Brønsted analysis of aspartate aminotransferase via exogenous catalysis of reactions of an inactive mutant

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Abstract

Primary amines functionally replace lysine 258 by catalyzing both the 1,3-prototropic shift and external aldimine hydrolysis reactions with the inactive aspartate aminotransferase mutant K258A. This finding allows classical Brønsted analyses of proton transfer reactions to be applied to enzyme-catalyzed reactions. An earlier study of the reaction of K258A with cysteine sulfinic acid (Toney, M.D. & Kirsch, J.F., 1989, *Science* 243, 1485) provided a β value of 0.4 for the 1,3-prototropic shift. The β value reported here for the transamination of oxalacetate to aspartate is 0.6. The catalytic efficacy of primary amines is largely determined by basicity and molecular volume. The dependence of the rate constants for the reactions of K258A and K258M on amine molecular volume is nearly identical. This observation argues that the alkyl groups of the added amines do not occupy the position of the lysine 258 side chain in the wild type enzyme. Large primary $C\alpha$ and insignificant solvent deuterium kinetic isotope effects with amino acid substrates demonstrate that the amine nitrogen of the exogenous catalysts directly abstracts the labile proton in the rate-determining step.

Keywords: Brønsted analysis; exogenous catalysis; kinetic isotope effects; site-directed mutagenesis

Aspartate aminotransferase (AATase) is a PLP-dependent enzyme whose structure and mechanism have been well studied (Christen & Metzler, 1985; Jansonius & Vincent, 1987; Hayashi et al., 1990). It catalyzes the reversible interconversion of dicarboxylic amino and keto acids by the mechanism outlined in Scheme 1. The central, chemically difficult step of the reaction is the 1,3-prototropic shift that interconverts the external aldimine (II) and ketimine (IV) intermediates. This step is subject to general base catalysis by the ϵ -amino group of LYS258 as demonstrated by studies on the K258A (Toney & Kirsch, 1989; M.D. Toney & J.F. Kirsch, unpubl.) and Y70F (Toney & Kirsch, 1987, 1991) mutants.

General acid/base catalysis is a common mechanism for reaction rate enhancement by enzymes. Examples in-

clude proteases (Baker & Drenth, 1987; Kossiakoff, 1987), dehydrogenases (Ehrig et al., 1991), glycosidases (Imoto et al., 1972), and isomerases (Knowles, 1991; see also Walsh, 1979). The delineation of the transition state structures for such reactions is a major goal of mechanistic enzymology.

Two major tools, kinetic isotope effects and structure-reactivity correlations, for transition-state structure analysis are available. The quantitative analysis of kinetic isotope effects has proven successful both in nonenzymatic (Westheimer, 1961; Melander & Saunders, 1980; Caldwell et al., 1991) and enzyme-catalyzed reactions (Northrop, 1981; Rosenberg & Kirsch, 1981; Markham et al., 1987; Caldwell et al., 1991). The physical organic chemist has an additional tool for transition state structure delineation in the linear free energy analysis of structure-reactivity correlations (e.g., Hammett and Brønsted analyses). The Hammett plot depends on systematic variations in reactant or catalyst structure and examines the linear relation (slope = ρ) between the logarithm of the reaction rate constant and of a quantitatively defined structural parameter of the reactant or catalyst (i.e., σ value). The utility of Hammett analyses with enzyme-catalyzed reactions is restricted, due to the often stringent substrate specificities of enzymes and the need for struc-

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Abbreviations: AATase, aspartate aminotransferase (E.C. 2.6.1.1); PLP, pyridoxal-5'-phosphate; PMP, pyridoxamine-5'-phosphate; E-PLP and E-PMP, PLP and PMP forms of AATase, respectively; K258A, K258M, AATase in which LYS258 has been changed to alanine or methionine by site-directed mutagenesis; KIE, kinetic isotope effect; superscript D (e.g., $D_{k_{cat}}$), deuterium KIE on the specified parameter; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; CHES, 2-[*N*-cyclohexylamino]-ethanesulfonic acid.

A) Wild Type **B) K258A**

Scheme 1. Reaction mechanism of aspartate aminotransferase. In wild-type enzyme (**A**), PLP-enzyme (**I**) reacts with amino acid substrate via transamination to form the covalent external aldimine intermediate (**II**). LYS258 is the general base catalyst for the external aldimine to ketimine (**IV**) tautomerization, which possibly includes the quinonoid (**III**) as an intermediate. The ketimine is hydrolyzed to PMP-enzyme (**V**) and keto acid product. The transamination cycle is completed when a second keto acid reacts through the reverse of this sequence to give PLP-enzyme and amino acid product. The K258A reaction (**B**) as shown includes catalysis by an exogenous amine. External aldimine (**II**) formation and hydrolysis, as well as the 1,3-prototropic shift interconverting the aldimine and ketimine (**IV**), are subject to amine catalysis.

tural variations in the substrate. Limited success has been achieved, particularly with nonspecific enzymes (Kirsch, 1972; Klinman, 1976; Ulevitch & Kallen, 1977).

General acid/base-catalyzed reactions are often analyzed with the linear free energy relation known as the Brønsted plot where the logarithm of the catalytic rate

constant is plotted against the pK_a of the catalyst. The slope of such a plot (α or β value) is frequently interpreted as a measure of the degree of proton transfer, or of the amount of charge development, in the transition state (Kresge, 1975). The near ubiquity of general acid or base catalysis in proposed enzymatic mechanisms brings

out the potential importance of enzymatic Brønsted analysis, but this method has previously been impossible to employ because the catalytic moieties are built into the enzyme structures and therefore cannot be varied. It was recently demonstrated with the K258A mutant of AA-Tase that site-directed mutagenesis combined with exogenous catalysis by small molecules can overcome this barrier, enabling direct Brønsted analyses of enzyme-catalyzed proton transfer reactions (e.g., Scheme 1B, II $\rightarrow$ IV) (Toney & Kirsch, 1989).

This report extends earlier work on the direct Brønsted analysis of the 1,3-prototropic shift occurring in the reaction of K258A with cysteine sulfinic acid to the reverse reaction (Scheme 1B, IV $\rightarrow$ II) with oxalacetate as substrate. The measurement of C_α and solvent ^2H KIEs for amino acid substrates defines the rate-determining step and resolves the kinetic ambiguity inherent in general acid/base catalysis. Finally, it is demonstrated that hydrolysis of the Schiff base of the external aldimine intermediate formed with K258A (Scheme 1B, II $\rightarrow$ I) is also subject to catalysis by exogenous amines.

Results

The rates of the 1,3-prototropic shifts catalyzed by K258A are increased by exogenous amines

Figure 1 illustrates the dependence of the observed pseudo-first order rate constants for the 1,3-prototropic shift on ethylamine concentration for the reaction of K258A-

Fig. 1. Dependence of the observed pseudo-first order rate constant for the 1,3-prototropic shift of the K258A-oxalacetate ketimine on the concentration of ethylamine. The pH dependence of the reaction shows that the rate constants depend linearly on the concentration of the free base form of the amine. The reaction rates were monitored by the increase in 430-nm absorbance due to formation of the external aldimine. Conditions: 0.1 M CHES-KOH, 25 °C, ionic strength = 0.5 (tetramethylammonium chloride).

PMP with oxalacetate (Scheme 1B, IV $\rightarrow$ II). Qualitatively similar results were obtained for the reactions of K258A with cysteine sulfinic acid (Toney & Kirsch, 1989), aspartate, glutamate, cysteine, and α -ketoglutarate (data not shown). The observed rate constants are linearly dependent on the concentration of the free base form of the amine catalyst. Values of k_B for catalysis of the K258A-PMP reaction with oxalacetate by 11 amines are collected in Table 1. A limited set of k_B values for the reaction of the K258M mutant with oxalacetate is also reported in Table 1.

Both amine basicity and molecular volume are significant determinants of the k_B values. The role of basicity is discussed below. The effect of amine volume on the K258A-PMP reaction with oxalacetate is isolated by the plot shown in Figure 2 (circles), where the $\log k_B$ values of methyl-, ethyl-, propyl-, and butylamine (which have nearly identical basicities) are plotted against amine molecular volumes. The slope of the best-fit line is $-0.048 \pm 0.004 \text{ \AA}^{-3}$, which agrees well with the value of V obtained from multiple regression analysis (see below). The limited set of k_B values for the K258M mutant included in Table 1 is two- to threefold less than that for K258A. A plot of $\log k_B$ vs. amine volume for the K258M data (Fig. 3, filled circles) generates a slope of $-0.046 \pm 0.005 \text{ \AA}^{-3}$, which is statistically indistinguishable from that obtained for the K258A reaction (Fig. 3, open circles). Likewise, the reactions of these two mutants with

Fig. 2. Dependence of the logarithm of the catalytic rate constant, $\log k_B$, for the transamination reactions of the K258A-cysteine sulfinic acid (Scheme 1B, II $\rightarrow$ IV) (dashed line, from Toney & Kirsch [1989]) and K258A-oxalacetate (Scheme 1B, IV $\rightarrow$ II) (circles) complexes, and the K258A-aspartate aldimine hydrolysis reaction (Scheme 1B, II $\rightarrow$ I) (squares) on amine molecular volume. This subset of amines contains only methyl-, ethyl-, propyl-, and butylamine, which have nearly equal basicities. The variation in k_B is therefore due only to the molecular volume. The slopes of the best-fit lines are $-0.062 \pm 0.006 \text{ \AA}^{-3}$ (cysteine sulfinic acid), $-0.048 \pm 0.004 \text{ \AA}^{-3}$ (oxalacetate), and $-0.038 \pm 0.002 \text{ \AA}^{-3}$ (aspartate aldimine hydrolysis).

Table 1. Rate constants for the amine-assisted transamination of the oxalacetate ketimine by the aspartate aminotransferase mutants K258A and K258M^a

Amine	pK _a	Molecular volume (Å ³)	K258A k _B (M ⁻¹ s ⁻¹)	K258M k _B (M ⁻¹ s ⁻¹)
Methyl-	10.6	42.1	322 (7) 304 (17)	115 (11)
Ethyl-	10.6	60.9	64.0 (0.5)	20 (1)
Propyl-	10.5	79.8	9.4 (0.3)	4.4 (0.1)
Butyl-	10.6	98.7	0.46 (0.02)	0.23 (0.07)
Ethylenedi-	10.0	74.6	1.08 (0.08) ^b	
Ethanol-	9.5	71.5	2.1 (0.2)	
Ammonia	9.2	23.2	117 (1)	
2-Fluoroethyl-	9.0	64.4	8.0 (0.2) 11.2 (0.6)	
2-Cyanoethyl-	7.7	70.5	0.032 (0.002) 0.033 (0.002)	
2,2,2-Trifluoroethyl-	5.7	71.6	0.018 (0.002) 0.017 (0.009)	
Cyanomethyl-	5.3	51.1	0.07 (0.01) 0.076 (0.007)	

^a The k_B values are for the free base. Standard errors are given in parentheses. Conditions: pH 9.3, 0.1 M CHES-KOH, ionic strength = 0.5 (tetramethylammonium chloride), 25 °C. The pK_a values and molecular volumes were obtained as described in Toney and Kirsch (1989). Duplicate values are for replicate experiments.

^b k_B has been divided by 2 to correct for statistical effects.

Fig. 3. Comparison of the dependencies of the rate constants for amine catalysis of the reactions of K258A (open circles) and K258M (filled circles) with oxalacetate on amine molecular volume. These amines (methyl-, ethyl-, propyl-, and butylamine) have nearly identical basicities (Table 1); thus, the slopes of the lines through the data are measures of the sensitivities of the reactions to amine molecular volume. The slopes of the best-fit lines are $-0.046 \pm 0.005 \text{ Å}^{-3}$ for K258M and $-0.048 \pm 0.004 \text{ Å}^{-3}$ for K258A.

cysteine sulfinat display indistinguishable sensitivities to amine volume.

The rate constants for the transamination of the cysteine sulfinat aldimine (Scheme 1B, II → IV) are linearly dependent on ammonia buffer concentration up to at

Fig. 4. Dependence of the observed pseudo-first order rate constants for the transamination of the K258A-cysteine sulfinat aldimine on ammonia buffer concentration. Conditions: 25 °C, pH 9.3, ionic strength = 2.0 (tetramethylammonium chloride).

least 2 M (Fig. 4). The value of 16 s^{-1} observed at 1 M NH_3 (2 M buffer) is 2×10^4 -fold larger than that observed in the absence of amines and represents 2% of the wild-type enzyme rate constant value (M.D. Toney & J.F. Kirsch, unpubl.). The value of k_B obtained from these data ($16.6 \pm 0.5 \text{ M}^{-1} \text{ s}^{-1}$) is nearly identical to that measured at low (0.1–1 mM) ammonia concentrations ($21 \pm 2 \text{ M}^{-1} \text{ s}^{-1}$; Toney & Kirsch, 1989).

Table 2. Kinetic isotope effects for the 1,3-prototropic shift reactions of the wild-type enzyme and the K258A mutant of aspartate aminotransferase with amino acids^a

Amino acid	Wild type ^b	K258A ^c
C α -H primary KIE		
Glutamate	2.1	5.5 (0.2)
Aspartate	2.2	5.2 (0.9)
D ₂ O solvent KIE		
Cysteine sulfinat		1.0 (0.1)

^a The KIEs are on $k_{\max}$ (see footnote 1). All measurements were made at 25 °C. Standard errors are given in parentheses.

^b Taken from Kuramitsu et al. (1990). Conditions: pH 8.0, 0.05 M HEPES-KOH, 0.1 M potassium chloride.

^c Conditions: pH 9.3, 0.1 M CHES-KOH, 0.5 M tetramethylammonium chloride, 1–5 mM methylamine added as catalyst, 25 °C.

The k_B value for dimethylamine catalysis of the cysteine sulfinat 1,3-prototropic shift is $0.23 \pm 0.01 \text{ M}^{-1} \text{ s}^{-1}$, a figure sixfold lower than that for ethylamine (Toney & Kirsch, 1989), which has the same molecular volume and basicity. No acceleration of the K258A reaction with cysteine sulfinat was detected with either imidazole or diaminobicyclooctane (DABCO) up to a concentration of 250 mM.

Kinetic isotope effects

In Table 2 the values for KIEs on wild-type enzyme and K258A reactions are presented. The value of $^Dk_{\max}$ with wild-type enzyme is 2.1 whereas that with K258A is 5.5 using glutamate as substrate.¹ The values of the aspartate KIEs are $^Dk_{\max} = 2.1$ for wild-type enzyme and 5.2 for K258A. There is no significant $^2\text{H}_2\text{O}$ KIE for the reaction of K258A with cysteine sulfinat.

Acetate and formate ion catalysis of the K258A–cysteine sulfinat 1,3-prototropic shift

Figure 5 shows the dependence of k_{obs} for the K258A–PLP reaction with cysteine sulfinat on the concentrations of acetate and formate ions. Both induce small but significant increases in k_{obs} over the concentration range 0–1.5 M. The catalytic rate constants, k_B , are $(1.6 \pm 0.1) \times 10^{-4} \text{ M}^{-1} \text{ s}^{-1}$ and $(1.0 \pm 0.1) \times 10^{-4} \text{ M}^{-1} \text{ s}^{-1}$ for acetate and formate, respectively.

¹ In these single turnover half-reaction experiments, $[\text{substrate}] \gg [\text{enzyme}]$ giving pseudo-first order reaction conditions. The $k_{\max}$ is the maximal first order rate constant, i.e.,

$$k_{\text{obs}} = \frac{k_{\max}[\text{S}]}{K_{\text{app}} + [\text{S}]}$$

where k_{obs} is the observed pseudo-first order rate constant, K_{app} is the apparent Michaelis constant, and S is the substrate.

Fig. 5. Dependence of the observed pseudo-first order rate constant for the 1,3-prototropic shift reaction of the K258A–cysteine sulfinat aldimine on the concentrations of formate and acetate ions. Conditions: 0.1 M HEPES-KOH, pH 7.5, 25 °C, ionic strength = 1.5 (sodium chloride).

Catalysis of K258A Schiff base hydrolysis by exogenous amines

Added amines also increase the rates of hydrolysis of the K258A–aspartate aldimine complex to amino acid and K258A–PLP (Scheme 1B, II $\rightarrow$ I). This reaction was conveniently studied by preparing the adduct with $[\alpha\text{-}^2\text{H}]$ aspartate to slow the competing azaallylic rearrangement by the value of the KIE (=5.2; Table 2) and by trapping the nascent free aspartate with the wild-type AATase and malate dehydrogenase (MDH) reactions. The values of the rate constants are linearly dependent on the concentration of the free base form of the added amine (Fig. 6). The magnitudes of the rate constants obtained at pH 9.2 are approximately 20-fold greater than those recorded for the competing 1,3-prototropic shift with the same catalyst and substrate.

Values of k_B for methyl-, ethyl-, propyl-, and butylamine catalysis of hydrolysis of the K258A–aspartate aldimine are presented in Table 3. The logarithms of these k_B values, like those of the 1,3-prototropic shift, are linearly correlated with amine molecular volume (Fig. 2, squares). The slope of the best-fit line for the aldimine hydrolysis data is $-0.038 \pm 0.002 \text{ \AA}^{-3}$.

Discussion

Brønsted analysis of the reactions of K258A with oxalacetate

The rate constants for the interconversions of external aldimines and ketimines are reduced 10^6 – 10^8 -fold by the K258A mutation (Toney & Kirsch, 1989; M.D. Toney & J.F. Kirsch, unpubl.), due to deletion of the LYS258

Fig. 6. Dependence of the observed pseudo-first order rate constant for hydrolysis of the K258A-aspartate external aldimine (II; Scheme 1B) on the concentration of added methylamine. Conditions: 0.1 M CHES-KOH, 25 °C, ionic strength = 0.5 (tetramethylammonium chloride).

Table 3. Rate constants for the amine-catalyzed hydrolysis of the Schiff base formed between the aspartate aminotransferase mutant K258A and aspartate^a

Amine	k _B (M ⁻¹ s ⁻¹)
Methyl-	3.71 (0.25)
Ethyl-	0.885 (0.024)
Propyl-	0.177 (0.002)
Butyl-	0.026 (0.001)

^a Schiff base hydrolysis was assayed by trapping freed aspartate with wild-type AATase in a coupled assay with MDH. The k_B values are for the free base. The pK_a values of these amines are 10.5–10.6. See Table 1 for molecular volume values. Conditions: pH 9.3, 0.1 M CHES-KOH, ionic strength = 0.5 (tetramethylammonium chloride), 20 μM K258A aldimine, 10 U/mL each of wild-type AATase and MDH, 5 mM α-ketoglutarate, 0.15 mM NADH, 25 °C. Standard errors are given in parentheses.

ε-amino group, the general base catalyst. It was reported previously that exogenous, primary amines increase the rate of reaction of K258A with cysteine sulfinic acid (Toney & Kirsch, 1989).² The data in Figure 1 extend these results to include the oxalacetate ketimine to aldimine transformation (IV → II; Scheme 1B). The following lines of evidence indicate that the reactions of the amines occur at the K258A active site: (1) There is no excess coen-

² The cysteine sulfinic acid transamination product, β-sulfinylpyruvate, rapidly decomposes irreversibly to pyruvate and bisulfite. The unusually high stability of the K258A-aspartate external aldimine complex (M.D. Toney & J.F. Kirsch, unpubl.), the product of the reaction of oxalacetate with K258A-PMP, coupled with the large excess of oxalacetate present effectively makes the reaction of this substrate irreversible as well.

zyme present, and the protein binds coenzyme tightly (Kochhar et al., 1987; M.D. Toney & J.F. Kirsch, unpubl.). (2) The rate constants for nonenzymatic model systems are slower than those observed with K258A (Auld & Bruce, 1967). (3) Steric influences on the catalytic rate constants are much larger than those observed in nonenzymatic reactions.

The pH dependence of the reaction demonstrates that the rate constants depend linearly on the concentration of the free base form of the amine.³ The rate law is

$$k_{\text{obs}} = \frac{k_B[\text{amine}]_{\text{total}}}{1 + ([\text{H}^+]/K_a)} + k_{\text{solvent}} \quad (1)$$

where K_a is the proton dissociation constant for the conjugate acid of the amine, and k_{solvent} is the rate constant observed in the absence of added amines. Equation 1 is kinetically indistinguishable from Eq. 2

$$k_{\text{obs}} = \frac{k_B[\text{R-NH}_3^+][\text{OH}^-]K_a}{K_w} + k_{\text{solvent}} \quad (2)$$

where K_w is the ion product constant of water (see below).

The data in Table 1 for the reaction of K258A-PMP with oxalacetate were initially fitted to the classical Brønsted equation, Eq. 3.

$$\log k_B = \beta \cdot \text{p}K_a + \text{constant} \quad (3)$$

The minimizing parameter values are β = 0.59 ± 0.12 and constant = -4.9 ± 1.1. The large errors in the values of the parameters show that the data are only moderately well accommodated by Eq. 3. Steric effects contribute prominently to the values of k_{obs} in the amine-assisted reactions of K258A with cysteine sulfinic acid (Toney & Kirsch, 1989), and a similar dependence is observed for the amine-catalyzed reactions of oxalacetate with K258A-PMP (Fig. 2). The four amines shown in Figure 2 (methyl-, ethyl-, propyl-, and butylamine) have nearly equal basicities so that the decrease in k_B is only due to steric effects (represented by amine molecular volume). The full data set was thus fitted to a modified form of the classical Brønsted equation, Eq. 4, to account for the large steric contribution to the rate constant values.

$$\log k_B = \beta \cdot \text{p}K_a + V \cdot \text{molecular volume} + \text{constant} \quad (4)$$

The minimizing values of the parameters for the multiple linear regression fit of the oxalacetate data are β = 0.62 ± 0.06, V = -0.047 ± 0.007 Å⁻³, and constant =

³ The rate of reaction of K258A with substrates under saturating conditions and in the absence of added amine catalysts is independent of pH (M.D. Toney & J.F. Kirsch, unpubl.). The variation with pH of the rate constants obtained by extrapolation to zero concentration of amine (e.g., Fig. 1) is thus not due to specific base catalysis. The source of this variation is presently undetermined.

-2.1 ± 0.6 . This value of V agrees with that obtained in Figure 2 using only methyl-, ethyl-, propyl-, and butyl-amine. The overall fit of the data to Eq. 4 is much poorer when solvent-accessible molecular surface area is used in place of molecular volume; therefore, the latter is used throughout the present analysis. The data are plotted as $\log k_B - (V \times \text{molecular volume})$ versus pK_a (Fig. 7) to permit a two-dimensional representation, which emphasizes the effect of basicity on the k_B values.

The F_χ statistical test (Bevington, 1969) was used to examine the fit of the oxalacetate data to Eq. 4. F_χ examines a specific term in the regression equation, and gives the likelihood that its coefficient is zero based on the reduction in χ^2 due to its inclusion. The calculation gives >99.9% confidence that the values of both V and β are nonzero ($F_\chi = 47.1$ and 107 for the inclusion of V and β , respectively).

The structures of AATase complexed with inhibitors (Kirsch et al., 1984; Jansonius & Vincent, 1987) show that the enzyme-bound aldimine is fully removed from bulk solvent due to a ligand-induced conformational change. Amines must, therefore, penetrate the K258A protein structure in order to effect catalysis (accounting for the large steric effects) and, in the process, undergo a transfer from the aqueous milieu to a new solvent (i.e., the K258A active site). The transfer of LYS258 from aqueous solution to its location within the external aldimine complex causes the pK_a value of the ϵ -amino group to decrease from 10.5 to less than 5, as demonstrated by the independence of the value of k_{cat} on pH between 5 and

Fig. 7. Brønsted plot for catalysis of the 1,3-prototropic shift in the reaction of K258A-PMP with oxalacetate by exogenous amines. The slope of the line, β , through the data is 0.62 ± 0.06 . The dashed line is for the reaction of K258A-PLP with cysteine sulfinat catalyzed by the same amines (Toney & Kirsch, 1989). The values of β and V were determined by linear regression on Eq. 4, using pK_a and molecular volume as independent variables.

10 (Kiick & Cook, 1983; W.L. Finlayson & J.F. Kirsch, unpubl.). Similar large decreases in the pK_a values of the amine catalysts are expected upon their assumption of the catalytically effective position in the external aldimine complex. Bell (1958) reviews relevant data on the solvent-induced perturbation of primary amine pK_a values. His conclusion (p. 58) that "the relative strengths of acids of the same charge and chemical type are independent of the solvent" supports the assumption implicit in the present Brønsted analysis that the basicities of all the amine catalysts are equally perturbed by their localization to the active site. The β values obtained directly from the experiments are thus the true values even though the amine pK_a values used were measured in aqueous solution.

The transition state structure for the azaallylic rearrangement

The pH dependence of the amine-catalyzed reactions does not distinguish between true general base catalysis, Eq. 1, and the kinetically indistinguishable specific base-general acid catalysis mechanisms, Eq. 2 (Jencks, 1969) (Scheme 2). For reactions with amino acids, general base catalysis (Scheme 2A) is represented by direct attack of the free base form of the amine on the C_α proton (as shown) or attack through an intervening water molecule (not shown). Specific base-general acid catalysis (Scheme 2B) involves the loss of a proton from the substrate to form a resonance-stabilized carbanion in a preequilibrium step with general acid catalysis of product formation by the conjugate acid of the amine as the rate-determining step.

The resolution of this kinetic ambiguity requires data other than that obtainable from pH or solvent-dependence studies (Jencks, 1969). The true general base mechanism for reactions of amino acids involves C_α proton abstraction only in the rate-determining step. This proton is equilibrated with solvent in the first step of the specific base-general acid mechanism and is therefore lost from the substrate. Thus, a C_α - 2H KIE should be observed only in the general base mechanism. The large values of the C_α - 2H KIEs measured for both aspartate and glutamate (5.2 and 5.5, respectively) with methylamine as catalyst decisively resolve the kinetic ambiguity in favor of the true general base mechanism (Scheme 2A).

The remaining mechanistic ambiguity is whether or not general base catalysis occurs through an intervening water molecule. This question is addressed by D_2O KIEs as this mechanism involves proton transfers with water in the transition state. The value of the D_2O KIE for the reaction of K258A with cysteine sulfinat is 1.0 ± 0.1 (Table 2). This result supports a mechanism of direct abstraction of the C_α proton by the amine nitrogen lone electron pair.

The large KIE observed for the aspartate aldimine to ketimine transformation (Scheme 1B, II $\rightarrow$ IV) suggests

A) General base catalysis

B) Specific base - general acid catalysis

Scheme 2. The two kinetically indistinguishable mechanisms of catalysis of the K258A-cysteine sulfinic shift by exogenous amines. **A:** General base catalysis with the amine free base abstracting the $\text{C}\alpha$ proton in the rate-determining step. **B:** Specific base-general acid catalysis where the substrate loses a proton in a preequilibrium step and reacts with the conjugate acid of the amine in the rate-determining step.

(but does not prove, see below) that removal of the $\text{C}\alpha$ proton is the rate-determining step. Within this kinetic model, an interpretation of the β values of 0.62 ± 0.06 and 0.39 ± 0.05 for the oxalacetate and cysteine sulfinic reactions, respectively, follows. The law of microscopic reversibility dictates that the transition state for the reverse reaction of the oxalacetate ketimine to aldimine be the same as that of the forward reaction.⁴ The transition state for the reverse reaction is therefore not represented by proton abstraction from the 4'-carbon, but by protonation of the α -carbon of the pyridoxyl-derived carbanion (formed in a preequilibrium proton dissociation from $\text{C}4'$) by the conjugate acid of the amine leading to the aldimine of L-aspartate. The transition state structures for

this mechanism are illustrated in Scheme 3. For the oxalacetate/aspartate pair, the transition state occurs with $\sim 60\%$ of a positive charge on the amine nitrogen and $\sim 60\%$ of a negative charge on the pyridoxyl moiety, which is delocalized through orbital overlap in the π -bonding system. The β value of 0.39 for cysteine sulfinic is interpreted similarly; the amine catalyst has acquired $\sim 40\%$ of a positive charge and the pyridoxyl moiety $\sim 40\%$ of a negative charge in the transition state. The lower value of β for the cysteine sulfinic reaction might be explained by the 125-fold higher reactivity of this amino acid, as compared to aspartate, with K258A (M.D. Toney & J.F. Kirsch, unpubl.). The α -carbon of the aldimine intermediate formed with cysteine sulfinic must be more acidic, enabling the transition state to be reached when the $\text{C}\alpha$ -H bond is only $\sim 40\%$ broken, rather than the $\sim 60\%$ inferred for the aspartate reaction.

A viable alternative to the simpler kinetic model above is one in which the aldimine to ketimine transformation encompasses two partially rate-determining steps, proton transfer to and from both the α - and 4'-carbons, with a

⁴ It is possible that the reverse reaction of K258A-PMP with oxalacetate would preferentially produce D-aspartate as product, in which case the transition state structure would likely differ completely from that of the forward reaction with L-aspartate. The finding that D-aspartate and K258A-PLP form a Schiff base that does not transaminate in either the presence or absence of amines (M.D. Toney & J.F. Kirsch, unpubl.) eliminates this possibility.

Scheme 3. Schematic interpretation of the β values of 0.4 and 0.6 obtained for the reactions of K258A with cysteine sulfinic acid and oxalacetate. The negative charges on the C α and C4' carbanions are distributed over the pyridoxyl adduct.

quinonoid intermediate. This more complex kinetic model, in contrast to that above, does not allow an unambiguous interpretation of the β values without detailed knowledge of the free energy profile of the reaction.

Integrity of the K258A active site

The 10^6 – 10^8 -fold decrease in activity caused by the mutation could, in principle, be due to structural changes at the active site. The crystal structure of the mutant reveals no gross perturbations (Smith et al., 1989), making this alternative improbable. The fact that a proton transfer rate constant 2×10^4 -fold larger than that observed in the absence of amines and equal to 2% of that of the wild-type enzyme rate constant could be observed with cysteine sulfinic acid in 1 M NH_3 (Fig. 4) further supports the contention that the K258A active site is fully functional except for the absence of the essential general base catalyst, LYS258.

Magnitude of the protein-induced enhancement of aldimine reactivity toward general base catalysis

Auld and Bruice (1967) report a value of $k_B = 6.5 \times 10^{-4} \text{ M}^{-1} \text{ s}^{-1}$ for the imidazole-catalyzed transamination of the model aldimine formed from 3-hydroxypyridine-4-aldehyde (a PLP analog) and alanine. A k_B value of $107 \text{ M}^{-1} \text{ s}^{-1}$ for the reaction of K258A with cysteine sulfinic acid is calculated from the previously described Brønsted correlation (Toney & Kirsch, 1989) for a base with molecular volume = 0 and pK_a for the conjugate acid = 7 ($=\text{pK}_a$ of imidazole). The use of a volumeless base in this calculation is necessary to equalize the two reactions with respect to steric properties; steric determinants of the values of the rate constants for the K258A reaction are large whereas those for the solution reaction are insignificant. Thus, the protein-bound aldimine is approximately 2×10^5 -fold ($=107 \text{ M}^{-1} \text{ s}^{-1}/6.5 \times 10^{-4}$

$\text{M}^{-1} \text{ s}^{-1}$) more reactive with general base catalysts than is the free aldimine in aqueous solution.

There are two obvious sources of the protein-derived activation of the aldimine. The first is specific orientation of the labile C α -H aldimine bond such that it is perpendicular to the pyridine ring, which Dunathan (1966) proposed to be the most reactive conformation. The structure of the mitochondrial AATase complex with α -methylaspartate does indeed have the analogous C α -methyl bond oriented perpendicular to the ring system (Jansonius & Vincent, 1987). The second source may reside in the numerous specific hydrogen-bonding and ionic interactions the enzyme makes with the aldimine (Jansonius & Vincent, 1987; Toney & Kirsch, 1987; Goldberg et al., 1991).

Does the added catalyst utilize the LYS258 side chain position?

Knowledge of the site(s) from which amines catalyze the 1,3-prototropic shifts is important for a complete understanding of the mechanism of external catalysis. The 80-Å³ difference in volume between lysine and alanine (Chothia, 1975) makes the 258 side chain position in K258A a good candidate for this site. This hypothesis was tested by investigation of the amine volume dependence of the rate constant for the 1,3-prototropic shift in the K258M mutant, as the methionine side chain should fill any cavity that might be present in K258A.

The comparison of the amine volume sensitivities of the K258A and K258M reactions with oxalacetate given in Figure 3 shows that there is no large difference in steric discrimination between these two mutants. This suggests that, in the majority of transformations, the alkyl group on the catalyzing amine does not occupy the position of the LYS258 side chain in the wild-type enzyme. Conversely, the results of the KIE measurements demonstrate that the amino groups of both LYS258 and the ex-

ogenous amines do function via the same location within the external aldimine complex (see above).

Proton transfer catalysis by carboxylate ions

Catalysis of AATase proton transfer reactions by exogenous molecules allows variation in catalyst type as well as in basicity. The data in Figure 5 demonstrate that small increases in the rate constant for transamination of the K258A–cysteine sulfinic aldimine are observed with high concentrations (0–1.5 M) of formate or acetate ions. The observed catalytic rate constants are 100-fold less than those predicted from basicity and volume from the amine data and the constants obtained from fits to Eq. 4 (Toney & Kirsch, 1989). The ionic strength in these reactions was held constant at 1.5 M by the addition of sodium chloride. The high concentrations of anions required raises the possibility that the observed catalysis is a specific ion effect. It is also conceivable that the azomethine linkage is well shielded from anions due to charge or structural restrictions imposed by the active site.

Catalysis of external aldimine hydrolysis by exogenous amines

A natural question arising from the above demonstration of exogenous amine catalysis of K258A 1,3-prototropic shifts is whether or not these amines can also catalyze the hydrolysis of K258A external aldimine intermediates (Scheme 1B, II $\rightarrow$ I). The data shown in Figure 6 and the rate constants collected in Table 3 establish that methyl-, ethyl-, propyl-, and butylamine catalyze the decomposition of the K258A–aspartate aldimine to K258A–PLP and aspartate. The value of V for these data, the amine volume parameter (Eq. 4), is $-0.038 \pm 0.002 \text{ \AA}^{-3}$, significantly less than the figures of $-0.062 \pm 0.006 \text{ \AA}^{-3}$ for the cysteine sulfinic and $-0.048 \pm 0.004 \text{ \AA}^{-3}$ for the oxalacetate 1,3-prototropic shifts (Fig. 2).

There are two mechanisms of amine-catalyzed hydrolysis of the aldimine to be considered. The first proceeds through direct nucleophilic displacement of the amino acid by the amine to yield a transient imine of enzyme-bound PLP, which is subsequently hydrolyzed. This mechanism of external aldimine decomposition is similar to that operative in wild-type enzyme where LYS258 enforces a transamination mechanism that yields the stable LYS258–PLP imine as final product. The second mechanism is one of general base-catalyzed attack of water on the aldimine, directly yielding the reaction products. Although the present experiments do not distinguish between these possibilities, the lower value of V might be taken to support the latter mechanism as the distance of closest approach of the attacking amine to the sterically crowded aldimine is less through an intervening water molecule.

Generality of the method

It had previously been impossible to perform Brønsted analyses of enzymatic proton transfer reactions as it is necessary to vary the catalyst, which is an integral part of the protein structure. Site-directed mutagenesis offers only a limited set of substitutions that are capable of catalyzing proton transfers. The concomitant large structural variations inherent in these substitutions severely limit their applicability to enzymatic Brønsted analyses. Two recent examples of the effects of small structural variations in catalytic residues on reactivity bear on this point. A 1,500-fold reduction in k_{cat} for triosephosphate isomerase was observed when GLU165, the putative general base catalyst, was mutated to aspartate (Raines et al., 1986). The two carboxylate side chains have very similar basicities, but the aspartate is one methylene group ($\sim 1 \text{ \AA}$) shorter. Planas and Kirsch (1990) and Yoshimura et al. (1990) have demonstrated that replacement of the catalytically essential PLP-binding lysine in AATase with an unnatural lysine analog (*S*-aminoethyl-cysteine) results in a 4–20-fold reduction in k_{cat} . These small differences in active site structure accompanied by large changes in catalytic power emphasize the importance of systematic catalyst variation.

Only primary amines are used in the present Brønsted analyses. The rates of reaction with other catalyst types are very much reduced. For example, no detectable acceleration of the K258A reaction with cysteine sulfinic was noted at 0.25 M imidazole free base. The predicted k_B value based on pK_a and molecular volume is $0.012 \text{ M}^{-1} \text{ s}^{-1}$, which is ≥ 60 -fold larger than the smallest observable value under the conditions used. The value of k_B for dimethylamine, a secondary amine, is sixfold less than that exhibited by ethylamine, which has the same basicity and molecular volume. Thus, the need to use a structurally homologous systematically varied series of catalysts in Brønsted analyses of sterically restricted enzymatic reactions is, at least in this case, clear.

Catalysis of enzymatic proton transfer reactions by external acids or bases is not unprecedented. Some examples from the literature follow. The PLP-dependent enzyme serine hydroxymethyltransferase reacts with the noncognate substrate D-alanine to form a quinonoid intermediate. Schirch and Jenkins (1964) demonstrated general base catalysis of this reaction by added ammonia, and a k_B value of $2 \text{ M}^{-1} \text{ s}^{-1}$ can be calculated from their data taken at 4°C . Burgner and Ray (1984) found general acid catalysis of decomposition of the pyruvate– NAD^+ adduct on lactate dehydrogenase by aminium ions. Rate constants for the eight catalysts were poorly correlated with pK_a values. The catalysts varied widely in structure (e.g., Tris-[hydroxymethyl]aminomethane, imidazole, histamine, piperazine), which probably accounts for the poor correlation observed. Carter and Wells (1987) found that a subtilisin mutant in which the histidine of the cat-

alytic triad is replaced with alanine preferentially catalyzes the hydrolysis of peptides with histidine at the P1 position. The pH dependence of this reaction confirmed the conclusion deduced from model-building experiments that the substrate histidine substitutes for the deletion. Silverman and Tu (1975) demonstrated that carbonic anhydrase reactions are accelerated by low (up to ~10 mM) concentrations of buffers and proposed that they assist in a rate-limiting proton transfer reaction. Later they mutated the active-site histidine, which is purported to be the mediator of the proton relay to solvent in carbonic anhydrase (Tu et al., 1989). This less active enzyme is more susceptible than wild-type enzyme to buffer-assisted catalysis. Smith and Hartman (1991) found that aminomethanesulfonate specifically increases the activity of the inactive K191C mutant of ribulosebiphosphate carboxylase/oxygenase in which the carbamate-forming lysine was removed. Ehrig et al. (1991) demonstrated that the sixfold decrease in k_{cat}/K_M observed with the H51Q mutant of alcohol dehydrogenase could be completely restored by the addition of various catalysts, and a linear Brønsted relationship ($\beta = 0.53$) was observed. These varied examples of exogenous catalysis of enzymatic reactions show that the powerful method of Brønsted analysis of transition state structure can now be conveniently imported from the realm of physical organic chemistry into mechanistic enzymology.

Materials and methods

Materials

The construction of K258A has been reported (Malcolm & Kirsch, 1985). K258M was prepared similarly, except that mutagenesis and expression of the protein were performed with the *aspC* gene in pUC119 (J.J. Onuffer & J.F. Kirsch, unpubl.). Purified AATase was obtained as described by Cronin and Kirsch (1988). Typically, 12 g of cell paste yielded 200–300 mg of pure protein (purity >95% as judged by sodium dodecyl sulfate–polyacrylamide gel electrophoresis [SDS-PAGE]). K258A–PLP was prepared as previously described (M.D. Toney & J.F. Kirsch, unpubl.). K258A–PMP was prepared by reaction of K258A–PLP with cysteine sulfinic acid followed by dialysis. L-Cysteine sulfinic acid and all amines were obtained from Aldrich Chemical Co. Amines were purchased as the hydrochloride salts, except for *n*-butylamine, which was obtained as the free base, and were stored over calcium sulfate. Amine hydrochlorides that were less than 99% pure (as specified by the vendor) were recrystallized from ethanol before use. D₂O (99.8%) was from BioRad. Other chemicals and MDH were purchased from Sigma. D,L-Aspartic acid and [α -²H]D,L-aspartic acid were prepared as follows: Either 217 mg of sodium borohydride or 240 mg sodium borodeuteride (98% ²H, Sigma) were reacted with 0.5 g oxalacetic acid in 10 mL

concentrated aqueous ammonia (stirred 10 min before addition of borohydride) for 15 h at room temperature. The ammonia was removed by rotary evaporation, the pH lowered to 1.5, and the acid isolated by chromatography on BioRad AG50W×2 with 1.5 M hydrochloric acid as eluent. The acids were recrystallized from aqueous ethanol. [α -²H]L-glutamic acid was a gift from Dr. D.A. Julin (Julin & Kirsch, 1989).

Conventional kinetic data were taken with either a Perkin-Elmer λ -4B or a Kontron Uvikon 860 spectrophotometer. Reaction cuvettes were maintained at 25 °C with circulating water baths. Curve fitting was performed with either the NLIN program of the SAS statistical package (SAS Institute, Cary, North Carolina), or the program ENZFITTER (R.J. Leatherbarrow; Biosoft Publishing Co.) on an IBM-compatible personal computer. Fast reactions were monitored with an Applied Photophysics SF.17MV stopped-flow spectrophotometer, and curve fitting was performed using the software provided with the instrument. Amine molecular volume and pK_a values were obtained as described previously (Toney & Kirsch, 1989).

Kinetic analysis of amine catalysis of the 1,3-prototropic shift

AATase 1,3-prototropic shifts are readily monitored by the change in the coenzyme absorption spectrum in single turnover half-reactions (Cronin & Kirsch, 1988). The reaction of K258A with oxalacetate was monitored at 430 nm (formation of K258A–aspartate aldimine) under the conditions previously described for the cysteine sulfinic reaction (Toney & Kirsch, 1989). The fast reaction of K258A with cysteine sulfinic in ammonia buffer at high concentrations was monitored with the stopped-flow apparatus at 334 nm. All reactions were conducted under pseudo-first order conditions: K258A, oxalacetate, and cysteine sulfinic concentrations were 10 μ M, 5 mM, and 25 mM, respectively. Ionic strength was held constant at 0.5 (except in the stopped-flow experiment with cysteine sulfinic where it was 2.0) with tetramethylammonium chloride. The absorbance data obeyed first order kinetics well for 3–4 half-lives. The pH values of the reaction solutions were measured after data collection and the concentrations of free bases calculated from the pK_a values. The observed rate constants were divided by the fraction free base to give the reported k_B values.

Kinetic isotope effects

Half-reactions were monitored as described above. The KIEs for reactions of K258A with amino acids were obtained by dividing the k_B value for the protiated substrate by that for the deuterated one. Methylamine was used as catalyst.

The D₂O isotope effect on the K258A–cysteine sul-

finite reaction was also determined with methylamine as catalyst. Buffers were prepared directly in D₂O. No correction was made for the small amount of protium introduced by this procedure. The pK_a for methylamine in D₂O was assumed to be 0.6 units greater than that in H₂O, as found for other amines, and the pD was taken as the observed meter reading + 0.4 (Schowen & Schowen, 1982).

Amine catalysis of aldimine hydrolysis

Kinetic analysis of K258A-[α -²H]aspartate aldimine hydrolysis was performed by trapping the released aspartate with wild-type AATase in the presence of MDH, and monitoring the loss of NADH absorbance at 340 nm. The aldimine was formed with K258A-PLP and a two-fold molar excess of [α -²H]aspartate. The solution was passed over a Sephadex G25 column in 10 mM HEPES-KOH, pH 7.5, to remove excess amino acid. Malate dehydrogenase and wild-type AATase were extensively dialyzed against 2 mM potassium phosphate, pH 7.5, before use to remove ammonium sulfate. Wild-type AATase and MDH concentrations were 10 units/mL, and the NADH and α -ketoglutarate concentrations were 0.15 mM and 5 mM, respectively, giving pseudo-first order conditions. Doubling the concentrations of the coupling enzymes and cosubstrates did not change the values of k_B in control reactions.

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