A Reaction Mechanism from Steady State Kinetic Studies for O-Acetylserine Sulfhydrylase from Salmonella typhimurium LT-2

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It has been determined from steady state kinetic studies using the sulfide ion selective electrode that O-acetylserine sulfhydrylase catalyzes a Bi Bi Ping Pong reaction between O-acetyl-L-serine and sulfide. Both O-acetyl-L-serine (OAS) and sulfide exhibit strong competitive substrate inhibition. A fit of all the data to the equation for the mechanism yields $K_{OAS} = 0.149 \pm 0.059$ mM and $K_{OASS} = 46.91 \pm 10.06$ mM for O-acetyl-L-serine and $K_{s,S^-} = 0.066 \pm 0.004$ mM and $K_{s,S^-} = 0.013 \pm 0.006$ mM for sulfide.

Product inhibition studies varying either substrate at changing fixed levels of cysteine demonstrate that cysteine combines with enzyme at two places along the reaction sequence to produce inhibition with $K_{ICys} = 1.048 \pm 0.048$ mM and $K_{ICys} = 11.4 \pm 0.5$ mM. Relatively high concentrations of acetate are required to produce inhibition and at least part of the acetate inhibition is due to ionic strength.

However, the ability of acetate to reverse the spectral shift produced from the binding of O-acetyl-L-serine to enzyme and the isotope exchange between $[^{14}C]$acetate and O-acetyl-L-serine does demonstrate that the O-acetyl-L-serine to acetate half-reaction is reversible. There is some doubt as to the specificity of acetate as a product inhibitor, since propionate can also be used to reverse the spectral shift.

Spectral studies using the spectral shift produced from binding O-acetyl-L-serine to enzyme confirms the assignment of a ping-pong mechanism since the spectral intermediate produced is α-aminoacrylic acid in Schiff base with pyridoxal phosphate and, therefore, the acetyl moiety has been β eliminated. Isotope exchange has been demonstrated for both the O-acetyl-L-serine to acetate and sulfide to cysteine half-reactions which also confirms a ping-pong mechanism.

The pathway for the biosynthesis of L-cysteine from L-serine, in the enteric bacteria, Escherichia coli and Salmonella typhimurium, was first demonstrated by Kredich and Tomkins (1). They determined that the synthesis proceeded via a two step enzymatic process which may be represented as:

$$\text{L-Serine} + \text{acetyl-CoA} \xrightarrow{\text{transacetylase}} \text{O-acetyl-L-serine} + \text{CoA-SH}$$ (1)

$$\text{O-Acetyl-L-serine} + \text{S}^2^- \xrightarrow{\text{sulfhydrylase}} \text{L-cysteine} + \text{acetate}$$ (2)

It was also observed that there is a physical association of serine transacetylase with O-acetylserine sulfhydrylase (2), such that a complex could be isolated which catalyzed the synthesis of L-cysteine from L-serine, acetyl-CoA, and H₂S. This multienzyme complex, which contained about 5% of the total cellular O-acetylserine sulfhydrylase, was given the trivial name cysteine synthetase. The sulfhydrylase activity found in the cell, not associated with serine transacetylase, is identical with that bound to the transacetylase, as far as its physical and kinetic properties are concerned. Michaelis constants obtained for O-acetylserine sulfhydrylase were $5 \times 10^{-4}$ M for O-acetyl-L-serine and less than $10^{-4}$ M for sulfide.

O-Acetylserine sulfhydrylase is highly specific for O-acetyl-L-serine but both methyl mercaptan and cyanide can replace sulfide as substrate (3, 4). The absorption spectrum for the highly purified O-acetylserine sulfhydrylase (3) exhibits a prominent absorption maximum at 412 nm which is due to the cofactor, pyridoxal phosphate. This maximum is shifted to 470 nm on binding of the substrate O-acetyl-L-serine, but in the presence of sulfide, O-acetyl-L-serine causes no spectral shift. Using this spectral shift, Becker et al. (3) obtained a dissociation constant of $6 \times 10^{-3}$ M, which is 4 orders of magnitude lower than the $K_m$. This large difference was attributed to the fact that the $K_m$ is not an accurate indication of substrate binding affinity.

O-Acetylserine sulfhydrylase catalyzes a β substitution reaction, but nothing is known about the order of addition of substrates and release of products. Other PLP enzymes which

1 The abbreviations used are: PLP, pyridoxal phosphate; OAS, O-acetyl-L-serine; OASS, O-acetylserine sulfhydrylase; TON, turnover number.
catalyze this general type of reaction have been well characterized. Among these is the B protein of tryptophan synthetase from E. coli, which is responsible for the formation of tryptophan from L-serine and indole, and S-methylcysteine or S-ethylcysteine from L-serine and methyl mercaptan or ethyl mercaptan. The B protein has also been reported to synthesize L-cysteine from L-serine and H₂S. This protein has been well characterized by spectral studies (7), as well as rapid kinetic studies (8), both of which indicate a sequential type mechanism. Tryptophanase, from E. coli (9) and Bacillus alvei (10), forms tryptophan from L-serine, L-cysteine, or S-methylcysteine and indole. Steady state kinetic evidence indicates both enzymes have a sequential mechanism. In all of the cases above, the amino acid has been found to add to the enzyme first. It is of interest, therefore, to determine whether the kinetic mechanism for O-acetylserine sulfhydrylase is of the sequential or the ping-pong type.

MATERIALS AND METHODS

Enzymes—O-Acetylserine sulfhydrylase was purified from Salmonella typhimurium LT-2 by the method of Becker et al. (3). The enzyme was homogeneous by the criterion of disc gel electrophoresis. Total purification was 280-fold with a specific activity of 8300 μmol/min/mg of protein, as compared to the preparation of Becker et al. with a 58-fold purification and a specific activity of 1100 μmol/min/mg of protein. The enzyme was stored at −20°C in 0.1 M Tris-HCl, pH 7.6, with 1 mg/ml of bovine serum albumin. The enzyme is stable under these conditions for more than a year with no loss in activity.

Enzyme Assay—The fixed time colorimetric assay used by Becker et al. has serious limitations for steady state kinetic studies because the assay produces an underestimate of the enzyme velocity. Therefore, in these studies O-acetylserine sulfhydrylase was assayed by continuously monitoring the disappearance of sulfide with a sulfide ion selective electrode (11). The sulfide ion selective electrode (model 94-16A) and the double junction reference electrode (model 90-02-00) were obtained from Orion Research, Inc. The electrodes were directly attached to a precalibrated Heath Schlumberger model EU-205-11 recorder with an EU-200-01 DC offset module and an EU-200-02 potentiometric amplifier. A 1.0-ml reaction chamber consisting of Lucite was under-jacketed to maintain a constant cell temperature of 25°C using a Haake type FJ circulating water bath.

The sulfide ion selective electrode, which has been thoroughly evaluated by Hseu and Rechnitz (12), produces a potential as a logarithmic function of sulfide concentration. A standard curve for sulfide is shown in Fig. 1. Assays were conducted in 0.1 M Tris-HCl, pH 7.6, at 25°C. A 1.0-ml volume of the standard assay contained final concentrations of: O-acetyl-L-serine, 10 mM; sodium sulfide (added in 0.1 M Tris-HCl, pH 7.6, 0.25 mM); Tris-HCl, pH 7.6, 0.1 M, and the reaction was initiated with 0.2 i.u. of enzyme. A unit is defined as the amount of enzyme required to utilize 1 μmol of sulfide in 1 min at pH 7.6 and 25°C.

A point by point conversion of potential to sulfide concentration, as described by Ngo and Shargool (11), using the equation for the standard curve shown in Fig. 1, accurately describes the time course of the reaction, and thus, the point at which the reaction ceases to be linear is readily determined. The linear portion of the time course, fitted by the method of least squares to a linear regression equation, gives the rate as the slope of the regression line. The rate of sulfide loss from the assay mixture by volatilization (beginning at the same sulfide concentration determined by the above procedure) and subtracted from the rate after the addition of enzyme to yield the true enzyme velocity. All features of the rate determination, which include conversion of potential to sulfide concentration, linear regression, calculation of volatility rate, and finally of the enzymatic rate, were incorporated into a program for a Wang 720C programmable calculator with a plotter and an input/output writer. Since, even by the point by point conversion, done by hand it is difficult to locate the linear portion of the rate at low substrate concentrations, the entire time course was put on paper tape using a Dates 308 analog to digital converter interfaced to the Heath Schlumberger recorder. The time course on paper tape was then converted to enzyme velocity by a program written for the Wang 720C programmable calculator including all the features listed above. A plot of velocity, determined by the above method, versus enzyme concentration in milligrams per ml is linear.

The accuracy of velocity measurements using the sulfide electrode was checked by coupling O-acetylserine sulfhydrylase to cysteine desulphydrylase, isolated by the method of Kredich et al. (13), and lactate dehydrogenase. Therefore, the disappearance of NADH at 340 nm could be monitored. Velocities determined by this method correlated well with those determined by the sulfide electrode.

Spectral Studies—Spectra were obtained using either a Varian Cary model 118 spectrophotometer with a repetitive scan attachment or a Varian model 635 spectrophotometer. The Cary model 118 spectrophotometer was used with a scan rate of 25 nm/minute and recorded at 1 nm/s. The Varian model 635 spectrophotometer was used with a scan rate of 25 nm/minute and recorded at 1 cm/min. A light path of 1 cm was used for both instruments.

Isotope Exchange—The cysteine to sulfide half-reaction was measured in a system containing a reaction vessel (consisting of a 5.0-ml test tube sealed with a serum cap) and a 10% silver nitrate trap (also consisting of a 5.0-ml test tube sealed with a serum cap) which was used to trap sulfide as insoluble silver sulfide. A 1.0-ml volume of the reaction mixture contained the following: O-acetylserine sulfhydrylase, 0.2 i.u.; [35S]cysteine, 6 mM (0.6 mCi/mmol); sodium sulfide, 0.25 mM; Tris-HCl, pH 7.6, 0.1 M. The control mixture contained the same components listed above minus O-acetylserine sulfhydrylase. After a 1-hour incubation period, the reaction was terminated by adding 5 N HCl and nitrogen was gently bubbled through the mixture for several minutes. An aliquot from the silver nitrate trap was added to 10 ml of scintillation fluid containing 1/5 volume of Triton X-100, 1/10 volume of toluene, 4.0 g/liter of 2,5-diphenyloxazole (POPOP) and 0.2 g/liter of 1,4-bis(2-(4-methyl-5-phenyloxazolyl) benzene (dimethyl-POPOP). Radioactivity was measured in a Beckman model LS 230 liquid scintillation system.

The acetate to O-acetyl-L-serine half reaction was measured by incubating O-acetylserine sulfhydrylase for 1 hour with [14C]acetate and O-acetyl-L-serine. A 0.1 ml volume of the reaction mixture contained the following: O-acetylserine sulfhydrylase, 0.2 i.u.; [14C]acetate, 100 mM (2.95 mCi/100 mmol); O-acetyl-L-serine, 10 mM; Tris-HCl, pH 7.6, 0.1 M. The control mixture contained the same components listed minus O-acetylserine sulfhydrylase. The reaction was terminated with 0.01 N HCl and samples were applied to small (Pasteur pipette) Dowex 50-W columns which were eluted with 0.1 N HCl until the counts per min eluting from the column reached background. Radioactivity in O-acetyl-L-serine was determined by suspending the contents of the small column in 1.5 ml of water and adding 10 ml of the same scintillation fluid used for the 35S exchange. Radioactivity was measured with a Beckman model LS 230 liquid scintillation system.

Chemicals—AR grade sodium sulfide, sodium acetate, and silver nitrate were obtained from Mallinckrodt. Scintillation grade Triton X-100 and toluene were obtained from Eastman Kodak Co. Crystalized, fatty acid-free bovine serum albumin, A grade L-cysteine, and 1,4-bis[2-(4-methyl-5-phenyloxazolyl)benzene (dimethyl-POPOP). Radioactivity was measured in a Beckman model LS 230 liquid scintillation system.
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Dowex 50W cation exchange resin were obtained from Sigma Chemical Co. A grade O-acetyl-L-serine was obtained from Calbiochem. Enzyme grade Tris base was obtained from Schwarz/Mann. Scintillation grade 2,5-diphenyloxazole and 1,4-bis[2-(4-methyl-5-phenyloxazolyl)]benzene, L-[35S]cysteine hydrochloride (0.6 mCi/mmol), and [2-3H]acetate (59 mCi/mmol) were all obtained from Amersham/Searle Corp.

Data Processing—Reciprocal initial velocities were plotted against reciprocal substrate concentrations. All calculations and fits were carried out using programs written for the Wang 720C programmable calculator except the data fitted to Equation 5 which was carried out by a program adapted for the Wang 2200 programmable calculator from a Fortran program developed by W. W. Cleland.

Saturation curves for both substrates were fitted to an equation which describes substrate inhibition assuming substrate combines twice with enzyme (14). The fit is by the method of least squares to a multiple linear regression equation of the reciprocal of Equation 4.

The data for double competitive substrate inhibition in a ping-pong mechanism were fitted to Equation 5 by the method of Cleland (15).

Data for S parabolic, I linear noncompetitive inhibition, varying O-acetyl-L-serine were fitted, line by line, to Equation 3 by the method of Cleland (15):

\[ v = \frac{V_A}{K_a + A} \]

(3)

Data for S parabolic, I linear noncompetitive inhibition varying sulfide, were fitted, line by line, by the method of least squares to a multiple linear regression equation of the reciprocal of Equation 4.

Replots of intercept and slope versus inhibitor concentration were fitted by the method of least squares, using a second order parabola or linear function, as appropriate.

RESULTS

The activity of O-acetylserine sulfhydrylase as a function of O-acetyl-L-serine concentrations up to 50 mM is shown in Fig. 2. Some substrate inhibition becomes apparent at O-acetyl-L-serine concentrations higher than 7.5 x 10^-4 M. Kinetic parameters derived from the fit according to Equation 4 are

\[ v = \frac{V_A}{K_a A + A} \]

(4)

listed in Table I. The apparent \( K_a \) for O-acetyl-L-serine is in reasonable agreement with the 5 x 10^-3 M previously reported (3).

The saturation curve for the other substrate, sulfide, as shown in Fig. 3, indicates a marked substrate inhibition at concentrations above 2.5 x 10^-4 M. Kinetic parameters for sulfide derived from these data are listed in Table I and the apparent \( K_a \) for sulfide is again in reasonable agreement with that previously reported.

Initial Velocity Studies in Absence of Products—When O-acetyl-L-serine is used as the variable substrate, at fixed varying levels of sulfide, over concentrations of sulfide from 2 x 10^-4 M to 3.6 x 10^-3 M and of O-acetyl-L-serine concentrations from 2 x 10^-\( \mu \)M to 5 x 10^-3 M, an unusual but revealing pattern is obtained (Fig. 4, A and B). The pattern has been shown by Cleland (14) to be indicative of strong competitive inhibition by both substrates in a ping-pong mechanism.

When the initial velocity as a function of sulfide as the variable substrate at fixed varying levels of O-acetyl-L-serine is plotted as in Fig. 5, A and B, the same pattern is found although with more pronounced inhibition due to sulfide as would be expected from the relative inhibitory effects of O-acetyl-L-serine and sulfide already determined.

The kinetic parameters shown in Table II were obtained by fitting all the data of Figs. 4 and 5 to Equation 5 (15):

\[ v = \frac{V_A}{K_{IA} \left( 1 + \frac{A}{K_{IA}} \right) + \frac{V_A}{K_{IB} \left( 1 + \frac{B}{K_{IB}} \right)}} + \frac{A}{b} \]

(5)

for double competitive substrate inhibition in a ping-pong mechanism.

Initial velocity studies were confined to the forward reaction since the reaction is for all practical purposes, irreversible. No reverse reaction could be detected with 0.4 M acetate, 0.1 M cysteine, and 1600 times the normal assay concentration of enzyme.

| Table I |
|-------------------|------------------|
| Kinetic constants derived from saturation curves obtained for O-acetyl-L-serine and sulfide |
| O-Acetyl-L-Serine | Sulfide |
|-------------------|--------|
| \( k_{0AS} \) | 2.15 ± 0.45 |
| \( k_{AG} \) | 0.130 ± 0.02 |
| \( k_{0AS} \) | 27.67 ± 5.25 |
| \( k_{BG} \) | 0.347 ± 0.06 |

Fig. 2. Dependence of O-acetylserine sulfhydrylase activity on O-acetyl-L-serine concentration. Standard assays were used except that O-acetyl-L-serine was varied as indicated and 4.0 i.u. of enzyme were used per assay. Inset shows the Lineweaver-Burk plot of rate versus O-acetyl-L-serine concentration.

Fig. 3. Dependence of O-acetylserine sulfhydrylase activity on sulfide concentration. Standard assays were used except that sulfide was varied as indicated and 1.0 i.u. of enzyme was used. Inset shows the Lineweaver-Burk plot of rate versus sulfide concentration.
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Fig. 4. Initial velocity pattern varying O-acetyl-L-serine at fixed varying levels of sulfide. The standard assay was used except that O-acetyl-L-serine and sulfide were varied as indicated. A, sulfide concentrations are: O, 2 x 10^{-4}; B, 1 x 10^{-4}; +, 5 x 10^{-4}; A, 1 x 10^{-4}; ○, 5.6 x 10^{-4}; ×, 1.44 x 10^{-4}; ■, 2.38 x 10^{-4}; □, 3 x 10^{-4}. B, sulfide concentrations are: ☄, 5.6 x 10^{-4}; ×, 1.44 x 10^{-4}; Δ, 2.38 x 10^{-4}; ■, 3 x 10^{-4}; ◆, 3.6 x 10^{-4}. All curves are numbered in order of increasing concentration.

Fig. 5. Initial velocity pattern varying sulfide at fixed varying levels of O-acetyl-L-serine. The standard assay was used except that O-acetyl-L-serine and sulfide were varied as indicated. A, O-acetyl-L-serine concentrations are: ○, 1 x 10^{-4}; B, 1 x 10^{-4}; +, 2 x 10^{-4}; A, 5 x 10^{-4}; Γ, 2 x 10^{-4}. All curves are numbered in order of increasing concentration.

Fig. 6. Reciprocal plot of velocity varying O-acetyl-L-serine with L-cysteine as the inhibitor. The standard assay was used except that O-acetyl-L-serine was varied and L-cysteine was added to the assays indicated. L-Cysteine concentrations are: O, control; Δ, 5 x 10^{-3}; ○, 1 x 10^{-2}; ×, 2 x 10^{-3}. Right, replot of slope versus L-cysteine concentration.

Inhibition constants for cysteine obtained from these data are listed in Table III.

With sulfide as the variable substrate, at concentrations which produced low and high levels of inhibition, and a

**Table II**

|                  | Kinetic constants derived from initial velocity data |
|------------------|-----------------------------------------------------|
| O-Acetyl-L-Serine| K_{OAS} 0.149 ± 0.059 K_{S2} 0.0066 ± 0.004 |
|                  | K_{OAS} 46.9 ± 10.06 K_{S2} 0.013 ± 0.006 |

**Product Inhibition Studies**—When O-acetyl-L-serine was varied at a constant level of sulfide, i.e., one which produced the maximum velocity observed in the sulfide saturation curve (0.25 mM), and changing fixed cysteine, the pattern shown in Fig. 6 was obtained. Since both O-acetyl-L-serine and cysteine combine to free enzyme, it would be expected that cysteine would be a competitive inhibitor of O-acetyl-L-serine. But the pattern shown in Fig. 6 indicates a noncompetitive inhibition and the replot of slope versus cysteine concentration, Fig. 6, is parabolic rather than linear.

Inhibition of the S parabolic, I linear type as found here indicates that the inhibitor combines with the enzyme at two places along the reaction sequence to produce an inhibition.
TABLE III

Inhibition constants derived from product inhibition studies varying O-acetyl-L-serine at different fixed levels of L-cysteine

|                      | L-Cysteine |
|----------------------|------------|
| mM                   |            |
| $K_{i1}$             | 10.48 ± 0.048 |
| $K_{i2}$             | 10.85 ± 0.3  |

Constants were obtained from least squares fits to the appropriate equation of the replots of slope and intercept versus L-cysteine concentration.

The replot of slope versus cysteine concentration in Fig. 7 again shows a parabolic function, but the relatively low rates obtained in these experiments produce standard errors for the slopes that are too large to allow calculation of any meaningful constants. The interpretation of this relationship is the same as that for cysteine inhibition in the presence of varying O-acetyl-L-serine concentration.

The mechanism supported by the steady state data presented requires that the intermediate species be II, i.e. α-aminoacrylic acid in Schiff base with PLP. A ping-pong mechanism (16) requires that the first product must be released prior to binding the second substrate. Therefore, the binding of O-acetyl-L-serine and the release of acetate should generate II.

Recently, work by Schnackerz* has shown fairly conclusively, the identity of the species produced on the binding of O-serine to O-serine dehydratase which produces absorption maxima at 460 and 330 nm. The incubation of transition state analogs of II, specifically III, with apodehydratase produced a shift of the absorption maximum of the analog from 420 nm to 460 nm as well as a shoulder at 330 nm.

The spectrum of purified O-acetylserine sulfhydrylase is shown in Fig. 8. Binding of O-acetyl-L-serine to enzyme (Fig. 9) gives rise, not only to a species at 470 nm, but also to one at 330 nm. When acetate is added to enzyme and O-acetyl-L-serine (Fig. 9) there is a decrease in the absorbance at 470 nm and 330 nm accompanied by an increase in the absorbance at 412 nm. Although the amount of acetate used here, 0.4 M, is very high, other ions, such as chloride and sulfate, in comparable concentrations do not cause this same shift. This decrease in the absorption maximum due to the binding of O-acetyl-L-serine to the enzyme suggests that at least part of the inhibition observed when acetate is used as a product inhibitor.

Spectral Studies—On binding of O-acetyl-L-serine to free enzyme, there is a shift in the absorption maximum from 412 to 470 nm. The species responsible for the shift has been identified as the quinonoid structure shown as I (7) and also as α-aminoacrylic acid in Schiff base with PLP (6), II (Scheme 1).

*K. D. Schnackerz, manuscript in preparation.
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Fig. 9. Difference spectrum for the addition of O-acetyl-L-serine and acetate to O-acetylserine sulfhydrylase. The abscissa represents enzyme alone. ● addition of 3 x 10^{-6} M O-acetyl-L-serine to enzyme in 0.1 M Tris-HCl, pH 7.6, and 25°C; ○, addition of 0.4 M acetate to enzyme plus O-acetyl-L-serine in 0.1 M Tris-HCl, pH 7.6, and 25°C. In all cases the reference cell contained no enzyme.

is attributable to a reversal of the first half-reaction rather than to the high level of ionic strength.

The specificity of acetate in inducing a decreased absorbance at 470 nm was tested with sodium propionate. The results are shown in Fig. 10 where it may be seen that propionate produces an effect very similar to that caused by acetate.

Isotope Exchange—If the reaction mechanism for O-acetylserine sulfhydrylase is of the ping-pong type, then two exchange reactions should be possible since the mechanism requires two half-reactions. These are as follows:

\[ E + OAS \leftrightarrow F + Acetate \]  
\[ E + Cysteine \leftrightarrow F + S^{2-} \]

where \( F \) is a second stable enzyme form, in this case \( \alpha \)-aminoacrylic acid in Schiff base with PLP.

The O-acetyl-L-serine to acetate exchange was tested using \(^{2-14}C\text{acetate} \) and the cysteine to sulfide exchange was tested using \(^{[35}S\text{cysteine} \). The results of these experiments, shown in Table IV, indicate that both exchange reactions are catalyzed by O-acetylserine sulfhydrylase.

**DISCUSSION**

Initial velocity studies produce a distinctive pattern which can only be interpreted as competitive inhibition by both substrates in a ping-pong mechanism. Competitive substrate inhibition is indicative of such a mechanism, where inhibition arises from a combination of substrate with a stable enzyme form other than that with which it normally combines as a substrate.

Using this information, the mechanism can be written as shown in Equation 8.

\[
\begin{align*}
E + OAS & \rightarrow F + Acetate \\
E + Cysteine & \rightarrow F + S^{2-}
\end{align*}
\]

The TON for O-acetylserine sulfhydrylase in these studies has been determined as \(1.86 \times 10^5\) amol/min/μmol of O-acetylserine sulfhydrylase from \(V_{\text{max}}\) obtained from initial velocity studies (0.1741 amol/min) and the concentration of O-acetylserine sulfhydrylase sites per assay. The TON reported previously (2) was \(4.3 \times 10^4\) amol/min/μmol of O-acetylserine sulfhydrylase. The difference is attributed to the underestimate of velocity produced by the fixed time assay.

It can be seen by comparison of the kinetic parameters in Tables I and II that quantitative data are not always very meaningful unless the mechanism for the enzyme is known. In the case of O-acetylserine sulfhydrylase, all four constants are different, but only \(K_{\text{OAS}} \) and \(K_{\text{Ac}} \) are significantly different.

The double competitive substrate inhibition together with identification of the species absorbing at 470 nm as \( \alpha \)-aminoacrylate provide strong support for a ping-pong mechanism. When O-acetyl-L-serine adds to the enzyme, the mechanism predicts that acetate, the first product, must be released prior to the addition of sulfide, the second substrate. This would leave, after the \( \beta \) elimination of acetate, the second stable enzyme form (\( F \)), \( \alpha \)-aminoacrylate acid in Schiff base with PLP.

Conclusive support for a ping-pong mechanism comes from the isotope exchange experiments. Both the A-P and B-Q exchanges must proceed if the assignment of this mechanism is correct. The fact that both exchanges do occur is proof that O-acetylserine sulfhydrylase catalyzes a Ping Pong Bi Bi reaction.

Cysteine, as a product inhibitor, produces S parabolic, I linear noncompetitive inhibition varying both substrates. This
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suggests that cysteine combines with the enzyme at two points along the reaction sequence for O-acetylserine sulfhydrylase. Thus, cysteine must combine with both of the stable enzyme forms; first as a product inhibitor to free enzyme with a $K_{i,cys}$ of 1.05 mM, competing with O-acetyl-L-serine and second as a dead end inhibitor to $F$ with a $K_{i,cys}$ of about 11.4 mM, competing with sulfide.

The effects of acetate are difficult to determine due to the added complication of high ionic strength. However, the reversal of the spectral shift, produced by addition of O-acetyl-L-serine to enzyme, by acetate and not by other ions, suggests that at least some of the inhibition is not due to the ionic strength. This fact is confirmed by the isotope exchange experiments which show that the acetate to O-acetyl-L-serine half reaction is reversible. However, propionate can also be used to reverse the spectral shift, so there is some doubt as to the specificity of acetate, thus, suggesting at least three possibilities. The first is that propionate also fits the site of enzyme occupied by the acetyl moiety of O-acetyl-L-serine and acetate, and thus, O-propionyl-L-serine should also be used as a substrate. Second, there is no site on enzyme for the acetyl moiety or acetate, but it simply serves as a good leaving group. Third, there is a site on enzyme for the acetyl moiety of O-acetyl-L-serine, but upon formation of the Schiff base of O-acetyl-L-serine with enzyme and $\beta$ elimination of acetate, there is a conformational change, destroying or partially destroying the acetate binding site. In the latter two hypotheses, both acetate and propionate inhibition would simply be a matter of statistics, dependent on the number of collisions of acetate with the $\alpha$-aminoacyclic acid Schiff base. These possibilities are in need of further investigation.

With the addition of cysteine product inhibition, the schematic for the mechanism of O-acetylserine sulfhydrylase can be written as shown in Equation 9.

$$
\begin{align*}
\text{DAS} & \rightarrow \text{Acetate} \\
\text{S} & \rightarrow \text{Cysteine} \\
\end{align*}
$$

There are two other cases reported of enzymes which catalyze ping-pong mechanisms in which both substrates produce strong competitive inhibition. One of these is $\beta$-ketothiolase from yeast (17) which is responsible for the synthesis of $\beta$-hydroxy-$\beta$-methylglutaryl coenzyme A. Product inhibition studies have not been done with this system but it is similar to O-acetylserine sulfhydrylase in that the reaction is essentially irreversible. Another enzyme which produces similar initial velocity patterns is nucleotide diphosphate kinase from yeast (18) which is responsible for the conversion of AMP to ATP.

It is interesting that O-acetylserine sulfhydrylase catalyzes a ping-pong mechanism while the other two PLP enzymes catalyzing $\beta$ substitution reactions have sequential mechanisms. The reason for this may become clear when the mechanism for the complex, cysteine synthetase, has been investigated.

Cysteine synthesis appears to be regulated not only coarsely, by induction-repression mechanisms, as reported by Kredich (19), but also with great sensitivity within strict finite limits of sulfide and cysteine concentrations. As sulfide concentration increases, there is a pronounced substrate inhibitory effect on O-acetylserine sulfhydrylase, thus limiting the quantities of cysteine produced. As cysteine concentration increases, there are several regulatory effects: one, an induction of cysteine desulfhydrylase (20), at concentrations above $10^{-4}$ M, and a subsequent degradation of cysteine to sulfide (which can now inhibit O-acetylserine sulfhydrylase, dependent on the levels), pyruvate, and ammonia, two, cysteine can act to inhibit in two ways along the reaction sequence of O-acetylserine sulfhydrylase, once as a product inhibitor and another as a dead end inhibitor; three, cysteine feedback inhibits serine transacetylase, the first enzyme in its synthetic pathway, thereby preventing the futile use of acetyl-CoA which is required in several other synthetic pathways beside cysteine synthesis.

In conclusion, it has been determined by initial velocity, spectral and isotope exchange experiments that O-acetylserine sulfhydrylase catalyzes a Bi Bi Ping Pong reaction. Both substrates form dead end complexes with stable enzyme forms other than the one with which they normally combine. Product inhibition studies with cysteine indicate two sites for combination of the product along the reaction sequence, once as a product inhibitor and another as a dead end inhibitor. Acetate inhibition was difficult to detect because of the added problem of ionic strength. There is some doubt as to whether there is a binding site on enzyme for acetate since it appears that propionate can replace acetate, at least in the reversal of the spectral shift produced by addition of O-acetyl-L-serine to enzyme.

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