Intrinsic Primary, Secondary, and Solvent Kinetic Isotope Effects on the Reductive Half-Reaction of D-Amino Acid Oxidase: Evidence against a Concerted Mechanism†

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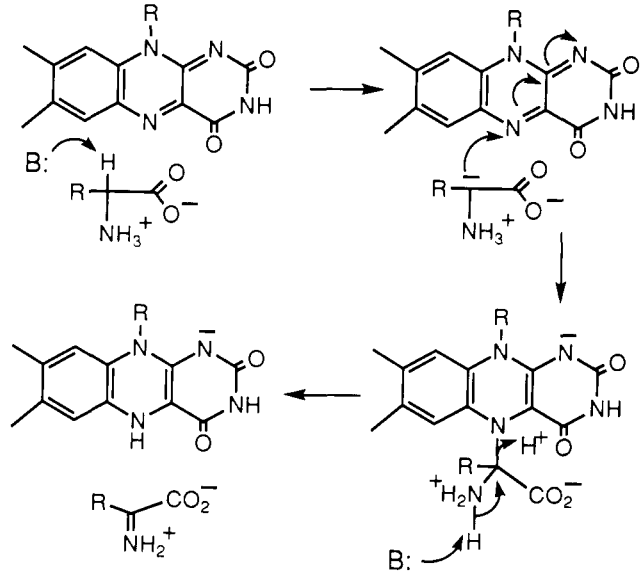
Received September 24, 1993; Revised Manuscript Received January 14, 1994®

ABSTRACT: D-Amino acid oxidase catalyzes the oxidation of D-amino acids to imino acids with subsequent transfer of the electrons to molecular oxygen. Proposed mechanisms for the mode of cleavage of the substrate CH bond include stepwise formation of a carbanion, followed by attack of the carbanion on the enzyme-bound FAD, direct hydride transfer of the substrate α-hydrogen to the FAD, and transfer of a hydride from the substrate amino group to the FAD. Conditions have previously been established under which large, limiting, primary deuterium kinetic isotope effects can be measured with D-alanine, D-serine, and glycine as substrates for D-amino acid oxidase [Denu, J. M., & Fitzpatrick, P. F. (1992) *Biochemistry 31*, 8207–8215]. To determine whether these values are the intrinsic isotope effects, primary tritium kinetic isotope effects have been determined with these three substrates. The values are 12.6, 8.6, and 6.4, respectively. These values are consistent with expression of the intrinsic isotope effects under these conditions, allowing for determination of the values of the intrinsic deuterium effects as 5.7, 4.5, and 3.6 for D-alanine, D-serine, and glycine, respectively. Under these conditions, the α-secondary tritium kinetic isotope effect with glycine, the β-secondary deuterium kinetic isotope effect with D-alanine, and the solvent kinetic isotope effect with D-serine are all indistinguishable from unity. These results are not consistent with concerted mechanisms for CH bond cleavage with this enzyme, but are fully consistent with the involvement of a carbanion intermediate.

The flavoprotein D-amino acid oxidase (DAAO[1]) catalyzes the oxidation of D-amino acids to their respective imino acids. The chemical mechanism of the reductive half-reaction of DAAO has been proposed to involve the initial formation of a carbanion by abstraction of the α-proton from the amino acid (Scheme 1) (Ghisla, 1982). Evidence for such a carbanion intermediate comes from the elimination of HCl from β-chloroalanine and β-chloroaminobutyrate catalyzed by DAAO (Walsh et al., 1971, 1973) and the inactivation of several flavoprotein oxidases by acetylenic and vinylic substrates (Marcotte & Walsh, 1976). Once formed, the carbanion is proposed to add to the N(5) position of the FAD. Imino acid elimination from this adduct would then generate reduced flavin and the imino acid. Nitroalkanes form adducts with DAAO at the N(5) position of the flavin (Porter et al., 1972, 1973), in support of such a model. Additionally, a stable flavin–substrate adduct is reported to be formed after the abstraction of the glyoxylate *si* proton by lactate oxidase (Ghisla & Massey, 1980), a similar flavoprotein oxidase.

However, Hersh and Jorns (1975) have shown that when the native FAD is replaced by 5-deaza-FAD, the α-hydrogen of the amino acid substrate is transferred from the amino acid to the C(5) position of 5-deaza-FAD. This result is consistent with a direct hydride-transfer mechanism (Scheme 2). In contrast, Miura and Miyake (1988) have proposed a concerted mechanism in which α-proton abstraction is coupled to the transfer of a hydride from the amino acid nitrogen, rather than from the α-hydrogen, to the flavin (Scheme 3). This last model is also consistent with the experimental evidence for a carbanion mechanism. It is thus clear that the question of the chemical mechanism of DAAO is still unsettled.

Scheme 1



Previously, we reported a detailed study of the effects of pH on primary deuterium kinetic isotope effects for the reductive half-reaction of DAAO with glycine, D-serine and D-alanine as substrates (Denu & Fitzpatrick, 1992). An amino acid residue with a pK_a value of 8.7 must be unprotonated, and a residue with a pK_a value of 10.7 must be protonated for

† This research was supported in part by National Science Foundation Grant DMB-9204698. P.F.F. is an Established Investigator of the American Heart Association.

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® Abstract published in *Advance ACS Abstracts,* March 15, 1994.

[1] Abbreviations: DAAO, D-amino acid oxidase; ACES, *N*-(2-acetamido)-2-aminoethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane.

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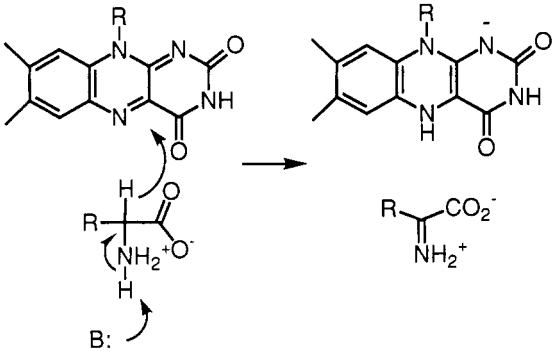
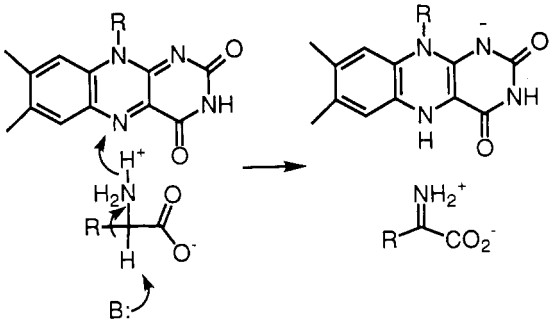
Scheme 2



Scheme 3



reduction. Of particular importance to the chemical mechanism, conditions were identified under which the observed primary deuterium kinetic isotope effects were maximal and possibly intrinsic with all three substrates. One would predict that for concerted hydride transfer there would be a significant secondary isotope effect when the observed primary kinetic effect is intrinsic. A lack of an observed secondary isotope effect would be consistent with a stepwise carbanion mechanism. However, it is first critical to establish that the previously reported values are indeed intrinsic. In the current investigation, primary tritium isotope effects were determined with all three substrates under conditions that afforded the maximal deuterium effects, allowing calculation of the intrinsic effects. In addition, α- and β-secondary isotope effects as well as solvent isotope effects on the reductive half-reaction of DAAO were determined.

MATERIALS AND METHODS

Materials. [1-^{14}C]Glycine (51 mCi/mmol) and [1-^{14}C]-D-alanine (46 mCi/mmol) were purchased from ICN Biomedicals. [1-^{14}C]-D-Serine (55 mCi/mmol) was from American Radiolabeled Chemicals, Inc. [^{3}H]Sodium borohydride (469–490 mCi/mmol) and [^{3}H]H_2O (1 mCi/g) were from NEN. [3,3,3-$^2H_3$]-DL-Alanine (99.7%) was from MSD Isotopes. [2-^{3}H]-DL-Serine was prepared by the method of Miles and McPhie (1974). Deuterium oxide (99.9%) was from Cambridge Isotope Laboratories. Catalase was from Boehringer Mannheim; lactate dehydrogenase was obtained from Sigma. D-Amino acid oxidase was purified from porcine kidney by the method of Fitzpatrick and Massey (1982), 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 DAAO was determined using an ϵ_{455} value of 11.3 mM^{-1} cm^{-1} (Massey & Ganther, 1965). All other compounds from commercial sources were of the highest purity available.

[2-^{3}*H*]-DL-*Glycine.* A modification of the procedure of White (1983) was used to synthesize [2-^{3}H]glycine. Sodium glyoxylate (52 mg) was dissolved in 15 mL of concentrated ammonia/water (14.8 M) and heated to 55 °C for 10 min. [^{3}H]Sodium borohydride (0.38 mg, 5 mCi) was dissolved in 0.5 mL of concentrated ammonia/water and added to the glyoxylate solution. The solution was kept in a sealed vessel at 55 °C for 10 min, after which time 23 mg of unlabeled sodium borohydride was added, and the reaction was allowed to proceed for 2 h at 55 °C. The complete reaction mixture was then frozen and lyophilized. The residue was dissolved in 8 mL of distilled water and lyophilized again. This time the residue was dissolved in 13 mL of 0.1 N HCl and loaded onto a 10-mL column of Dowex-50W. The column was washed with 100 mL of distilled water, and the glycine was eluted with a 0–5 M ammonium hydroxide gradient (50 mL). The ninhydrin-positive fractions were pooled and lyophilized. The yield of [2-^{3}H]-DL-glycine was 11 mg (18%), with a specific activity of 6.4 mCi/mmol. The purity was analyzed by HPLC and paper chromatography. A μBondaPak NH_2 cartridge housed in a radial compression module (RCM 8 × 10, Waters) was employed for the HPLC analysis. The mobile phase was acetonitrile/water/25 mM potassium phosphate (pH 4) (500/70/85) at a flow rate of 2 mL/min. The carboxylate of the amino acid was detected at 210 nm. The mobile phase for paper chromatography was butanol/acetic acid/water (12/3/5). Only one radioactive compound was detected by either method, and its chromatographic behavior was identical to that of authentic glycine. NMR spectra of control experiments with unlabeled reactants confirmed that glycine was the only product.

[2-^{3}*H*]-DL-*Alanine.* The general method of White (1983) was adapted for the synthesis of [2-^{3}H]alanine. Sodium pyruvate (96 mg) was dissolved in 10 mL of concentrated ammonia/water and heated at 55 °C for 10 min. After cooling, [^{3}H]sodium borohydride (5 mCi, 0.38 mg) was added. The reaction vessel was sealed and the solution heated to 55 °C for 30 min. Eight milligrams of unlabeled sodium borohydride was then added, and the reaction was allowed to proceed for 1.5 h at 55 °C. The solution was then frozen and lyophilized. The residue was redissolved in 3 mL of distilled water and lyophilized. The final residue was dissolved in 6 mL of 0.33 N HCl, and the solution was loaded onto a 5-mL Dowex-50W column. The column was washed with 200 mL of distilled water, and the amino acid was eluted with a 0–1 M linear gradient of ammonium hydroxide. Ninhydrin-positive fractions were pooled and dried under a stream of dry nitrogen. The residue was dissolved in 1 mL of distilled water and analyzed for purity by HPLC, using the same conditions that were used for the analysis with glycine. The single radioactive peak had the same retention time as authentic alanine. NMR analysis of unlabeled control experiments indicated that alanine was the only product. The yield was 16 mg (22%), with a specific activity of 6.1 mCi/mmol.

Deuterium Isotope Effects. DAAO activity was measured either by following the decrease in oxygen concentration with a YSI Model 5300 biological oxygen monitor or by using a coupled enzymatic assay with lactate dehydrogenase and NADH as described previously (Denu & Fitzpatrick, 1992). Concentrations of both deuterated and nondeuterated substrates were determined using an oxygen electrode end-point assay as described earlier (Denu & Fitzpatrick, 1992). The uncertainties in the substrate concentrations (typically less than 1%) were taken into account in the determination of the uncertainties of the observed isotope effects. Data from the deuterium kinetic isotope effect experiments were fit directly to eq 1 using the programs of Cleland (1979) in the KinetAsyst

software (IntelliKinetics, State College, PA). The $^{D}V/K$ values were calculated by direct comparison of the V/K values.

$$v = VA/(K + A) \quad (1)$$

Tritium Isotope Effects. At pH 10.8, DAAO (34–100 μM) was incubated at 25 °C with 3 mM glycine containing [2-^{3}H]-DL-glycine (19 μCi/mL) and [1-^{14}C]glycine (1.9 μCi/mL) in 150 mM sodium bicarbonate, 20 mM hydroxylamine, and 0.06 mg/mL catalase at 25 °C in a final volume of 0.6–0.7 mL. At pH 6.5, the reaction mixture contained 50 mM sodium pyrophosphate, 10 mM hydroxylamine, DAAO (44–62 μM), 0.3 mM glycine containing [2-^{3}H]-DL-glycine (2.13 μCi/mL) and [1-^{14}C]glycine (0.28 μCi/mL), and catalase (0.06 mg/mL) at 25 °C in a final volume of 0.6–0.7 mL. The initial concentration of oxygen was 0.266 mM. Sodium bicarbonate was chosen as buffer instead of EDTA, as was used previously (Denu & Fitzpatrick, 1992), because of its low affinity for the stationary phase used in the HPLC separations. The pH was monitored over the course of the reaction with pH paper. All reaction mixtures were stirred with the aid of a stir bar and magnetic stirrer.

Control experiments were done without DAAO to correct for any tritium exchange with solvent. Control samples were taken at the same time as the experimental samples, allowing correction for tritium exchange at each fractional conversion. It was found that, at pH 10.8, the exchange was small (0.1–0.9%) but significant, and correction was necessary. However, below pH 8 there was no detectable exchange with solvent, and no correction was made.

At pH 7.5, DAAO (1.8, 8, or 26 μM) was incubated with 195 mM DL-serine containing [1-^{14}C]-D-serine (0.2 μCi/mL) and [2-^{3}H]-DL-serine (1 μCi/mL) in 0.1 M sodium phosphate, 120 mM hydroxylamine, 10 μM FAD, and 0.06 mg/mL catalase at 25 °C in a final volume of 0.6–1.0 mL. The oxygen concentration was kept as high as possible by saturating the air above the reaction solution with humidified 100% oxygen. This was accomplished with the use of a 25-mL three-necked round- bottom flask fit with rubber septa; one neck was used for oxygen entry, one for gas exit, and one for sample removal.

At pH 4, DAAO (22–40 μM) was incubated with 1.8 mM DL-alanine containing [1-^{14}C]-D-alanine (1.25 μCi/mL) and [2-^{3}H]-DL-alanine (10 μCi/mL) in 0.1 M sodium acetate, 10 mM hydroxylamine, and 0.06 mg/mL catalase at 25 °C in a final volume of 0.6–1.0 mL. As with D-serine, the oxygen concentration was kept as high as possible. Additional aliquots of enzyme were added at 2–3-h intervals until no [1-^{14}C]-D-alanine remained.

At various extents of reaction, aliquots (50–200 μL) of the reaction mixtures were removed and passed through an ULTRAFREE-MC filter (Millipore) with a nominal MW cutoff of 10 000 to quench the reaction. The protein-free filtrate (10–50 μL) was then injected for HPLC separation of the substrates and products. HPLC separations were conducted with the same mobile phase and conditions as described above. The oxime products and substrates were detected at 210 nm. Fractions (1–2 mL) were collected, and the amount of ^{3}H and ^{14}C in each fraction was determined with a Beckman LS 5000TA liquid scintillation counter. The counter was programmed to correct for the spillover of ^{3}H into the ^{14}C channel, as well as ^{14}C spillover into the ^{3}H channel. A quench correction curve was established for each radioisotope by adding increasing amounts of the HPLC solvent employed in the separations to a known amount of ^3H_2O or [1-^{14}C]glycine. When D-alanine was used, additional 10 mM hydroxylamine was added after an aliquot of the reaction mixture was filtered. Also, phenethylamine was added to the scintillation vials to ensure reproducibility of the ^{14}C dpm from the pyruvate fraction. All samples were counted for 5 min; counting was repeated five times. Background counts were subtracted before any calculations were made. Recovery of ^{3}H and ^{14}C counts from HPLC separations was always 97% or greater.

The $^{T}V/K$ values were calculated using eqs 2 and 3 by nonlinear least-squares fitting with *NonLin for Macintosh*, a Macintosh version (Brenstein) of a computer code developed by Johnson and Frasier (1985); the confidence probability for the optimized parameters was set at 67%. Tritium isotope effects with glycine were calculated at each fractional conversion using eq 2; the individually calculated isotope effects were then averaged. With D-alanine and D-serine, the data from several experiments were fit directly to eq 3. Here, f is the fractional conversion of substrate to products, R_t is the ratio of ^{3}H in products to ^{14}C in products at time t, and $R_{100\%}$ is the value of $^3H/^{14}C$ in products after complete reaction (Northrop, 1991). Alternatively, R_0 can be used instead of $R_{100\%}$, unless there is a radioactive contaminant in the substrate. Because both the commercial [^{14}C]-D-serine and [^{14}C]-D-alanine contained small amounts of the L isomer, it was necessary to use $R_{100\%}$ values. The fractional conversion f was determined from the ratio of ^{14}C in product to total ^{14}C in product and substrate.

$$^{T}V/K = \frac{\ln(1-f)}{\ln(1-f(R_t/R_{100\%}))} \quad (2)$$

$$\frac{R_{100\%}}{R_t} = \frac{f}{1 - e^{\ln(1-f)/^{T}k}} \quad (3)$$

Solvent Isotope Effects. A buffer consisting of 0.1 M ACES, 0.052 M Tris, and 0.052 M ethanolamine was used in the determination of the $^{D}(V/K_{Ser})$ and V/K_{Ser} values in deuterium oxide. The dry buffer components were added directly to D_2O, and the pD was adjusted with either acetic acid-d_4 or NaOD. The reaction was started by the addition of no more than 10 μL of enzyme (in H_2O) to a final volume of 3 mL. The pD value was determined by adding 0.4 to the pH electrode reading (Schowen & Schowen, 1982). Equation 4 was used in fitting the V/K_{Ser} pD data.

$$\log Y = \log(C/(1 + H/K_1)) \quad (4)$$

RESULTS

Glycine. $^{T}V/K$ values were determined with glycine, D-serine, and D-alanine as substrates for DAAO. Preliminary experiments with glycine demonstrated the need for the addition of hydroxylamine during turnover. This prevented product inhibition by glyoxylate, afforded better resolution in the HPLC separations, and prevented loss of ^{14}C upon repeated scintillation counting. Free [1-^{14}C]glyoxylate in acetonitrile and scintillation fluid exhibited a loss of ^{14}C with time, presumably as a result of the decarboxylation of glyoxylate.

The use of racemic monotritiated glycine allowed the determination of both the primary and secondary tritium kinetic isotope effects in the same experiment. In order to determine the secondary tritium kinetic isotope effect, the $^3H/^{14}C$ value in the product glyoxylate was used, whereas the primary kinetic isotope effect was determined from the ratio of ^{3}H in water to ^{14}C in glyoxylate. For these analyses, it was critical that no ditritiated glycine be present since significant amounts of ditritiated glycine would artificially increase the

Table 1: Agreement among Calculated and Measured $^{D}V/K$ Values for D-Amino Acid Oxidase

substrate	$^{T}V/K$ experimental	$^{D}V/K$ calculated[a]	$^{D}V/K$ experimental
glycine			
pH 10.8	6.41 (5.95–6.87)[b]	3.63 (3.44–3.81)	3.86 (3.45–4.27)
			3.4[c] (3.0–3.8)
pH 6.5	1.80 (1.73–1.87)	1.50 (1.46–1.54)	1.26[d] (1.06–1.46)
D-serine			
pH 7.5	8.57 (8.44–8.69)	4.44 (4.39–4.48)	4.48[d] (4.16–4.80)
D-alanine			
pH 4	12.55 (12.41–12.69)	5.78 (5.73–5.82)	5.08[d] (4.39–5.77)

[a] Values calculated from eq 5. [b] Confidence intervals. [c] The isotope effect on the limiting rate of reduction of pH 10.5 (Denu & Fitzpatrick, 1992). [d] Denu and Fitzpatrick (1992).

apparent secondary isotope effect. The method used for the synthesis of tritiated glycine was chosen because it produces monotritiated and not ditritiated glycine. Furthermore, the value of $^{3}H/^{14}C$ in the products (either $^{3}H_2O$/[^{14}C]glyoxylate or [^{3}H]glyoxylate/[^{14}C]glyoxylate) at 100% conversion was identical to the value of $^{3}H/^{14}C$ in glycine at $t = 0$, after correction for a racemic mixture; i.e., it was determined from complete conversion of three separate experiments that $R_0/2 = R_{100\%}$.

At pH 10.8, the observed primary isotope effects at fractional conversions from 0.05 to 0.37 were corrected to $f = 0$ according to eq 2 to yield an average $^{T}(V/K_{Gly})$ value of 6.41 ± 0.46. Using the Swain–Schaad relationship (Swain et al., 1958) (eq 5), one can calculate the expected $^{D}V/K$ value from the experimental $^{T}V/K$ value, assuming that both forward and reverse commitments are negligible, to obtain a value of 3.63. Under the same experimental conditions, a steady-state $^{D}(V/K_{Gly})$ value of 3.86 ± 0.4 was found. Previous rapid reaction kinetic data gave a value of 3.4 for the primary deuterium isotope effect on the limiting rate of reduction (Denu & Fitzpatrick, 1992). These results are all consistent with a value of 3.6 ± 0.2 for the intrinsic deuterium kinetic isotope effect with glycine as a substrate for DAAO (Table 1).

$$^{T}V/K = (^{D}V/K)^{1.442} \quad (5)$$

At pH 10.8, $^{\alpha\text{-}T}(V/K_{Gly})$ values were measured at fractional conversions ranging from 0.05 to 0.373. The values were corrected to zero fractional conversion using eq 2 to give an average $^{\alpha\text{-}T}(V/K_{Gly})$ value of 1.03 ± 0.02. $^{T}(V/K_{Gly})$ and $^{\alpha\text{-}T}(V/K_{Gly})$ values were also measured at pH 6.5 to obtain average values of 1.80 ± 0.07 and 0.99 ± 0.02, respectively. Using eq 5, the calculated $^{D}(V/K_{Gly})$ is 1.5 at pH 6.5. The observed and calculated isotope effects are summarized in Table 1.

D-Serine. With [2-^{3}H]-DL-serine as substrate for DAAO, $R_{100\%}$ was used to calculate the isotope effect since the commercial [1-^{14}C]-D-serine contained a few percent of the L isomer. However, this was not a problem with the [^{3}H]-serine; upon complete reaction of [2-^{3}H]-DL-serine with DAAO, 50% of the total tritium was found in the product water and 50% remained as the L isomer. At pH 7.5, the $^{T}(V/K_{Ser})$ values were measured over a wide range (0.07–0.73) of fractional conversions (Figure 1). The complete set of observed $^{T}(V/K_{Ser})$ values was fit to eq 3, yielding a value of 8.57 with a range of 8.44–8.69. The calculated $^{D}(V/K_{Ser})$ value (eq 5) was 4.44 (Table 1). Previous steady-state data gave an average pH-independent value for $^{D}(V/K_{Ser})$ of 4.47

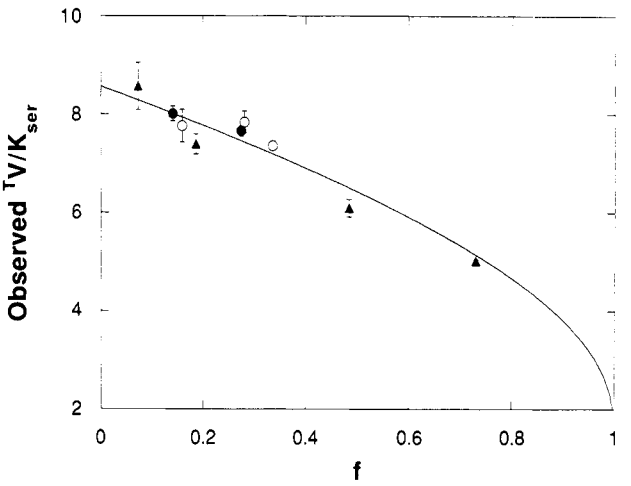


FIGURE 1: Dependence of the observed $^{T}(V/K_{Ser})$ values on fractional conversion. Conditions: 195 mM D,L-serine, 120 mM hydroxylamine, 10 μM FAD, 60 μg/mL catalase, 0.1 M sodium phosphate, pH 7.5, 25 °C. The concentrations of DAAO were (▲) 1.8 μM, (○), 8 μM, and (●) 26 μM. The line is a fit of the data to eq 3. Error bars indicate the standard deviations of five separate countings.

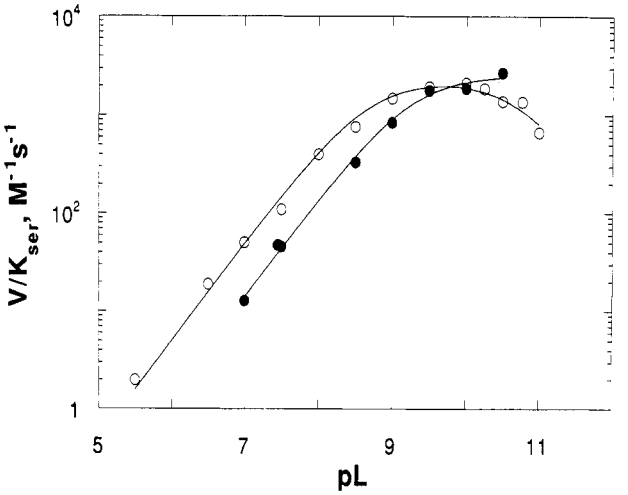


FIGURE 2: pL dependence of the V/K_{Ser} value. Conditions: 0.1 M ACES, 0.052 M Tris, 0.052 M ethanolamine, 0.266 mM oxygen, 25 °C. (●) Values determined in D_2O; (○) values in H_2O from Denu and Fitzpatrick (1992). The line is a fit of the data in D_2O to eq 4.

(Denu & Fitzpatrick, 1992), which is consistent with 4.4–4.5 as the value of the intrinsic deuterium kinetic isotope effect with D-serine.

To establish whether there are any slow proton transfers from exchangeable sites on DAAO during the rate-limiting step of reduction, the solvent isotope effect on the V/K_{Ser} value was determined. V/K_{Ser} values were measured in D_2O over a wide range of pH values, and the pH profile was compared with the previously observed profile in H_2O (Denu & Fitzpatrick, 1992). Each data set was corrected for protonation of the substrate amino group (pK_a = 9.2 in H_2O and 9.7 in D_2O) since only the zwitterionic form of the amino acid will react with DAAO (Purdy, 1982). The V/K_{Ser} data in D_2O were fit to eq 4, which describes a decrease in rate with decreasing pH, to yield a pK_a value of 9.3 ± 0.1 and a pH-independent value of 2570 ± 400 M^{-1} s^{-1} (Figure 2). Previous results in H_2O indicated that a group with a pK_a value of 8.7 must be unprotonated and a group with a pK_a value of 10.7 must be protonated for activity. The difficulty in measuring enzymatic rates at high pH precluded defining the second pK_a value reliably in D_2O; it has a predicted value of 11.3. The pH-independent V/K_{Ser} value in H_2O is 2370 ± 500; thus, the

calculated solvent isotope effect is 0.92 ± 0.27.

The $^{D}(V/K_{Ser})$ value was also measured in 100% D_2O at pD 7.5 to obtain a value of 4.55 ± 0.23. This agrees well with the value of 4.48 for $^{D}(V/K_{Ser})$ in H_2O and the value of 4.44 calculated from the $^{T}(V/K_{Ser})$ value.

D-Alanine. With [2-^{3}H]-DL-alanine as substrate, the $R_{100\%}$ value was used to calculate the isotope effect since the commercial [1-^{14}C]-D-alanine also contained a few percent of the L isomer. In contrast, upon complete reaction of [2-^{3}H]-DL-alanine with DAAO, 50% of the total tritium was found in the product water and 50% remained as the L isomer.

The observed $^{D}(V/K_{Ala})$ value changes with pH, and only when the pH drops to 4 or below does the $^{D}(V/K_{Ala})$ value reach its apparent limiting value of 5.5 (Denu & Fitzpatrick, 1992). Therefore, values of $^{T}(V/K_{Ala})$ were measured at pH 4. The observed isotope effects at fractional conversions from 0.05 to 0.47 were fit to eq 3, yielding a value of 12.55 ± 0.14 for $^{T}(V/K_{Ala})$. Using eq 5, the predicted value for $^{D}(V/K_{Ala})$ is 5.78 ± 0.05, which is not significantly different from the experimental value of 5.08 ± 0.69 observed at pH 4.

The $^{\beta\text{-}D3}(V/K_{Ala})$ value was measured at pH 5 and 4.5, where the $^{D}(V/K_{Ala})$ values are 4.0 and 5.0, respectively (Denu & Fitzpatrick, 1992). Initial rates with DL-alanine and [3,3,3-$^2H_3$]-DL-alanine were compared at concentrations far below the K_m values. The value at pH 4.5 was determined by directly comparing the initial rates at 10, 25, and 50 mM D-alanine. At pH 5 the initial rates over a wider range of concentrations were fit directly to eq 1, and the resulting V/K_{Ala} values were compared. Values for $^{\beta\text{-}D3}(V/K_{Ala})$ of 0.99 ± 0.02 at pH 5 and 1.03 ± 0.04 at pH 4.5 were found. In neither case is the value significantly different from 1.

DISCUSSION

A variety of experimental approaches have been employed to probe the mechanism of carbon–hydrogen bond cleavage in flavoprotein oxidases. Almost without exception, these have utilized substrates with unusual reactivities or nonphysiological flavin analogues. Support for a carbanion intermediate comes from the catalysis by DAAO of chloride elimination from β-chloroalanine and β-chloroaminobutyrate (Walsh et al., 1971, 1973) and from the inactivation of some flavoprotein oxidases by vinylic or acetylenic substrates (Marcotte & Walsh, 1976). Support for a direct hydride-transfer mechanism comes from the observation that the substrate α-hydrogen is transferred directly to the flavin in 5-deaza-FAD-containing DAAO (Hersh & Jorns, 1975). The result obtained with the latter has been rationalized as a result of the very different reactivity of 5-deazaflavins, resulting in a new mechanistic pathway becoming available (Hemmerich et al., 1977). However, the possibility of alternative pathways must be considered for artificial substrates as well as for artificial cofactors. A major advantage in the use of kinetic isotope effects to probe enzyme mechanisms is that the perturbation in substrate structure is so minor that no change in the catalytic mechanism is expected.

The mechanism of Scheme 1 differs from those of Schemes 2 and 3 in that CH bond cleavage and rehybridization of the α-carbon are stepwise in the former and concerted in the latter. In all three cases, cleavage of the CH bond is expected to exhibit a primary kinetic isotope effect and bond rehybridization a secondary isotope effect. The approach we have taken is to establish conditions under which the intrinsic primary kinetic isotope effect for CH bond cleavage is fully expressed and to measure secondary isotope effects under these conditions. The mechanism of Scheme 1 predicts that no significant secondary effects will be observed, while the mechanisms of Schemes 2 and 3 predict that significant secondary effects will be seen. In addition, because a proton attached to the substrate amino group is in flight in the transition state for CH bond cleavage in Scheme 3, a solvent isotope effect should also be found if this mechanism is correct.

Because enzymatic reactions usually involve multiple steps, catalysis may not be the slowest step, rendering the observed kinetic isotope effect closer to unity. The equation for the deuterium isotope effect on the V/K value is shown in eq 6 (Northrop, 1991). ^{D}k is the intrinsic kinetic isotope effect on

$$^{D}V/K = \frac{^{D}k + c_f + c_r\,^{D}K_{eq}}{1 + c_f + c_r} \qquad (6)$$

catalysis, and $^{D}K_{eq}$ is the isotope effect on the equilibrium constant. The terms c_f and c_r are commitments to catalysis and will lower the observed isotope effect. The forward commitment c_f is the ratio of the net rate constant for the isotope-sensitive step to the net rate constant for dissociation of the substrate, and the reverse commitment c_r is the ratio of the net rate constant for the isotope-sensitive step in the reverse reaction to the net rate constant for release of product. Effectively, when the isotope-sensitive step is fully rate-limiting, the commitments are zero and the observed effect becomes the intrinsic effect. Therefore, it is important to establish conditions where the commitments are minimized. This can often be done by using slow substrates or by operating off the pH optimum (Cook & Cleland, 1981). In previous studies, we have utilized pH extremes and slow substrates to eliminate external forward commitments and to obtain large primary isotope effects with glycine, D-serine, and D-alanine as substrates for DAAO (Denu & Fitzpatrick, 1992). However, the possibility of significant internal commitments still remained. To determine whether the previously reported isotope effects were indeed the intrinsic values, tritium isotope effects have now been determined. Comparison of the deuterium isotope effects calculated from the latter using the Swain–Schaad relationship with the experimental values was then used to determine whether the intrinsic values are indeed being measured. These conditions could then be used in the measurement of secondary and solvent isotope effects.

D-Serine. With D-serine, the pH-independent primary deuterium isotope effect of 4.47 and the value of 4.44 calculated from the $^{T}(V/K_{Ser})$ value agree quite well. This close agreement establishes that the value of 4.4–4.5 is the intrinsic deuterium kinetic isotope effect with this substrate and therefore reflects the true structure of the transition state. The classical limit is about 7 for a symmetrical transition state, suggesting that the transition state in this case is not symmetrical, but is more like either the product or the reactant.

At pH 9, the $^{D}(V/K_{Ser})$ value is sensitive to the concentration of DAAO (Denu & Fitzpatrick, 1992). This has been attributed to a decrease in the rate of substrate dissociation at enzyme concentrations that promote the formation of oligomers, resulting in a small forward commitment and a decrease in the observed primary isotope effect. At pH 7.5, this forward commitment is abolished, and the value of 4.5 is observed at enzyme concentrations as high as 30 μM. It was therefore important to demonstrate that the $^{T}(V/K_{Ser})$ values reported here were not dependent on DAAO concentration. The $^{T}(V/K_{Ser})$ value was measured at pH 7.5 at three different DAAO concentrations with no significant difference in the resulting isotope effect.

The solvent isotope effect on the V/K_{Ser} value was then measured. A pD profile was constructed for the V/K_{Ser} value

by measuring this value in deuterium oxide over a wide range of pD values. The calculated solvent isotope effect is not significantly different from 1, and the shift in pK_a is 0.6 for the group that must be unprotonated for activity. A typical shift in the pK_a value is about 0.5 for "well-behaved" acids (Schowen & Schowen, 1982). Showen (1977) has stated that "normal" behavior of the pH profile indicates that structural changes are most likely not being induced by deuteration, nor are the pK_a values a result of mechanistic features other than ionization, such as changes in the rate-determining step. The results presented here are consistent with those expected for normal behavior.

The lack of a solvent effect on V/K_{Ser} where the primary deuterium isotope effect is intrinsic at 4.5 is consistent with the proposed carbanion mechanism for reduction (Scheme 1). There is no requirement for the transfer of exchangeable protons during the transition state of CH bond cleavage in this mechanism. Miura and Miyake (1988) have proposed that CH bond cleavage is concerted with amino proton abstraction by flavin (Scheme 3). In such a mechanism, a significant solvent isotope effect would be expected since the substrate amino protons would be exchanged for deuterons in D_2O.[2] Similarly, if direct hydride transfer of the α-hydrogen were involved, as in Scheme 2, a solvent effect could be observed on the same step as CH cleavage.

D-*Alanine*. The value of 12.55 for the $^{T}(V/K_{Ala})$ value was determined at a pH value of 4. The previously reported value of 5.08 ± 0.69 was measured under similar conditions. Using the Swain–Schaad relationship, the calculated $^{D}(V/K_{Ala})$ value is 5.78 ± 0.05 which is in reasonably good agreement, suggesting that 5.7 is the intrinsic deuterium isotope effect and that all commitments are negligible under these conditions.

Few β-secondary kinetic isotope effects have been measured for enzymatic reactions, and to our knowledge, none have been reported for reactions catalyzed by flavoprotein oxidases. β-Secondary isotope effects result from a change in hyperconjugation on nonbonded atoms, which can occur in the transition state (Cleland, 1987). Delocalization of the negative charge of the carbanion to the β-hydrogen atoms is less likely than delocalization to the protonated nitrogen, and thus no observed effect is expected for a carbanion. The $^{\beta\text{-}D3}(V/K_{Ala})$ values of 1.03 ± 0.04 and 0.99 ± 0.01 were measured at pH values of 4.5 and 5.0, respectively. The primary deuterium isotope effect values are 4.97 at pH 4.5 and 4.04 at pH 5 (Denu & Fitzpatrick, 1992). For comparison, Cook et al. (1981) determined a β-secondary effect of 1.21 for the reaction of lactate-d_3 with lactate dehydrogenase, which uses a hydride-transfer mechanism. We can compare the observed secondary isotope effects determined here with the expected values calculated from the previously determined forward commitments at these pH values. Setting 1.21 as the limiting effect, we would expect effects of 1.17 at pH 4.5 and 1.15 at pH 5. Thus, there is no significant β-secondary isotope effect under conditions that show a large primary deuterium isotope effect. Although the lack of observed β-effects does not prove a carbanion mechanism, it is certainly consistent with that mechanism and potentially offers an alternative method that can be applied in the study of similar enzymatic reactions.

Glycine. The $^{D}(V/K_{Gly})$ value shows pH behavior opposite from that of the $^{D}(V/K_{Ala})$ value: i.e., increasing with increasing pH until a limiting $^{D}(V/K_{Gly})$ value of 3.4 ± 0.3 is reached at high pH. This behavior results from a pH-dependent reverse commitment. The limiting rate of reduction measured in rapid reaction experiments shows a similar primary deuterium isotope effect of 3.4 ± 0.4 (Denu & Fitzpatrick, 1992). The $^{T}(V/K_{Gly})$ values reported here are 6.41 at pH 10.8 and 1.8 at pH 6.5. The value of 6.41 allows one to calculate a $^{D}(V/K_{Gly})$ value of 3.63 from the Swain–Schaad relationship; this agrees reasonably well with the directly measured values of 3.86 and 3.4. Thus, the value of 3.6 is the intrinsic value and reflects a transition-state structure that is less symmetrical than the transition states with D-serine and D-alanine.[3]

At pH 6.5, the $^{T}(V/K_{Gly})$ value of 1.8 allows one to calculate a $^{D}(V/K_{Gly})$ value of 1.50 ± 0.04. The previously reported $^{D}(V/K_{Gly})$ value at pH 6.5 is 1.26 ± 0.20. Our steady-state kinetic model with glycine proposes that at pH 6.5 CH bond cleavage is reversible, and the equilibrium effect is observed. Glycine is not a sticky substrate, so that the observed isotope effect will contain contributions only from the intrinsic kinetic isotope effect and the equilibrium isotope effect (eq 7). The pH-dependent reverse commitment at this pH is approximately 22. If the α-hydrogen from the amino acid is transferred to a hydroxyl residue, the $^{D}K_{eq}$ value can be calculated to be 1.13; if transfer is to an uncharged nitrogen, the $^{D}K_{eq}$ value is 1.17 (Cleland, 1980). Using a value of 1.46 for $^{D}(V/K_{Gly})$, the calculated $^{D}K_{eq}$ value (eq 7) is 1.23. This value is consistent with the value of 1.17 for transfer to an uncharged nitrogen, although the uncertainty is large.

$$^{D}V/K = \frac{^{D}k + c_r\,^{D}K_{eq}}{1 + c_r} \qquad (7)$$

The $^{\alpha\text{-}T}(V/K_{Gly})$ isotope effect values of 1.03 ± 0.02 at pH 10.8 and 0.99 ± 0.02 at pH 6.5 are indistinguishable from unity. Thus, no significant α-secondary isotope effect was observed in the same experiment, which yielded an intrinsic $^{T}(V/K_{Gly})$ value of 6.41. This experiment indicates that the transition state for CH bond breakage involves little or no bond rehybridization at the α-carbon and does not support a concerted mechanism for reduction. The calculated secondary tritium isotope effect for a hydride mechanism is 1.50 (Cleland, 1980). On the basis of this value, the upper limit on the amount of bond rehybridization in the transition state for CH cleavage with glycine is 8.4%.[4]

[2] A stepwise mechanism similar to that of Scheme 3 has been suggested by a reviewer. Here, carbanion formation would be followed by hydride transfer from the amino group to the flavin in a stepwise fashion. Such a mechanism would be consistent with the data presented here, in that no solvent isotope effect would be seen. The difference from the mechanism of Scheme 1 lies in the mode of transfer of electrons to the flavin. This aspect of the mechanism of this group of enzymes is much less understood than the mechanism of CH bond cleavage. The results of Ghisla and Massey (1980) with lactate oxidase provide the strongest evidence for formation of the adduct proposed in Scheme 1.

[3] The limiting rate of reduction with these substrates can be compared with the magnitudes of the intrinsic isotope effects. The values at pH 8.5 for D-alanine, D-serine, and glycine are 700–1000, 560, and 28 s^{-1}, respectively. It is tempting to conclude from these data that there is a late transition state for CH bond cleavage, so that the transition state becomes less symmetrical as it becomes slower, resulting in a decrease in the isotope effect. However, in the case of formate dehydrogenase, where there has been a detailed analysis of transition-state structure as a function of the redox potential of the pyridine nucleotide, the effect on the rates is opposite that predicted from isotope effects (Hermes et al., 1984). Clearly, further studies are required to determine whether the transition state for CH bond cleavage by DAAO is truly late.

[4] Carbanions of hydroxy or amino acids are frequently assumed to be stabilized by formation of the *aci*-acid. This necessarily involves some rehybridization of the α-carbon to sp^2. The small secondary effect observed with glycine may indeed be due to such an effect, but the level of precision does not allow definitive conclusions to be drawn.

Conclusions. The values of the intrinsic primary deuterium isotope effects are 3.6 with glycine, 4.5 with D-serine, and 5.7 with D-alanine as substrates for DAAO. The α-secondary, β-secondary, and solvent isotope effects with glycine, D-alanine, and D-serine, respectively, are not significantly different from unity under the conditions that yielded the intrinsic primary effects; these results are not consistent with a concerted mechanism for CH bond cleavage and flavin reduction.

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