Pre-Steady-State Kinetics of *Escherichia coli* Aspartate Aminotransferase Catalyzed Reactions and Thermodynamic Aspects of Its Substrate Specificity†

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ABSTRACT: The four half-transamination reactions [the pyridoxal form of *Escherichia coli* aspartate aminotransferase (AspAT) with aspartate or glutamate and the pyridoxamine form of the enzyme with oxalacetate or 2-oxoglutarate] were followed in a stopped-flow spectrometer by monitoring the absorbance change at either 333 or 358 nm. The reaction progress curves in all cases gave fits to a monophasic exponential process. Kinetic analyses of these reactions showed that each half-reaction is composed of the following three processes: (1) the rapid binding of an amino acid substrate to the pyridoxal form of the enzyme; (2) the rapid binding of the corresponding keto acid to the pyridoxamine form of the enzyme; (3) the rate-determining interconversion between the two complexes. This mechanism was supported by the findings that the equilibrium constants for half- and overall-transamination reactions and the steady-state kinetic constants (K_m and k_{cat}) agreed well with the predicted values on the basis of the above mechanism using pre-steady-state kinetic parameters. The significant primary kinetic isotope effect observed in the reaction with deuterated amino acid suggests that the withdrawal of the α-proton of the substrates is rate determining. The pyridoxal form of *E. coli* AspAT reacted with a variety of amino acids as substrates. The Gibbs free energy difference between the transition state and the unbound state (unbound enzyme plus free substrate), as calculated from the pre-steady-state kinetic parameters, showed a linear relationship with the accessible surface area of amino acid substrate bearing an uncharged side chain. The substrate specificity of the *E. coli* enzyme was much broader than that of pig isoenzymes, reflecting some subtle but distinct difference in microenvironment accommodating the side chain of the substrate between *E. coli* and mammalian AspATs.

Aspartate aminotransferase (L-aspartate:2-oxoglutarate aminotransferase, EC 2.6.1.1) (AspAT[1]) catalyzes the following transamination reaction via the ping-pong bi-bi mechanism (Velick & Vavra, 1962; Kiick & Cook, 1983; Jenkins & Fonda, 1985).

L-aspartate + 2-oxoglutarate ⇌
oxalacetate + L-glutamate (1)

E. coli AspAT has only a 40% amino acid sequence homology with AspATs from higher animals, but the active site residues are well conserved (Kondo et al., 1987). Recent X-ray crystallographic studies on *E. coli* AspAT (Smith et al., 1986; Kamitori et al., 1987, 1988; Kamitori, 1989) have shown a high degree of similarity in three-dimensional structure among *E. coli* and animal AspATs (Jansonius et al., 1985; Arnone et al., 1985b; Borisov et al., 1985; Harutyunyan et al., 1985; Jansonius & Vincent, 1987).

Recently the nucleotide sequence of the *E. coli aspC* gene encoding AspAT has been determined (Kuramitsu et al., 1985; Malcolm & Kirsch, 1985). The *aspC* gene was inserted into a high-copy plasmid and AspAT was overproduced several hundred-fold in *E. coli* (Kamitori et al., 1987). This expression system has opened up opportunities to design specific mutant enzymes by oligonucleotide-directed in vitro mutagenesis and to explore systematically the structural basis of enzymic catalysis.

It is desirable to ascribe the catalytic consequence of an amino acid substitution to a particular step of the reaction sequence of enzymatic transamination. However, the enzymatic transamination process is extremely complex and each elementary step is very rapid. This makes it almost impossible to analyze each intermediary step. Steady-state kinetics of overall reactions in both forward and reverse directions can be performed routinely to yield kinetic parameters such as V_{max} and K_m for each of the four substrates. However, the physical understanding of these parameters is so complex that a simple comparison of these values between a mutant enzyme and the wild-type enzyme would not define the critical step affected by the mutation. A more informative approach is to determine the effect of a mutation on the reactivity with each of the four substrates by performing four sets of half-transamination reactions, i.e., the pyridoxal 5′-phosphate (PLP) form of the enzyme with amino acid substrates and the pyridoxamine 5′-phosphate (PMP) form with keto acid substrates. The present study demonstrates that these half-reactions with wild-type *E. coli* AspAT could be analyzed by stopped-flow techniques, thereby eliciting important kinetic parameters for individual substrates. Based on these kinetic parameters, thermodynamic aspects of enzymatic transamination will be described in the light of the transition-state theory and

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[1] Abbreviations: AspAT, aspartate aminotransferase; c-AspAT, cytosolic AspAT; m-AspAT, mitochondrial AspAT; EDTA, ethylenediaminetetraacetic acid; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; PLP, pyridoxal 5′-phosphate; PMP, pyridoxamine 5′-phosphate.

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equilibrium thermodynamics. Some of these kinetic analyses have been utilized for the study of mutant enzymes with amino acid substitution at Arg386[2] (Inoue et al., 1989) or Arg292 (Hayashi et al., 1989).

Experimental Procedures

Preparation of E. coli AspAT. *E. coli* W3110 was transformed with pKDHE19, which carries the *aspC* gene and overproduces AspAT (Kamitori et al., 1987). The transformed *E. coli* cells were grown in the medium of Kuramitsu et al. (1981). The enzyme was purified as described previously (Kamitori et al., 1987). Minor contamination was removed by further purification with a Sephacryl S-200 column equilibrated with 10 mM potassium phosphate, 10 mM potassium succinate, 0.1 M KCl, 10 μM EDTA, and 1 mM dithiothreitol at pH 7.0. The PLP (PMP) form of AspAT was prepared in the presence of 50 mM 2-oxoglutarate (10 mM cysteine sulfinate) and about a 10 M excess of PLP (PMP) over AspAT at pH 8.0 and 25 °C for 10 min. Small molecules were removed by Sephadex G-25.

Synthesis of L-[2-^{2}H]*Aspartic Acid.* DL-Asparagine (3 g) was dissolved in 50 mL of ^2H_2O (99.8%) and then evaporated to dryness. This procedure was repeated three times. The resulting powder was dissolved in 100 mL of 10 mM phosphate buffer in ^2H_2O, pH 7.5, and reacted with 100 IU of *Aeromonas caviae* amino acid racemase (Inagaki et al., 1987) at 25 °C for 12 h in a sealed bottle. The NMR (JEOL FX-90) spectrum showed more than 99% selective replacement of 2-H by deuterium (data not shown). The resulting mixture was passed through an Amicon PM10 membrane to remove the racemase and treated with 2.5 mg of *E. coli* L-asparaginase at 25 °C for 30 min. The solution was applied immediately to a Dowex 1-X8 column (15 × 150 mm, formate form). D-[2-^{2}H]Asparagine was washed out with water, and the bound L-[2-^{2}H]aspartic acid was eluted with 0.2 M formic acid and recrystallized twice from water/ethanol, yielding 1.1 g (41%) of the product [mp 330 °C (dec), $[\alpha]^{24}_D$ −26.2° (*c* 1, 6 M HCl)].

Synthesis of L-[2-^{2}H]*Glutamic Acid.* L-Glutamic acid (5 g) was deuterated as described for L-[2-^{2}H]aspartic acid synthesis. The resulting powder was dissolved in 25 mL of 10 mM phosphate buffer in ^2H_2O at pH 7.5 and reacted with 0.5 μmol of *E. coli* AspAT at 25 °C for 10 h. The reaction mixture was then applied to a Dowex 1-X8 column (15 × 150 mm, formate form). The column was washed with 100 mL of water, and L-[2-^{2}H]glutamic acid was eluted with 0.2 M formic acid and recrystallized twice from water/ethanol, yielding 3.5 g (70%) of the product [mp 188 °C, $[\alpha]^{24}_D$ −31.6° (*c* 0.9, 6 M HCl)]. The NMR spectrum showed that over 99% of 2-H was exchanged with deuterium and that the peak area for 3-H remained unchanged (data not shown), indicating that the $^1H/^2H$ exchange occurred specifically at position 2.

Spectrophotometric and pH Measurements. Absorption spectra of the protein and PLP were recorded with a Hitachi spectrophotometer, Model 557, at 25 °C. A buffer solution containing 50 mM HEPES, 0.1 M KCl, and 10 μM EDTA was used to control the pH of the enzyme solution. pH was measured at the temperature where spectrophotometric measurements were performed. pH values were the direct meter readings uncorrected for deuterium isotope effect.

Determination of Protein Concentration. The concentration of *E. coli* AspAT was determined spectrophotometrically by using a molar extinction coefficient $\epsilon_M = 4.7 \times 10^4$ M^{-1} cm^{-1} ($A_{1cm}^{1mg/mL} = 1.07$) at 280 nm and pH 8 for the PLP form of the enzyme or $\epsilon_M = 4.6 \times 10^4$ M^{-1} cm^{-1} ($A_{1cm}^{1mg/mL} = 1.04$) for the PMP form. The ϵ_M values for the coenzyme moiety were determined from the absorption change on binding of a coenzyme to apoenzyme: 2000 M^{-1} cm^{-1} for the Schiff base formed between PLP and Lys258 or 700 M^{-1} cm^{-1} for PMP at 280 nm. The value of ϵ_M for the apoprotein moiety at 280 nm was estimated to be 4.5×10^4 M^{-1} cm^{-1} on the basis of the numbers of tryptophan and tyrosine residues in *E. coli* AspAT (Kondo et al., 1987). The contributions of tryptophan and tyrosine to the ϵ_M values were estimated on the basis of 5800 and 1450 M^{-1} cm^{-1} for the two residues, respectively. These values were averages calculated from the data for several other proteins (Wetlaufer, 1962; Glazer & Smith, 1971; Hess, 1971; Imoto et al., 1972; Keil, 1971; Markland & Smith, 1971; Donovan, 1973).

Stopped-Flow Kinetic Studies. The half-transamination reaction of the PLP form of AspAT with an amino acid substrate, or that of the PMP form with a keto acid substrate, was followed by monitoring the change in absorption of the bound coenzyme at 333 (the PMP form) or 358 nm (the PLP form) at pH 8.0 and 25 °C with a Union Giken RA-1300 stopped-flow spectrophotometer. The dead time of the apparatus was 1.5 ms under the present operating conditions (5 kg/cm^2 N_2 gas pressure). A Union Giken RA-451 data-processing unit was used for curve fitting. The enzyme concentration in the reaction mixture was about 5 μM, and the concentration of substrates ranged from 0.02 to 25 mM.

Measurement of Enzymic Activity. The overall transamination reaction of AspAT proceeds via the ping-pong bi-bi mechanism (eq 1) and was measured spectrophotometrically at 280 nm by using the method described by Velick and Vavra (1962) at pH 8.0 and 25 °C, and the steady-state kinetic parameters, K_m and k_{cat}, were determined. The activity was calculated by using molar extinction coefficients of 530, 21, 0, and 0 M^{-1} cm^{-1} for oxalacetate, 2-oxoglutarate, aspartate, and glutamate, respectively.

Amino Acid Analysis. The equilibrium constant for the overall transamination reaction between aspartate and 2-oxoglutarate was measured by amino acid analysis. The reaction mixture contained 1 mM aspartate, 1 mM 2-oxoglutarate, and about 10 μM *E. coli* AspAT in 50 mM HEPES, 0.1 M KCl, and 10 μM EDTA, at pH 8.0 and 25 °C. Aliquots were withdrawn every 5 min, and the concentrations of aspartate and glutamate in the reaction mixture were determined with a JEOL Model JLC-200A amino acid analyzer. Since oxalacetate was unstable, the experimental conditions were set up so that an equilibrium state was reached within 10 min.

Results

Stopped-Flow Measurements of Half-Transamination Reactions. The PLP form of *E. coli* AspAT showed an absorption band at 358 nm. Upon addition of an amino acid substrate, the 358-nm band shifted to 333 nm, representing the conversion of the PLP form of the enzyme into the PMP form (Kallen et al., 1985). Conversely, the addition of a keto acid substrate converted the PMP form of the enzyme back to the PLP form. Thus the rapid reaction of the enzyme with a substrate was monitored by the change in absorbance at either 333 or 358 nm with a stopped-flow spectrophotometer. Every reaction studied at pH 8.0 showed fits to a monophasic exponential curve as described previously (Inoue et al., 1989), and an identical value of pseudo-first-order rate constant (k_{app}) was obtained for a particular reaction at either 333 or 358 nm (data not shown).

[2] Amino acid residues are numbered according to the sequence of cytosolic AspAT from a pig.

Table I: Kinetic Parameters of *E. coli* AspAT Catalyzed Reactions with Natural Substrates[a]

substrate	k_{max} (s^{-1})	K_d (mM)	k_{max}/K_d (M^{-1} s^{-1})
aspartate	550	4.5	1.2×10^5
glutamate	700	38	1.8×10^4
oxalacetate	800	0.035	2.3×10^7
2-oxoglutarate	600	1.3	4.6×10^5

[a] pH 8.0, 25 °C.

Table II: Values of k_{max}/K_d for *E. coli* AspAT Catalyzed Reactions with Various Amino Acids[a]

substrate	k_{max}/K_d	substrate	k_{max}/K_d
phenylalanine[b]	200	asparagine	1.4
tyrosine[b]	400	glutamine	0.74
tryptophan[b]	880	serine	0.047
leucine	2.4	threonine	0.0085
isoleucine	<0.001	glycine	<0.001
valine	<0.001	histidine	13
alanine	0.77	arginine	0.0086
methionine	22	lysine	0.012

[a] pH 8.0, 25 °C. [b] Determined by stopped-flow spectrometry.

The k_{app} value was dependent on the concentration of substrate ([S]), and the concentration dependency conformed well to the relation (eq 3) derived on the basis of the scheme (eq 2) (data not shown):

$$E + S \underset{}{\overset{K_d}{\rightleftharpoons}} ES \xrightarrow{k_{max}} ES' \quad (2)$$

$$k_{app} = k_{max}[S]/([S] + K_d) \quad (3)$$

where E is the enzyme; ES, the enzyme-substrate complex; K_d, the dissociation constant of ES to E and S; and k_{max}, the rate constant of the conversion of ES into ES'. In this scheme (eq 2), the intermolecular binding process from E + S to ES is in rapid equilibrium, and the intramolecular conversion from ES to ES' is rate determining. The values of k_{max}, K_d, and k_{max}/K_d thus obtained for each substrate are summarized in Table I. The k_{app} values for the half-reaction with aspartate were seriously affected by the reverse reaction that readily took place due to the small dissociation constant for the complex between oxalacetate and the PMP form of AspAT, which are products of the forward reaction. This contribution of oxalacetate formation to the k_{app} value was estimated as described under Appendix[3] on the basis of the kinetic parameters for oxalacetate (Table I) and the reaction sequence (eq 4) as described by Fasella and Hammes (1967) and Hammes and Schimmel (1970).

For comparison with the natural dicarboxylic substrates, the other amino acids were tested for their reactivity with the PLP form of the enzyme (Table II). In contrast to the reactions with aspartate and glutamate, none of the reactions with these amino acids exhibited saturation kinetics, showing a linear dependence of the k_{app} value on substrate concentration within the concentration range studied (up to 100 mM). Under this situation ($[S] \ll K_d$), eq 3 can be transformed into $k_{app} = (k_{max}/K_d)[S]$. Thus, the specificity constant, k_{max}/K_d, was obtained as $k_{app}/[S]$ for these nonsaturating substrates. *E. coli* AspAT showed fairly high activities for hydrophobic amino acids, particularly aromatic amino acids, as described by other workers (Gelfand & Steinberg, 1977; Powell & Morrison, 1978; Yagi et al., 1979; Gehring, 1985).

Steady-State Kinetics. Steady-state kinetic parameters, K_m and k_{cat}, for the forward reaction with the aspartate–2-oxoglutarate pair as substrates and the reverse reaction with the glutamate–oxalacetate pair were determined by the method of Velick and Vavra (1962) at pH 8.0 and 25 °C (Table III).

[3] The method for estimating the contribution of oxalacetate to the observed rate constant for aspartate is described under Appendix.

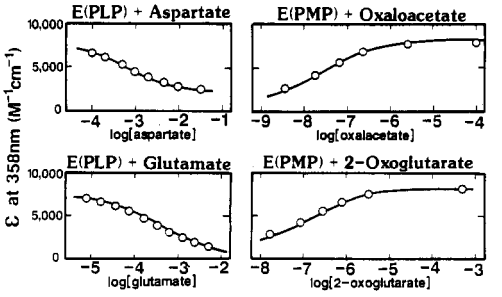


FIGURE 1: Dependence of molar extinction coefficient in *E. coli* AspAT at 358 nm on the free concentration of aspartate (A), oxalacetate (B), glutamate (C), or 2-oxoglutarate (D) at pH 8.0, 25 °C. The total protein concentrations were 1.2×10^{-5} M (A), 9.5×10^{-6} M (B), 1.9×10^{-5} M (C), and 9.2×10^{-6} (D). The solid lines show the theoretical curves obtained according to eq 5 by using the parameters shown in Table I (see the text for details).

Equilibrium Measurement of the Half-Transamination Reaction. The ratio of the PLP form of AspAT to the PMP form of AspAT at equilibrium varied with the substrate concentration. The value of the apparent molar extinction coefficient (ϵ_{app}) at 358 nm in an equilibrium state was plotted against the free substrate concentration, as shown in Figure 1.

An equilibrium scheme for the half-transamination reaction is expressed by

$$\underset{(\epsilon_{EL})}{EL} + aa \overset{K_{aa}}{\rightleftharpoons} \underset{(\epsilon_{EL\cdot aa})}{EL\cdot aa} \overset{k_i}{\underset{k_{-i}}{\rightleftharpoons}} \underset{(\epsilon_{EM\cdot ka})}{EM\cdot ka} \overset{K_{ka}}{\rightleftharpoons} \underset{(\epsilon_{EM})}{EM} + ka \quad (4)$$

where EL denotes the PLP form, EM the PMP form, aa an amino acid substrate, and ka a keto acid substrate and K_{aa} and K_{ka} are dissociation constants for amino acid and keto acid substrates, respectively, in rapid-binding processes; k_i and k_{-i} rate constants for the interconversion between EL·aa and EM·ka, which is assumed to be a rate-determining step in each direction of the reaction; and ϵ_{EL}, $\epsilon_{EL\cdot aa}$, $\epsilon_{EM\cdot ka}$, and ϵ_{EM} molar extinction coefficients of EL, EL·aa, EM·ka, and EM, respectively.

The apparent molar extinction coefficient (ϵ_{app}) for an equilibrium mixture is expressed as follows

$$\epsilon_{app} = (\epsilon_{EL}[EL] + \epsilon_{EL\cdot aa}[EL\cdot aa] + \epsilon_{EM\cdot ka}[EM\cdot ka] + \epsilon_{EM}[EM])/[E]_t \quad (5)$$

where $[E]_t$ is the total concentration of the enzyme. Dependence of ϵ_{app} on substrate concentration (Figure 1) was analyzed by using eq 5 as described by Inoue et al. (1989) on the basis of the equilibrium reaction scheme (eq 4) and pre-steady-state kinetic parameters (Table I). When the reaction of the PMP form of the enzyme with a keto acid substrate was analyzed, [ka] = [EM] was replaced by [aa] = [EL]. The experimental data fitted well the theoretical curves as shown in Figure 1, supporting the kinetic scheme shown in eq 4. The equilibrium constants for the half-transamination reactions ($K_{eq,half}$) could be also calculated from the pre-steady-state kinetic parameters (see eq 13 under Discussion).

Equilibrium Measurement of Overall Reactions. The equilibrium constant for the overall transamination reaction in eq 1, $K_{eq,overall}$ = ([glutamate][oxalacetate])/([aspartate][2-oxoglutarate]), was determined to be 0.17 by amino acid analysis.

The Primary Isotope Effect on the Half-Transamination Reaction. The isotope effect on the half-reaction of the PLP

Table III: Kinetic Parameters for the Overall-Transamination Reactions of *E. coli* AspAT[a]

	$k_{cat,f}$ (s^{-1})	$k_{cat,r}$ (s^{-1})	K_m (mM)				$k_{cat,f}/K_m$ (M^{-1} s^{-1})		$k_{cat,r}/K_m$ (M^{-1} s^{-1})	
			Asp	2OG	OA	Glu	Asp	2OG	OA	Glu
obsd	220	330	2.0	0.60	0.018	24	1.1×10^5	3.7×10^5	2.2×10^7	1.4×10^4
calcd	290	370	2.3	0.62	0.016	20	1.2×10^5	4.6×10^5	2.3×10^7	1.8×10^4

[a] pH 8.0, 25 °C. Abbreviations: Asp, aspartate; 2OG, 2-oxoglutarate; OA, oxalacetate; Glu, glutamate; obsd, observed values for the reaction with the aspartate–2-oxoglutarate pair (f, forward reaction) or the glutamate–2-oxalacetate pair (r, reversed reaction) obtained directly from spectrophotometric measurement; calcd, values calculated from the kinetic parameters of the half-reaction (see text for details).

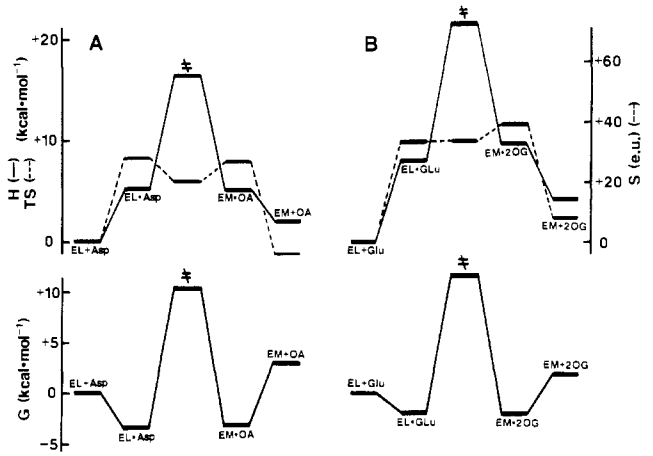


FIGURE 2: Energy profile for the transamination reaction of AspAT at pH 8.0, 25 °C. The half-transamination reaction between aspartate and oxalacetate (A) or between glutamate and 2-oxoglutarate (B). EL, PLP form of AspAT; EM, PMP form of AspAT; Asp, aspartate; OA, oxalacetate; Glu, glutamate; 2OG, 2-oxoglutarate; ‡, transition state. The free energy change (ΔG_s) for the fast binding step is $-RT$ ln $(1/K_d)$, and the activation free energy ($\Delta G^{\ddagger}$) for the rate-determining step is $RT(\ln (k_BT/h) - \ln k_{max})$. R is the gas constant; T, the absolute temperature; k_B, the Boltzmann constant; and h, the Planck constant.

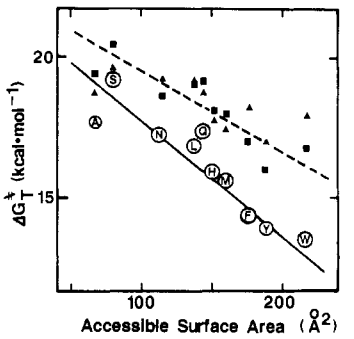


FIGURE 3: $\Delta G_T^{\ddagger}$ value for the reaction of *E. coli* (○), pig c-AspAT (▲), and pig m-AspAT (■) with uncharged amino acid substrate was calculated from eq 16 and was plotted against the accessible surface area of the substrate (Miller et al., 1987); pH 8.0, 25 °C. The single letters in the circles in the plot for *E. coli* AspAT denote amino acid substrates: S, serine; A, alanine; N, asparagine; L, leucine; Q, glutamine; H, histidine; M, methionine; F, phenylalanine; Y, tyrosine; W, tryptophan. The free energy difference ($\Delta G_T^{\ddagger}$) between the transition state (‡) and unbound enzyme plus substrate (E + S) for various uncharged amino acid substrate was calculated from the k_{max}/K_d value by the equation $\Delta G_T^{\ddagger} = \Delta G_s + \Delta G^{\ddagger} = RT(\ln (k_BT/h) - \ln (k_{max}/K_d))$ (Fersht, 1985).

form of the enzyme with an amino acid substrate was examined by using [2-^{2}H]aspartate or [2-^{2}H]glutamate as a substrate. The k_{max} value for aspartate was found to decrease from 550 to 250 s^{-1} upon deuteration at position 2 of the substrate. Similarly, the corresponding value for glutamate decreased from 700 to 330 s^{-1}. The dissociation constants (K_d) for these substrates remained unaltered. The k_{max} and K_d values for nondeuterated substrates were not affected when the reactions were performed in ^2H_2O solution. The large primary isotope effect observed in the reaction with deuterated aspartate or glutamate is consistent with the contention that abstraction of the α-hydrogen of the amino acid substrate is a rate-determining step in the half-transamination reaction with an amino acid substrate.

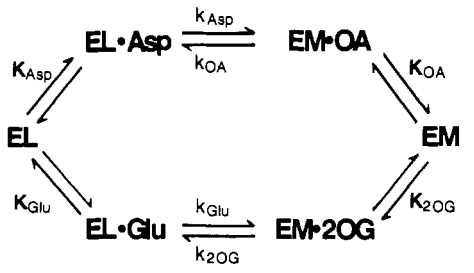
Thermodynamic Parameters for Each Reaction Step in the Half-Transamination Reaction. The pre-steady-state kinetics of AspAT with aspartate, glutamate, and their keto acids were studied at 10, 15, 20, and 25 °C to define the effect of temperature on the kinetic parameters (K_d and k_{max}) (Glasstone et al., 1941; Fersht, 1985) for each substrate (data not shown). Thermodynamic parameters for the fast binding (ΔG_s, ΔH_s, and ΔS_s) and the rate-determining steps ($\Delta G^{\ddagger}$, $\Delta H^{\ddagger}$, and $\Delta S^{\ddagger}$) were obtained from the above kinetic parameters and were combined to construct the energy profiles (Figure 2) for the half-transamination reactions.

Substrate Specificity of AspATs. AspAT is particularly active toward dicarboxylic acid substrates, aspartate and glutamate. According to X-ray crystallographic studies, the proximal and distal carboxylate groups of these substrates are in electrostatic interaction with Arg386 and Arg292, respectively (Jansonius et al., 1985; Arnone et al., 1985b; Borisov et al., 1985; Harutyunyan et al., 1985; Jansonius & Vincent, 1987; Kamitori, 1989). In fact, the mutation of these arginine residues in *E. coli* leads to a striking decrease in the reactivity with dicarboxylic substrates (Cronin & Kirsch, 1988; Hayashi et al., 1989; Inoue et al., 1989), thus providing unequivocal evidence for the critical importance of these residues in determining the substrate specificity of this enzyme. Meanwhile, the *E. coli* enzyme shows higher activity toward aromatic amino acids than animal isoenzymes (Kagamiyama et al., 1984). This may result from a subtle difference among *E. coli* and animal isoenzymes in the microenvironment of the active-site pocket. In order to compare the substrate binding sites among these AspATs, the $\Delta G_T^{\ddagger}$ values of three AspATs (*E. coli* AspAT, pig c-AspAT, and pig m-AspAT) for amino acid substrates with uncharged side chain were plotted against their accessible surface areas (Miller et al., 1987; Eisenberg & Mclachlan, 1986) (Figure 3). Interestingly, such plots gave two groups of lines: the one for *E. coli* AspAT having a steeper slope (40 cal·Å^{-2}·mol^{-1}) than the other for animal AspATs (30 cal·Å^{-2}·mol^{-1}). Since the slope may represent a measure of the hydrophobic nature of the binding pocket accommodating the side chain of a substrate, it seems likely that the binding pocket of *E. coli* AspAT is more hydrophobic than that of animal enzymes.

Discussion

Kinetics of E. coli AspAT. Fasella and Hammes (1967) analyzed the reactions of pig c-AspAT with natural substrates using mainly the temperature-jump method and found many relaxation processes. They also described difficulty in following the reactions by the stopped-flow method. However, the reactions of *E. coli* AspAT with natural substrates could be reasonably followed by a stopped-flow apparatus with a dead time of 1.5 ms as described in the present study. The direct observation of the reaction of the enzyme with α-deuterated

Scheme I



substrates allowed us to determine the primary isotope effect that resulted from the substitution with deuterium at the α-position of the amino acid substrate.

The kinetic and equilibrium data on aspartate, glutamate, and their keto acids (oxalacetate and 2-oxoglutarate) can be analyzed on the basis of the minimum mechanism shown in Scheme I, where EL represents the PLP form of AspAT and EM, the PMP form of AspAT; Asp, aspartate; OA, oxalacetate; Glu, glutamate; and 2OG, 2-oxoglutarate. In this mechanism it is assumed that the intermolecular dissociation process of EL·aa or EM·ka is in rapid equilibrium and that the intramolecular reaction process between EL·aa and EM·ka is rate determining. Steady-state kinetic parameters for overall reactions (k_{cat} and K_m) are related to the pre-steady-state kinetic parameters (k_{max} and K_d) as follows:[4]

$$k_{cat,f} = k_{Asp}k_{2OG}/(k_{Asp} + k_{2OG}) \quad (6)$$

$$K_{m,Asp} = K_{Asp}k_{2OG}/(k_{Asp} + k_{2OG}) \quad (7)$$

$$K_{m,2OG} = K_{2OG}k_{Asp}/(k_{Asp} + k_{2OG}) \quad (8)$$

$$k_{cat,r} = k_{OA}k_{Glu}/(k_{OA} + k_{Glu}) \quad (9)$$

$$K_{m,Glu} = K_{Glu}k_{OA}/(k_{OA} + k_{Glu}) \quad (10)$$

$$K_{m,OA} = K_{OA}k_{Glu}/(k_{OA} + k_{Glu}) \quad (11)$$

Then the rate constants of the overall transamination reaction ($k_{cat,f}$ and $k_{cat,r}$) are governed by the rate constants k_{Asp}, k_{OA}, k_{Glu}, and k_{2OG}. The use of eq 6–11 permits calculation of the k_{cat} and K_m values for the overall transamination reactions from the kinetic parameters obtained in the stopped-flow analysis of the half-reactions (Table I). These calculated values were found to be in good agreement with the corresponding observed values (Table III). This could be taken as showing the internal consistency of the proposed minimum mechanism. From eq 6–11, the k_{max}/K_d value determined from the pre-steady-state kinetics of the half-transamination reaction (Table I) is shown to be identical with the k_{cat}/K_m value obtained from the steady-state kinetics of the overall transamination reaction.

The minimum mechanism shown in Scheme I was also ascertained by examining the equilibrium constants for the half- ($K_{eq,half}$) and overall- ($K_{eq,overall}$) transamination reactions. The $K_{eq,half}$ value was determined precisely, as described under Results. When the equilibrium reaction was started, either with amino acid and the PLP form of AspAT or with keto acid and the PMP form of AspAT, the accumulation of intermediate species (EL·aa and EM·ka) was found to be small in the above calculation [see the Appendix in the paper by Inoue et al. (1989)]. This conclusion is also supported by the presence of an isosbestic point near 340 nm (data not shown). In this situation, the $K_{eq,half}$ value was also estimated from the spectrum at each substrate concentration (eq 12). Here $[aa]_t$ is

$$K_{eq,half} = ([EM][ka])/([EL][aa]) \simeq ([E]_t(\epsilon_{EL} - \epsilon_{app})^2)/((\epsilon_{app} - \epsilon_{EM})([aa]_t(\epsilon_{EL} - \epsilon_{EM}) - [E]_t(\epsilon_{EL} - \epsilon_{app}))) \quad (12)$$

the initial concentration of an amino acid substrate. The average $K_{eq,half}$ values thus obtained directly from spectrophotometric measurements were 0.033 for the glutamate–2-oxoglutarate pair and 0.0056 for the aspartate–oxalacetate pair. The equilibrium constant $K_{eq,half}$ is related to the pre-steady- (eq 13) and steady-state kinetic parameters (eq 14).

$$K_{eq,half} = (1/K_{Glu})(k_{Glu}/k_{2OG})K_{2OG} \quad (13)$$

$$K_{eq,half} = (k_{cat,r}/K_{m,Glu})/(k_{cat,f}/K_{m,2OG}) \quad (14)$$

In the case of the glutamate–2-oxoglutarate pair, the $K_{eq,half}$ values calculated from the pre-steady- and the steady-state kinetic parameters were 0.039 and 0.037, respectively, in agreement with the observed value of 0.033. In the case of the aspartate–oxalacetate pair, corresponding values were 0.0053, 0.0050, and 0.0056, respectively. The equilibrium constant for the overall transamination reaction (eq 1), $K_{eq,overall}$, was determined to be 0.17 by amino acid analysis. The $K_{eq,overall}$ value calculated from the ratio of the two $K_{eq,half}$ values ($K_{eq,half,Asp\text{-}OA}/K_{eq,half,Glu\text{-}2OG}$) was 0.17. The equilibrium constant $K_{eq,overall}$ is also related to the pre-steady- (eq 15) and steady-state kinetic parameters (eq 16). The calculated values

$$K_{eq,overall} = (k_{Asp}/K_{Asp})(K_{OA}/k_{OA})(k_{2OG}/K_{2OG})(K_{Glu}/k_{Glu}) \quad (15)$$

$$K_{eq,overall} = (k_{cat,f}/K_{m,Asp})(K_{m,OA}/k_{cat,r})(k_{cat,f}/K_{m,2OG})(K_{m,Glu}/k_{cat,r}) \quad (16)$$

from eqs 15 and 16 were both 0.14. The agreement of the observed value with the values calculated on the basis of Scheme I could also be considered to show the internal consistency of the proposed minimum mechanism shown in Scheme I.

Energy Profile for AspAT Catalysis. The free energy levels of enzyme–substrate complexes were lower than those of unbound enzyme plus free substrate by 2–6 kcal·mol^{-1} (Figure 2). The activation free energy changes (ΔG^*) were +13–14 kcal·mol^{-1}. Formation of these intermediate enzyme–substrate complexes would serve to reduce the high free energy of activation for the nonenzymic reaction (Fersht, 1985).

The enthalpy changes (ΔH) upon binding of substrate were +3 to +8 kcal·mol^{-1}. These values agreed well with those obtained from recent calorimetric measurements (unpublished observation). The increase in ΔH may be attributed partly to the dissociation of bound water molecules from both the substrate and the active site of the enzyme. When the entropy change (ΔS) was expressed in unitary units ($\Delta S_u = \Delta S + R$ ln 55.5), the ΔS_u values upon binding of the dicarboxylic keto acid substrates were about +40 eu ($T\Delta S_u$ = +12 kcal·mol^{-1}). The ΔS_u values for the protonation of two carboxylate groups in dicarboxylic acid ($^-OOC\text{–}(CH_2)_n\text{–}COO^-$, n = 1–5) have been reported to be +57–66 eu, and ΔH is found to be −1 to +1 kcal·mol^{-1} (Christensen et al., 1967). The large entropy changes for these model compounds have been ascribed to the release of the water molecules, which are oriented around the negatively charged carboxylates and positively charged protons. The ΔS values for model compounds are larger than that for the binding of substrate to the enzyme. This difference may arise from the fact that the number of hydrating water molecules on the side chains of Arg292 and Arg386, substrate-binding residues, are small (Jansonius et al., 1987) as compared with that for a side chain of the arginine molecule

[4] See Table V in the paper by Velick and Vavra (1962).

in water. Therefore, the large increase in both enthalpy and entropy upon substrate binding will be due partly to the release of hydrating water molecules from the free substrate and the substrate-binding site in the enzyme.

The activation enthalpy changes ($\Delta H^{\ddagger}$) were +11–14 kcal·mol^{-1}. These large values would reflect, for instance, the withdrawal of the α-hydrogen atom from the amino acid substrates. This step may be a rate-determining one in the half-transamination reaction, because a fairly large primary isotope effect was observed upon α-deuteration of aspartate or glutamate. In contrast, the activation entropy changes ($\Delta S^{\ddagger}$) were small, indicating that the activation to the transition state involves an intramolecular process.

Catalytic Mechanism of E. coli AspAT. The present results of kinetic analyses on *E. coli* AspAT catalyzed reactions appear to conform to the catalytic mechanism derived mainly from the studies on animal AspATs (Kirsch et al., 1984; Arnone et al., 1985a). The stopped-flow kinetic data for pig c-AspAT catalyzed half-reactions were analyzed on the basis of the minimum mechanism (Scheme I) and were found to show essentially the same kinetic behaviors as those of *E. coli* AspAT catalyzed reactions except that there were some differences in the values of kinetic parameters between pig c-AspAT and *E. coli* AspAT (data not shown). Thus the transamination reaction catalyzed by the *E. coli* enzyme could be explained by a catalytic mechanism virtually identical with that proposed for the reaction catalyzed by animal AspATs (Kirsch et al., 1984; Arnone et al., 1985a). This mechanism can be described briefly according to Scheme I, EL represents the PLP form of the enzyme, in which Lys258 forms the internal aldimine with PLP. When an amino acid substrate (aa) binds to the PLP form of AspAT (EL), a new aldimine bond is formed between the substrate and PLP (EL·aa). The next step is the withdrawal of the 2-hydrogen atom of the substrate. The deprotonation leads to the formation of a quinonoid intermediate, which should represent one transition-state structure. The next reaction is the addition of a proton to the coenzyme C4′, leading to the formation of the ketimine intermediate (EM·ka). The ketimine intermediate is then hydrolyzed to form a keto acid substrate (ka) and the PMP form of the enzyme (EM).

The issue of rate-determining step(s) in the transamination reaction remains controversial (Arnone et al., 1985a; Jansonius & Vincent, 1987). The present finding that a significant kinetic isotope effect was observed in the rate of reaction with α-deuterated glutamate and aspartate supports the contention that the withdrawal of the α-proton of these substrates is a rate-determining step in the half-transamination reaction with aspartate or glutamate.[5]

Several mutant enzymes of *E. coli* AspAT so far designed have been analyzed for half-transamination reactions under pre-steady-state conditions according to the minimum mechanism (Scheme I) in an attempt to define any independent effect of a mutation on the reactivity with each substrate and to elicit kinetic parameters amenable to thermodynamic consideration based on the transition-state theory. This approach for kinetic analysis of the Arg386 → Lys mutant enzyme of *E. coli* AspAT has been reported (Inoue et al., 1989). Details of such analysis on the other mutant enzymes will appear elsewhere.

Substrate Specificity of AspATs. The plots of the $\Delta G_T^{\ddagger}$ values for uncharged amino acid substrates against their accessible surface areas gave two groups of lines. *E. coli* AspAT gave a slope of 40 cal·Å^{-2}·mol^{-1} and pig isozymes of 30 cal·Å^{-2}·mol^{-1} (Figure 3). The free energy difference for the transfer of an amino acid side chain from water to an organic solvent (Cornette et al., 1987) is roughly proportional to their accessible surface area (Chothia, 1974; Miller et al., 1987). The free energy obtained from hydrophobic interaction is 24–26 cal·Å^{-2}·mol^{-1} when ethanol and dioxane are used as solvents. The hydrophobic surfaces of an unbound enzyme and an unbound substrate are both accessible to water when they are in a free state, but both surfaces are involved in hydrophobic interactions in the bound state. Thus a hydrophobic interaction can confer free energy of about 50 cal·Å^{-2}·mol^{-1} upon the binding of the substrate to the enzyme. This value is slightly larger than the values obtained in the present study for *E. coli* and animal AspATs. The difference in the slope among *E. coli* and animal enzymes suggests that the binding pocket of the *E. coli* enzyme may be more hydrophobic than that of pig isoenzymes. Arg292 constitutes part of the active-site pocket. When this residue was replaced by hydrophobic amino acids such as valine or leucine, the mutant enzymes exhibited a striking preference for aromatic amino acids as substrates (Hayashi et al., 1989). This finding provides support for the presence of a correlation between the hydrophobicity of the pocket and the substrate specificity.

Appendix

Analysis of the Half-Transamination Reaction of AspAT with Aspartate. The half-transamination reaction of the PLP form of *E. coli* AspAT (EL) with aspartate (Asp) was followed by the stopped-flow method, and the contribution of the reverse reaction of the PMP form of AspAT (EM) with oxalacetate (OA) was estimated as follows:

$$\mathrm{EL} + \mathrm{Asp} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \mathrm{EL{\cdot}Asp} \underset{k_{OA}}{\overset{k_{Asp}}{\rightleftharpoons}} \mathrm{EM{\cdot}OA} \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} \mathrm{EM} + \mathrm{OA} \tag{a1}$$

The rate equations for EL and EL·Asp are

$$d[EL]/dt = -k_1[EL][Asp] + k_{-1}[EL{\cdot}Asp] \tag{a2}$$

$$d[EL{\cdot}Asp]/dt = k_1[EL][Asp] - k_{-1}[EL{\cdot}Asp] + k_{OA}[EM{\cdot}OA] - k_{Asp}[EL{\cdot}Asp] \tag{a3}$$

The concentration for each species ([A]) at a given time is expressed by the final equilibrium concentration ([$\bar{A}$]) and the deviation from the equilibrium (Δ[A]).

$$[A] = [\bar{A}] + \Delta[A] \tag{a4}$$

From eqs a2–a4, eq a5 is obtained.

$$d(\Delta[EL] + \Delta[EL{\cdot}Asp])/dt = k_{OA}\Delta[EM{\cdot}OA] - k_{Asp}\Delta[EL{\cdot}Asp] \tag{a5}$$

If the binding processes are in rapid equilibria, the following equations hold:

$$k_1[EL][Asp] = k_{-1}[EL{\cdot}Asp] \tag{a6}$$

$$k_3[EM{\cdot}OA] = k_{-3}[EM][OA] \tag{a7}$$

By substitution of eq a4 for [EL], [Asp], and [EL·Asp] in eq a6 under the condition $[\overline{EL}] + [\overline{Asp}] \gg \Delta[EL]$, Δ[Asp] and by use of $\Delta[EL] = \Delta[Asp]$, $\Delta[EL{\cdot}Asp]$ is expressed as follows:

$$\Delta[EL{\cdot}Asp] = (k_1([\overline{EL}] + [\overline{Asp}])/k_{-1}){\cdot}\Delta[EL] \tag{a8}$$

[5] An attempt to examine a possible isotope effect on the rate of reaction of the PMP form of the enzyme with a keto acid substrate was made by preparing [4′-^{2}H]PMP-reconstituted *E. coli* AspAT. However, a fairly rapid exchange of the 4′-^{2}H with solvent protons even in the absence of a keto acid substrate (Tobler et al., 1986) hampered a precise determination of the primary isotope effect on the reaction of the [4′-^{2}H]PMP-reconstituted enzyme with a keto acid substrate.

Similarly, introduction of eq a4 and [EM] = [OA] into eq a7 yields

$$k_3(\overline{[EM{\cdot}OA]} + \Delta[EM{\cdot}OA]) = k_{-3}(\overline{[EM]} + \Delta[EM])^2 \quad (a9)$$

Under the condition $\overline{[EM{\cdot}OA]} \gg \Delta[EM{\cdot}OA]$

$$\Delta[EM] = (k_3/(2k_{-3}\overline{[EM]}))\Delta\overline{[EM{\cdot}OA]} \quad (a10)$$

Mass conservation requires

$$\Delta[EL] + \Delta[EL{\cdot}Asp] + \Delta[EM{\cdot}OA] + \Delta[EM] = 0 \quad (a11)$$

Eqs a8, a10, and a11 give

$$\Delta[EM{\cdot}OA] = -((1 + k_1(\overline{[EL]} + \overline{[Asp]})/k_{-1})/(1 + k_3/(2k_{-3}\overline{[EM]})))\Delta[EL] \quad (a12)$$

Finally, by substituting eqs a8 and a12 for $\Delta[EL{\cdot}Asp]$ and $\Delta[EM{\cdot}OA]$ in eq a5, respectively, the following equation is obtained:

$$(1 + k_1(\overline{[EL]} + \overline{[Asp]})/k_{-1}))(d(\Delta[EL])/dt) = -(k_{OA}(1 + k_1(\overline{[EL]} + \overline{[Asp]})/k_{-1})/(1 + k_3/(2k_{-3}\overline{[EM]})) + (k_1k_{Asp}/k_{-1})(\overline{[EL]} + \overline{[Asp]}))\Delta[EL] \quad (a13)$$

Solution of the differential equation eq a13 yields

$$k_{app} = (k_{Asp}(\overline{[EL]} + \overline{[Asp]})/(K_{Asp} + \overline{[EL]} + \overline{[Asp]}) + 2k_{OA}\overline{[EM]}/(K_{OA} + 2\overline{[EM]}) \quad (a14)$$

where k_{app} is the observed rate constant; K_{Asp}, k_{-1}/k_1; and K_{OA}, k_3/k_{-3}.

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Studies of the Cryptic Allylic Pyrophosphate Isomerase Activity of Trichodiene Synthase Using the Anomalous Substrate 6,7-Dihydrofarnesyl Pyrophosphate†

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ABSTRACT: Two enantiomeric analogues of farnesyl pyrophosphate (**1**) were tested as inhibitors and anomalous substrates of trichodiene synthase, which catalyzes the cyclization of *trans,trans*-farnesyl pyrophosphate (**1**) to the sesquiterpene hydrocarbon trichodiene (**2**). The reaction has been shown to involve preliminary isomerization of **1** to the tertiary allylic isomer nerolidyl pyrophosphate (**3**) which is cyclized without detectable release of the intermediate from the active site of the cyclase. Both (7*S*)-*trans*-6,7-dihydrofarnesyl pyrophosphate (**7a**) and (7*R*)-*trans*-6,7-dihydrofarnesyl pyrophosphate (**7b**), prepared from (3*R*)- and (3*S*)-citronellol (**9a** and **9b**), respectively, proved to be modest competitive inhibitors of trichodiene synthase. The values of K_i(**7a**), 395 nM, and K_i(**7b**), 220 nM, were 10–15 times the observed K_m for **1** and half the K_i of inorganic pyrophosphate alone. Incubation of either **7a** or **7b** with trichodiene synthase resulted in formation of a mixture of products which by radio/gas–liquid chromatographic and GC/selected ion mass spectrometric analysis was shown to be composed of 80–85% isomeric trienes **19–21** and 15–20% allylic alcohols **12** and **18**. Examination of the water-soluble products resulting from incubation of **7a** also revealed the generation of 24% of the isomeric *cis*-6,7-dihydrofarnesyl pyrophosphate (**26**). The combined rate of formation of anomalous alcoholic and olefinic products was 10% the V_{max} determined for the conversion of **1** to **2**. The results can be explained by initial enzyme-catalyzed isomerization of dihydrofarnesyl pyrophosphate (**7**) to the corresponding tertiary allylic isomer dihydronerolidyl pyrophosphate (**8**). Since the latter intermediate is unable to cyclize due to the absence of the 6,7-double bond, ionization of **8** and quenching of the resulting ion pair by deprotonation, capture of water, or collapse to the isomeric primary pyrophosphate esters will generate the observed spectrum of anomalous products.

Trichodiene synthase catalyzes the cyclization of *trans,trans*-farnesyl pyrophosphate (**1**) (FPP)[1] to trichodiene (**2**), the parent hydrocarbon of the trichothecane family of antibiotics and mycotoxins (Cane et al., 1981a, 1985). The enzyme has been isolated from a variety of sources, including *Fusarium sambucinum* (*Gibberella pulicaris*) (Hohn & Beremand, 1989a) and *Fusarium sporotrichioides* (Hohn & VanMiddlesworth, 1986), producers of the potent mycotoxins diacetoxyscirpenol and T-2 toxin, respectively, as well as the apple mold fungus *Trichothecium roseum* (Cane et al., 1981a, 1985; Evans et al., 1973), the source of the antibiotic trichothecin. The *F. sporotrichioides* cyclase has been purified to

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[1] Abbreviations: FFAP, free fatty acid phase; FPP, farnesyl pyrophosphate; GC/MS, capillary gas chromatography/mass spectrometry; GLC, gas–liquid chromatography; Hepes, *N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethanesulfonate; HPLC, high-pressure liquid chromatography; HRMS, high-resolution mass spectrometry; MPLC, medium-pressure liquid chromatography; NMR, nuclear magnetic resonance; PP_i, inorganic pyrophosphate; THF, tetrahydrofuran.

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