Characterization of the Allosteric Anion-Binding Site of *O*-Acetylserine Sulfhydrylase†

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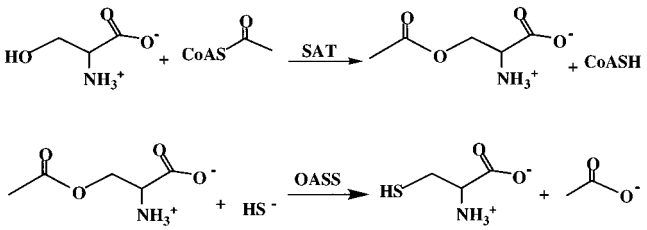
ABSTRACT: A new crystal structure of the A-isozyme of *O*-acetylserine sulfhydrylase-A (OASS) with chloride bound to an allosteric site located at the dimer interface has recently been determined [Burkhard, P., Tai, C.-H., Jansonius, J. N., and Cook, P. F. (2000) *J. Mol. Biol. 303*, 279−286]. Data have been obtained from steady state and presteady-state kinetic studies and from UV−visible spectral studies to characterize the allosteric anion-binding site. Data obtained with chloride and sulfate as inhibitors indicate the following: (i) chloride and sulfate prevent the formation of the external aldimines with L-cysteine or L-serine; (ii) chloride and sulfate increase the external aldimine dissociation constants for *O*-acetyl-L-serine, L-methionine, and 5-oxo-L-norleucine; (iii) chloride and sulfate bind to the allosteric site in the internal aldimine and α-aminoacrylate external aldimine forms of OASS; (iv) sulfate also binds to the active site. Sulfide behaves in a manner identical to chloride and sulfate in preventing the formation of the L-serine external aldimine. The binding of chloride to the allosteric site is pH independent over the pH range 7−9, suggesting no ionizable enzyme side chains ionize over this pH range. Inhibition by sulfide is potent (K_d is 25 μM at pH 8) suggesting that SH^- is the physiologic inhibitory species.

The biosynthesis of L-cysteine in enteric bacteria, such as *Salmonella typhimurium* and *Escherichia coli*, and in plants proceeds via a two-step pathway (see below). The amino acid precursor of L-cysteine is L-serine, which is first acetylated at its β-hydroxyl group by acetyl-CoA to give *O*-acetyl-L-serine (OAS),[1] a reaction catalyzed by the enzyme serine acetyltransferase (SAT; E.C. 2.3.1.30) (*1*). The final step in cysteine synthesis, replacement of the acetate side chain, is catalyzed by OASS (E.C. 4.2.99.8) with inorganic sulfide as the thiol donor (*2*).



At the level of enzyme activity, regulation is by feedback inhibition of SAT by the end product L-cysteine. Cysteine is a potent inhibitor, with a K_i of 1 μM at 0.1 mM acetyl CoA for SAT either free or in complex with OASS (*1*). No regulation of OASS has been reported, although sulfide is a competitive substrate inhibitor of OASS with a K_i of 50 μM (*1*, *3*, *4*). For more details on the mechanism of OASS, see the recent review by Tai and Cook (*5*).

Recently, a structure of the A-isozyme of OASS was solved with chloride bound to an allosteric site located at the enzyme's dimeric interface (*6*). The conformation of the enzyme with chloride bound differs from that of the free enzyme (no ligand bound) or open form (*7*), and that of the methionine external Schiff base of the K41A mutant enzyme or closed form (*8*). Formation of the external Schiff base is accompanied by an interaction of the substrate α-carboxylate with a portion of the moveable domain (residues 87−131) called the asparagine loop, resulting in a closure of the active site. In the chloride-inhibited conformation, the moveable domain is in a new position, and the α-carboxylate is prevented from interacting with the asparagine loop. The external Schiff base is destabilized in the inhibited conformation.

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[1] Abbreviations: BCA, β-chloro-L-alanine; OAS, *O*-acetyl-L-serine; OASS-A, the A-isozyme of *O*-acetylserine sulfhydrylase (the A is omitted for convenience); PLP, pyridoxal 5′-phosphate; *S*-CNP-cysteine, *S*-(5-carboxy-4-nitrophenyl)-L-cysteine; TNB, 5-thio-2-nitrobenzoate; ONL, 5-oxo-L-norleucine; SH^-, sulfide is used instead of hydrosulfide for convenience, Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; Ches, 2-(*N*-cyclohexylamino)ethanesulfonic acid; HPLC, high performance liquid chromatography.

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Chloride is likely not the physiologic effector of OASS-A ($K_I \sim 40$ mM). Sulfide is a substrate for the OASS reaction and is also an inhibitor competitive with OAS with a K_I of 50 μM (see above). Although there is no obvious binding site for sulfide as a substrate (*8*), there must be an inhibitory site. The ionic radii of chloride (1.67 Å) and sulfide (1.7 Å) are nearly identical and the allosteric binding site could easily accommodate sulfide.

In the present study, the allosteric anion-binding site of OASS-A was characterized utilizing steady state and pre-steady-state kinetic and UV−visible spectral studies. Data indicate that sulfide behaves in a manner identical to chloride, suggesting that the substrate sulfide is the physiologic inhibitor of the OASS-A reaction. Regulation of the cysteine biosynthetic pathway in enteric bacteria must be extended to include OASS-A.

MATERIALS AND METHODS

Chemicals. *O*-Acetyl-L-serine, L-cysteine, L-serine, and L-methionine were obtained from Sigma. Hepes and Ches were from Research Organics. The 5,5′-dithiobis-(2-nitrobenzoate) was from Aldrich. All other chemicals and reagents were obtained from commercial sources and were of the highest purity available.

The OAS analogue, 5-oxo-L-norleucine (ONL), was prepared by Professor David Gani, and its preparation will be described elsewhere. The methyl ketone was purified prior to use by treatment with activated charcoal in methanol. The concentration of ONL was determined by ^{1}H NMR with ethanol as an internal standard. The concentration of ethanol was determined by endpoint assay using alcohol and aldehyde dehydrogenases in the presence of excess of NAD^+.

Enzyme Preparation. OASS-A was prepared from a plasmid-containing overproducing strain using the method of Hara et al. (*9*) adapted to HPLC (*4*).

Enzyme Assay. *O*-Acetylserine sulfhydrylase activity was monitored using TNB as the nucleophilic substrate. The intrinsic absorbance of TNB and its disappearance was monitored continually at 412 nm (ϵ_{412}, 13 600 M^{-1} cm^{-1}) using a recording spectrophotometer (*4*). All assays were performed in 100 mM Hepes, pH 7, at 25 °C. Initial rates were obtained at varying concentrations of OAS or TNB with the other reactant fixed and at different fixed concentrations of either chloride or sulfate (including zero).

Presteady-State Kinetic Studies. Single-wavelength absorbance stopped-flow studies were carried out using an OLIS RSM stopped-flow spectrophotometer. Single-wavelength time courses were fitted using the software provided with an equation of the following general form.

$$A_t = A_\infty \pm \sum A_i \exp^{(-t/\tau_i)} \quad (1)$$

In eq 1, A_t and A_∞ are the absorbance values at time t and time ∞, respectively, and A_i and τ_i represent the ith amplitude and relaxation time, respectively. All time courses were collected under pseudo-first-order conditions. Data for the substrate dependence of the pseudo-first-order rate constant were fitted using eq 2.

$$1/\tau_i = k_{AA}[\text{OAS}]/(K_{EST} + [\text{OAS}]) \quad (2)$$

In eq 2, the constants k_{AA} and K_{EST} represent the first-order rate constant for the formation of the α-aminoacrylate intermediate from the external Schiff base and the dissociation constant for the external Schiff base, respectively.

Spectral Measurements. All UV−visible spectra were collected at 25 °C as previously described (*10*). The pH dependence of the appK_d for chloride dissociating from the allosteric site was determined by fixing L-serine at a concentration sufficient to form the external aldimine based on the pH dependence of its dissociation constant reported previously (*10*), and varying the concentration of chloride. The recovery of absorbance at 412 nm reflects the binding of chloride to the allosteric site. Data for change in A_{412} vs [chloride] were fitted using eq 3 below. The appK_d for chloride is then corrected by dividing by (1 + [L-serine]/$K_{d\ L-serine}$). Data were collected at pH 7, 8, and 9 using 100 mM Hepes or Ches buffer at 25 °C. Similar experiments were carried out with sulfide at pH 8.

Data Processing. Data were fitted using the FORTRAN programs developed by Cleland (*11*). Data for spectral titrations were fitted using eq 3. Data for chloride inhibition with either TNB or OAS as the variable substrate were fitted using eq 4. Data for sulfate inhibition with TNB as the variable substrate were fitted using eq 5, while those obtained with OAS as the variable substrate were fitted using eq 6.

$$\Delta A_{412} = A_{412}[\text{I}]/(\text{app}K_d + [\text{I}]) \quad (3)$$

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

$$v = VA/[K_a(1 + \text{I}/K_{iN})/(1 + \text{I}/K_{iD}) + A] \quad (5)$$

$$v = VA/[K_a(1 + \text{I}/K_{is1} + \text{I}^2/K_{is1}K_{is2}) + A(1 + \text{I}/K_{iN})/(1 + \text{I}/K_{iD})] \quad (6)$$

In eq 3, ΔA_{412} represents the observed change in A_{412} at any anion concentration in the presence of a fixed concentration of L-serine, A_{412} reflects the initial absorbance of OASS in the absence of anion and L-serine, appK_d represents the apparent dissociation constant for inhibitor dissociating from the allosteric site in the presence of a fixed L-serine concentration, and [I] is the concentration of anionic inhibitor. In eqs 4−6, v and V are initial and maximum velocities, respectively, A represents the variable reactant, and I represents the inhibitor, chloride or sulfate. The constant K_a represents the K_m for A, while K_{is} and K_{ii} represent slope and intercept inhibition constants, respectively. In the case of sulfate inhibition vs TNB, the slope is hyperbolic indicating partial inhibition. The K_{iN} and K_{iD} are inhibition constants for numerator and denominator of the slope term, where K_{iD} reflects the dissociation constant for the EI complex, and K_{iN} is a term that is responsible for ensuring partial inhibition. In the case of sulfate inhibition vs OAS, the slope is parabolic indicating two distinct inhibitor binding sites to give EI and EI_2 complexes, and the intercept is hyperbolic indicating partial inhibition as for the slope in eq 4.

RESULTS

Steady-State Kinetic Studies. To understand the chloride-inhibited structure of OASS (*6*), it is important to obtain kinetic data on inhibition by chloride and sulfate as dead-

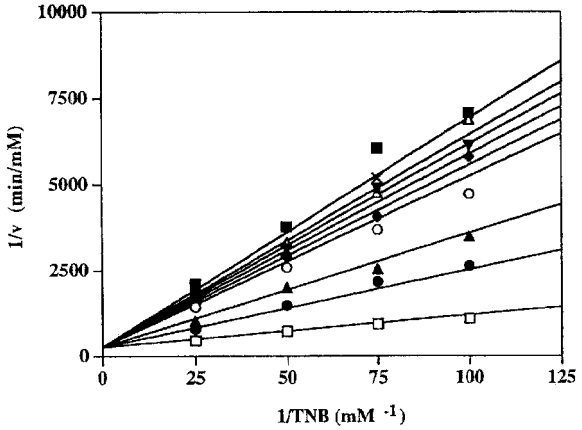


FIGURE 1: Dead-end inhibition of OASS by sulfate at varying levels of TNB. The concentration of OAS was fixed at 2 mM, and all rates were measured at pH 6.5, 100 mM Mes, and 25 °C. The following fixed sulfate concentrations in (mM) were used: 0 (◆); 60 (●); 120 (▲); 240 (○); 270 (◆); 300 (▼); 330 (△); 360 (×); and 420 (■). Curves are theoretical based on a fit using eq 5, while points are experimental.

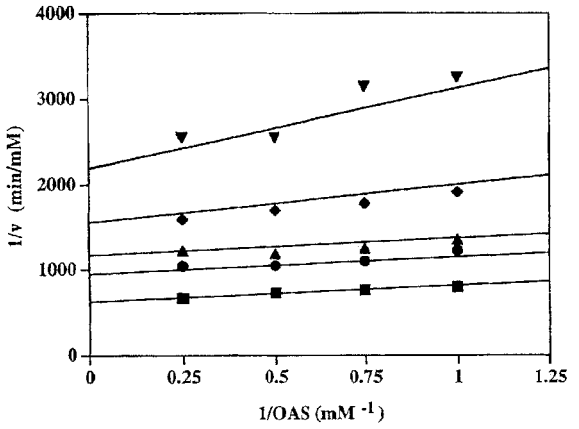


FIGURE 2: Dead-end inhibition of OASS by sulfate at varying levels of OAS. The concentration of TNB was fixed at 0.1 mM, and all rates were measured at pH 6.5, 100 mM Mes, and 25 °C. The following fixed sulfate concentrations in (mM) were used: 0 (■); 30 (●); 60 (▲); 120 (◆); and 240 (▼). Curves are theoretical based on a fit using eq 6, while points are experimental.

end inhibitors of the amino acid and nucleophilic substrates, as well as data with sulfide as an inhibitor. A continuous assay monitoring the disappearance of the absorbance maximum at 412 nm resulting from 5-thio-2-nitrobenzoate (TNB) was used with OAS as the amino acid substrate.

Product inhibition by chloride, using BCA as the amino acid substrate, has been reported previously (*4*). Although chloride is a product when BCA is used as the amino acid substrate, no reversal of the reaction is observed under any conditions when Cl^- and *S*-(5-carboxy-4-nitrophenyl)-L-cysteine (*S*-CNP-cysteine) are used as substrates for the reverse of the OASS reaction, and thus, Cl^- can be considered a dead-end inhibitor. With TNB as the nucleophilic substrate, chloride is noncompetitive vs both BCA and TNB, suggesting two binding sites for chloride, one on the internal Schiff base form of OASS, and a second on the α-aminoacrylate external Schiff base form of OASS. Furthermore, the binding constants for combination with either enzyme form are, within error limits, identical. The reason for the noncompetitive inhibition patterns obtained with Cl^- was not addressed previously and will be discussed below. A more extensive study of the dead-end inhibition by chloride and sulfate was carried out, and results are presented in Figures 1 and 2, and Table 1.

Chloride is also noncompetitive vs OAS as shown in Table 1. The K_i values obtained with OAS as the amino acid substrate are within experimental error identical to those obtained previously with BCA as the amino acid substrate (*4*). The K_i values are within error limits identical to the K_i value estimated from presteady-state data and spectral titrations (see below).

Sulfate, on the other hand, shows a very complex behavior as an inhibitor. Inhibition vs OAS is noncompetitive, but with a parabolic slope effect and a hyperbolic intercept effect. The former is indicative of two sulfate ions binding to the internal Schiff base form of the enzyme, while the hyperbolic intercept reflects combination of sulfate to the α-aminoacrylate external aldimine form of the enzyme, resulting in a slowdown of the second half-reaction. In agreement with the inhibition pattern vs OAS, the inhibition pattern by sulfate vs TNB exhibits a hyperbolic slope effect reflecting combination of sulfate to the α-aminoacrylate external aldimine form of the enzyme, slowing down the second half-reaction. The K_i of about 25 mM estimated from presteady-state data (see below) is somewhat lower than that obtained from the steady-state K_{is} of about 100 mM. However, given the complexity of the steady-state data, this difference is not surprising.

Table 1: Dead-End Inhibition by Chloride and Sulfate[a]

inhibitor	variable substrate	type of inhibition	$K_{is} \pm$ SE (mM)	$K_{ii} \pm$ SE (mM)
chloride	OAS[b]	NC[d]	21 ± 8	40 ± 30
	TNB[c]	NC	37 ± 5	50 ± 10
sulfate	OAS[b]	S-parabolic	135 ± 20	300 ± 90
		I-hyperbolicNC	115 ± 20	50 ± 5
	TNB[c]	hyperbolicC	540 ± 80	
			36 ± 2	

[a] All data were obtained with 300 μg/mL OASS at pH 6.5, 100 mM Mes, and 25 °C. [b] OAS was fixed at 2 mM ($K_{OAS} = 15$ mM; *4*). [c] TNB was fixed at 0.1 mM ($K_{TNB} = 0.6$ mM; *4*). [d] NC, noncompetitive; hyperbolicC, competitive with a hyperbolic slope effect (the first and second K_{is} values are for K_{iD} and K_{iN} per eq 4); S-parabolic, I-hyperbolic NC, noncompetitive inhibition with a parabolic slope effect (the first and second K_{is} values represent K_{is1} and K_{is2} per eq 5) and a hyperbolic intercept effect (the first and second K_{ii} values are for K_{iD} and K_{iN} per eq 5).

Characterization of 5-oxo-L-Norleucine. An analogue of OAS with the bridging ester oxygen replaced by a methylene, 5-oxo-L-norleucine (ONL), was prepared as a potential inhibitor vs OAS. Reaction of ONL with OASS produces the external aldimine with a λ_{max} of 419 nm (data not shown), and the difference spectrum has a λ_{max} of 445 nm. A plot of ΔA_{445} vs ONL at pH 9 gives a K_d of 120 ± 10 μM. 5-Oxo-L-norleucine is also a competitive inhibitor vs BCA with a K_I of 2.4 ± 0.4 mM at pH 8.8. The difference in K_I for competitive inhibition and K_d for external aldimine dissociation can be explained by the mechanism shown in eq 7, where K_I reflects the initial interaction complex between ONL and OASS, and K_d reflects dissociation from the isomerized complex, ESB. On the basis of this mechanism

$$\mathrm{ESB} \underset{K_{iso}}{\rightleftharpoons} \mathrm{E{:}ONL} \underset{K_i}{\rightleftharpoons} \mathrm{E} \overset{k_1\mathrm{BCA}}{\underset{k_2}{\rightleftharpoons}} \mathrm{E{:}BCA} \xrightarrow{k_3} \mathrm{F} \qquad (7)$$

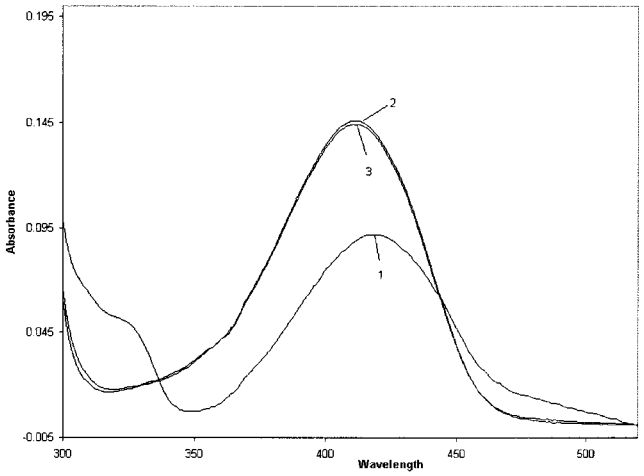


FIGURE 3: UV-visible spectra of OASS (20 μM) in the presence of 150 mM L-serine. Spectra were recorded at pH 9, 100 mM Ches, and 25 °C in the absence of anions (1) and in the presence of either 500 mM KCl (2) or 250 mM K_2SO_4 (3).

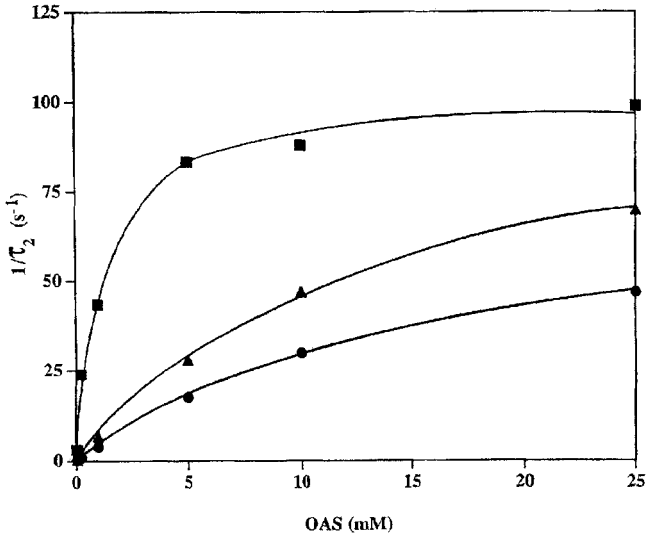


FIGURE 4: Dependence of the pseudo-first-order rate constant on the concentration of OAS. The data were recorded at pH 6.5, 100 mM Mes, and 25 °C. All time courses were measured with 1 μM concentration of OASS in the absence of anions (■) and presence of 500 mM chloride (●) or 250 mM sulfate (▲) at the OAS concentrations indicated. Points are experimental, while the solid curve is theoretical based on a fit using eq 2.

K_d will be equal to $K_{iso}K_I$, and thus K_{iso} must be 0.05, i.e., the isomerization step is 20-fold in favor of the external aldimine. The isomerization may reflect the conformational change required to close the site as the external aldimine is formed, but at a slower rate than observed during steady-state turnover of the substrate (*6*).

Spectral Studies. To obtain further information on the effects of chloride and sulfate, UV−visible spectra were measured for OASS, in the presence of chloride or sulfate, either with or without OAS, L-cysteine, or L-serine. In the presence of OAS, the α-aminoacrylate intermediate is formed as evidenced by the shift in the visible λ_{max} from 412 to 470 nm (*3*, *12*). A repeat of the experiment in the presence of either chloride or sulfate gives identical results (data not shown). Addition of L-cysteine to OASS results in a shift in the visible λ_{max} from 412 to 418 nm (*10*), and this shift is prevented when chloride or sulfate are present (data not shown). The addition of L-serine results in a mixture of external aldimine species with λ_{max} values of 330 and 418 nm, respectively (*10*). These absorbance changes are also prevented with either chloride or sulfate present, Figure 3.

Spectra were also measured in the presence of L-methionine and 5-oxonorleucine, the best mimics of OAS, with pH-independent K_d values of 80 and 120 μM, respectively (*8*). Neither 500 mM chloride nor 250 mM sulfate prevents formation of the external aldimine with these two amino acids (data not shown). To determine the effect of the anions, the K_d for the dissociation of L-methionine external Schiff base was measured at pH 9 in the presence of 500 mM chloride or 250 mM sulfate. The K_d for external aldimine dissociation is 750 ± 100 μM in the presence of chloride and 200 ± 30 μM in the presence of sulfate (data not shown).

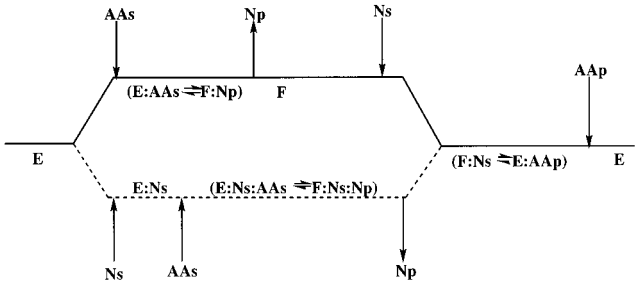
pH Dependence of the Chloride and Sulfide K_d. The dissociation constant for chloride dissociating from the allosteric inhibitory site was measured as discussed in Methods. A pH independent value of 10 ± 1 mM was obtained, in reasonable agreement with the kinetic data reported in Table 1.

Sulfide has been reported to be a substrate inhibitor of OASS-A with a K_I around 50 μM (*3*, *4*). At pH 8, inhibition by sulfide is observed with a K_I of 25 ± 10 μM. At pH values below 8, inhibition is also quite potent, but difficult to quantitate because of the small amount of serine external aldimine that can be formed.

Other monovalent anions also prevent formation of the serine external aldimine at pH 9 and a concentration of 500 mM. These include NaN_3, KSCN, and KF, while KBr and KI exhibit much less inhibition.

Presteady-State Kinetic Studies. The rate of formation of the α-aminoacrylate intermediate was measured as a function of OAS concentration using the stopped-flow method and monitoring the reaction at 470 nm. In Figure 4, the dependence of the rate on the concentration of OAS is shown in the absence of added anions and in the presence of either 500 mM Cl^- or 250 mM SO_4^{2-}. The maximum rate of formation of the α-aminoacrylate intermediate (k_{AA}) is not affected by SO_4^{2-}, with a value of 110 ± 4 s^{-1} in the presence as compared to the value of 101 ± 3 s^{-1} in the absence of SO_4^{2-}. Chloride, however, decreases the rate of α-aminoacrylate formation by 20% (80 ± 2 s^{-1}). The dissociation constant of substrate from the external Schiff base, K_{ESB}, is increased in the presence of both anions with values of 17 ± 1 mM (Cl^-) and 14 ± 1 mM (SO_4^{2-}) as compared to a value of 1.2 ± 0.2 mM in the absence of anions. The resulting second-order rate constants ($k_{AA}/(K_{EST})$ in the absence of anions, and in the presence of Cl^- or SO_4^{2-} are (8 ± 1) × 10^4 M^{-1} s^{-1}, (4.7 ± 0.3) × 10^3 M^{-1} s^{-1}, and (7.9 ± 0.6) × 10^3 M^{-1} s^{-1}, respectively. Thus, decreases in the second order rate constant of about 17- and 10-fold, respectively, are observed in the presence of Cl^- and SO_4^{2-}. Preincubation of OASS with these anions has no effect on the results. Using the above data, one can calculate the competitive inhibition constant for the two anions vs OAS, according to the equation appK_{EST} = $K_{EST}(1 + [anion]/K_{is})$ where appK_{EST} is the value of K_{EST} measured in the presence of anion (17 mM with Cl^- and 14 mM with SO_4^{2-}, respectively), K_{EST} is the value in the absence of anion (1.2 mM) and [anion] is the concentration of anion used.

Scheme 1: Ping Pong Kinetic Mechanism with Alternative Sequential Pathway[a]



[a] The alternative pathway in which sulfide binds prior to OAS is shown as a dotted line. AA_s, AA_p, N_s, and N_p represent the amino acid substrate, amino acid product, nucleophilic substrate and nucleophilic product, respectively.

Estimates of K_i of 38 ± 2 mM and 23 ± 2 mM are obtained for Cl^- and SO_4^{2-}, respectively.

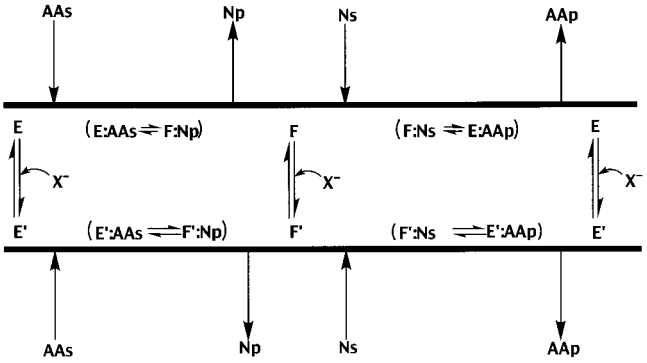
DISCUSSION

Kinetic Mechanism. The kinetic mechanism of OASS is of the classical ping pong BiBi type with OAS binding as first substrate to the internal aldimine form of the enzyme (E), and acetate released as the first product. Sulfide then binds as the second substrate to the α-aminoacrylate form of the enzyme (F), Scheme 1, and L-cysteine is released as the final product (*3*, *4*). Competitive inhibition by both substrates is observed, presumably as a result of binding to the incorrect enzyme form, OAS to F and sulfide to E.

The kinetic mechanism of OASS represents an enigma. In the large majority of enzymes that have a classical ping pong kinetic mechanism with the exception of the oxidoreductases, the reactants and products are structurally similar. The prototypical example of such a reaction is that catalyzed by aspartate aminotransferase, for which reactants (L-aspartate and α-ketoglutarate) and products (oxalacetate and L-glutamate) are dicarboxylic α-amino or α-oxo acids. In the case of OASS, one of the substrates is a *β*-substituted amino acid (OAS) and the second is a small inorganic ion (SH^-). There are several pieces of evidence that are difficult to reconcile in the context of a classical one-site ping pong mechanism for OASS. First, the second half-reaction is very fast with a first-order rate constant $>1000\ s^{-1}$ at 5 *μ*M giving an estimated second-order rate constant of $>10^8\ M^{-1}\ s^{-1}$ (*13*). Second, the structure of the K41A mutant enzyme [methionine external Schiff base (*8*)], indicates that there is no obvious binding site for sulfide as a substrate, consistent with the diffusion-limited second half reaction. However, competitive inhibition by sulfide is observed, so that an inhibitory binding site must exist (*4*). Third, competitive inhibition by sulfide is not complete, which could not easily be explained by any active site combination of sulfide. The above observations can now be explained based on the structure of OASS with chloride bound to an allosteric inhibitory site and the fact that sulfide at inhibitory concentrations behaves identically to chloride.

First, the site for inhibition by substrate, and especially sulfide, is likely the newly identified binding site for chloride as will be discussed further below. Sulfide has no binding site as a substrate (see above) and likely adds to the α-aminoacrylate intermediate directly upon diffusion into the active site as suggested previously (*13*). Second, partial inhibition is common for allosteric inhibitors, and thus the partial competitive substrate inhibition by sulfide is consistent with an allosteric inhibition. The slow alternative pathway proposed previously (see below; ref *4*) is confirmed, but the explanation differs slightly from that proposed. The slow pathway observed at high sulfide concentration likely results from binding of sulfide to the allosteric site, thereby generating a partially inhibited enzyme.

Scheme 2: Ping Pong Kinetic Mechanism with Inhibition upon Anion-Binding to an Allosteric Site[a]



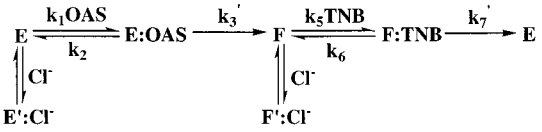
[a] The allosteric ligand can bind to either the internal aldimine (E) or the α-aminoacrylate external aldimine (F) to generate an inhibited enzyme. Reactants and products are as defined in the legend to Scheme 1.

The previously proposed kinetic mechanism is illustrated in Scheme 1 (*4*). In this proposal, the nucleophilic substrate binds to the active site prior to the amino acid substrate, resulting in an apparent sequential kinetic mechanism. It was suggested that the nucleophilic substrate might bind in a hole behind the cofactor in the vicinity of H152 (*7*). However, changing H152 by site-directed mutagenesis does not affect substrate inhibition by sulfide (C.-H. Tai, unpublished results). A modified kinetic mechanism for OASS-A is shown in Scheme 2. In this modified mechanism, binding of an anion (X^-) to the allosteric anion-binding site generates a less active enzyme designated by E′ and F′. However, whether the allosteric site is occupied, the overall kinetic mechanism remains ping pong. The allosteric site appears to be relatively nonspecific and will allow binding of a number of small anions as outlined in Results. In addition, the substrate inhibition by OAS binding to the α-aminoacrylate form of the enzyme (where the active site is largely occupied) may potentially be explained by binding of the α-carboxylate of OAS to the allosteric site. The affinity for the latter combination of OAS is very low; $K_{I\ OAS}$ as a substrate inhibitor is 60 mM, while the K_m for OAS is 2 mM (*4*).

Inhibition Studies. Chloride and sulfate act as dead-end analogues of the nucleophilic substrate. Each of these inhibitors binds with different affinity, dependent on the anion considered, to the active and/or allosteric sites. Each of the inhibitors will be considered independently below.

There is no structural evidence that chloride binds to the active site of OASS, either to the E form of the enzyme, occupying the α-carboxylate subsite, or to the F form of the enzyme, competing with the nucleophilic substrate (e.g., sulfide). Thus, the effects of chloride can be interpreted in

Scheme 3: Kinetic Mechanism for Inhibition by Chloride[a]



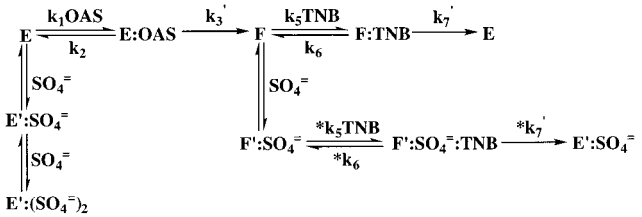
[a] The ' indicates the inhibited forms of the enzyme. The constants k_3' and k_7' are net rate constants for formation of F and acetate, and E and S-CNP-cysteine, respectively.

terms of interaction with the allosteric site alone. Chloride is noncompetitive, regardless of whether OAS or TNB is the varied reactant, and the slope and intercept inhibition constants are identical. These data indicate that chloride combines with both the E and F forms of the enzyme (as pictured for X^- in Scheme 2) with equal affinity. In both cases, chloride is in apparent competition with the varied reactant (slope inhibition), indicating that when chloride is bound to the allosteric site, the reactant either does not combine with the enzyme or it combines but does not react to give product. This interpretation is confirmed by the UV−visible spectral studies that show that when chloride is present at saturating concentrations, the external Schiff bases with L-serine or L-cysteine cannot be formed, i.e., one observes only the free enzyme spectrum. Although the external Schiff bases with OAS, L-methionine, and 5-oxonorleucine can be formed in the presence of chloride, the anion competes effectively with these amino acids. Thus, the mechanism of chloride inhibition can best be described as shown in Scheme 3, where k_3' and k_7' are net rate constants for the conversion of E:OAS and F:TNB to F and E, respectively, and the E′ and F′ forms represent the inhibited forms of the enzyme.

Sulfate, unlike chloride, does bind to the active site in the amino acid substrate α-carboxylate subsite. Inhibition patterns indicate combination with both the E and F forms of the enzyme. In the α-aminoacrylate intermediate, the amino acid's α-carboxylate subsite is occupied, and sulfate cannot be bound to this site. In addition, just as there is no binding site for bisulfide, there is no evidence for an acetate binding site. Thus, the obvious site on F for combination of sulfate would then be the allosteric anion-binding site. The UV−visible spectral data again confirm the interpretation. The external Schiff bases with L-serine or L-cysteine cannot be formed at saturating concentrations of sulfate.

The mechanism of sulfate inhibition with OAS as the amino acid reactant is complex and very informative. Noncompetitive inhibition is obtained for sulfate vs OAS, indicating combination of sulfate with the internal Schiff base and α-aminoacrylate intermediate forms of the enzyme as stated above. However, the slope effect for the net noncompetitive inhibition for binding to the internal Schiff base is parabolic, and indicative of the sequential binding of two molecules of sulfate, both of which compete with the binding of OAS. The most reasonable sites for combination of sulfate on E are the active and allosteric sites. The intercept effect for the net noncompetitive inhibition reflecting binding to the α-aminoacrylate intermediate is hyperbolic indicating a partial inhibition. This suggests that although sulfate slows down the second half reaction upon binding to the allosteric site (the active site is occupied by α-aminoacrylate) it does not completely inhibit it. Thus, the overall kinetic mechanism for sulfate inhibition is given in Scheme 4 where all of the

Scheme 4: Kinetic Mechanism for Inhibition by Sulfate[a]



constants are as defined for Scheme 3 with the addition of the rate constants with an asterisk (*) representing the inhibited pathway.

As a test of the mechanism, the inhibition data for sulfate obtained vs TNB should be accommodated. The inhibition pattern is hyperbolic competitive, consistent with the inhibition of the reaction at low TNB as a result of switching to the slower pathway, and elimination of the inhibition at saturating TNB, restricting the reaction to the uninhibited pathway. However, one would predict an intercept effect as a result of combination to E to give net noncompetitive inhibition. The lack of an intercept effect can in this case be accommodated. The second half-reaction, i.e., F to E is much slower than the first with TNB as the nucleophilic substrate (*4*), and the first half-reaction is irreversible at low acetate, resulting in regeneration of the F form of the enzyme via Le Chatelier's Principle. As a result, the E form of the enzyme does not build up to any significant concentration in the steady state.

Interestingly, addition of OAS to OASS gives the α-aminoacrylate intermediate even in the presence of sulfate or chloride. Initially, this was thought to be due to the thermodynamic sink that is generated by the practically irreversible formation of the α-aminoacrylate intermediate; the equilibrium constant at pH 7 is about 15 000 in favor of the intermediate (*14*). No such irreversible reaction occurs in the presence of L-serine or L-cysteine, which generate the external aldimine species of L-serine and L-lanthionine, respectively (*10*, *13*). There is an effect of both anions on the formation of the α-aminoacrylate intermediate with OAS. The pre-steady-state data indicate a 10- and 17-fold decrease, respectively, in the second-order rate constant $[k_{AA}/(K_{EST})]$ for formation of the external aldimine in the presence of chloride or sulfate. However, the irreversibility of the reaction alone is insufficient to explain the discrepancy, since one observes external aldimine formation with either L-methionine or ONL in the presence of chloride or sulfate, albeit with higher dissociation constants. There must also be a difference in the structure of the external aldimine complex that is formed with OAS (or its mimics) as compared to those formed with either L-cysteine or L-serine. The obvious difference lies in the side chain of the amino acid used.

Inhibition by Sulfide. It was recently suggested that the chloride allosteric inhibitory site was the site for substrate inhibition by sulfide, the physiologic inhibitor (*6*). Data obtained in these studies are consistent with the interpretation. Sulfide prevents the formation of the L-serine external aldimine with a K_I of 25 μM, very similar to the value of 50 μM estimated from initial velocity studies (*3*, *4*). The similarity in behavior of sulfide and chloride, along with their nearly identical ionic radii (chloride, 1.67 Å; sulfide, 1.70 Å) is evidence that sulfide binds to the allosteric site at the dimeric interface of OASS.

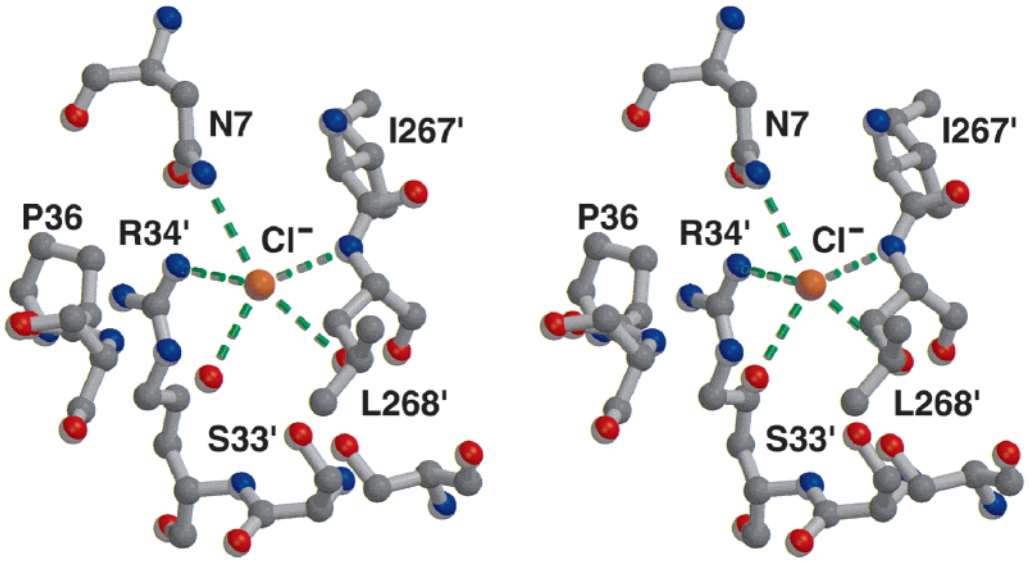


FIGURE 5: Stereoview of the allosteric site at the dimer interface of OASS. Residues that make up the site are contributed from both subunits (distinguished by '). The chloride is shown as an orange sphere, while two water molecules are shown as red spheres.

pH Dependence of Inhibitor Binding. A view of the allosteric inhibitory site is shown in Figure 5. Note that the only potentially ionizable group is Arg-34′, which is expected to have a p*K* greater than 10. To determine whether an enzyme side chain changes protonation state in the allosteric site over the pH range accessible to study of OASS, the pH dependence of the chloride dissociation constant was determined. The chloride K_d is pH independent over the range 7−9, indicating that no enzyme side chain changes protonation state in the accessible pH range. Thus, Arg-34′ is almost certainly ionized to neutralize the negative charge on Cl^- or SH^-.

Sulfide as an inhibitor binds with a K_d of about 25 μM at pH 8 where activity is optimal (*14*). On the basis of the low K_d and the known p*K* values for H_2S ionization ($pK_1 = 7$, $pK_2 = 12$), the ionization state of sulfide as an inhibitor can be determined. At pH 8, the concentrations of H_2S, SH^-, and S^{2-} are 0.09, 0.91, and zero, respectively. Thus, it is not surprising to learn that the monoanion (SH^-), consistent with the charge on Cl^-, is the inhibitory form of sulfide.

Regulation of the Cysteine Biosynthetic Pathway. The cysteine biosynthetic pathway in enteric bacteria is regulated by feedback inhibition of SAT by L-cysteine at the level of enzyme activity. Regulation of OASS has not been reported previously, but sulfide gives partial competitive substrate inhibitor of OASS with a K_i of about 10 μM (*1*, *3*, *4;* this study). The question arises as to why one would want to down-regulate the activity of OASS by its substrate SH^-? There is the possibility of runaway cysteine synthesis when cysteine concentration is low. Under these conditions, SAT is not inhibited and thus OAS will build up. An increase in sulfide concentration would then produce an increase in cysteine concentration equal to the concentration of OAS available. Cysteine is a reducing agent and thus is potentially toxic to the cell. As an estimate of the in vivo concentration of cysteine, its K_I for feedback inhibition of SAT is 1 μM (*1*). The allosteric inhibition of OASS by SH^- would serve to prevent an uncontrolled increase in the concentration of cysteine.

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