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Kinetics and Mechanism of Mutant O-acetylserine Sulfhydrylase-A (C43S) from *Salmonella typhimurium* LT-2

Moon-Young Yoon

Department of Chemistry, Hanyang University, Seoul 133-791, Korea
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Abstract: The pH dependence of the kinetic parameters of mutant O-acetylserine sulfhydrylase (OASS) from *Salmonella typhimurium* LT-2 has been determined in order to obtain information on the chemical mechanism. The initial velocity pattern obtained by varying the concentrations of OAS at several fixed concentrations of TNB, shows an intersection on the left of the ordinate at pH 7.0, indicating that the kinetic mechanism is a sequential mechanism in which substrate inhibition by OAS is observed while the wild type enzyme showed a ping pong mechanism. The values of V/E_t, $V/K_{OAS}E_t$ and $V/K_{TNB}E_t$ decreased by about 68%, 14% and 16% as compared with the wild type enzyme. The V/K_{OAS} is a pK of 6.5 on the acid side of the pH profile, and the V/K_{TNB} is pH independent. As compared with the wild type enzyme, the pKs in the V/K profiles are shifted, reflecting that binding of the cofactor in free E:OAS is less asymmetric.
Key words: kinetics, mechanism, O-acetylserine sulfhydrylase.

Cysteine biosynthesis in the enteric bacterium *Salmonella typhimurium* is catalyzed in two steps as shown in Eqs. (1) and (2) (Kredich and Tomkins, 1966). The first step is carried out by the enzyme serine transacetylase (STA, EC 2.3.1.30), which catalyzed the formation of O-acetyl-L-serine from acetyl CoA and L-serine, while the second step is carried out by O-acetylserine sulfhydrylase (EC 4.2.99.8; O-acetylserine (thiol)-lyase), which catalyzes the formation of L-cysteine from a sulfide and O-acetyl-L-serine (Becker *et al.*, 1969).

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 (OASS-A) and B (OASS-B) isozymes are PLP-dependent and homodimeric with subunit MWs of 34,450 (Levy and Danchin, 1988) and 27,500 (Nakamula *et al.*, 1984), respectively. The gene for the A isozyme from *Salmonella typhimurium* LT-2 has been sequenced (Byrne *et al.*, 1988). The second isozyme, O-acetylserine sulfhydrase-B, catalyzes the same reaction (Kredich, 1971; Hulanicka *et al.*, 1979). A large intracellular excess of the A isozyme over the B isozyme is required during aerobic growth (Filutowicz, 1982).

The kinetic mechanisms for both isozymes have recently been determined using 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 complexed (Tai *et al.*, 1993). The major difference between the two isozymes is in substrate specificity, with the A-isozyme much more specific than the B-isozyme.

OASS-A has been shown to have a ping pong kinetic mechanism. The first half-reaction requires the β-elimination of acetate from O-acetyl-L-serine to generate an α-aminoacrylate intermediate in Schiff base with the active site Pyridoxal-5-phosphate (PLP) (Cook and 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. The ultraviolet-visible spectrum of OASS-A exhibits an absorption maximum at 412 nm attributed to a protonated Schiff base between an active site lysine residue and the PLP (Cook and Wedding, 1976; Nalabolu *et al.*, 1992). Additon 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 indicative of the formation of a protonated Schiff base between PLP and α-aminoacrylate upon the β-elimination of acetate from OAS (Cook and Wedding, 1976; Schnackerz *et al.*, 1979; Nalabolu *et al.*, 1992).

UV-visible spectra of the mutant OASS (C43S) exhibits the same spectral change in the presence of OAS as seen with the wild type enzyme, indicating C43S

*To whom correspondence should be addressed.
Tel and Fax: 82-2-290-0946.

will form the α-aminoacrylate Schiff base intermediate; however, at pH 6.5, the deacetylase activity of C43S is much higher than the wild type enzyme indicating that cysteine 43 plays a role in stabilizing the α-aminoacrylate intermediate. In addition, the circular dichroic and fluorescence studies suggest binding of the cofactor is less asymmetric in C43S than in the wild type enzyme (Park *et al.*, 1996).

In the present work, in order to further understand the chemical mechanism of mutant O-acetylserine sulfhydrylase-A from *Salmonella typhimurium* LT-2, we report pH studies with the mutant OASS-A in which serine replaces cysteine 43. The initial velocity pattern and pH dependence of the kinetic parameters are examined. Data are discussed in terms of the chemical mechanism.

Materials and Methods

Chemicals and enzymes

O-Acetyl-L-serine was obtained from Sigma. All other reagents and chemicals were obtained from commercial sources and were of the highest quality available. OASS-A is a gene product of the *S. typhimurim* cysK gene which has been previously isolated and cloned into pRSM40 (Byrne *et al.*, 1988). The serine-43 mutant was constructed by the method described by Colyer and Kredich (1994). Mutation was confirmed by DNA sequence analysis. Wild type OASS-A was purified by the method of Hara *et al.* (1990) as modified by Tai *et al.* (1993). The purification procedures for the mutant enzyme were essentially the same as those for the wild type enzyme. The enzyme preparation was judged 95% pure on the basis of SDS-PAGE. The protein concentration of the purified enzyme was determined from the enzyme absorbance at 412 nm using an extinction coefficient of 7,600 $M^{-1}cm^{-1}$ (Kredich *et al.*, 1969; Cook *et al.*, 1992). The activity of the enzyme is stable over the pH range of 5.5 to 10 (Hara *et al.*, 1990).

Enzyme assay

The disappearance of 5-thio-2-nitrobenzoate (TNB) as an alternative substrate, resulting from formation of S-(3-carboxy-4-nitrophenyl)-L-cys-teine, was monitored at 412nm. 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 13600 $M^{-1}cm^{-1}$ for TNB (Ellman, 1959).

pH studies

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 could be made for spurious buffer effects. The pH of reaction mixtures was determined before and after sufficient data collection with a Radiometer PHM 82 pH meter with a combined microelectrode at 25℃ 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 cuvette. Initial velocity data were collected using a Gilford 2600 spectrophotometer connected to a flated plotter. The initial velocity pattern was obtained at pH 5.5, 7.0 and 9.5 to determine the pH dependence of the kinetic mechansim and obtain an estimate of the K_m values for reactants. The pH dependencies of V/K_{OAS} and V/K_{TNB} were obtained by varying one substrate at fixed unsaturating concentration of the second substrate and measuring the initial velocity. The maximum rate was estimated by varying substrate concentrations in constant ratio and extrapolating to infinite substrate concentration.

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. (1), while initial velocity data conforming to a sequential mechanism were fitted using Eq. (2).

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

$$v=VAB/(K_{ia}K_b+K_aB+K_bA+AB) \quad (2)$$

In Eqs. (1), (2), v and V represent initial and maximum velocities; K_a and K_b are K_m values for A and B; and A and B represent reactant concentrations, respectively. K_{ia} is the substrate inhibition constant for A.

Data for pH profiles, which decrease with a slope of 1 at low pH, were fitted using Eq. (3).

$$\log y=\log [C/(1+H/K_1)] \quad (3)$$

In Eq. (3), K_1 represents 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, and H is the hydrogen ion concentration.

Results and Discussion

Initial velocity studies

Enzymatic reactions in which there are two substrates

(bisubstrate reactions) usually involve transfer of an atom or a functional group from one substrate to the other. Such reactions proceed by one of several different pathways. In some cases, both substrates are bound to the enzyme at the same time at some point in the course of the reaction, forming a ternary complex. Such a complex can be formed by substrates binding in a random sequence or in a specific order. No ternary complex is formed when the first substrate is converted to the product and dissociates before the second substrate binds. An example of this is the ping-pong or double-displacement mechanism. Steady-state kinetics can help distinguish among these possibilities.

The initial velocity pattern obtained with OAS/TNB for wild type OASS-A is consistent with a ping pong mechanism in which substrate inhibition by both substrates is observed (Tai *et al.*, 1993). In the ping pong mechanism, the reactant $V/K_{substrate}$ reflects the individual half reactions (Cleland, 1977). And the individual half-reaction is 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. So, the V/K_{OAS} reflects the conversion of free enzyme and free OAS to F and acetate, while the V/K_{TNB} reflects the conversion of free F and TNB to E and S-CNP-cysteine. In addition, the V/K is a measure of the rate at limiting reactant concentration, and thus it is the uncomplexed forms of reactant and enzyme that predominate under these conditions.

Kinetic mechanistic data have been obtained for mutant OASS-A (C43S) using O-acetyl-L-serine as amino acid substrate with TNB as nucleophilic substrate. In this mutant OASS-A case, initial velocity obtained by varying one reactant at different fixed levels of the second gave a series of non-parallel lines. The initial velocities obtained with OAS and TNB are shown for mutant OASS-A in Fig. 1. Kinetic parameters obtained for OAS/TNB are listed in Table 1 for C43S. The initial velocities pattern obtained with OAS/TNB for C43S is consistent with a sequential mechanism in which substrate inhibition by OAS is observed. In addition, the values of V/E_t, $V/K_{OAS}E_t$, and $V/K_{TNB}E_t$ were seen to decrease by about 68%, 14% and 16% compared to the wild type enzyme.

The UV-Visible spectrum of OASS-A has shown a λ_{max} at 412 nm, representing a hydrogen-bonded species in which the proton donor in the protonated Schiff base and the proton acceptor is the ionized phenolic oxygen at C-3 of PLP (Kallen *et al.*, 1985). The λ_{max} at 470 nm observed in the presence of OAS represents

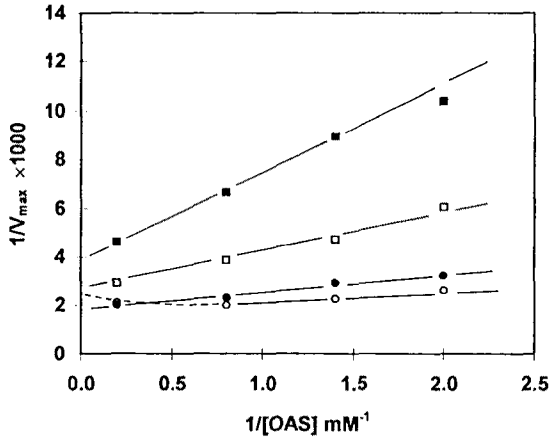


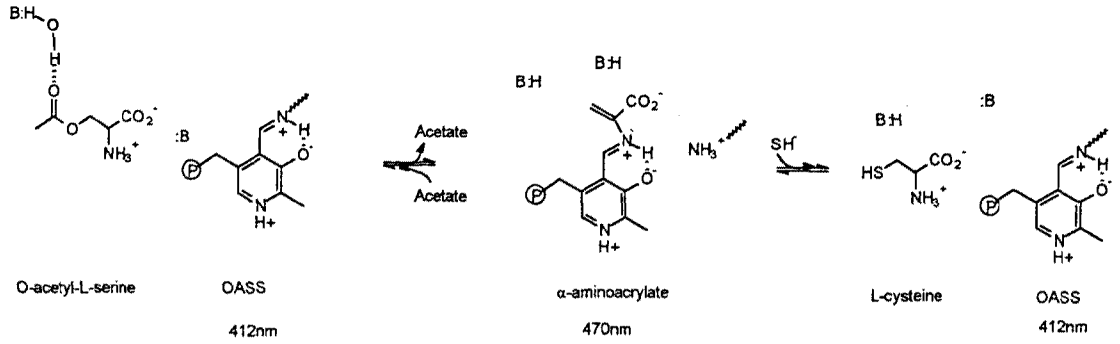
Fig. 1. Initial velocity pattern of the mutant O-acetylserine sulfhydrylase-A (C43S) from *Salmonella typhimurium* LT-2 at pH 6.7 and 25℃. The TNB concentration used was as follows: 0.014 mM (■); 0.025 mM (□); 0.05 mM (●); 0.1 mM (○). The data were fitted to Eq. (2).

Table 1. Kinetic parameters for wild type and mutant of O-acetylserine sulfhydrylase-A obtained at pH 7 and 25℃

Parameter	OAS-TNB	
	Wild type[a]	Mutant
V/E_t (S^{-1})	0.56± 0.08	0.39± 0.001
$V/K_{OAS}E_t$ ($M^{-1}s^{-1}$)	37± 5	5± 1
$V/K_{TNB}E_t$ ($M^{-1}s^{-1}$)	950± 55	150± 12
K_{OAS} (mM)	15± 3	1.3± 1.0
K_{TNB} (mM)	0.6± 0.1	0.043± 0.0008
$K_{I,OAS}$ (mM)	67± 15	9.5± 1.5
$K_{I,TNB}$ (mM)	0.41± 0.16	0.31± 0.2

[a] The value of wild type was obtained from Tai *et al.* (1993).

a hydrogen-bonded species in which the proton donor is the protonated form of the Schiff base of the α-amino of the aminoacrylate intermediate and the proton acceptor is the ionized phenolic oxygen at C-3 of PLP (Schnakerz *et al.*, 1979). The mutant enzyme, C43S, shows that the protonated internal Schiff base of PLP and the ε-amino group of a lysine 42 in the presence of OAS undergo a transaldimination reaction to generate the external Schiff base of OAS and PLP (Park *et al.*, 1996). The α-aminoacrylate in Schiff base with the active site PLP is produced from the external Schiff base at 470 nm. However, the λ_{max} disappearance at 470 nm versus time is much higher than for the wild type enzyme (Park *et al.*, 1996). Such a phenomenon is attributed to an unstable α-amino-acrylate intermediate. In such a case, it is possible that both substrates are bound to the enzyme at the same time at some point in the course of the reaction, forming a ternary complex. Therefore, this complex is able to



Scheme 1. Reaction of O-acetylserine sulfhydrylase-A.

be formed by OAS and TNB binding in a random sequence or in a specific order.

pH dependence of the kinetic parameters

The use of pH studies to determine the chemical mechanism of an enzyme-catalyzed reaction provides knowledge of the chemical interconversions that occur on the enzyme surface once reactants are available, that is a characterization of the catalytic steps responsible for the conversion of reactants to products. Therefore, experiments are designed to define the groups on the enzyme or reactant that are responsible for catalysis and/or binding. In a ping pong kinetic mechanism, the reactant V/Ks reflect the individual half-reactions (Cleland, 1977). That is the V/K_{OAS} reflects the conversion of free enzyme and free OAS to F and acetate, while the V/K_{TNB} reflects the conversion of free F and TNB to E and S-CNP-cysteine. In addition, the V/K is a measure of the rate at limiting reactant concentration, and thus it is the uncomplexed forms of reactant and enzyme that predominate under these conditions.

In the wild type enzyme OASS-A, a group with a pK of about 7.0 is observed on the acidic side of the pH profile suggesting that it should be unprotonated for catalytic activity and/or binding of OAS, while the base of the profile is undefined as a result of the instability of OAS (Nalabolu *et al.*, in press). It was suggested that this general base is required to polarize the carbonyl of the acetyl depositing group to facilitate β-elimination, perhaps by hydrogen bonding to a water molecule and positioning it to hydrogen bond to the carbonyl oxygen (Scheme 1). The V/K for a given substrate in a ping pong kinetic mechanism is independent of the second substrate. The pK_as for two groups are reflected in the profile with values of about 7 on the acidic side and 8.2 on the basic side. The group with a pK_a of 8.2 is attributed to the α-amino group of the enzyme active site lysine that originally formed the internal Schiff base with PLP. The lysine likely donates a proton to the α-carbon to form the cysteine external

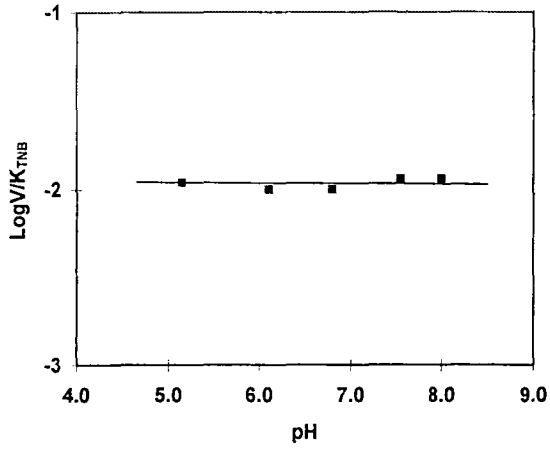


Fig. 2. pH dependence of V/K for the mutant O-acetylserine sulfhydrylase-A (C43S) from *Salmonella typhimurium* LT-2 by varing the concentration of TNB substrate. The concentration of OAS was saturated. The data were fitted to Eq. (3).

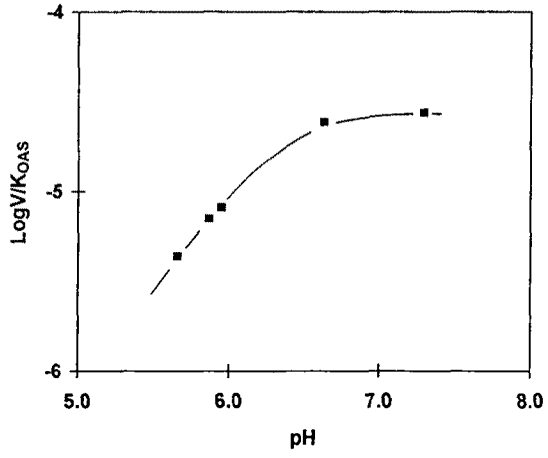


Fig. 3. pH dependence of V/K for the mutant O-acetylserine sulfhydrylase-A (C43S) from *Salmonella typhimurium* LT-2 by varing the concentration of OAS substrate. The concentration of TNB was saturated.

Schiff base. 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

Table 2. Summary of pK values obtained from the pH dependence of kinetic parameters

Parameter	$pK_1 \pm$ S.E.		$pK_2 \pm$ S.E.	
OAS/TNB	Wild type[a]	Mutant	Wild type[a]	Mutant
$V/K_{OAS}E_t$	7.5± 0.7	6.5± 0.1	ND	ND
$V/K_{TNB}E_t$	7.1± 0.1	ND	8.2± 0.1	ND

[a] This data obtained from Nalabolu *et al.* (1994).
ND : not determined.
pK_1 indicates that the group must be protonated for enzyme activity and pK_2 indicated that the group must be deprotonated.

and ammonia catalyzed by OASS-A. The group with a pK 7.0 observed in the V/K_{TNB} profile is likely the same one that is seen in the V/K_{OAS} profile (Nalabolu *et al.*, in press).

In the mutant enzyme C43S, using the OAS/TNB substrate pair, the pH dependences of V/K_{OAS} and V/K_{TNB} are shown in Fig. 2 and Fig. 3, respectively. The V/K_{OAS} profile decreases at low pH with a slope of 1, while the V/K_{TNB} profile is independent of pH. A group of V/K_{OAS} with a pK of about 6.5 is observed on the acidic side of the pH profile suggesting that it should be unprotonated for catalytic activity and/or binding of OAS. The group with a pK of 6.5 observed in the V/K_{OAS} pH profiles is identical to the group with a pK 7.0 observed in the wild type enzyme. The group with a pK of 7.0 observed in the V/K_{OAS} pH profiles in the wild type enzyme is perturbed to a pK of 6.5 observed in the mutant enzyme.

The V/K_{TNB} profile is pH independent. In the mutant enzyme, C43S, the acidic pK and the basic pK are apparently perturbed to a value of <5.0 and <9.0, respectively. The circular dichroic and fluorescence studies have shown that binding of the cofactor is less asymmetric in C43S than the wild type enzyme (Park *et al.*, in press). In this study, the initial velocity pattern obtained with OAS/TNB for the C43S is consistent with sequential mechanism in which OAS inhibition is slightly observed. The V/K for a reactant is the second-order rate constant for conversion of free enzyme and free reactant to products. The pKs observed in the V/K_{TNB} pH profiles reflect acid-dissociable functional groups in free E:OAS in the presence of saturating OAS. Therefore, the perturbation of the pKs in the V/K_{TNB} pH profile might be attributed to the binding of the cofactor in free E:OAS.

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