Kinetic Isotope Effects as a Probe of the β-Elimination Reaction Catalyzed by *O*-Acetylserine Sulfhydrylase†

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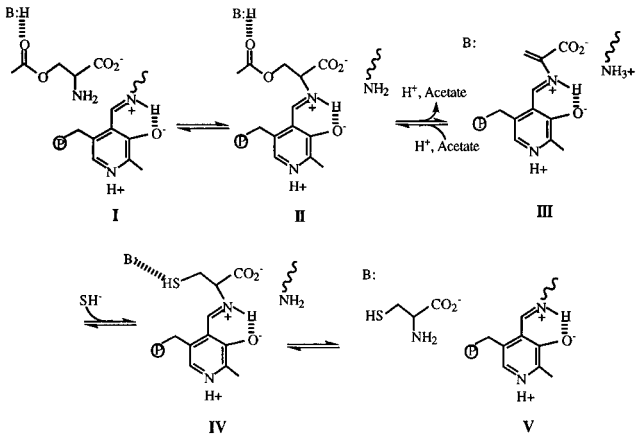
ABSTRACT: Primary and α-secondary deuterium kinetic isotope effects have been measured for the *O*-acetylserine sulfhydrylase from *Salmonella typhimurium* using both steady-state and single-wavelength stopped-flow studies. Data suggest an asymmetric transition state for α-proton abstraction by the active site lysine and the elimination of the acetyl group of *O*-acetyl-L-serine (OAS) to form the α-aminoacrylate intermediate. The value of $^{D}(V/K_{OAS})$ using OAS-2-*d* is dependent on pH from 5.8 to 7.0 with independent values of 2.8 and 1.7 estimated at low and high pH, respectively. Thus, OAS is sticky, and a value of 1.5 is calculated for the forward commitment to catalysis, indicating that the OAS external Schiff base preferentially partitions toward the α-aminoacrylate intermediate compared to OAS being released from enzyme. The intrinsic primary deuterium isotope effect determined from single-wavelength stopped-flow studies of α-proton abstraction by the active site lysine is about 2.0. $^{D}(V/K_{OAS})$ and $^{T}(V/K_{OAS})$ were determined as 2.6 ± 0.1 and 4.2 ± 0.2 at pH 6.1, respectively, giving a calculated intrinsic deuterium isotope effect of 3.3 ± 0.9, consistent with the $^{D}(V/K_{OAS})$ obtained from steady-state studies at low pH. The α-secondary deuterium kinetic isotope effect using OAS-3,3-d_2 is 1.11 ± 0.06 obtained by direct comparison of initial velocities and 1.2 obtained by single-wavelength stopped-flow experiments. Data can be compared to a value of 1.81 ± 0.04 using OAS-3,3-d_2 for $^{\alpha\text{-}D}K_{eq}$ for the first half-reaction.

The biosynthesis of L-cysteine in the enteric bacterium *Salmonella typhimurium* is carried out by serine transacetylase (EC 2.3.1.30), the enzyme responsible for acetylating the β-hydroxyl of L-serine using acetyl-Co A as the donor to form *O*-acetyl-L-serine, and the PLP-dependent *O*-acetylserine sulfhydrylase (OASS,[1] EC 4.2.99.8), the enzyme which catalyzes the β-replacement of the acetate in OAS by sulfide to give the product, L-cysteine. OASS-A is one of two OASS isozymes, composed of two identical subunits with a molecular weight of 34450 (Byrne et al., 1988), and each subunit has one tightly bound PLP (Becker et al., 1969; Nakamura et al., 1984).

The reaction catalyzed by OASS-A has been shown by initial rate studies and isotope exchange to adhere to a ping-pong kinetic mechanism (Cook & Wedding, 1976; Tai et al., 1993). In the first half-reaction, OASS catalyzes the α,β-elimination of the elements of acetic acid from OAS to form α-aminoacrylate in Schiff base with the active site PLP. In the second half-reaction, Michael addition of sulfide to the α-aminoacrylate intermediate results in the formation of the product L-cysteine. Two intermediates in the first half-reaction, the OAS external Schiff base and α-aminoacrylate Schiff base, have been identified by rapid-scanning stopped-flow and UV−visible spectroscopic studies (Woehl et al., 1996; Schnackerz et al., 1995). Addition of OAS to OASS results in a rapid shift in the λ_{max} from 412 nm (reflecting the internal Schiff base) to 418 nm (reflecting the OAS external Schiff base) with the rate constant around 800 s^{-1}, and this is followed by the appearance of absorbance at 470 and 320 nm reflecting the α-aminoacrylate intermediate (Woehl et al., 1996). In addition, the cysteine external Schiff base has been identified in the second half reaction using UV−visible spectroscopy (Schnackerz et al., 1995).

Scheme 1



A general mechanism (Scheme 1) has been proposed by Tai et al. (1995) based on initial velocity studies as a function

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[1] Abbreviations: OASS, *O*-acetylserine sulfhydrylase; Mes, 2-(*N*-morpholino)ethanesulfonic acid; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; PLP, pyridoxal 5′-phosphate; OAS, *O*-acetyl-L-serine; DTT, dithiothreitol; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); TNB, 5-mercapto-2-nitrobenzoic acid; S-CNP-cysteine, *S*-(3-carboxy-4-nitrophenyl)-L-cysteine; CD, circular dichroism.

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of pH. In Scheme 1, OAS binds with its α-amine unprotonated, and an enzyme group forms a hydrogen bond to the acetyl group of OAS. The amino group of OAS attacks C4′ of the protonated internal Schiff base (I) to form the external Schiff base (II) via a *gem*-diamine intermediate. *β*-Elimination of the acetyl group with the α-proton abstraction by the lysine at the active site forms the α-aminoacrylate intermediate (III) at the end of the first half-reaction. The first half-reaction is then reversed upon nucleophilic addition of sulfide to the α-aminoacrylate intermediate to form cysteine external Schiff base (IV), followed by release of the product L-cysteine to regenerate the internal Schiff base (V).

Recently, Woehl et al. (1996) have shown by rapid scanning stopped-flow and single-wavelength stopped-flow studies that the first half-reaction limits the overall reaction with the slow step being α-proton abstraction and *β*-elimination of acetate to form the α-aminoacrylate intermediate. However, nothing is known concerning the nature of the transition state in the α,*β*-elimination reaction catalyzed by *O*-acetylserine sulfhydrylase. Kinetic isotope effects have been useful as a probe of enzyme mechanism and transition state geometry (Cook, 1991). In order to determine the nature of the transition state for α-proton abstraction and the *β*-elimination of acetate, primary deuterium and tritium and secondary deuterium kinetic isotope effects have been measured by competitive (tritium) and noncompetitive (deuterium) methods to probe the *β*-elimination reaction catalyzed by *O*-acetylserine sulfhydrylase.

MATERIALS AND METHODS

Chemicals. L-Serine, D-serine (98%), sodium sulfide, and OAS were from Sigma, KOD (99 atom % D) and HCl gas were from Aldrich, and D_2O (99.8 atom % D) and *O*-acetyl-L-serine-2,3,3-d_3 (>98 atom % D) were from Cambridge Isotope Lab. Tritiated H_2O (100 mCi/mL) and [1-^{14}C]acetic anhydride (10 mCi/mmol) were from American Radiolabeled Chemicals, Inc. Scintillation cocktail (Ecolite) was from INC Biomedicals, Inc. The TNB was freshly prepared prior to use by reduction of DTNB with DTT in slight molar excess. The buffers, Mes and Hepes, were from Research Organic, Inc. All other reagents and chemicals were obtained from commerically available sources and were of the highest quality available.

Enzymes. *O*-Acetylserine sulfhydrylase-A from *S. typhimurium* LT-2 was purified according to the procedure of Hara et al. (1990) and modified by Tai et al. (1993). The concentration of enzyme was determined by the absorbance of the internal Schiff base using an extinction coefficient 7600 of M^{-1} cm^{-1} at 412 nm (Kredich et al., 1969).

L-Serine-2-d. L-Serine and OASS-A were lyophilized against D_2O to remove trace H_2O. To a solution of L-serine (2.06 g, 19.5 mmol) in D_2O (40 mL), OASS-A (5.3 mg) was added to a solution adjusted to pD 9.0 to catalyze the deuterium exchange. The progress of deuterium incorporation at the C-2 position in L-serine was monitored via the disappearance of the signal at 3.80 ppm using a 300 MHz ^{1}H NMR (Varian XL-300). The reaction was almost complete after 5 h with about 2.2% of the α-proton resonance remaining. The reaction was allowed to stand overnight and was stopped by the removal of the enzyme by Amicon ultrafiltration through a PM10 membane. The solution was adjusted to pH 6 with 3 N HCl, and ethanol (90 mL) was gradually added to the solution to crystallize the L-serine-2-*d*, which was obtained as a white crystalline solid (95.4%, 1.97 g, 18.6 mmol). The final deuterium incorportation was measured by ^{1}H NMR and gave about 3% of the α-proton resonance remaining. ^{1}H NMR (300 MHz, HDO 4.80 ppm) shows *β*-protons: δ 3.91 (d, J = 12.3 Hz, 1H), 3.96 (d, J = 12.3 Hz, 1H). ^{13}C NMR (75 MHz) δ 175.0, 62.8, 58.8 (t, J = 22.6 Hz). CD, $[\theta]_{204}$ = 2329 deg cm^2 $dmol^{-1}$.

L-Serine-3,3-d$_2$. The synthesis of L-serine-3,3-d_2 is similar to the procedure for the synthesis of L-serine-2-*d*. To a solution of L-serine-2,3,3-d_3 (280 mg, 2.6 mmol) in H_2O (40 mL), the enzyme OASS-A (5.3 mg) was added at pH 8.4 to catalyze the protium exchange at the α-position. The reaction was then treated as for L-serine-2-*d* and the L-serine-3,3-d_2 was crystallized with ethanol, again giving white crystals. ^{1}H NMR (300 MHz, HDO 4.80 ppm) shows only the α-proton: δ 3.80 (s). ^{13}C NMR (75 MHz) δ 175.0, 58.9 (t, J = 22.6 Hz). CD, $[\theta]_{204}$ = 2026 deg cm^2 $dmol^{-1}$.

Deuteriated O-Acetylserine. The general procedure for acetylation of L-serine was according to Sheehan et al. (1956). Dry hydrogen chloride gas was bubbled through a suspension of L-serine-2-*d* (1.26 g, 12 mmol) in 15 mL of glacial acetic acid at room temperature until the suspension was saturated. The suspension was stirred overnight, and acetylation was then stopped by removing the solvent and HCl *in vacuo*. The white powder obtained gives two spots on TLC ($CHCl_3$/EtOH/AcOH/H_2O, 25:26:10:5) with a small amount of serine remaining. The acetylation was repeated to complete the reaction. *O*-Acetyl-L-serine-2-*d* (96%, 2.11 g, 11.5 mmol) was obtained as white power. ^{1}H NMR (300 MHz, HDO 4.80 ppm), δ 4.57 (d, J = 12.5 Hz, 1H), 4.51 (d, J = 12.5 Hz, 1H), 2.0 (s, 3 H). ^{13}C NMR (75 MHz) δ 175.9, 172.3, 64.7, 59.9 (t, J = 21 Hz). *O*-Acetyl-L-serine-3,3-d_2 (98%) and OAS-2,3,3-d_3 (88%) were obtained by the same synthetic procedure. ^{1}H NMR (300 MHz, HDO 4.80 ppm) for OAS-3,3-d_2: δ 4.35 (s, 1 H), 2.10 (s, 3H). ^{13}C NMR (75 MHz) δ 175.9, 172.2, 64.2 (p), 54.9, 22.7. ^{1}H NMR (300 MHz, HDO 4.80 ppm) for OAS-2,3,3-d_3: δ 2.1 (s). ^{13}C NMR (75 MHz) δ176.0, 172.3, 64.2 (p, J = 23 Hz), 54.8 (t, J = 21 Hz).

[2-^{3}H,5-^{14}C]O-Acetyl-L-serine. [2-^{3}H]L-Serine (503 mg, 4.8 mmol, 1.4 μCi/mmol) was synthesized by tritium exchange of the α-protium of unlabeled L-serine (631 mg, 6 mmol) with OASS-A (2.9 mg) in ^3H_2O (10 mL, 2 mCi) at pH 8.2. Acetylation of the obtained [2-^{3}H]L-serine was carried out with [1-^{14}C]acetic anhydride (5.3 mmol, 18.9 μCi/mol) in glacial acetic acid saturated with HCl gas (9.5 mL) to give [2-^{3}H,5-^{14}C]*O*-acetyl-L-serine. Acetylation was then repeated to complete the reaction. The specific activity of ^{3}H and ^{14}C in labeled OAS is 1.4 and 1.3 μCi/mmol, respectively.

Nomenclature. Nomenclature for kinetic isotope effects is according to Northrop (1977) and Cook and Cleland (1981). The isotope is indicated by a leading superscript and the following subscript indicates the varied substrate. $^D(V/K_{OAS})$ is the ratio of kinetic parameter (V/K_{OAS}) for the unlabeled and deuteriated substrate. ^DV is the ratio of maxium rate for unlabeled and deuteriated substrate. A "sticky" substrate is one that reacts to give product as fast or faster than it dissociates from the enzyme complex. C_f and C_r are commitments to catalysis for forward and reverse reaction and represent the ratio of the rate constant for the

isotope-sensitive step to the net rate constant for reactant release from the complex undergoing the isotope-sensitive step.

Kinetic Studies. The OASS-A catalyzed reaction was monitored by the disappearance of sulfide ion by using a computer-assisted sulfide ion electrode assay (Hara et al., 1990). All kinetic assays were carried out at 25 °C, and the pH of the assay solution was controlled with 100 mM of the following buffers: Mes, pH 5.7−6.8; Hepes, pH 7.0−7.5. Initial rates were measured at varied concentrations of unlabeled or deuteriated OAS and fixed sulfide concentration (25 μM) at the indicated pH. The enzyme was added last to initiate the reaction. The concentration of unlabeled and deuteriated OAS were determined by converting it with excess OASS-A into the α-aminoacrylate intermediate which has an ϵ_{470} of 9760 M^{-1} cm^{-1} (Cook et al., 1992). Sulfide concentration was determined by reduction of DTNB to molecules of TNB and using an effective extinction coefficient of 27 200 M^{-1} cm^{-1}, since the stoichiometry is 2 TNB/sulfide (Ellman, 1958).

Single-Wavelength Stopped-Flow. Single-wavelength stopped-flow studies were performed according to the method of Woehl et al. (1996) using the light source and monochromator and photomultiplier system from a Durrum D-110 stopped-flow device with the sample handling unit replaced by a unit from Applied Photophysics. A single wavelength time course for the formation of the α-aminoacrylate intermediate, monitoring the appearance of absorbance at 470 nm were single-exponential and were fitted using eq 1 to

$$A_t = A_\infty \pm A \exp(-t/\tau) \quad (1)$$

$$1/\tau = k_5 K_{ext}[\mathrm{OAS}]/(1 + K_{ext}[\mathrm{OAS}]) \quad (2)$$

obtain the relaxation time τ, where A_t and A_∞ are the absorbance value at time t and infinity, respectively, and A is the amplitude of exponential process. Since α-proton abstraction is slow compared to the formation of external Schiff base, a function of $1/\tau$ vs the concentration of OAS was fitted using eq 2 to obtain the rate constant (k_5) for the α-proton abstraction and the association constant K_{ext} for formation of the external Schiff base.

Secondary Equilibrium Isotope Effect. Equilibrium constants for the OASS-A catalyzed β-elimination of OAS to form the α-aminoacrylate intermediate in the first half-reaction were determined at pH 6.1 and 25 °C from the known concentrations of OAS (or OAS-3,3-d_2; S), acetate (OAc), OASS (E), and the amount of α-aminoacrylate intermediate (F) formed at equilibrium and fitted using eq 3. The reaction mixture contained 29.52 μM OASS-A, 32.70

$$K_{eq} = [\mathrm{F}][\mathrm{OAc}]/[\mathrm{S}][\mathrm{E}] \quad (3)$$

μM OAS (or 33.45 μM OAS-3,3-d_2), and 22.5 mM acetate in the 100 mM Mes, pH 6.1. The extinction coefficient (ϵ_{470}) for unlabeled and deuteriated α-aminoacrylate intermediates was determined using OAS or OAS-3,3-d_2 in excess of OASS-A, so that all of the enzyme was present as the α-aminoacrylate intermediate. The measured ϵ_{470} values were then used to calculate the concentration of substrates (OAS or OA-3,3-d_2) by converting all of it to the the α-aminoacrylate intermediate in the presence of excess of enzyme. The equilibrium isotope effect was determined as the ratio of the equilibrium constants for unlabeled OAS and deuteriated OAS.

Deuterium Wash-in. Deuterium wash-in was studied for the OAS/TNB substrate pair at pD 6.90 and for the OAS/sulfide substrate pair at pD 6.64 with 0.1 M phosphate buffer in 0.5 mL of D_2O. The pD was adjusted by 40% KOD. The final concentrations of OAS, TNB, and sulfide were 20, 20, and 10 mM, respectively. *O*-Acetyl-L-serine shows an AA′X pattern in the proton NMR with a triplet for C-2 and a doublet for C-3. Deuterium incorporation was measured by the integrated ratio of proton resonances at C-3 and C-2.

Tritium Kinetic Isotope Effect. The tritium kinetic isotope effect on the OASS-A-catalyzed reaction was obtained at room temperature in a volume of 1 mL containing 10 mM [2-^{3}H,5-^{14}C]OAS and 15 mM sulfide in 100 mM Mes, pH 6.1. The reaction was initiated by adding OASS-A (7.3 μg) and quenched at the proper time with 2 N HCl (200 μL). The enzyme was removed by passing the solution through a 0.2 μm filter. An aliquout of 1 mL was applied to 1.5 g of Dowex 50Wx8 in the H^+ form in an Econo column. The products (^3H_2O and [1-^{14}C]acetic acid) were eluted with 3 mL of H_2O, followed by washing with 2 mL of H_2O and 2 mL of 2 N pyridine. The unreacted OAS was finally eluted with 5 mL of 2 N pyridine. A 1 mL aliquot of the product solution was transferred to 10 mL of scintillation fluid and counted using a Beckman LS 6000IC scintillation counter corrected for channel ratios and quenching effects due to solvents. Radioactivities of *N*-acetylserine and ^3H_2O resulting from the hydrolysis of OAS were substracted based on control experiments. Control experiments were performed in triplicate using the same conditions as above in the absence of OASS-A. Complete conversion of OAS to products was done in triplicate with 10 times the enzyme (73 μg) used in the low conversion samples. However, about 3% of the total radioactivity eluted with 2 N pyridine, and increasing the amount of enzyme or reaction time did not eliminate the remaining 3%. For the determination of channel ratio and quenching effects, unlabeled OAS was employed under the same condition as in the control experiments. An external standard of [1-^{14}C]acetic acid was then added to the appropriate fraction from the chromatographic separation and channel ratio and quenching effects determined for the ^{14}C-labeled compound. The experiment was then repeated using ^3H_2O as the external standard when determining the channel ratio and quenching effect for tritium.

Data Processing. Reciprocal initial velocities were plotted as a function of reciprocal substrate concentrations. Data were fitted using the appropriate rate equations and the Fortran programs of Cleland (1979). Data for substrate saturation curves at a fixed concentration of the second substrate were fitted using eq 4. Kinetic isotope effects, ^{D}-

$$v = VA/(K_a + A) \quad (4)$$

$$v = VA/[K_a(1 + F_i E_{V/K}) + A(1 + F_i E_V)] \quad (5)$$

$$\log y = \log[Y_L + Y_H(H/K)]/(1 + H/K) \quad (6)$$

$$^T(V/K_{OAS}) = \log(1 - F_1)/\log[1 - F_1(R_p/R_\infty)] \quad (7)$$

(V/K_{OAS}) and ^DV, obtained by direct comparison of initial rates using unlabeled and deuteriated substrate were fitted using eq 5. The pH variation of $^D(V/K_{OAS})$ was fitted using

eq 6. In eqs 4 and 5, v and V represent the initial and maximum velocities, K_a is the Michaelis constant for substrate A, F_i is the fraction of deuterium label in the substrate, and E_V and $E_{V/K}$ are the isotope effects minus one for $^D(V/K_{OAS})$ and ^DV, respectively. For the pH dependence of isotope effects, y is the value of $^D(V/K_{OAS})$ at any pH. Y_L and Y_H are constant values of $^D(V/K_{OAS})$ at low and high pH, respectively; H is the hydrogen ion concentration, and K is the acid dissociation constant for a functional group on enzyme or substrate. The value of $^T(V/K_{OAS})$ was obtained using eq 7, where F_1 is the fractional conversion of unlabeled OAS determined by the ratio of ^{14}C counts in the product acetic acid obtained at partial reaction to that obtained at complete reaction. R_p and R_∞ are $^3H/^{14}C$ ratio for the products (^3H_2O and [1-^{14}C]acetic acid) at partial and complete reaction, respectively.

RESULTS

Synthesis of Deuteriated Compounds. L-Serine-2-*d* was prepared from unlabeled L-serine exchanged with D_2O, and L-serine-3,3-d_2 was obtained from L-serine-2,3,3-d_3 exchanged with H_2O catalyzed by the enzyme OASS-A. The rate of deuterium exchange of the α-proton measured by following the disappearance with time of the α-proton signal at 3.80 ppm is second order with a rate constant of 66 ± 3 M^{-1} s^{-1}. In both cases, addition of serine results in the formation of the external Schiff base, as shown by changes in the UV−visible spectrum (Schnackerz et al., 1995). Addition of D-serine to OASS-A in D_2O shows similar changes in the UV−visible spectrum, but there was no deuterium incorporated into the D-serine at either the α or β positions after 48 h at pD 8.46. Data indicate that, although D-serine forms an external Schiff base, the α-proton is not properly positioned for exchange by the active site lysine. The CD spectra show a positive Cotton effect with L-serine-2-h or L-serine-2-*d*, while a negative Cotton effect is observed with D-serine.

Deuteriated OAS was synthesized according to Sheehan et al. (1956). L-Serine was not completely dissolved in the glacial acetic acid. Upon saturation with HCl gas, serine was acetylated, and the product, OAS, was precipitated. After repetition of reaction, deuteriated serine was 97% acetylated with a quantitative yield of deuteriated OAS. The proton NMR spectra of OAS-2-*d* shows 97 atom % D incorporation at the C-2 position, while no proton signal is detected at C-3 for OAS-3,3-d_2 or at C-2 and C-3 for OAS-2,3,3-d_3 (data not shown). In all cases, about 3% contaminating L-serine is present.

Deuterium Wash-in. The wash-in experiments were carried out for the OAS/TNB pair at pD 6.9 and the OAS/sulfide pair at pD 6.6 with 0.1 M phosphate buffer. *O*-Acetyl-L-serine gives an AA′X pattern in the neutral buffer solution with a triplet at C-2 and a doublet at C-3 (Tai et al., 1993) instead of the ABX pattern observed for the hydrogen chloride form (data not shown). If deuterium is incorporated at C-2 of OAS, the doublet will collapse into a singlet with a concomitant disappearance of the α-proton. The integral ratio of the resonances for the protons at C-3 to those at C-2 of unlabeled OAS should be 2 within intrstrumental integration errors and should decrease if deuterium is incorporated. Without enzyme present, the measured integral ratio is 2.2 for OAS in the OAS/TNB pair and 2.9 for the OAS/sulfide pair. After 17 h in the presence of 60 μg of OASS-A, about 60% conversion to product for OAS/TNB pair, the ratio is still 2.2, and the β-protons still give a doublet even after 44 h. The doublet has become a singlet after 64 h with only around 4% of the OAS remaining. For the OAS/sulfide pair, the signal for the OAS α-proton with about 14% of the substrate remaining is still a doublet after 27 h in the presence of 60 μg of enzyme. On the other hand, the products S-CNP-cysteine for the OAS/TNB pair and L-cysteine and the thiolether byproduct (Woehl et al., 1996) for the OAS/sulfide pair were completely deuteriated at the C-2 position. Data indicate that the rate of deuterium incorporation from solvent is not significant, while the rate of dissociation of acetate from the F−acetate complex must be rather rapid.

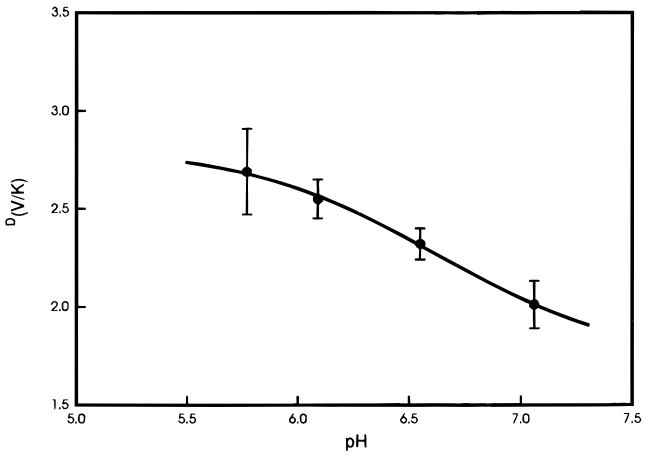


FIGURE 1: pH dependence of the primary kinetic isotope effects, $^D(V/K_{OAS})$. The isotope effects were obtained by varing the concentration of unlabeled OAS and deuteriated OAS at fixed concentration of sulfide at 25 μM at 25 °C and fit using eq 5. Points are experimental values, while the curve is from a fit using eq 6.

Kinetic Isotope Effects from Steady-State Studies. A value of 2.6 ± 0.1 was determined for $^D(V/K_{OAS})$ at pH 6.1, 25 °C. The secondary isotope effect using OAS-3,3-d_2 is 1.11 ± 0.06, while the combined effect, obtained with OAS-2,3,3-d_3, is identical to the value obtained with OAS-2-*d*. The primary kinetic isotope effect was further studied as a function of pH (Figure 1). The isotope effect is constant with a value of 2.8 at low pH and is also constant with a value of 1.72 at high pH. A pK_a of 6.6 ± 0.2 is obtained for the change, consistent with the steady-state studies, which give a p*K* of about 7 for the OAS/sulfide substrate pair and a p*K* of 6.7 for the β-chloroalanine/TNB substrate pair (Tai et al., 1995).

Kinetic Isotope Effects from Single Wavelength Stopped-Flow Studies. Stopped-flow studies on the OASS-A catalyzed reaction have indicated that the first half-reaction limits the overall reaction (Woehl et al., 1996). In order to determine the kinetic isotope effect for α-proton abstraction in the first half-reaction, the first-order rate constant was measured with deuteriated OAS using single-wavelength stopped-flow. The relaxation rate ($1/\tau$) was obtained by following the appearance of the α-aminoacrylate intermediate at 470 nm upon mixing OAS with OASS. The dependence of reaction rate ($1/\tau$) on the concentration of OAS at pH 6.5 was fitted using eq 2 to obtain the rate constant k_5 for α-aminoacrylate formation from the OAS external Schiff base and K_{ext}, the association equilibrium constant for formation of the external Schiff base (Figure 2). Note that

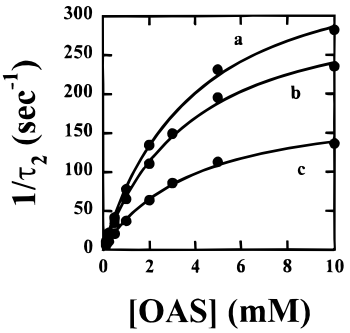


FIGURE 2: Single-wavelength stopped-flow studies of OASS-A monitoring the appearance of absorbance at 470 nm as a function of OAS concentration. The curves are theoretical based on fits using eq 2 for OAS (a), OAS-3,3-d_2 (b), and OAS-2,3,3-d_3 (c), while points are experimental.

Table 1: Tritium Kinetic Isotope Effect [$^T(V/K_{OAS})$] on the OASS-A-Catalyzed Reaction[a]

	^{3}H (cpm)	^{14}C (cpm)	^{3}H/^{14}C (R_p)	F_1	$^T(V/K_{OAS})$[b]
OAS (stock solution)			0.413		
A	2970	7087	0.419	1.0	
B	2890	6932	0.417	1.0	
C	2900	7000	0.414	1.0	
av	2920 ± 40	7010 ± 40	0.417 ± 0.001 (R_∞)		
D	150	1394	0.108	0.199	4.2
E	124	1358	0.091	0.194	5.0
F	170	1358	0.129	0.188	3.5
G	208	1959	0.106	0.280	4.4
H	211	1864	0.113	0.266	4.1
I	203	1846	0.110	0.264	4.2
av					4.2 ± 0.2

[a] Experimental details are described in Materials and Methods. [b] Data were fitted using eq 7.

in the presence of acetate a single isosbestic point is not obtained as a result of the equilibrium established including external Schiff base and the α-aminoacrytlate intermediate (Woehl et al., 1996). In the absence of acetate all of the enzyme is present as the α-aminoacrylate intermediate (Schnackerz et al., 1995). The value of K_{ext} is 0.26 mM^{-1} and independent of isotopic substitution, while values of 400 ± 80,[2] 330 ± 65, and 200 ± 40 s^{-1} are estimated for k_5 with OAS, OAS-3,3-d_2, and OAS-2,3,3-d_3, respectively. Estimates of the primary and secondary isotope effects obtained from the extrapoltated rate constants measured above are 2.0 ± 0.6 and 1.2 ± 0.3, respectively. The primary isotope effect is within error of the value of 2.6 measured at pH 6.1 using initial rate studies, and although the secondary deuterium effect is undefined, it must also be small in agreement with the steady state data.

Tritium Kinetic Isotope Effect. Experimental data for determination of the tritium kinetic isotope effect are presented in Table 1. The reaction was quenched at either 19% or 27% (F_1) with 2 N HCl. Products (^3H_2O and [1-^{14}C]-acetic acid) were completely separated from unreacted OAS by Dowex chromatography. The tritium kinetic isotope effect $^T(V/K_{OAS})$ is 4.2 ± 0.2. Using the experimentally obtained value of 2.6 ± 0.1 for $^D(V/K_{OAS})$ at pH 6.1, the intrinsic deuterium kinetic isotope effect ^Dk and tritium kinetic isotope effect ^Tk can be calculated using eq 8

$$[^D(V/K_{OAS}) - 1]/[^T(V/K_{OAS}) - 1] = (^Dk - 1)/(^Dk^{1.442} - 1) \quad (8)$$

(Northrop, 1975) to give 3.3 ± 0.9 and 5.6 ± 1.5, respectively.[3]

Secondary Equilibrium Isotope Effect. Equilibrium constants for the α,β-elimination of acetate from OAS or OAS-3,3-d_2 catalyzed by OASS-A were determined at pH 6.1, 25 °C (Figure 3). Using a known concentration of OASS-A (determined from Figure 3a), ϵ_{470} values of 9057 ± 32 and 8904 ± 69 M^{-1} cm^{-1} were obtained for unlabeled and deuteriated α-aminoacrylate intermediates (Figure 3b,c). Addition of OAS or OAS-3,3-d_2 to OASS-A in the presence of acetate, gives the spectra shown in Figure 3d,e. Equilibrium constants of 2056 ± 15 and 1136 ± 23 were obtained for the OAS and OAS-3,3-d_2, respectively. The equilibrium isotope effect contributed by two deuterons at C-3 of OAS-3,3-d_2 is 1.81 ± 0.04. The measured value of 1.81 can be compared to the value of 1.77 estimated from the fractionation factors for the protons at C-1 of ethanol representing the protons at C-3 of OAS and the protons at C-3 of phosphoenolpyruvate representing the protons at C-3 of the α-aminoacrylate Schiff base (Cleland, 1980).

DISCUSSION

Kinetic Isotope Effects. The kinetic mechanism of the OASS-A-catalyzed reaction is ping-pong with conversion of OAS to the α-aminoacrylate Schiff base and acetate comprising the first half-reaction (Tai et al., 1993). In a ping-pong mechanism, the reactant *V*/*Ks* reflect the individual half-reactions, so that the macroscopic rate constant V/K_{OAS} will include microscopic rate constants for all steps from addition of OAS to enzyme to release of the first product acetate. The isotope effect measured with OAS-2-*d* as the labeled reactant, the primary isotope effect on α-proton abstraction, will reflect only V/K_{OAS}, since the deuteron, once abstracted by the active site lysine will exchange with solvent prior to the addition of sulfide to begin the second half-reaction. A secondary isotope effect measured with OAS-3,3-d_2 can be obtained for each of the two half-reactions, but in the present case consideration will be restricted to the first half-reaction.

In the case where the isotope-sensitive step is not solely rate-determining and substrate is sticky, the observed isotope effect can be enhanced by changing the pH so that it is in the region where *V*/*K* is decreasing (Cook & Cleland, 1981b,c). The latter results in the EA complex being incorrectly protonated for binding and/or catalysis, giving a decrease in the rate of the catalytic step. V_{max} is known from stopped-flow experiments to reflect the first half-reaction of the OASS-A reaction (Woehl et al., 1996). Table 2 lists the isotope effects measured by steady-state and pre-steady-state studies. The V/K_{OAS} and V_{max}, with OAS and sulfide as substrates, have the same pH dependence (Tai et al., 1995), and, as shown above, $^D(V/K_{OAS})$ increases as the pH is decreased from a value of 1.7 at neutral pH to a value of 2.8 at low pH. The pH dependence of kinetic parameters thus adhere to case 1 of Cook and Cleland (1981b), which can be described using Scheme 2. In Scheme 2, k_3'

[2] The difference between this value and the value of 300 s^{-1} obtained by Woehl et al. (1996) is attributed to the absence of the monovalent cations Na^+ and K^+.

[3] The estimated values are determined using a computer program developed by D. B. Northrop which provides an error analysis to generate the reported standard errors.

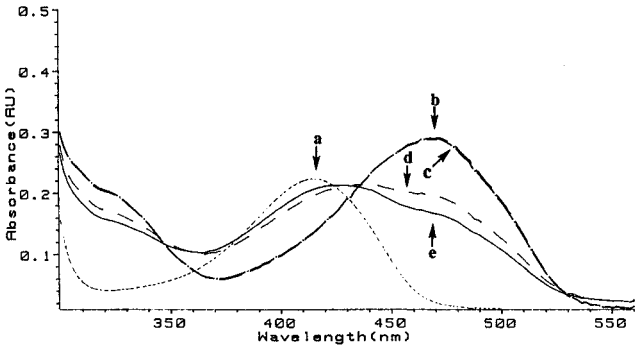


FIGURE 3: UV−visible spectra for the reaction of OASS-A and OAS or OAS-3,3-d_2: (a) 29.52 ± 0.04 μM OASS-A alone; (b) (a) with an excess of OAS; (c) (a) with an excess of OAS-3,3-d_2; (d) OASS-A (29.52 ± 0.04 μM) plus OAS (32.70 ± 0.03 μM) in the presence of 22.5 mM acetate; (e) OASS-A (29.52 ± 0.04 μM) plus OAS-3,3-d_2 (33.45 ± 0.30 μM) in the presence of 22.5 mM acetate.

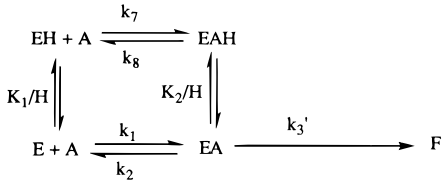
Table 2: Kinetic Isotope Effecs in the OASS-Catalyzed Reaction

	steady state		
labeled OAS	$^{D}(V/K)$	$^{T}(V/K)$	$^{\alpha\text{-}D}K_{eq}$
OAS-2-*d*	2.81 ± 0.02[a]		
	(3.3 ± 0.9)[b]		
	1.72 ± 0.07[a]		
OAS-3-t		4.2 ± 0.2	
		(5.6 ± 1.5)[b]	
OAS-3,3-d_2	1.11 ± 0.06[c]		1.81 ± 0.04
OAS-2,3,3-d_3	2.5 ± 0.1[c]		

	pre-steady state		
	k_5 (s^{-1})	K_{ext} (mM^{-1})	$^{D}k_5$
OAS	400 ± 80	0.26	
OAS-3,3-d_2	330 ± 65	0.26	1.2 ± 0.3
OAS-2,3,3-d_3	200 ± 40	0.26	2.0 ± 0.6

[a] Constant values of $^{D}(V/K)$ at low (2.8) and high (1.7) pH, respectively. [b] Estimates of intrinsic deuterium and tritium isotope effects. [c] pH 6.1.

Scheme 2



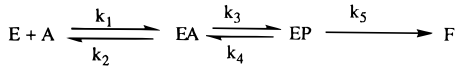
represents the net rate constant for the isotope-sensitive step and includes formation of the α-aminoacrylate intermediate (F), while k_1 and k_2 are the association and dissociation rate constants for the E−OAS complex, respectively. The expression for the observed kinetic isotope effect $^{D}(V/K_{OAS})$ is given by eq 9, where the ratio of k_3'/k_2 is the forward

$$^{D}(V/K_{OAS}) = (^{D}k_3' + k_3'/k_2)/(1 + k_3'/k_2) \quad (9)$$

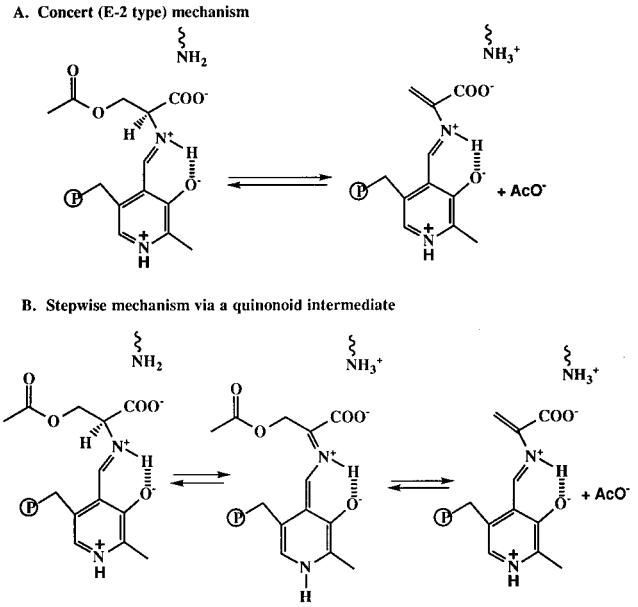
commitment to catalysis, C_f.

At low pH, substrate stickiness is eliminated and eq 9 reduces to $^{D}(V/K_{OAS}) = {}^{D}k_3'$, Thus, using the value of 1.7 for $^{D}(V/K_{OAS})$ and 2.8 for $^{D}k_3'$, a value of 1.5 is estimated for k_3'/k_2. Expansion of the mechanism in Scheme 2 to include the rate processes within k_3' provides the mechanism shown in Scheme 3, where k_3 and k_4 reflect formation of the external Schiff base (EP) and the E−OAS complex (EA), respectively, and k_5 reflects formation of the α-aminoacrylate

Scheme 3



Scheme 4



intermediate (F). Based on Scheme 3, the net rate constant k_3' is equal to $k_3k_5/(k_4 + k_5)$, and the expression for $^{D}k_3'$ is

$$^{D}k_3' = (^{D}k_5 + k_5/k_4)/(1 + k_5/k_4) \quad (10)$$

In eq 10, k_5/k_4 is again termed the forward commitment to catalysis, C_f. The C_f can be divided into an external commitment to catalysis, $C_{f\text{-}ex}$, k_3k_5/k_2k_4, and an internal commitment to catalysis, $C_{f\text{-}in}$, k_5/k_4. The expanded expression for $^{D}(V/K_{OAS})$ is thus provided in

$$^{D}(V/K_{OAS}) = (^{D}k_5 + C_{f\text{-}in} + C_{f\text{-}ex})/(1 + C_{f\text{-}in} + C_{f\text{-}ex}) \quad (11)$$

The intrinsic primary deuterium isotope effect on α-aminoacrylate formation, $^{D}k_5$, is estimated to be 3.3 ± 0.9 calculated from the Swain−Schaad relationship (eq 8) from the observed $^{T}(V/K_{OAS})$ and $^{D}(V/K_{OAS})$, consistent with the extrapolated value of 2.8 obtained for $^{D}k_3'$ at low pH from steady-state studies and the crude estimate of about 2 obtained from rapid-scanning stopped-flow studies at pH 6. Data thus suggest that $C_{f\text{-}in}$ (k_5/k_4) is close to 0, i.e., $k_5 \ll k_4$. The external commitment to catalysis $C_{f\text{-}ex}$ (k_3k_5/k_2k_4) is 1.5 using the value of 2.8 estimated from extrapolation of the observed deuterium effect to low pH. The latter requires that k_3/k_2 be much greater than k_5/k_4 to give the final value of 1.5. In agreement with these data, a value of 800 s^{-1} has been estimated from rapid-scanning stopped-flow studies for the first order rate constant for formation of EP.

The secondary isotope effect measured on α-aminoacrylate formation using single-wavelength stopped-flow experiments is slightly larger than that obtained by steady-state studies at pH 6, as predicted by the pH dependence of the primary isotope effect (see above). The maximum isotope effect is expected to be expressed below pH 5.

Although a pK value of 7 has been estimated from the pH dependence of kinetic parameters using OAS and sulfide as reactants, an accurate value could not be determined

because of the instability of OAS. The group with pK of around 7 is suggested to assist in elimination of the acetate side chain of OAS (Tai et al., 1995). The pH dependence of the kinetic isotope effects $^{D}(V/K_{OAS})$ gave a well-defined pK of 6.6. Since substrate is sticky, however, the intrinsic pK is obtained as 7.0 once corrected for the perturbation to lower pH by the factor $\log(1 + k_3'/k_2)$ (Cleland, 1975).

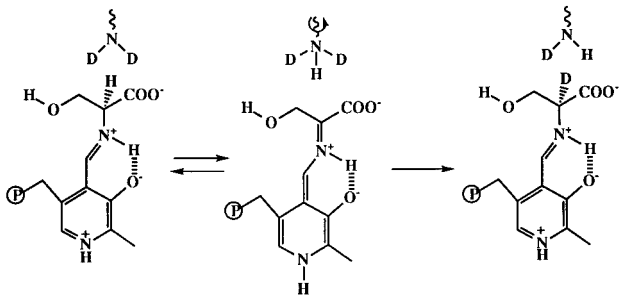
Mechanistic Implications. The isotope effect data have implications concerning the kinetic and chemical mechanisms of OASS-A. A primary isotope effect of 2.0 is obtained on $^{D}(V/K_{OAS})$ at pH 7.0, while an isotope effect of 1.5 is obtained on the apparent V (sulfide was not saturating), suggesting that the first half-reaction limits the overall reaction, and that α-proton abstraction is largely rate-limiting within the first half-reaction. These data are in agreement with previously measured pre-steady-state data (Woehl et al., 1996).

The α,β-elimination of OAS, catalyzed by the active site lysine (α) and the enzyme group that is thought to hydrogen-bond the acetyl carbonyl of OAS (β) can occur via a concerted reaction (Scheme 4A) or a stepwise reaction in which the active site lysine abstracts α-proton to form a quinonoid intermediate, followed by the elimination of acetate to form the α-aminoacrylate intermediate (Scheme 4B). A quinonoid intermediate would be expected to exhibit a λ_{max} higher than the 470 nm maximum of the α-aminoacrylate intermediate. No such intermediate has been observed by either rapid-scanning stopped-flow (Woehl et al., 1996) or equilibrium UV−visible studies (Schnackerz et al., 1995), but these results do not necessarily rule out a quinonoid intermediate. Because acetate is such a good leaving group, there is no reason, *a priori*, to believe that a quinonoid intermediate exists along the OASS-A reaction pathway. As long as the acetate leaving group is orthogonal to the imine of the external Schiff base, the reaction would be expected to be concerted. Tai et al. (1995) have proposed that the enzyme group that interacts with the acetate leaving group functions to hold it in position for elimination. The above arguments are in favor of a concerted reaction as shown in Scheme 4A. However, since a stepwise mechanism cannot be ruled out, such α-proton abstraction limits overall, and thus data will be interpreted for both of the possible mechanisms.

The primary and secondary deuterium kinetic isotope effects potentially provide information on the transition state for the α,β-elimination reaction. The primary effect will be small for either an early or a late transition state and will be maximal for a symmetric transition state with the proton halfway between the α-carbon and the ϵ-amine of the active site lysine (Cook, 1991). A maximum value of 6−8 is expected for a symmetric transition state.[4] The observed value of about 2.8 could thus reflect an early transition state with the proton closer to the α-carbon or a late transition state with the α-proton closer to the ϵ-amine.

The secondary isotope effect can be used to help distinguish between early and late transition states. The secondary effect reflects changes in the bending vibrations that occur upon changes in hybridization at or adjacent to the isotope-substituted carbon. If the transition state is early, little or no hybridization change occurs, and a secondary isotope effect of 1 is observed, while if the transition state is late, the hybridization change will be complete, and the secondary effect will be equal to the full equilibrium isotope effect (Cook, 1991). In the case of a stepwise mechanism, the equilibrium effect will reflect the hybridization change at the α-carbon. An estimate of 1.1 is obtained for the value of the equilibrium isotope effect for two deuterons at the β-carbon of OAS from the fractionation factors of the protons at C-1 of ethanol (representing the protons at C-3 of OAS) and the α-protons of unhydrated dihydroxyacetone phosphate (representing the protons at C-3 of the α-aminoacrylate Schiff base) (Cleland, 1980). In the case of the concerted mechanism, the equilibrium isotope effect will reflect the hybridization change at the β-carbon, measured experimentally in these studies as 1.8. The value of 1.11 ± 0.06 obtained for the secondary isotope effect thus suggests either a late transition state for α-proton abstraction in a stepwise reaction or an early transition state for a concerted α,β-elimination reaction.

Scheme 5



Serine Exchange. The reaction of L-serine with OASS-A has been studied using a number of spectral probes (Schnackerz et al., 1995). Addition of L-serine to OASS-A, which absorbs maximally at 412 nm, results in the appearance of absorption maxima at 418 and 330 nm. The authors suggested that the 330 and 418 nm bands reflect an equilibrium mixture of two tautomeric forms of the serine external Schiff base between serine and the active site PLP. The CD spectrum of the starting L-serine shows a positive Cotton effect, as does the resulting L-serine-2-*d*, and both have the same molar ellipticity within error. A negative Cotton effect is observed with D-serine, and no exchange of the α-proton is observed after extended periods of time in the presence of enzyme in D_2O. Thus, OASS-A abstracts the α-proton of L-serine but not of D-serine; the reaction is stereospecific. In addition, no exchange of the β-protons is observed, and thus the reaction is regiospecific. Results differ significantly from the exchange reaction catalyzed by alanine aminotranferase, for example, which exchanges both the α- and β-protons of L-alanine in D_2O, but at different rates (Cooper, 1976).

The exchange of the L-serine α-proton likely proceeds via the intermediacy of a quinonoid intermidiate (Scheme 5), although no evidence of such an intermediate was observed in the absorbance spectra of OASS-A plus L-serine (Schnackerz et al., 1995). Arguments will be made below for the absence of a quinonoid intermediate in the reaction where OAS is converted to the α-aminoacrylate intermediate because the leaving group is activated for elimination. In the case of L-serine, the leaving group is hydroxide, which is a poor leaving group in comparison to acetate, and thus the exchange reaction could be pictured proceeding via a quinonoid intermediate. Data require that if a quinonoid

[4] These estimates are in the absence of hydrogen tunneling.

exists, however, the equilibrium constant for its formation be well in favor of the external Schiff base. In Scheme 5, the α-proton of the external Schiff base is abstracted by the active site lysine that originally participated in the internal Schiff base, and exchange requires that the lysine then stereospecifically replace the proton with a deuteron. Schnackerz et al. (1995) have proposed that the active site of OASS-A is closed upon formation of the external Schiff base, and, if correct, the lysine would be unable to exchange its protons with bulk solvent. However, since the reaction is carried out in D_2O, the Schiff base nitrogen of the internal Schiff base will be deuteriated, as will the α-amine of the bound L-serine. For all intents and purposes, the only proton present on lysine will then be the abstracted α-proton. As long as the lysine has freedom of motion in the active site and ignoring isotope effects, there will be an approximately 2/3 chance of the putative quinonoid intermediate being deuteriated at the α-carbon as L-serine is reformed.

Once L-serine-2-*d* is released from the enzyme surface, the enzyme will favor abstraction of an α-proton over an α-deuteron by the magnitude of the primary isotope effect on the proton abstraction step. The estimated second-order rate constant for deuterium exchange, 66 ± 3 M^{-1} s^{-1}, is quite slow compared to the rate of the enzyme-catalyzed α-proton abstraction from the OAS external Schiff base, 300 s^{-1} (Woehl et al., 1996), and $V/K_{OAS}E_t$, $(1.4 \pm 0.7) \times 10^5$ M^{-1} s^{-1} (Tai et al., 1993). However, it is not possible to determine whether the low rate constant reflects removal of the α-proton from the serine external Schiff base alone or some other step(s) in the overall exchange reaction.

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