Use of Site-Directed Mutagenesis and Alternative Substrates To Assign the Prototropic Groups Important to Catalysis by *Escherichia coli* Aspartate Aminotransferase†

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ABSTRACT: The pH dependence of *Escherichia coli* aspartate aminotransferase (AATase) has been investigated by the use of site-directed mutants and alternative substrates. Inhibition of the enzyme by CHES and variations in ionic strength are proposed to explain some of the qualitative differences in the published pH dependence of pig cytosolic AATase kinetics [Velick, S. F., & Vavra, J. (1962) *J. Biol. Chem. 237,* 2109−2122; Kiick, D. M., & Cook, P. F. (1983) *Biochemistry 22,* 375−382]. The pK_a values of the basic limbs in the k_{cat}/K_M profiles for the amino acids, L-Asp and L-cysteinesulfinate (L-CS), are identical, within error, to those of free substrates, (L-Asp, pK_a = 9.6; L-CS, pK_a = 9.0). This pK_a therefore is assigned to the α-amino group of the substrate. Replacement of the active site base, Lys-258, with the weaker base, γ-thia-Lys, does not alter the intrinsic pK_a for the profiles of the K_i values for the maleate−E·PMP complexes or the $k_{cat}/K_M^{\alpha\text{-KG}}$ values. The mutation Y225F results in an alkaline shift of the pK_a in the $k_{cat}/K_M^{\alpha\text{-KG}}$ profile. This pK_a is assigned to the C4′ amino group of PMP. *E. coli* AATase, unlike pig cytosolic AATase, shows a pH dependence on k_{cat} between pH 5 and 10 that arises from a change in the rate-determining step at pH extremes. C_α proton abstraction is partially rate-determining at neutral pH values, but not at pH extremes. The pH dependence of the ^2H-C_α kinetic isotope effects demonstrates that ketimine hydrolysis, oxalacetate dissociation, or the α-KG half-reaction is rate-determining at the pH extremes.

Aspartate aminotransferase (AATase)[1] is a PLP-dependent enzyme that catalyzes the transfer of the α-amino group from the amino acids L-Asp, L-Glu, and L-CS to the α-keto acids α-KG and OAA. The basic reaction is summarized in eq 1.

$$\text{E·PLP} + \text{L-aspartate} \rightleftharpoons \text{E·PMP} + \text{oxalacetate} \quad (1)$$

$$\text{E·PMP} + \alpha\text{-ketoglutarate} \rightleftharpoons \text{E·PLP} + \text{L-glutamate}$$

The mechanistic details of the first half-reaction, with L-Asp, are shown in Scheme 1.

The reaction mechanism of the enzyme from vertebrate sources has been studied extensively by kinetics (Velick & Vavra, 1962; Kiick & Cook, 1983; Julin & Kirsch, 1989) and by X-ray crystallography [reviewed in Jansonius and Vincent (1987)]. The enzyme from *E. coli* is closely related to the cytosolic and mitochondrial vertebrate enzymes (~40% sequence identity; Mehta *et al.*, 1989). Site-directed mutagenesis has been employed to examine the contributions to catalysis of many of the active site residues of *E. coli* AATase. Lys-258 forms a Schiff base with the PLP cofactor and acts as the general base for the 1,3-prototropic shift (Scheme 1). Mutations at this position (K258A, K258M, K258R, K258C, K258H) have been studied in detail (Toney & Kirsch, 1989, 1991, 1992, 1993; Planas & Kirsch, 1991; Ziak *et al.*, 1990, 1993).

Recently, site-directed mutagenesis was combined with chemical modification to replace Lys-258 with γ-thia-Lys, a nearly isosteric substitution (Planas & Kirsch, 1991). This was accomplished by modification of the K258C mutant with Br-EA. The WT enzyme has five native cysteines that had to be protected from modification in K258C. Subsequently, the five native cysteines were mutated to Ala (Gloss *et al.*, 1992). Lys-258 was converted to a now unique Cys ($K258C_Q$) and reacted with Br-EA to yield an apparently

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[1] Abbreviations: AATase, aspartate aminotransferase; WT, wild-type *E. coli* AATase; Quint, *E. coli* AATase without cysteine, containing the mutations C82A, C191A, C192A, C270A, and C401A; $K258C_Q$, Quint *E. coli* AATase containing a unique cysteine at the 258 position in place of the WT Lys residue; Br-EA, 2-bromoethylamine; $K258C_Q$-EA, the $K258C_Q$ enzyme modified with 2-bromoethylamine to generate γ-thia-Lys at position 258; Y225F, AATase in which Tyr-225 has been mutated to Phe; pcAATase, pig heart cytosolic AATase; α-KG, α-ketoglutarate; AMPSO, 3-[(1,1-dimethyl-2-hydroxyethyl)amino]-2-hydroxypropanesulfonic acid; CAPS, 3-(cyclohexylamino)propanesulfonic acid; CAPSO, 3-(cyclohexylamino)-2-hydroxypropanesulfonic acid; CHES, 2-(cyclohexylamino)ethanesulfonic acid; L-CS, L-cysteinesulfinate; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; KIE, primary C_α kinetic isotope effect; LDH, lactate dehydrogenase; MDH, *E. coli* malate dehydrogenase; MES, 2-morpholinoethanesulfonic acid; MHP buffer, a tricomponent buffer with constant ionic strength (μ = 0.2, see Materials and Methods); OAA, oxalacetate; TAPS, *N*-[tris(hydroxymethyl)methyl]-3-aminopropanesulfonic acid; PLP, pyridoxal 5′-phosphate; PMP, pyridoxamine 5′-phosphate; E, enzyme.

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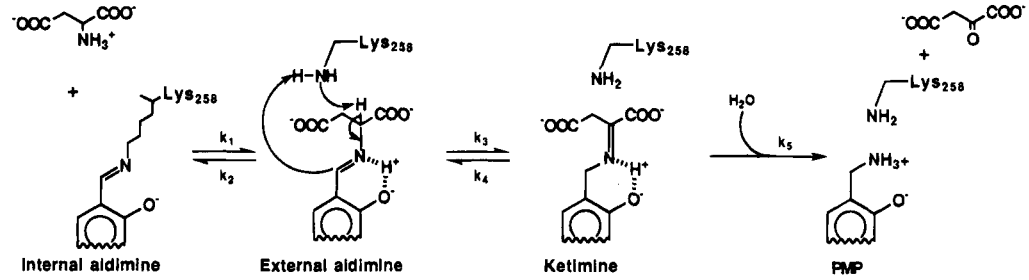
Scheme 1: Partial Mechanism for the Conversion of L-Asp to Oxalacetate by Aspartate Aminotransferase[a]



[a] Reaction of the PLP enzyme (the internal aldimine) with L-Asp produces the external aldimine. The ϵ-amino group of Lys-258 catalyzes the 1,3-prototropic shift that gives rise to the ketimine. Hydrolysis of the ketimine yields the α-keto acid (OAA) and the PMP form of the enzyme. The second half-reaction, with α-KG, regenerates the PLP enzyme and produces L-Glu.

homogeneous enzyme, K258C$_Q$-EA, containing γ-thia-Lys (Gloss & Kirsch, 1995). The pK_a of the ϵ-amino group of γ-thia-Lys is 1.1 pH units lower than that of Lys (Hermann & Lemke, 1968). The ionization of Lys-258, in an internal aldimine with the PLP cofactor, is reflected in the acidic pK_a of the pH profile of k_{cat}/K_M^{Asp}. Therefore, replacement of the WT Lys-258 with this lysine analogue lowers this pK_a (Planas & Kirsch, 1991) and could possibly have additional effects on the pH dependence of AATase kinetic parameters.

The previous detailed studies on the pH dependence of catalysis by AATase were carried out on the pig heart cytosolic enzyme (Velick & Vavra, 1962; Kiick & Cook, 1983). There are significant qualitative differences in some of the findings from the two groups, and the identities of the titrating groups responsible for the observed pH-dependent behavior were not established. The data presented here constitute the first report of the pH dependence of catalysis by the *E. coli* AATase, which is the current benchmark for on-going site-directed mutagenesis and mechanistic studies of transaminases. This report shows that the effect of pH on the rate constants of the *E. coli* and pcAATase differs, particularly in their k_{cat} profiles, which show no variation with pH for pcAATase. The identity of the titrating groups has been investigated by the use of three AATase variants: wild type (WT), Y225F, and K258C$_Q$-EA. The pH-dependent activity of these enzymes was determined with α-KG and either L-Asp or L-CS.

MATERIALS AND METHODS

Materials

[$C_α$-^{2}H]-D,L-CS was a gift from Dr. J. J. Onuffer. [$C_α$-^{2}H]-D,L-Asp was synthesized and purified as described previously (Julin & Kirsch, 1989). MDH was purified from an AATase-deficient *E. coli* strain (Onuffer & Kirsch, 1994). LDH was from Boehringer Mannheim. Pig heart cytosolic AATase was from Sigma. 4-Hydroxy-*N*-methylpiperidine was purchased from Lancaster and vacuum-distilled before use as a buffer. All other chemicals and reagents were of the highest purity available from either Sigma or Aldrich.

Methods

The site-directed mutagenesis, protein purification, and quantification of mutant AATases have been described previously (Goldberg *et al.*, 1991; Gloss *et al.*, 1992; Gloss & Kirsch, 1995).

Steady-State Kinetic Conditions. Steady-state kinetics was monitored by the decrease in NADH absorbance at 340 nm at 25 °C with a Kontron Uvikon 860 spectrophotometer, except where noted for Y225F. MHP buffer was used for kinetic measurements. It is a tricomponent system designed to give a constant ionic strength with varying pH (Ellis & Morrison, 1982) and contains 25 mM MES, 25 mM HEPES, 50 mM 4-hydroxy-*N*-methylpiperidine, and 150 mM KCl (μ = 0.2), with 150 μM NADH and 5−30 units/mL of the coupling enzyme (MDH for L-Asp assays; LDH for L-CS assays). Matrices of data were fitted by nonlinear regression with the NLIN program of the SAS statistical package (SAS Institute, Cary, NC). Data sets with only x and y variables were fitted by nonlinear regression with the program, *Enzfitter* (Biosoft Publishing Co.).

The kinetic parameters of WT and K258C$_Q$-EA reactions with the L-Asp/α-KG and L-CS/α-KG substrate pairs were determined from matrices of initial rate data at 5−7 concentrations of each substrate. The data were fitted to the ping-pong bi-bi equation (Velick & Vavra, 1962):

$$\frac{v}{E_t} = \frac{k_{cat}}{1 + K_M^{AA}/[AA] + K_M^{\alpha\text{-}KG}/[\alpha\text{-}KG]} \quad (2)$$

where AA is the respective amino acid.

The low $K_M^{\alpha\text{-}KG}$ values of Y225F (~10 μM; Goldberg *et al.* 1991; this study, data not shown) make it difficult to collect initial rate data (<10% of the substrate consumed) at substrate concentrations ≤K_M. The Y225F steady-state kinetic data for $k_{cat}/K_M^{\alpha\text{-}KG}$ were collected with an Applied Photophysics stopped-flow spectrophotometer (SF.17 MV) to monitor the decrease in absorbance at 340 nm from 0.5 to 50 s. The concentration of α-KG was varied from 5 to 200 μM, and [L-Asp] was held constant at 1 mM. For the parameters k_{cat}/K_M^{Asp} and k_{cat}, manual mixing techniques were used with a Kontron Uvikon 860. The concentration of L-Asp was varied, and [α-KG] was held constant at 1.5 mM (~100K_M). At all pH values employed, [α-KG] was varied from 1 to 2.5 mM to show that (1) there is no substrate inhibition by α-KG and (2) the velocities showed little or no dependence on [α-KG]. Both the α-KG and L-Asp data were fitted to:

$$\frac{v}{E_t} = \frac{k_{cat}[S]}{K_M + [S]} \quad (3)$$

where S is either L-Asp or α-KG.

The competitive inhibition constants for maleate were determined at pH values above the pK_a of the internal aldimine, where maleate binds preferentially to the PMP−enzyme (Kiick & Cook, 1983; Gloss & Kirsch, 1995). Inhibitor concentrations were varied from 0 to 100 mM

(exact range depending on the enzyme and pH), and substrate concentrations were held constant: [L-Asp] = (3.6−5.6) K_M; [α-KG] = K_M. The data were fitted to eq 4 with fixed K_M values for both substrates:

$$\frac{v}{E_t} = \frac{k_{cat}}{1 + K_M^{Asp}/[Asp] + (K_M^{\alpha\text{-}KG}/[\alpha\text{-}KG])(1 + [I]/K_i)} \quad (4)$$

where K_i is the PMP•maleate dissociation constant, and [I] is the concentration of maleate.

[C_α-^{2}H]Amino Acid Kinetic Isotope Effects. The data were collected at the concentrations of α-KG indicated in the footnotes to Table 2 and varied concentrations of [C_α-^{1}H]- and [C_α-^{2}H]amino acids. The [L-amino acid] values were the same for both the ^{1}H and ^{2}H data sets. The data were fitted to eq 3.

The deuterated aspartate was a 50:50 mixture of the D and L isomers, but the stated concentration refers to that of the L isomer only. The D isomer does not bind to AATase (Planas & Kirsch, 1991). The [C_α-^{2}H]CS was a 35:65 mixture of the L and D enantiomers. The calculations are based on the fraction of the L enantiomer.

Evaluation of the pH Dependence of the Kinetic Parameters. The data were fitted with the *Enzfitter* program. The adequacy of the models was assessed by *F*-tests where indicated, and the results were compared with *F*-distributions to accept or reject the null hypothesis (Draper & Smith, 1981).

The pH dependence of k_{cat}/K_M^{Asp} and k_{cat}/K_M^{CS} was obtained from fits to the bell-shaped curve described by

$$Y = \frac{Y_{lim}}{1 + 10^{(pK_1 - pH)} + 10^{(pH - pK_2)}} \quad (5)$$

The value of $k_{cat}/K_M^{\alpha\text{-}KG}$ for K258C$_Q$-EA is dependent on a single pK_a with one active (protonated) species:

$$k_{cat}/K_M^{\alpha\text{-}KG} = \frac{(k_{cat}/K_M)_{lim}}{1 + 10^{(pH - pK_a)}} \quad (6)$$

The pH dependence of the WT $k_{cat}/K_M^{\alpha\text{-}KG}$ value was determined from fits to both eqs 6 and 7. The latter describes a model with two active species, a limiting value at lower pH, lim_{acid}, one pK_a, and a limiting value at high pH, lim_{basic}:

$$Y = \frac{lim_{acid}(10^{(pK_a - pH)}) + lim_{basic}}{1 + 10^{(pK_a - pH)}} \quad (7)$$

The pH dependence of the K_i (maleate) values (for the PMP−enzyme forms of WT and K258C$_Q$-EA) were fitted to

$$K_i = (K_i)_{lim}(1 + 10^{(pH - pK_a)}) \quad (8)$$

The Y225F k_{cat} values are adequately described by eq 5. The other k_{cat} *vs* pH profiles were fitted to a modified bell curve equation:

$$k_{cat} = \frac{(k_2)_{lim} + (k_1)_{lim}(10^{(pK_1 - pH)})}{1 + 10^{(pK_1 - pH)} + 10^{(pH - pK_2)}} \quad (9)$$

which describes limiting values at low (k_1) and neutral (k_2) pH.

RESULTS

Buffer Selection. Preliminary pH profiles with the WT and Quint enzymes, in which ionic strength was not held constant over the pH range, yielded data exhibiting a strong dependence on ionic strength, including artifactual pK_a values. Significant substrate inhibition, by both L-Asp and α-KG, was observed in the absence of KCl, which is a competitive inhibitor of pcAATase (Jenkins, 1989). For *E. coli* AATase, KCl is a weak, pH-dependent, competitive inhibitor of amino and keto acid substrates (A. Planas and J. F. Kirsch, unpublished results). Constant ionic strength is maintained in a three-component buffer system (Ellis & Morrison, 1982), without adjusting the [KCl] to compensate for the titration of the buffer components. In the present studies, [KCl] was held constant at 150 mM from pH 5 to 10. The rates of reaction are relatively insensitive to [KCl] around this concentration range (A. Planas and J. F. Kirsch, unpublished results).

Buffer effects on AATase were examined by varying the buffer concentration from 25 to 200 mM (pH = pK_a of buffer, ionic strength not controlled; [L-Asp] and [α-KG] = (1−2)K_M). The velocity was independent of the buffer concentration for MES, MOPSO, MOPS, HEPES, TAPS, and AMPSO buffers. In contrast, increasing the concentration of CHES, CAPS, and CAPSO, from 25 to 200 mM decreased reaction rates by 40−50%.

As earlier studies (Kiick & Cook, 1983; Goldberg *et al.*, 1991) employed CHES as a buffer in pH variation studies of AATase kinetics, the pH dependence of its inhibition was examined. The kinetic constants for the L-Asp/α-KG substrate pair were determined at different concentrations of CHES (pK_a = 9.3) at pH 9.0 and 9.5, with AMPSO (pK_a = 9.0) added to maintain constant ionic strength as the [CHES] was varied. For *E. coli* AATase, CHES gave pH-dependent inhibition. At pH 9.0, the values of $k_{cat}/K_M^{\alpha\text{-}KG}$ and k_{cat}/K_M^{Asp} were decreased by 30 and 10%, respectively (0 *vs* 100 mM CHES). At pH 9.5, the values of $k_{cat}/K_M^{\alpha\text{-}KG}$ and k_{cat}/K_M^{Asp} were decreased by 42 and 23%, respectively. At pH values above the pK_a of CHES, the decreases in absorbance with time were not linear at substrate combinations of [Asp] ≥ 4K_M and [α-KG] ≤ K_M, but they were linear in the presence of 100 μM PMP or higher [α-KG]. CHES appears to promote the dissociation of PMP from this enzyme.

For cytosolic AATase as well, pH-dependent effects on $k_{cat}/K_M^{\alpha\text{-}KG}$ and k_{cat}/K_M^{Asp} were observed in CHES buffers. As AMPSO inhibited this AATase, the parameters were compared at 50 *vs* 200 mM CHES at pH 9.0 and 9.75. The value of k_{cat}/K_M^{Asp} was decreased by 30 and 52% in 200 mM CHES at pH 9.0 and 9.75, respectively. The value of $k_{cat}/K_M^{\alpha\text{-}KG}$ was decreased by 85 and 62% at pH 9.0 and 9.75, respectively.

A three-component buffer system requires that the conjugate bases of the two components with the lowest pK_a values have a charge of −1 (Ellis & Morrison, 1982). MES (pK_a = 6.2) and HEPES (pK_a = 7.5) were chosen because they were not inhibitory. The conjugate base of the component with the highest pK_a must be neutral. A number of amines with pK_a values from 9 to 10 were included in AMPSO-buffered assays to determine whether they inhibited *E. coli* AATase. 4-Hydroxy-*N*-methylpiperidine (pK_a = 9.7)

Table 1: pH Dependence of the Kinetic and Inhibition Constants of WT, K258C$_Q$-EA, and Y225F Aspartate Aminotransferases[a]

constant	enzyme	substrate	pK_1	pK_2	limit (M^{-1} s^{-1})
k_{cat}/K_M(amino acid)	WT	L-Asp	6.87 (0.06)	9.64 (0.05)	68000 (2000)
	WT	L-CS	6.79 (0.05)	9.00 (0.05)	39000 (1000)
	K258CQ-EA	L-Asp	5.79 (0.03)	9.61 (0.03)	5810 (80)
	Y225F[b]	L-Asp	8.59 (0.05)	9.72 (0.05)	3800 (200)

constant	enzyme	substrate	pK_1	acidic limit (M^{-1} s^{-1})	basic limit[c] (M^{-1} s^{-1})
k_{cat}/K_M(α-KG)	WT	α-KG	9.51 (0.07)	192000 (5000)	one-limit fit
	WT	α-KG	8.9 (0.1)	200000 (4000)	59000 (7000)
	K258CQ-EA	α-KG	9.65 (0.04)	74000 (900)	one-limit fit
	Y225F[b]	α-KG	>10	25500 (2000)	

constant	enzyme	substrate	pK_1	acidic limit (mM)
K_i(maleate) for E·PMP	WT	α-KG	9.62 (0.04)	6.0 (0.2)
	K258CQ-EA	α-KG	9.47 (0.02)	6.8 (0.1)

constant	enzyme	substrate	pK_1	pK_2	acidic limit (s^{-1})	basic limit (s^{-1})
k_{cat} with α-KG	WT	L-Asp	7.2 (0.2)	10.3 (0.1)	105 (5)	162 (4)
	WT	L-CS	6.7 (0.1)	9.36 (0.07)	175 (32)	540 (20)
	K258CQ-EA	L-Asp	5.5 (0.2)	10.3 (0.04)	18 (2)	34.4 (0.4)
	Y225F	L-Asp	5.55 (0.03)	10.3 (0.1)	not fit[d]	0.292 (0.004)

constant	enzyme	substrate	pK_1	pK_2	acidic limit	basic limit
$^D k_{cat}$	WT	L-Asp	6.7 (0.2)	10.6 (0.1)	1.01 (0.06)	1.60 (0.04)

[a] The data fitted by these constants are shown in Figures 1−4. Standard errors are in parentheses. Conditions: MDH- or LDH-coupled assays in MHP buffer, μ = 0.2, 25 °C. [b] The values reported by Goldberg *et al.* (1991) are given in the text. [c] The WT data set was fit to both one-limit and two-limit models (eqs 6 and 7, respectively). The K258C$_Q$-EA data were accommodated sufficiently by a one-limit model. See text. [d] The Y225F data were fitted to the one-limit model described by eq 5.

had the smallest effect. The addition of 50 mM 4-hydroxy-*N*-methylpiperidine to 200 mM HEPES (pH 7.5) and 200 mM AMPSO (pH 9.7) buffers resulted in only a 14 ± 1% decrease in either $k_{cat}/K_M^{\alpha\text{-KG}}$ or k_{cat}/K_M^{Asp}. The effect was pH-*independent.*

pH Profiles of k_{cat}/K_M for L-*Asp and* L-*CS.* pK_1 (acidic limb) of the k_{cat}/K_M^{Asp} profile is due to titration of the internal aldimine formed between PLP and Lys-258 (deprotonated structure shown in Scheme 1) and is independent of the amino acid substrate (Table 1). This pK_a can also be measured by direct spectral titration of the aldimine. The WT spectrophotometric pK_a (6.95 ± 0.03; Gloss & Kirsch, 1995; Goldberg *et al.*, 1991) agrees with that determined kinetically (Figure 1, Table 1).

Lys-258 is replaced with γ-thia-Lys in K258C$_Q$-EA. Potentiometric titrations of α-*N*-acetyl-Lys and α-*N*-acetyl-γ-thia-Lys demonstrate that the thio ether bond lowers the pK_a of the ε-amino group by 1.1 pH units (Hermann & Lemke, 1968). This difference is reflected in the internal aldimines, as seen by spectrophotometric titration (Gloss & Kirsch, 1995), and in the pH dependence of k_{cat}/K_M^{Asp} (Figure 1, Table 1). The mutation Y225F eliminates the hydrogen bond between the hydroxyl of Tyr-225 and O3′ of the cofactor, with a resulting increase in the spectrophotometrically determined pK_a of 1.6 pH units to 8.60 ± 0.02 (Goldberg *et al.*, 1991). A similar alkaline shift (1.7 pH units) from the WT value is observed in the kinetic pK_a reported here (Table 1) and in that reported by Goldberg *et al.* (1991), 8.4 ± 0.2.

Only one alkaline pK_a is observed in the WT k_{cat}/K_M^{Asp} profiles, and it is not affected by any of the mutations examined in this study. This pK_a, however, is dependent on the amino acid substrate. The L-Asp data fit to a pK_a of 9.64 ± 0.05, and the L-CS data fit to one of 9.00 ± 0.05, a ΔpK_a of 0.6 pH unit. Literature values for the pK_a of the α-amino groups of L-Asp and L-CS under similar conditions

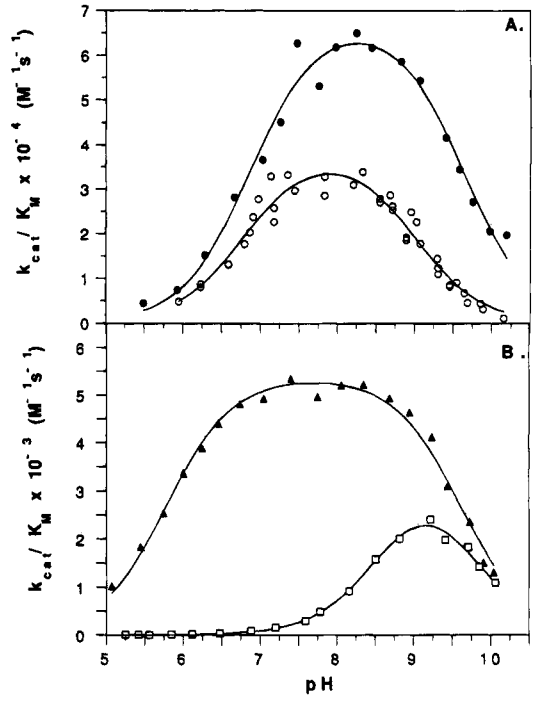


FIGURE 1: pH dependence of k_{cat}/K_M for amino acid reactions with the WT, K258C$_Q$-EA, and Y225F aspartate aminotransferases. The lines represent the nonlinear regression fits to eq 5. (A) WT with L-Asp (●) and L-CS (○); (B) K258C$_Q$-EA (▲) and Y225F (□) with L-Asp. Conditions for Figures 1−5: see Table 1, footnote *a*.

of temperature (25 °C) and ionic strength (μ = 0.2) are 9.8 and 9.2, respectively (Jencks & Regenstein, 1968; Palmieri *et al.*, 1979). Titration of the two amino acids under the conditions of the kinetic assay (μ = 0.2, 150 mM KCl, 25 °C) yielded pK_a values of 9.78 ± 0.01 and 9.17 ± 0.01 for the α-amino groups of L-Asp and L-CS, respectively.

pH Profiles of $k_{cat}/K_M^{\alpha\text{-KG}}$. The pH dependence of the $k_{cat}/K_M^{\alpha\text{-KG}}$ values of WT and K258C$_Q$-EA (Figure 2, Table 1)

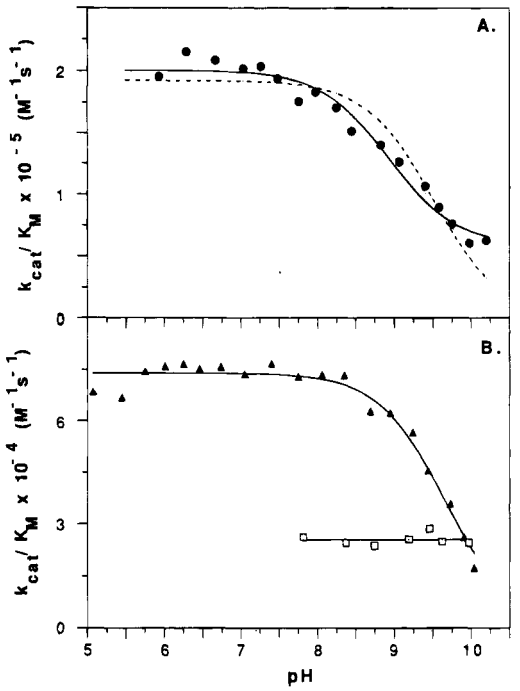


FIGURE 2: pH dependence of $k_{cat}/K_M^{\alpha\text{-KG}}$ for the WT, K258C$_Q$-EA, and Y225F aspartate aminotransferases. The amino acid was L-Asp. (A) WT data fitted to eq 6 (single limit, dashed line) and eq 7 (two limits, solid line); (B) K258C$_Q$-EA (▲) fitted to eq 6 and Y225F (□). The line is drawn through the weighted mean of the Y225F data.

were fitted to a single, alkaline pK_a value with one active protonation state (eq 6). The WT data, however, are better accommodated by a model in which both the protonated and deprotonated species are active (eq 7), with $\lim_{acid} > \lim_{basic}$. The statistical validity of the additional fitted parameter was verified by an *F*-test (Draper & Smith, 1981). The F_{calcd} value is 14.4, which allows a rejection of the null hypothesis with >99% confidence (corresponding to an *F*-distribution of 6.51).

The pH dependence of the WT $k_{cat}/K_M^{\alpha\text{-KG}}$ data fit to a pK_a value of 8.9 for both L-Asp (Figure 2) and L-CS (data not shown) as the amino acid substrate. The K258C$_Q$-EA substitution results in an alkaline shift of the observed pK_a value to 9.6, a ΔpK_a of 0.7 pH unit. No pH dependence on $k_{cat}/K_M^{\alpha\text{-KG}}$ for Y225F was observed. The pK_a of 9.4 observed in an earlier study of Y225F (Goldberg *et al.*, 1991) was an artifact of the pH-dependent inhibition of CHES, which predominantly affects the $k_{cat}/K_M^{\alpha\text{-KG}}$ values of WT.

pH Profiles of K_i(Maleate) for the PMP Enzymes. The pH dependence of the K_i values for the maleate complexes with WT and K258C$_Q$-EA are shown in Figure 3. The pK_a values observed in K_i *vs* pH profiles are the intrinsic ones for the enzyme–dicarboxylate inhibitor/substrate complexes, as the K_i values are true dissociation constants. The pK_a values obtained from the K_i (maleate) profiles of WT and K258C$_Q$-EA are similar (9.5 and 9.6). This suggests that the same prototropic group is responsible, and it cannot be the ϵ-amino group of Lys-258. The pH profiles of $K_M^{\alpha\text{-KG}}$ for WT and K258C$_Q$-EA (data not shown) fit to pK_a values of 8.6 and 9.8, respectively. Comparison of the pK_a values obtained from the $K_i^{maleate}$ and $K_M^{\alpha\text{-KG}}$ profiles supports the conclusion that $K_M^{\alpha\text{-KG}}$ is a true dissociation constant for the K258C$_Q$-EA enzyme, but not for WT.

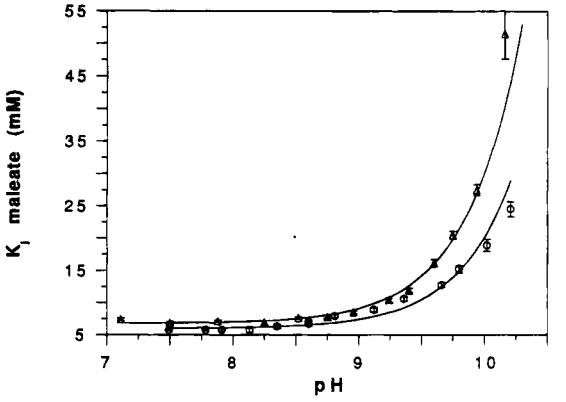


FIGURE 3: pH dependence of K_i(maleate) for the PMP forms of the WT and K258C$_Q$-EA aspartate aminotransferases. WT (○) and K258C$_Q$-EA (Δ) data were fitted to eq 8.

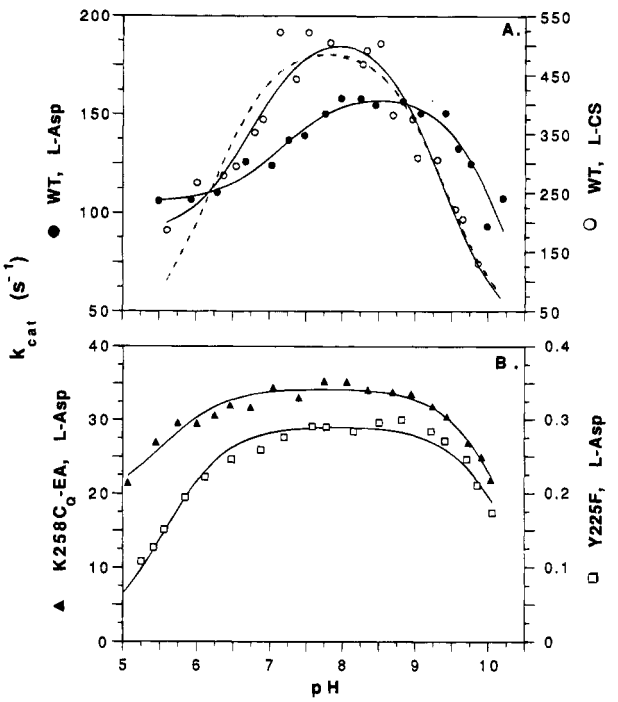


FIGURE 4: pH dependence of k_{cat} for the WT, K258C$_Q$-EA, and Y225F aspartate aminotransferases. The amino acid was either L-Asp or L-CS, as indicated, and the keto acid was α-KG. The solid lines represent the nonlinear regression fits to eq 9 (WT with L-Asp and L-CS, K258C$_Q$-EA) and eq 5 (Y225F). (A) WT with L-Asp (●) and L-CS (○); the dashed line is the fit of the WT L-CS data to eq 5. (B) K258C$_Q$-EA with L-Asp (▲) and Y225F with L-Asp (□).

pH Profiles of k_{cat}. The pH dependence of k_{cat} for WT with the L-Asp/α-KG and L-CS/α-KG substrate pairs is shown in Figure 4A. The WT data are not well fitted by a simple bell curve model (eq 5), but are satisfactorily accommodated by one with an acidic limiting value of k_{cat} (eq 9). The F_{calc} values for the L-Asp and L-CS data sets are 14.31 and 3.53, respectively. This allows >99% confidence (*F*-distribution, 5.56) and >90% confidence (*F*-distribution, 2.46) for the inclusion of a non-zero $(k_1)_{lim}$ in the model for the L-Asp and L-CS fits, respectively. Both of the ionization constants in the L-CS profile are more acidic than those of the L-Asp profile, by 0.5 and 0.9 pH unit for the pK_1 and pK_2 values, respectively.

The k_{cat} *vs* pH profiles for K258C$_Q$-EA and Y225F with the L-Asp/α-KG substrate pair are shown in Figure 4B. The

K258C$_Q$-EA data were fitted only marginally better by eq 9 than by the reduced eq 5. The F_{calc} value is 2.18, intermediate between the F-distributions of 1.52 and 2.49 for 75 and 90% confidence levels, respectively. However, the data were fitted to eq 9 by analogy to the WT k_{cat} data. The lower pK_1 of K258C$_Q$-EA (relative to WT) makes it too difficult to collect data at sufficiently low pH to demonstrate unambiguously the existence of an acidic limit for this enzyme. The Y225F data were well fitted by the simpler bell curve model of eq 5. K258C$_Q$-EA (fit to eq 9) and Y225F have very similar values for both pK_1 and pK_2. The value of pK_2 is the same as that for the WT L-Asp data, but pK_1 is lower than the WT value by 1.7 pH units. The value of k_{cat} for Y225F was found to be constant from pH 7.5 to 10 in an earlier study (Goldberg *et al.*, 1991). The difference from the current pH profile may be the result of perturbation by CHES of the pK_a to a higher value (J. M. Goldberg, personal communication).

The k_{cat} of pcAATase has also been reported to be pH-independent (Kiick & Cook, 1983). The possibility that the decreases in k_{cat} at the high and low pH extremes observed here were due to irreversible denaturation was investigated. As WT and mutant AATases are purified at pH 5.0, it is not likely that the enzyme is irreversibly inactivated at this pH. A 10 min preincubation of AATase in the reaction cocktail did not affect the velocity at either pH 5 or 10.2. The typical assay time was 2 min.

The pH dependences of the C$_\alpha$ proton kinetic isotope effects are given in Table 2. The $^Dk_{cat}$ and $^D(k_{cat}/K_M)$ values for the WT reaction with L-Asp are shown in Figure 5, and the fitted constants describing the pH dependence are given in Table 1. The reactions of WT and K258C$_Q$-EA with L-Asp show a pH dependence on $^Dk_{cat}$, but none on $^D(k_{cat}/K_M)$. Y225F exhibits no C$_\alpha$ kinetic isotope effect on either k_{cat} or k_{cat}/K_M, in agreement with earlier studies at pH 7.5 and 9 (Goldberg, 1992; J. M. Goldberg and J. F. Kirsch, manuscript in preparation). The present study shows that C$_\alpha$ proton abstraction is not rate-determining at the pH extremes (Table 2). The values in Table 2 are the first reported C$_\alpha$ kinetic isotope effects for the L-CS substrate. The rate of the WT reaction with L-CS is less determined by proton abstraction than is that of WT with L-Asp. This statement is based on the reasonable assumption that there is no large difference in the intrinsic isotope effects for C$_\alpha$ proton abstraction for the two amino acids. The L-CS $^Dk_{cat}$ and $^D(k_{cat}/K_M)$ values are pH-independent. The KIE values of L-CS with K258C$_Q$-EA are similar to or larger than those observed with K258C$_Q$-EA and L-Asp.

Table 2: C$_\alpha$-^{2}H Kinetic Isotope Effects for WT, K258C$_Q$-EA, and Y225F Asparate Aminotransferases with L-ASP AND L-CS[a]

pH	$^Dk_{cat}$	$^Dk_{cat}/K_M$
	WT and [C$_\alpha$-^{2}H]Asp[b]	
5.06	1.1 (0.2)	2.1 (0.2)
5.61	1.0 (0.1)	2.0 (0.2)
6.42	1.17 (0.03)	2.2 (0.2)
6.90	1.46 (0.04)	1.8 (0.1)
7.60	1.45 (0.05)	2.1 (0.1)
8.08	1.55 (0.07)	2.1 (0.2)
8.56	1.65 (0.07)	1.9 (0.1)
8.91	1.56 (0.05)	2.4 (0.2)
9.31	1.56 (0.05)	2.3 (0.1)
9.69	1.41 (0.06)	2.1 (0.1)
10.23	1.17 (0.05)	2.0 (0.2)
	WT and [C$_\alpha$-^{2}H]CS[c]	
7.89	1.16 (0.07)	1.55 (0.1)
9.08	1.15 (0.06)	1.34 (0.1)
9.91	1.01 (0.07)	1.43 (0.1)
	K258C$_Q$-EA and [C$_\alpha$-^{2}H]Asp[b]	
5.07	3.3 (0.1)	6.1 (0.4)
8.08	3.5 (0.2)	5.7 (0.8)
10.2	1.6 (0.2)	4.5 (0.5)
	K258C$_Q$-EA and [C$_\alpha$-^{2}H]CS[d]	
8.14	4.0 (0.3)	6.4 (0.5)
	Y225F and [Ca-^{2}H]Asp[e]	
4.99	1.1 (0.1)	1.2 (0.2)
8.08	1.06 (0.02)	1.03 (0.06)
9.92	1.00 (0.04)	1.03 (0.17)

[a] Errors are given in parentheses. The data were collected and fitted as described in Materials and Methods. Conditions: MDH- or LDH-coupled assays in MHP buffer, μ = 0.2, 25 °C. [b] [α-KG] = 2K_M; [L-Asp] = 0.5−20 mM. [c] [α-KG] = 2K_M; [L-CS] = 1.5−15 mM. [d] [α-KG] = 7K_M; L-CS] = 5−40 mM. [e] [α-KG] = 100K_M; [L-Asp] = 5−100 mM (pH 4.99), 0.05−2.5 mM (pH 8.08), or 0.05−7.5 mM (pH 9.92). Values determined by Goldberg (1992) for Y225F for $^Dk_{cat}$ and $^D(k_{cat}/K_M)$ at pH 9.0 are 1.06 ± 0.04 and 1.0 ± 0.1, respectively, and those at pH 7.5 are 1.0 ± 0.08 and 1.1 ± 0.3, respectively.

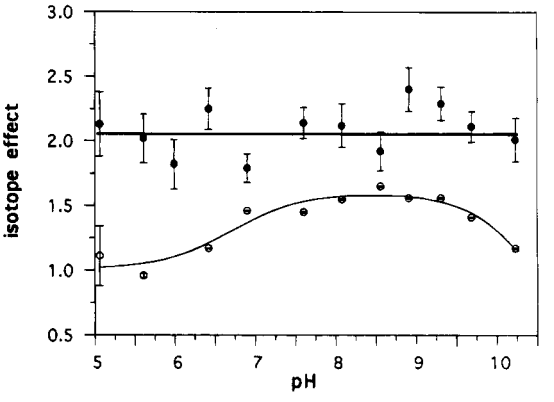


FIGURE 5: pH dependence of the [C$_\alpha$-^{2}H]Asp kinetic isotope effects for WT aspartate aminotransferase: (●) $^Dk_{cat}/K_M$ and (○) $^Dk_{cat}$ values. The upper line is drawn through the weighted mean of the $^Dk_{cat}/K_M$ data. The line drawn through the $^Dk_{cat}$ values represents the nonlinear regression fit to eq 9.

DISCUSSION

Buffer Effects on pH Profiles. An artifactual pK_a of 7.5 was observed in the $k_{cat}/K_M^{\alpha\text{-KG}}$ profile of *E. coli* AATase when the ionic strength was allowed to vary from 0.2 to 0.55 over the pH range (data not shown). The rate constant increased below this pK_a, mirroring the $k_{cat}/K_M^{\alpha\text{-KG}}$ profile of pcAATase published by Velick and Vavra (1962), in which ionic strength was not controlled. Ionic strength effects may explain the discrepancy between the pcAATase $k_{cat}/K_M^{\alpha\text{-KG}}$ profiles of Velick and Vavra (1962) and those of Kiick and Cook (1983).

A standard test for specific buffer effects is to perform the assays at pH values in two buffers at overlapping ranges of pH (Kiick & Cook, 1983; Goldberg *et al.*, 1991). While usually adequate, this test fails if buffer inhibition is pH-dependent, as seen for the CHES inhibition of AATase (see Results). The k_{cat}/K_M^{Asp} pH profile of Kiick and Cook (1983) fit to two pK_a values on the alkaline limb (9.3 and 10.1). The experiments described in the Results section strongly suggest that the pK_a of 9.3 is due to CHES, which shows pH-dependent inhibition of this rate constant for pcAATase.

pH Dependence of the E·PLP + Amino Acid Half-Reaction. The only pK_a observed in the pH dependence of AATase activity, which had been previously assigned unambiguously, was that of the acidic limb on the k_{cat}/K_M^{Asp} profile (Kiick & Cook, 1983), attributed to the internal aldimine (deprotonated structure shown in Scheme 1). Therefore, given that pK_a values in k_{cat}/K_M profiles reflect ionizations of the free enzyme and free substrate (e.g., Velick & Vavra, 1962), the similarity of the WT values for the acidic pK_a of the k_{cat}/K_M profiles with L-Asp and L-CS (Table 1) is consistent with this assignment. Mutations that alter the spectrophotometric pK_a of the internal aldimine (K258C_Q-EA and Y225F) shift the kinetic pK_a values similarly (Gloss & Kirsch, 1995; Goldberg *et al.*, 1991).

The group responsible for the alkaline pK_a observed in the k_{cat}/K_M^{Asp} profile has not been assigned previously. For the pcAATase, it was postulated to be the pyridine nitrogen atom (Kiick & Cook, 1983; Kirsch *et al.*, 1984). Subsequent NMR studies of exchangeable protons of the pcAATase, however, were interpreted to show that the PLP pyridine nitrogen atom remains protonated up to pH 10.8 (Kintanar *et al.*, 1991).

Mutations of AATase (e.g., K258C_Q-EA, Y225F) do not affect the observed alkaline pK_2, but changes in the amino acid substrate do. Thus, the ionization must be associated with the free substrate. The α-amino group of L-CS is more acidic than that of L-Asp, and a shift of similar magnitude and direction is observed in the k_{cat}/K_M^{CS} pK_a relative to that of the k_{cat}/K_M^{Asp} profile. This allows clear assignment of the alkaline pK_a of k_{cat}/K_M for the amino acid half-reaction to the titration of the α-amino group of the substrate. As the kinetic pK_a values for L-Asp and L-CS closely agree with those determined potentiometrically, it can be concluded that these are not sticky substrates (Cleland, 1977).

pH Dependence of the E·PMP + Keto Acid Half-Reaction. The $k_{cat}/K_M^{\alpha\text{-KG}}$ profile for pcAATase is described by a bell curve with acidic and alkaline pK_a values of 5.8 and 9.2, respectively. The acidic pK_a was tentatively assigned to the protonation of the diamine system of the ϵ-amino group of Lys-258 and the amino group of PMP (Kiick & Cook, 1983). No acidic pK_a was observed in the $k_{cat}/K_M^{\alpha\text{-KG}}$ profile of *E. coli* AATase (Figure 2). The alkaline pK_a of pcAATase was assigned to the ionization of the pyridine nitrogen (Kiick & Cook, 1983) or to the first protonation of the diamine system of Lys-258 and PMP (Kirsch *et al.*, 1984). ^{1}H NMR data show that the PMP pyridine nitrogen atom is protonated to at least pH 9.25 (Kintanar *et al.*, 1991), demonstrating that the alkaline pK_a in the $k_{cat}/K_M^{\alpha\text{-KG}}$ profile is not associated with the pyridine nitrogen. The alkaline pK_a values observed in the present studies must be due to the titration of an enzyme group because α-KG has no ionizable groups with pK_a values in this range.

If a substrate is sticky, i.e., the rate constant for substrate dissociation is less than the net rate constant for the reaction to give products, the pK_a values observed in k_{cat}/K_M profiles may be affected. A sticky substrate will give an anomalously low pK_a value if the protonated species is the active one, while the observed pK_a is increased where the deprotonated species is the functional one (Cleland, 1977). Intrinsic pK_a values can be determined from K_i profiles where K_i is a true dissociation constant. The results of a study for the E·PMP forms of WT and K258C_Q-EA with the competitive inhibitor, maleate, are given in Figure 3. Substrate stickiness can be ruled out if the pK_a values for the k_{cat}/K_M and K_i profiles are the same (Kiick & Cook, 1983). By this criterion, the data in Table 1 show that $K_M^{\alpha\text{-KG}}$ is not a true dissociation constant for WT. For pcAATase, α-KG is not a sticky substrate (Kiick & Cook, 1983). The data in Table 1 demonstrate that α-KG is not a sticky substrate for K258C_Q-EA. However, K258C_Q-EA has a $K_M^{\alpha\text{-KG}}$ similar to that of WT (Gloss & Kirsch, 1995), showing that this mutant protein has a higher affinity for α-KG than does WT.

The intrinsic pK_a values (deduced from the K_i (maleate) profiles) for WT and K258C_Q-EA are virtually the same. Because the pK_a of the ϵ-amino group of γ-thia-Lys is 1.1 pH units more acidic than that of Lys, the ϵ-amino group of Lys-258 cannot be the group responsible for the observed pK_a. If the proton is shared by the amino groups of Lys-258 and PMP, the pK_a of PMP must be at least 1 pH unit greater than that of the pK_a of Lys-258 (Gloss, 1994). Therefore, the proton must be predominantly associated with the PMP amino group. Further support for this assignment is provided by the $k_{cat}/K_M^{\alpha\text{-KG}}$ profile of Y225F. This mutation increases the pK_a of the internal aldimine by the loss of a hydrogen bond to O3′ (Table 1, Figure 1; Goldberg *et al.*, 1991). The hydrogen bond between the hydroxyl of Tyr-225 and O3′ of the cofactor is likely to be present in the E·PMP form as well. In the X-ray crystal structure of the PMP form of chicken mitochondrial AATase (McPhalen *et al.*, 1992), the oxygen−oxygen distance is 2.4 Å, which is similar to the distance observed in the E·PLP structure of this AATase, 2.6 Å (McPhalen *et al.*, 1992). Therefore, Y225F should have an effect on the pK_a of the amino group of PMP similar to that on the pK_a of internal aldimine: an alkaline shift of ~1 pH unit. No variation with pH was observed in the $k_{cat}/K_M^{\alpha\text{-KG}}$ values of Y225F from pH 7.5 to 10 (Figure 2B). This is interpreted as a shift in the pK_a beyond the measurable range of steady-state kinetics. An alternative interpretation is that the ionizable group is the hydroxyl of Tyr-225, which was removed by the mutation. It would be surprising if the pK_a of Tyr-225 were as low as 9.6. Deprotonation would juxtapose two negative phenolic oxygens (Tyr-225 and O3′, its hydrogen bond partner) within ~2.5 Å of each other.

In both the unliganded E·PLP and E·PMP enzymes, one proton is required for efficient turnover. In the former case, the proton is on the incoming amino acid substrate (rather than on the internal aldimine); in the latter form, the proton resides on the C4′ amino group of the cofactor (rather than on Lys-258). In both enzyme forms, the proton is localized to the nucleophile of the subsequent transaldimination/transimination reaction. Upon binding dicarboxylate ligands, the enzyme closes (by movement of the small domain) with concomitant shielding of the positive charges of the arginine side chains (Arg-292 and Arg-386), which bind the carboxylates of the substrate (Kirsch *et al.*, 1984). This structural change alters the pK_a values of the active site ionizable groups to permit at least partial deprotonation of the nucleophile. This has been demonstrated for the E·PLP·maleate complex (Yano *et al.*, 1993), where the pK_a of the internal aldimine is increased by 2 pH units relative to the unliganded enzyme.

pH Dependence of k_{cat}. Velick and Vavra (1962) reported an acidic pK_a in the pH dependence of k_{cat} for pcAATase,

with activity decreasing at pH values below this pK_a (Velick & Vavra, 1962). No assignment was suggested to account for the observed pK_a. Later studies showed that k_{cat} and $^Dk_{cat}$ from the C_α kinetic isotope effects are pH-independent (Kiick & Cook, 1983; Julin & Kirsch, 1989).

Two steps are partially rate-determining in the WT reaction with L-Asp at pH 8: C_α proton abstraction (k_3 in Scheme 1) and OAA dissociation (a component of k_5) (Kirsch *et al.*, 1990). Ketimine hydrolysis may also be partially rate-determining (Goldberg, 1992). The pH dependence of the KIE for the WT L-Asp reaction demonstrates that there is a change in the rate-determining step at the pH extremes (Table 2). k_{cat}/K_M^{Asp} includes all steps up to the first irreversible step, OAA dissociation, but $^D(k_{cat}/K_M)$ contains only those steps up to the loss of the C_α hydron. $^D(k_{cat}/K_M)$ is pH-independent; therefore, the rate of C_α proton abstraction must be relatively constant from pH 5 to 10 (Figure 5). The value of $^Dk_{cat}$ approaches unity at both pH extremes. Planas and Kirsch (1991) showed that when $^D(k_{cat}/K_M) > ^Dk_{cat}$, $k_2 > k_5$ (Scheme 1). Given the pH dependence of these KIE values, in the overall ping-pong reaction, some step after C_α proton abstraction, i.e., a component of k_5, becomes more fully rate-determining at the pH extremes. If the deuteron is not lost to solvent during the half-reaction, it could be deduced from the observed relation of $^Dk_{cat} < ^D(k_{cat}/K_M)$ that the component of k_5 that has become rate-determining is a step in k_{cat}, but not in k_{cat}/K_M^{Asp} (i.e., the keto acid half-reaction). The mechanism of AATase involves a diprotic base (Lys-258) and may proceed with a concerted 1,3-prototropic shift (Julin & Kirsch, 1989; Goldberg, 1992). Because the hydron is washed out in the AATase reaction after C_α hydron abstraction (Julin & Kirsch, 1989), $^D(k_{cat}/K_M)$ does not reflect any steps after k_3. Therefore, ketimine hydrolysis and OAA dissociation cannot be eliminated as candidates for the rate-determining step at the pH extremes.

$^Dk_{cat}$ is essentially unity for the reaction of WT with L-CS (Table 2). C_α proton abstraction therefore is not rate-determining for the overall reaction of the L-CS/α-KG substrate pair. The value of $^D(k_{cat}/K_M^{CS})$ is about two-thirds of the WT value for $^D(k_{cat}/K_M^{Asp})$. The major rate-determining step of the WT/L-CS reaction is a component of k_5, and there is no evidence for a pH-dependent change in the rate-determining step. However, C_α KIEs do not report on steps subsequent to C_α proton abstraction. Viscosity variation experiments on k_{cat} and k_{cat}/K_M have shown that the WT L-CS reaction is not diffusion-controlled (Goldberg, 1992). In contrast, the L-Asp reaction is partially rate-limited by OAA dissociation. The rate-determining step for the L-CS/α-KG reaction is either ketimine hydrolysis or the keto acid half-reaction. At pH 8, it can be deduced that the rate-determining step is the α-KG half-reaction. A k_{max} value for the L-CS reaction can be calculated from the relationship of k_{cat} to the k_{max} values (determined from single-turnover kinetics) for the two half-reactions,

$$k_{max}^{CS} = \frac{k_{max}^{\alpha\text{-KG}} k_{cat}}{k_{max}^{\alpha\text{-KG}} - k_{cat}} \qquad (10)$$

and the observed values for k_{cat} (pH 8.2, 500 ± 17 s^{-1}, Figure 4A) and $k_{max}^{\alpha\text{-KG}}$ (600 ± 40 s^{-1}; Kuramitsu *et al.*, 1990). The calculated value of k_{max} for L-CS is 3000 ± 1000 s^{-1}. A similar analysis for L-Asp, using k_{cat} = 158 ± 2 s^{-1} (pH 8.0, Figure 4A), yields a k_{max} value of 214 ± 24 s^{-1}. This rate constant is much lower than the published value (550 s^{-1}; Kuramitsu *et al.*, 1990). The discrepancy arises because the single-turnover kinetics in the PLP → PMP direction measured by Kuramitsu *et al.* (1990) only monitors the rate of steps up to ketimine formation. There is no cofactor absorbance change for the steps of ketimine hydrolysis and OAA dissociation, which are partially rate-determining for the L-Asp reaction.

It is, therefore, reasonable to conclude that the WT k_{cat} pH profile with L-CS/α-KG (Figure 4) examines prototropic groups in the second half-reaction. The value of the pK_a from this k_{cat} profile (9.4) is in reasonable agreement with the intrinsic pK_a of the $k_{cat}/K_M^{\alpha\text{-KG}}$ and K_i (maleate) profiles (9.6). This suggests that pK_2 arises from the titration of the amino group of PMP in the free enzyme, as established earlier for the $k_{cat}/K_M^{\alpha\text{-KG}}$ profiles. pK_1 may be the second protonation of the PMP·Lys-258 diamine system in the unliganded E·PMP species or a histidine (143, 189, or 193). pK_1 cannot be the pK_a of Lys-258 in the ketimine with α-KG, since the activity does not go to zero upon protonation, as would be anticipated for the protonation of the general base that abstracts the C4′ proton.

K258C_Q-EA has a 5−7-fold lower k_{cat} value (L-Asp/α-KG substrate pair) than does WT, due to the decreased basicity of the general base, γ-thia-Lys relative to Lys (Gloss & Kirsch, 1995). This mutation results in a higher C_α proton KIE than that of WT (Table 2). The $^D(k_{cat}/K_M)$ values of K258C_Q-EA at pH 8.1 for L-Asp and L-CS approach the intrinsic KIE values for this reaction, ~7 (Onuffer & Kirsch, 1994). The less than intrinsic KIE values exhibited by WT demonstrate that proton abstraction is only partially rate-determining, while this step appears to be nearly fully rate-determining for K258C_Q-EA. Unlike WT, the K258C_Q-EA KIEs for L-CS are equal to or slightly higher than those measured with L-Asp.

The pH dependence of $^Dk_{cat}$ for the K258C_Q-EA/L-Asp reaction demonstrates that there is a change in the rate-determining step at high pH. Data could not be collected at a pH sufficiently below the K258C_Q-EA k_{cat} pK_a to determine whether $^Dk_{cat}$ changes below this pH. The rate-determing step for K258C_Q-EA at high pH must be some component of k_5 (see preceding discussion of WT/L-Asp k_{cat}).

The rate-determining step for Y225F at pH 8−9 is ketimine hydrolysis (Goldberg, 1992). The k_{cat} *vs* pH profile of this enzyme therefore reflects prototropic groups in the ketimine complex. There is no evidence for a change in the rate-determining step with pH, but as discussed earlier for the WT/L-CS reaction, this possibility cannot be excluded from the data presented here.

It is possible that each of the k_{cat} *vs* pH profiles presented in Figure 4 reflects ionizations of different enzyme·substrate or enzyme·product complexes. However, the L-Asp k_{cat} profiles of WT, K258C_Q-EA, and Y225F all fit to a value of 10.3 for pK_2. The simplest explanation would be that the titrating group is in the same enzyme complex for the three enzymes. The k_{cat} value of Y225F is limited by the rate of ketimine hydrolysis, and both WT and K258C_Q-EA are rate-limited by some component of k_5 at high pH, which would include ketimine hydrolysis. Therefore, a reasonable assignment for pK_2 is the titration of the proton shared by the C4′ nitrogen and O3′ in the ketimine (protonated structure

shown in Scheme 1). The Y225F mutation does not alter this pK_a, as it does for the corresponding proton in the internal aldimine (k_{cat}/K_M^{Asp} profile) or the PMP enzyme ($k_{cat}/K_M^{\alpha\text{-KG}}$ profile). The effect of the Tyr-225 hydroxyl hydrogen bond is substantially decreased in the enzyme·substrate complexes, due to the rotation of the cofactor (Goldberg *et al.*, 1991). The O3′ ↔ O (Tyr-225 hydroxyl) distance in the *E. coli* E·PLP X-ray crystal structure is 2.7 Å (Jäger *et al.*, 1994). It is increased to 3.5 and 3.2 Å in the α-Me-Asp and PLP·maleate structures, respectively.

Given the differences in the k_{cat} models employed in fitting the low pH data of WT, K258C_Q-EA (eq 9), and Y225F (eq 5), it is not clear that pK_1 of the L-Asp/α-KG k_{cat} profiles reflects an ionization of the same enzyme·product complex for all three enzymes. This pK_a value is affected by modifications of both Lys-258 and Tyr-225. A tentative assignment is the ϵ-amino group of Lys-258 in the ketimine complex. Other possibilities include one of the three histidines (143, 189, or 193) in the hydrogen bond network around the pyridine nitrogen and Asp-222.

The pH dependence of k_{cat} and the pH-dependent change in the rate-determining step (or lack thereof) are striking differences between the pig heart cytosolic and *E. coli* AATases. Because k_{cat} does not go to zero at low pH, the pK_a of Lys-258 must be less than 5 in those external aldimine and ketimine complexes where it acts as the general base for the 1,3-prototropic shift. This is in agreement with an earlier deduction for pcAATase (Julin & Kirsch, 1989). Other significant prototropic differences between the two enzymes are the more acidic pK_a of the internal aldimine of pcAATase (6.3 *vs* 6.9) and the stickiness of α-KG. This study shows the inherent danger in assuming that subtle details are similar for the kinetic mechanisms of enzymes from evolutionarily distant sources, despite their significant sequence conservation.

CONCLUSIONS

The important new assignments from this work are as follows: (1) The alkaline pK_a (9.6 and 9.0) of the k_{cat}/K_M amino acid profile is that of the α-amino group of the substrate. (2) The single pK_a (ca. 9.6) observed in the $k_{cat}/K_M^{\alpha\text{-KG}}$ and K_i PMP·maleate profiles is assigned to the amino group of PMP. (3) Ionization of the ketimine is reflected in the pK_a of 10.3 observed in the L-Asp/α-KG k_{cat} profile.

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