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Tyrosine 70 Fine-Tunes the Catalytic Efficiency of Aspartate Aminotransferase†

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ABSTRACT: The aspartate aminotransferase mutant Y70F exhibits k_{cat} = 8% and k_{cat}/K_M = 2% of the wild type values for the transamination of aspartate and α-ketoglutarate. The affinity of the enzyme for the noncovalently bound inhibitor maleate is reduced 17-fold by the mutation, while only a 2.5-fold reduction is observed for α-methylaspartate, which forms a stable, covalent external aldimine. The high population of the quinonoid intermediate formed in the reaction of the wild type with β-hydroxyaspartate is more than 75% diminished by the mutation. The values of the Y70F C^{α}-H kinetic isotope effects for the aspartate reaction are larger than those of wild type (^{D}V = 2.4 vs 1.52; $^{D}(V/K)$ = 2.5 vs 1.7). Conversely, the Y70F value of $^{D}(V/K)$ for the glutamate reaction is decreased compared to wild type (1.75 vs 2.5). These results, combined with previous studies of Lys258 mutants, eliminate Tyr70 as an essential component of the catalytic apparatus, with the caveat that the functionality of the deleted hydroxyl group is possibly replaced by a water molecule.

Aspartate aminotransferase is a pyridoxal 5′-phosphate (PLP)[1] dependent enzyme that preferentially catalyzes the reversible interconversion of the dicarboxylic α-amino acids, L-aspartate and L-glutamate, and the corresponding α-keto acids, oxalacetate and α-ketoglutarate. It is the most extensively studied PLP-dependent enzyme involved in amino acid metabolism. Not only have numerous classical solution studies elucidated details of its mechanism but also X-ray crystallographic analyses have now provided three-dimensional atomic models around which this extensive body of data can be organized [for reviews see Jansonius and Vincent (1987) and Christen and Metzler (1985)]. The X-ray structure provided a basis for the formulation of a detailed mechanistic proposal (Kirsch et al., 1984), which is currently being tested by analyses of active site mutants generated by site-directed mutagenesis techniques (Cronin & Kirsch, 1988; Toney & Kirsch, 1989; Kuramitsu et al., 1987; Inoue et al., 1989).

The reaction pathway for aspartate aminotransferase includes several distinct intermediates (Scheme I). The chemically difficult step, and the one that differentiates transamination from other PLP-catalyzed reactions, is the 1,3 prototropic shift. It involves abstraction of a proton from C^{α} of the amino acid and its transfer to C4′ of the coenzyme. This step was originally proposed to be catalyzed by the active site residue Lys258 in concert with the hydroxyl group of Tyr70 (Kirsch et al., 1984). Considered most likely was the involvement of Tyr70 as a member of a charge-relay network between Lys258 and the coenzyme phosphate.

A direct probe of the function of Tyr70 in the AATase mechanism is provided by the site-directed mutant Y70F, in which Tyr70 is changed to Phe. Described herein are analyses of this mutant constructed with the *Escherichia coli* AATase. The bacterial enzyme facilitates molecular biological experiments. All active site residues in the prokaryotic and euka-

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[1] Abbreviations: AATase, aspartate aminotransferase (EC 2.6.1.1); wild type, wild type *E. coli* AATase; Y70F, *E. coli* AATase in which Tyr70 has been changed to phenylalanine by site-directed mutagenesis; E-PLP and E-PMP, pyridoxal 5′-phosphate and pyridoxamine 5′-phosphate forms of AATase, respectively; HEPES, *N*-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid; β-hydroxyaspartate, *erythro*-β-L-hydroxyaspartate; α-methylaspartate, α-methyl-D,L-aspartate; KIE, kinetic isotope effect.

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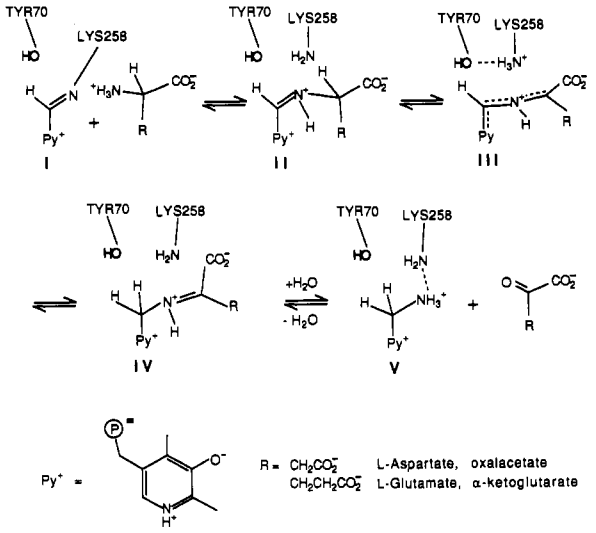
Scheme I: Mechanism of Transamination by Aspartate Aminotransferase[a]



[a] E-PLP (I) reacts with amino acid substrate to form the covalent external aldimine intermediate (II). The external aldimine is tautomerized to the ketimine (IV) possibly via the quinonoid (III). The ketimine is hydrolyzed to E–PMP (V) and keto acid product. The transamination cycle is completed when a second keto acid reacts through the reverse of this sequence to give E–PLP and amino acid product. The hydroxyl moiety of Tyr70 is highlighted to emphasize the chemical group being examined by the Y70F mutation.

ryotic isozymes, as well as the overall and active site structures, are conserved (Kondo et al., 1987; Fotheringham et al., 1986; Smith et al., 1989).

The present paper is concerned with the reactions of the holoenzymes of wild type and Y70F with substrates and inhibitors. The following one analyzes the interactions of the apoenzymes with coenzymes. A preliminary report has been published (Toney & Kirsch, 1987).

Experimental Procedures

A Perkin-Elmer λ4B spectrophotometer was used for most absorbance measurements. Stopped-flow studies were carried out with a Union-Giken Model RA-401 instrument interfaced with an OLIS data acquisition/reduction system. All experiments were conducted at 25 °C in 200 mM HEPES–KOH, pH 7.5/100 mM potassium chloride, unless noted otherwise. Concentrations of pure proteins were determined spectrophotometrically with use of A_{205} (ϵ = 31 mL mg^{-1}; Scopes, 1974) or A_{280} (ϵ = 0.86 mL mg^{-1}; Yagi, et al., 1979). The AATase concentrations are given for subunits (MW = 43 500). Error propagation was performed according to standard procedures (Bevington, 1969).

Materials. Malate dehydrogenase, NADH, aspartate, α-ketoglutarate, glutamate, oxalacetate, α-methyl-D,L-aspartate, Trizma base, bromopyruvic acid, PLP, and PMP were purchased from Sigma. HEPES and cysteinesulfinic acid were from Aldrich. Maleic acid was obtained from Matheson, Coleman, and Bell. 5′-Phosphopyridoxyl-L-aspartate was prepared essentially by the procedure of Severin et al. (1969). *erythro*-β-Hydroxyaspartate was prepared by the method of Jenkins (1979). [α-^{2}H]-L-Aspartate and [α-^{2}H]-L-glutamate were gifts from Dr. D. A. Julin and were prepared by the method of Dougherty et al. (1972). The construction of Y70F has been described (Toney & Kirsch, 1987).

Enzyme Preparations. Wild type and Y70F enzymes were prepared according to Cronin and Kirsch (1988). All preparations were greater than 95% pure as judged by SDS–PAGE analysis.

Transamination Kinetics. AATase was assayed by coupling the released oxalacetate to malate dehydrogenase, which was present at 20 units/mL. The malate dehydrogenase preparation, as supplied by the vendor, has AATase activity that only becomes manifest when excess coenzyme (necessary for the assay of Y70F but not wild type) is added to the reaction mixtures. The malate dehydrogenase supplied AATase activity was greatly reduced by reacting the enzyme preparation with 50 mM bromopyruvate and 5 mM cysteinesulfinate for 4 h at room temperature in 100 mM Tris-HCl, pH 8.0 (Morino & Okamato, 1970). Care was taken to measure the blank rate in the absence of AATase in initial rate studies.

Kinetic parameters for the substrate pair aspartate and α-ketoglutarate were obtained by measuring initial rates for five concentrations of aspartate at each of five concentrations of α-ketoglutarate, generally ranging from 0.5 to 5K_M. These 25-point data sets were fitted by nonlinear regression to the equation describing the ping-pong mechanism (Velick & Vavra, 1962), eq 1, where K_{Asp} and $K_{\alpha KG}$ are Michaelis con-

$$\frac{v}{[E]_t} = \frac{k_{cat}}{1 + K_{Asp}/[Asp] + K_{\alpha KG}/[\alpha KG]} \quad (1)$$

stants for aspartate and α-ketoglutarate, respectively. The disposable parameters in the regression are k_{cat}, K_{Asp}, and $K_{\alpha KG}$. No substrate-dependent inhibition was noted at the highest concentrations employed.

Inhibition constants for maleate and α-methylaspartate were determined by holding the concentration of one substrate constant at its K_M and varying the concentrations of inhibitor and the second substrate. For the maleate experiments, the aspartate concentration was constant, and the α-ketoglutarate concentration was constant for the α-methylaspartate studies. The data sets obtained were fitted to the equations describing inhibition of the ping-pong mechanism. Equation 2 was used

$$\frac{v}{[E]_t} = k_{cat}/1 + K_{Asp}/[Asp](1 + [maleate]/K_i^{Asp}) + K_{\alpha KG}/[\alpha KG](1 + [maleate]/K_i^{\alpha KG}) \quad (2)$$

for maleate, which binds to both E–PLP and E–PMP. In eq 2, K_i^{Asp} and $K_i^{\alpha KG}$ are the inhibition constants against substrates aspartate and α-ketoglutarate, respectively. Equation 3 was

$$\frac{v}{[E]_t} = k_{cat}/1 + K_{Asp}/[Asp](1 + [\alpha\text{-methylaspartate}]/K_i^{Asp}) + K_{\alpha KG}/[\alpha KG] \quad (3)$$

fitted to the data for α-methylaspartate, which binds significantly only to E–PLP. In each case, the independently determined K_M values for both substrates were held constant, and the values of the K_i and k_{cat} parameters were adjusted.

Measurement of the steady-state activity with β-hydroxyaspartate was performed as described by Jenkins and Harruff (1979). The α-ketoglutarate concentration was constant at 5 mM, which ensures that the β-hydroxyaspartate half-reaction is fully rate-determining.

Stopped-flow experiments were performed by monitoring either 330-nm absorbance due to enzyme-bound PMP or 360-nm absorbance due to enzyme-bound PLP, in single-turnover half-reactions (Cronin & Kirsch, 1988). The absorbance vs time data conformed well to single-exponential processes. The k_{obs} vs substrate concentration data were fitted to a rectangular hyperbola, eq 4 (Wilkinson, 1961) to obtain

$$k_{obs} = \frac{k_{max}[S]}{K_{app} + [S]} \quad (4)$$

values of k_{max} and K_{app}, the limiting first-order rate constant

Table I: Kinetic Parameters for Wild Type and Y70F *E. coli* Aspartate Aminotransferase Reactions with the Substrate Pair L-Aspartate and α-Ketoglutarate[a]

	k_{cat} (s^{-1})	K_{Asp} (mM)	$K_{\alpha KG}$ (mM)	k_{cat}/K_{Asp} × 10^{-3} (M^{-1} s^{-1})	$k_{cat}/K_{\alpha KG}$ × 10^{-3} (M^{-1} s^{-1})
wild type	160 (2)	1.83 (0.04)	0.47 (0.01)	87 (2)	340 (10)
Y70F	13 (1)	5.6 (0.5)	2.1 (0.2)	2.3 (0.3)	5.9 (0.8)
Y70F/wild type	0.08 (0.01)	3.1 (0.3)	4.6 (0.5)	0.026 (0.003)	0.017 (0.002)

[a] Conditions: 20 mM HEPES–KOH/100 mM potassium chloride, pH 7.5, 25 °C. The AATase activity was measured with the malate dehydrogenase coupled assay. Standard errors are given in parentheses.

Table II: Competitive Inhibition of *E. coli* Aspartate Aminotransferases by Maleate and α-Methyl-D,L-aspartate[a]

	maleate		α-methyl-aspartate
	K_i^{Asp} (mM)[b]	$K_i^{\alpha KG}$ (mM)[c]	K_i^{Asp} (mM)[b]
wild type	5.1 (0.2)	5.6 (0.5)	1.2 (0.1)
Y70F	87 (9)	29 (5)	3.0 (0.1)
Y70F/wild type	17 (2)	5 (1)	2.5 (0.2)

[a] Conditions: 200 mM HEPES–KOH/100 mM potassium chloride, pH 7.5, 25 °C. The AATase activity was measured by the malate dehydrogenase coupled assay. Standard errors are given in parentheses. [b] Inhibition constant for E–PLP. The α-methylaspartate concentrations given are for the L isomer. [c] Inhibition constant for E–PMP.

at infinite substrate concentration and the apparent dissociation constant, respectively. The aspartate and α-ketoglutarate concentrations were varied from 0.5 to 30 mM and 0.1 to 16 mM, respectively, for Y70F. Five to seven traces were monitored for each concentration of substrate.

Kinetic Isotope Effects. The concentrations of prepared stock solutions of [α-^{2}H]aspartate and [α-^{1}H]aspartate were determined by adding small aliquots to 1-mL solutions containing 200 mM HEPES–KOH buffer, pH 7.5, 5 units each of wild type enzyme and malate dehydrogenase, 200 μM NADH, and 10 mM α-ketoglutarate. The 340-nm absorbance was measured first before aspartate addition and again after full consumption (when stable absorbance readings were obtained). Transamination of aspartate is irreversible under these conditions. The concentrations were calculated from the changes in 340-nm absorbance by use of an extinction coefficient of 6220 M^{-1} cm^{-1} for NADH. Cocktails containing all substrates and enzymes except aspartate were prepared for each wild type and Y70F sample. The α-ketoglutarate concentrations were 5-fold larger than K_M for each enzyme. The reactions were monitored in a palindromic sequence to minimize the effects of enzyme inactivation on the analysis. The initial rates were fitted to the Michaelis–Menten equation.

The reverse reactions, with glutamate and oxalacetate as substrates, were monitored by the decrease in absorbance due to the disappearance of oxalacetate (Velick & Vavra, 1962; Julin & Kirsch, 1989). Oxalacetate concentrations were held constant at 1.0 mM for both enzymes. Glutamate-catalyzed decarboxylation of oxalacetate became problematic at glutamate concentrations greater than 10 mM; thus, low concentrations were used in order to obtain rates that were linearly dependent on glutamate concentration. The rate vs glutamate concentration data were fitted to straight lines, the slopes of which were taken as the values of V/K.

Results

Kinetic Parameters. The kinetic parameters for wild type and Y70F enzymes are given in Table I. Y70F has a k_{cat} value that is 8% that of wild type. The Michaelis constants for Y70F are increased 3-fold for aspartate and 4.5-fold for α-ketoglutarate relative to wild type. The second-order rate constants (k_{cat}/K_M) describing the reactions of aspartate and α-ketoglutarate with Y70F are significantly less than those for wild type, the relative magnitudes being 2.6 and 1.7%, respectively. The parameters reported here are 2–3-fold lower than those reported previously (Toney & Kirsch, 1987) due to the AATase activity in the malate dehydrogenase preparation, which was not previously taken into account.

The kinetic parameters for the aspartate and α-ketoglutarate half-reactions of Y70F were measured by stopped-flow spectrophotometry. The Y70F (E–PLP) half-reaction with aspartate is described by k_{max} = 37 ± 1 s^{-1} and K_{app} = 4.1 ± 0.5 mM. The Y70F (E–PMP) half-reaction with α-ketoglutarate has k_{max} = 105 ± 9 s^{-1} and K_{app} = 9.6 ± 1.6 mM. For comparison, the corresponding values for wild type measured at pH 8.0, I_c = 0.1 (Inoue et al., 1989), are, for aspartate, k_{max} = 530 ± 30 s^{-1} and K_{app} = 4.0 ± 0.5 mM and, for α-ketoglutarate, k_{max} = 500 ± 50 s^{-1} and K_{app} = 0.8 ± 0.1 mM.

Inhibition by Maleate and α-Methyl-D,L-aspartate. Table II contains the values of the inhibition constants for the competitive inhibitors maleate and α-methylaspartate. The ratio of Y70F to wild type maleate dissociation constants is 17 for E-PLP (K_i^{Asp}) and 5 for E–PMP ($K_i^{\alpha KG}$). α-Methylaspartate, which binds only to E-PLP under these conditions, is bound 2.5-fold more weakly to Y70F.

The Quinonoid Species. The absorption band near 490 nm in AATase solutions containing homologous amino acid/keto acid pairs at equilibrium is generally attributed to the *p*-quinonoid resonance form of carbanionic intermediates (III, Scheme I) (Jenkins, 1964). Neither wild type nor Y70F exhibits significant absorption near 490 nm when incubated with 100 mM glutamate and 5 mM α-ketoglutarate. Figure 1A compares the absorption spectra of wild type and Y70F, each with 50 mM aspartate and 5 mM OAA. A small, ~490-nm absorption band is present in the wild type sample, whereas it is absent in that of Y70F under these conditions.

The reaction of E–PLP with β-hydroxyaspartate gives rise to a long-lived quinonoid intermediate with mammalian AATase (Jenkins, 1964). It is also found in *E. coli* wild type (Inoue et al., 1989). The contrasting absence of a quinonoid band in Y70F solutions containing aspartate and oxalacetate with its presence in wild type solutions prompted an examination of the interaction of β-hydroxyaspartate with Y70F. The spectra of Figure 1B show dramatic differences between the enzyme-bound species for wild type and Y70F. Wild type has a major absorption band at 490 nm, whereas Y70F exhibits only modest absorbance at this wavelength.

The steady-state kinetic parameters for the reactions of wild type and Y70F with β-hydroxyaspartate and α-ketoglutarate as substrates are given in Table III. The rate-limiting half-reaction with 5 mM α-ketoglutarate employed is the one involving β–hydroxyaspartate. Wild type has a nearly 4-fold larger k_{cat} and a 15-fold larger k_{cat}/K_M with this substrate than does Y70F.

[α-^2H]-L-*Amino Acid Kinetic Isotope Effects.* The values of the kinetic isotope effects (KIE's) for wild type and Y70F with both aspartate and glutamate are listed in Table IV. The effect of aspartate deuteration at the C^α position for wild type is small and similar to that found for cytosolic and mito-

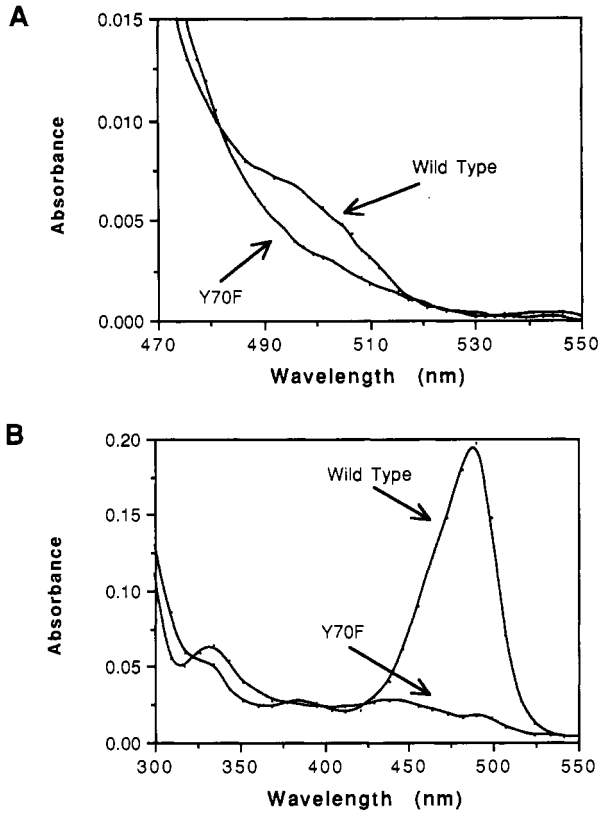


FIGURE 1: Absorbance spectra of quinonoid species formed in wild type and Y70F reactions with (A) aspartate/oxalacetate, and (B) β-hydroxyaspartate/α-ketoglutarate. Solutions contained 200 mM HEPES-KOH, pH 7.5, 100 mM KCl, 10 μM AATase, and either (A) 50 mM aspartate and 5 mM oxalacetate or (B) 5 mM each β-hydroxyaspartate and α-ketoglutarate, all at 25 °C. The reactions with β-hydroxyaspartate were scanned immediately after initiation.

Table III: Kinetic Parameters for the Reactions of *erythro*-β-Hydroxyaspartate with Wild Type and Y70F Aspartate Aminotransferases[a]

	k_{cat} (s^{-1})	K_M (mM)	k_{cat}/K_M (M^{-1} s^{-1})
wild type	0.93 (0.01)	0.25 (0.01)	3700 (200)
Y70F	0.25 (0.01)	0.89 (0.05)	245 (10)
Y70F/wild type	0.27 (0.02)	3.4 (0.3)	0.067 (0.004)

[a] Conditions: 200 mM HEPES-KOH/100 mM potassium chloride, pH 7.5, 25 °C. The transaminase activity was measured by coupling the production of dihydroxyfumarate to malate dehydrogenase. Standard errors are given in parentheses.

chondrial AATase (Julin & Kirsch, 1989). Y70F exhibits larger KIE's with aspartate relative to wild type. Conversely, Y70F has a smaller KIE than does wild type for the glutamate reaction.

Discussion

Tyr70 Is Not Essential for Transaminase Activity. Tyr70 is conserved in all AATases yet examined (Kondo et al., 1987). The phenolic oxygen atom is close (4.3 Å) to the C^{α} proton of the model aldimine formed by phosphopyridoxyl-L-aspartate and apoenzyme, but slightly more distant than is the ϵ-NH_2 of Lys258 (3.7 Å) (Kirsch et al., 1984). It was originally postulated that Tyr70 functions critically in helping Lys258 to effect the 1,3 prototropic shift (see Scheme I). Considered most likely were models in which the phenolic hydroxyl group of Tyr70 relays charge from Lys258 to the 5′-phosphate

Table IV: Primary α-^{2}H Kinetic Isotope Effects for the Reactions of Wild Type and Y70F Aspartate Aminotranferases with L-Aspartate and L-Glutamate[a]

	aspartate		glutamate
	^DV	$^D(V/K)$	$^D(V/K)$
wild type	1.52 (0.01)	1.70 (0.04)	2.53 (0.04)
Y70F	2.4 (0.1)	2.5 (0.1)	1.75 (0.01)
Y70F/wild type	1.6 (0.1)	1.5 (0.1)	0.69 (0.01)

[a] Conditions: 20 mM HEPES-KOH/100 mM potassium chloride, pH 7.5, 25 °C. Standard errors are given in parentheses.

and/or orients the ϵ-NH_2 group of Lys258. Considered plausible, but less likely due to the greater distance of the hydroxyl group vs the Lys258 ϵ-NH_2 moiety from C^{α} in the aldimine model, was the possibility that Tyr70 is the primary general base responsible for catalyzing the 1,3 prototropic shift. Each of these possibilities predicts that the elimination of the phenolic hydroxyl group of Tyr70 by substitution of phenylalanine would produce an enzyme with greatly diminished activity.

Y70F retains a large percentage (8%, as measured by k_{cat}) of the wild type transamination activity. Recent experiments with highly active enzymes demonstrate the large magnitude (ca. 10^5-fold) of reductions in activity, which can be expected from the elimination of a catalytically essential group. Substitutions in the catalytic triads of serine proteases (Carter & Wells, 1988; Craik et al., 1987) and at the reactive center of staphylococcal nuclease (Serpersu et al., 1987) are clear examples. Unlike Y70F, the K258A mutant discussed below exhibits just such a large reduction in activity. These results demonstrate that Tyr70 is not an essential component of the catalytic apparatus. It remains possible that the function of the deleted Tyr70 hydroxyl group is replaced by a water molecule in the mutant. However, the extra entropy required to localize the water molecule to the active site and the extra steric bulk due to the combined volume of the water molecule and the Phe70 para hydrogen, argue against this hypothesis.

Are Steps Subsequent to Proton Transfer Kinetically Significant for the Aspartate Half-Reaction? The steady-state value of k_{cat} for a mechanism involving two serial half-reactions can be calculated from the k_{max} values of the individual half-reactions (Cronin & Kirsch, 1988). Consider the minimal AATase mechanism, eq 5. The value of k_{cat} derived from the

$$E + A \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} EA \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} E' + P \qquad (5a)$$

$$E' + B \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} E'B \underset{k_{-4}}{\overset{k_4}{\rightleftharpoons}} E + Q \qquad (5b)$$

velocity expression given by Segel (1975) is given by eq 6.

$$k_{cat} = \frac{k_2 k_4}{(k_2 + k_4)} \qquad (6)$$

Analyses of individual half-reactions provide values of k_{max} that correspond to either k_2 or k_4, depending on the substrate employed. It is thus a simple task to predict the steady-state k_{cat} from the k_{max} values of the constituent half-reactions.

It has been found that measured *E. coli* AATase steady-state k_{cat} values are lower than those predicted from the results of half-reactions analyses (Cronin & Kirsch, 1988; Inoue et al., 1989; present work). In the case of Y70F reacting with aspartate and α-ketoglutarate, the calculated value of the steady-state k_{cat} using half-reaction k_{max} values from stop-

ped-flow experiments is 27 ± 3 s^{-1}, whereas the measured value is 13 ± 1 s^{-1}. Discrepancies observed with wild type follow: (1) calculated k_{cat} = 183 ± 14 s^{-1}, observed k_{cat} = 139 ± 7 s^{-1} at I_c = 1.0 (Cronin & Kirsch, 1988); (2) calculated k_{cat} = 260 ± 15 s^{-1}, observed k_{cat} = 220 ± 7 s^{-1} at I_c 0.1 (Inoue et al., 1989). The difference between calculated and observed values of k_{cat} can be accounted for by a consideration of the elementary chemical steps constituting each half-reaction. The stopped-flow experiments monitor a particular chromogenic step, the proton transfer between C^{α} and C4′ in the case of AATase. No intermediate between external aldimine (II; A_{360}/A_{430}) and ketimine (IV; A_{330}) is kinetically significant (Gehring, 1986), and external aldimine formation is rapid and at equilibrium compared to proton transfer (Julin & Kirsch, 1989; Kiick & Cook, 1983). If a nonchromogenic step subsequent to proton transfer (e.g., ketimine hydrolysis or oxalacetate dissociation) were to have a rate constant of comparable magnitude to the 1,3 prototropic shift, the steady-state k_{cat} value predicted from the half-reactions can be made to agree with the observed value. Corroborative evidence for the existence in wild type of kinetically significant steps subsequent to proton transfer is provided by recent measurements of the viscosity dependence of *E. coli* AATase reactions. It was found for wild type that the aspartate half-reaction is approximately 30% diffusion-controlled, i.e., the rate constant for oxalacetate release from E–PMP is kinetically significant (J. M. Goldberg, E. Neymark, and J. F. K., unpublished results). The larger discrepancy for Y70F wild type enzyme between predicted and observed k_{cat} values thus argues that the mutation has altered not only the kinetic barrier for the 1,3 prototropic shift but also the barriers for subsequent steps.

Competitive Inhibitors. The values of the dissociation constants for the AATase competitive inhibitors maleate and α-methylaspartate were measured for both wild type and Y70F. The aromatic ring of Tyr70 makes contacts with maleate in the X-ray structure of the E–PLP–maleate complex (Jansonius & Vincent, 1987). The 17-fold larger value of maleate K_i^{Asp} in the mutant (Table II) is possibly due to a higher mobility, originating from hydrogen-bond loss, of Phe70 vs Tyr70 in the constrained environment of the complex. α-Methylaspartate reacts further along the reaction pathway than does maleate in forming a stable external aldimine. The latter step is accompanied by a coenzyme rotation (Jansonius & Vincent, 1987). The relatively unfavorable interaction in the noncovalent complex of ligand with the mutant enzyme is partially relieved upon external aldimine formation, as the α-methylaspartate K_i values are more nearly the same for the mutant and wild type. The possibility that the differences in K_i values are due to an altered pH dependence for the mutant is ruled out by the similarity in internal aldimine pK_a values (Toney & Kirsch, 1987). The association of inhibitors with the enzyme near neutral pH is dependent on this ionization (Kiick & Cook, 1983).

Quinonoid Species Are Less Stable in Y70F. A structurally based hypothesis for the unusual stability of the quinonoid formed from β-hydroxyaspartate proposed that the β-hydroxyl group of the substrate makes hydrogen bonds with oxygen OP2 of the coenzyme phosphate and with the hydroxyl group of Tyr70 (Kirsch et al., 1984). The model thus predicts that the quinonoid formed from β-hydroxyaspartate and Y70F should be less stable than the corresponding species derived from wild type due to the absence of one of the stabilizing hydrogen bonds. This prediction is borne out by the experiments shown in Figure 1. Under aspartate/oxalacetate equilibrium conditions, wild type exhibits a small absorption band near 490 nm, characteristic of the quinonoid intermediate. No such absorbance appears in the corresponding reaction with Y70F (Figure 1A). A more dramatic contrast appears in the reactions of β-hydroxyaspartate (Figure 1B). A very strong absorption band at 490 nm results from the reaction of wild type with this amino acid, but Y70F forms only a small one under identical conditions, indicating that the quinonoid intermediate is less stable on Y70F. A similar disruption in the stability of this intermediate derived from β-hydroxyaspartate was found with the R386K mutant (Inoue et al., 1989). Both the R386K and the Y70F data are consistent with a model in which the β-hydroxyl group of the substrate enforces the stability of the quinonoid through hydrogen bonding and steric interactions. The Tyr70 quinonoid-stabilizing contacts are removed by the mutagenic elimination of the hydroxyl group in Y70F. With R386K, the decrease in overall binding constraints of the quinonoid complex, due to the mutation of the α-carboxylate-anchoring Arg386 to lysine, must indirectly disrupt the quinonoid-stabilizing hydrogen-bonding interactions of the β-hydroxyl group. In line with these considerations, both Y70F and R386K show less discrimination between aspartate and β-hydroxyaspartate as substrates than does wild type (Tables I and III; Inoue et al., 1989): for wild type, $k_{cat}^{Asp}/k_{cat}^{\beta\text{-OH-Asp}}$ = 172, and for Y70F, $k_{cat}^{Asp}/k_{cat}^{\beta\text{-OH-Asp}}$ = 52.

Kinetic Isotope Effects. Small but significant C^{α} KIE's are observed for the reactions of aspartate with both wild type and Y70F (Table IV). Similar results were obtained for the corresponding reactions of cytosolic and mitochondrial AATases (Julin & Kirsch, 1989). The reaction between Y70F and aspartate exhibits 50% greater KIE's on both V and V/K than does the wild type reaction. Conversely, the reaction of glutamate with Y70F yields a 30% lower $^{D}(V/K)$ than does the wild type reaction. Changes in KIE's for a complex mechanism, such as that for AATase, can result from a change in the intrinsic KIE for the isotope-sensitive step as well as from a change in the balance of rate-determining steps. A self-consistent interpretation for both the aspartate and glutamate reactions requires the proposition that the reaction pathways for the two substrates differ significantly in transition state structure and/or relative kinetic barrier heights and that these factors are diferentially altered by the mutation Y70F. Substrate- and isozyme-dependent variation in mechanism has previously been documented in cytosolic and mitochondrial AATase reactions (Julin & Kirsch, 1989).

Thus, the conserved Tyr70 of AATases is seen to play a subtle, fine-tuning rather than essential role in the overall catalytic efficiency of the enzyme. The conservative substitution of phenylalanine at position 70 produces an enzyme that binds substrates, inhibitors, and coenzymes more weakly than does wild type (Toney & Kirsch, 1991). Conversely, replacement of Lys258 with alanine results in a $>10^7$-fold decrease in the aspartate half-reaction k_{max} (Toney & Kirsch, 1989; M.D.T. and J.F.K., submitted for publication). Catalytic activity can be partially restored to K258A by the addition of small, primary amines (Toney & Kirsch, 1989). Together, these results demonstrate unequivocally that the critical residue involved directly in general base catalysis of the central 1,3 prototropic shift in AATase reactions is Lys258.

Registry No. α-KG, 328-50-7; AATase, 9000-97-9; Asp, 56-84-8; Tyr, 60-18-4; Phe, 63-91-2; malate, 110-16-7; α-methyl-DL-aspartate, 2792-66-7; *erythro*-β-hydroxyaspartic acid, 1186-90-9; glutamate, 56-86-0; deuterium, 7782-39-0.

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Kinetics and Equilibria for the Reactions of Coenzymes with Wild Type and the Y70F Mutant of *Escherichia coli* Aspartate Aminotransferase†

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ABSTRACT: The Y70F mutant of aspartate aminotransferase has reduced affinity for coenzymes compared to the wild type. The equilibrium dissociation constants for pyridoxamine phosphate (PMP) holoenzymes, K_{diss}^{PMP}, were determined from the association and dissociation rate constants to be 1.3 nM and 30 nM for the wild type and mutant, respectively. This increase in K_{diss}^{PMP} for Y70F is due to a 27-fold increase in the dissociation rate constant. Pyridoxal phosphate (PLP) association kinetics are complex, with three kinetic processes detectable for wild type and two for Y70F. A directly determined, accurate value of K_{diss}^{PLP} for wild type enzyme has been difficult to obtain because of the low value of this constant. The values of K_{diss}^{PLP} for the holoenzymes were determined indirectly through the measured values for K_{diss}^{PMP}, glutamate–α-ketoglutarate half-reaction equilibrium constants, and the equilibrium constant for the transamination of PLP by glutamate catalyzed by Y70F. The values of K_{diss}^{PLP} obtained by this procedure are 0.4 pM for wild type and 40 pM for Y70F. The increases in K_{diss}^{PMP} and K_{diss}^{PLP} for Y70F correspond to $\Delta\Delta G$ values of 1.9 and 2.7 kcal/mol, respectively, and are directly attributed to the loss of the hydrogen bond from the phenolic hydroxyl group of Tyr70 to the coenzyme phosphate. The ΔG for association of PLP with wild type enzyme is 4.7 kcal/mol more favorable than that for PMP.

The previous paper (Toney & Kirsch, 1991) describes the properties of the aspartate aminotransferase (AATase)[1] active site mutant Y70F in its reactions with substrates. The present paper is concerned with the reactions of this mutant and the WT enzyme with coenzymes.

AATases from several species, including *Escherichia coli*, bind PLP and PMP with very high affinity such that under

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[1] Abbreviations: AATase, aspartate aminotransferase (EC 2.6.1.1); wild type, wild type *E. coli* AATase; Y70F, *E. coli* AATase in which Tyr70 has been changed to phenylalanine by site-directed mutagenesis; PLP, pyridoxal phosphate; PMP, pyridoxamine phosphate; E–PLP and E–PMP, PLP and PMP forms of AATase, respectively; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid.

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