pH and Kinetic Isotope Effects on the Reductive Half-Reaction of D-Amino Acid Oxidase

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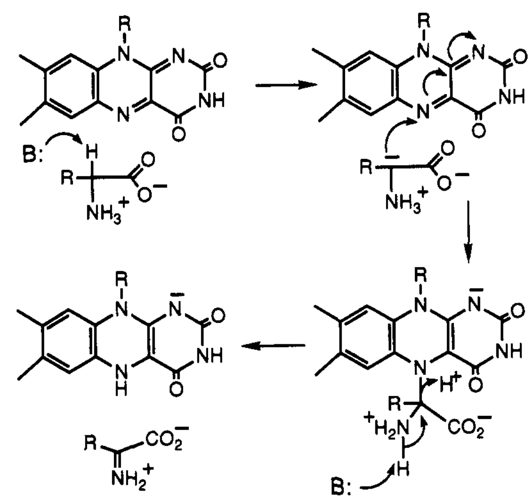
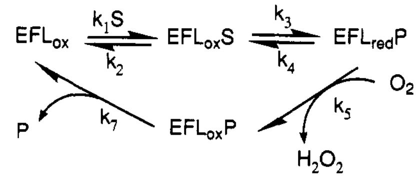
ABSTRACT: Primary deuterium kinetic isotope and pH effects on the reduction of D-amino acid oxidase by amino acid substrates were determined using steady-state and rapid reaction methods. With D-serine as substrate, reduction of the enzyme-bound FAD requires that a group with a pK_a value of 8.7 be unprotonated and that a group with a pK_a value of 10.7 be protonated. The $^DV/K_{ser}$ value of 4.5 is pH-independent, establishing that these pK_a values are intrinsic. The limiting rate of reduction of the enzyme shows a kinetic isotope effect of 4.75, consistent with this as the intrinsic value. At high enzyme concentration ($\sim$15 μM) at pH 9, D-serine is slightly sticky ($k_3/k_2 = 0.8$), consistent with a decrease in the rate of substrate dissociation. With D-alanine as substrate, the pK_a values are perturbed to 8.1 and 11.5. The $^DV/K_{ala}$ value increases from 1.3 at pH 9.5 to 5.1 at pH 4, establishing that D-alanine is sticky with a forward commitment of $\sim$10. The effect of pH on the $^DV/K_{ala}$ value is consistent with a model in which exchange with solvent of the proton from the group with pK_a 8.7 is hindered and is catalyzed by H_2O and OH^- above pH 7 and by H_3O^+ and H_2O below pH 7. With glycine, the pH optimum is shifted to a more basic value, 10.3. The $^DV/K_{gly}$ value increases from 1.26 at pH 6.5 to 3.1 at pH 10.7, consistent with fully reversible CH bond cleavage followed by a pH-dependent step. At pH 10.5, the kinetic isotope effect on the limiting rate of reduction is 3.4.

D-Amino acid oxidase (DAAO)[1] catalyzes the two-electron oxidation of D-amino acids to their respective imino acids by transfer of a hydride equivalent to the tightly bound FAD (reductive half-reaction) and subsequent transfer of electrons to molecular oxygen to form hydrogen peroxide (oxidative half-reaction). The imino acid is hydrolyzed nonenzymatically to the keto acid and ammonia. Reduction has been proposed to involve initial formation of a carbanion by α-proton abstraction from the D-amino acid (Scheme I) [Ghisla (1982) and references therein]. Evidence for a carbanion comes from the DAAO-catalyzed elimination of HCl from β-chloroalanine (Walsh et al., 1971) and β-chloroaminobutyrate (Walsh et al., 1973) and the inactivation of several flavoprotein oxidases by acetylenic substrates (Marcotte & Walsh, 1976). The carbanion is proposed to attack the N(5) of FAD to form a covalent adduct. Imino acid elimination from such an adduct would then generate reduced flavin. Support for adduct formation comes from the observation that a stable flavin–substrate adduct is formed after abstraction of the glycolate Si proton when glycolate reacts with L-lactate oxidase (Ghisla, 1980). In addition, nitroalkane anions form adducts with DAAO at the N(5) position of flavin (Porter et al., 1972, 1973). However, when the native FAD is replaced with 5-deaza-FAD, the α-hydrogen of alanine is transferred to the C(5) position of 5-deaza-FAD, suggesting a hydride-transfer mechanism (Hersh & Jorns, 1975). The relevance of the latter has been questioned on the grounds that 5-deazaflavins are analogues of pyridine nucleotides rather than flavin analogues (Hemmerich et al., 1977). Recently, Miura and Miyake (1988) proposed that α-proton abstraction is concerted with transfer of electrons from the amino acid nitrogen to the flavin on the basis that discrete carbanion formation is energetically unlikely.

Scheme I



Scheme II



Previous kinetic work on DAAO is consistent with the kinetic mechanism shown in Scheme II. The most complete kinetic results have been obtained with D-alanine (Palmer & Massey, 1968; Porter et al., 1977) and D-α-aminobutyrate (Fitzpatrick & Massey, 1982b) as substrates. Both give parallel line patterns in double-reciprocal plots, indicating that the steady-state rate equation is eq 1. With some substrates such as phenylalanine and glycine, the initial velocity pattern is intersecting, indicating the steady-state rate equation has an additional term (eq 2). The intersecting line pattern is the

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[1] Abbreviations: DAAO, D-amino acid oxidase; FAD, flavin adenine dinucleotide; ACES, *N*-(2-acetamido)-2-aminoethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetate; LDH, lactate dehydrogenase; NADH, nicotinamide adenine dinucleotide (reduced); CAPS, 3-(cyclohexylamino)propanesulfonic acid.

$$1/v = 1V + K_a/VA + K_{O_2}/V[O_2] \quad (1)$$

$$1/v = 1/V + K_a/VA + K_{O_2}/V[O_2] + K_{ia}K_{O_2}/VA[O_2] \quad (2)$$

result of k_4 in Scheme II being significant in the overall reaction (Palmer & Massey, 1968). With D-alanine and D-α-aminobutyrate as substrates, the rate-limiting step in turnover is the release of imino acid from oxidized enzyme (Fitzpatrick & Massey, 1982b; Porter et al., 1977). Consistent with rate-limiting product release, no kinetic isotope effect on turnover has been observed. Primary deuterium effects on V/K_a values of 1–4 for amino acid substrates have indicated that carbon–hydrogen bond cleavage is partially rate-limiting in reduction (Fitzpatrick & Massey, 1982b; Porter et al., 1977; Yagi et al., 1970). Other than identifying the presence or absence of an isotope effect, an extensive study of kinetic isotope effects on DAAO has not previously been done and would yield considerably more information on mechanistic details of this model flavoprotein oxidase.

Chemical modification studies [D'Silva and Massey (1987) and references cited therein] have identified methionine-110, lysine-204, histidine-217 and -307, tyrosine-55, -224, and -228, cysteine, and arginine residues as essential. Identification of these residues has spurred interest in the application of site-directed mutagenesis to elucidate their specific roles. Watanabe et al. (1989) reported on the preliminary kinetics of a few site-directed mutants of hog DAAO. The difficulty of interpreting their results clearly emphasizes the need for a more complete kinetic characterization of DAAO. Moreover, the step-wise model of Scheme I predicts a more complicated kinetic model for reduction than is shown in Scheme II. Alternative methods such as kinetic isotope effects should prove helpful in answering these questions. Kinetic isotope effects are a powerful tool in defining a more complete kinetic mechanism, in assigning relative rates of various catalytic steps, in interpreting the chemical mechanism, and in describing the nature of the transition state that cannot be obtained by any other method. In this paper, we present a detailed study and interpretation of kinetic isotope effects as a function of pH with several substrates for this archetypal flavoprotein oxidase.

MATERIALS AND METHODS

Materials. Glucose oxidase and lactate dehydrogenase were from Sigma Chemical Co. DL-[2,3,3-$^2H_3$]Serine (98%) and [$^2H_5$]glycine (98%) were from Cambridge Isotope Laboratories. DL-[2-$^2H_1$]Alanine (98.7%) was from MSD Isotopes. Hydroxyapatite was made according to the procedure of Tiselius et al. (1956). D-Amino acid oxidase was purified as described by Fitzpatrick and Massey (1982a), and benzoate was removed by the method of Brumby and Massey (1968). The enzyme was stored at −70 °C in 20 mM sodium pyrophosphate, pH 8.5, until use. The concentration of purified DAAO was determined using an ϵ_{455} value of 11.3 mM^{-1} cm^{-1} (Massey & Ganther, 1965).

Steady-State Kinetics. Between pH 5.5 and 10, a buffer consisting of 0.1 M ACES, 0.052 M Tris, and 0.052 M ethanolamine was used. This buffer maintains a constant ionic strength of 0.1 M over this pH range (Ellis & Morrison, 1982) and exhibits no inhibitory effects on the kinetics of DAAO. Above pH 10.0, 0.1 M EDTA was used to buffer the solution, and below pH 5.5, 0.1 M sodium acetate was used. Kinetic results in both EDTA and sodium acetate buffers were identical to those in the constant ionic strength buffer. The pH was adjusted with NaOH or acetic acid as necessary. Preliminary experiments showed that sodium bicarbonate (50 mM), CAPS (50 mM), diethylamine (200 mM), and dimethylamine (100 mM) inhibited DAAO.

Two different assays were used: (1) A YSI Model 5300 biological oxygen monitor (modified to increase the sensitivity by 10-fold) thermostated at 25 ± 0.1 °C was used to follow oxygen consumption. Variable oxygen concentrations were achieved by bubbling the appropriate O_2/N_2 gas mix directly into the assay solution (2–3 mL) for 10 min. (2) Alternatively, a Gilford Model 260 spectrophotometer thermostated at 25 ± 0.1 °C connected to a Gilford 6051 chart recorder was used to follow NADH oxidation in a coupled enzymatic assay when D-alanine was the substrate for DAAO below pH 9. Lactate dehydrogenase and NADH were present to convert the product pyruvate to lactate while the loss of absorbance at 340 nm due to NADH oxidation was monitored. Concentrations of LDH (30–75 units/mL) and NADH (0.12–0.24 mM) were chosen such that this reaction was not rate-limiting and the traces showed no lags. All assays solutions contained 10 μM FAD. Assays were initiated by the addition of 3–25 μL from a DAAO stock solution (0.015–0.29 mM). In order to obtain V/K_{gly} values, oxygen and glycine concentrations were both varied. The V/K_a values with D-serine and D-alanine were measured in air-saturated solutions ([O_2] = 0.266 mM) since these values were independent of the oxygen concentration. Most traces were linear over the first 5 min. Initial velocities were determined from the linear portion.

Concentrations of both deuterated and nondeuterated substrates were determined using enzymatic end-point assays. The LDH-coupled end-point assay was initiated by the addition of 3–20 μL of the stock substrate solution to the assay solution containing 15 μM DAAO, 30 units/mL LDH, 0.18 mM NADH, and 20 mM sodium pyrophosphate, pH 8.5. The oxygen electrode end-point assay was initiated in the same way. The total change in either oxygen or NADH concentration was then used to calculate the actual substrate concentration.

Rapid Reaction Kinetics. Anaerobic rapid reactions were followed with an Applied Photophysics Limited Model DX.17MV stopped-flow spectrofluorometer interfaced with an Acorn Archimedes 420/1 computer. Removal of oxygen from enzyme solutions was achieved by repeated flushing and evacuation with purified nitrogen from which oxygen was removed by passage through a column of BASF catalyst heated to 120 °C. Substrate solutions were made anaerobic by bubbling nitrogen directly into the solution for 10 min. Glucose oxidase (6 ng/mL) and glucose (2 mM) were present in the enzyme solution to scavenge any residual oxygen. Prior to an experiment, oxygen was scrubbed from the stopped-flow apparatus using the following procedure: nitrogen was bubbled through the water bath thermostated at 25 ± 0.1 °C, a solution of 600 ng/mL glucose oxidase and 10 mM glucose in 100 mM sodium acetate, pH 5, was flushed through the internal plumbing, and 1 g of sodium dithionite was added to the bath. After 1 h, deoxygenated buffer was then used to flush out the glucose oxidase solution. Under these anaerobic conditions, enzyme and substrate were rapidly mixed in the stopped-flow apparatus and the flavin absorbance was followed either as a decrease at 455 nm or as an increase at 550 nm.

Enzyme Concentration Dependence of $^DV/K_{ser}$ The effect of enzyme concentration on the $^DV/K_{ser}$ value was determined as follows. At 15 and 13.5 μM enzyme, the V/K_{ser} values were determined using the stopped-flow apparatus as described above. At 15 μM, 10 μM, and below, the V/K_{ser} values were determined using the oxygen electrode assay. Above 3 μM enzyme, the assay was initiated by the addition of D-serine to give a final [D-serine] in the micromolar range. Below 3 μM enzyme, the assays were initiated by the addition of enzyme to give the appropriate final concentration. The total volume of each assay was 2 mL. Between three and six different substrate concentrations were used, and the initial velocities were fit to eq 3 to obtain the V/K_{ser} value.

Data Processing. Data were fit to eqs 3–5 and 7 of Cleland (1979) using the KinetAsyst software (IntelliKinetics, State College, PA). When only one substrate was varied, the data were fit to eq 3. In cases where both substrates were varied, data were fit to eqs 4 and 5. $^{D}V/K_a$ values were determined by direct comparison of the V/K_a values for the nondeuterated and deuterated substrates. Fitting of pH-dependent data to eqs 6–12 was accomplished with nonlinear least-squares fitting using NonLin for Macintosh, a Macintosh version of a computer code developed by Johnson and Frasier (1985). The confidence limits of the optimized parameters were determined using a 67% confidence probability. If rates decreased at low pH, eq 6 was used, and if the rates decreased at both high and low pH, eq 7 was used to fit the data. Data that showed limiting values at both high and low pH were fit to eq 8, where YL is the limiting value at low pH while YH is the limiting value at high pH.

$$v = \frac{V_{max}[A]}{K_a + [A]} \quad (3)$$

$$v = \frac{V_{max}[AB]}{K_a[B] + K_b[A] + [AB]} \quad (4)$$

$$v = \frac{V_{max}[AB]}{K_{ia}K_b + K_a[B] + K_b[A] + [AB]} \quad (5)$$

$$\log Y = \log\left(\frac{[C]}{1 + [H]/K_1}\right) \quad (6)$$

$$\log Y = \log\left(\frac{[C]}{1 + K_2/[H] + [H]/K_1}\right) \quad (7)$$

$$\log Y = \log\left(\frac{YL + YH(K_1/[H])}{1 + K_1/[H]}\right) \quad (8)$$

Various mathematical approaches were employed to test the validity of the resulting fits. The nonlinear least-squares program returned the same optimized values independent of the initial values. Also, a theoretical data set was created with the optimized $^{D}V/K_{ala}$ values obtained from a fit to eq 11 and was then subjected to a subsequent fit to eq 11. The rationale was to determine whether there were more than one mathematical solution for that given equation. In a more stringent test, the theoretical values were randomized within a specified percent error of the theoretical value. Up to 15% error, the fit returned values within the range of the fitted values of the experimental data.

The rapid reaction data were analyzed according to Scheme III, where A is oxidized enzyme, B is the reduced enzyme–imino acid charge-transfer complex, and C is free reduced enzyme. Alcock et al. (1970) derived an expression (eq 9) for

Scheme III

$$A \xrightarrow{k_1} B \xrightarrow{k_2} C$$

$$A_{total} = (A_A - A_C)e^{-k_1 t} + \frac{k_1(A_B - A_C)}{(k_1 - k_2)}(e^{-k_2 t} - e^{-k_1 t}) + A_C \quad (9)$$

$$A_{550} = \frac{k_1(A_B)}{(k_1 - k_2)}(e^{-k_2 t} - e^{-k_1 t}) + A_C \quad (10)$$

describing the kinetics of Scheme III, utilizing the different spectroscopic signals of each species. A_A, A_B, and A_C are the respective absorbances due to species A, B, and C. For our purposes, eq 9 simplifies to eq 10 when the reaction is followed at 550 nm. At this wavelength, the rapid exponential increase in absorbance of the charge-transfer complex is followed by the slow exponential loss in absorbance due to the dissociation of the complex. The D-serine and D-alanine data were fit to eq 10 using the nonlinear least-squares fitting capability of the stopped-flow software. In many cases k_1 and k_2 were well-resolved and the data were fit separately to single exponentials. The glycine data were analyzed at 455 nm, where the data were best fit as a single-exponential loss of absorbance of the oxidized enyzme.

RESULTS

Steady-State Kinetics. (*a*) *D-Serine.* When the concentrations of both oxygen and D-serine were varied, the initial rate data were best fit by eq 4, which describes a parallel line pattern in double-reciprocal plots, consistent with k_4 (Scheme II) being small or zero. The V/K_a value is an apparent second-order rate constant for the reaction of free DAAO with free substrate and includes all steps involved in flavin reduction. The V/K_{ser} value was determined over the pH range 5.5–11. The results are shown in Figure 1. The V/K_a values have been corrected for protonation of the amino group since the zwitterion binds DAAO to form the catalytically active complex (Purdy, 1982). The V/K_{ser} values decreased at both low and high pH and were therefore fit to eq 7. The data indicate that a group with an apparent pK_a value of 8.7 ± 0.1 must be unprotonated and a group with an apparent pK_a value of 10.7 ± 0.2 must be protonated for activity. Primary deuterium kinetic isotope effects were determined with D-serine as substrate over the pH range 5–9.5. The $^{D}V/K_{ser}$ value was pH-independent with an average value of 4.47 ± 0.18 (Table I). This pH-independence of the $^{D}V/K_{ser}$ value establishes that D-serine is not a sticky substrate ($k_3 < k_2$) and that the pK_a values of 8.7 and 10.7 from the V/K_{ser} profile are intrinsic values. The results are consistent with Scheme IV in which two ionizations describe the pH dependency of the free enzyme.

(*b*) *D-Alanine.* D-Alanine has been the most widely employed substrate for studying the properties of DAAO. It has been shown previously that k_4 in Scheme II is effectively zero with this substrate (Palmer & Massey, 1968). As a result, the V/K_{ala} value shows no oxygen dependence and measurements of V/K_{ala} could be made in air-saturated solutions. The effects of pH on the V/K_{ala} and $^{D}V/K_{ala}$ values were determined. As with D-serine, the V/K_{ala} values decreased at low pH with a pK_a value of 8.1 and at high pH with a pK_a value of ~11.5 (Figure 1 and Table II). These pK_a values are clearly perturbed outward relative to the pK_a values obtained with D-serine, consistent with D-alanine being a sticky substrate ($k_3 > k_2$). The pK_a value of 11.5 is poorly defined because of the inability to obtain data above pH 11; therefore, the data points above pH 10.5 were not utilized in subsequent analyses.

The stickiness of D-alanine was clearly established by the pH dependence of the $^{D}V/K_{ala}$ value, which increased from

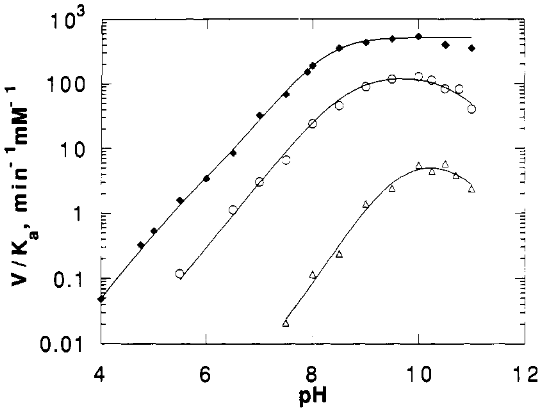


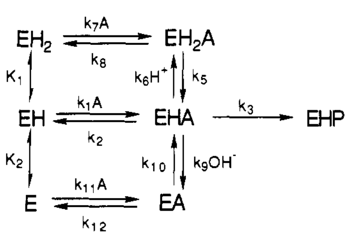
FIGURE 1: pH dependence of the V/K_a values for D-amino acid oxidase at 25 °C: (◆) D-alanine; (○) D-serine; (△) glycine. Initial velocities were determined with the LDH-coupled and the oxygen electrode assays by varying the concentration of the amino acid at 266 mM O_2. The enzyme concentration varied from 0.03 to 30 μM. The FAD concentration was at least 10 μM. At and below pH 10 the concentrations of both glycine and oxygen were varied. The buffers used were 0.1 M sodium acetate (pH 4–5.5), 0.1 M ACES, 0.052 M Tris, 0.052 M ethanolamine (pH 5.5–10), and 0.1 M EDTA (pH 10–11). The solid lines are fits to eqs 11, 7, and 13, respectively.

Table I: Effect of pH on the $^{D}V/K_{ser}$ Value for D-Amino Acid Oxidase at 25 °C[a]

pH	$^{D}V/K_{ser}$	pH	$^{D}V/K_{ser}$
5.0	4.61 ± 0.51	9.0	4.17 ± 0.25
7.0	4.40 ± 0.47	9.5	4.70 ± 0.38
7.5	4.48 ± 0.32	average	4.47 ± 0.18
8.5	4.43 ± 0.43		

[a] Conditions: 0.1 M ACES, 0.052 M Tris, and 0.052 M ethanolamine; and 0.1 M acetate at pH 5.0; 10 μM FAD. The enzyme concentration varied from 0.03 μM at pH 9.5 to 30 μM at pH 5.

Scheme IV



1.15 ± 0.04 at pH 10.5 to 5.08 ± 0.69 at pH 4.0 (Figure 2). When the $^{D}V/K_{ala}$ data were fit to eq 8, values of 5.2 for the isotope effect at low pH, 1.5 for the isotope effect at high pH, and 5.4 for the apparent pK_a were obtained. The kinetic mechanism of Scheme IV predicts that the pK_a value determined from the $^{D}V/K$ profile will agree with that of 8.1 from the V/K_a profile (Cook & Cleland, 1981a), in contrast to the experimental results. In addition, the experimental $^{D}V/K_{ala}$ values were significantly less than 1.5 between pH 8.5 and 10.5. Finally, the $^{D}V/K_{ala}$ values do not increase monotonically between pH 8 and 5, as predicted by Scheme IV, but instead appear to level off around pH 6.5 at a value of 2.3 before increasing further below pH 6. Consequently, an explanation was sought for the results with D-alanine which was still consistent with the results with D-serine. Adding the assumption that binding of D-alanine hindered the exchange with solvent of the proton on the amino acid residue with pK_a 8.7 did not increase the quality of the fit of the data and did not account for the effect of pH on the $^{D}V/K_{ala}$ value (results not shown). However, Cleland (1977, 1986) has pointed out that the common assumption that protonation of an amino acid residue involves H_3O^+ is not likely to be valid for a residue with a pK_a value significantly above 7, as is the case here. Instead, protonation above pH 7 will involve water. The mechanism of Scheme IV was therefore modified to make explicit the possibility of protonation of EHA by either water or H_3O^+ (Scheme V). The relevant equations for the effects of pH on the V/K_{ala} and $^{D}V/K_{ala}$ values are eqs 11 and 12. The values resulting from fitting the experimental data to these equations are given in Table II, and the theoretical lines from these analyses are drawn in Figures 1 and 2. By a number of criteria the model of Scheme V provides a better fit of the data than that of Scheme IV. The variances for these fits are significantly smaller than those obtained from the mechanism of Scheme IV. The pK_a value of 8.9 obtained from the fit to eq 11 agrees with the pK_a value of 8.7 seen with D-serine. The fit to eq 12 results in an intrinsic isotope effect ($^{D}k_3$) of 5.5 and a pK_a

Table II: Analyses of the pH Dependence of V/K_{ala} and $^{D}V/K_{ala}$ Values for D-Amino Acid Oxidase at 25 °C

	V/K_{ala}			$^{D}V/K_{ala}$	
parameter	eq 7	eq 6	eq 11	eq 8	eq 12
C or k_1, min^{-1} mM^{-1}	465	444	590		
	(390–550)[a]	(366–530)	(540–640)		
pK_1	8.1	8.0	8.9	5.4	8.1
	(7.9–8.2)	(7.9–8.2)	(8.7–9.1)	(5.1–5.6)	(7.7–8.7)
pK_2	11.5				
	(11.2–12.0)				
YL or $^{D}k_3$				5.2	5.5
				(4.8–5.7)	(5.2–5.6)
YH				1.5	
				(1.4–1.7)	
k_3/k_2			7.8		16
			(4.8–12)		(10–26)
k_6'/k_2			6.0		4.4
			(4.2–14)		(2.8–7.5)
k_6/k_2, μM^{-1}			8.3		2.7
			(3.7–20)		(1.6–4.6)
variance	0.0102	0.0104	0.00232	0.0775	0.0206

[a] Range for 67% confidence.

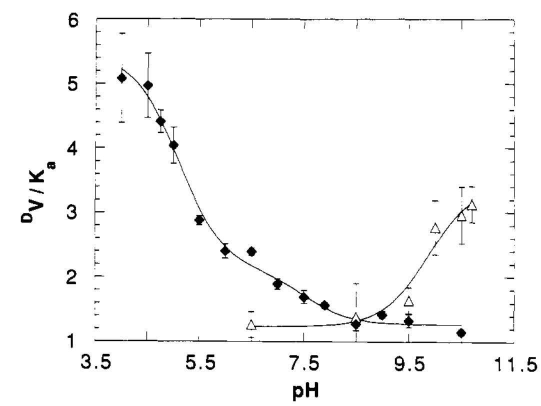
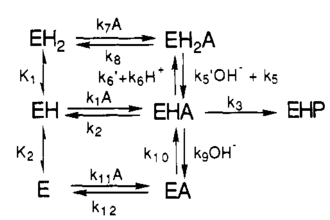


FIGURE 2: pH dependence of the $^{D}V/K_a$ values for D-amino acid oxidase at 25 °C: (◆) D-alanine; (△) glycine. The solid lines are fits to eqs 12 and 14, respectively. Conditions as described for Figure 1.

Scheme V



$$V/K_{\mathrm{ala}} = \frac{\left(\dfrac{k_1(k_3/k_2)}{1 + k_3/k_2}\right)\left(1 + \dfrac{[\mathrm{H}]k_7(1 + k_2/k_6' + k_6[\mathrm{H}]/k_6')}{K_1k_1(1 + k_6[\mathrm{H}]/k_6')}\right)}{(1 + [\mathrm{H}]/K_1)\left(1 + \dfrac{[\mathrm{H}]k_7(1 + k_2/k_6' + k_3/k_6' + k_6[\mathrm{H}]/k_6')}{K_1k_1(1 + k_3/k_2)(1 + k_6[\mathrm{H}]/k_6')}\right)} \quad (11)$$

$$^{\mathrm{D}}V/K = \frac{^{\mathrm{D}}k_3 + c_{\mathrm{f}}}{1 + c_{\mathrm{f}}}$$

$$c_{\mathrm{f}} = \frac{(k_3/k_2)\left(1 + [\mathrm{H}]/K_1\left(\dfrac{1}{k_6'/k_2 + k_6[\mathrm{H}]/k_2}\right)\right)}{\left(1 + [\mathrm{H}]/K_1\left(1 + \dfrac{1}{k_6'/k_2 + k_6[\mathrm{H}]/k_2}\right)\right)} \quad (12)$$

value of 8.06, which is not significantly different from 8.7,[2] compared to the previous value of 5.4. The values obtained from fits of eqs 11 and 12 show good agreement (Table II). Finally, the $^{\mathrm{D}}V/K_{\mathrm{ala}}$ data are now well fit, with a clearly bimodal increase in the value of the observed isotope effect. A slight hollow is also seen in the V/K_{ala} profile, resulting in a better fit of the data, but that is less convincing.

(*c*) *Glycine.* Intersecting lines in double-reciprocal plots were obtained when oxygen and glycine concentrations were both varied. Thus, it was necessary to vary both concentrations to determine kinetic parameters; the data were fit to eq 5 in order to obtain the V/K_{gly} values. At pH values greater than 10, there was a switch to a parallel line pattern; the data were fit to eq 3. These patterns are consistent with all steps between glycine and oxygen binding being reversible below pH 10, with one or more steps becoming irreversible at high pH. The V/K_{gly} pH optimum is shifted to 10.3 compared to the value of 9.7 for D-serine (Figure 1). Assuming minimum separation of two closely occurring pK_a values near 10, the rates decrease below an apparent pK_a value of 10.0 and decrease above a pK_b value of 10.6. These results are in stark contrast to the pH dependencies of both D-serine and D-alanine. A shift in the pH optimum of a V/K value with a change in substrate is not a result of stickiness but indicates instead a mechanistic change.

The $^{\mathrm{D}}V/K_{\mathrm{gly}}$ value *increased* at high pH, from 1.26 ± 0.20 at pH 6.5 to 3.13 ± 0.30 at pH 10.5, in direct contrast to the behavior seen with D-alanine, further confirming the contrast between glycine and the other two substrates. The change in kinetic patterns with pH indicates that the reversibility of a step between the binding of glycine and oxygen is pH-dependent. This is made explicit in Scheme VI, in which reversible carbon–hydrogen bond cleavage is followed by a pH-sensitive step. The steps with rate constants k_{13} and k_{15} contain all steps subsequent to carbon–hydrogen bond cleavage through oxygen binding. At pH values below pK_3, flux will be via the upper pathway and HE′HX will partition between EHA and HE′HP. Since all steps between glycine and oxygen binding are reversible, it must be the case that $k_4 \gg k_{13}$. Under these conditions only a very small primary kinetic isotope effect will be seen. At high pH, deprotonation of HE′HX will be sufficiently rapid that formation of HE′HX from EHA will be effectively irreversible. This will generate an irreversible step between the binding of glycine and oxygen, as is required by the steady-state kinetic results, and result in an increase in the $^{\mathrm{D}}V/K_{\mathrm{gly}}$ value. The effects of pH on the V/K_{gly} and $^{\mathrm{D}}V/K_{\mathrm{gly}}$ values predicted by Scheme VI are described by eqs 13 and 14, respectively. The results of fitting

Scheme VI

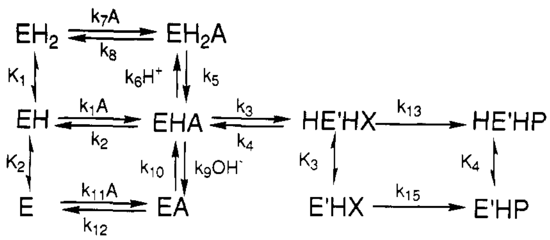


Table III: Analyses of the pH Dependence of V/K_{gly} and $^{\mathrm{D}}V/K_{\mathrm{gly}}$ Values for D-Amino Acid Oxidase at 25 °C

	V/K_{gly}		$^{\mathrm{D}}V/K_{\mathrm{gly}}$	
parameter	eq 7	eq 13[a]	eq 8	eq 14[b]
C, min^{-1} mM^{-1}	9.9 (8.8–11.0)	8.7 (6.9–11.0)		
pK_1	10.0 (9.9–10.1)	8.7		
pK_2	10.6 (10.5–10.7)	10.7		
pK_3		8.3 (8.0–8.6)	9.9 (9.6–10.2)	8.5 (8.0–8.8)
YL			1.2 (1.0–1.4)	
$^{\mathrm{D}}k_3$ or YH			3.4 (3.1–3.8)	3.4 (3.2–3.8)
k_4/k_{13}		24 (16–33)		21 (8.9–44)
variance	0.0807	0.0106	0.0483	0.0483

[a] The pK_1 and pK_2 values were fixed at 8.7 and 10.7, and the value of k_{13}/k_{15} was fixed at 1. [b] Fixed k_{13}/k_{15} equal to 1 and $^{\mathrm{D}}K_{\mathrm{eq}}$ = 1.13.

$$V/K_{\mathrm{gly}} = \left(\frac{k_3k_1/k_2(1 + k_{13}[\mathrm{H}]/k_{15}K_3)}{(1 + K_2/[\mathrm{H}] + [\mathrm{H}]/K_1)(1 + [\mathrm{H}]/K_3(k_{13}/k_{15} + k_4/k_{15}))}\right) \quad (13)$$

$$^{\mathrm{D}}V/K = \frac{^{\mathrm{D}}k_3 + c_{\mathrm{r}}{}^{\mathrm{D}}K_{\mathrm{eq}}}{1 + c_{\mathrm{r}}} \qquad c_{\mathrm{r}} = \frac{k_4/k_{13}}{1 + \dfrac{k_{15}K_3}{k_{13}H}} \quad (14)$$

the data with glycine as substrate to the model of Scheme VI are given in Table III and Figures 1 and 2.[3] As expected, the model of Scheme VI results in an increase in the $^{\mathrm{D}}V/K_{\mathrm{gly}}$ value with increasing pH. Furthermore, the inclusion of K_3 for the pH-dependent partitioning between two catalytically viable steps results in a shift in the V/K_{gly} pH optimum to higher values with pK_a values for the free enzyme which are consistent with the pH dependencies of both D-serine and D-alanine. Thus, the mechanism of Scheme VI can explain the kinetics with glycine as substrate in a manner that is fully consistent with the results obtained with the other substrates.

Rapid Reaction Kinetics. Under anaerobic conditions the limiting rate of reduction DAAO by amino acid substrates can be determined with a stopped-flow apparatus (Palmer &

[2] The small discrepancy between the optimized values of 8.1 and 8.9 obtained from fits to eqs 12 and 11, respectively, is best explained by the bias between the two fits. The pK_1 value of 8.1 from the $^{\mathrm{D}}V/K_{\mathrm{ala}}$ fit (eq 12) is heavily influenced by the $^{\mathrm{D}}V/K_{\mathrm{ala}}$ values at high pH. Since these values are quite low, the accuracy is inherently poorer. This is borne out by the wide confidence interval, 7.7–8.7, for the pK_1 value (eq 12).

[3] In fitting the isotope effect data to eq 14, the $^{\mathrm{D}}K_{\mathrm{eq}}$ value was fixed at 1.13. This value was calculated by assuming the proton from the amino acid is transferred to a hydroxyl residue (Kresge et al., 1987). Transfer to an uncharged nitrogen would give a value of 1.15, with no significant effect on the overall fit of the data.

Scheme VII

$$E + A \underset{}{\overset{K_d}{\rightleftharpoons}} EA \xrightarrow{k_{red}} EP \xrightarrow{k_5} E + P$$

Table IV: Deuterium Kinetic Isotope Effects on the Rate of Reduction of D-Amino Acid Oxidase at 25 °C[a]

substrate	pH	${}^{D}k_{red}$	${}^{D}(k_{red}/K_d)$
D-alanine	9.0	1.15 ± 0.07	1.13 ± 0.14
glycine	10.5	3.36 ± 0.43	2.33 ± 0.20
D-serine	9.0	4.75 ± 0.59	2.23 ± 0.28
D-serine	7.5	2.49 ± 2.49	4.45 ± 0.38

[a] ${}^{D}k_{red}$ is the isotope effect on the limiting rate of reduction according to Scheme VII. The buffer consisted of 0.1 M ACES, 0.052 M Tris, and 0.052 M ethanolamine except with glycine at pH 10.5, where 0.1 M EDTA was used. The enzyme concentration was 30 μM with D-serine at pH 7.5 and was ~15 μM with all others.

Massey, 1968). The loss of oxidized flavin absorbance at 455 nm or the increase in absorbance of the charge-transfer complex at 550 nm is used to follow reduction of the enzyme. The mechanism of Scheme VII describes the kinetics observed in the stopped flow. Our rationale for employing the stopped-flow method was to determine the deuterium isotope effect on the limiting rate of reduction and compare these values to the limiting values obtained in the steady state. When the enzyme is saturated with substrate, the external forward commitment is abolished just as it is at the pH extremes, if the models of Schemes IV–VI are correct. This approach therefore provides an independent check on the validity of the limiting values of ${}^{D}k_3$ obtained from the steady-state data. This was especially critical in the case of glycine, where the limiting value of 3.4 is not well-defined. Rates of reduction were determined under different substrate concentrations, and the data were fit to eq 3. The limiting rate of reduction (k_{red}) at high substrate concentration and the apparent second-order rate constant (k_{red}/K_d) at low substrate concentration were determined with glycine, D-alanine, and D-serine as substrates.

With glycine as substrate, a value of 3.4 ± 0.4 at pH 10.5 was found for the deuterium isotope effect on the limiting rate of reduction (Table IV). The value of 3.4 is identical to the value of ${}^{D}k_3$ obtained from fitting the steady-state data (Table III) to eq 14. With D-alanine at very low pH, it was difficult to obtain precise values of ${}^{D}k_{red}$ because of the difficulty in saturating the enzyme with this substrate ($K_m > 0.5$ M). At pH 9.0, the ${}^{D}k_{red}$ value with D-alanine was 1.15 ± 0.07. At pH 9.0 with D-serine, the ${}^{D}k_{red}$ value was 4.75 ± 0.59, in excellent agreement with the steady-state value. However, the ${}^{D}(k_{red}/K_d)$ value was 2.2 ± 0.3, significantly lower than the average value of 4.5 ± 0.2 for ${}^{D}V/K_{ser}$ observed in the steady-state analysis, and the k_{red}/K_d values were consistently 3–4-fold larger than the V/K_{ser} values. One difference between the conditions for the rapid reaction experiments and the steady-state assays was the much higher concentration of enzyme used in the former. D-Amino acid oxidase is known to polymerize with increasing enzyme concentration (Antonini et al., 1966; Tojo et al., 1986a,b); the enzyme activity is also reported to change with increasing enzyme concentration (Shiga & Shiga, 1972; Horiike et al., 1977; Fitzpatrick & Massey, 1982b). To examine whether the anomalously low ${}^{D}V/K_{ser}$ value at pH 9 could be the result of high enzyme concentration, we determined the effect of enzyme concentration on the ${}^{D}V/K_{ser}$ value (Figure 3). Near 15 μM enzyme, the isotope effect reaches a limiting value of ~3. Below 1 μM, the isotope effect increases to an average value of 4.5. Values of 4.45 ± 0.38 for ${}^{D}(k_{red}/K_d)$ and 2.5 ± 2.5 for ${}^{D}k_{red}$ were obtained with D-serine at pH 7.5. The large error in ${}^{D}k_{red}$ was a consequence of the large uncertainty in the k_{red} value with the deuterated substrate ($K_m \sim 230$ mM).

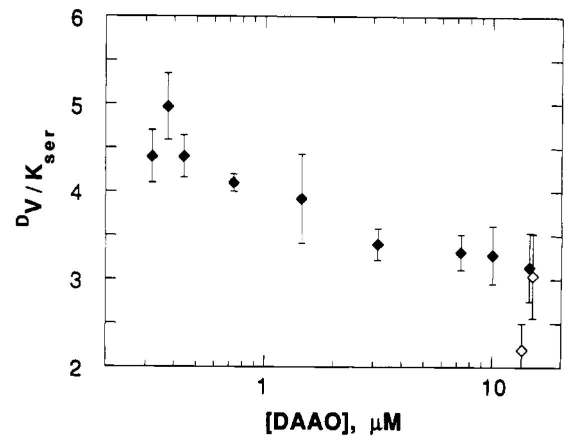


FIGURE 3: Dependence of the ${}^{D}V/K_{ser}$ value on the concentration of D-amino acid oxidase at pH 9 and 25 °C: (◆) data determined using the stopped-flow spectrophotometer; (◇) determined using the oxygen electrode assay. The buffer consisted of 0.1 M ACES, 0.052 M Tris, and 0.052 M ethanolamine.

DISCUSSION

We have determined the effect of pH on the V/K_a and ${}^{D}V/K_a$ values with several substrates for D-amino acid oxidase. We have determined the primary deuterium isotope effect on the limiting rate of reduction under anaerobic conditions. The effect of D-amino acid oxidase concentration on the ${}^{D}V/K_{ser}$ value was also examined. By studying the pH dependency of the V/K_a rate constant, we gain insight into the protonation states of groups on the substrate and enzyme which are critical for binding and/or catalysis. Also, the pH dependency of the kinetic isotope effect on V/K_a allows us to determine or at least place limits on the values of the various catalytic steps in reduction.

Competitive inhibitors and various D-amino acid substrates have been used previously to identify the ionization states of critical groups in DAAO. Quay and Massey (1977) determined the effect of pH on benzoate binding and concluded that three enzyme ionizations were involved: an ionizable group with a pK_a of 6.3 that must be unprotonated and two groups with pK_a values of 9.6 and 9.2 that must be protonated for maximal binding. Purdy (1982) concluded that three noncooperative ionizations with pK_a values of 6.6, 8.9, and 9.7 sufficiently describe the pH dependency. Benzoate binding requires that the group with a pK_a value of ~8.9 be protonated, while catalysis requires that this group be unprotonated.

D-*Serine*. All of our results can be explained by the mechanism of Scheme VI. The most unambiguous results were obtained with D-serine as substrate. The pH-independent value of 4.5 for ${}^{D}V/K_{ser}$ establishes this substrate as nonsticky ($k_2 > k_3$), and therefore, the pK_a values of 8.7 of a group which must be unprotonated and of 10.7 of a group which must be protonated for activity are the intrinsic values. With the rapid reaction analysis, the value of 4.75 ± 0.59 for the isotope effect on the limiting rate of reduction agrees nicely with the steady-state ${}^{D}V/K_{ser}$ value of 4.5, suggesting that 4.5 is the limiting isotope effect.[4] However, the low value of 2.2 for ${}^{D}V/K_{ser}$ observed with the rapid reaction method at pH 9 was unanticipated. To examine whether the anomalously low ${}^{D}V/K_{ser}$ value at pH 9 could be the result of high enzyme concentration, we determined the effect of enzyme concentration on the $^{D}V/K_{ser}$ value. At enzyme concentrations near 15 μM, the average $^{D}V/K_{ser}$ is 2.8. Below 1 μM, the isotope effect increases to an average value of 4.5. Thus, the anomalously low values can be explained by the enzyme concentration dependency of $^{D}V/K_{ser}$. At high enzyme concentrations at pH 9, D-serine is slightly sticky ($k_3 \sim k_2$) and the observed isotope effect follows eq 15. A decrease of ~3-fold in the rate of substrate dissociation (k_2) from the oxidized enzyme at high enzyme concentrations would explain the smaller observed isotope effect. From the difference in the observed isotope effect at the concentration extremes, a value of 0.8 ± 0.2 can be calculated for k_3/k_2 at pH 9 and high enzyme concentrations.

$$^{D}V/K_{ser} = (^{D}k_3 + k_3/k_2)/(1 + k_3/k_2) \qquad (15)$$

A number of studies have established that the molecular weight of D-amino acid oxidase depends upon enzyme concentration (Charlwood et al., 1961; Antonini et al., 1966). At 25 °C, the enzyme behaves as a monomer at low concentrations, dimerizing with increasing concentration; at very high enzyme concentrations, larger oligomers are formed (Tojo et al., 1985a,b). The transition from monomer to dimer occurs between 1 and 5 μM, consistent with the concentration dependence of the $^{D}V/K_{ser}$ value reported here. There have been a number of previous reports that the activity of D-amino acid oxidase is concentration-dependent. Both catalytic rates and dissociation constants for inhibitors decrease 2–4-fold when the enzyme concentration is varied between 50 nM and 50 μM (Shiga & Shiga, 1972; Yagi et al., 1973; Horiike et al., 1977). A detailed investigation of the effect of enzyme concentration on kinetic parameters with D-alanine as substrate found that the V/K values for both oxygen and D-alanine were independent of enzyme concentration, but the V_{max} decreased 2–4-fold as the enzyme concentration was increased from 30 nM to 15 μM (Fitzpatrick & Massey, 1982b). The V_{max} value for D-alanine equals the rate of product release (Porter et al., 1977) and the V/K_{ala} value is essentially k_1, the association rate constant for the amino acid binding to the enzyme. Consequently, the effect of enzyme concentration on the kinetics of D-amino acid oxidase has been interpreted as a decrease in the value of dissociation of ligands from the enzyme as dimerization occurs (Fitzpatrick & Massey, 1982b). The results reported here with D-serine are fully consistent with such a model. Since D-serine is not a very sticky substrate, $V/K_{ser} = k_1k_3/(k_2 + k_3)$, and the magnitude of the $^{D}V/K_{ser}$ value will be determined by the value of the forward commitment, k_3/k_2. A decrease in the value of k_2 at the concentrations of enzyme used in stopped-flow experiments will increase the value of the commitment and decrease the value of the observed isotope effect. Consistent with this interpretation, the V/K_{ser} values obtained using the stopped-flow spectrophotometer were consistently 2–4-fold higher than those obtained in steady-state experiments using 10–30 nM enzyme. Thus, the effects of enzyme concentration upon $^{D}V/K_{ser}$ values are consistent with the known effect of enzyme concentration on the kinetics of D-amino acid oxidase.

If D-serine is indeed slightly sticky at high enzyme concentrations, the model of Scheme IV predicts that the substrate will become nonsticky off the pH optimum. This was tested by repeating the stopped-flow measurements at pH 7.5, 2 pH units below the optimum. A value of 4.45 ± 0.38 for the $^{D}(k_{red}/K_d)$ value was determined at pH 7.5 under these conditions. The enzyme concentration was 30 μM, or 2-fold higher than in the identical experiment at pH 9. Thus, under these conditions, D-serine is not sticky and the limiting isotope effect of 4.5 is observed. In effect, the forward commitment is pH-dependent (increasing with pH) and is negligible by pH 7.5, consistent with the pH dependency of the V/K_{ser} value. This slight stickiness means that the pK_a values determined with this substrate are slightly perturbed. However, a k_3/k_2 value of 0.2 will perturb a pK_a value by log (1 + 0.2) or 0.08 unit. Such a small perturbation is less than the precision with which enzyme pK_a values can confidently be measured.

D-Alanine. With D-alanine as substrate, the observed pK_a values of 8.1 and 11.5 are perturbed outward compared with the intrinsic values of 8.7 and 10.7 observed with D-serine. The $^{D}V/K_{ala}$ value increases from 1.3 at pH 9.5 to 5.1 at pH 4.0. These results establish that D-alanine is sticky, with k_3/k_2 approximately equal to 10. If Scheme IV described the results with D-alanine, the fit to eq 8 should be a good one and the pK_a value from the $^{D}V/K_{ala}$ profile should be near 8 (Cook & Cleland, 1981a). However, the fit of $^{D}V/K_{ala}$ data was poor and yielded a pK_a value of 5.4. These disparities called for an alternative analysis of the pH data with D-alanine. Cleland (1977, 1986) has pointed out that a residue with a pK_a value greater than 7 will be protonated by water rather than H_3O^+ above pH 7. Only below pH 7 will the concentration of H_3O^+ be sufficient. This treatment requires that EH_2A and EHA not be in equilibrium over the pH range where the isotope effect is pH-dependent.[5] It is the biphasic nature of the increase in the $^{D}V/K_{ala}$ value with decreasing pH which is most diagnostic of the switch from protonation of EHA by H_2O to protonation by H_3O^+. Introducing this qualification results in minimal changes in the model developed for D-serine yet accounts for the drastically different results obtained with D-alanine.

At the pH optimum, the forward commitment with D-alanine as substrate is essentially k_3/k_2, which has a value of ~10. Thus, the observed isotope effect is small at pH 9.5. Below the pK_a value of 8.7, most of the catalytic flux will be through EH_2 and EH_2A rather than through EH. The forward commitment under these conditions is therefore essentially k_3/k_6', where k_6' is the rate of reaction of H_2O and EAH to form EH_2A. The value of k_3/k_6' is ~2 (Table II), so the value of the observed isotope effect does not increase to a maximum but instead levels off around pH 7. At pH values significantly below pH 7, the concentration of H_3O^+ becomes sufficiently high to allow EHA and EH_2A to equilibrate and the forward commitment does approach zero, so that the $^{D}V/K_{ala}$ value increases to a limiting value of 5.5. Fits to the proposed model (Scheme V) accurately describe the D-alanine pH data. The values of k_3/k_2, k_6/k_2, and k_6'/k_2 obtained from fits to both the V/K_{ala} and $^{D}V/K_{ala}$ equations (11 and 12) show good agreement (Table II).

[4] More rigorously, the agreement between the $^{D}k_{red}$ value and the $^{D}V/K$ value establishes that the forward commitment is effectively zero under these conditions. The limiting isotope effects of 5.5, 4.5, and 3.4 determined with D-alanine, D-serine, and glycine, respectively, may be the intrinsic values and may reflect the different nature of each transition state. However, it is possible that the values are not intrinsic and are lowered by the presence of an internal reverse commitment.

[5] Protonation of a group with a pK_a value of 8.7 by H_3O^+ would be expected to have a rate constant of $\sim 2 \times 10^{10}$ M^{-1} s^{-1} (Eigen, 1964). The highest rate of reduction by D-alanine we were able to measure was 730 s^{-1}, setting a lower limit for k_3 with this substrate. The extrapolated value at infinite concentrations of D-alanine was ~1000 s^{-1}, setting this as an upper limit. Using the latter value for k_3, the values in Table II set k_6 at $\sim 6 \times 10^8$ M^{-1} s^{-1}, consistent with hindered access of this residue to the solvent.

While the steady-state and rapid reaction data give limiting isotope effects for both D-serine and glycine, only a very small effect was observed for the limiting rate of reduction of DAAO by D-alanine. This low value (1.15 at pH 9.0) is most likely due to the difficulty of truly saturating an enzyme with a very sticky substrate in presteady-state kinetics. As discussed by Strickland et al. (1975) and by Fitzpatrick and Massey (1982b), at most substrate concentrations the rate constant for reduction will be k_1 for a very sticky substrate. No isotope effect is expected on k_1. Extrapolation to an infinite concentration of substrate will not give a valid measure on the limiting rate.

Glycine. The pH optimum of the V/K_{gly} profile is higher by ~0.5 pH unit than that of the other substrates. Since glycine is such a slow substrate, it is unlikely to be sticky. Even so, stickiness does not alter the pH optimum of a pH profile. The changed pH optimum, the switch in steady-state kinetic patterns at high pH, and the reversed pH dependency of the $^{D}V/K_{gly}$ value are all consistent with a pH-dependent step following the carbon–hydrogen bond cleavage step in the overall reduction of the flavin. Such a model has previously been proposed by Cook and Cleland (1981b) to explain a similar change in the pH dependency of isotope effects with alcohol dehydrogenase. The intersecting line patterns seen in double-reciprocal plots with glycine below pH 10 establishes that all steps in the reductive half-reaction must be reversible under these conditions. With a nonzero k_4 value, the pH dependence of the V/K_{gly} value is then affected by pK_1, pK_2, and pK_3 (Scheme VI). The behavior of the $^{D}V/K_{gly}$ value with varied pH is due to the presence of a pH-dependent reverse commitment. The reverse commitment is abolished at high pH, resulting in the limiting $^{D}V/K_{gly}$ value of 3.4. At pH 10.5, the isotope effect on the limiting rate of reduction is 3.4 (Table IV) in excellent agreement with the steady-state results. Upon dissecting eqs 13 and 14, it is evident that the true pK_a value and the ratio k_{13}/k_{15} cannot be resolved. The apparent pK_a occurs at the pH where it is equal to $p(K_a(k_{15}/k_{13})) + \log(1 + k_4/k_{13})$. From the two fits, it was determined that k_4/k_{13} is ~20. With an apparent pK_a of 10, $p(K_a(k_{15}/k_{13}))$ is then equal to 8.5. If we assume k_{15}/k_{13} is 1, then a pK_a value of 8.3 is obtained from a fit to eq 13 and a value of 8.5 from a fit to eq 14.

The small size of glycine may render the base with an apparent pK_a value of 10 accessible to solvent when this amino acid is bound. At high pH values, loss of the proton to solvent is irreversible, explaining the switch to parallel lines in double-reciprocal plots at high pH. Also, at very high pH, the reverse commitment becomes negligible and the observed isotope effect approaches the limiting value. As the pH is lowered, the reverse commitment becomes very large and the observed isotope effect approaches the equilibrium effect. In contrast, D-serine and D-alanine probably form tight complexes (HE′HX in Scheme V) with DAAO, prohibiting the group with pK_3 from transferring its proton to bulk solvent. This is consistent with the results of Walsh et al. (1973), which showed the α-proton of β-chloro-α-aminobutyrate is shielded from solvent after its removal from the substrate. In addition, we find that the V/K_{O_2} values with D-alanine and D-serine are pH-independent over this pH range, while the value with glycine exhibits a bell-shaped pH dependency.[6] These latter results are consistent with active site residues being more accessible to solvent when glycine is the substrate.

Given a step-wise model for reduction (Scheme I), the additional base observed with glycine may be the base responsible for pulling off the amino proton, facilitating release of the imino acid from reduced flavin. It is also appealing to envisage that the group with a pK_a value of 8.7 is the base involved in α-proton abstraction and the group with a pK_b of 10.7 is the flavin N(3) position, which when protonated has been shown to increase the electrophilicity of the N(5) of oxidized flavin (Moonen et al., 1984).

Purdy (1982) and Quay and Massey (1977) have reported that a group with a pK_a value of 6.3–6.6 must be unprotonated for benzoate binding. We find no evidence from the V/K_a profiles for a pK_a near 6.3–6.6 in the free enzyme. The rate of product release is pH-dependent with both D-alanine (pK_a 8.7) and glycine (pK_a 9.4).[6] The pK_a of this group will vary depending upon the substrate because an enzyme–product complex is involved. Thus, it is quite possible that benzoate acts as a product analogue and binds most tightly to the form of the enzyme with this group unprotonated.

We have presented a detailed study of the pH effects on the V/K_a values and the observed kinetic isotope effects for D-amino acid oxidase. The results with all three substrates can be explained by a single model. Our major conclusions are as follows:

(1) D-Serine is slightly sticky only under very high enzyme concentration at pH 9; otherwise, the limiting isotope effect of 4.5 is observed. High enzyme concentration appears to slow the rate of substrate dissociation (k_2). The intrinsic pK_a values are 8.7 and 10.7.

(2) D-Alanine is a sticky substrate; k_3/k_2 is approximately 10. The transfer of a proton mediated by the solvent between EH_2A and EHA is not in rapid equilibrium.

(3) With glycine, the results are consistent with reversible CH bond cleavage followed by a pH-dependent step. This group with an apparent pK_a value of 10 is accessible to solvent when glycine, a relatively small substrate, is bound.

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