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Mechanism of O-acetylserine sulfhydrylase from *Salmonella typhimurium* LT-2

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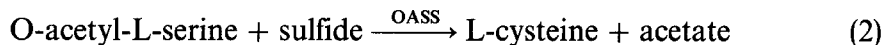
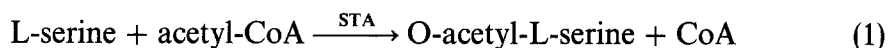
Summary. O-Acetylserine sulfhydrylase is a pyridoxal 5'-phosphate (PLP) dependent enzyme that catalyzes the final step of L-cysteine biosynthesis in *Salmonella*, viz. the conversion of O-acetyl-L-serine (OAS) and sulfide to L-cysteine and acetate. A spectrophotometric assay is available using 5-thio(2-nitrobenzoate) (TNB) as an analog of sulfide and monitoring the disappearance of absorbance at 412 nm. The enzyme catalyzes a ping pong mechanism with α -aminoacrylate in Schiff base with the active site PLP as a covalent intermediate. Using data obtained from the pH dependence of kinetic parameters, the acid-base chemical mechanism and the optimum protonation state of enzyme and substrate functional groups necessary for binding has been determined. The Schiff base and the α -amine of the substrate OAS are unprotonated for binding. There also appears to be a requirement for one active site general base to accept a proton from the α -amine and to donate a proton to form cysteine. The enzyme also catalyzes an OAS hydrolase activity, and the pH dependence of this reaction suggests that the active site lysine that participated in the Schiff base linkage is protonated to start the second half reaction, and has a pK of about 8.2. The stereochemistry of ³H-borohydride reduction of the Schiff base in free enzyme has been determined by degradation of the resulting pyridoxyllysine to pyridoxamine and measuring ³H-release with apo-aspartate aminotransferase. The sequence around the active site lysine is AsnProSerPheSerValLysCysArg.

Keywords: Amino acids – O-Acetylserine sulfhydrylase – Pyridoxal 5'-phosphate – 5-thio(2-nitrobenzoate) – Acid-base chemistry pH dependence – Stereochemistry – Borohydride reduction – Active site sequence

Introduction

O-Acetylserine sulfhydrylase (EC 4.2.99.8) catalyses the final step of L-cysteine biosynthesis in *Escherichia coli* and *Salmonella typhimurium*. The biosynthetic

pathway for L-cysteine consists of two enzyme-catalyzed reactions as illustrated below:



The first reaction is catalyzed by the enzyme serine transacetylase and the second is catalyzed by O-acetylserine sulphydrylase. The latter enzyme contains 1 mol of pyridoxal 5'-phosphate per subunit covalently bound as a Schiff base (Kredich and Tomkins, 1966). The enzyme has a native molecular weight of 68,000 with two identical subunits (Becker et al, 1969).

The sulphydrylase has been shown to proceed via a Ping Pong Bi Bi kinetic mechanism (Cook and Wedding, 1976). The stable intermediate generated in the first half reaction has been suggested to be a Schiff base between PLP and α -aminoacrylate. In the present study we present data from the pH dependence of kinetic parameters on the acid-base chemistry catalyzed by OASS. In addition, data are presented on the stereochemistry of reduction of the internal Schiff base.

Materials and methods

Chemicals and enzymes

O-Acetyl-L-serine, β -chloro-L-alanine, DTNB¹, DTT, NaSCN, Na₂S, acetate, pyridoxamine dihydrochloride, CNBr, NaBH₄, D₂O, KOD (99 atom % D) were from Sigma. Endoproteinase Arg-C was from Boehringer Mannheim Biochemicals. Sodium ³H-borohydride (100 mCi, 9.5 Ci/mmol) was purchased from Amersham. All other reagents and chemicals were obtained from commercially available sources and were of the highest quality available.

O-Acetylserine sulphydrylase from *Salmonella typhimurium* was purified by the method of Hara et al. (1991). The OASS had a final specific activity of 800 units/mg assayed with sulfide as the second substrate using the sulfide ion selective electrode (Hara et al., 1991).

Enzyme assay

The sulphydrylase was monitored using 5-thio(2-nitrobenzoate) (TNB) as an alternative substrate. The disappearance of TNB is monitored continuously at 412 nm as the reaction proceeds. Initial rates were calculated using ϵ_{412} of 13,600 M⁻¹cm⁻¹ for TNB (Ellman 1959).

Initial velocity studies

A typical assay contained the following in final concentrations: 100 mM Hepes, pH 7.5, 5 mM OAS, 0.1 mM TNB and 25 μ g of OASS. Inhibition patterns were obtained with thiocyanate, β -chloro-L-alanine, and acetate by varying one substrate at a fixed non-saturating concentration of the second substrate and different fixed levels of the inhibitors.

¹ Abbreviations: Mes, 2-(N-morpholino)ethanesulfonic acid; Hepes, N-(2-hydroxyethyl)piperazine-N'-2-ethanesulfonic acid; Taps, 3-[[tris(hydroxymethyl)-methyl]amino]propanesulfonic acid; Ches, 2-(N-cyclohexylamino)-ethanesulfonic acid; Na₂S, Sodium sulfide; NaSCN, Sodium thiocyanate; DTNB, 5,5'-DithioBis-(2-Nitrobenzoic acid); DTT, Dithiothreitol; CNBr, cyanogen bromide; TFA, Trifluoroacetic acid.

pH studies

Initial velocity patterns were obtained at pH 5.5, 7, and 9.5 to obtain estimates of the K_m values for OAS and TNB. The pH dependence of V/K_{OAS} , $V/K_{\beta\text{-chloro-L-alanine}}$ and V/K_{TNB} was obtained by varying one substrate at a fixed nonsaturating concentration of the second substrate and measuring the initial velocity. The V_{max} was then measured by varying substrates in constant ratio and extrapolating to infinite substrate concentration. 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 to pH with KOH).

CNBr and endo Arg-C cleavage

24 mg of OASS was reduced by the addition of 0.1 M NaBH₄ containing 100 mCi of sodium ³H-borohydride in 20 mM Hepes at pH 8 until the yellow color disappeared. The reduced protein was digested in 70% TFA with 205 mg of CNBr for 24 hours and the reaction was quenched by lyophilization. Lyophilized CNBr fragments were digested at pH 8 for two days in 0.1 M NH₄HCO₃ and 0.1% SDS by endoproteinase Arg-C with a weight ratio of 20 to 1.

Purification of the pyridoxyl peptide

The pyridoxylpeptide was isolated and purified using a Beckman System Gold HPLC with a model 171 radioisotope detector. Mobile phases were as follows: solvent A consisted of 0.1% TFA in HPLC grade H₂O, while solvent B consisted of 0.1% TFA in HPLC grade CH₃CN. Aliquots of the digest were loaded onto a C₁₈ reversed phase column (4.6 × 250 mm, Beckman) equilibrated with solvent A. The column was eluted with 0% solvent B for 5 min followed by a 90 min linear gradient of 0–60% solvent B at a flow rate of 1.0 ml/min. Elution of peptide was simultaneously monitored using radioactivity and the absorbance at 215 nm.

Reduction of the internal Schiff base

24 mg of OASS in 20 mM Hepes, pH 8 was reduced by the addition of 0.1 M NaBH₄ containing 100 mCi NaBT₄ until the yellow color disappeared. The enzyme was hydrolyzed using 6 N HCl for 24 hours at 110°C and the hydrolysate chromatographed using HPLC and a SP-5PW (21.5 × 150 mm, TosoHaas) cation exchange column to isolate the pyridoxyl-lysine. The chromatograph was developed using a linear gradient of 0–100% solvent B for 60 min at a flow rate of 5 ml/min. Solvent A consisted of 50 mM each of triethylamine and formic acid at pH 3, while solvent B consisted of 50 mM ammonium formate, pH 10. The elution profile was monitored at 294 nm and the pyridoxyllysine identified using a standard synthesized according to Miles et al. (1982). The pyridoxyllysine was then converted to pyridoxamine by oxidation with sodium hypochlorite. Briefly, 10–20 mg of pyridoxyllysine was dissolved in 0.5 ml water at 4°C and treated with 0.06 ml 1 N NaOH followed by 0.05 ml 5% sodium hypochlorite. After 15 minutes the solution was added dropwise to a flask of boiling water (15 ml) and heated for 10 min. The pyridoxamine was then purified by HPLC as above, again using a standard for identification.

Stereochemical analysis of [4'-³H]-pyridoxamine

In a typical analysis, 0.5 mg of pyridoxamine was dissolved in 0.3 ml 20 mM Tris, pH 8.1 and combined with 10 μ l each of 3.4 mM α -ketoglutarate and 150 mM L-glutamate. The pH of the solution was then adjusted to 8 with 1 M sodium carbonate, pH 8.7, 1.5 mg of apo-aspartate aminotransferase was added and the mixture incubated in a closed vessel for 3 days at 37°C, and subsequently distilled to obtain the ³H₂O.

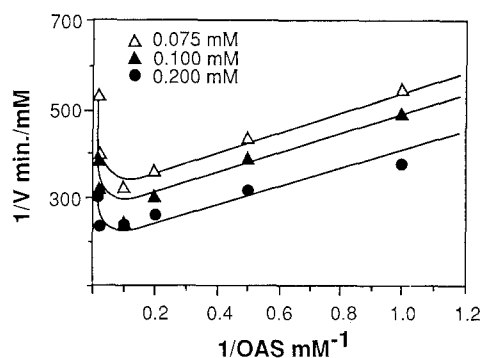


Fig. 1. Initial velocity pattern for OASS varying the concentration of OAS at different concentrations of TNB as indicated. Data were fitted to $v = VAB/(K_aB + K_bA[1 + A/K_{IA}] + AB)$ using the FORTRAN program of Cleland (1979). In the above equations, v is the initial velocity, V is the maximum velocity, K_a and K_b are K_m values, K_{IA} is the substrate inhibition constant for A , while A and B are reactant concentrations. Solid curves are theoretical and points are experimental values

Results and discussion

Initial velocity studies

O-Acetylserine sulfhydrylase has been shown to exhibit double competitive substrate inhibition (Cook and Wedding, 1976). Initial velocity patterns obtained by varying OAS at different fixed levels of TNB exhibit parallel lines with competitive substrate inhibition by OAS alone, Fig. 1. Kinetic parameters are as follows: V/E_t , 0.045 s^{-1} ; K_{OAS} , 2.16 mM ; K_{TNB} , $67 \mu\text{M}$. These can be compared with the following values with sulfide as a substrate: V/E_t , 430 s^{-1} ; K_{OAS} , $140 \mu\text{M}$; K_{s2-} , $70 \mu\text{M}$ (Cook and Wedding, 1976). The effective concentration range of TNB is limited because of the high extinction coefficient for the chromophore, with a maximum of about 0.2 mM allowed. The lack of observed substrate inhibition by TNB is likely a result of the inability to use high enough concentrations of this substrate to see the inhibition. Inhibition by acetate, a product of the first half reaction, is competitive with TNB and noncompetitive against OAS. Dead-end inhibition patterns were obtained with thiocyanate (an analog of TNB) and with β -chloro-L-alanine (an analog of OAS). Thiocyanate is competitive with TNB and noncompetitive with OAS, while β -chloro-L-alanine, a slow substrate, is competitive vs OAS and noncompetitive against TNB. Data are summarized in Table 1. These data are consistent with a ping pong kinetic mechanism in which OAS adds first, acetate is released prior to the binding of TNB, and the final product is S-(3-carboxy, 4-nitrophenyl)-L-cysteine. Substrate inhibition by OAS is a result of binding to the intermediate α -aminoacrylate Schiff base form of the enzyme. Thus, substitution of TNB for sulfide does not result in a change in the kinetic mechanism of OASS and the V/K values reflect the two separate half reactions of the ping pong reaction.

pH dependence of kinetic parameters

Preliminary data collected on the pH dependence of V , V/K_{OAS} , and V/K_{TNB} were obtained as outlined in Materials and methods, Fig. 2. The V/K_{OAS} , and

Table 1. Product and dead-end inhibition of O-acetylserine sulphydrylase^a

Variable substrate ^b	Inhibitor	Type of inhibition	$K_{is} + SE$ (mM)	$K_{ii} + SE$ (mM)
<i>Product inhibition</i>				
TNB	Acetate	C	33 ± 5	130 ± 24
OAS		NC	22 ± 6	
<i>Dead-end inhibition</i>				
TNB	SCN	C	0.15 ± 0.02	0.29 ± 0.05
OAS		NC	0.093 ± 0.038	
TNB	β -chloro-L-Alanine	NC	20 ± 3.3	96 ± 29
OAS		C	12 ± 3	

^a All initial velocities were obtained at 25°C as described under Materials and methods.

^b The fixed substrate was maintained at or below its K_m ; TNB, 0.067 ± 0.013 mM; OAS, 2.16 ± 0.44 mM.

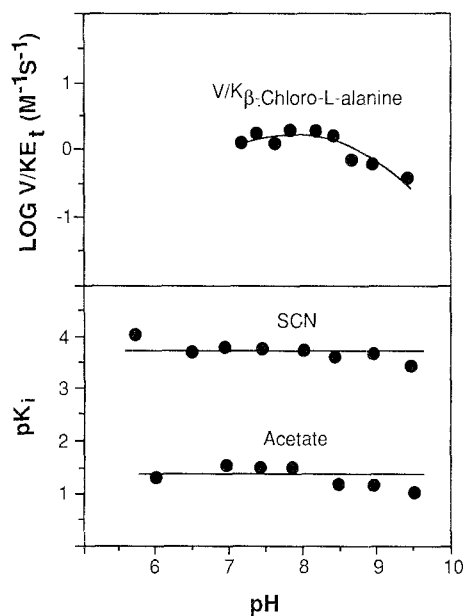


Fig. 2. pH dependence of the kinetic parameters V , V/K_{OAS} , and V/K_{TNB} . Data for V/K s were fitted to $\log y = \log[C/(1 + H/K_1 + K_2/H)]$, while data for V were fitted to $\log y = \log[(Y_L + Y_H K_1/H)/(1 + K_1/H)]$ using the FORTRAN program of Cleland (1979). In the above equations, y is the observed value at any pH, C , Y_H and Y_L are pH independent values, K_1 and K_2 are acid dissociation constants, and H is the hydrogen ion concentration. Solid curves are theoretical and points are experimental values

V/K_{TNB} profiles decrease at both high and low pH with a slope of -1 and 1 respectively. The V/K for OAS exhibits pK values of 7 and 8.7, while the V/K for TNB decreases below a pK of 7.1 and above a pK of 8.2. The V profile increases from a constant value at low pH to another constant value at high pH

giving a pK at or above 9. True pK values are observed in the pH profiles for the dissociation constants for competitive inhibitors or slow substrates (Cleland, 1977). To determine whether intrinsic pK values are observed in the V/K profiles for OAS and TNB, pK_i profiles for SCN^- and the V/K profile for β -chloro-L-alanine (10% the rate with OAS) were measured. The inhibition constants for thiocyanate and acetate were pH independent, Fig. 3, which indicates that the groups present in the V/K profiles were not involved in binding. The $V/K_{\beta\text{-chloro-L-alanine}}$ decreases at high pH with a slope of -1 , giving a pK value of about 8.9.

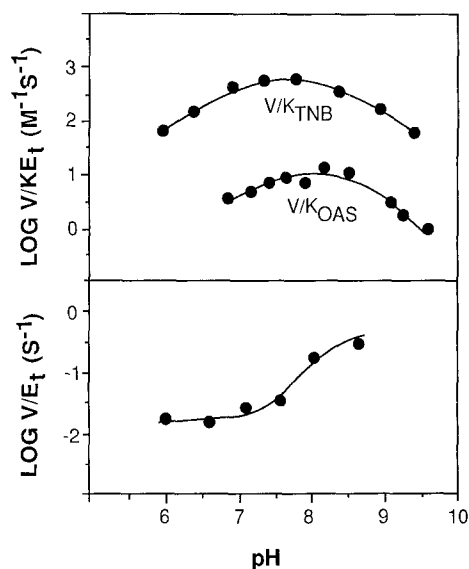


Fig. 3. pH dependence of the reciprocal of the dissociation constant for inhibitors and the V/K for β -chloro-L-alanine. Data for the V/K were fitted to $\log y = \log[C/(1 + K_2/H)]$ using the FORTRAN program of Cleland (1979), where y is the observed value at any pH, C is the pH independent value, K_2 is the acid dissociation constant, and H is the hydrogen ion concentration. Solid curves are theoretical and points are experimental values

The pK of 8.7–8.9 observed in the V/K_{OAS} and $V/K_{\beta\text{-chloro-L-alanine}}$ profiles likely represents the α -amine of the amino acid substrate, while the pK of 7 probably represents a group responsible for accepting the proton from the α -amine of the substrate. In the first half reaction, it is the lysine that formed the internal Schiff base that likely acts as the general base required to accept a proton from the α -carbon in the α, β -elimination reaction. The pK of 7 is also observed in the V/K_{TNB} profile and would reflect the same group as that seen in the first half reaction this time is required as a general acid, while the group with a pK of about 8.2 is most likely the lysine which has lost a proton to solvent at the end of the first half reaction. Since the group with a pK of 7 must be protonated and the lysine must be unprotonated for nucleophilic attack on $C4'$ of the pyridoximine, these groups are in a protonation state opposite that suggested by the pH profile (Cleland, 1977). The pK of the lysine has been determined to 8.2 from the pH dependence of an OAS hydrolase activity observed for this enzyme (unpublished results from this laboratory).

Active site peptide

The radiolabeled peptide containing the active site lysine (see Materials and Methods) is 9 amino acids long and had all of the radioactivity in position 7. The sequence is as follows: AsnProSerPheSerValLysCysArg. The above sequence exactly matches one of those predicted based on the nucleotide sequence (Levy and Danchin, 1988; Byrne et al., 1988), but is not the one predicted based on homology to other PLP enzymes (Levy and Danchin, 1988). The cysteine immediately C-terminal to the active site lysine is the only one present in the polypeptide, and the tryptophan at position 51 is one of two with the other present at position 162.

Stereochemistry

The [4'-³H]-pyridoxamine generated by reduction of the internal Schiff base with sodium ³H-borohydride retained most of its tritium after incubation with apo-aspartate aminotransferase. Of the 30,000 cpm added to the apo-aspartate aminotransferase incubation, 3,280 cpm were released to solvent compared to 2,245 cpm for the minus apo-enzyme control. These results agree with the hypothesis put forth by Dunathan (Dunathan 1971; Dunathan and Voet, 1974) that a single surface (re face) of the active site PLP is accessible to solvent.

Acknowledgement

This work was supported by grants to PFC from the National Science Foundation (DMB 8912053), Robert A. Welch Foundation (B-1031), and to KDS from Deutsche Forschungsgemeinschaft (Schn 139/11-1) and jointly to PFC and KDS from NATO Collaborative Research Grants Program (CRG 900519).

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