7 pH unit) of Arg\(^{292}\) and Arg\(^{386}\) on the aldimine \(pK_a\) value as described above, we must consider other mechanisms for the upward \(pK_a\) shift in this complex, such as alterations in the torsion angle of the aldimine and the hydrogen bond network surrounding it. Furthermore, although the increase in the aldimine \(pK_a\) in the Michaelis complex has been claimed to accelerate the transaldimination step (\(E_2H^+S\rightarrow K\) in Scheme I) by shifting the equilibrium from \(E_2H^+SH\) to \(E_2H^+S\rightarrow K\) in the Michaelis complex (10), its exact role in catalysis is obscure, because the transaldimination step is not rate-determining at all in the catalytic reaction (16).

To solve these problems associated with the Michaelis complex, we set out to carry out structural and mechanical analysis of the complex. Upon formation of the Michaelis complex, AspAT undergoes a conformational change (2–7), in which the small domain approaches the large domain. Together with the domain rotation, the side chains of the residues aligned at the entrance of the active site are reorganized and correctly packed to form a lid over the active site. These changes are expected to cause alterations in the conformation of the aldimine and the hydrogen-bonding pattern around the aldimine in the Michaelis complex. Therefore, we have chosen the mutation of Val\(^{39}\) (19), which exists at the interface of the large and small domains, to a more bulky residue Phe, with the anticipation of hampering the domain rotation and side-chain packing. This mutant enzyme was analyzed together with the wild-type enzyme, to explore the effect of the substrate-induced conformational change on the important catalytic groups.

**EXPERIMENTAL PROCEDURES**

**Materials**—The medium used for bacterial growth contained 0.5% yeast extract, 1% peptone, and 0.5% NaCl, pH 7.0. All other chemicals were of the highest grade commercially available.

**Site-directed Mutagenesis**—The V39F (19) and N194A (18) mutants of pUC19 containing the \(E.\) coli AspAT gene (aspC) were prepared as described. The double mutant V39F/N194A was constructed using the QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). The V39F plasmid was used as a template. The primers for mutagenesis used were 5′-CAT GGC TGC TGC CAT GCC CCA ACC GGT ATC-3′ and 5′-GAT ACC GGT TGG GGC ATG GCA GCA GCC ATG-3′. The obtained mutant plasmids were expressed as described previously (20).

**Spectroscopic Measurements**—Absorption spectra were measured using a Hitachi U-3300 spectrophotometer (Tokyo, Japan) at 298 K. The buffer solution contained 50 mM buffer components (27) and 0.1 M KCl. The buffer components used were MES-NaOH, HEPES-NaOH, and TAPS-NaOH. The concentration of the AspAT subunit in solution was determined spectrophotometrically using the apparent molar extinction coefficient of \(\varepsilon_{358} = 4.7 \times 10^{4}\) \(\text{M}^{-1}\) cm\(^{-1}\) for the PLP form of the enzyme at 280 nm (20). The \(pK_a\) value of the aldimine N of the PLP-Lys\(^{258}\) Schiff base was calculated by fitting the data as follows in Equation 3.

\[
\varepsilon_{\text{app}} = \varepsilon_p + \frac{\varepsilon_{p} - \varepsilon_{n}}{1 + 10^{pK_a - \text{app}}}
\]

Here, \(\varepsilon_p\) and \(\varepsilon_{\text{app}}\) represent the molar absorptivity of the basic (\(E_p\)) and the acidic (\(E_n\)) forms of the PLP form of the enzyme, respectively, at a fixed wavelength.

**Crystalization of the Mutant Enzymes**—Crystalization of V39F AspAT was performed by the hanging drop vapor diffusion method. A 5-ml drop containing 40 mg of protein/ml, 10 mM potassium phosphate, pH 7.0, 10 \(\mu\)L PLP, and 0.3 mM Na\(_2\) was added to 5 \(\mu\L\) reservoir solution containing 10 mM potassium phosphate, pH 7.0, and 40% saturated ammonium sulfate and was equilibrated against 400 \(\mu\L\) of reservoir solution at 293 K. After 2 days, the drop was seeded with a small crystal of the wild-type (WT) AspAT obtained previously. Crystals with a size (about 0.8 \(\times\) 0.5 \(\times 0.4\) mm) suitable for x-ray experiments were grown for 7 days. Crystals of V39F/N194A AspAT were obtained using the same procedure for V39F AspAT except that the reservoir solution contained 45% saturated ammonium sulfate. Crystals of maleate-bound V39F AspAT were obtained using the same crystallization method using the same reservoir solution as that for V39F AspAT except that 1 \(\mu\L\) of 1 mM maleate was mixed with 5 \(\mu\L\) of enzyme solution, and a small crystal of V39F AspAT was used for seeding. The diffraction data for V39F AspAT were collected on the BL6A station at the Photon Factory, High Energy Accelerator Research Organization, Tsukuba, Japan using an x-ray beam of wavelength 1.0 A at 293 K and Fuji imaging plates with a screenless Weissenberg camera for macromolecular crystallography (21). Data collection was performed using two crystals. The diffraction data for V39F/N194A AspAT and maleate-bound V39F AspAT were collected with a Rigaku R-AXIS Ic image plate detector mounted on a Rigaku RU-200 rotating anode generator operated at 40 kV and 100 mA with monochromatized CuK\(_\alpha\) radiation at room temperature. Each data collection was performed using one crystal. All data were processed and scaled using the programs DENZO and SCALEPACK (22) (see Table I). The diffraction data for V39F and V39F/N194A AspATs showed good isomorphism with those of WT AspAT; therefore, the coordinates of 1ARS (7) were used as the first model for refinement. The crystals of maleate-bound V39F AspAT were isomorphous with those of WT AspAT and the 2-methylaspartate complex; thus, the coordinates of 1ART (7) were used as the first model for refinement. Simulated annealing and several subsequent rounds of least squares refinement using X-PLOR (23) were carried out. After each round of refinement, the model obtained was refitted to an electron density map using program O (24). The mutated residues and solvent molecules were modeled on the basis of \(2F_o - F\) and \(F_o - F\) electron density maps. The solvent molecules whose thermal factors were above 100.0 \(\AA^2\) after refinement were removed from the model. The quality of the model for each structure was evaluated using PROCHECK (25). Table I contains a summary of the refinement statistics. The mean positional discrepancy factors from the Luzzati plot was 0.32 A for both V39F and WT AspATs.

**RESULTS**

**Aldimine \(pK_a\) Values**—The absorption spectra of the V39F mutant AspAT (V39F) show an absorption maximum at 358 nm in the alkaline pH region and at 430 nm in the acidic pH region (data not shown), which are identical to those of the WT AspAT and correspond to the unprotonated (\(E_p\); see Scheme I) and protonated (\(E_n^+\); see Scheme I) structures of the PLP-Lys\(^{258}\) aldimine, respectively (13, 17). The pH dependence of the apparent molar absorptivity at 430 nm is shown in Fig. 1. The aldimine \(pK_a\) value of V39F is obtained by fitting the data to Equation 3 to be 6.1, which is 0.7 unit lower than that of WT (6.8; see Refs. 9 and 27). Because the aldimine \(pK_a\) value is closely related to the presence of the hydrogen bond between PLP O3′ and Asn\(^{194}\) (14, 15), the effect of the V39F mutation
was studied on the N194A mutant AspAT (Fig. 1). The aldimine pKₐ value of the V39F/N194A double mutant AspAT is 8.7, essentially identical to that of the N194A mutant AspAT (pKₐ = 8.6; see Ref. 18).

The binding of maleate to WT causes an increase in the 430-nm absorption band with a concomitant decrease in the 360-nm band, reflecting the increase in the aldimine pKₐ value (17). Similar spectral changes are observed for the binding of maleate to V39F (data not shown). The aldimine pKₐ value of the maleate-bound V39F (V39F/maleate) is 7.9 (Fig. 1), which is 0.9 unit lower than the value 8.8 (18) of the maleate-bound WT (WT/maleate). The aldimine pKₐ values of WT and mutant AspATs in the presence and absence of maleate are summarized in Table II, together with the aldimine torsion angle (χ) obtained from the crystallographic analysis.

Structure of V39F—The structure of V39F is shown in Fig. 2A, in comparison with the structure of WT. Most of the small domain and a part of the large domain are shown. The large domains of the two enzymes are almost superimposable. However, the position of the small domain of V39F deviates from that of WT. The deviations are most pronounced at the N-terminal part (Met5-Pro48) of the small domain and the two helices at the C terminus. The remaining part of the small domain, containing the last part of the long helix and the following helix, shows little deviation.

Val₁⁰⁰ is one of the residues that locate at the entrance of the active site (6) (Fig. 2A). In V39F, the space between the Tyr⁷⁰* and Asn⁶⁹* side chains cannot accommodate the bulky side chain of Phe₃⁹, and the phenyl ring is excluded from the space. The phenyl ring has a direct steric effect on the side chain of Ile₃⁷ and pushes it outward from the active site. This displacement causes the movement of the main chain of the N-terminal part of the small domain outward of the active site, providing a more open conformation of V39F as described above (Fig. 2A). Together with the rotation of the small domain induced by the

| Table I Data collection and refinement statistics |
|-----------------------------------------------|
|                      V39F | V39F · maleate | V39F/N194A |
|------------------------|----------------|------------|
| Data collection        |                |            |
| Space group            | C222₁          | C222₁      | C222₁      |
| Cell parameters (Å)    |                |            |
| a                      | 156.1          | 158.5      | 155.0      |
| b                      | 87.8           | 85.8       | 88.7       |
| c                      | 80.5           | 79.6       | 80.3       |
| Temperature (K)        | 293            | 298        | 298        |
| Wavelength (Å)         | 1.00           | 1.542      | 1.542      |
| Resolution range (Å)   | 10.2 – 2.2     | 10.2 – 2.2 | 10.2 – 2.2 |
| No. of unique reflections | 26,929        | 25,797     | 26,903     |
|Completeness (%)        | 95.7           | 93.4       | 95.5       |
| R_merge (%)            | 6.8            | 7.7        | 7.3        |
| R_factor               | 20.5           | 19.6       | 20.2       |
| R_free                 | 25.8           | 24.7       | 25.9       |
| Deviations             |                |            |
| Bond length (Å)        | 0.008          | 0.007      | 0.007      |
| Bond angles (deg)      | 1.4            | 1.4        | 1.4        |
| Average B factor       |                |            |
| Main chains (Å²)       | 27.3           | 24.4       | 24.4       |
| Side chains (Å²)       | 31.3           | 27.7       | 29.2       |
| Hetero atoms (Å²)      | 17.8           | 16.4       | 15.9       |
| Waters (Å²)            | 35.1           | 34.8       | 33.4       |

a R_merge = Σ[Iₐobs]−[Iₐcalc]/ΣIₐobs, where I = observed intensity, and [I] = average intensity for multiple measurements.
The pK<sub>a</sub> values of the PLP-Lys<sup>358</sup> aldimine are obtained by fitting the 430-nm absorbance at various pH values to Equation 3. The C3-C4-C4'-N<sub>3</sub> torsion angles are obtained from the crystallographic data.

| Enzyme | Aldimine pK<sub>a</sub> | Torsion angle | Species | PDB |
|--------|------------------------|---------------|---------|-----|
| WT     | 6.8                    | 90            | E<sub>1</sub> | 1ARS |
| V39F   | 6.1                    | 101           | E<sub>1</sub> | 1IX6 |
| N194A  | 8.6                    | —             | E<sub>1</sub> | 1X8  |
| V39F/N194A | 8.7               | 38            | E<sub>H</sub> | 1IX8 |
| WT + maleate | 8.3<sup>a</sup> | 18            | E<sub>H</sub> | 1ASM |
| V39F + maleate | 7.9               | 55            | E<sub>H</sub> | 1X7  |

<sup>a</sup> No crystallographic data are available for N194A AspAT.

<sup>b</sup> Taken from Ref. 18.

V39F mutation, the side chain of Arg<sup>386</sup> is moved by 0.63 Å outward from the active site (see Fig. 2B and Fig. 4A). This movement caused a small shift (0.33 Å) in the position of the side chain of Asn<sup>194</sup>, which is hydrogen-bonded to the guanidinium group of Arg<sup>386</sup>. The entire structure of the V39F/N194A double mutant AspAT is essentially identical to that of V39F (data not shown; see PDB 1IX6).

Structure of the Maleate-bound V39F—As in the case of WT, maleate forms a complex with V39F with its two carboxylate groups bound to Arg<sup>292*</sup> and Arg<sup>386</sup>, causing a significant rotation of the small domain (Fig. 3). Although the improper packing of the side chains around Phe<sup>39</sup> hampers the completion of the small domain as observed for WT, the difference in the structure around Arg<sup>386</sup> is not significant between V39F-maleate and WT-maleate. As a result, the guanidinium groups bound to Arg<sup>386</sup>, the entire structure of the V39F/N194A; V39F in the presence of a saturating concentration of maleate. The theoretical lines are drawn using Equation 3. The ε<sub>K</sub> value is set to zero, because the unprotonated aldimine has no absorbance over 400 nm (13).

Reaction of V39F with 2-Methylaspartate—As in the case of WT, the pK<sub>a</sub>-values of dissociation groups at the active site of enzymes are generally considered to be regulated mainly by the polarity of the microenvironment and the electrostatic effects (including that of hydrogen bonds) of the surrounding residues. In this study on AspAT, we observed that the conformational change in the enzyme protein significantly affects the pK<sub>a</sub>-values for its amino acid substrates, aspartate and glutamate (19). To analyze the effect of the V39F mutation on the elementary steps, we studied the kinetic isotope effect of the V39F/N194A double mutant AspAT (8), which stops the catalytic reaction at the external aldime (8), we assigned the two species to be the Michaelis complex (MC) and the external aldime (EA), as shown in Equation 4 (14).

\[ K_a^E \overset{E+S \rightarrow MC \rightarrow EA}{\rightarrow} \frac{k_{cat}}{k_{-1}} \]  

The apparent rate constant for the spectral change (k<sub>app</sub>) is fitted to the equation k<sub>app</sub> = (([MeAsp]/[K<sub>d</sub> + [MeAsp]]) k<sub>-1</sub> + k<sub>-1</sub>) and the parameters are obtained as follows (the value of WT is in parenthesis, taken from Ref. 14): K<sub>d</sub> = 12 ± 4 mM (1.4 ± 0.1 mM); k<sub>-1</sub> = 27 ± 4 s<sup>-1</sup> (200 ± 4 s<sup>-1</sup>); k<sub>-1</sub> = 130 ± 5 s<sup>-1</sup> (110 ± 4 s<sup>-1</sup>).

**DISCUSSION**

Protein Conformation and the Aldimine pK<sub>a</sub>—The pK<sub>a</sub>-values of dissociation groups at the active site of enzymes are generally considered to be regulated mainly by the polarity of the microenvironment and the electrostatic effects (including that of hydrogen bonds) of the surrounding residues. In this study on AspAT, we observed that the conformational change in the enzyme protein significantly affects the pK<sub>a</sub> of the PLP-Lys<sup>358</sup> aldime at the active site. The V39F mutation causes movement of the small domain outward from the active site (Fig. 2A). Together with this domain rotation, Arg<sup>386</sup> of the small domain residue located near the PLP-Lys<sup>358</sup> aldime, moves away from the aldime. Contrary to the expectation that the electrostatic effect of the positive charges of Arg<sup>386</sup> on the aldime would be attenuated in V39F, the aldime pK<sub>a</sub> of V39F is 0.7 unit lower than that of WT (see Fig. 1 and Table II). If Asn<sup>194</sup>, which bridges PLP O<sup>3*</sup> and Arg<sup>386</sup>, is removed in advance, the V39F mutation does not decrease the aldime pK<sub>a</sub> (see Fig. 1 and Table II), although V39F/N194A shows an essentially identical conformation as V39F (comparison of 1IX6 and 1X8). These results indicate that the effect of the displacement of Arg<sup>386</sup> on the V39F mutation is transmitted through Asn<sup>194</sup> to the PLP-Lys<sup>358</sup> aldime and affects its pK<sub>a</sub>.
sible mechanism for the pK_a shift is that the torsion angle \(\chi\) of PLP-Lys^{258}, which is the main factor controlling the aldimine pK_a (14), is altered. However, because the resolution of V39F AspAT is not high (2.2 Å), the change in \(\chi\) is not assessed structurally. The modeled structure of V39F AspAT (1IX6) has a \(\chi\) value only 10° larger than that of WT AspAT (1ARS); the difference is not significant. However, it should be noted that what determines the internal aldimine pK_a value is the \(\chi\) value of the protonated PLP aldimine (14). This structure is not obtained under the crystallization conditions used for 1ARS and 1IX6 (pH 7). A model study indicates that the direction of the movement of the residues Arg^{292}, Arg^{386}, and Asn^{194} increases the \(\chi\) value of the protonated internal aldimine, by pulling the O3' atom to the re face of the aldimine. This is consistent with the decreased pK_a value of the aldimine.

**Maleate Binding and the Conformational Change**—The above discussion shows the importance of the PLP-Asn^{194}, Arg^{386} linkage system that controls the aldimine pK_a. The structure of V39F-maleate shows that Arg^{292}, Arg^{386}, Asn^{194}, and maleate are almost superimposable on those of WT-maleate. Therefore, the contribution of the PLP-Asn^{194}, Arg^{386} system to the control of the aldimine pK_a is the same between V39F-maleate and WT-maleate. Accordingly, the 0.9-unit decrease in the pK_a of V39F-maleate should be ascribed to alterations in the other part of the structure. The most remarkable change is seen for residues 37–39. In V39F-maleate, because of the steric hindrance of the Phe^{39} side chain, Gly^{38} carbonyl O does not approach Tyr^{225} O (Fig. 4B). Assuming that this change between V39F-maleate and WT-maleate is the cause of the lowered aldimine pK_a value of V39F-maleate, we can interpret the results as follows. In the unprotonated aldimine, Tyr^{225} O forms a hydrogen bond with PLP O3' (2.90 Å for 1ARS and 2.86 Å for 1IX6), in which Tyr^{225} is the hydrogen donor. The lone pair electrons of Tyr^{225} O are pointed toward the Gly^{38} main chain. On the other hand, if the aldimine is protonated, the proton is expected to be shared by PLP O3', imine N, and Tyr^{225} O, and the hydrogen atom of the Tyr^{225} OH is pointed toward the Gly^{38} main chain. Therefore, if the
Fig. 3. Superimposed structures (parallel stereo view) of WT maleate and V39F maleate. The large domains are colored gray (WT) and light orange (V39F), and the small domains are light gray (WT) and yellow (V39F). The side chains of the residues Ile37, Val/Phe39, Arg292, and Arg386 are shown, and the carbon atoms are colored light grey (wt) and yellow (V39F). The Asp42-Val49 main chains are drawn with dashed lines.

Gly38 main chain approaches Tyr225 on the binding of maleate, the lone pair electrons of Gly38 carbonyl O cause repulsion with Tyr225 O in the unprotonated aldimine (E,SH; see Fig. 5A), whereas a hydrogen bond is formed between Tyr225 O and Gly38 carbonyl O in the protonated aldimine (E,S-H; see Fig. 5A). In E,S-H, the hydrogen bond lifts the Tyr225 side chain and allows rotation of the PLP pyridine ring, reducing the aldime torsion angle χ and thereby stabilizing the structure. As a consequence, the approach of Gly38 main chain approaches Tyr225 on binding of maleate to WT is considered to destabilize E,SH relative to E,S-H and increases the aldime pKₐ in the Michaelis complex. The binding of maleate to V39F will not increase the aldime pKₐ, as much, because the movement of Gly38 main chain on the maleate binding is restricted.

Effect of V39F Mutation on the Elementary Steps—For the analysis of the reaction of AspAT with aspartate at high pH, we consider the information shown in Equation 5 below (9), where MC, EA, and K are the Michaelis complex of the PLP form of AspAT and aspartate, the external aldime, and the ketimine, respectively (for the structures, see Scheme I).

\[
\begin{align*}
E + \text{Asp} & \rightleftharpoons \text{MC} \\
\text{MC} & \rightleftharpoons \text{EA} \\
\text{EA} & \rightleftharpoons \text{K} \\
\text{K} & \rightleftharpoons [\text{E,SH}] \rightleftharpoons [\text{E,S-H}] \\
\text{E,SH} & \rightleftharpoons \text{E,S-H} \\
\text{E,S-H} & \rightleftharpoons \text{OA} \\
\text{MC} \rightarrow \text{OA} & \rightleftharpoons \text{E,S-H} + \text{OA} \\
\text{OA} \rightarrow \text{E,S-H} + \text{OA} & \rightleftharpoons \text{E,SH} + \text{OA} \\
\text{E,SH} + \text{OA} & \rightleftharpoons \text{E,S-H} + \text{OA} \rightleftharpoons \text{E} + \text{OA}
\end{align*}
\] (Eq. 5)

E₄ OA is the Michaelis complex of E₄ and oxalacetate. The study of Goldberg and Kirsch (16) showed that the rate-determining steps in the forward reaction are the 1,3-prototropic shift between the external aldime and the ketimine (k₅), the ketimine hydrolysis (k₄), and the release of oxalacetate (k₃). Because the forward transaldimination rate (k₂) is considered to be much larger than other rate constants (16), \( \frac{k_{\text{cat}}}{k_{\text{cat}}^*} \) is expressed as shown in Equation 6, where \( k_{\text{cat}}^* = |k_{\text{cat}}^*(k_{\text{cat}} + k_{-2})k_{-3}L/(k_{-3} + k_{-2} + k_{\text{cat}})k_{-3}L + \) \( k_{-2} + k_{-3}L \) and \( L = (k_{-3}k_{-4} + k_{-5}k_{-3} + k_{-4}k_{-3})/(k_{-3} + k_{-4} + k_{-5}) \).

\[
\frac{k_{\text{cat}}}{k_{\text{cat}}^*} = (k_{-3}^2L + k_{-3}L)(k_{-3}^2L + 1)
\] (Eq. 6)

Because \( k_{-2} + k_{-3} \) are insensitive to isotopic substitution, \( k_{\text{cat}}^* \) is equal to \( k_{\text{cat}} \). Using the observed \( k_{\text{cat}}^* \) value of 2.0 for WT (20) and 4.6 for V39F (this study), and the reported value of \( k_{\text{cat}}^* = 7.3 \) for the primary kinetic isotope effect of the 1,3-prototropic shift (28), \( k_{\text{cat}}^*/k_{\text{cat}} \) is calculated to be 5.3 for WT and 0.75 for V39F. These values indicate that the V39F muta-
In the Michaelis complex, $E_L\cdot S$ and $E_L\cdot SH^+$, showing the hydrogen-bonding pattern. Lone pair electrons are expressed by shaded lobes. $b$, free energy levels of the reaction intermediates in the reaction pathway starting from the PLP form of AspAT and aspartate to the transition state of the 1,3-prototropic shift. Horizontal bars indicate the free-energy levels expressed in kJ mol$^{-1}$. The labels attached to the bars are defined as shown in Scheme I. The horizontal coordinate shows the chemical species sorted by the protonation state. Perpendicular to this one is the reaction coordinate. The broken bars are those of V39F. The energy levels of the transition state are adjusted be-

tween WT and V39F. The energy levels are calculated based on the data obtained in this study and Ref 14, corrected for the difference in the pK$^a$ value of the amino acid α-amino group (10.6 for MeAsp and 9.6 for aspartate). The rate of the 1,3-prototropic shift of WT is estimated to be 3500 s$^{-1}$, based on the consideration that this step is 16% rate-determining (calculated using the equation $(k_{cat} - 1/k_{-1})$; see Ref. 28) in the half-reaction.

Thus, a change in essentially a single energy level ($E_L^\text{SH^+}$) accounts for all the changes in the acid-base and kinetic parameters caused by the V39F mutation. This conclusion is consistent with the comparative structures of WT-maleate and V39F-maleate, the model for the Michaelis complex. That is, if the ligand-induced conformational change of residues 37–39 covering the active site and the concomitant approach of Gly$^{38}$ main chain to Tyr$^{225}$ is blocked by the V39F mutation, the enzyme is unable to destabilize the species $E_L\cdot S$. The reorientation of the PLP ring in the external aldimine lowers the side chain of Tyr$^{225}$, and Tyr$^{225}$ Ω is distant from the carbonyl O of Gly$^{38}$ (2, 7) (1ART and 1AJS). We consider that the lack of the Gly$^{38}$-Tyr$^{225}$ interaction in the external aldimine is the reason why the V39F mutation does not affect the energy levels of the external aldimine species.

**Strain in the Michaelis Complex**—Structural and kinetic analyses of the V39F mutant AspAT described as above provide important findings as follows. First, the conformation of AspAT, i.e. the position of the small domain relative to that of the large domain, affects the aldimine pK$^a$ value, and the effect of the conformational change on the aldimine is mediated through the PLP-Asn$^{194}$-Arg$^{288}$ linkage system. Second, despite the incorporation of the bulky Phe side chain to the domain interface, which causes outward rotation of the small domain, the position and conformation of the bound maleate and the residues that interact with it, i.e. Arg$^{292}$, Asn$^{194}$, and Arg$^{288}$, are the same as those in the WT-maleate, showing the flexibility of the small domain. Therefore, the lowered aldimine pK$^a$ of V39F-maleate as compared with that of WT-maleate is not because of the outward domain rotation but with the reduction in the Gly$^{38}$-Tyr$^{225}$ interaction provided by the ligand-induced

---

4. H. Hayashi and H. Kagamiyama, unpublished results.
packing of the residues at the active site. Together with the kinetic analysis of V39F, it was proposed that the Gly38-Tyr225 interaction destabilizes the unprotonated aldimine species in the Michaelis complex (i.e., ground-state destabilization), and contributes to the increase in $k_{cat}$. The role of the substrate-induced strain shown in this study is in contrast to that of the torsional strain of the PLP-Lys258 aldimine in the unliganded enzyme, which reduces the energy gap between the starting state (unliganded enzyme plus unbound substrate) and the transition state ES‡ by elevating the free energy level of the starting state and contributes to the increase in $k_{cat}/K_m$ (14).

REFERENCES

1. Kiick, D. M., and Cook, P. F. (1983) *Biochemistry* 22, 375–382
2. Rhee S., Silva, M. M., Hyde, C. C., Rogers, P. H., Metzler, C. M., Metzler, D. E., and Arnone, A. (1997) *J. Biol. Chem.* 272, 17293–17302
3. McPhalen, C. A., Vincent, M. G., and Jansonius, J. N. (1992) *J. Mol. Biol.* 225, 495–517
4. Malashkevich, V. N., Toney, M. D., and Jansonius, J. N. (1993) *Biochemistry* 32, 13451–13462
5. Malashkevich, V. N., Strakosch, B. V., Barisov, V. V., Dauter, Z., Wilson, K. S., and Torchinsky, Y. M. (1995) *J. Mol. Biol.* 247, 111–124
6. Jagar, J., Moser, M., Sauder, U., and Jansonius, J. N. (1994) *J. Mol. Biol.* 239, 285–305
7. Okamoto, A., Higuchi, T., Hirotsu, K., Kuramitsu, S., and Kagamiyama, H. (1994) *J. Biochem.* 116, 95–107
8. Fasella, P., and Hammes, G. G. (1967) *Biochemistry* 6, 1796–1804
9. Hayashi, H., and Kagamiyama, H. (1997) *Biochemistry* 36, 13558–13569
10. Ivanov, V. I., and Karpeisky, M. Y. (1969) *Adv. Enzymol. Relat. Areas Mol. Biol.* 32, 21–53
11. Kirsch, J. F., Eichele, G., Ford, G. C., Vincent, M. G., Jansonius, J. N., Gehring, H., and Christen, P. (1984) *J. Mol. Biol.* 174, 497–525
12. Ovchinnikov, Yu., Egorov, C. A., Aldanova, N. A., Feigina, M. Yu., Lipkin, V. M., Abduluaev, N. G., Grushin, E. V., Kiselev, A. P., Modyanov, N. N., Braunstein, A. E., Poyanyovsky, O. L., and Nosikov, V. V. (1975) *FEBS Lett.* 29, 31–34
13. Kallen, R. G., Korpela, T., Martell, A. E., Matsushima, Y., Metzler, C. M., Metzler, D. E., Morozov, Yu. V., Raletson, I. M., Savin, F. A., Torchinsky, Yu. M., and Ueno, H. (1985) in *Transaminases* (Christen, P., and Metzler, D. E., eds) pp. 37–108, John Wiley & Sons, Inc., New York
14. Hayashi, H., Mizuguchi, H., and Kagamiyama, H. (1988) *Biochemistry* 36, 15076–15085
15. Mizuguchi, H., Hayashi, H., Okada, K., Miyahara, I., Hirotsu, K., Kagamiyama, H. (2001) *Biochemistry* 40, 353–360
16. Goldberg, J. M., and Kirsch, J. F. (1996) *Biochemistry* 35, 5280–5291
17. Jenkins, W. T., and Sizer, I. W. (1957) *J. Am. Chem. Soc.* 79, 2655–2656
18. Yano, T., Mizuno, T., and Kagamiyama, H. (1993) *Biochemistry* 32, 1810–1815
19. Hayashi, H., Kuramitsu, S., and Kagamiyama, H. (1991) *J. Biochem.* 109, 699–704
20. Kuramitsu, S., Hiromi, K., Hayashi, H., Morino, Y., and Kagamiyama, H. (1990) *Biochemistry* 29, 5469–5476
21. Sakabe, N. (1991) *Nucl. Instrum. Methods Phys. Res.*, A303, 448–463
22. Otwinowski, Z. (1989) in *Data Collection and Processing, Proceedings of CCP4 Study Weekend* (Sawyer, L., Isaacs, N., and Bailey, S., eds) pp. 56–62, S.E.R.C. Daresbury Laboratory, United Kingdom
23. Brunger, A. T., Kuriyan, J., and Karplus, M. (1987) *Science* 235, 458–461
24. Jones, T. A., Zou, J.-Y., and Kjeldgaard, M. (1991) *Acta Crystallogr. Sect. A* 47, 110–119
25. Lawkowski, R. A., MacArthur, M. W., Moss, D. S., and Thornton, J. M. (1993) *J. Appl. Crystallogr.* 26, 283–291
26. Koradi, R., Billeter, M., and Wuthrich, K. (1996) *J. Mol. Graphics* 14, 51–55
27. Glass, L. M., and Kirsch, J. F. (1996) *Biochemistry* 35, 3993–3998
28. Ounlifier, J. J., Ton, B. T., Klement, I., and Kirsch, J. F. (1995) *Protein Sci.* 4, 1743–1749
Conformational Change in Aspartate Aminotransferase on Substrate Binding Induces Strain in the Catalytic Group and Enhances Catalysis
Hideyuki Hayashi, Hiroyuki Mizuguchi, Ikuko Miyahara, Yoshitaka Nakajima, Ken Hirotsu and Hiroyuki Kagamiyama

J. Biol. Chem. 2003, 278:9481-9488.
doi: 10.1074/jbc.M209235200 originally published online December 17, 2002

Access the most updated version of this article at doi: 10.1074/jbc.M209235200

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 26 references, 2 of which can be accessed free at http://www.jbc.org/content/278/11/9481.full.html#ref-list-1