Kinetic Mechanisms of the A and B Isozymes of *O*-Acetylserine Sulfhydrylase from *Salmonella typhimurium* LT-2 Using the Natural and Alternative Reactants†

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ABSTRACT: The resonance-stabilized quinonoid 5-mercapto-2-nitrobenzoate (TNB) is a substrate for *O*-acetylserine sulfhydrylase-A (OASS-A) and -B (OASS-B), giving rise to the product *S*-(3-carboxy-4-nitrophenyl)-L-cysteine (S-CNP-cysteine) as confirmed by ultraviolet–visible and ^{1}H NMR spectroscopies. A comparison of the kinetics of OASS-A and OASS-B indicates that the mechanism proceeds predominantly via a bi–bi ping pong kinetic mechanism as suggested by an initial velocity pattern consisting of parallel lines at low concentrations of reactants, but competitive inhibition by both substrates as the reactant concentrations are increased. Thus, in the first half-reaction, *O*-acetyl-L-serine (OAS) or β-chloro-L-alanine (BCA) is converted to α-aminoacrylate in Schiff base with the active site pyridoxal 5′-phosphate, while in the second half-reaction cysteine (with sulfide as the reactant) or S-CNP-cysteine (with TNB as the reactant) is formed. The ping pong mechanism is corroborated by a qualitative and quantitative analysis of product and dead-end inhibition. Product inhibition by acetate is S-parabolic noncompetitive. These data are consistent with acetate reversing the first half-reaction and producing more free enzyme to which acetate may also bind. Thus, there may be some randomness to the mechanism at high concentrations of the nucleophilic substrate.

The PLP[1]-dependent *O*-acetylserine sulfhydrylase catalyzes the formation of L-cysteine from sulfide and *O*-acetyl-L-serine (Becker & Tomkins, 1969). The A and B isozymes are homodimeric with subunit MWs of 34 450 (Levy & Danchin, 1988) and 27 500 (Nakamura *et al.*, 1984), respectively. The gene for the A isozyme has been sequenced (Byrne *et al.*, 1988). The second isozyme, *O*-acetylserine sulfhydrylase-B, is also PLP-dependent and catalyzes the same reaction, although it will use thiosulfate as an alternative reactant in *Salmonella typhimurium* (Hulanicka *et al.*, 1979; Kredich, 1971). The large excess of the A isozyme over the B isozyme during aerobic growth indicates that the former accounts for the majority of L-cysteine synthesis from *O*-acetyl-L-serine and sulfide (Hulanicka *et al.*, 1979; Kredich, 1971). Unlike the A isozyme, the B isozyme is required for efficient cysteine biosynthesis during anaerobic growth (Filutowicz *et al.*, 1982).

The OASS-A has previously been shown to have a ping pong kinetic mechanism that requires the β-elimination of acetate from OAS in the first half-reaction to generate α-aminoacrylate in Schiff base with the active site PLP (Cook & Wedding, 1976). The Michael addition of sulfide to the α-aminoacrylate intermediate then occurs in the second half-reaction to produce the final product L-cysteine. Two dead-end complexes, E:sulfide and F:OAS, were observed consistent with the ping pong nature of the mechanism (where F is α-aminoacrylate in Schiff base with the active site PLP). The identities of the reactions catalyzed by *O*-acetylserine sulfhydrylase-A and -B make it of interest to determine the kinetic mechanisms and substrate specificities of the two enzymes.

A computer-assisted sulfide ion selective electrode assay has recently been developed for *O*-acetylserine sulfhydrylase (Hara *et al.*, 1990). A comparison of the data obtained from the early study (Cook & Wedding, 1976) with those obtained using the computer-assisted assay suggests that the old assay overestimated the rates of the OASS reaction as a result of inadequate calibration of the electrode. Thus, it is imperative that the kinetic mechanism of the sulfhydrylase be redetermined. In addition, a number of substrate analogs of both sulfide and OAS have been identified, and the kinetic mechanism has also been determined with some of these to be certain that it does not change with the reactant used.

In this paper, we report the kinetic mechanisms of *O*-acetylserine sulfhydrylase-A and -B from *Salmonella typhimurium* using the natural substrates and two alternative substrates. The mechanism of the B isozyme is determined for the first time. Finally, a new spectrophotometric assay has been developed using 5-mercapto-2-nitrobenzoate as an alternative substrate in place of sulfide.

MATERIALS AND METHODS

Chemicals. *O*-Acetyl-L-serine, β-chloro-L-alanine, DTNB,[1] DTT, NaSCN, Na_2S, maleic acid, acetate, D_2O, and KOD

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[1] Abbreviations: PLP, pyridoxal 5′-phosphate; Na_2S, sodium sulfide; NaSCN, sodium thiocynate; OAS, *O*-acetyl-L-serine; NAS, *N*-acetyl-L-serine; DTT, dithiothreitol; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); TNB, 5-mercapto-2-nitrobenzoic acid; DSS, sodium 2,2-dimethyl-2-silapentane-5-sulfonate; BCA, β-chloro-L-alanine; *S*-CNP-cysteine, *S*-(3-carboxy-4-nitrophenyl)-L-cysteine; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; Taps, 3-[[tris(hydroxymethyl)methyl]-amino]propanesulfonic acid; Mes, 2-(*N*-morpholino)ethanesulfonic acid; Ches, 2-(*N*-cyclohexylamino)ethanesulfonic acid.

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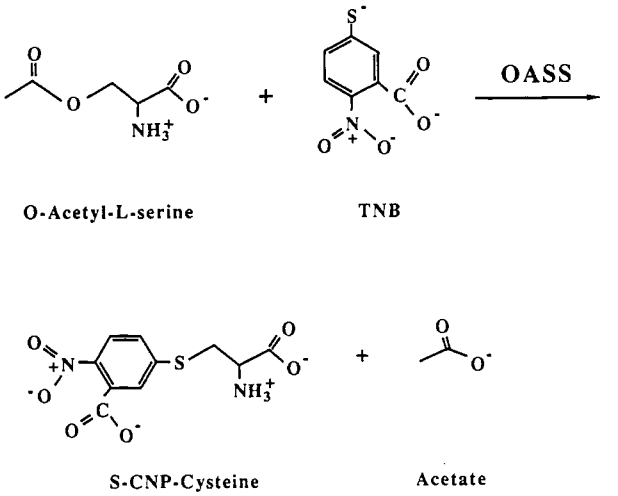
(99 atom % D) were obtained from Sigma. The reduced form of DTNB, TNB, was prepared fresh prior to use by reduction with DTT in slight molar excess to DTNB. The TNB was then used without further treatment. Neither reduced nor oxidized DTT has any effect on the OASS reaction. All other reagents and chemicals 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 an adaptation of the method of Hara *et al.* (1990) using HPLC. The present procedure differs from that developed previously in the column chromatographic procedures. The modified procedure makes use of a Phenyl 5-PW column (Toso Haas, 21.5 mm × 15 cm) after the phenyl sepharose chromatography. The sample from the phenyl sepharose column was pooled, concentrated, and dialyzed against 1.0 M $(NH_4)_2SO_4$ in 10 mM Hepes, pH 8.0, overnight, and applied to the Phenyl 5-PW column with a flow rate of 4 mL/min. The column was then developed using a linear reverse gradient from 100% A to 0% A for 60 min, where eluant A is 1 M $(NH_4)_2SO_4$, 10 mM Hepes, pH 8, and eluant B is 10 mM Hepes, pH 8. Fractions were collected every minute, monitoring the absorbance at 280 and 412 nm. Fractions with a ratio of 4 for A_{280}/A_{412} were pooled. The enzyme obtained was >95% pure based on a 12% SDS gel. A final specific activity of about 800 units/mg measured with OAS and sulfide using the sulfide ion selective electrode assay (Hara *et al.*, 1990) was obtained. The protein concentration of the purified enzyme was determined from the 412-nm absorbance of the Schiff base between the active site lysine and PLP, using an extinction coefficient of 7600 M^{-1} cm^{-1} (Kredich *et al.*, 1969).

A convenient method for the assay of complete column effluents was developed on the basis of the use of the chromophoric TNB as a substrate for the sulfhydrylase. The system consists of a 96-well microtiter plate and an Elisa plate reader (Bio-Rad Model 2550) with a 414-nm-wavelength filter. Reaction mixtures were 0.25 mL in volume per well with the following final reaction component concentrations: 100 mM Hepes, pH 7; 50 μM TNB; and 10 mM OAS. The reaction was initiated by the addition of 100 μL of water plus sample. The water, buffer, sample, and TNB were added to each well and allowed to react at room temperature for 15 min. After 15 min, a reading was obtained at 0 and 2 min to measure a background rate (usually not significant). The OAS was then added, and the plate was read at 0-, 4-, and 8-min intervals. A rate was calculated for each well from the disappearance of absorbance at 414 nm/min.

O-Acetylserine sulfhydrylase-B from *Salmonella typhimurium* LT-2 was purified by a procedure identical to that for OASS-A through the Q-Sepharose column, where OASS-B elutes at a slightly higher salt concentration. Column fractions were assayed using TNB as a substrate using the 96-well ELISA plate reader assay. Fractions containing OASS-B activity were pooled, concentrated, and dialyzed against 10 mM Hepes, pH 8. The sample was loaded onto a DEAE-5PW (Toso Haas, 21.5 mm × 15 cm) column and developed as follows. The column was first washed with 100% A for 10 min followed by a linear gradient from 0% to 20% B for 10 min, from 20% to 80% B for 90 min, and from 80% B to 100% B for 10 min. Eluant A was 10 mM Hepes, pH 8, and eluant B consisted of eluant A with 0.5 M NaCl. A flow rate of 3 mL/min was maintained, and fractions were collected every 1.5 min. The OASS-B activity was pooled, concentrated, and dialyzed overnight against 1.2 M $(NH_4)_2SO_4$, 10 mM Hepes, pH 8.

Scheme I: Reaction Catalyzed by OASS with TNB as the Nucleophilic Substrate



The pooled fractions were loaded onto an Ether-5PW (Toso Haas, 21.5 mm × 15 cm) column. The column was washed with 100% A for 10 min, followed by a linear gradient from 0% to 30% B for 90 min and from 30% to 100% B for 10 min. Eluant A was 1.2 M $(NH_4)_2SO_4$ and 10 mM Hepes, pH 8, while eluant B was 10 mM Hepes, pH 8. The flow rate was again maintained at 3 mL/min, and fractions were collected every 1.5 min. Fractions were assayed as above, and those containing OASS-B activity were pooled, concentrated, and dialyzed against 10 mM Hepes, pH 8, overnight.

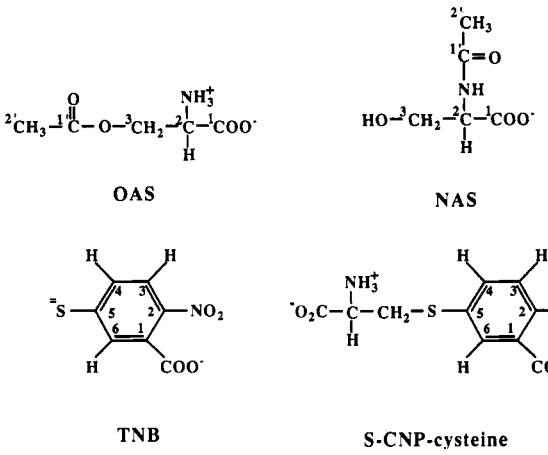
The pooled sample was then loaded onto a HP-PEI (Interaction Chemicals Inc., 7.8 mm × 10 cm) column. The column was washed with 100% A for 10 min, followed by a linear gradient from 0% to 20% B for 10 min, from 20% to 80% B for 90 min, and from 80% to 100% B for 10 min. Eluant A was 10 mM Hepes, pH 8, while eluant B was A plus 0.5 M NaCl. A flow rate of 1 mL/min was maintained, and fractions were collected every 2 min. Fractions were assayed as above, and those containing OASS-B were pooled. A 12% SDS gel was run to check for purity. The gel showed a band corresponding to a subunit molecular weight of approximately 31 000–33 000, similar to that of OASS-A.

Enzyme Assays. Two assays were used to monitor the OASS reaction dependent on the identity of the second substrate. Either the disappearence of sulfide was monitored using a computer-assisted sulfide ion selective electrode assay (Hara *et al.*, 1990) or the disappearance of TNB was followed spectrophotometrically at 412 nm (Cook *et al.*, 1991).

The sulfide electrode has been automated as described previously (Hara *et al.*, 1990). It has been observed that the sulfide standard curve has a tendency to drift slightly with the age and condition of the Ag_2S membrane of the sulfide ion selective electrode. As a result, the standard curve was repeated daily as described previously (Hara *et al.*, 1990) as a check of the equation used by the computer to calculate sulfide concentration. In addition, it is important to be certain the membrane is polished prior to use. This can be accomplished using ORION polishing strips, as suggested in the accompanying literature. After polishing, the electrode was rinsed and soaked in a standard sulfide solution for about 5 min prior to use.

The disappearance of TNB at 412 nm resulting from formation of *S*-(3-carboxy-4-nitrophenyl)-L-cysteine, Scheme I, was monitored continuously as the reaction proceeded. Data were collected using a Gilford 2600 spectrophotometer with

Chart I: Chemical Structures of OAS, TNB, S-CNP-cysteine, and *N*-Acetylserine



a flatbed plotter. A typical assay in a final volume of 1 mL contained the following: Hepes, pH 7, 100 mM; OAS, 2 mM; TNB, 0.05 mM. The reaction was initiated with OASS. Initial rates were calculated using an ϵ_{412} of 13 600 M^{-1} cm^{-1} for TNB (Ellman, 1959). The pH of the reaction mixture was measured with a Radiometer PHM 82 pH meter with a combined microelectrode before and after sufficient data were collected for determination of initial velocities. 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. Data were collected using a 1-cm light path for data collected to 0.15 mM TNB and a 0.1-cm light path at higher concentrations with overlapping concentrations used in both path length cells.

Spectral Studies. All UV–vis spectra were obtained using a Hewlett-Packard Model 8452A photodiode array spectrophotometer recording the absorbance at wavelengths from 240 to 600 nm using a light path of 1 cm. A reaction mixture was prepared containing the following: Hepes, pH 7, 100 mM; OASS-A, 3 mg; OAS, 2 mM. A spectrum was recorded at zero time with this reaction mixture and repetitively every 30 s following the addition of 50 μM TNB.

Spectra for the formation of the α-aminoacrylate intermediate for OASS-B were obtained using 0.18 mg/mL enzyme in the absence and in the presence of 2 mM OAS as a function of pH. The following buffers were used over the pH range indicated at a final concentration of 100 mM: Mes, 5.5–6.5; Hepes, 6.5–8.0; Ches, 8.5–9.5.

NMR Studies. ^{1}H NMR spectra of the reaction mixture with OAS and TNB were measured using a Varian XL-300 NMR spectrometer in the absence of OASS-B. All reactants were prepared in D_2O, and the pH was adjusted with KOD. In a 0.8-mL reaction mixture the following were present: a trace amount of DSS, 10 mM TNB, 100 mM OAS, and 20 mM Hepes, pH 7. The reaction was initiated by the addition of 8 μg of OASS-B, and spectra were recorded in 5-mm NMR tubes using the following parameters: spectral width 3333.4 Hz, acquisition time 2.465 s, relaxation delay 1 s, and pulse width 50°. A total of 64 scans were averaged to obtain the spectra. Complete conversion of S-CNP-cysteine occurred in 24.5 h. Standard spectra of TNB and DTNB were also obtained under identical conditions at a fixed concentration of 10 mM.

^{1}H NMR chemical shifts of OAS (Chart I) are as follows: δ 2.13 (s, 3H, 2′-H), 4.07 (t, 1H, J = 4.5 Hz, 2-H), 4.50 (d, 2H, J = 4.5 Hz, 3-H) ppm. ^{1}H NMR chemical shifts of TNB are as follows: δ 7.26 (d, 1H, J = 2.1 Hz, 6-H), 7.38 (dd, 1H, J = 8.6 and 2.1 Hz, 4-H), 7.83 (d, 1H, J = 8.9 Hz, 3-H) ppm. ^{1}H NMR chemical shifts of DTNB are as follows: δ 7.62 (d, 1H, J = 2.3 Hz, 6-H), 7.69 (dd, 1H, J = 8.9 and 2.3 Hz, 4-H), 8.08 (d, 1H, J = 8.9 Hz, 3-H) ppm. ^{1}H NMR chemical shifts of aromatic protons on S-CNP-cysteine are as follows: δ 7.46 (d, 1H, J = 1.9 Hz, 6-H), 7.56 (dd, 1H, J = 8.9 and 2.1 Hz, 4-H), 8.10 (d, 1H, J = 8.9 Hz, 3-H) ppm. ^{1}H NMR chemical shifts of *N*-acetylserine are as follows: δ 2.06 (s, 3H, 2′-H), 3.81 (dd, 1H, J = 11.5, 5.9 Hz, 3-Ha), 3.87 (dd, 1H, J = 11.5, 4.1 Hz, 3-Hb), 4.29 (dd, 1H, J = 5.9, 4.1 Hz, 2-H) ppm. Numbers listed for protons are according to Chart I.

Initial Velocity Studies. Initial velocity patterns were obtained by varying one reactant over a range of concentrations less than K_m and if possible greater than the K_I for substrate inhibition and several fixed concentrations of the second reactant (less than K_m to greater than K_I). Product and dead-end inhibition patterns were obtained by measuring the initial velocity at varying concentrations of one reactant with the second maintained at K_m at several different fixed concentrations of inhibitor including zero.

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 linear competitive, uncompetitive, and noncompetitive inhibition were fitted using eqs 1–3, respectively, while data for acetate product inhibition *vs* OAS with TNB as the second substrate were fitted using eq 4, reflecting S-parabolic noncompetitive inhibition. Initial velocity data conforming to a ping pong mechanism with double competitive substrate inhibition were fitted using eq 5. In eqs

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

$$v = VA/[K_a + A(1 + I/K_{ii})] \quad (2)$$

$$v = VA/[K_a(1 + I/K_{is}) + A(1 + I/K_{ii})] \quad (3)$$

$$v = VA/[K_a(1 + I/K_{is} + I^2/K_{is}K_{is2}) + A(1 + I/K_{ii})] \quad (4)$$

$$v = VAB/(K_bA[1 + A/K_{IA}] + K_aB[1 + B/K_{IB}] + AB) \quad (5)$$

1–5, v and V represent initial and maximum velocities, respectively, K_a and K_b are K_m values for A and B, respectively, K_{is} and K_{ii} are slope and intercept inhibition constants, while K_{is2} is the slope inhibition constant for the combination of acetate to enzyme in other than its normal capacity as a product, K_{IA} and K_{IB} are substrate inhibition constants for A and B, respectively, and A, B, and I represent reactant and inhibitor concentrations, respectively.

RESULTS

Spectral Studies. The absorbance spectra for OASS-B have λ_{max} values at 280 and 412 nm with a ratio of about 4.8 for A_{280}/A_{412}. Addition of OAS produces a decrease in the absorbance at 412 nm with resultant increases at 320 and 470 nm, respectively. The extinction coefficient at 412 nm is about 6700 M^{-1} cm^{-1}, while the extinction coefficient at 470 nm is about 7700 M^{-1} cm^{-1} (Figure 2A). The extinction coefficients at 412 and 470 nm are pH-independent from 6 to 9.5. At pH 9.5, OASS-B also catalyzes a deacetylase activity as shown by a decrease in the A_{470} with time (data not shown). The first-order rate constant for the deacetylase activity at pH 9.5 is ca. 2 min^{-1}.

Mercaptonitrobenzoate as an Alternative Substrate. A new continuous spectrophotometric assay has been developed using

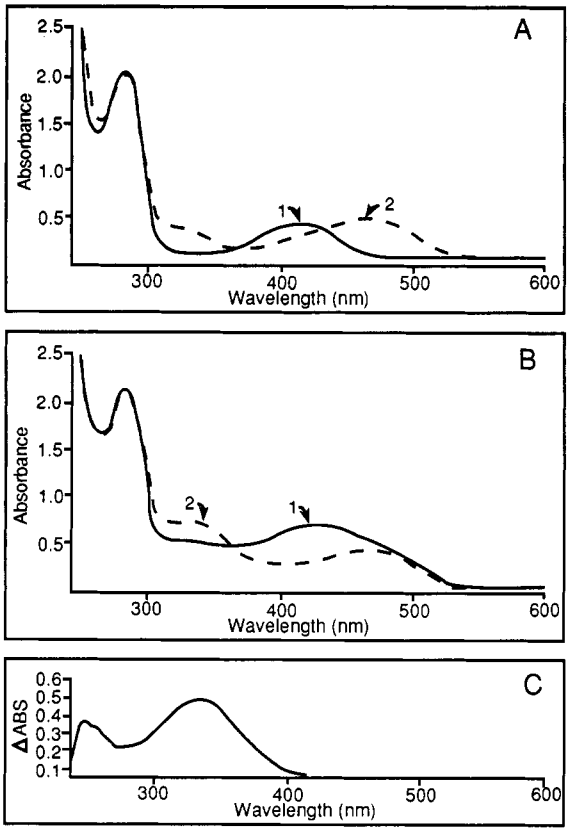


FIGURE 1: Formation of *S*-(3-carboxy-4-nitrophenyl)-L-cysteine by *O*-acetylserine sulfhydrylase-A: (A) ultraviolet–visible spectrum of OASS-A at pH 7, 100 mM Hepes, (1) OASS-A alone, (2) OASS-A in the presence of 2 mM OAS; (B1) (A2) plus 0.05 mM TNB after 10 s; (B2) (B1) at the completion of the reaction; (C) difference spectrum between (A2) and (B2).

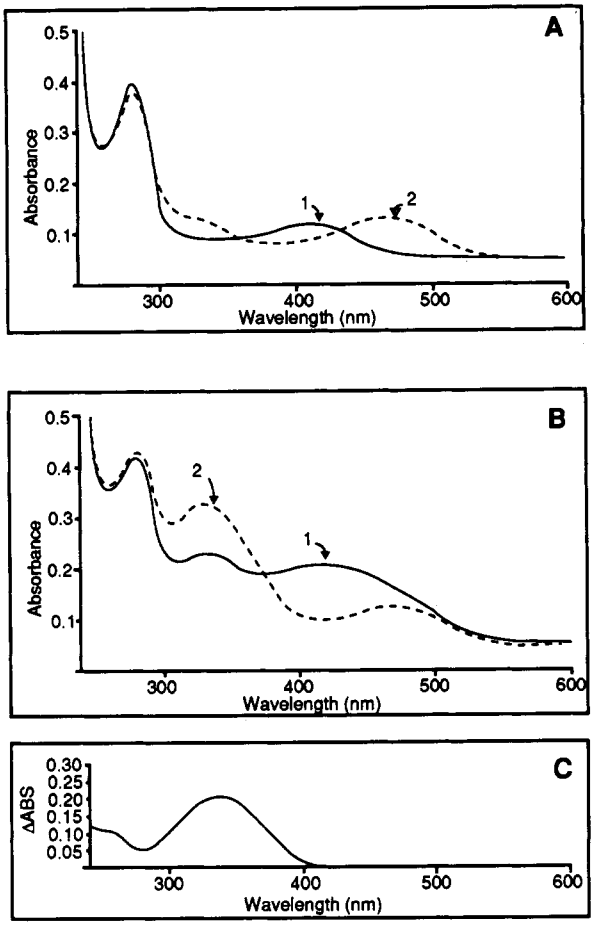


FIGURE 2: Formation of *S*-(3-carboxy-4-nitrophenyl)-L-cysteine by *O*-acetylserine sulfhydrylase-B: (A) ultraviolet–visible spectrum of OASS-B at pH 7, 100 mM Hepes, (1) OASS-B alone, (2) OASS-B in the presence of 1 mM OAS; (B1) (A2) plus 0.02 mM TNB after 5 s; (B2) (B1) at the completion of the reaction; (C) difference spectrum between (A2) and (B2).

TNB as an alternative substrate for sulfide. *O*-Acetylserine sulfhydrylase-A converts OAS to α-aminoacrylate in Schiff base with PLP which absorbs maximally at 320 and 470 nm (Cook & Wedding, 1976; Cook *et al.*, 1991), Figure 1A. Addition of TNB to the α-aminoacrylate intermediate results in the disappearance of absorbance at 470 nm with a concomitant increase in absorbance at 340 nm, Figure 1B. A difference spectrum recorded on a Hewlett-Packard Model 8452A photodiode array spectrophotometer shows the disappearance of TNB at 412 nm and the concomitant appearance of a species absorbing at 340 nm, Figure 1C, due to the formation of the presumed product, *S*-(3-carboxy-4-nitrophenyl)-L-cysteine, Scheme I. Similar spectra were also obtained for the α-aminoacrylate intermediate (Figure 2A) and S-CNP-cysteine formation (Figure 2B) catalyzed by OASS-B. The difference spectrum between parts A and B of Figure 2 is shown in part C. The identity of the product was corroborated using ^{1}H NMR (see Discussion).

The velocity *vs* enzyme concentration is linear for any given concentration of reactants for both isozymes. The saturation curves for either OAS or TNB at a fixed concentration of the other are hyperbolic, but exhibits substrate inhibition at high concentrations (data not shown). The maximum rate obtained with TNB and OAS is 10^{-2}-fold and 10^{-1}-fold that measured with sulfide and OAS as substrates, for OASS-A and -B respectively. However, the high extinction coefficient for TNB (13 600 M^{-1} cm^{-1}) and the high turnover numbers for OASS-A and -B make TNB useful as a continuous monitor of the sulfhydrylase reaction.

The stoichiometry of the reaction was determined under conditions where OAS and TNB concentrations were maintained at a final concentration of 60 μM in 100 mM Hepes, pH 7. A 1-mg sample of OASS-A or 0.7 μg of OASS-B was added to rapidly convert all of the TNB present in the reaction mixture to product, and the reaction was monitored using the diode array spectrophotometer. Of 60 μM TNB added only 20 μM product was formed in the OASS-A reaction, while 60 μM of product was formed in the OASS-B reaction.

Initial Velocity Studies in the Absence of Added Products. Although the kinetic mechanism for the sulfhydrylase has been determined previously with OAS and sulfide as substrates (Cook & Wedding, 1976), the quantitative data are in error as a result of improper calibration of the sulfide ion selective electrode. In addition, a number of alternative reactants have been identified, replacing both OAS and sulfide, and it is important to show that the kinetic mechanism does not change dependent on the reactant being used. Kinetic mechanistic data have been obtained for OASS-A and -B using *O*-acetyl-L-serine or β-chloro-L-alanine as amino acid substrates with either sulfide or TNB as nucleophilic substrates. In all cases, initial velocities obtained varying one reactant at different fixed levels of the second gave a series of parallel lines with competitive substrate inhibition by both substrates. Examples of initial velocity patterns obtained with OAS and TNB and with BCA and TNB are shown for OASS-A in parts A1 and A2 of Figure 3 and for OASS-B in parts B1 and B2. Kinetic parameters obtained for all reactant/product pairs are listed in Tables I and II for OASS-A and OASS-B, respectively. (In the case of BCA/TNB, no substrate inhibition by TNB is

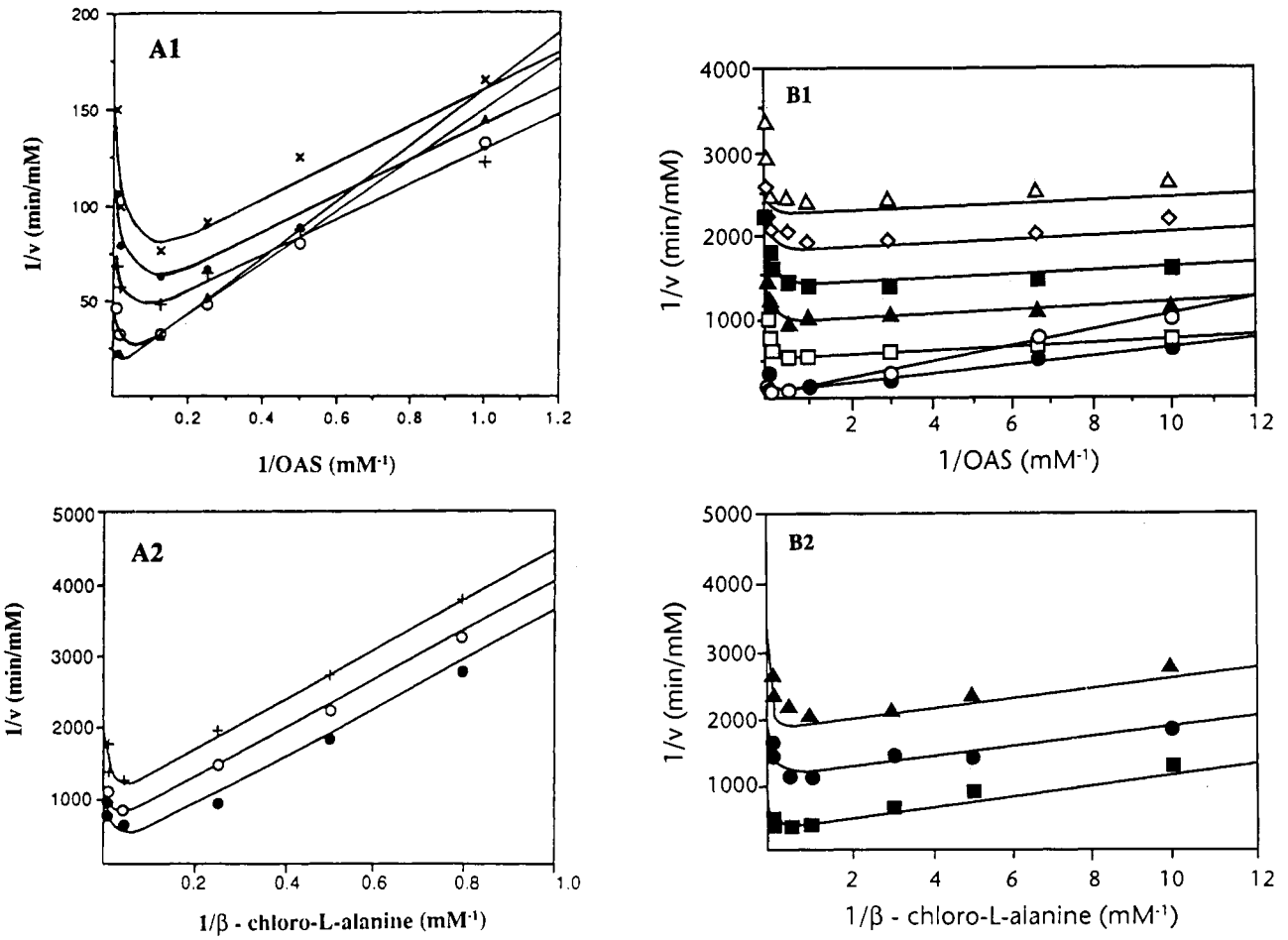


FIGURE 3: (A) Initial velocity patterns obtained for 25 μg of OASS-A by varying the concentration of OAS/BCA at different fixed levels of TNB at pH 7, 100 mM Hepes, and 25 °C. (1) Pattern obtained by varying OAS. The OAS concentrations are as indicated and TNB concentrations are as follows: 0.05 mM (×), 0.07 mM (●), 0.1 mM (+), 0.2 mM (○), 0.4 mM (▲). (2) Pattern obtained by varying BCA. The BCA concentrations are as indicated, and TNB concentrations are as follows: 0.02 mM (+), 0.04 mM (○), 0.06 mM (●). (B) Initial velocity patterns obtained for 0.7 μg of OASS-B by varying the concentration of OAS/BCA at different fixed levels of TNB at pH 7, 100 mM Hepes, and 25 °C. (1) Pattern obtained by varying OAS. The OAS concentrations are as indicated, and TNB concentrations are as follows: 0.02 mM (▲), 0.025 mM (◇), 0.0333 mM (■), 0.05 mM (▲), 0.1 mM (□), 0.5 mM (●) 1 mM (○). (2) Pattern obtained by varying BCA. The BCA concentrations are as indicated, and TNB concentrations are as follows: 0.0143 mM (▲), 0.05 mM (●), 0.1 mM (■). In all cases, points are experimental and the solid lines are from a fit using eq 5.

Table I: Kinetic Parameters for *O*-Acetylserine Sulfhydrylase-A Obtained at pH 7 and 25 °C

	substrate pair			
parameter	OAS/sulfide	OAS/TNB	BCA/sulfide	BCA/TNB
V/E_t (s^{-1})	130 ± 17	0.56 ± 0.08	2.0 ± 0.1	0.016 ± 0.002
$V/K_{OAS}E_t$ (M^{-1} s^{-1})	(1.4 ± 0.7) × 10^5	37 ± 5		
$V/K_{BCA}E_t$ (M^{-1} s^{-1})			(3.7 ± 0.6) × 10^3	1.2 ± 0.2
$V/K_{sulfide}E_t$ (M^{-1} s^{-1})	(2.4 ± 1.8) × 10^7		(3.9 ± 0.2) × 10^6	
$V/K_{TNB}E_t$ (M^{-1} s^{-1})		950 ± 55		590 ± 130
K_{OAS} (mM)	1.0 ± 0.6	15 ± 3		
K_{BCA} (mM)			0.50 ± 0.07	13 ± 2
$K_{sulfide}$ (mM)	0.006 ± 0.003		0.0005 ± 0.0001	
K_{TNB} (mM)		0.6 ± 0.1		0.03 ± 0.01
$K_{I,OAS}$ (mM)		67 ± 15		
$K_{I,sulfide}$ (mM)	0.05 ± 0.03			
$K_{I,BCA}$ (mM)				70 ± 20
$K_{I,TNB}$ (mM)		0.41 ± 0.16		

observed likely as a result of the restricted concentration range that could be used.)

Product Inhibition Studies. Acetate is competitive *vs* sulfide or TNB and noncompetitive *vs* OAS whether sulfide or TNB is the second substrate for either OASS-A or -B. In both cases the noncompetitive pattern obtained *vs* OAS is not reflective of simple linear noncompetitive inhibition, Figure 4, but rather is indicative of S-parabolic noncompetitive inhibition. Cysteine is noncompetitive *vs* both OAS and sulfide in the OASS-A reaction. With OASS-A, chloride, a product when BCA is used as the amino acid substrate, is noncompetitive *vs* both BCA and TNB, and noncompetitive *vs* sulfide at fixed BCA, but competitive *vs* BCA at a fixed sulfide concentration. With OASS-B, chloride is uncompetitive *vs* BCA and competitive *vs* TNB. Results are summarized to Tables III and IV for OASS-A and -B, respectively.

Dead-End Inhibition Studies. For OASS-A, thiocyanate is competitive *vs* sulfide, but noncompetitive *vs* TNB whatever

Table II: Kinetic Parameters for *O*-Acetylserine Sulfhydrylase-B Obtained at pH 7 and 25 °C

parameter	substrate pair			
	OAS/sulfide	OAS/TNB	BCA/sulfide	BCA/TNB
V/E_t (s^{-1})	115 ± 12	8 ± 2	22 ± 4	7 ± 1
$V/K_{OAS}E_t$ (M^{-1} s^{-1})	(1.3 ± 0.1) × 10^5	(4.1 ● 1.9) × 10^4		
$V/K_{BCA}E_t$ (M^{-1} s^{-1})			(1.0 ● 0.2) × 10^4	(1.2 ● 0.3) × 10^4
$V/K_{sulfide}E_t$ (M^{-1} s^{-1})	(1.2 ± 0.1) × 10^7		(1.8 ± 0.3) × 10^6	
$V/K_{TNB}E_t$ (M^{-1} s^{-1})		(1.8 ± 0.1) × 10^4		(3.2 ± 0.1) × 10^4
K_{OAS} (mM)	0.9 ± 0.1	0.19 ± 0.08		
K_{BCA} (mM)			2.2 ± 0.4	0.5 ± 0.1
$K_{sulfide}$ (mM)	0.010 ± 0.002		0.012 ● 0.002	
K_{TNB} (mM)		0.43 ● 0.09		0.20 ● 0.02
$K_{I,OAS}$ (mM)		51 ± 4		
$K_{I,BCA}$ (mM)				33 ± 3

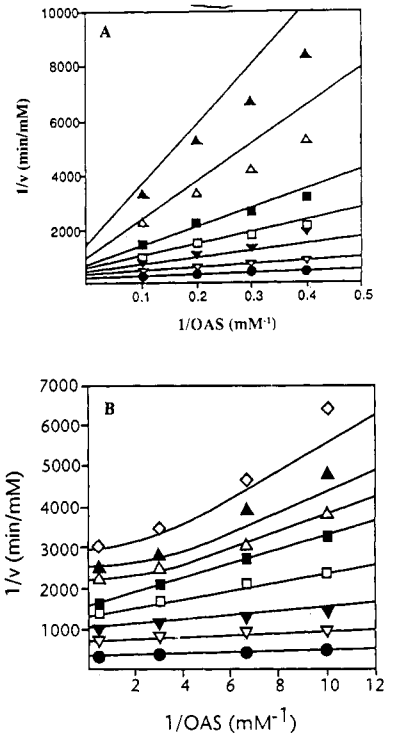


FIGURE 4: Product inhibition by acetate at varied levels of OAS. The concentration of TNB was fixed at 0.05 mM, and all initial rates were measured at pH 7, 100 mM Hepes, and 25 °C. The inhibition pattern was obtained for OASS-A (A) and OASS-B (B). (A) The following acetate concentrations were added: 0 mM (●), 55 mM (▽), 110 mM (▼), 165 mM (□), 220 mM (■), 330 mM (△), 440 mM (▲). (B) The following acetate concentrations were used: 0 mM (●), 74 mM (▽), 148 mM (▼), 222 mM (□), 296 mM (■), 333 mM (△), 370 mM (▲), 444 mM (◇). In both cases OAS is varied as indicated. Points are experimental values, while the solid lines are from a fit using eq 4.

Table III: Product Inhibition Constants for *O*-Acetylserine Sulfhydrylase-A at pH 7 and 25 °C

inhibitor	variable substrate	type of inhibition	K_{is} ± SE (mM)	K_{ii} ± SE (mM)
		OAS/Sulfide		
acetate	sulfide	C	39 ± 1	
	OAS	NC	13 ± 4	6.0 ± 1.5
cysteine	sulfide	NC	7.5[c]	50 ± 10
	OAS	NC	3.0 ● 0.6	7 ± 2
		OAS/TNB		
acetate	TNB	C	30 ± 3	
	OAS	S-ParaNC[b]	95 ± 13	88 ● 5
			K_{is2} = 55 ● 8	
		BCA/Sulfide		
chloride	sulfide	NC	335 ± 30	61 ● 19
	BCA	C	43 ● 5	
		BCA/TNB		
chloride	TNB	NC	48 ± 5	51 ± 11
	BCA	NC	41 ± 10	38 ± 3

[a] Graphically estimated. [b] Data adhere to slope parabolic noncompetitive inhibition and were fitted using eq 4.

Table IV: Product Inhibition Constants for *O*-Acetylserine Sulfhydrylase-B at pH 7 and 25 °C

inhibitor	variable substrate	type of inhibition	K_{is} ± SE (mM)	K_{ii} ± SE (mM)
		OAS/TNB		
acetate	TNB	C	73 ± 4	
	OAS	S-ParaNC[a]	300 ± 100	60 ± 1
			K_{is2} = 30 ± 10	
		BCA/TNB		
chloride	TNB	C	12 ± 1	
	BCA	UC		18 ± 1

[a] Data adhere to slope parabolic noncompetitive inhibition and were fitted using eq 4.

the second substrate. When OAS or BCA is the varied substrate, thiocyanate is uncompetitive with sulfide as the second substrate, but noncompetitive when TNB is the second substrate. For OASS-B, thiocyanate is competitive *vs* TNB whether the other substrate is OAS or BCA, and uncompetitive *vs* OAS or BCA. Maleate, an analog of OAS, is noncompetitive *vs* TNB and OAS. All inhibition patterns and inhibition constants are summarized in Tables V and VI for OASS-A and -B, respectively.

DISCUSSION

NMR Studies. In Figure 5 spectra A and C are obtained for TNB, while spectrum D is for DTNB. The difference between spectra A and C is the presence of OAS. The resonance at 7.26 ppm is due to the proton at C-6 with long-range coupling to C-4, while the doublets at 7.38 and 7.83 ppm reflect C-3 and C-4, respectively, which couple to each other. All of these resonances are shifted downfield on formation of DTNB as a result of reestablishing the aromaticity of the ring and further deshielding the protons at C-3, C-4, and C-6. After addition of enzyme to the reaction mixture depicted in spectrum A, the three proton resonances are again shifted downfield, now as a result of formation of S-CNP-cysteine. Those at C-3 and C-6 are not shifted as far downfield in S-CNP-cysteine as they are in DTNB. Proton NMR spectra were also obtained for OASS-A with qualitatively identical results (data not shown). Since the activity of OASS-A is 13-fold less than that of OASS-B with TNB as a substrate, the latter was used to collect the NMR data.

Interestingly, as listed in the Materials and Methods the α- and β-protons of OAS appear as a triplet and doublet in spectrum A (data not shown). They were expected to reflect

Table V: Dead-End Inhibition Constants for *O*-Acetylserine Sulfhydrylase-A at pH 7 and 25 °C

inhibitor	variable substrate	type of inhibition	K_{is} ± SE (mM)	K_{ii} ± SE (mM)
		OAS/Sulfide		
thiocyanate	sulfide[a]	C	0.055 ● 0.007	
	OAS[b]	UC		0.06 ± 0.01 (0.028 ● 0.005)
		OAS/TNB		
thiocyanate	TNB[c]	NC	0.3 ± 0.1	0.28 ± 0.08 (0.25 ± 0.07)
	OAS[d]	NC	0.13 ± 0.01	0.58 ± 0.05 (0.53 ± 0.05)
		BCA/Sulfide		
thiocyanate	sulfide[e]	C	1.1 ± 0.3	
	BCA[f]	UC		4.9 ± 0.2 (0.45 ± 0.02)
		BCA/TNB		
thiocyanate	TNB[g]	NC	0.4 ± 0.1	0.6 ± 0.1 (0.34 ± 0.06)
	BCA[d]	NC	1.11 ± 0.33	0.8 ± 0.14 (0.37 ± 0.06)

[a] Data were obtained at 10 mM OAS. [b] Data were obtained at 0.007 mM sulfide. [c] Data were obtained at 2 mM OAS. [d] Data were obtained at 0.05 mM TNB. [e] Data were obtained at 2 mM BCA. [f] Data were obtained at 0.005 mM sulfide. [g] Data were obtained at 10 mM BCA.

Table VI: Dead-End Inhibition Constants for *O*-Acetylserine Sulfhydrylase-B at pH 7 and 25 °C

inhibitor	variable substrate	type of inhibition	K_{is} ± SE (mM)	K_{ii} ± SE (mM)
		OAS/TNB		
maleate	TNB[a]	NC	64 ± 8	17 ± 4 (9 ± 2)
	OAS[b]	NC	26 ± 5	82 ± 11 (62 ± 8)
thiocyanate	TNB[a]	C	0.014 ± 0.001	
	OAS[c]	UC		0.0125 ± 0.0004 (0.0110 ± 0.0003)
		BCA/TNB		
thiocyanate	TNB[d]	C	0.010 ± 0.001	
	BCA[c]	UC		0.016 ± 0.001 (0.013 ± 0.001)

[a] Data were obtained at 0.2 mM OAS. [b] Data were obtained at 0.15 mM TNB. [c] Data were obtained at 0.05 mM TNB. [d] Data were obtained at 0.5 mM BCA.

an ABX pattern. The absence of such a pattern is indicative of magnetic equivalence of the two β-protons. In addition to OAS, the product of the nonenzymatic conversion of OAS to *N*-acetylserine was also shown in spectrum A (data not shown). The resonance of protons on the methyl group is shifted slightly upfield from 2.13 to 2.06 ppm on formation of *N*-acetylserine. The α-proton and β-protons are all nonequivalent with a large geminal coupling constant of 11.5 Hz between the β-protons and smaller coupling constants of 4.1 and 5.9 Hz between the α-proton and β-protons. The α-proton resonance is downfield with respect to those of the β-protons of *N*-acetylserine, while it is upfield with respect to those of the β-protons of OAS. Apparently the *N*-acetyl group has a rather dramatic effect on the chemical shifts of the α-proton.

Initial Velocity Patterns. The initial velocity patterns obtained with all reactant pairs for OASS-A and -B are consistent with a ping pong mechanism in which one and probably both reactants exhibit competitive substrate inhibition. The latter is not seen in all cases as a result of the concentration range used. In the case of OAS/TNB, however, the substrate inhibition by both substrates is observed. The mechanism then generally adheres to that shown in Scheme II, where AAs, NP, NS, and AAp are amino acid substrate, nucleophilic product, nucleophilic substrate, and amino acid product, respectively.

In a ping pong mechanism, the individual half-reactions are independent of the concentration of the other substrate; for example, the conversion of the E and OAS to F and acetate should be independent of the concentration or even the identity of the second substrate, i.e., whether it is sulfide or TNB. Examination of the data in Table I obtained for OASS-A indicates that the V/K values for BCA are not identical when sulfide or TNB is the nucleophilic substrate, while those for TNB are identical whether OAS or BCA is the amino acid substrate. The V/K for OAS is 4000-fold higher when sulfide is the nucleophilic substrate compared to that measured with TNB, while the V/K for sulfide is only 6-fold higher when OAS is used as the amino acid substrate compared to that measured with BCA. The disagreement in the V/K values for the amino acid substrate is quite pronounced with the A isozyme, while those for the nucleophilic substrate are in reasonable agreement. A number of factors likely contribute to the 3 order of magnitude disagreement in the V/K values of OAS and BCA with the two nucleophilic substrates. As stated above in the Results, there is a stoichiometry of 0.33 OAS being converted to product at low TNB concentration. Upon further characterization it was found that the remaining OAS is converted to pyruvate and ammonia (data not shown); that is, the deacetylase activity (Cook *et al.*, 1992) of OASS also occurs under these conditions. A calculation of the first-order rate of conversion of F to S-CNP-cysteine using the second-order rate constant and the TNB concentration used to determine stoichiometry (60 μM) gives a first-order rate constant of 2 s^{-1}, while a value of about 0.01 s^{-1} is observed for the first-order decay of F at pH 7 (Cook *et al.*, 1991). Thus, there must be an increase in the deacetylase activity for the A isozyme when TNB binds and prior to nucleophilic attack on the α-aminoacrylate; that is, F:TNB partitions one-third toward product and two-thirds toward elimination of α-aminoacrylate to give pyruvate and ammonia. The latter may be a result of a decrease in the pK of the active site lysine, thereby facilitating transaldimination and release of α-aminoacrylate. This will certainly contribute to a decrease in the V/K for the amino acid substrate but will only account for a 2–3-fold difference, leaving still a 3 order of magnitude difference. The V/K for TNB under these conditions will be unaffected independent of the amino acid substrate used since the same partitioning of F:TNB will be seen in either case. The remainder is then likely a combination of several additional factors. First, because of the similarity between the A and B isozymes, there is likely to be substrate activation with TNB. Although the latter can be observed with the B isozyme using OAS and TNB as substrates (see below), the TNB concentration apparently cannot be raised to high enough levels to see the activation with the A isozyme. Second, there appears to be the possibility that some randomness can exist for both enzymes with the second substrate combining to E to give an E:S complex that is still productive. This putative randomness (see acetate product inhibition below) will have the effect of providing a dependence of V/K for the amino acid substrate on the concentration of the nucleophilic substrate. (The reverse is also true.)

Interestingly, the A isozyme appears to be more selective than the B isozyme with respect to both amino acid and nucleophilic substrates. The V/K for OAS is 10^2-fold higher with sulfide as a substrate than that for BCA, while the V/K for sulfide with OAS as a substrate is 10^4-fold higher than that of TNB.

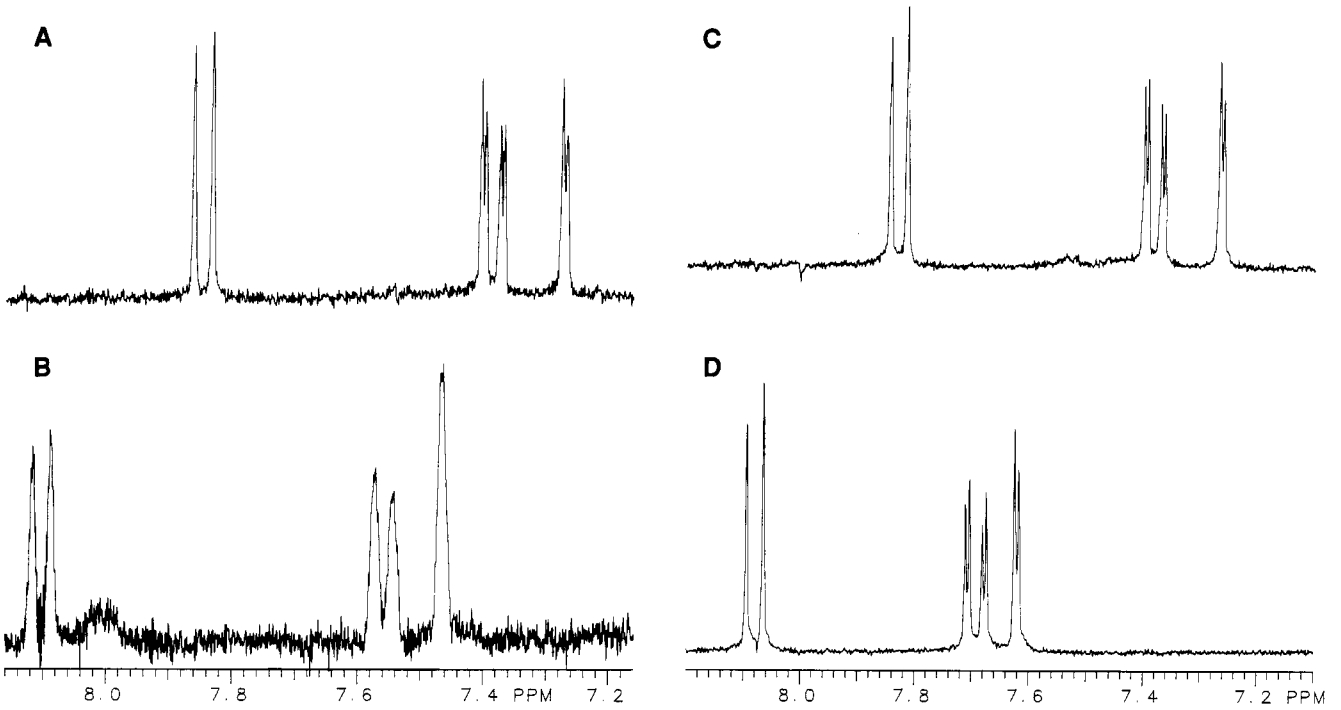
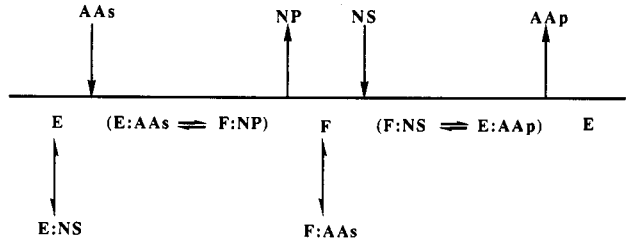


FIGURE 5: ^{1}H NMR of the *O*-acetylserine sulfhydrylase-B catalyzed reaction of OAS and TNB: (A) reaction before enzyme addition containing at pH 7, 100 mM Hepes, 100 mM OAS, and 10 mM TNB; (B) spectra recorded 24.5 h after addition of 8 μg of OASS-B; (C, D) spectra of TNB and DTNB recorded under conditions identical to those in (A).

Scheme II: Proposed Kinetic Mechanism for OASS



A comparison of the data obtained in these studies with those observed from earlier studies (Cook & Wedding, 1976) in which the electrode was not properly calibrated yields some quantitative differences but gives a mechanism that is qualitatively identical. The largest difference appears to be in V/E_t which is smaller by 10-fold (already reported in Hara *et al.*, 1990) and the K_m for OAS which is actually higher by 5-fold.

Spectral data for OASS-B are consistent with a ping pong mechanism similar to that proposed for OASS-A. However, the spectral shift from 412 to 470 nm upon binding of OAS is not as pronounced as for OASS-A. As a result it is likely that other tautomeric species of the α-aminoacrylate Schiff base contribute to the absorbance spectrum in the presence of OAS. In addition, OASS-B catalyzes the deacetylation of OAS as does OASS-A, with an increase in rate as the pH is increased. Thus, it appears that qualitatively the mechanism for OASS-B is identical to that of OASS-A.

Examination of Table II indicates that the V/K values for BCA are identical whether sulfide or TNB is the nucleophilic substrate and those for TNB are identical whether OAS or BCA is the amino acid substrate. However, the V/K for OAS is 3-fold higher when sulfide is the nucleophilic substrate compared to that measured with TNB, and the V/K for sulfide is 6-fold higher when OAS is used as the amino acid substrate compared to that measured with BCA. There is some

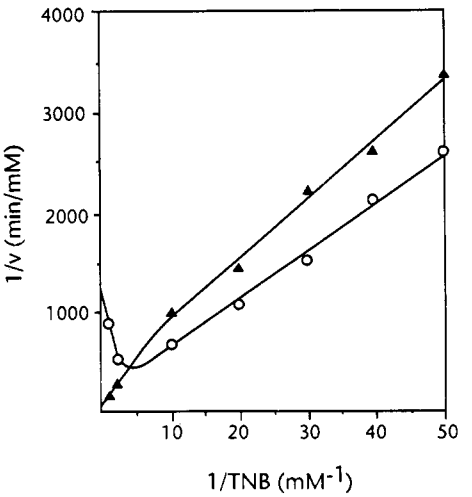
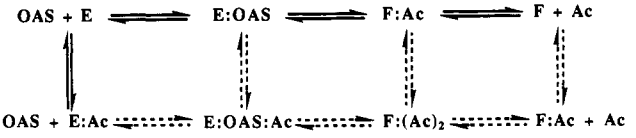


FIGURE 6: Initial velocity pattern for OASS-B at high concentrations of TNB. Shown are data for two concentrations of OAS for ease of interpretation. Data are shown for 0.1 mM (○) and 50 mM OAS (▲) at the TNB concentrations indicated. Curves are drawn by the eye, and points are experimental.

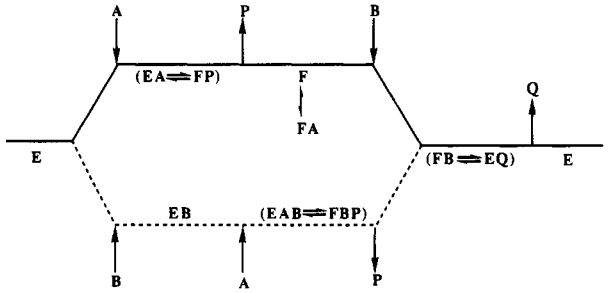
indication as pointed out in the Results that substrate activation is observed at high concentrations of TNB (Figure 6). When the latter is taken into account, the values of all of the kinetic parameters are within a factor of 2. The reason for the substrate activation of TNB is at present not known.

Product Inhibition. A single site ping pong kinetic mechanism predicts competitive inhibition between acetate and the nucleophilic substrate and between the final product and the amino acid substrate, but noncompetitive inhibition between the pairs acetate/substrate amino acid and product amino acid/nucleophilic substrate. Inhibition of OASS-A by acetate is competitive *vs* TNB as expected since both bind to F and noncompetitive *vs* OAS, also as expected since acetate can reverse the first half-reaction, and this aspect can be overcome by OAS, but F is still available for acetate to bind

Scheme III: Proposed Mechanism for S-Parabolic Noncompetitive Inhibition by Acetate



Scheme IV: Proposed Kinetic Mechanism for OASS with Random Addition of Substrate



even at high concentrations of OAS (except at substrate inhibitory levels). (The more complex nature of the inhibition by acetate will be discussed below.) Qualitatively identical acetate inhibition patterns are observed for OASS-B. Cysteine, however, is noncompetitive against both OAS and sulfide, an indication that it binds to both E and F. The latter is not surprising since the amino acid substrate which binds to E in its normal capacity also binds to F as a substrate inhibitor. Chloride is noncompetitive *vs* sulfide, suggesting that it binds both E and F, but competitive *vs* BCA, suggesting that it binds only to E. The apparent discrepancy results from the use of too high a sulfide concentration in the BCA experiment. With the weaker binding TNB, the noncompetitive patterns *vs* both substrates are observed. For OASS-B, with Cl^- as the product of the BCA reaction, the inhibition is again as expected *vs* TNB, but an uncompetitive pattern is observed against OAS. The latter is consistent with the fact that Cl^- cannot serve as a nucleophilic substrate, and thus acts as a dead-end inhibitor.

For both isozymes, the inhibition by acetate *vs* OAS with TNB as a reactant is S-parabolic noncompetitive. The parabolic slope effect suggests the presence of a second enzyme form to which acetate can bind at high concentrations. These data are consistent with Scheme III. Acetate binding to F reverses the first half-reaction, resulting in an increase in E to which acetate also binds. This mechanism is corroborated by the elimination of the parabolic slope effect at saturating OAS. Under these conditions OAS effectively competes for E, eliminating the second form to which acetate binds.

In the case of OASS-B (Figure 4B) partial substrate inhibition by OAS is also observed at concentrations of acetate where binding to E is observed. These data suggest (broken arrows, Scheme III) that OAS can still bind to E:Ac in a productive mode but that the reaction is slowed by the presence of acetate. (At what point acetate is released is not known, so all species are included in Scheme III). As a result there is an allowance for some random sequential nature to the reaction catalyzed by OASS. The overall mechanism for OASS is thus shown in Scheme IV. The predominant ping pong pathway is depicted with a solid line while the sequential pathway is depicted with broken lines. In Scheme IV, A, B, P, and Q are the amino acid substrate, nucleophilic substrate, nucleophilic product, and amino acid product, respectively.

Dead-End Inhibition. The dead-end inhibition patterns predicted by the ping pong mechanism are as follows. Competitive inhibition is predicted between a substrate and its analog since they bind to the same enzyme form, while an uncompetitive pattern is predicted for the analog *vs* the other substrate since they bind to different enzyme forms. The expected inhibition patterns by SCN^- are observed when sulfide is the nucleophilic substrate, but with TNB as the nucleophilic substrate, noncompetitive patterns are observed as both OAS and TNB, suggesting combination by SCN^- to both E and F. Thus, sulfide must bind to E under conditions used to obtain the dead-end inhibition pattern, interfering with SCN^- binding. In agreement with this suggestion, the K_I for sulfide substrate inhibition is quite low. These data are consistent with the proposed randomness discussed above. The expected patterns are observed for both OASS-A and -B, although it should be noted that SCN^- binds much more tightly to the B isozyme. Maleate, thought to be an analog of the amino acid substrate, binds poorly to OASS-B and does so to both E and F.

Quantitative Analysis of Dead-End Inhibition Data. A good test of the assigned ping pong kinetic mechanism is the quantitative agreement of K_i values for dead-end inhibition observed by varying either of the two reactants. In a ping pong kinetic mechanism dead-end analogs can bind to either of the two stable enzyme forms, E and F. If an inhibitor is an analog of A, it will bind to E and compete with A, giving a true K_i. The inhibition will be uncompetitive *vs* B, giving an apparent K_i equal to the true K_i, modified by the factor $1 + B/K_b$. If an inhibitor is an analog of B, the converse will be true; that is, it will bind to F, competing with B, but will be uncompetitive with A, giving an apparent K_i equal to $K_i(1 + A/K_a)$. A noncompetitive inhibitor binds to both E and F, with K_{is} being a true K_i and the K_{ii} being apparent and corrected as above. The corrected K_i values are shown in parentheses below the observed K_i values in Tables V and VI.

As can be seen from the data in Tables V and VI, the calculated true K_i values agree within experimental error. For example, using OAS and TNB as substrates for OASS-A, SCN^- is noncompetitive *vs* TNB and noncompetitive *vs* OAS as a result of binding of the inhibitor to E and F. The true K_i values are 300 and 530 μM for binding to F and 130 and 250 μM for binding to E. Values of the true K_i are independent of the substrate pair used as expected for a ping pong mechanism; an exception is the data obtained when OAS and sulfide are substrates. In this case, the K_i values are an order of magnitude lower. The reasons for this discrepancy is not known at present.

For OASS-B, using OAS and TNB as substrates, SCN^- is competitive *vs* TNB as a result of binding of the inhibitor to F and uncompetitive *vs* OAS as a result of binding of the inhibitor to F. The K_i values for SCN^- inhibition agree within experimental error and are more than an order of magnitude lower than those estimated for OASS-A. The maleate inhibition data are also internally consistent with values of 64 and 62 mM for binding to F and 26 and 10 mM for binding to E. The affinity for maleate binding to F is weaker as a result of the site being partially occupied by α-aminoacrylate.

Comparison of OASS-A and -B. The data obtained from the initial velocity patterns for all reactant pairs are consistent with the proposed ping pong mechanism for both isozymes in which one and probably both reactants exhibit competitive substrate inhibition *vs* the other reactant. However, the S-parabolic noncompetitive product inhibition *vs* OAS displayed by acetate suggests there is allowance for some random sequential nature to the reaction catalyzed by both isozymes. While there is agreement in the V/K values for the amino acid substrate for the B isomer, the V/K values vary by several

orders of magnitude as a function of the nucleophilic substrate for OASS-A. This discrepancy may be accounted for in part by an increase in the deacetylase activity induced by the binding of TNB. The reason that the latter activity is less for the B isozyme is at present unknown but may correlate with the increase in efficiency of utilization of TNB by the B isozyme compared to that by the A isozyme. A comparison of the kinetic parameters V and V/K of both isozymes indicates that the A isozyme is more selective than the B isozyme with respect to both the amino acid and nucleophilic substrates. Both substrate types, i.e., nucleophilic and amino acid, bind more tightly to OASS-B. Although the two isozymes are similar in many respects, there are a number of differences that require further investigation.

Other Enzymes. The finding of the double competitive substrate inhibition in a ping pong mechanism is not unique. Other enzymes that also have a ping pong kinetic mechanism and exhibit double competitive substrate inhibition include β-ketothiolase (Stewart & Rudney, 1966), nucleoside diphosphate kinase (Garces & Cleland, 1969), and aspartate aminotransferase (Kiick & Cook, 1983), which have single site ping pong mechanisms, and dihydropyrimidine dehydrogenase (Podschun *et al.*, 1990), which has a nonclassical two site ping pong mechanism. In addition, the reductive half-reaction of glutamate synthase which has a nonclassical two site ping pong mechanism exhibits double competitive substrate inhibition in the reductive half-reaction when NADPH is used as a reductant of the active site flavin and thioNADP is used as an oxidant of the reduced flavin (Rendina & Orme-Jonson, 1978). There is a difference, however, between the observation of double competitive substrate inhibition in the OASS reaction as opposed to the other enzymes listed. In the case of OASS, the reactants do not resemble one another structurally, while they do for all of the other reactions. In the case of the dihydropyrimidine dehydrogenase this may not appear to hold since uracil does not appear to resemble NADPH. However, the former does appear to mimic the dihydropyridine portion of NADPH.

It is not known presently why OASS exhibits double competitive substrate inhibition. The inhibitions by sulfide and OAS have a similar affinity relative to their respective K_m values. For example, an inhibition constant of 50 μM is observed for sulfide, an order of magnitude greater than $K_{sulfide}$ (6 μM), while the K_I for OAS is about 40 mM (data not shown; K_{OAS} is 1 mM). The strong substrate inhibition may have important implications to the kinetic mechanism, allowing the enzyme to be primed with sulfide prior to binding OAS. In other words, at high sulfide concentrations the enzyme mechanism may approach a sequential pathway, making it less likely that OAS will be degraded, and will rather yield product.

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