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Role of Asp222 in the Catalytic Mechanism of *Escherichia coli* Aspartate Aminotransferase: The Amino Acid Residue Which Enhances the Function of the Enzyme-Bound Coenzyme Pyridoxal 5'-Phosphate†

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ABSTRACT: Asp222 is an invariant residue in all known sequences of aspartate aminotransferases from a variety of sources and is located within a distance of strong ionic interaction with N(1) of the coenzyme, pyridoxal 5'-phosphate (PLP), or pyridoxamine 5'-phosphate (PMP). This residue of *Escherichia coli* aspartate aminotransferase was replaced by Ala, Asn, or Glu by site-directed mutagenesis. The PLP form of the mutant enzyme D222E showed pH-dependent spectral changes with a pK_a value of 6.44 for the protonation of the internal aldimine bond, slightly lower than that (6.7) for the wild-type enzyme. In contrast, the internal aldimine bond in the D222A or D222N enzyme did not titrate over the pH range 5.3-9.5, and a 430-nm band attributed to the protonated aldimine persisted even at high pH. The binding affinity of the D222A and D222N enzymes for PMP decreased by 3 orders of magnitude as compared to that of the wild-type enzyme. Pre-steady-state half-transamination reactions of all the mutant enzymes with substrates exhibited anomalous progress curves comprising multiphasic exponential processes, which were accounted for by postulating several kinetically different enzyme species for both the PLP and PMP forms of each mutant enzyme. While the replacement of Asp222 by Glu yielded fairly active enzyme species, the replacement by Ala and Asn resulted in 8600- and 20 000-fold decreases, respectively, in the catalytic efficiency (k_{max}/K_d value for the most active species of each mutant enzyme) in the reactions of the PLP form with aspartate. In contrast, the catalytic efficiency of the PMP form of the D222A or D222N enzyme with 2-oxoglutarate was still retained at a level as high as 2-10% of that of the wild-type enzyme. The pre-steady-state reactions of these two mutant enzymes with [2-^{2}H]aspartate revealed a deuterium isotope effect (k^H/k^D = 6.0) greater than that [k^H/k^D = 2.2; Kuramitsu, S., Hiromi, K., Hayashi, H., Morino, Y., & Kagamiyama, H. (1990) *Biochemistry 29*, 5469-5476] for the wild-type enzyme. These findings indicate that the presence of a negatively charged residue at position 222 is particularly critical for the withdrawal of the α-proton of the amino acid substrate and accelerates this rate-determining step by about 5 kcal·mol^{-1}. Thus it is concluded that Asp222 serves as a protein ligand tethering the coenzyme in a productive mode within the active site and stabilizes the protonated N(1) of the coenzyme to strengthen the electron-withdrawing capacity of the coenzyme.

The catalytic mechanism for the AspAT[1]-catalyzed reaction proposed by Ivanov and Karpeisky (Karpeisky & Ivanov, 1966; Ivanov & Karpeisky, 1969) was in principle supported by the X-ray crystallographic studies of animal AspATs and their complexes with substrate analogues (Jansonius et al., 1985; Arnone et al., 1985b; Borisov et al., 1985; Harutyunyan et al., 1985; Jansonius & Vincent, 1987). The recent X-ray crystallographic studies on *Escherichia coli* AspAT (Kamitori et al., 1988, 1990; Smith et al., 1989) indicated that the positions of the active-site residues of *E. coli* AspAT were virtually identical with those of the animal AspATs. In the active site of all these AspATs, Asp222[2] is situated within a salt-bridge formation and/or hydrogen-bonding distance to N(1) of the coenzyme PLP or PMP (Figure 1). Hence it has been postulated that the negative charge of its side chain would stabilize the positive charge at N(1) of the coenzyme and would thus

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[1] Abbreviations: AspAT, aspartate aminotransferase; D222E, D222N, or D222A, AspAT in which Asp222 is replaced by Glu, Asn, or Ala, respectively; CD, circular dichroism; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*'-2-ethanesulfonic acid; MES, 2-(*N*-morpholino)-ethanesulfonic acid; PLP, pyridoxal 5'-phosphate; PMP, pyridoxamine 5'-phosphate.

[2] The amino acid residue is numbered according to the sequence of cytosolic AspAT from pig (Ovchinnikov et al., 1973).

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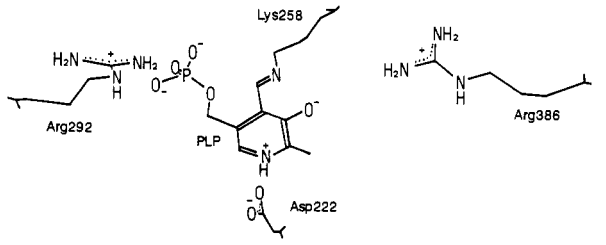


FIGURE 1: The active site of *E. coli* aspartate aminotransferase, showing the relationship between the bound PLP molecule and Asp222 (Kamitori et al., 1990).

enhance the function of the coenzyme as an "electron sink" during enzymic catalysis (Arnone et al., 1985a).

In the present paper, this residue has been replaced by Glu, Asn, or Ala by site-directed mutagenesis to define the functional role of Asp222 in catalysis. The mutation Asp222 → Glu retains the carboxyl group but introduces one additional methylene group into the side chain; Asp222 → Asn leads to a loss of the negative charge at position 222 but may retain the ability to form a hydrogen bond with N(1) of the coenzyme; Asp222 → Ala results in the loss of both the negative charge and the hydrogen-bonding ability.

Experimental Procedures

Materials

Bacterial Strains. E. coli TY103 (Yano et al., 1991), which is a derivative of *E. coli* JM103 (*Δlacpro, supE, thi, strA, sbcB15, endA, hsdR4*, F′ [*traD36, proAB*$^+$, *LacI*^{q}*ZΔM15*]) (Messing et al., 1981) and which has the genotype of *aspC, tyrB*, and *recA*, was used for the expression of the mutant *aspC* genes.

Medium. The medium used for the bacterial growth contained 0.5% yeast extract (Nacalai Tesque, Kyoto, Japan), 0.8% polypepton (Nihon Pharmaceutical Co., Tokyo, Japan), and 0.25% NaCl at pH 7.2–7.4. About 6 μg/mL pyridoxine hydrochloride (Nacalai Tesque) was added to the culture medium for the production of wild-type and mutant AspATs.

Chemicals. Enzymes for DNA manipulations were obtained from Takara Shuzo Co. (Kyoto, Japan) except for the oligonucleotide-directed in vitro mutagenesis system (Amersham International plc., Buckinghamshire, England). MES and HEPES were from Dojin Laboratories (Kumamoto, Japan); cysteine sulfinate was from Sigma Chemical Co. (St. Louis, MO); NADH was from Oriental Yeast Co. (Tokyo, Japan); porcine mitochondrial malate dehydrogenase was from Boehringer Mannheim (Mannheim, Germany). L-[2-^{2}H]-Aspartate was synthesized as described previously (Kuramitsu et al., 1990).

Methods

Site-Directed Mutagenesis. Site-directed mutagenesis was performed by the method of Nakamaye and Eckstein (1986). The following 22-mer synthetic oligonucleotides were used to direct the mutations (asterisks indicate the mismatches): CCG-CTG-TTT-XXX-TTC-GCT-TAC-C (XXX, GC*C for D222A; GAA* for D222E; A*AC for D222N). The expression plasmid for each mutant AspAT was constructed as described previously (Yano et al., 1991). Briefly, plasmid pKDHE19 (Kamitori et al., 1987), in which the nucleotide sequence of the *aspC* gene had been ascertained, and the M13 phage DNA containing the mutant *aspC* gene were digested with *Nco*I and *Nsi*I, to produce the small restriction fragments corresponding to the coding region for His189–His247 of AspAT. This small fragment from the mutant M13 DNA was ligated with the large fragment from pKDHE19 lacking the His189–His247 region. The nucleotide sequence of the newly inserted region of the resultant mutant plasmid was ascertained by the method of Messing (1983) with a slight modification (Kuramitsu et al., 1985).

E. coli TY103 carrying the expression plasmid overproduced the mutant AspAT, and the enzyme was purified as described previously (Inoue et al., 1989).

Spectrophotometric Measurements. Absorption and CD spectra of the PLP form of AspAT were recorded under the same conditions as those described (Yano et al., 1991).

Determination of Protein Concentration. The concentration of AspAT was determined spectrophotometrically using molar extinction coefficients of ϵ_M = 47 000, 46 000, and 45 000 M^{-1} cm^{-1} at 280 nm for the PLP-enzyme, PMP-enzyme, and apoenzyme, respectively (Kuramitsu et al., 1990).

Construction of the PMP Form of AspAT. The PLP form of AspAT was converted to the PMP form by adding cysteine sulfinate, and the excess cysteine sulfinate was removed by Sephadex G-25 (Jenkins & D'Ari, 1966). Since PMP was completely dissociated from D222A and D222N during the G-25 chromatography, the PMP forms of these mutant enzymes were reconstituted by adding PMP to the apo-forms.

Determination of the Dissociation Constants of D222A and D222N AspATs for PMP. The dissociation constant (K_d value) of D222A or D222N for PMP was determined by CD spectrometric titration of the apo-form with PMP at a protein concentration of 20 μM in a 1-cm cell. The spectral changes accompanied by the reconstitution were observed in the region 250–330 nm. Similar K_d values were obtained at any wavelength in this region. The experiments were performed in the buffer solution, pH 8.0, containing 50 mM HEPES, 0.1 M KCl, and 10 μM EDTA at 25 °C.

Measurement of Steady-State Overall-Transamination Reaction. AspAT catalyzes the reversible transamination reaction

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

via the "ping-pong bi–bi" mechanism (Velick & Vavra, 1962; Kiick & Cook, 1983; Jenkins & Fonda, 1985). The overall-transamination reaction of AspAT was measured by the malate dehydrogenase-coupled method (Karmen, 1955) at pH 8.0, 25 °C. The activity was calculated by using a molar extinction coefficient of 6220 M^{-1} cm^{-1} at 340 nm for NADH. Because PMP was readily dissociated from D222A and D222N during the reaction, the assay was performed in the presence of a saturating amount of PMP (100 μM). The reaction was initiated by addition of the PMP form of AspAT to the reaction mixture. The concentration of the enzyme in the reaction mixture was about 1 μM (D222A or D222N) or 0.01 μM (D222E or wild-type enzyme).

Measurement of the Pre-Steady-State Half-Transamination Reaction. The above overall-transamination reaction of AspAT consists of the following two half-reactions (Kuramitsu et al., 1990)

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

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

where EL and EM are the PLP form and PMP form of AspAT, respectively; Asp is L-aspartate; OA is oxalacetate; Glu is L-glutamate; and OG is 2-oxoglutarate. The half-transamination reactions were measured by using a Hitachi spectrophotometer, Model 557 (for slow reactions, $k_{max} < 0.5$ s^{-1}), or a stopped-flow apparatus, Applied Photophysics SF17MV equipped with a data-processing unit (for the more rapid reactions) at pH 8.0, 25 °C. The conversion of the PLP form

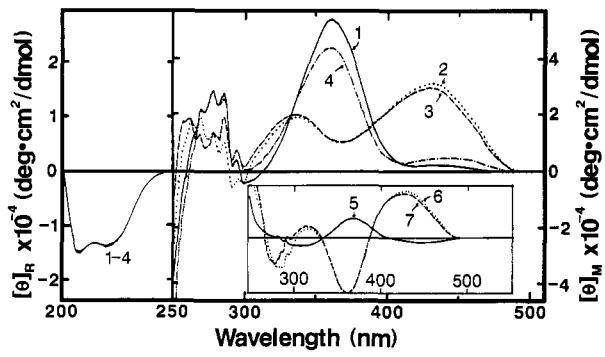


FIGURE 2: CD spectra of the PLP form of D222E (curve 1), D222N (2), D222A (3), and the wild-type enzyme (4) at pH 8.0, 25 °C. The inset shows the difference spectra between each of the mutant enzymes [D222E (curve 5), D222N (6), D222A (7)] and the wild-type enzyme in the region above 250 nm. The ordinate on the left side shows the mean residue ellipticity ($[\theta]_R$) for the region 200–250 nm; the ordinate on the right side shows the molar ellipticity ($[\theta]_M$) for the region above 250 nm. The spectra in the region 200–250 nm were obtained in 10 mM borate and 15 mM phosphate buffer containing 0.1 M KCl and 10 μM EDTA at protein concentrations of 1–1.5 μM in a 1-mm cell. The spectra in the region above 250 nm were obtained in 50 mM HEPES buffer containing 0.1 M KCl and 10 μM EDTA at protein concentrations of 10–20 μM in a 1-cm cell.

into the PMP form, or the PMP form into the PLP form, was monitored by following the absorption change at 358 nm (for D222E or wild-type enzyme), 427 nm (for D222N), or 428 nm (for D222A). The reactions of the PMP forms of AspATs with 2-oxoglutarate were performed in the presence of a saturating amount of PMP (100 μM). All data, including those obtained by a conventional spectrophotometer, were analyzed by a nonlinear regression curve-fitting program (Bevington, 1969) used in the data-processing unit of the stopped-flow apparatus. The concentration of the enzyme in the reaction mixture was 10–20 μM. The concentrations of aspartate and 2-oxoglutarate ranged from 0.50 to 25 mM and 0.25 to 20 mM, respectively.

RESULTS

CD and Absorption Spectra of AspATs. There was no significant difference in the CD spectra between the wild-type and mutant enzymes in the region of 200–250 nm (Figure 2), suggesting that no large conformational change has occurred upon the mutations at position 222.

The absorption spectra of wild-type AspAT at pH 5.4, 7.1, and 9.0 are shown in Figure 3A (curves 1–3). D222E gave pH-dependent spectral changes (curves 4–6) similar to those of the wild-type enzyme: absorption maxima at 430 nm (wild type) or 425 nm (D222E) in an acidic pH range and at 358 nm in an alkaline pH range with an isosbestic point at 385 nm. These pH-dependent spectral changes reflect the ionization state of the imine nitrogen of the internal aldimine bond formed between Lys258 and PLP (Jenkins & Sizer, 1957; Kallen et al., 1985). The plots of the apparent molar extinction coefficients of each spectral band against pH values were in good agreement with the theoretical titration curves (Yano et al., 1991) that were constructed using pK_a values of 6.44 for D222E and 6.70 for the wild-type enzyme (Figure 4). In contrast, neither D222A nor D222N showed any pH-dependent spectral change (Figure 3B,C); two absorption bands of D222A and D222N around 330 and 430 nm did not titrate with pH over the range from 5.3 to 9.5.

The CD spectra of the wild-type and mutant enzymes in the region 250–550 nm at pH 8.0 are shown in Figure 2. All of the four AspATs showed positive CD bands at positions corresponding to absorption maxima in the region above 300

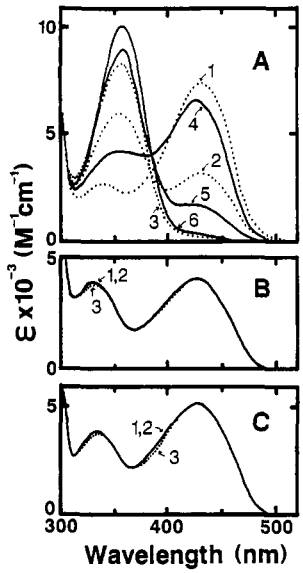


FIGURE 3: Absorption spectra of the PLP form of mutant and wild-type AspATs at various pH values, 25 °C. (A) Wild type (dotted lines) at pH 5.4 (curve 1), 7.1 (2), and 9.0 (3) and D222E (solid lines) at pH 5.6 (curve 4), 7.2 (5), and 9.0 (6). (B) D222A at pH 5.3 (curve 1), 7.2 (2), and 9.2 (3). (C) D222N at pH 5.4 (curve 1), 7.2 (2), and 9.2 (3). All these spectra were obtained in a buffer solution of 50 mM MES, 50 mM HEPES, or 50 mM borate containing 0.1 M KCl and 10 μM EDTA at protein concentrations of 10–20 μM in a 1-cm cell.

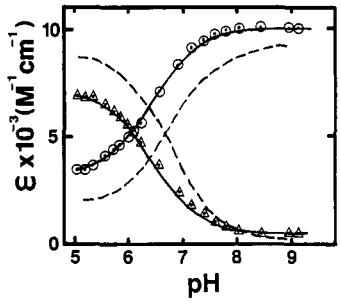


FIGURE 4: pH dependence of molar extinction coefficients at 358 (○) and 425 nm (△) for D222E at 25 °C. The solid (for D222E) and broken (for wild type) lines represent the theoretical curves (see text). The absorption bands of D222A and D222N around 330 and 430 nm did not titrate with pH over the range from 5.3 to 9.5 (see text and Figure 3B,C).

nm, reflecting the state of the bound PLP molecule in the active site. The spectrum of D222E was very similar to that of the wild-type enzyme in the region 250–300 nm. By contrast, significant differences were observed between the wild-type enzyme and either of the other two mutant enzymes. The difference spectra (Figure 2, inset) revealed a negative peak centered around 280 nm. This might result from either a difference in the binding mode of PLP or a perturbation of some aromatic side chains.

Dissociation Constants of D222A and D222N for PMP. Addition of PMP to apoenzyme samples of the wild-type and mutant enzymes gave rise to multiple positive CD bands at around 330, 290, 280, and 270 nm (Figure 5A). Titration of D222A and D222N apoenzymes with PMP at 287 nm where the highest CD intensity was given yielded saturation curves from which a K_d value of 1.0×10^{-5} was elicited for either of the mutant enzymes (Figure 5, inset). A similar value was also obtained from the data at 320 nm. In contrast, the CD titration of both the wild-type and D222E apoenzymes with PMP did not produce typical saturation curves, but it instead yielded straight lines with a deflection at the concen-

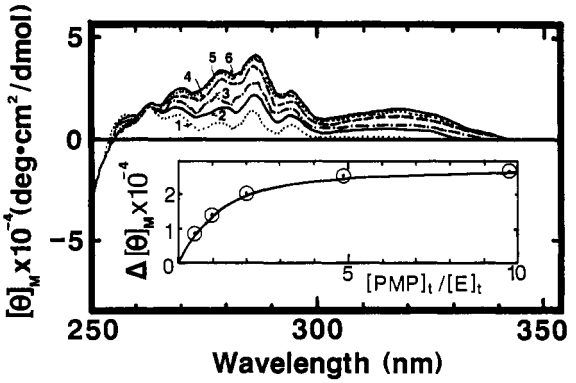


FIGURE 5: CD titration of apo-D222A with PMP at pH 8.0, 25 °C. The spectra were measured at a protein concentration of 20 μM in the presence of 0 (curve 1), 10 (2), 22 (3), 43 (4), 100 (5), and 200 (6) μM PMP. Free PMP lacks CD. The buffer solution contained 50 mM HEPES (pH 8.0), 0.1 M KCl, and 10 μM EDTA. The inset shows the plots of the changes in the apparent molar ellipticities (deg·cm²·dmol⁻¹) at 287 nm against $[PMP]_{total}/[protein]_{total}$. The solid line represents the theoretical curve constructed using a K_d value of 1.0×10^{-5} M.

Table I: Steady-State Kinetic Parameters for the Overall-Transamination Reactions of AspATs (pH 8.0, 25 °C)

AspAT	k_{cat} (s^{-1})	K_m (mM) aspartate	K_m (mM) 2-oxoglutarate
wild type	210	1.5	0.45
D222E	61[a]	5.0	0.17
D222A	0.27[b]	20[b]	0.0076[b]
	0.073[c]	5.5[c]	3.9[c]
D222N	0.079[b]	13[b]	0.0076[b]
	0.041[c]	6.8[c]	3.4[c]

[a] The observed k_{cat} value (53 s^{-1}) was corrected to 61 s^{-1}, according to Scheme IV (see Appendix). [b] Calculated values for the EM_1 route in Scheme III (see Appendix). [c] Calculated values for the EM_2 route in Scheme III (see Appendix).

tration of PMP that was equivalent to that of the subunit of these dimeric apoenzymes (data not shown). This indicates that the affinity of these apoenzymes was too high to calculate the K_d values from the titration data. The binding of PMP to the apoenzyme can also be monitored at a much lower protein concentration by using a strong quenching of tryptophan fluorescence as described by Bertland and Kaplan (1968). To determine the K_d value by this method, it would be necessary to correct for the effect of energy transfer from tryptophan residues of the apo-form subunit to the coenzyme bound to the holo-form subunit in the hybrid dimer composed of one subunit in apo-form and the other in holo-form (Arrio-Dupont & Vergé, 1982). However, such a hybrid dimer of *E. coli* AspAT has not been available. Neglecting the effect of energy transfer, K_d values of 1×10^{-8} and 1×10^{-6} M were obtained for the wild-type and D222E enzymes, respectively (data not shown). Thus, the K_d value for PMP was increased by 3 orders of magnitude upon replacement of Asp222 by Ala or Asn.

Steady-State Overall-Transamination Reactions Catalyzed by D222 Mutant AspATs. The overall-transamination reaction between aspartate and 2-oxoglutarate catalyzed by D222E followed a typical "ping-pong bi–bi" mechanism. In contrast, the overall reactions catalyzed by D222A and D222N showed anomalous kinetic behaviors (Figure 6), which were analyzed by assuming the reaction schemes described in the Appendix. The kinetic parameters thus obtained (Table I) show that the catalytic activity was considerably retained upon replacing Asp222 by a negatively charged residue, glutamate, while the replacement by a neutral residue, such as asparagine or alanine, resulted in an extensive decrease in the catalytic activity.

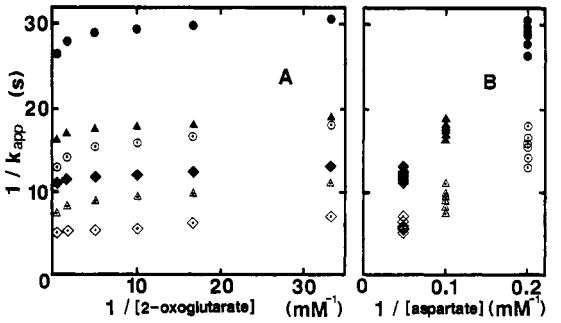


FIGURE 6: Double-reciprocal plots of the initial velocities versus the concentrations of the substrate for the overall reaction catalyzed by D222A. The concentration of 2-oxoglutarate is varied at fixed concentrations of aspartate (A) and vice versa (B). Observed (open) and calculated (closed) values are shown (see text). The calculated values were obtained according to Scheme III (see Appendix). The initial velocities were obtained at 18 pairs of substrate concentrations; 5 (○), 10 (△), or 20 (◇) mM aspartate and 0.03, 0.06, 0.1, 0.2, 0.6, or 2 mM 2-oxoglutarate. The overall reaction was initiated by addition of the PMP form of the enzyme and was performed in the presence of 100 μM PMP at 25 °C. The buffer solution contained 50 mM HEPES (pH 8.0), 0.1 M KCl, and 10 μM EDTA.

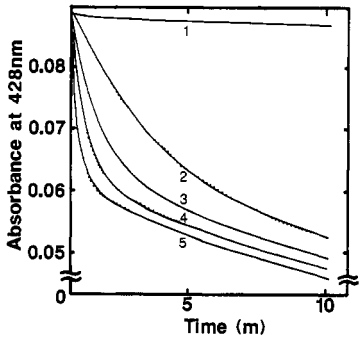


FIGURE 7: Typical profiles of the pre-steady-state reactions of the PLP form of D222A with aspartate in the absence (curve 1) and presence (curves 2–5) of malate dehydrogenase (240 units) and NADH (0.25 mM) at pH 8.0, 25 °C. The reactions were monitored at 428 nm. The concentration of the enzyme in the reaction mixture was 22 μM. Curve 2 shows the reaction with 2.5 mM [2-²H]aspartate; curves 1, 3, 4, and 5 show the reactions with 5.0, 1.3, 2.5, and 5.0 mM aspartate, respectively. The buffer solution contained 50 mM HEPES (pH 8.0), 0.1 M KCl, and 10 μM EDTA. The dotted lines indicate the theoretical curves constructed by the curve-fitting program (see text).

Pre-Steady-State Half-Transamination Reactions Catalyzed by D222A. The conversion of the PLP form into the PMP form of D222A upon the addition of an amino acid substrate was monitored by following the decrease in absorbance at 428 nm. When the PLP forms of this mutant enzyme was mixed with aspartate, the reaction proceeded at a slow rate and appeared to reach an equilibrium after a short period (Figure 7, curve 1). However, the reaction profile was strikingly changed, when it was performed in the presence of a saturating amount of malate dehydrogenase (240 units) and NADH (0.25 mM) (Figure 7, curve 5). This finding was interpreted as showing that the equilibrium of the reaction shifted far toward the side of the PLP form and aspartate in comparison with the case of the wild-type enzyme (Inoue et al., 1989; Kuramitsu et al., 1990) and that a particularly large contribution by the reverse reaction between the products, the PMP form of the enzyme and oxalacetate, was indeed eliminated by removing oxalacetate by the malate dehydrogenase reaction. The curve fitting of the progress curves (Figure 7, curves 3–5) revealed that the reaction comprised two exponential processes: a fast phase followed by a slow phase. The

Table II: Pre-Steady-State Kinetic Parameters of AspATs[a] (pH 8.0, 25 °C)

	aspartate			2-oxoglutarate		
AspAT	k_{max} (s^{-1})	K_d (mM)	k_{max}/K_d ($s^{-1}\cdot mM^{-1}$)	k_{max} (s^{-1})	K_d (s^{-1})	k_{max}/K_d ($s^{-1}\cdot mM^{-1}$)
wild type	550	4.5	120	600	1.3	460
D222E	64[b]	5.2[b]	12	1300	2.6	500
D222A	0.27	20	0.014	120[c]	3.4[c]	35
				0.10[d]	5.4[d]	0.019
D222N	0.079	13	0.0061	50[c]	4.8[c]	10
				0.086[d]	7.1[d]	0.012

[a] Rate constants for the substrate-independent phases observed in the analyses of the mutant enzymes were omitted. [b] Calculated values according to Scheme IV (see Appendix). [c] Values for EM_1 in Scheme III (see Appendix). [d] Values for EM_2 in Scheme III (see Appendix).

rate constants for the fast phase varied with substrate concentrations, while those for the slow phase did not. The relative amplitudes in absorption change for the fast and slow phases were 3:7. Variations of apparent rate constants with substrate concentrations for the fast phase conformed to the Michaelis–Menten equation, yielding the k_{max} value of 0.27 s^{-1} and the K_d value of 20 mM (Table II). The rate constant for the slow substrate-independent phase was 0.00060 s^{-1}.

The reaction with [2-^{2}H]aspartate also gave rise to a biphasic progress curve with the relative amplitudes for the two phases similar to those for the reaction with nondeuterated aspartate (Figure 7, curve 2). The fast phase exhibited a deuterium kinetic isotope effect of $k^H/k^D = 6.0$, while the slow phase did not. The K_d value (20 mM) for deuterated aspartate was identical to that for nondeuterated aspartate. This finding indicated that the abstraction of the α-hydrogen of the substrate is a rate-determining step in the reaction catalyzed by this mutant enzyme as demonstrated previously for the wild-type enzyme, $k^H/k^D = 2.2$ (Kuramitsu et al., 1990).

The reaction of the PLP form of D222A with glutamate exhibited a profile very similar to that observed in the reaction with aspartate performed in the absence of malate dehydrogenase and NADH (data not shown). Also in this case, it appears highly likely that a large contribution by the reverse reaction interfered with the progress of the forward reaction. Unfortunately, this was not confirmed experimentally because no appropriate system to remove the product, 2-oxoglutarate, from the reaction mixture was available. By contrast, cysteine sulfinate, a good substrate for the wild-type enzyme, was found to react readily with the PLP form of this mutant enzyme. The reaction profile was very similar to that observed in the reaction with aspartate in the presence of malate dehydrogenase and NADH. This finding was understood in light of the fact that the transamination product of this amino acid, sulfinylpyruvate, is rapidly decomposed to give rise to pyruvate, a very poor keto-acid substrate for AspAT, and hence does not cause any significant reverse reaction. The k_{max} and K_d values for the fast phase were 0.14 s^{-1} and 12 mM, respectively. The rate constant for the slow phase and the relative amplitudes of these two phases were identical with those observed for the reaction with aspartate.

The conversion of the PMP form of D222A into the PLP form upon the addition of 2-oxoglutarate was monitored by following the increase in absorbance at 428 nm. A saturating amount of PMP (100 μM) was included in the reaction mixture because the affinity of this mutant enzyme for PMP was considerably low (K_d = 10 μM) as described earlier. Reaction profiles at varied concentrations of 2-oxoglutarate are shown in Figure 8. All of the progress curves were accounted for by the presence of three increasing exponential processes: substrate-dependent fast and middle phases and

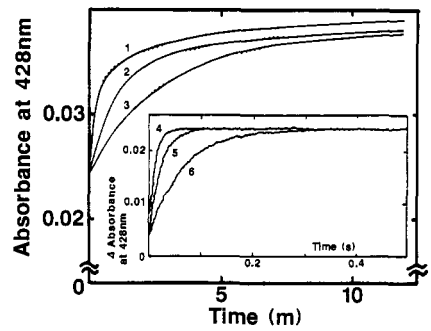


FIGURE 8: Typical profiles of the pre-steady-state reactions with the PMP form of D222A with 2-oxoglutarate. All the reactions were performed in the presence of 100 μM PMP at 25 °C and monitored at 428 nm. The inset shows the early stages of the reactions as monitored by the stopped-flow spectrophotometer (see text). The concentrations of the enzyme in the conventional and stopped-flow spectrophotometric measurements were 11 and 16 μM, respectively. The concentrations of 2-oxoglutarate were 0.50 (curves 3 and 6), 2.0 (2 and 5), and 10 mM (1 and 4). The buffer solution contained 50 mM HEPES (pH 8.0), 0.1 M KCl, and 10 μM EDTA. The dotted lines indicate the theoretical curves constructed by the curve-fitting program (see text).

a substrate-independent slow phase. The relative amplitudes for the fast, middle, and slow phases were 6.5:2.0:1.5. In the fast and middle phases, the variation of the apparent rate constants with the concentrations of 2-oxoglutarate followed the Michaelis–Menten equation, yielding the following kinetic parameters: k_{max} = 120 s^{-1} and K_d = 3.4 mM for the fast phase and k_{max} = 0.10 s^{-1} and K_d = 5.4 mM for the middle phase (Table II). The rate constant for the substrate-independent slow phase was 0.0030 s^{-1}.

In summary, the substitution of alanine for Asp222 resulted in an over 8600-fold decrease in the catalytic efficiency (k_{max}/K_d) in the reaction of the PLP form of the enzyme with aspartate, while the major, most active species of the PMP form of the mutant enzyme still retained the catalytic efficiency with 2-oxoglutarate of as high as 10% that of the wild-type enzyme (Table II). Thus, the contribution of Asp222 to catalysis appears to be greater in the reaction of the PLP form of the enzyme with an amino acid substrate than in the reaction of the PMP form with a keto-acid substrate.

Catalytic Activity of D222N. The kinetic properties of the pre-steady-state half-reactions as well as of the steady-state overall reactions were quite similar to those described for D222A. The reactions of the PLP form of D222N with aspartate or cysteine sulfinate represented two exponential processes: a fast phase and a slow phase with the relative amplitudes of 1:9 (data not shown). The reactions of the PMP form of this mutant enzyme with 2-oxoglutarate were also accounted for by the presence of three exponential processes: substrate-dependent fast and middle phases and a substrate-independent slow phase, with relative amplitudes of 4.5:2.0:3.5 (data not shown). These kinetic behaviors could be analyzed by using the same reaction schemes as those for D222A. Comparison of the kinetic parameters between the wild-type enzyme and D222N (Table II) showed that the replacement of Asp222 by asparagine resulted in a 20 000-fold decrease in the catalytic efficiency (k_{max}/K_d) in the reaction of the PLP form of the enzyme with aspartate while the catalytic efficiency was retained still at a level of 2% of that of the wild-type enzyme in the reaction of the PMP form with 2-oxoglutarate. It is also noted that D222N showed somewhat lower catalytic efficiency than D222A did.

Catalytic Activity of D222E. The pre-steady-state half-transamination reaction of the PLP form of this mutant enzyme with aspartate was not feasible for experimentation for

the following two reasons: (1) the contribution by the reverse reaction was too large for correcting the observed rate constants by the treatment employed for the reaction catalyzed by the wild-type enzyme (Kuramitsu et al., 1990), and (2) an attempt to prevent the reverse reaction by using the malate dehydrogenase and NADH system was unsuccessful, because the reaction was so rapid that the malate dehydrogenase reaction became rate-limiting under the practicable conditions. By contrast, the use of cysteine sulfinate as the substrate in the reaction with the PLP form of this mutant enzyme circumvented these experimental difficulties. The reaction with this amino acid substrate revealed the presence of two exponential time courses: a substrate-dependent fast phase and a substrate-independent slow phase with relative amplitudes of 2.5:1.0. The k_{max} and K_d values for the fast phase were 4.8 s^{-1} and 4.2 mM, respectively. The rate constant for the slow phase was 0.0059 s^{-1}.

The reaction of the PMP form of D222E with 2-oxoglutarate showed two increasing exponential time courses: a substrate-dependent fast phase and a substrate-independent slow phase, with relative amplitudes of 7:1. The k_{max} and K_d values for the fast phase were 1300 s^{-1} and 2.6 mM, respectively (Table II). The rate constant for the slow phase was 0.060 s^{-1}. It should be noted that the k_{max} value for 2-oxoglutarate was greater by 2-fold than that of the wild-type enzyme.

Comments on the Kinetic Analysis of D222 Mutant AspATs. Reactions catalyzed by the mutant enzymes showed anomalous kinetic behaviors as mentioned above. These anomalous profiles were reproducible with different preparations for each mutant enzyme. Some of the attempts to analyze these anomalies are described in the Appendix, where several enzyme species with different kinetic properties are postulated for both the PLP and PMP forms of each mutant enzyme. The peculiar profiles (see, for instance, Figure 6) observed in the steady-state overall reactions catalyzed by the mutant enzymes D222A and D222N are also considered in terms of the kinetic parameters obtained from the kinetic analyses of the multiphasic processes in the pre-steady-state half-reactions catalyzed by these mutant enzymes. The data presented in Tables I and II are based on such kinetic considerations as described in the Appendix.

Discussion

The present work shows that the replacement of the aspartate residue at position 222 of *E. coli* AspAT by a neutral amino acid leads to a pronounced decrease in the reactivity of the PLP form with an amino acid substrate, while the influence of the substitution is much less on the reactivity of the PMP form with a keto-acid substrate. The change to glutamate showed a representative conservative mutation. D222E still retained as high as 10% of the catalytic activity in the reaction of the PLP form with aspartate, and that in the reaction of the PMP form with 2-oxoglutarate was even higher than that of the wild-type enzyme. These findings clearly show that the presence of a negatively charged residue at position 222 is required for catalysis by AspAT, particularly in the reaction with an amino acid substrate. In addition, the fact that the replacement by asparagine with potential hydrogen-bonding capacity produced an enzyme even lower in catalytic activity than that by alanine suggests that the interaction with N(1) of the coenzyme ring should not be only via hydrogen bonding but, more importantly, via electrostatic attraction.

The negatively charged residue at position 222 likely contributes to the binding of the coenzyme. Although the effects of the mutations on the binding affinity for PLP were not explored in the present study, the binding affinity of D222A or D222N for PMP was found to be decreased by 3 orders of magnitude compared with that of the wild-type enzyme. This situation is also clear from the fact that, when one examines the reaction of the PMP form of D222A or D222N with a keto-acid substrate, one has to include a saturating amount of PMP in the reaction mixture. These findings are all consistent with the X-ray data on *E. coli* AspAT showing that the carboxylate group of Asp222 is situated within a salt-bridge formation and/or hydrogen-bonding distance to N(1) of the coenzyme. The binding affinity for PMP was decreased by 2 orders of magnitude upon replacement by Glu. However, this finding is not thought to conflict with the importance of the negative charge for the binding of the coenzyme, because one additional methylene group introduced into position 222 would cause some steric hindrance.

D222E showed pH-dependent spectral changes similar to those of the wild-type enzyme: a 430-nm band for the protonated form of the internal aldimine bond predominates at acidic pH while a 360-nm band for the unprotonated form does at alkaline pH. In contrast, no spectral changes were observed for D222A and D222N in the pH range from 5.3 to 9.5. Persistence of the 430-nm band indicates that the internal aldimine bond exists in a protonated form even in the alkaline pH region, suggesting that the loss of a negative charge at position 222 would lead to a rise in the pK_a value of the imine nitrogen of the internal aldimine bond above 9.5. This is consistent with the experimental results on model compounds in which the pK_a value for an aldimine bond formed between PLP and an amine is lowered by more than 2 pH units upon protonation at N(1) of PLP (Johnston et al., 1963; Kallen et al., 1985). Thus, it is highly likely that the presence of a negative charge at position 222 stabilizes the protonated N(1) of the bound coenzyme and lowers the pK_a value for the internal aldimine bond of the wild-type enzyme or D222E to the titratable level.

The PLP form of D222A and D222N showed another pH-independent band around 330 nm. It was of interest to examine a possible change in this spectral band upon the addition of substrates. However, attempts to observe the spectral change during the reaction with aspartate or cysteine sulfinate were hampered by the appearance of overlapping absorbance resulting from the added NADH or the arising PMP form of the enzymes. The pH-independent 330-nm bands observed in CD (Figure 2) and absorption (Figure 3) spectra of these mutant enzymes may represent an enolimine type of the internal aldimine. This type of the bound coenzyme was observed also in the absorption spectra of *E. coli* tryptophanase and was assigned to a catalytically inactive/slowly reactive species (Metzler et al., 1991).

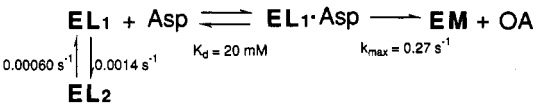
All the mutant enzymes examined in the present study showed more or less anomalous kinetic behaviors in the reactions with either amino acid or keto-acid substrates. These anomalous multiphasic processes were kinetically accounted for by postulating several enzyme species having different catalytic activities (see Appendix). The chemical and structural entities of these enzyme species remain unknown. However, they might reflect enzyme molecules in which the mode of coenzyme binding differs from one another; i.e., a substitution for Asp222 allows the coenzyme to bind in different modes and generates multiple enzyme species with different kinetic properties. The wild-type enzyme exhibited a monophasic reaction in the pre-steady-state half-reaction with each substrate (Kuramitsu et al., 1990). Among the

mutant *E. coli* AspATs so far examined, the mutant enzymes at Arg386 and Arg292, the residues apparently not involved in binding the coenzyme, showed a monophasic progress curve in the pre-steady-state reaction with each substrate studied (Inoue et al., 1989; Hayashi et al., 1989). The mutant enzymes at Tyr225, the residue forming a hydrogen bond with the coenzyme O(3′), and those at Trp140, the residue stacking with the coenzyme pyridine ring, did not show any anomalous multiphasic processes in the half-reactions (Inoue et al., 1991; Hayashi et al., 1990). Such anomalies were observed only for the reactions catalyzed by the mutant enzymes at position 222. Thus, it is highly likely that the very presence of aspartate at a position sufficiently close to N(1) of the coenzyme warrants a supreme binding mode of the coenzyme that entails its functions so as to optimize the overall catalytic efficiency by regulating properly the electronic distributions and positions of the substrate–coenzyme conjugate during the transamination reaction comprising multiple elementary steps.

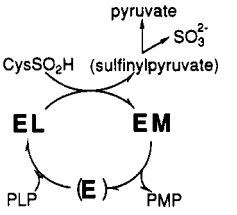
In the pre-steady-state reaction of the PLP form with aspartate, the k_{max} value decreased by 2000- or 6000-fold (4.5 or 5.2 kcal·mol^{-1} as an increase in the activation free energy for the rate-determining step) upon replacement of Asp222 by Ala or Asn, respectively (Table II). The reactions of both mutant enzymes with [2-^{2}H]aspartate revealed a deuterium isotope effect (k^H/k^D = 6.0) considerably greater than that for the reaction catalyzed by the wild-type enzyme (k^H/k^D = 2.2; Kuramitsu et al., 1990). This suggests that the removal of the α-proton of the amino acid substrate, a partially rate-determining step in the reaction of the wild-type enzyme, was impaired upon the mutation of the residue to a neutral amino acid. These findings would support the postulated role of Asp222 in stabilizing the positive charge at N(1) of PLP and thus enhancing the electron-withdrawing capacity of the coenzyme to facilitate the removal of the α-proton of the amino acid substrate. In the reaction of the PMP form of AspAT with a keto-acid substrate, the coenzyme might act as an electron sink at the step for abstraction of the proton at C(4′) in the ketimine complex. However, the most active species (EM_1 in Scheme III) of the PMP form of D222A still retained 20% of the k_{max} value of the wild-type enzyme (Table II). Thus, the requirement for a negatively charged residue at position 222 does not appear to be stringent in the reactivity of the PMP form of the enzyme toward a keto-acid substrate. To substantiate the above arguments, it should be important to learn the ionization state of N(1) of the coenzyme at least for the PLP form, PMP form, external aldimine complex, and ketimine intermediate of the wild-type and mutant enzymes. For instance, ^{15}N-NMR spectroscopy may be useful for this purpose.

The PLP form of AspAT reacts with 2-methylaspartate to form a stable external aldimine complex. On the other hand, the reaction of the PLP form of the enzyme with *erythro*-β-hydroxyaspartate gives rise to an intense absorption band around 500 nm, which is assigned to a quinonoid intermediate (Kallen et al., 1985; Arnone et al., 1985a). The reactions with these substrate analogues were compared between the wild-type and mutant enzymes (data not shown). D222E yielded spectral changes essentially similar to those of the wild-type enzyme upon the addition of these substrate analogues. The addition of 2-methylaspartate to the PLP form of D222A and D222N resulted instantaneously in an increase in the 430-nm band with a concomitant decrease in the 330-nm band, suggesting that the external aldimine may be formed also in these two mutant enzymes. The reactions of D222A and D222N with *erythro*-β-hydroxyaspartate were examined in the absence

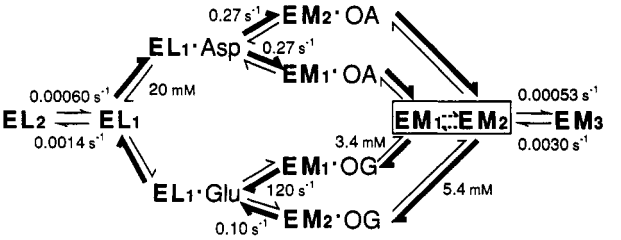
Scheme I



Scheme II



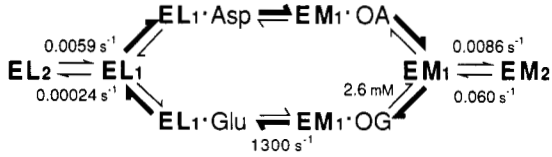
Scheme III



or presence of NADH and malate dehydrogenase, which also reacts with the transamination product of this aspartate analogue. The difference in the reaction profile between the two conditions was similar to that in the reaction with aspartate. The reactions proceeded slowly in the presence of NADH and malate dehydrogenase; the absorbance around 430 nm was decreased without giving rise to any absorption band around 500 nm; i.e., the quinonoid intermediate did not accumulate during the conversion of the PLP form into the PMP form. This may indicate that, upon the mutations, the reaction step(s) for the quinonoid formation [for instance, deprotonation at C(α)] is impaired more seriously than that for the subsequent protonation at C(4′). These findings seem to be consistent with the idea that the presence of the negative charge at position 222 would contribute much more to the abstraction of the α-proton from an amino acid substrate than to the other reaction steps such as the formation of the external aldimine intermediate or the protonation at C(4′).

Initial formation of an external aldimine complex between the enzyme-bound PLP and an amino acid substrate and subsequent cleavage of a covalent bond to the α-carbon of the substrate are common to the reactions catalyzed by all PLP-dependent enzymes. The latter reaction is thought to be facilitated by the electron-withdrawing capacity of the bound coenzyme through the positive charge at N(1) and the π-electron system extending from the pyridine ring to the α-carbon of the bound substrate (Metzler et al., 1954). The present study has clearly shown that the negative charge at position 222 enhances the catalytic function of the bound PLP molecule by stabilizing the positive charge at N(1), facilitating the cleavage of the substrate α-proton, a rate-determining step of the reaction catalyzed by AspAT, by as much as 5 kcal·mol^{-1}. A negatively charged residue or the negatively charged end (C-terminal) of an α-helix, interacting with and stabilizing the positive charge at N(1), might be needed by most of PLP-dependent enzymes to "fully" exert the electron-withdrawing ability of the bound PLP molecule. In this context, it is noted that tryptophan synthase from *Salmonella typhimurium*, another PLP-dependent enzyme whose three-dimensional structure is known, has a glutamate residue at a

Scheme IV



position interacting with N(1) of PLP (Hyde et al., 1988).

ACKNOWLEDGMENTS

We thank Nippon Mining Co. (Tokyo, Japan) for help in the stopped-flow measurements.

APPENDIX: KINETIC ANALYSIS OF D222 MUTANT ASPATS

Kinetic Schemes for Reactions Catalyzed by D222A and D222N. The pre-steady-state reaction of the PLP form of these mutant enzymes with an amino acid substrate (aspartate, cysteine sulfinate, and, presumably, glutamate) was depicted as a biphasic process. The anomalous kinetics could be explained by the reaction sequence shown in Scheme I, where only one (EL_1) of the two enzyme species can bind the substrate and interconversion between the two species ($EL_1 \rightleftharpoons EL_2$) is very slow. The parameters in Scheme I are those for D222A. Another mechanism is also possible, where both of the two species can bind the substrate but only one can catalyze the reaction further. However, these two mechanisms are kinetically equivalent under the condition of excess substrate. Hence, Scheme I was employed to simplify the story. The estimation of the rate constant for $EL_1 \rightarrow EL_2$ in Scheme I is based on the assumption that the molar extinction coefficient of EL_1 at 428 nm is equal to that of EL_2, so the concentrations of the two species at equilibrium correspond to the relative amplitudes of the two phases observed in the pre-steady-state reaction.

To examine the validity of Scheme I, the steady-state reaction of the PLP form of D222A with cysteine sulfinate was analyzed. During the reaction, PMP dissociates from this mutant enzyme, and PLP binds readily to generate the PLP form. Thus, the reaction proceeds under a steady-state condition as shown in Scheme II. The reaction was examined spectrophotometrically by following the conversion of the free PLP into PMP during the reaction of the enzyme (17 μM) with cysteine sulfinate in the presence of excess PLP (140 μM). However, the spectrophotometrical observation of the reaction was found to be complicated by the spectral changes accompanying the Schiff base formation between free PLP and cysteine sulfinate. In order to avoid the interfering effect of the Schiff base formation, the reaction was monitored at 409 nm, the position of an isosbestic point for the spectral changes resulting from the Schiff base formation. The rate constant was calculated by using a molar extinction coefficient for free PLP at 409 nm: $\epsilon^{409}_{PLP} = 3800\ M^{-1}\ cm^{-1}$ in the present buffer system (pH 8.0) as determined by using $\epsilon^{388}_{PLP} = 4900\ M^{-1}\ cm^{-1}$ in 0.1 M phosphate buffer (pH 7.0) (Peterson et al., 1954). The observed rate constant was dependent on the concentration of cysteine sulfinate ($k_{cat} = 0.030\ s^{-1}$ and $K_m = 8.1$ mM), indicating that the dissociation of PMP would not be a rate-determining step under the present condition and also that the reaction corresponding to the slow phase ($k = 0.000\,60\ s^{-1}$) observed in the pre-steady-state reaction would not significantly contribute to the steady-state turnover of the reaction. According to Scheme I, the observed k_{cat} value (0.030 s^{-1}) for the steady-state reaction with cysteine sulfinate would be corrected to 0.10 s^{-1} because the "working" species, EL_1, is 30% of the total enzyme concentration in the reaction mixture (see above). The corrected parameters, $k_{cat} = 0.10\ s^{-1}$ and $K_m = 8.1$ mM, were in good agreement with the parameters for the fast phase of the pre-steady-state reaction, $k_{max} = 0.14\ s^{-1}$ and $K_d = 12$ mM (see Results), indicating that the proposed mechanism shown in Scheme I is valid.

The pre-steady-state half-transamination reaction of the PMP form of D222A with 2-oxoglutarate exhibited a triphasic process (Figure 8). The steady-state overall-transamination reaction between aspartate and 2-oxoglutarate showed a substantial deviation from the typical "ping-pong bi–bi" mechanism (Figure 6). To analyze the anomalous kinetic behaviors of these reactions, we propose the reaction sequences shown in Scheme III. This scheme is essentially an extension of Scheme I and would be the simplest one to match with the kinetic behaviors of D222A. The following four assumptions are employed to simplify the scheme: (1) Three enzyme species in the PMP form (EM_1, EM_2, and EM_3) are defined; EM_1 and EM_2 are responsible for the fast and middle phases, respectively, and EM_3 represents an inactive species. (2) The overall reaction consists of two half-reactions, each of which comprises two intermediary aldimine and ketimine complexes as shown previously for the wild-type enzyme (Kuramitsu et al., 1990). (3) The molar ratio of EM_1, EM_2, and EM_3 corresponds to the relative amplitudes for the three phases observed in the reaction with 2-oxoglutarate (6.5:2.0:1.5) and the ratio does not change during the steady-state overall reactions. (4) The rate constants for interconversion among these three species are unknown but are presumably very slow. Thus, EL_1, EM_1, and EM_2 are catalytically active and lie on two kinetic pathways, the EM_1 route and EM_2 route, as indicated by the bold arrows in the scheme. The k_{cat} and K_m values for the overall reaction via each route could be expressed by using the pre-steady-state kinetic parameters (Kuramitsu et al., 1990) as follows:

$$k_{cat} = k_{Asp}k_{OG}/(k_{Asp} + k_{OG})$$

$$K_{m,Asp} = K_{Asp}k_{OG}/(k_{Asp} + k_{OG}) \qquad \text{(A1)}$$

$$K_{m,OG} = K_{OG}k_{Asp}/(k_{Asp} + k_{OG})$$

where $K_{m,x}$ is K_m for x and k_x and K_x are the pre-steady-state kinetic parameters k_{max} and K_d for x, respectively (x is a substrate). The calculated k_{cat} and K_m values for each route are shown in Table I. To test the validity of Scheme III, the apparent rate constants (k_{app}) for the overall reaction were calculated from these k_{cat} and K_m values at a variety of substrate concentrations, on the basis of the "ping-pong bi-bi" mechanism (Cleland, 1970). When the overall reaction is initiated by adding the PMP-form enzyme,

$$k_{app} = [(0.27[Asp][OG] \times 0.65)/(20[OG] + 0.0076[Asp] + [Asp][OG])] + [(0.073[Asp][OG] \times 0.20)/(5.5[OG] + 3.9[Asp] + [Asp][OG])] \qquad \text{(A2)}$$

where [Asp] and [OG] are the concentrations (mM) of aspartate and 2-oxoglutarate, respectively. The double-reciprocal plots for the calculated values are shown in Figure 6. Although each observed value is 2-fold larger than the corresponding calculated value, the profiles of the calculated k_{app} values versus substrate concentrations are very similar to those of the observed values, suggesting that Scheme III would be a reasonable one. The apparent "activation" by 2-oxoglutarate may be accounted for by the existence of two pathways with largely different K_m values for 2-oxoglutarate (7.6 μM for the EM_1 route, 3.9 mM for the EM_2 route). The very small K_m value for 2-oxoglutarate in the EM_1 route is mainly due to the high

activity of EM_1 for 2-oxoglutarate in comparison with the activity of EL_1 for aspartate ($k_{Asp} \ll k_{OG}$ in eq A1).

Kinetic Schemes for Reactions Catalyzed by D222E. Although the pre-steady-state half-transamination reaction of the PLP form of this mutant enzyme with aspartate was not feasible for direct observation due to unavoidable experimental difficulties as described under Results, the kinetic parameters for the reaction were estimated according to the reaction sequence shown in Scheme IV. The rate constant for the interconversion between the two species of the PLP form ($EL_1 \rightleftharpoons EL_2$) were assumed to be the same as those for the reaction with cysteine sulfinate. Scheme IV is only speculative and is based on the assumption that the slow phases (k = 0.060 and 0.0059 s^{-1}) observed in the pre-steady-state reactions are not significantly involved in the pathway of the steady-state turnover (k_{cat} = 53 s^{-1}) indicated by the bold arrows in Scheme IV. Among the possible kinetic models, Scheme IV would be a reasonably minimum mechanism to explain the kinetic behaviors of D222E. According to this scheme, the observed k_{cat} value (53 s^{-1}) for the steady-state overall reaction should be corrected to 61 s^{-1} (Table I) because the reaction was initiated by addition of the PMP form of the enzyme and the active species (EM_1) is seven-eights of the total enzyme concentration in the reaction mixture. The pre-steady-state k_{max} and K_d values for the reaction with aspartate were estimated at 64 s^{-1} and 5.2 mM (Table II), respectively, from the parameters for the steady-state reaction (k_{cat} = 61 s^{-1} and $K_{m,Asp}$ = 5.0 mM) and the rate constant for 2-oxoglutarate in the pre-steady-state reaction (k_{OG} = 1300 s^{-1}), using eq A1.

Registry No. Asp, 56-84-8; PLP, 54-47-7; AspAT, 9000-97-9; Ala, 56-41-7; Asn, 70-47-3; Glu, 56-86-0; PMP, 529-96-4; 2-oxoglutaric acid, 328-50-7; deuterium, 7782-39-0; cysteine sulfinate, 1115-65-7.

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Role of CO_2 in Proton Activation by Histidine Decarboxylase (Pyruvoyl)†,‡

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ABSTRACT: The amino acid decarboxylases that use an intrinsic pyruvoyl cofactor have been viewed in terms of the pyridoxal-P paradigm whereby a Schiff base is formed between the enzyme-bound cofactor and the substrate, setting up a cation sink for electrons of the C_α–CO_2^- bond, ejecting CO_2, and the reversal of these steps with a proton with overall *retention* stereochemistry. With histidine decarboxylase (pyruvoyl) it is found that the presence of CO_2 is required for T-exchange between histamine and water. Since the forward reaction including formation of the C–H bond does not require added CO_2, it might be assumed that the CO_2 that is formed in the fragmentation step is retained by the enzyme perhaps to assist in proton transfer. No such requirement for CO_2 has been reported for the pyridoxal-P-dependent decarboxylases which are generally thought to liberate CO_2 prior to proton transfer. In seeking a connection between bound CO_2 and proton transfer in the histidine decarboxylase reaction, one is reminded of the carboxybiotin enzymes also known for an invariant stereochemistry of retention and for the requirement that the biotin be in the carboxylated form for H-exchange to occur. Perhaps the bound CO_2 of histidine decarboxylase forms a carbamate by addition to Lys155 or to an amide group of the active site. The new carboxy group could then be the vehicle for protonating the carbon from which it originated, giving overall retention of the stereochemistry at the α-C. Carboxybiotin may serve a similar role in reverse, using the carboxylate to abstract the proton from the substrate, giving the carbanionic center to which carboxybiotin-derived CO_2 would then be transferred. Two other models are considered to explain the requirement for CO_2 for proton exchange from histamine by the decarboxylase. One of these assumes a conformational effect of CO_2 that leads to proper orientation of the proton donor. This is implied by a significant effect of CO_2 on the apparent affinity of imidazole comparing its K_i in the decarboxylase and the T-exchange reactions under the same conditions. In another model CO_2 is required for the complete reversal of reaction, histamine + CO_2 → histidine. This model requires that the abstracted T remains sequestered on the enzyme during formation of all normal reaction intermediates and can only be liberated at some step subsequent to carboxylation. This will require an extraordinary immobilization of the abstracted proton in the absence of CO_2, $k_{exchange}$ $<10^{-5}$ s^{-1}.

Histidine decarboxylase of *Lactobacillus* 30a is the best studied amino acid decarboxylase for which an intrinsic pyruvoyl residue serves as a cofactor, filling the role of pyridoxal-P in other enzymes of this class [for reviews see Boeker and Snell (1972), Gallagher et al. (1989), Recsei and Snell (1984), and Van Poelje and Snell (1990)]. Notable are extensive steady-state and inhibition studies (Recsei & Snell, 1970; Alston & Abeles, 1987), heavy atom kinetic isotope effect studies (Abell & O'Leary, 1988), specific amino acid replacements (Gelfman et al., 1991), a 2.5-Å resolved X-ray map of the enzyme alone and with histidine methyl ester, and a 3-Å map with histamine (Gallagher et al., 1989). The substrate binding site is composed of an imidazole binding pocket contributing both hydrophobic and H-bonding interactions and a substrate carboxyl binding pocket made up of seven hydrophobic residues and Lys155 and Glu197 as the only conspicuous acid/base reagents that might contribute to enzyme-mediated proton transfer to the α-carbon.

The mechanism shown in Scheme I [adopted from Recsei and Snell (1984)] uses the carbonyl of the N-terminal pyruvoyl residue to form a Schiff base with histidine, SB1.[1] Decarboxylation of SB1 produces CO_2 and the central intermediate, X, the enzyme-bound imine of histamine. The Schiff base of histamine, SB2, is formed by protonation, and histamine is liberated by its hydrolysis. Both SB1 and SB2 were established by chemical identification of sodium borohydride trapped products derived from a single incubation with histidine (Recsei & Snell, 1970). Observation of the derivative of the histidine Schiff base indicates that decarboxylation is a slow step. Consistent with this is a substantial V/K ^{13}C isotope effect

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[1] Abbreviations: SB1 and SB2, Schiff base intermediates of Scheme I; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid.

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