Acid−Base Chemical Mechanism of *O*-Acetylserine Sulfhydrylases-A and -B from pH Studies†

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ABSTRACT: The pH dependence of kinetic parameters using natural and alternative reactants was determined in order to obtain information on the chemical mechanisms of the A and B isozymes of *O*-acetylserine sulfhydrylase (OASS) from *Salmonella typhimurium*. A general mechanism is proposed for OASS in which OAS binds with its α-amine unprotonated to carry out a nucleophilic attack on C4′ of the protonated Schiff base and with the acetyl carbonyl hydrogen-bonded to a protonated enzyme group (or a water molecule), which aids in the β-elimination of acetate. The enzyme lysine that was in Schiff base linkage with the active site pyridoxal 5′-phosphate deprotonates the α-carbon in the β-elimination reaction, and a proton is likely released with the acetate product. Sulfide likely binds as HS^- to undergo nucleophilic attack on the α-aminoacrylate intermediate, followed by protonation of the α-carbon by the enzyme lysine. In OASS-A, HS^- is hydrogen-bonded to the enzyme group that assists in the β-elimination of acetate, but this is not the case for OASS-B. The pH independent equilibrium constant for the first half-reaction of OASS-A is 1.6×10^{-3}, while the second half-reaction is practically irreversible.

L-Cysteine biosynthesis in the enteric bacterium *Salmonella typhimurium* is catalyzed in two steps, as shown in eqs 1 and 2. The first step is carried out by the enzyme serine transacetylase (STA, EC 2.3.1.30),[1] which catalyzes the formation of *O*-acetyl-L-serine from acetyl-CoA and L-serine, while the second step is carried out by *O*-acetylserine sulfhydrylase (OASS, EC 4.2.99.8; *O*-acetylserine(thiol)-lyase), which catalyzes the formation of L-cysteine from sulfide and *O*-acetyl-L-serine.

L-serine + acetyl-CoA → *O*-acetyl-L-serine + CoA (1)

O-acetyl-L-serine + sulfide → L-cysteine + acetate (2)

Two OASS isozymes, A and B, have been described in *Salmonella typhimurium* (Becker et al., 1969). The A and B isozymes are thought to be required for aerobic and anaerobic growth, respectively. In this regard, OASS-B will utilize thiosulfate as a substrate in addition to sulfide (Filutowicz et al., 1982). The A and B isozymes are both dimeric, with molecular weights of 68 900 (Byrne et al., 1988) and about 64 000 (Tai et al., 1993), respectively, and each has one tightly bound pyridoxal 5′-phosphate (PLP) per subunit (Becker et al., 1969; Nakamura et al., 1984).

The kinetic mechanisms for both isozymes have recently been determined using several alternative substrates. The isozymes share a common ping-pong kinetic mechanism, with competitive inhibition by both substrates indicative of E:sulfide and F:OAS dead-end complexes (Tai et al., 1993). The major difference between the two isozymes is in substrate specificity, with the A isozyme being much more specific than the B isozyme.

The ultraviolet−visible spectrum of *O*-acetylserine sulfhydrylase-A exhibits an absorption maximum at 412 nm due to the formation of a protonated Schiff base between an active site lysine and the active site PLP (Cook & Wedding, 1976; Cook et al., 1992). Addition of OAS to the native enzyme results in the disappearance of absorbance at 412 nm and the appearance of new absorption maxima at 320 and 470 nm, which is indicative of the formation of a protonated Schiff base between PLP and α-aminoacrylate upon the β-elimination of acetate from OAS (Cook & Wedding, 1976; Schnackerz et al., 1979; Cook et al., 1992). Michael addition of sulfide to the β-carbon of the α-aminoacrylate Schiff base results in the formation of L-cysteine.

The pH dependence of the ultraviolet−visible spectrum in the absence and presence of OAS indicates that the pK_a values for the Schiff base in the free enzyme and the α-aminoacrylate intermediate are greater than 10 (Cook et al., 1992). In addition, these authors showed, via ^{31}P NMR, that the phosphate is dianionic over the pH range 6−10. Finally, an OAS deacetylase activity was identified in which the active site lysine that was in Schiff base with PLP displaces α-aminoacrylate to regenerate free E and produce pyruvate and ammonia. The pH dependence of the deacetylase activity gave a pK_a for the active site lysine[2] of 8.2.

Although the two isozymes are similar in many respects, there are quantitative differences between them. Thus, it is

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[1] Abbreviations: Mes, 2-morpholinoethanesulfonic acid; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; Taps, 3-[[tris(hydroxymethyl)methyl]amino]propanesulfonic acid; Ches, 2-(*N*-cyclohexylamino)ethanesulfonic acid; DTT, dithiothreitol; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); TNB, 5-thio-2-nitrobenzoate; *S*-CNP-cysteine, *S*-(3-carboxy-4-nitrophenyl)-L-cysteine; OAS, *O*-acetyl-L-serine; BCA, β-chloro-L-alanine; NAS, *N*-acetyl-L-serine; OASS, *O*-acetylserine sulfhydrylase; PLP, pyridoxal 5′-phosphate; STA, serine transacetylase.

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of interest to determine whether differences exist in the protonation state of the enzyme and in the reactant functional groups required for binding and catalysis of OASS-A and -B. In the present study, the pH dependence of kinetic parameters for the two isozymes has been obtained, and an acid−base chemical mechanism is proposed.

MATERIALS AND METHODS

Chemicals. *O*-Acetyl-L-serine, BCA, DTNB, DTT, sodium thiocyanate, sodium acetate, and Na_2S were obtained from Sigma. The reduced form of DTNB, TNB, was freshly prepared prior to use by reduction with DTT in slight molar excess of DTNB. The TNB was then used without further treatment. Neither reduced nor oxidized DTT had any effect on the OASS reaction. All other reagents and chemicals were obtained from commercially available sources and were of the highest quality available.

Enzymes. *O*-Acetylserine sulfhydrylase-A from *Salmonella typhimurium* LT-2 was purified by the method of Hara et al. (1990), as modified by Tai et al. (1993). The enzyme preparation used in this study was 95% pure on the basis of SDS−PAGE. The enzyme had a specific activity of 800 units/mg with sulfide as the second substrate and was assayed using a sulfide ion selective electrode (Hara et al., 1990). The protein concentration of the purified enzyme was determined from the absorbance of the Schiff base between the active site lysine and PLP at 412 nm, using an extinction coefficient of 7600 M^{-1} cm^{-1} (Kredich et al., 1969; Cook et al., 1992). The activity of the enzyme is stable over the pH range 5.5−10 (Hara et al., 1990).

O-Acetylserine sulfhydrylase-B from *Salmonella typhimurium* LT-2 was purified by the method of Tai et al. (1993). The enzyme obtained was >95% pure on the basis of a 12% SDS gel. Enzyme had a final specific activity of about 2 units/mg measured with 2 mM OAS and 0.05 mM TNB.

Enzyme Assay. *O*-Acetylserine sulfhydrylase activity was monitored by using two different assays. The disappearance of sulfide was followed using a computer-assisted sulfide ion selective electrode assay (Hara et al., 1990). In addition, 5-thio-2-nitrobenzoate (TNB) was used as an alternative substrate in the present studies (Tai et al., 1993). At high pH (>7) the reaction was initiated by adding OAS to minimize its degradation, while in all other cases enzyme was added to initiate the reaction. Initial rates at pH > 7 were calculated using ϵ_{412} = 13 600 M^{-1} cm^{-1} for TNB (Ellman, 1958). Initial rates measured below pH 7 were calculated using the apparent extinction coefficient at 412 nm, correcting for the protonation state of TNB.

Initial Velocity Studies. *O*-Acetylserine sulfhydrylase was assayed spectrophotometrically by using a Gilford 2600 spectrophotometer. The temperature was maintained at 25 °C using a circulating water bath with the capacity to heat and cool the thermospacers of the cell compartment. Reaction cuvettes were 1.0 mL in volume with a 1.0 cm path length. A typical assay contained the following at final concentration: 100 mM Hepes (pH 7), 5 mM OAS, 0.1 mM TNB, and 25 μg of OASS. Initial velocity patterns were obtained by varying OAS or BCA at several different fixed concentrations of TNB. Data were also obtained using a 0.1 cm path length cuvette at high concentrations of TNB. Studies in the presence of product and dead-end inhibitors were carried out after determining the inhibition constant from a Dixon plot maintaining the substrate concentration constant and varying the inhibitor concentration. All inhibition patterns were obtained by fixing the concentration of one substrate and varying the concentration of the other substrate at several different fixed concentrations of inhibitor, including zero.

pH Studies. Initial velocity patterns were obtained at pH 5.5 and 7.0 to determine the pH dependence of the kinetic mechanism and to obtain an estimate of the K_m values for reactants. The pH dependencies of V/K_{OAS}, V/K_{BCA}, and V/K_{TNB} were obtained by varying one substrate at a fixed, nonsaturating concentration of the second substrate and measuring the initial velocity. The maximum rate was estimated by varying substrate concentrations in a constant ratio and extrapolating to infinite substrate concentration. The pH dependencies of kinetic parameters for the OAS/sulfide substrate pair were also obtained from initial velocity patterns as a function of pH from 5.5 to 7.5 by using the sulfide ion selective electrode assay. Initial velocity patterns were obtained as a function of pH by varying OAS at different fixed concentrations of sulfide. Data were not well-conditioned to be fitted using the overall equation for a ping-pong mechanism and thus were fitted using eq 3 to obtain slopes and intercepts for each line in an initial velocity pattern. The V_{max} and $V/K_{sulfide}$ values were obtained as the intercept and slope of the intercept replot, while the V/K_{OAS} was estimated graphically as the intercept of the slope replot. Inhibition data were obtained from pH 5.5 to 9.5 for acetate and thiocyanate at nonsaturating concentrations of the fixed substrate. Acetate and thiocyanate are noncompetitive inhibitors, and the K_{is} values are reported. For OASS-A, buffers at 100 mM final concentration were used over the following pH ranges: Mes, 5.5−6.5; Hepes, 7−8; Taps, 8.5; Ches, 9−10; all buffers were titrated with KOH. In all cases, sufficient overlaps were obtained when buffers were changed so that correction should be made for spurious buffer effects. For OASS-B, the pH was maintained using a mixed buffer at 50 mM each of the following: Mes, Hepes, and Ches.

The pH of all reaction mixtures was measured before and after initial velocity data were collected with a Radiometer PHM 82 pH meter with a combined microelectrode. All experiments were carried out at 25 °C, using a circulating water bath to maintain a constant temperature of the thermospacers in the cell compartment.

Titration of O-Acetyl-L-serine. The first-order rate constant for the nonenzymatic degradation of *O*-acetyl-L-serine to *N*-acetyl-L-serine was obtained from pH 6.5 to 9.5. A stock solution of OAS was prepared at pH 6.0 in 100 mM Mes buffer. From this a second stock solution was prepared at the pH of interest. Then an aliquot from the second stock solution, estimated to have a final concentration of 0.075 mM OAS, immediately was added to a cuvette containing, in a final volume of 1 mL, 3 mg of OASS-A (0.088 mM) and 100 mM Hepes (pH 6.5). The resulting absorbance at 470 nm was then recorded, and the OAS concentration was calculated using an ϵ_{470} of 9760 M^{-1} cm^{-1} (Nalabolu et al., 1992). The preceding experiment was then repeated at different time intervals by assaying identical aliquots of the stock solution incubated at the desired pH. The zero time point was obtained as the concentration of the stock solution at pH 6.5, where OAS is stable. A plot of ln(concentration

[2] The identity of the active site lysine has been established as Lys42 (unpublished work of V. D. Rege in this laboratory).

of remaining OAS) vs time gives the rate constant for the degradation of OAS, calculated as the absolute value of the slope of the first-order plot. The first-order rate constant for the degradation of OAS from pH 6.5 to 9.5 was plotted vs pH, and the data were fitted using eq 11 to obtain the pK_a value(s) of ionizable groups(s) involved in the rearrangement of OAS to NAS. Similar experiments were carried out with BCA; there is no significant change in the concentration of BCA incubated at pH 7, 8, 9, and 10 over 8 h, suggesting that BCA is quite stable over this pH range.

Titration of TNB. Thionitrobenzoate was titrated from pH 2.5 to 9.5 at 25 °C, and the extinction coefficient at 412 nm was determined as a function of pH. The following buffers were used at a concentration of 100 mM over the associated pH ranges: citrate, 2.5−4.5; Mes, 5−6.5; Hepes, 7−8; Taps, 8.5; Ches, 9−9.5. A plot of the log(fractional change in ϵ_{412}) was obtained from these data and fitted using eq 7 to obtain the pK value for the 5-thiol group of TNB.

Thermodynamic Measurements. A typical assay consisted of 25 μM OASS-A and 50 μM OAS in the appropriate buffer at room temperature. The absorbance spectrum of the enzyme was measured in the presence of OAS (that is, the α-aminoacrylate intermediate was formed), and this served as the zero acetate point. The spectrum was then measured after the addition of acetate from a 200 mM sodium acetate stock solution. The measurement for each addition was carried out in a separate cuvette. Spectra were obtained at pH 5.5−7.0. Measurements were not performed above pH 7 due to the *O*- to *N*-acetyl migration of OAS, resulting in the production of *N*-acetylserine at pH values of 7.6 and above (see above; Flavin et al., 1965). The disappearance of the α-aminoacrylate intermediate was followed by measuring the decrease in 470 and 320 nm absorbances and the increase in 412 nm absorbance with the addition of sodium acetate. Calculation of the equilibrium constant is discussed in the following.

Equilibrium measurements for the second half-reaction consisted of the addition of L-cysteine to OASS-A in an attempt to drive the reaction in the reverse direction, thus forming the α-aminoacrylate intermediate, which absorbs at 470 nm. Assays were performed at pH 6−9. A typical assay consisted of 50 μM OASS-A in the appropriate buffer, to which L-cysteine was added in a stepwise manner. Absorption spectra were measured at zero L-cysteine concentration and after each L-cysteine addition. No formation of the α-aminoacrylate intermediate was detected at concentrations of L-cysteine up to 50 mM.

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). Individual saturation curves were fitted using eq 3, while initial velocity data conforming to a ping-pong mechanism were fitted using eq 4. Data for linear noncompetitive inhibition were fitted using eq 5, while initial velocity data conforming to double competitive substrate inhibition in a ping-pong mechanism were fitted using eq 6. In eqs 3−6, v and V represent initial

$$v = VA/(K_a + A) \tag{3}$$

$$v = VAB/(K_bA + K_aB + AB) \tag{4}$$

$$v = VA/(K_a[1 + I/K_{is}] + A[1 + I/K_{ii}]) \tag{5}$$

$$v = VAB/(K_bA[1 + A/K_{IA}] + K_aB[1 + B/K_{IB}] + AB) \tag{6}$$

and maximum velocities, K_a and K_b are K_m values for A and B, K_{IA} and K_{IB} are substrate inhibition constants for A and B, K_{is} and K_{ii} are slope and intercept inhibition constants, and A, B, and I represent reactant and inhibitor concentrations, respectively.

Data for the pH dependence of the log of the fractional change in ϵ_{412} for TNB, which decreases with a slope of 1 at low pH, were fitted using eq 7, while data for pH profiles that decrease at high pH to a slope of −1 were fitted using eq 8. Data for pH profiles that decrease at low and high pH with slopes of 1 and −1 were fitted using eq 9. The V_{max} pH profile for OASS-A with TNB as a substrate were fitted using eq 10, which describes a curve with different constant values at low and high pH. Data for pH profiles with a limiting slope of +2 were fitted using eq 11. In eqs 7−11,

$$\log y = \log[C/(1 + H/K_1)] \tag{7}$$

$$\log y = \log[C/(1 + K_2/H)] \tag{8}$$

$$\log y = \log[C/(1 + H/K_1 + K_2/H)] \tag{9}$$

$$\log y = \log(Y_L + Y_H[H/K_1])/(1 + H/K_1) \tag{10}$$

$$\log y = \log[C/(1 + H/K_1 + H^2/K_1K_3)] \tag{11}$$

K_1−K_3 represent acid dissociation constants for enzyme or substrate functional groups, y is the value of V or V/K at any pH, C is the pH independent value of y, H is the hydrogen ion concentration, and Y_L and Y_H are constant values of V at low and high pH, respectively.

For thermodynamic measurements, the fractional change in A_{470} at each of the acetate concentrations used was calculated using

$$f = [(A_{470})^0 - (A_{470})]/(A_{470})^0 \tag{12}$$

where f is the fractional change in A_{470} at any acetate concentration, $(A_{470})^0$ is the absorbance in the absence of acetate, and A_{470} is the measured absorbance at a given acetate concentration. Data were plotted as f vs acetate concentration and were fitted using

$$f = A[1 + X/K_{IN}]/[1 + X/K_{ID}] \tag{13}$$

where f is the fractional change at any acetate concentration, A is $(A_{470})^0$, $(A)K_{ID}/K_{IN}$ is zero, X is the acetate concentration, K_{ID} is the concentration of acetate that gives one-half the change, and K_{IN} is a term that causes the fraction to stop at a value of zero at infinite acetate concentration.

The equilibrium constant obtained at any pH is given by

$$K_{eq} = [F][\text{acetate}]/[E][OAS] \tag{14}$$

where E is the unliganded enzyme that absorbs maximally at 412 nm and F is the α-aminoacrylate intermediate that absorbs maximally at 470 nm. When one-half of the 470 nm absorbance remains, the concentrations of E and F are equal, and the concentration of acetate that gives one-half of the change in $(A_{470})^0$ (K_{ID} from eq 13) is K_{eq}[OAS]. Thus, the value of K_{eq} is K_{ID}/[OAS]. The value of $\Delta G^{\circ\prime}$ is then

obtained by using the value of K_{eq} measured at pH 7 and

$$\Delta G^{\circ\prime} = -2.3RT \log(K_{eq}) \quad (15)$$

where the ′ indicates the value at pH 7. The pH independent value of K_{eq} (termed K_{eq}') was calculated at each pH by multiplying the calculated value of K_{eq} by $[H^+]$ and then averaging all of the values.

Theory. The majority of theoretical treatments of the pH dependence of kinetic parameters in a ping-pong mechanism focus on the *V/K* values. The *V/K* values for the individual reactants reflect the half-reaction of the ping-pong kinetic mechanism. The *V* value, however, may reflect the rate limitation of both half-reactions. If one or the other half-reaction limits, *V* will reflect that half-reaction, while if both are partially rate-limiting, *V* will reflect both half-reactions.

Consider the mechanism in Scheme 1 on the basis of the data presented in this paper. By using the method of Cha (1968) assuming that the segments in the dotted boxes are in rapid equilibrium, the mechanism is reduced to that shown at the bottom of Scheme 1. Expressions for f_3 and f_{11} are as follows:

$$f_3 = A/[K_a(1 + H/K_1 + H^2/K_1K_3) + A(1 + H/K_2)] \quad (16)$$

$$f_{11} = B/[K_b(1 + K_5/H) + B(K_6/H + 1)] \quad (17)$$

where K_a and K_b are dissociation constants for EA and FB, respectively. By using the net rate constant method of Cleland (1975) and substituting f_3 and f_{11}, the expression for the initial rate is given by

$$v/E_t = k_3k_5k_{11}k_{13}AB/\{K_b(k_3k_5k_{12} + k_3k_5k_{13})(1 + K_5/H)A + K_a(k_4k_{11}k_{13} + k_5k_{11}k_{13})(1 + H/K_1 + H^2/K_1K_3)B + [(k_4k_{11}k_{13} + k_5k_{11}k_{13})(1 + H/K_2) + (k_3k_5k_{12} + k_3k_5k_{13})(1 + K_6/H) + k_3k_5k_{11} + k_3k_5k_{13}]AB\} \quad (18)$$

From eq 18 the following expressions are obtained for V/E_t, V/K_aE_t, and V/K_bE_t:

$$V/E_t = k_3k_5k_{11}k_{13}/\{(k_{11}k_{13}(k_4 + k_5))(1 + H/K_2) + k_3k_5((k_{12} + k_{13})(1 + K_6/H) + k_{11} + k_{13})\} \quad (19)$$

$$V/K_aE_t = k_3k_5/K_a(k_4 + k_5)(1 + H/K_1 + H^2/K_1K_3) \quad (20)$$

$$V/K_bE_t = k_{11}k_{13}/K_b(k_{12} + k_{13})(1 + K_5/H) \quad (21)$$

Thus, V/K_a will decrease with an eventual slope of 2 at lower pH, while V/K_b will decrease with a slope of 1 at high pH. *V*, on the other hand, will decrease at high and low pH, as long as both half-reactions are partially rate-limiting. Limitation more by one half-reaction than the other will result in a perturbation in the p*K* value(s) of the least rate-limiting half-reaction greater than that of the more rate-limiting half-reaction. For example, a slow first half-reaction will, for the preceding model, give little decrease in the acidic p*K* observed in *V* compared to that observed in V/K_a, but will give a significant increase in the basic p*K* observed in *V* compared to that observed in V/K_b. If, however, one or the other half-reaction limits completely under *V* conditions, one or the other terms in the denominator will predominate. *V* will decrease at low pH if the first half-reaction limits [$V/E_t = k_3k_5/(k_4 + k_5)(1 + H/K_2)$] and at high pH if the second half-reaction limits [$V/E_t = k_{11}k_{13}/[(k_{12} + k_{13})(1 + K_6/H) + k_{11}k_{13}]$].

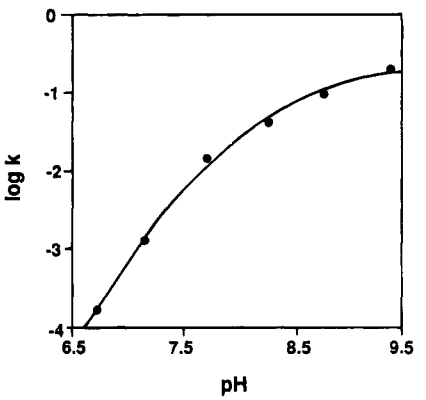
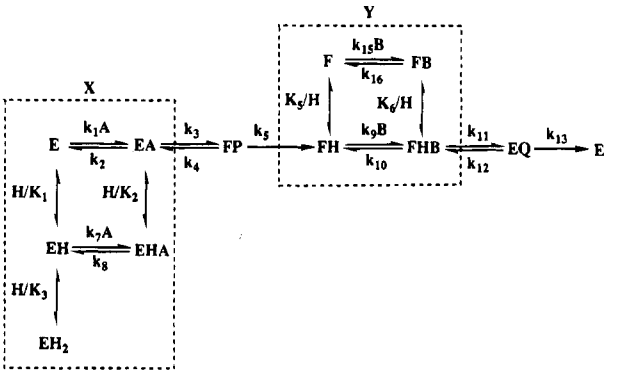
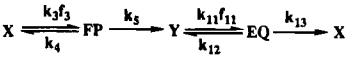


FIGURE 1: Dependence of the first-order rate constant (units are minute^{-1}) for rearrangement of OAS to NAS. Data were obtained at 25 °C. The points shown are experimentally determined values, while the curve is from a fit of the data using eq 11.

Scheme 1





The rapid equilibrium assumption is used in the preceding derivation. If the mechanism is not in rapid equilibrium, K_a and K_b will not be dissociation constant, but it may contain other rate constants for reactant or product release. In addition, measured p*K* values will not be intrinsic, but will be perturbed to lower pH for p*K*'s on the acid side or to higher pH for p*K*'s on the basic side.

RESULTS

Titration of TNB. Thionitrobenzoate was titrated from pH 2.5 to 9.5. A plot of the extinction coefficient at 412 nm as a function of pH shows a titration curve for a single group giving a p*K* of 4.8 ± 0.1 for the 5-thiol, making TNB a useful substrate for the entire pH-accessible range of OASS-A activity (data not shown).

*Titration of O-Acetyl-*L*-serine.* The first-order rate constant for the degradation of OAS was obtained as a function of pH over the range 6.5−9.5. A plot of log *k* vs pH is shown in Figure 1 and gives a limiting slope of 2 at low pH. The p*K* values estimated from Figure 1 are 8.7 ± 0.1 and 7.7 ± 0.1, while the pH independent value of the rate constant for degradation of OAS is 0.16 ± 0.018 min^{-1}. A plausible intramolecular rearrangement mechanism for the conversion of OAS to NAS is discussed in the following.

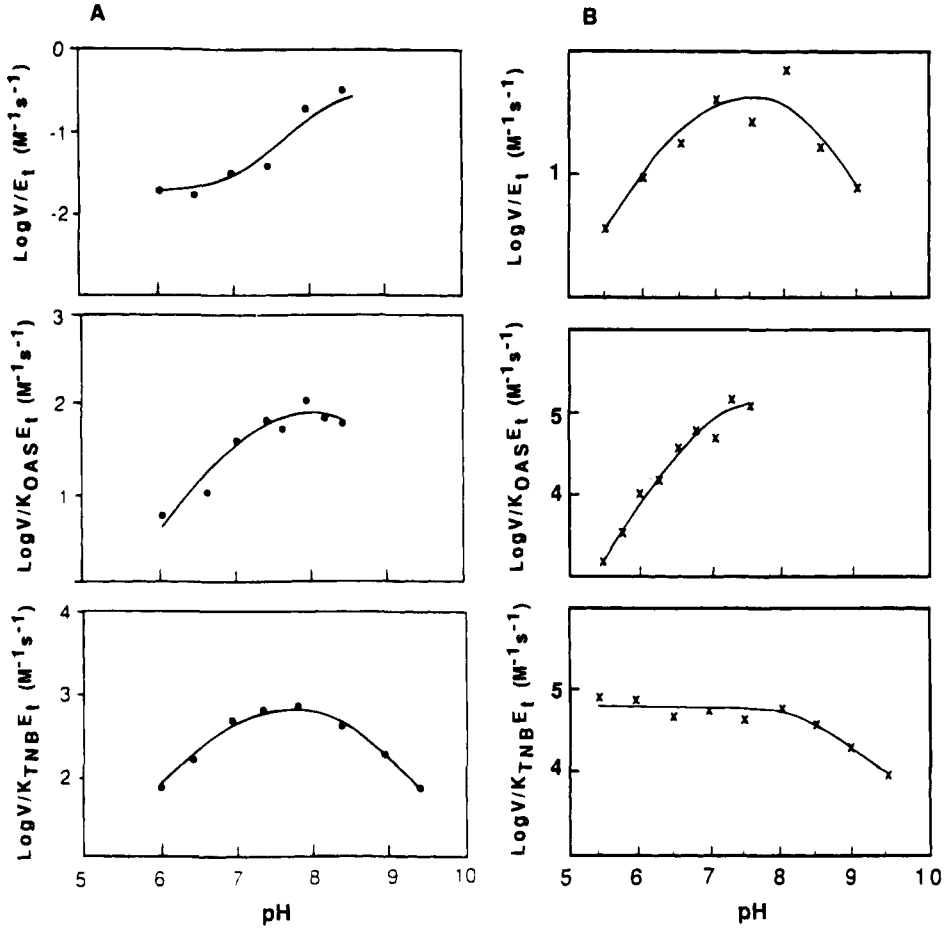


FIGURE 2: (A) pH dependence of kinetic parameters for the *O*-acetylserine sulfhydrylase-A reaction using the OAS/TNB substrate pair. Data were obtained at 25 °C for V, V/K_{TNB}, and V/K_{OAS}. The points shown are experimentally determined values, while the curves are from fits of the data using eq 10 for V and eq 9 for V/K_{OAS} and V/K_{TNB}. (B) pH dependence of kinetic parameters for *O*-acetylserine sulfhydrylase-B using OAS/TNB as substrate. Data were obtained at 25 °C for V, V/K_{OAS}, and V/K_{TNB}. The points shown are experimentally determined values, while the lines are theoretical curves for fits to the data using eq 9 for V, eq 11 for V/K_{OAS}, and eq 8 for V/K_{TNB}.

pH Dependence of Kinetic Parameters for OASS-A. It is important to know the pH dependence of a kinetic mechanism and, thus, the enzyme form(s) represented by the kinetic parameter measured as a function of pH. Initial velocity patterns obtained for all substrate pairs at the extremes of pH are consistent with a ping-pong kinetic mechanism, with competitive inhibition by both substrates. The pH dependence of kinetic parameters for the OAS/sulfide substrate pair was also determined from initial velocity patterns, as described in Materials and Methods. The V and $V/K_{sulfide}$ pH profiles decrease below pK's of 7.1 and 7.3, respectively (data not shown). Due to the degradation of OAS at high pH, data for V/K_{OAS} were only obtained from pH 5.5 to 7.5 and give a pK of about 7. Estimates of the values at pH 7.0 of V/E_t and $V/K_{sulfide}E_t$ are 280 ± 140 s^{-1} and (8 ± 3) × 10^7 M^{-1} s^{-1}, respectively. (An accurate estimate of the pH independent value for $V/K_{OAS}E_t$ could not be calculated.)

The V/K for one substrate is independent of the concentration of the other substrate in a ping-pong kinetic mechanism. Thus, for the OAS/TNB and BCA/TNB substrate pairs, the V/K for each substrate was determined by maintaining the concentration of one substrate constant and varying the concentration of the second. Since both reactants give substrate inhibition, the fixed substrate was maintained at a level one-tenth of its K_I and the other substrate was varied.

By using the OAS/TNB substrate pair, the pH dependencies of V, V/K_{OAS}, and V/K_{TNB} are shown in Figure 2A. The V/K_{OAS} and V/K_{TNB} profiles decrease at both high and low pH with slopes of −1 and 1, respectively. The V profile increases from a constant value at low pH to another constant value at high pH, giving a pK at or above 9. The V/K for OAS decreases below a pK of about 7, while the V/K for TNB decreases below a pK of 7.1 and above a pK of 8.2. The pH independent values of $V/K_{OAS}E_t$ and $V/K_{TNB}E_t$ are 125 ± 60 and 1000 ± 100 M^{-1} s^{-1}, respectively. All pK values are summarized in Table 1.

The pH dependencies of the kinetic parameters for the BCA/TNB substrate pair, V, V/K_{BCA}, and V/K_{TNB}, are shown in Figure 3A. The V/K_{BCA} profile decreases at low pH with a slope of 1 and at high pH with a slope of −1 and gives pK values of 6.7 and 7.4, which are similar to those observed for V/K_{OAS}. The V/K_{TNB} and V profiles are similar to those obtained with the OAS/TNB substrate pair. The V/K_{TNB} decreases below a pK of 6.9 and above a pK of 8.3, and the V profile increases from a constant value at low pH to an another constant value at high pH, giving a pK value at or above 9. The pH independent values of $V/K_{BCA}E_t$ and $V/K_{TNB}E_t$ are 5.0 ± 0.8 and 1000 ± 400 M^{-1} s^{-1}, respectively. In all cases the calculated pH independent values of the kinetic parameters are very similar to the values reported previously at pH 7 (Tai et al., 1993).

The inhibition constants for thiocyanate and acetate are pH independent (data not shown), indicating that the groups present in the V/K profiles reflecting the second half-reaction likely are not involved in binding of these inhibitors.[3]

Table 1: Summary of pK Values Obtained from the pH Dependence of Kinetic Parameters

O-Acetylserine Sulfhydrylase-A		
OAS/TNB		
V/E_t		9.0[a]
$V/K_{OAS}E_t$	7.45 ± 0.7	ND
$V/K_{TNB}E_t$	7.1 ± 0.1	8.2 ± 0.1
BCA/TNB		
V/E_t		9.0[a]
$V/K_{BCA}E_t$	6.7 ± 0.1	7.4 ± 0.1
$V/K_{TNB}E_t$	6.9 ± 0.2	8.3 ± 0.2
OAS/sulfide		
V/E_t	7.1 ± 0.3	ND
$V/K_{OAS}E_t$	7.0[a]	ND
$V/K_{sulfide}E_t$	7.3 ± 0.3	ND
O-Acetylserine Sulfhydrylase-B		
OAS/sulfide		
V/E_t		
$V/K_{OAS}E_t$	6[a]	
	7[a]	
$V/K_{sulfide}E_t$		
OAS/TNB		
V/E_t	6.7 ± 0.6	8.2 ± 0.7
$V/K_{OAS}E_t$	5.9 ± 0.1	
$V/K_{TNB}E_t$		8.7 ± 0.1
BCA/TNB		
V/E_t	6.5 ± 0.1	
$V/K_{BCA}E_t$	7.6 ± 0.3	9.0 ± 0.3
$V/K_{TNB}E_t$	7.6 ± 0.3	8.9 ± 0.1

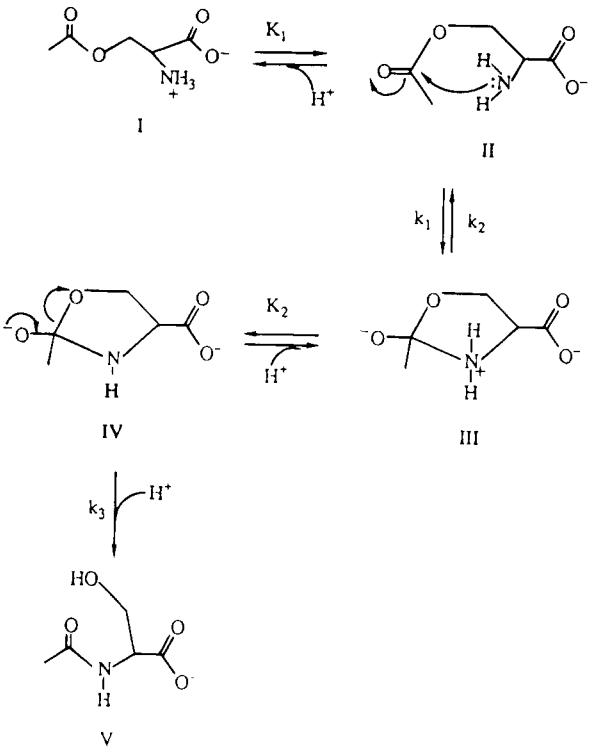
[a] Estimated values; ND, not determined.

Thermodynamics of the OASS-A Reaction. Addition of 50 μM OAS to 25 μM OASS results in the conversion of the free enzyme or E form to the α-aminoacrylate intermediate or F form of the enzyme that absorbs at 470 nm. As sodium acetate is added to F in incremental steps, a spectral shift occurs with a decrease in the absorbances at 320 and 470 nm concomitant with an increase in the absorbance at 412 nm, as a result of regenerating E. At infinite acetate concentration (or zero OAS concentration) the enzyme is entirely in the E form. An example of a titration of F with acetate is shown in Figure 4A. The decrease in A_{470} with increasing acetate represents a reversal of the first half-reaction. From the acetate dependence of A_{470}, the K_{eq} at pH 6 is calculated as discussed in Materials and Methods. A plot of log(K_{eq}) (calculated as in Materials and Methods) vs pH is shown in Figure 4B and gives a slope of 1, which is indicative of a single proton being involved in the equilibrium. A value of K_{eq}' (the pH independent value of K_{eq}) determined from the average values measured at each pH according to $K_{eq}' = (K_{eq})[H^+]$ is $(1.6 \pm 0.4) \times 10^{-3}$ M. From the latter, a value of −5.1 kcal/mol is obtained for $\Delta G^{o\prime}$. All attempts to measure the equilibrium constant for the second half-reaction, by driving the reaction in the reverse direction by the addition of L-cysteine to OASS-A, failed. On the basis of the amount of F that could have been formed but not detected (~5%), a lower limit of about 10^5 is calculated at pH 6 for the second half-reaction equilibrium constant in the direction of L-cysteine formation.

pH Dependence of Kinetic Parameters for OASS-B. Initial velocity patterns were measured for OASS-B using OAS and sulfide as substrates over the pH range 6−7.5. Over this pH range, initial velocity patterns are consistent with a ping-pong mechanism (Tai et al., 1993). Figure 5 shows the pH dependence of V/E_t, $V/K_{OAS}E_t$, and $V/K_{sulfide}E_t$. V and $V/K_{sulfide}$ are pH independent from pH 6 to 7.5, while the V/K for OAS decreases below estimated pK values of 7 and 6. The pH independent values of these parameters are $V/E_t = 190 \pm 50$ s^{-1}, $V/K_{OAS}E_t = (2.5 \pm 0.3) \times 10^5$ M^{-1} s^{-1}, and $V/K_{sulfide}E_t = (6.5 \pm 2.1) \times 10^6$ M^{-1} s^{-1}.

Figure 3B shows the pH dependencies of V/E_t (determined as discussed in Materials and Methods), $V/K_{BCA}E_t$, and $V/K_{TNB}E_t$. The V/K for BCA decreases below a pK of 8 and above a pK of 9, while V/K for TNB decreases above a pK of 8.9. V decreases below a pK of 6.5 and probably also decreases at higher pH with a pK of about 9. The pH independent values of these parameters are $V/E_t = 6.4 \pm 0.6$ s^{-1}, $V/K_{BCA}E_t = (3.2 \pm 0.6) \times 10^4$ M^{-1} s^{-1}, and $V/K_{TNB}E_t = (4 \pm 0.4) \times 10^4$ M^{-1} s^{-1}. Figure 2B shows the pH dependencies of V/E_t, $V/K_{OAS}E_t$, and $V/K_{TNB}E_t$. The value of V decreases below a pK of 6.7 and above a pK of 8.2. The V/K for OAS decreases below pK values of 5.9 and 7.1, while V/K for TNB decreases above a pK of 8.7. The pH independent values of these parameters are $V/E_t = 58 \pm 24$ s^{-1}, $V/K_{OAS}E_t = (2 \pm 0.3) \times 10^5$ M^{-1} s^{-1}, and $V/K_{TNB}E_t = (6.3 \pm 0.6) \times 10^4$ M^{-1} s^{-1}. All pK values are summarized in Table 1. In all cases, the calculated pH independent values of the kinetic parameters are very similar to the values reported previously at pH 7 (Tai et al., 1993).

Scheme 2: Proposed Mechanism for the Rearrangement of OAS to NAS



DISCUSSION

Titration of the α-Amine of O-Acetyl-L-serine. A search of the literature failed to produce a value for the α-amine pK of OAS. As a result, the pK of the α-amine of OAS was determined from the pH dependence of its stability. The anionic form of OAS is unstable and undergoes an intramolecular acyl migration from the β-hydroxyl to the α-amine (Scheme 2). The log of the first-order rate constant for the disappearance of OAS decreases as the pH decreases with a

[3] McClure and Cook (1994) have suggested that acetate binds to the α-carboxyl subsite of the amino acid substrate binding site, in addition to its combination as a product. It is likely that thiocyanate combines to the same site.

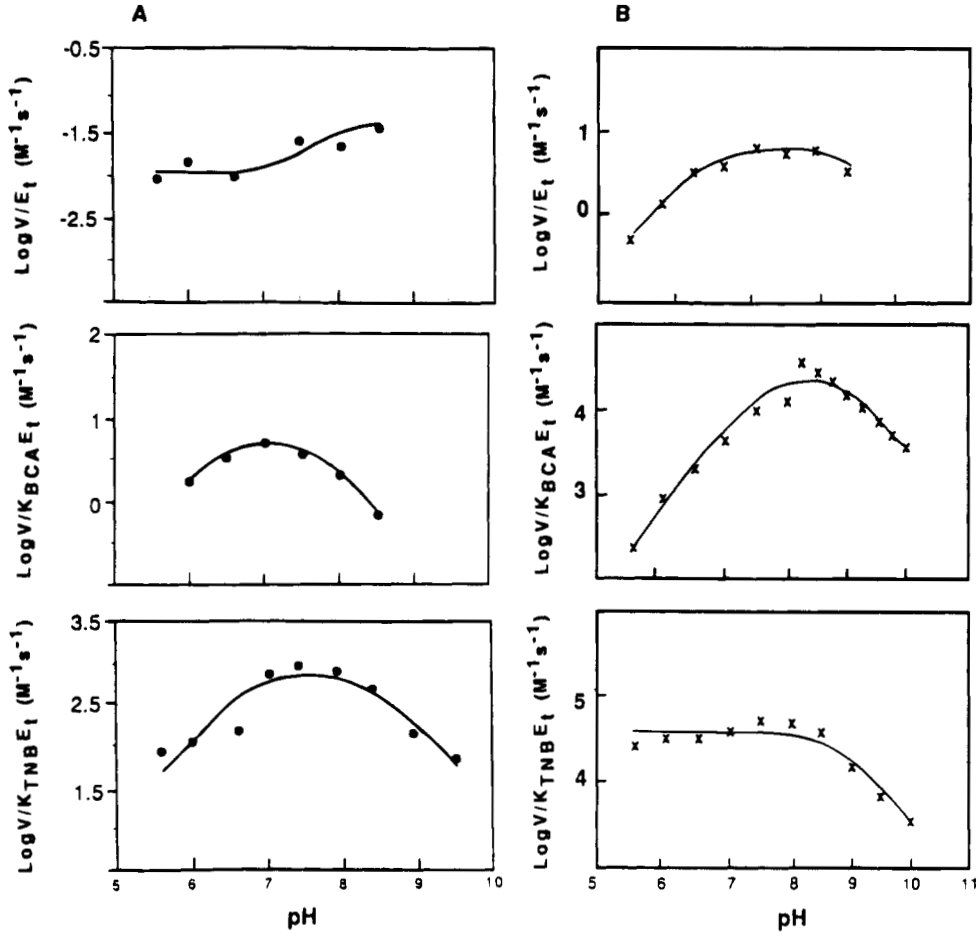


FIGURE 3: (A) pH dependence of kinetic parameters for the *O*-acetylserine sulfhydrylase-A reaction using the BCA/TNB substrate pair. Data were obtained at 25 °C for V, V/K_{TNB}, and V/K_{BCA}. The points shown are experimentally determined values, while the curves are from fits of the data using eq 10 for V, eq 8 for V/K_{TNB}, and eq 9 for V/K_{OAS} and V/K_{BCA}. (B) pH dependence of kinetic parameters for *O*-acetylserine sulfhydrylase-B using BCA/TNB as substrate. Data were obtained at 25 °C for V, V/K_{BCA}, and V/K_{TNB}. The points shown are experimentally determined values, while the lines are theoretical curves for fits to the data using eq 9 for V and V/K_{BCA} and eq 8 for V/K_{TNB}.

limiting slope of 2, giving pK values of 8.7 and 7.7. The pK of 7.7 likely reflects the pK_a for the α-amine of OAS. The value of 7.7 can be compared to a value of 9.2 for L-serine (Dawson et al., 1991). It is expected that the α-amine pK_a of OAS would be lower than that of L-serine as a result of the increased electron-withdrawing ability of the resonance-restricted acetyl side chain compared to that of a hydroxyl. The pK is somewhat higher than the value of 7.4 assigned to the α-amine of β-chloro-L-alanine, as expected. The pK_a of 8.7 then likely reflects deprotonation of the amine nitrogen in the tetrahedral intermediate formed after nucleophilic attack of the α-amine nitrogen on the acyl side chain carbonyl (Scheme 2).

O-Acetyl-L-serine is converted to NAS at a rate of 1% per minute at pH 7.6 (Flavin et al., 1965). At low pH, the α-amine of OAS is protonated, greatly decreasing the rate of nonenzymatic conversion of OAS to NAS. According to the proposed mechanism, the neutral α-amine of OAS (I, Scheme 2) results from deprotonation with a pK of 7.7 (II, Scheme 2). The neutral amine carries out a nucleophilic attack on the acyl side chain carbonyl, giving rise to a carbinolamine intermediate (III, Scheme 2). The tetrahedral intermediate must lose a proton in order to collapse to the product *N*-acetyl-L-serine (V, Scheme 2). The pK of 8.7 may not be an intrinsic value and may reflect the rate of formation and decomposition of the tetrahedral intermediate and the collapse of the unprotonated intermediate to NAS. The rate equation for the mechanism shown in Scheme 2 is given in eq 22, derived by assuming rapid equilibration of species I and II and species III and IV:

$$k_{obs} = k_1k_3/(k_2[1 + H/K_2] + k_3)(1 + H/K_1) \quad (22)$$

The values of k_{obs} under conditions in which $H \gg K_1$, K_2, $K_2 < H < K_1$, and $H \ll K_1$, K_2 are as follows: $k_1k_3K_1K_2/k_2H^2$, $k_1k_3K_2/(k_2 + k_3)H$, and $k_1k_3(k_2 + k_3)$. From these equations, the expressions for the observed pK values are pK_1 and pK_2 − log(1 + k_3/k_2). Thus, the intrinsic pK for the α-amine of OAS is observed, while the intrinsic pK for the oxocarbinolamine (III) will be greater than or equal to the observed value of 8.7, dependent on the ratio of decomposition of the protonated and unprotonated forms of the tetrahedral intermediate.

O-Acetylserine Sulfhydrylase-A

Interpretation of the V/K pH Profiles for the Amino Acid Substrates. In a ping-pong kinetic mechanism, the reactant V/K's reflect the individual half-reactions (Cleland, 1977). That is, the V/K_{OAS} (V/K_{BCA}) reflects the conversion of enzyme and OAS (BCA) to F and acetate (chloride), while the $V/K_{sulfide}$ (V/K_{TNB}) reflects the conversion of F and sulfide (TNB) to E and L-cysteine (*S*-CNP-cysteine). In addition, the V/K is a measure of the rate at limiting reactant

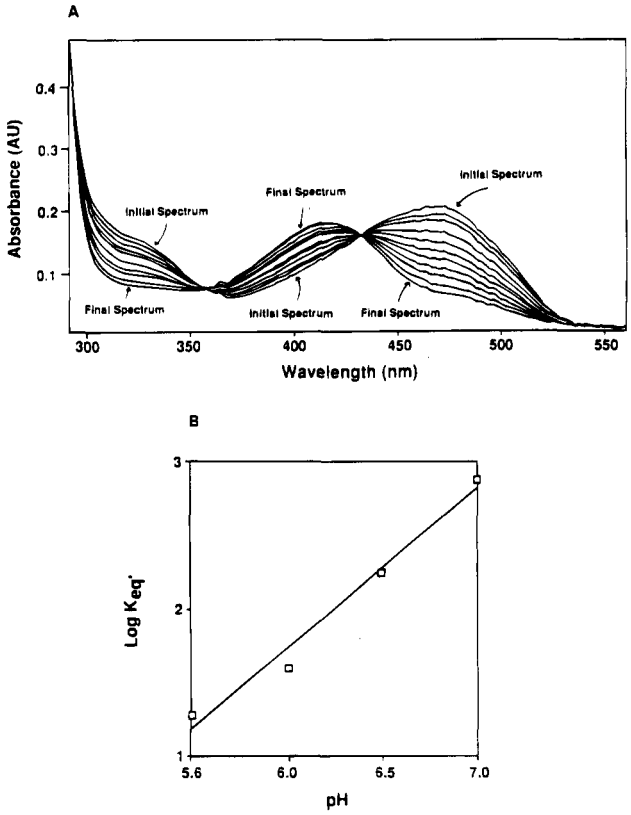


FIGURE 4: (A) Absorbance spectra of OASS-A and OAS with the stepwise addition of acetate at pH 6.0. The initial spectrum reflects the α-aminoacrylate intermediate. (B) Plot of log K_{eq}' vs pH for the first half-reaction catalyzed by OASS-A.

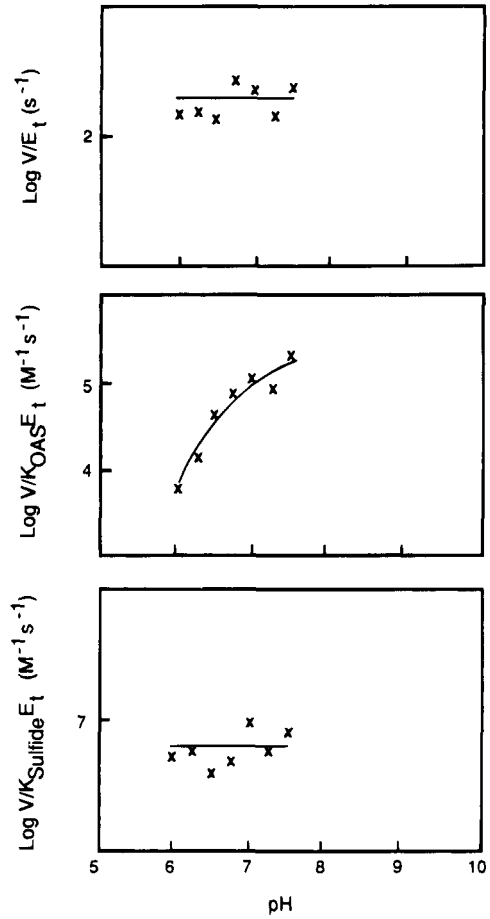


FIGURE 5: pH dependence of kinetic parameters of *O*-acetylserine sulfhydrylase-B using OAS/sulfide as substrate. Data were obtained at 25 °C for V, V/K_{OAS}, and $V/K_{sulfide}$. The points shown are experimentally determined values. The lines for V and $V/K_{sulfide}$ are average values, while the curve for V/K_{OAS} is drawn by eye.

concentration, and thus it is the uncomplexed forms of reactant and enzyme that predominate under these conditions.

With TNB as a common substrate, the V/K_{OAS} and V/K_{BCA} pH profiles (reflecting free E and free amino acid substrate) are obtained and can be compared with respect to the enzyme's interaction with the leaving group. In the case of V/K_{OAS} and V/K_{BCA}, a group with a pK of about 6.7−7 is observed on the acid side of the pH profile. The pH stability of BCA allows its use as a substrate at limiting concentrations over a wider pH range than can be used for OAS. The V/K_{BCA} decreases at high pH giving a pK_a of 7.4, which is suggested to be the pK for the α-amine of BCA. In agreement with the low value of the pK, a value of 7.9 is obtained from the pH dependence of the V/K for BCA in the β-elimination reaction catalyzed by alanine aminotransferase (Morino et al., 1979). Although OAS is unstable at high pH and data cannot be collected, it is expected on the basis of analogy to BCA that its α-amine would also be observed on the basic side of the pH profile.

Invaluable in interpreting the preceding profiles are data obtained from the pH dependence of the dissociation constant for the L-serine external Schiff base (Schnackerz et al., 1995). A plot of the log of $1/K_d$ for the L-serine Schiff base decreases with a limiting slope of +2 at low pH, with one of the pK's reflecting the α-amine of L-serine and the second reflecting an enzyme residue that must be unprotonated to hydrogen bond to the β-hydroxyl of L-serine upon binding and subsequent formation of the external Schiff base. There is no reason to believe that the α-amine of OAS (or BCA) would have a protonation state any different from that of L-serine. The side chain of OAS (or BCA), however, is a hydrogen bond acceptor, not a donor as for L-serine. As a result, the enzyme group would be expected to be protonated to interact with OAS (or BCA) and unprotonated to interact with L-serine. On this basis, the group with a pK of 6.7−7 would be protonated and the α-amine of OAS (or BCA) would be unprotonated for binding and reaction. That is, the groups on the acidic and basic sides of the V/K_{OAS} and V/K_{BCA} pH profiles have reverse protonation states, with the acidic side group required to be protonated and the group on the basic side (observed only for BCA because of the instability of OAS) required to be unprotonated (Cleland, 1977).

Data for OAS/sulfide are more difficult to obtain because of the tedious nature of the assay and the time (30 min/assay) and enzyme amounts (30 mL assay volume) involved. For example, it is difficult to complete an initial velocity pattern in a single day. Those data collected with OAS/sulfide, however, are qualitatively consistent with the preceding interpretation (data not shown). The OASS-A kinetic mechanism is known to have some random nature, and as stated previously (Tai et al., 1993), this is the most likely reason that the absolute values for $V/K_{OAS}E_t$ differ when sulfide and TNB are used as nucleophilic substrates.

Interpretation of the V/K pH Profiles for the Nucleophilic Substrates. The V/K for a given substrate in a ping-pong kinetic mechanism is independent of the concentration or identity of the second substrate, and thus the V/K_{TNB} pH profile should be identical whether OAS or BCA is the amino acid substrate. As can be seen in Figures 2 and 3, this

expectation is realized. The pK_a's for two groups are reflected in both profiles, with values of 6.9−7.1 on the acidic side and 8.2−8.3 on the basic side. The group with a pK_a of 8.2−8.3 is attributed to the ϵ-amino group of the enzyme active site lysine[2] that originally formed the internal Schiff base with PLP. The lysine likely donates a proton to the α-carbon to form the product external Schiff base (see the following). The value of 8.2 is in agreement with the reported pK value obtained by Cook et al. (1992) from a measurement of the pH dependence of the conversion of the α-aminoacrylate intermediate to pyruvate and ammonia catalyzed by OASS-A. These authors assigned the pK of 8.2 to the ϵ-amino group of the active site lysine that originally formed the internal Schiff base and must be unprotonated to displace α-aminoacrylate from the active site PLP by transimination.

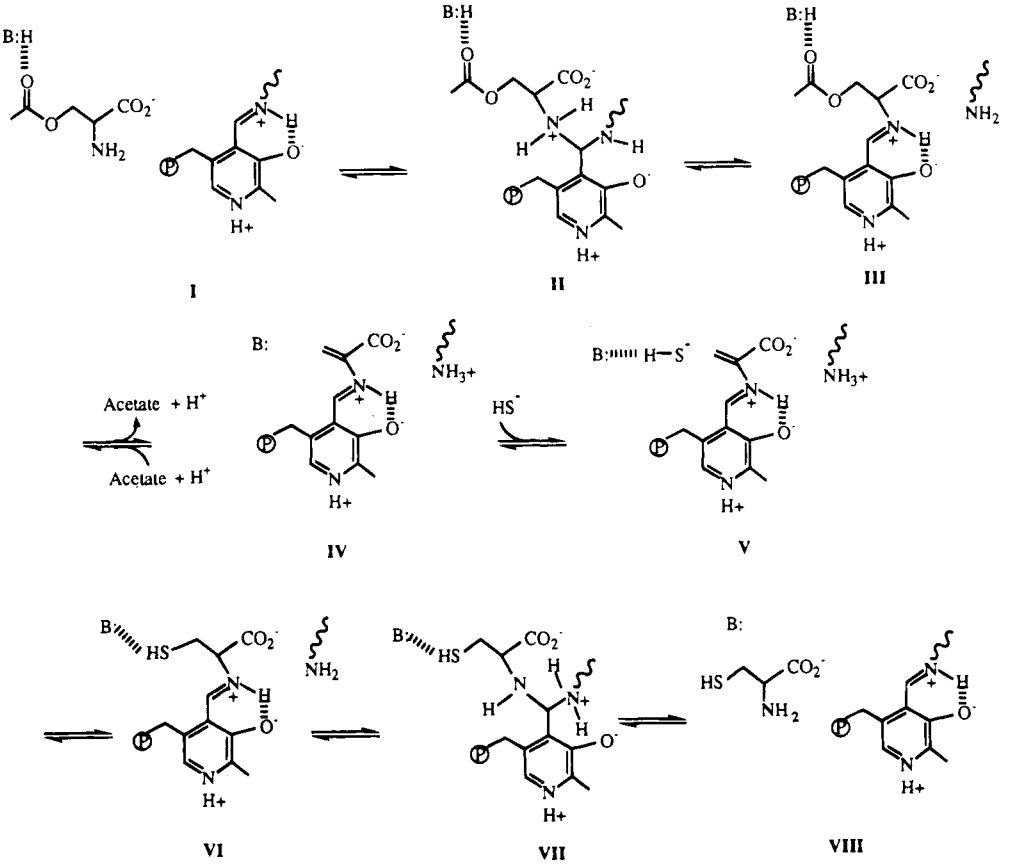
The group with a pK of about 7 observed in the TNB V/K pH profile is likely the same one that is seen in the OAS V/K pH profile. The group is required to be unprotonated to begin the second half-reaction and is suggested to interact with the nucleophilic substrate. Of use in interpreting these data are data obtained for the pH dependence of the K_d for the L-cysteine external Schiff base (Schnackerz et al., 1995). Data have been interpreted as for the L-serine Schiff base with the added requirement that the thiol side chain must be protonated to hydrogen bond to the unprotonated residue with a pK of about 7. By extrapolating these findings to the second half-reaction in which the α-aminoacrylate intermediate and HS^- are the reactants, the enzyme group with a pK around 7 observed in the V/K_{TNB} pH profile likely hydrogen bonds HS^- prior to nucleophilic attack on C3 of α-aminoacrylate. With TNB as the substrate, the group is still required to be unprotonated for binding and catalysis. There is no hydrogen bond donor on TNB, but it is an aromatic, hydrophobic compound. If bound such that the hydrophilic substituents are directed away from the enzyme residue, and if the residue is a cationic acid such as the imidazole side chain of histidine, one would expect enzyme with the neutral (unprotonated) form of imidazole to be favored upon binding of TNB. We would thus predict that the enzyme residue with a pK around 7 is a cationic acid. The data for the $V/K_{sulfide}$ pH profile, although limited, are consistent with this interpretation.

pH Dependence of Inhibitor Dissociation Constants. True pK values are observed in the pH profiles for the dissociation constants for competitive inhibitors or slow substrates (Cleland, 1977). To determine whether intrinsic pK values are observed in the V/K profiles for TNB (using the OAS/TNB substrate pair), pK_i profiles for SCN^- and acetate were obtained. The inhibition constants for thiocyanate and acetate show little, if any, pH dependence.

Interpretation of the V pH Profiles. The V_{max} profile obtained by using OAS/TNB and BCA/TNB substrate pairs exhibits a partial change in activity as the pH is decreased below 9. These data indicate the need for an enzyme group that must be unprotonated for optimum activity. However, even when this group is protonated the reaction proceeds at about 3% of the maximum rate observed above pH 9 with OAS and at about 20% of the rate with BCA. It is difficult to interpret the V profile in the absence of information on the location of rate-determining steps, but several possibilities can be ruled out. The pK for the α-amine of OAS will not be observed in the V profile since reactant is bound under V conditions. The groups responsible for the pK values observed in the V/K_{TNB} pH profiles are both observed with both amino acid substrates. The group with a pK of 8.2 is that of the active site lysine required to protonate the α-carbon upon the addition of TNB; this group is required to be unprotonated to remove a proton from the α-carbon of OAS in the first half-reaction. It is unlikely, however, that the reaction would proceed at a significant rate, even 3%, when the lysine is in the incorrect protonation state. The group with a pK of 7 observed in the V/K_{TNB} pH profile (suggested to be identical to that observed in the V/K_{OAS} pH profile) is thought to be required to be protonated for interaction with the acetyl side chain of OAS and unprotonated for TNB binding. With the latter function, the reaction might be expected to proceed at a finite rate when this group is in the incorrect protonation state. The group may still be able to hydrogen bond to the acetyl side chain of OAS via a water or directly hydrogen bond to TNB with a geometry that may not be optimum for the subsequent reaction. The increase in the pK from the value of 7 observed for F (V/K_{TNB}) or 7.4 observed for E (V/K_{OAS}) to a value of 9 observed for the complexes E:OAS to F or F:TNB to E:L-cysteine could easily result from environmental differences in these enzyme forms. Differences in the amount of remaining activity measured with OAS and BCA could be a result of the relative rate limitation of the first and second half-reactions. A more complete explanation will have to await a thorough study of rate limitation in the reaction.

Chemical Mechanism. On the basis of the preceding information, a reasonable chemical mechanism is proposed in Scheme 3. To begin the reaction sequence, the active site PLP internal Schiff base is protonated at both the imine and pyridinium nitrogens, and the PLP 5′-phosphate is the dianion (Cook et al., 1992). In addition, an enzyme group with a pK of 6.7−7 must be protonated for optimum activity, and the amino acid substrate (OAS is depicted) binds as the monoanion (I, Scheme 3). The neutral α-amine of the amino acid substrate (I, Scheme 3) attacks C4′ of the internal Schiff base, resulting in the formation of an external Schiff base (III, Scheme 3) via *gem*-diamine intermediates (one of these is pictured as II, Scheme 3). The lysine that initially formed the internal Schiff base with PLP is released in the process. The lysine then acts as a general base to accept a proton from the α-carbon of the external Schiff base, and the amino acid side chain is β-eliminated to form α-aminoacrylate in Schiff base with PLP (IV, Scheme 3). In agreement with the assignment of the lysine as the general base, the K42A mutant is inactive even though the enzyme's structure is unaltered: it binds PLP and can form an external Schiff base such as III (unpublished work of V. Rege in this laboratory). The α,β-elimination occurs in this manner for both OAS and BCA. With OAS as the substrate, the enzyme group with a pK of 6.7−7 is positioned near the carbonyl of the acetyl leaving group and is hydrogen-bonded to the carbonyl oxygen to facilitate the β-elimination of acetate, while with BCA it hydrogen bonds to the chlorine. The release of acetate (and a proton) thus ends the first half-reaction with the α-aminoacrylate external Schiff base protonated, as was the internal Schiff base to begin the first half-reaction. The first half-reaction is reversible with a pH independent equilibrium constant of 1.6×10^{-3} M. The $\Delta G^{\circ\prime}$ of the first half-reaction is −5.7 kcal/mol, and it is thus exergonic. The latter is consistent with elimination of the resonance-restricted acetate.

Scheme 3: Proposed Acid–Base Chemical Mechanism for OASS-A



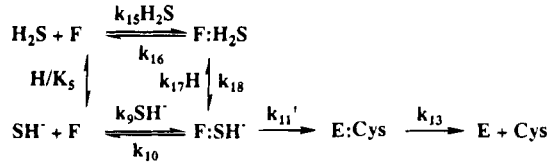
The lysine that originally formed the internal Schiff base is protonated, and the group with a pK of 7 is unprotonated to start the second half-reaction. Sulfide binds as HS^-, as discussed earlier (V, Scheme 3). The latter is aided by the group with a pK of about 7, which hydrogen bonds to the substrate. (When the substrate is TNB, the group likely remains unprotonated and neutral.) Nucleophilic attack by TNB or sulfide on C3 of the α-aminoacrylate Schiff base is accompanied by protonation of the α-carbon using the active site lysine (pK 8.2; Cook et al., 1992), generating the product external Schiff base (VI, Scheme 3). Finally, the unprotonated ε-amino group of the active site lysine attacks the C4′ carbon of the external Schiff base to generate a product *gem*-diamine (VII, Scheme 3), which collapses to regenerate free enzyme and amino acid product (VIII, Scheme 3). As described in the Results, the second half-reaction is essentially irreversible. The irreversible step appears to be the nucleophilic addition of the second substrate to the α-aminoacrylate intermediate, since the external Schiff base with L-cysteine can be formed from free enzyme and free L-cysteine (Schnackerz et al., 1995). [The reaction is not actually irreversible, since isotope exchange between sulfide and L-cysteine has been observed (Cook & Wedding, 1976).]-

At the end of the first half-reaction the α-aminoacrylate Schiff base is protonated, while the ε-amine of the active site lysine that formed the internal aldimine must also be protonated. As the pH increases such that the lysine is neutral, the α-aminoacrylate intermediate decomposes to pyruvate and ammonia in the absence of the nucleophilic substrate as a result of transaldimination to release α-aminoacrylate (Cook et al., 1992). The enzyme thus has apparently evolved to maximize the stability of the α-aminoacrylate intermediate at pH 7, while still allowing the lysine a low enough pK to act efficiently as a general base.

O-Acetylserine Sulfhydrylase-B

Interpretation of the V/K pH Profiles. With TNB as a common substrate, the V/K_{OAS} and V/K_{BCA} pH profiles (reflecting free E and free amino acid substrate) have been obtained and, as for OASS-A, can be compared with respect to the interaction of the enzyme with the substrate leaving group. In the case of OAS, pK's of about 6 and 7 are observed on the acidic side, while the basic side of the profile is undefined as a result of the instability of OAS. (Although the pK of 7 is reasonably well-defined in the V/K_{OAS} profile, it is doubtful because of the instability of OAS that it really is as low as pH 7.) The profile obtained with BCA, on the other hand, exhibits only a single pK of about 7.6 on the acid side, while it decreases above a pK of 9.0. These data are consistent with optimum binding of monoanionic BCA (with its α-amine unprotonated) to undergo nucleophilic attack on C4′ of the external Schiff base (see the following). The group with a pK of 9 likely reflects an enzyme group involved in binding the amino acid substrate, probably interacting with the α-carboxyl group. On the basis of analogy to the V/K_{BCA}, the V/K_{OAS} is expected to reflect the pK for OAS on the acid side of the pH profile if, as stated earlier, data could be collected to a high enough pH, and the group with a pK of 9 would also be expected to be observed to interact with the OAS carboxyl. The group with a pK of 6 observed in the V/K_{OAS} pH profile likely interacts with the acetyl leaving group, but for the B isozyme it apparently is not necessary when BCA is the substrate and chloride is the leaving group. It is suggested that the group with a pK of 6 is required to polarize the carbonyl oxygen of the acetyl leaving group to facilitate β-elimination. Since

Scheme 4



the group is likely required to donate a hydrogen bond for optimum binding and catalysis, it likely does so via an intervening water molecule. Data for OAS/sulfide are more difficult to obtain as stated earlier, but those data collected are consistent with the preceding interpretation.

As stated earlier for OASS-A, the V/K_{TNB} pH profiles should be identical whether OAS or BCA is the amino acid substrate, and this is true as seen in Figures 2 and 3. A single pK is observed on the basic side of each profile with an observed value of about 8.7−8.9. On the basis of analogy to OASS-A, the pK is attributed to the ϵ-amino group of the enzyme active site lysine that originally participated in internal Schiff base linkage, and this group, when protonated, likely donates a proton to the α-carbon to form the *S*-CNP-cysteine external Schiff base (see the following). The pK of 9 is less than a pH unit higher than the value of 8.2 reported for the ϵ-amino group of the active site lysine of OASS-A (Cook et al., 1992).

The $V/K_{sulfide}$ profile is pH independent. Surprisingly, the pK of 7, reflecting protonation of HS^- to H_2S, is not observed in the $V/K_{sulfide}$ profile. One possibility for the lack of observation of the pK is that it is perturbed to a value lower than pH 6, which is out of the accessible range of the $V/K_{sulfide}$ profile. This would suggest that the F form of OASS-B binds both HS^- and H_2S, but the former with much higher affinity. Either because HS^- is sticky or because the transaldimination of the final product L-cysteine is slow, the pK for HS^- is perturbed to lower pH. Of the latter, it is likely that HS^- is a sticky substrate. A mechanism describing the stickiness of sulfide is shown in Scheme 4. The rate equation after Cleland (1977) is given:

$$V/K = \{k_9k_{11}'E_t/(k_{10} + k_{11}')[1 + k_{18}k_{15}H/k_9(k_{18} + k_{16})K_5]\}/\{(1 + H/K_5)[1 + k_{10}k_{18}k_{15}H/k_9(k_{10} + k_{11}')(k_{18} + k_{16})K_5]\} \quad (23)$$

From eq 23, the following expression is obtained for app pK:

$$\text{app } pK = pK_5 - \log(1 + k_{11}'/k_{10}) \quad (24)$$

Thus, if $k_{11}' \gg k_{10}$, the pK of H_2S of is perturbed to a lower pH. In the case of sulfide, the pK is out of the accessible pH range.

Interpretation of V Profiles. The V profiles reflect the pK's of groups on enzyme in the rate-limiting half-reaction-(s) when reactants are saturating. In the case of OAS/sulfide, the maximum velocity is pH independent from pH 6 to 7.5 and thus provides little information. Examination of the V profiles for the OAS/TNB and BCA/TNB substrate pairs shows that they decrease at low and high pH. Calculated pK values are 6.5−6.7 and 8.2−9, respectively. These pK's likely reflect the pK for the Schiff base lysine in both half-reactions (see Theory), the lower pK representing that of the unprotonated lysine that must accept a proton from the α-carbon in the β-elimination of acetate and the higher pK representing that of the lysine in the second half-reaction, which must protonate the α-carbon in the Michael addition reaction. The pK values observed in the V profiles when compared to those observed in the V/K profiles reflect the amount of rate limitation of the other half-reaction on the basis of the amount of change in the acidic side pK to lower pH and the basic side pK to higher pH. For OAS/TNB and BCA/TNB, the second half-reaction appears to be more rate-limiting. In both cases, the V pK on the basic side (8.2−9.0) is close in value to that observed in the V/K_{TNB} profile (the pK of 8.2 estimated when OAS is the substrate has a large standard error). It is difficult to compare the pK on the acid side of the profile since the lysine pK is not observed in the V/K profiles, and it is not possible to estimate a value for the lysine in the external Schiff base complex.

Chemical Mechanism. A chemical mechanism for the OASS-B reaction consistent with the pH dependence of the V and V/K profiles is similar to that shown in Scheme 3, with the following exceptions: (1) the enzyme group that interacts with the substrate leaving group requires an intervening water molecule; (2) a group that likely interacts with the α-carboxyl is required; and (3) the enzyme group that interacts with the amino acid side chain in the first half-reaction apparently has no role in the second half-reaction. (The group that interacts with the α-carboxyl may also be present in the case of OASS-A, but is not within the accessible pH range.)

Comparisons of O-Acetylserine Sulfhydrylases-A and -B. The proposed kinetic mechanism for OASS-A and -B isozymes is Bi-Bi ping-pong with all amino acid and nucleophilic substrates used thus far (Tai et al., 1994). Although the two isozymes are qualitatively similar in many respects, there are differences in the proposed acid−base mechanism. In the first half-reaction, the PLP internal Schiff base for both isozymes is protonated at the imine nitrogen. The amino acid substrate (OAS) binds to both isozymes as the monoanion. However, in the case of OASS-A, an enzyme group must be protonated for optimum activity thought to assist in the β-elimination reaction by hydrogen bonding the acetyl leaving group, while for the B isozyme and enzyme group also assists in the β-elimination of acetate, likely by polarizing the carbonyl, but via the intervention of a water molecule. For OASS-B (and maybe also for OASS-A, although it is not observed over the pH range studied) another enzyme group is required to be protonated and likely interacts with the α-carboxyl. For both isozymes, the α-amine of the substrate then undergoes nucleophilic attack on C4′ of the internal Schiff base. At the end of the first reaction, both the α-aminoacrylate Schiff base and the active site lysine are protonated for both isozymes. (In the case of the A-isozyme, a proton is released from the general acid with acetate.) At the start of the second half-reaction, sulfide likely binds as the monoanion for both isozymes, followed by Michael addition of the nucleophilic substrate to the α-aminoacrylate intermediate and protonation of the α-carbon of the amino acid product using the active site lysine to generate a product external Schiff base. However, the enzyme group that interacts with the amino acid side chain also hydrogen bonds to the nucleophilic substrate for OASS-A, but not OASS-B. The biggest difference between the two isozymes is the mechanism for polarization of the acetyl carbonyl. Finally, the B isozyme ends the reaction in the same protonation state that it started the first half-reaction, while the A isozyme does not. It is unclear at this time why

the acid−base mechanisms of these highly homologous isozymes differ.

Other Enzymes. It is difficult to compare the results obtained from pH−rate profiles for OASS with those for other PLP dependent β-replacement enzymes, since very little information is available for other PLP enzymes. However, pH−rate data are available for PLP dependent β-eliminases that catalyze similar reactions. For example, tyrosine phenol lyase (Kiick & Phillips, 1988a) and tryptophan indole lyase (Kiick & Phillips, 1988b) catalyze reactions very similar to the deacetylase reaction catalyzed by OASS-A, that is, the elimination of a nucleophile from the β-carbon of an amino acid to generate α-aminoacrylate, which is then released. Both enzymes have evolved to use the unprotonated form of the internal Schiff base between PLP and an enzyme lysine to accept the α-proton of the incoming amino acid (Phillips, 1989). Transaldimination forms the protonated external Schiff base and unprotonated lysine that likely acts as a general base to accept a proton from the α-carbon. A second enzymic base then facilitates the elimination of the amino acid side chain by accepting a proton from the leaving group. The latter mechanism differs from that proposed for OASS-A in that the Schiff base is protonated and unable to accept a proton from the α-amine of OAS and differs in the function of the enzyme in the elimination reaction. In the case of OASS, the enzyme may activate the side chain by general acid catalysis, while the β-eliminases require general base catalysis to activate the leaving group.

Tryptophan synthases from bacterial sources have been shown to have an $\alpha_2\beta_2$-subunit composition (Yanofsky et al., 1972). The dimeric β_2-subunit has many similarities with OASS-A. These two enzyme activities are PLP-dependent and catalyze β-elimination and β-substitution reactions. Although the physiological role of the β_2-subunit is to catalyze the synthesis of L-tryptophan from indole and L-serine, the β_2-subunit has been shown to catalyze several other reactions (Miles, 1979; Miles et al., 1987). With L-serine as a substrate for the β_2-subunit, a Schiff base intermediate is formed similarly to OASS (III, Scheme 3). In both instances, the α-proton is removed to facilitate the elimination of an electronegative β-substituent to form the α-aminoacrylate Schiff base (III and IV, Scheme 3). The addition of a nucleophilic substrate (for example indole in the formation of tryptophan) at the β-carbon of the α-aminoacrylate Schiff base intermediate followed by donation of a proton to the α-carbon generates the product external Schiff base (VI, Scheme 3). The biggest difference between OASS and the tryptophan synthase β_2-subunit is that the latter must activate the nucleophilic substrate (indole) for attack on the α-aminoacrylate intermediate. The tryptophan synthase β_2-subunit (Miles, 1979) and OASS (Cook et al., 1992) also catalyze the formation of pyruvate and ammonia from the α-aminoacrylate Schiff base intermediate.

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