Resolution of \( \nu \)-Serine Dehydratase by Cysteine

AN ANALYTICAL TREATMENT*

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SUMMARY

A general method is presented for analysis of the resolution of pyridoxal-P-requiring enzymes by carbonyl reagents. The method is useful for accurately determining the very small equilibrium constants \( (K_\epsilon) \) which characterize the dissociation of cofactor from many pyridoxal-P-requiring enzymes. The analysis also establishes the minimum number and relative stabilities of distinct enzymic species involved in the resolution process. Analysis of the resolution of \( \nu \)-serine dehydratase by \( L \)- and \( D \)-cysteine resulted in the establishment of an enzyme bound thiazolidine derivative as an intermediate in the pathway for resolution. The overall equilibrium constant \( (K_F) \) for the reaction, \( \nu \)-serine dehydratase + cysteine \( \rightarrow \) thiazolidine derivative + \( \nu \)-serine apodehydratase was determined. At \( pH \) 7.80, \( K_F = 1.08 \times 10^{-3} \). A value of 7.0 nm for the equilibrium constant for the dissociation of \( \nu \)-serine dehydratase to apoenzyme and free pyridoxal-P was determined from the ratio \( K_F/K_P \), where \( K_P \) is the equilibrium constant for the formation of a thiazolidine derivative from free pyridoxal-P and cysteine. An estimate of 14 nm for \( K_P \) was also obtained from partial resolution of \( \nu \)-serine dehydratase by high dilution. The difficulties associated with this direct determination of \( K_P \) from the dependence on the enzyme concentration of the activity of very dilute solutions of enzyme are discussed.

EXPERIMENTAL PROCEDURE

Materials

Compounds used and the sources from which they were purchased are: EDTA (acid form) and \( \nu \)-penicillamine from Aldrich Chemical Co.; DEAE-cellulose (Cellex D) from Bio-Rad Laboratories; \( \nu \)-cysteine hydrochloride, \( L \)-cysteine hydrochloride hydrate, \( D \)-, \( L \)-, and \( \nu \)-serine, sodium pyruvate, dithiothreitol, protamine sulfate (salmine), \( \beta \)-NADH (reduced, disodium salt), rabbit muscle lactate dehydrogenase, deoxyribonuclease I (bovine pancreas, B grade), ribonuclease (bovine pancreas, A grade), and bovine serum albumin (crystallized, A grade) from Calbiochem Corp.; bromphenol blue (tracking dye) from Canalco; Bacto-peptone and Bacto-agar from Difco Laboratories; \( N, N', N'' \), \( N' \)-tetramethylthelyenediamine, acrylamide, \( N' \)-methylene-bisacrylamide and riboflavin from Eastman Organic Chemicals; ammonium persulfate from Fischer Scientific; casein hydrolysate (enzymatic) from Nutritional Biochemicals Corporation; Sephadex G-25, from Pharmacia Fine Chemicals; Coomassie brilliant blue, and ultrapure ammonium sulfate, special enzyme grade, from Schwarz/Mann; DTNB\(^1\) from Sigma. Pyridoxal 5'-phosphate monohydrate (originally from Calbiochem Corp.) was the generous

* The abbreviation used is: DTNB, 5,5'-dithiobis-(2-nitrobenzoic acid).

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Methods

Single distilled water was used to prepare culture media. All other buffers were prepared from potassium salts in distilled water which was deionized and redistilled in a Corning AG-2 all-glass apparatus. The acid component of the buffer at the appropriate concentration was mixed with equimolar conjugate base until the desired pH was obtained. In this paper, standard buffer is defined as 0.1 M potassium phosphate, 0.1 mM EDTA, pH 7.80, and Buffer A as 0.1 M potassium phosphate, 0.1 mM EDTA, 1 mM dithiothreitol, 5 mM pyridoxal-P, pH 7.80. Acetylated bovine serum albumin was prepared and tested for effectiveness of acetylation, according to the procedures used by Dowhan and Snell (14). Dialysis tubing (Union Carbide Corp.) was pretreated by heating 3 times in 0.1 M phosphate, 1 mM EDTA buffer at 70 C and 2 more times in the same buffer without EDTA. Each time the buffer was boiled for 10 min. The tubing was then stored in standard buffer at 4 C. Ultrafiltration of enzyme samples was performed in a Diaflo apparatus (Amicon Corp.) equipped with either a PM 10 or UM 10 membrane. Measurements of pH were made as described in the preceding paper (15). Absorption spectra were obtained using a Cary spectrophotometer, model 118. All reactions analyzed at a single wavelength were followed in a Gilford recording spectrophotometer, model 2000 or 240; both machines were equipped with jacketed cuvette compartments for temperature control. All measurements were taken at 25.0 ± 0.5 C.

Re-isolation of C6 Mutant

The C6 mutant of Escherichia coli K-12 was reisolated in the following manner. The organism was suspended and serially diluted in minimal medium (20), plated on agar, and allowed to grow at 37 C for 20 hours. Ten individual colonies were selected according to the following manner. The organism was suspended and serially diluted in minimal medium (20), plated on agar, and allowed to grow at 37 C for 20 hours. Ten individual colonies were selected at random. Each colony was transferred to 100 ml of sterile minimal medium and allowed to grow at 37 C to stationary phase. The specific activity of each colony was determined from a crude extract of the stationary phase cells prepared by sonic disruption of the bacterial cells with an HSL sonicator (Instruments Corporation of America). All reactions analyzed at a single wavelength were followed in a Gilford recording spectrophotometer, model 2000 or 240; both machines were equipped with jacketed cuvette compartments for temperature control. All measurements were taken at 25.0 ± 0.5 C.

Growth of C6 Mutant

Fraser's glycerol medium (23) was modified such that each liter of medium contained 8.4 g of Na2HPO4, 4.5 g of KH2PO4, 2.0 g of NH4Cl, 2.5 g of casein hydrolysate (enzymatic), 0.5 g of MgSO4-7H2O and 8.0 ml of reagent grade glycerol. Bacterial suspensions were grown from a 3 to 5% inoculum in 200 liters of modified Fraser's medium at 37 C in a Fernamann fermentor, model F-250 (New Brunswick Scientific Co.), which was continuously stirred at a speed of 200 rpm and aerated at a rate of 6.5 cubic feet air/min. Stationary phase cells were harvested 10 hours after inoculation by continuous centrifugation in a Sharples super centrifuge, type A312. The cell paste was frozen and stored at -20 C.

Enzyme Assays

One unit of D-serine dehydratase is defined as that amount of enzyme which catalyzes the formation of 1 mmol of pyruvate per min at 25 C in 0.1 M potassium phosphate buffer, pH 7.80, at a D-serine concentration of 25 mM. Specific activity is defined as units of enzyme per mg of protein. Solutions for enzyme assays were equilibrated at 25 C for a minimum of 10 min before initiation of the enzyme reaction. Loss of enzyme activity observed in dilute solutions due to protein adsorption to glass was prevented by addition of 0.3 mg of acetylated bovine serum albumin per ml which decreased enzyme concentration by 50% prior to the final dilution of enzyme into the assay cuvette. It was not necessary, however, to add acetylated bovine serum albumin to the final assay buffer.

The 280 nm Assay

D-Serine dehydratase was routinely assayed by observing the rate of formation of pyruvate at 220 nm on a Gilford recording spectrophotometer for a minimum of 30 s. A standard assay system contained 320 mmol of potassium phosphate, pH 7.80, 80 mmol of D-serine, and enzyme in a final volume of 3.2 ml. The assay was initiated by addition of enzyme such that the final enzyme concentration was 0.05 to 7 mg/ml. A molar absorptivity of 1050 M -1 cm -1 for pyruvate at 220 nm was used for calculations of enzymic activity. This value was determined in 0.1 M phosphate buffer, pH 7.4, and is identical to that calculated from spectra by Davis (25) which were run in 0.1 M phosphate, pH 7.2. Errera and Greenstein (26) provide evidence that the molar absorptivity of pyruvate does not vary with pH in the range 7 to 8. Absorbance of species other than pyruvate which interfered at 220 nm was offset up to a limit of 2.5 A units on the Gilford spectrophotometer.

Protein Determinations

Absorbance at 280 nm—Concentrations of pure D-serine dehydratase were calculated from the absorbance at 280 mm using the extinction coefficient of 10.5 determined by Dowhan and Snell (24) for a 1% enzyme solution.

Disc Gel Electrophoresis—Disc electrophoresis was carried out according to the procedure described by Brewer and Ashworth (27) for the standard system (Tris-glycine buffer, pH 8.3); analytical gels (5 x 50 mm) were run at 1.5 ma/gel for approximately 1.5 hours. Protein was fixed in 12.5% trichloroacetic acid (30 min) and stained in a 0.05% solution of Coomasie brilliant blue in 12.5% trichloroacetic acid (1 to 2 hours). Detergents were effectuated by soaking in several changes of 12.5% trichloroacetic acid over a period of 2 days.

Preparation of D-serine Dehydratase—D-Serine dehydratase was purified by a procedure (28) similar to that described by Dowhan and Snell (29). The preparation of D-serine dehydratase was judged homogeneous by the appearance of a single band on disc gel electrophorosis. Furthermore, final specific activities of our preparations ranged from 150 to 165 units/mg, which on correction to 37 C (290 to 314 units/mg) compare well with those of Dowhan and Snell (290 to 310 units/mg) (24).

Recrystallized enzyme was centrifuged (43,000 x g, for 30 min) and stored in the dark at 4 C as a crystalline suspension in a 1 M phosphate buffer, pH 7.0 to 7.2, which had 1 mM dithiothreitol, 10 mM pyridoxal-P, and ammonium sulfate at 50% saturation. The volume of buffer used was approximately 5 times the volume of settled crystalline enzyme.

Preparation of Enzyme for Experimental Use—To avoid problems

1 Previous publications from other laboratories (13, 14, 20, 24) have defined the unit of D-serine dehydratase activity at 37 C. Because all enzyme assays in this paper were performed at 25 C and because the international enzyme unit is defined at 25 C, the risk of possible confusion by changing the definition to 25 C was taken. The unit of enzymic activity at 25 C is approximately 0.52 times the unit defined at 37 C (24).

2 Slow recrystallization (12 days, 4 C, from 30% ammonium sulfate) was required to obtain electrophoretically pure protein after the final steps in the purification procedure (28). Crystallization can be induced to occur more quickly by seeding with enzyme crystals. Crystallization occurs more rapidly from more concentrated ammonium sulfate solutions, but it results in electrophoretically impure protein.
of interpretation of results due to the presence of dibiothreitol, excess free pyridoxal-P or ammonium sulfate, enzyme solutions were desalted prior to every experiment. The 50% ammonium sulfate suspension of crystalline enzyme was dialyzed against two changes of Buffer A and desalted by chromatography on a Sephadex G-25 column (1 X 45 cm) which had been equilibrated with standard buffer. The enzyme was eluted with the same buffer and then aliquots were concentrated to 0.5 ml.

Regeneration of Active Enzyme—Enzyme which had been inactivated by resolution with cysteine was recovered and converted to holoenzyme by overnight dialysis against standard buffer followed by dialysis against changes of Buffer A over a period of 3 days. Dialyzed enzyme was concentrated by ultrafiltration and stored at 4°. For low concentrations of active n-serine dehydratase (<0.4 \( \mu \)M) the assay was initiated by addition of 15 \( \mu l \) of 0.1 \( \mu M \) NADP to an enzyme aliquot of 1.0 ml. In such cases, the assay was completed in less than 45 minutes. Before the experimental use of this enzyme was described above, Enzyme regenerated in this manner had a specific activity which was at least 95% of the specific activity of freshly purified enzyme.

Analysis of Resolution of Holoenzyme by Cysteine—Assays of low concentrations of active n-serine dehydratase (2 to 32 \( \mu M \)) in the presence of cysteine were performed by the coupled system using lactate dehydrogenase according to the method of Dowhan and Snell (24) with the following modifications.

Prior to use, rabbit muscle lactate dehydrogenase was dialyzed exhaustively against standard buffer to remove an unidentified small molecule which inhibited both enzymes. A standard assay system contained, in a final volume of 1.1 ml: 110 \mu mol of potassium phosphate, pH 7.80; 0.275 pmol of NADH; 1.5 pmol of serine, and 20 pg of lactate dehydrogenase. Final assay concentrations were determined by a modified procedure of Ellman for thiol titration with 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB), as described previously (18). The absorbance at equilibrium, \( A_{\text{eq}} \), was negligible in comparison to \( A_i \) and \( A_o \), respectively.

Each resolution experiment consisted of 4 to 6 solutions in standard buffer at constant ionic concentration, constant cysteine concentration, and varying enzyme concentrations. The absorbance at equilibrium, \( A_{\text{eq}} \), was determined spectrophotometrically at 415 nm. The ratio \( (A_i - A_{\text{eq}})/(A_i - A_o) \) is equivalent to \( f_{\text{eq}} \). The absorbance at equilibrium, the initial absorbance prior to addition of cysteine and the absorbance if thiazolidine formation was complete is given by \( A_i \) and \( A_o \), respectively. The molar absorptivity of thiazolidine is vanishingly small at 415 nm, \( A_{\text{eq}} \) was negligible in comparison to \( A_i \) and \( A_o \).

Each resolution experiment consisted of 4 to 6 solutions in standard buffer at constant ionic concentration, constant cysteine concentration, and varying enzyme concentrations. The absorbance at equilibrium, \( [E]_o \), was in the range 5 to 60 \( \mu M \) when \( f_o \) was evaluated, and 0.02 to 25 \( \mu M \) enzyme when \( f_o \) was evaluated. Iodinal concentration, \( I_o \), was monitored by conductivity measurement with a Radiometer conductivity meter type CDM 2d, and controlled by addition of KCl. A standard curve was used to relate conductivity to \( [E]_o \) in solutions of KCl in standard buffer. Resolution was initiated by addition of cysteine to enzyme. Solutions were allowed to come to equilibrium (usually less than 3 to 4 hours) in the dark in a 25° water bath. For resolution experiments involving low enzyme concentrations (less than 1 \mu M), all solutions were made up in the presence of 0.3 mg of acetylated bovine serum albumin per ml to prevent loss of enzyme by adsorption to glass.

Resolution of n-serine Holodehydratase by Dilution—Enzyme in standard buffer at either 9.28 nm or 18.56 nm was sterilized by filtration through type HA Millipore filters and incubated in evacuated sealed tubes, which had been subjected to 3 cycles of evacuation and admission of nitrogen prior to final evacuation and sealing. After incubation in a 25° water bath for specified time intervals, the tubes were broken open, an aliquot removed, and the activity of the enzyme determined using a lactate dehydrogenase assay similar to the one described above. An aliquot of resolved enzyme was regenerated by adding an aliquot of pyridoxal-P in standard buffer to a final concentration of \( 10^{-4} \) M and allowing the solution to incubate in the dark at room temperature for 2 hours prior to assay. The fraction \( f'_{\text{eq}} \) of regeneratable enzyme remaining unresolved was obtained by dividing the activity of the sample before regeneration by its activity after regeneration. The fraction \( f_{\text{eq}} \) of enzyme which was recovered by addition of pyridoxal-P was obtained by dividing the activity of enzyme after regeneration by the activity of the enzyme prior to resolution.

RESULTS AND DISCUSSION

Analysis of Resolution of Holoenzyme—A series of schemes which describe possible equilibrium states in the resolution of pyridoxal-P enzymes by a carbonyl reagent, in this case cysteine, is given in Table I. Scheme 1 is the pathway proposed by Dowhan and Snell (14) for resolution of n-serine dehydratase in which the apoenzyme-thiazolidine complex does not dissociate. Scheme 2 is the simple alternative in which the apoenzyme-thiazolidine complex does not exist. The third scheme is the logical combination of Schemes 1 and 2 in which reaction of holoenzyme with cysteine results in an apoenzyme-thiazolidine complex which subsequently dissociates. Schemes 4 to 6 represent variations of Scheme 3 in which cysteine associates with holoenzyme prior to formation of thiazolidine. The holoenzyme cysteine complex can either be inactive (Scheme 4) or active (Scheme 5). Scheme 6 shows a pathway in which both active and inactive holoenzyme-cysteine complexes exist. Finally, Scheme 7 illustrates formation of an apoenzyme-cysteine complex. Clearly there are many possible pathways other than these listed in Table I. However, the schemes presented are representative of the major possible variations for the resolution of pyridoxal-P-requiring enzymes.

Algebraic expression of each of these schemes in terms of the experimentally determinable parameters of free cysteine concentration at equilibrium, total enzyme concentration, the fraction of total enzyme which remains catalytically active at equilibrium, \( f_o \), and the fraction of total enzyme which contains a Schiff base linkage at equilibrium, \( f'_{\text{eq}} \), results in the linear relationships of Equations 1 and 2.

\[
y = mx + b
\]

where \( y = \frac{1 - f}{f} [\text{Cys}] \) and \( x = \left( \frac{f [E]_o [\text{Cys}]}{a} \right)^{0.5} \)

\[
y' = m' x' + b'
\]

where \( y' = \frac{1 - f'}{f} [\text{Cys}] \) and \( x' = \left( \frac{f [E]_o [\text{Cys}]}{a} \right)^{0.5} \).

Derivation of Equations 1 and 2 for Scheme 4 of Table I is illustrated in the appendix to this paper. Plots of \( y \) versus \( x \) or \( y' \) versus \( x' \) at constant \([\text{Cys}]\) yield straight lines for each of the resolution schemes depicted in Table I. Examination of Table I reveals that the listed schemes are experimentally distinguishable from each other by their characteristic expressions for \( m', m, b, \) and \( b' \); and that the individual equilibrium constants associated with each scheme are obtainable from the values of these parameters and their dependence on the concentration of cysteine.

Resolution of n-serine Dehydratase by Cysteine—Figs. 1 and 2 present plots of \( y' \) versus \( x' \) for the resolution of n-serine dehydratase by L- and D-cysteine. The slopes and intercepts of...
| Scheme | Equation | Slope $m^d$ | Intercept $b^d$ |
|--------|----------|-------------|---------------|
| 1      | $E + Cys \xrightarrow{K_1} AT$ | 0           | $K_1$         |
| 2      | $E + Cys \xrightarrow{K_2} A + T$ | $(K_2)^{0.5}$ | 0             |
| 3      | $E + Cys \xrightarrow{K_1} AT \xrightarrow{K_3} A + T$ | $(K_1K_3)^{0.5}$ | $K_1$         |
| 4      | $E + Cys \xrightarrow{K_4} ECys_1 \xrightarrow{K_5} AT \xrightarrow{K_3} A + T$ | $(K_3K_4K_5)^{0.5}$ | $K_4(1 + K_5)$ |
| 5      | $E + Cys \xrightarrow{K_6} ECys_1 \xrightarrow{K_7} AT \xrightarrow{K_3} A + T$ | \(\frac{K_3K_6K_7}{1 + K_6[Cys]}\)^{0.5} | $\frac{K_6K_7}{1 + K_6[Cys]}$ |
| 6      | $E + Cys \xrightarrow{K_8} ECys_2 \xrightarrow{K_9} ECys_1 \xrightarrow{K_5} AT \xrightarrow{K_3} A + T + Cys$ | $(K_3K_5K_8)^{0.5}$ | $\frac{K_6K_8(1 + K_5)}{1 + K_6[Cys]}$ |
| 7      | $E + 2Cys \xrightarrow{K_1} AT \xrightarrow{K_3} A + T \xrightarrow{K_9} ACys + T$ | $(K_1K_3(1 + K_9[Cys]))^{0.5}$ | $K_1$         |

$^a$ AT = apoenzyme-thiazolidine complex
$^b$ ACys = apoenzyme-cysteine complex
$^c$ ECys$_1$ = active enzyme-cysteine complex
$^d$ ECys$_2$ = inactive enzyme-cysteine complex

The assumption is made in the derivations of equations 1 and 2 that the concentration of free pyridoxal-P is much smaller than the concentration of free thiazolidine derivative. If this assumption is invalid, the constant $K_3$ should be replaced by $K_3'$, where $K_3' = K_3(1 + \frac{1}{K_T[Cys]})$ and $K_T$ defines the equilibrium

$$Cys + PLP \xrightarrow{K_T} T.$$ For the studies presented in this work, $\frac{1}{K_T[Cys]} << 1.$

$^d$ $y = \frac{1 - fa}{fa[Cys]}$, $x = (fa[E_t][Cys])^{-0.5}$

$^c$ $y' = \frac{1 - fa}{fa[Cys]}$, $x' = (fa[E_t][Cys])^{-0.5}$

$^d$ $m' = m$ and $b' = b$ for Schemes 1-3, 5, 7.

For Scheme 4:

$$m' = \frac{K_3K_4K_5}{(1 + K_4[Cys])}^{0.5} \text{ and } b' = \frac{K_4K_5}{1 + K_4[Cys]}$$

For Scheme 6:

$$m' = \frac{K_3K_5K_8}{(1 + K_5[1 + K_8][Cys])}^{0.5} \text{ and } b' = \frac{K_5K_8}{1 + K_5(1 + K_8)[Cys]}$$

Peated determinations of the value of $b'$ at different ional concentrations and cysteine concentrations always yielded positive non-zero values for $b'$ having standard deviations similar to those in Table II. The large error in the intercept makes it difficult to analyze the cysteine dependence of the intercept. The observation that $m'$ was independent of the cysteine concentration, and the non-zero value of the intercept establishes Scheme 3 as the
listed in Table II. No significant differences could be detected for n-serine dehydratase. Free cysteine does not appear to interact with the substrate binding site on the enzyme (14). If the cysteine portion of the thiazolidine does not interact with the enzyme, the value of $K_3$ would be an estimate of the noncovalent interactions between the cofactor and the enzyme. The uncertainty associated with the measured value of $b'$ and therefore $K_3$ (Table II), caused us to devise an independent method for checking this estimate of the noncovalent interactions between pyridoxal-P and the enzyme (30).

Activity assays were also used to analyze the resolution of d-serine dehydratase by cysteine. However, this method was not as satisfactory as the spectral analysis. One problem associated with activity assays is that the substrate d-serine in the assay solution perturbs the resolution equilibrium. Table III presents data which show that $f_a$ calculated from the final lactate dehydrogenase assay slope is much larger than $f_a$ calculated from the initial slope. This effect diminishes but does not disappear as the d-serine concentration decreases. The fact that at the two low d-serine concentrations (1 mM and 5 mM) the values of $f_a$ calculated from initial slopes are the same suggests that the activity method of analyzing resolution is valid provided, (a) d-serine is less than 5 mM in the assay solution, and (b) the initial slope is used.

A major limitation of the activity method was the difficulty in determining the small values of the intercept $b$. The way to ensure a precise value of $b$ is to get experimental points as close to the ordinate as possible, a task which requires high enzyme concentrations. However, the activity assay cannot handle active enzyme concentrations greater than 20 nM. This fact puts a lower limit on the value of $x$ which can be obtained at a given cysteine concentration. Dilution of an enzyme-cysteine solution into the assay system in an attempt to overcome this problem is not satisfactory because dilution perturbs the very equilibrium the assay seeks to measure. This perturbation becomes more pronounced as the enzyme concentration increases. As total enzyme increases, the fraction of total enzyme existing as the AT complex increases. Dilution causes dissociation of AT to both active holoenzyme and inactive apoenzyme (Scheme 3).

In spite of its disadvantages, the activity assay is a much more sensitive method, capable of analyzing enzyme concentrations 100 times lower than the spectral method. Therefore, the activity method was used to show that Scheme 3 describes resolution of d-serine dehydratase over a very large enzyme concentration range (Fig. 3). A slope ($m$) of 0.0435 (Fig. 3) for resolution at $\Gamma/2 = 0.39$ correlates reasonably well with a value of 0.037 for $m'$ determined at $\Gamma/2 = 0.40$ using the spectral method.

To ensure that apoenzyme species AT and A had not been denatured during the resolution reaction (thereby invalidating the results), enzyme in final equilibrium mixtures (E, Cys, AT and A) was regenerated by treatment with pyridoxal-P. In all

minimum pathway for resolution. It should be pointed out, however, that Schemes 4 to 7 have not been excluded. These pathways may be operative and give rise to no observable dependence of $m'$ on [Cys] if the terms containing [Cys] in the expressions for $m'$ are small compared to unity. Whatever the pathway, the ratio $(m'/b')$ yields $K_3$, the dissociation constant for the thiazolidine from the enzyme, when the Cys terms are small compared to unity (Scheme 7). Values for this ratio are also listed in Table II. No significant differences could be detected for the value of $K_3$ with L- or D-cysteine. This result is consistent with the idea that the cysteine portion of the thiazolidine does not interact appreciably with n-serine dehydratase. Free cysteine does not appear to interact with the substrate binding site on the enzyme (14). Neither D- nor L-cysteine compete with d-serine for the enzyme (14). If the cysteine portion of the thiazolidine does not interact with the enzyme, the value of $K_3$ would be an estimate of the noncovalent interactions between the cofactor and the enzyme. The uncertainty associated with the measured value of $b'$ and therefore $K_3$ (Table II), caused us to devise an independent method for checking this estimate of the noncovalent interactions between pyridoxal-P and the enzyme (30).

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TABLE II

| [Cys] | $10^3 m'$ | $y'$ | $10^4 (m'/y')$ |
|-------|-----------|------|----------------|
| L-Cysteine | 18.2 | 3.05 ± 0.09 | 14.7 ± 2.4 | 0.63 ± 0.06 |
| | 42.3 | 3.26 ± 0.05 | 6.1 ± 1.6 | 1.7 ± 0.4 |
| Average | 3.16 ± 0.15 | 1.7 ± 0.8 |
| D-Cysteine | 19.3 | 3.52 ± 0.14 | 16.3 ± 5.2 | 0.8 ± 0.2 |
| | 44.8 | 3.34 ± 0.11 | 9.9 ± 2.6 | 1.1 ± 0.2 |
| Average | 3.43 ± 0.13 | 0.95 ± 0.2 |

$^a$ At $95^\circ$, $\Gamma/2 = 0.33$, pH 7.80.  
$^b$ The contribution of zwitterions to the ionic concentration was neglected. Errors are standard deviations. Averages are unweighted means.


determined at $\Gamma/2 = 0.40$ using the spectral method.

FIG. 1. Resolution of d-serine dehydratase at pH 7.80, $\Gamma/2 = 0.33$, 25° by 18.3 mM (O- - -O) and 42.3 mM (–•–•) L-cysteine. $y'$ and $x'$ are defined in Equation 2.

FIG. 2. Resolution of d-serine dehydratase at pH 7.80, $\Gamma/2 = 0.33$, 25° by 19.3 mM (O- - -O) and 44.8 mM (–•–•) D-cysteine. $y'$ and $x'$ are defined in Equation 2.

TABLE III

| [n-Serine] | 10$^3$ Initial $f_a$ | 10$^3$ Final $f_a$ |
|------------|---------------------|-------------------|
| 17.5       | 4.20                | 21.2              |
| 9.50       | 5.61                | 19.0              |
| 4.96       | 3.73                | 16.2              |
| 1.03       | 3.79                | 7.39              |
cases checked, a minimum specific activity of 95% of that of pure 
enzyme was regenerated.

Equilibrium Constant for Dissociation of Cofactor from Enzyme
—The dependence of the degree of resolution on the concentration 
of enzyme and cysteine can be used to determine the equilibrium 
constant for the dissociation of holoenzyme to apoenzyme and 
pyridoxal-P (Equation 3). When the cysteine-containing terms

\[
E \stackrel{k_D}{\longrightarrow} A + \text{pyridoxal-P} \tag{3}
\]

in the expression for \( m' \) listed in Table I are small compared to 
unity, the value of \((m')^2\) is equivalent to the equilibrium 
constant for the over-all resolution reaction (Equation 4) regardless 
of the resolution pathway. Since dissociation of cofactor from 
enzyme (Equation 3) is simply the difference between the reso-

\[
E + \text{Cys} \stackrel{k_R}{\longrightarrow} A + \text{T} \tag{4}
\]

and the reaction

\[
\text{Pyridoxal-P} + \text{Cys} \stackrel{k_T}{\longrightarrow} \text{T} \tag{5}
\]

\( K_F = K_R/K_T \). Values for \( K_T \) have been presented in the pre-
ceding paper (18). Applying this analysis to \( \alpha \)-serine dehydratase,
we obtain a value of 7.0 nM for \( K_F \) (at \( \Gamma/2 = 0.33 \), 25°, pH 7.80) 
using a value of \( 1.56 \times 10^3 \) M\(^{-1} \) for \( K_T \) and an average value of 
\( 3.30 \times 10^{-2} \) for \( m' \). Since little uncertainty is associated with the 
values of \( K_T \) and \( m' \) the value of \( K_F \) obtained from this analysis 
should be accurate.

The relationship \( K_R = K_F K_T \) suggests that the ability of an 
aminothiol to resolve pyridoxal-P-requiring enzymes to apo-
enzyme and thiazolidine depends on the stability of the thiazoi-
dine formed between pyridoxal-P and the aminothiol. The 
thiazolidine formed from penicillamine and pyridoxal-P is 10 
times more stable than the corresponding thiazolidine formed 
from cysteine (18). As expected penicillamine was found to be 
more effective than cysteine in resolving \( \alpha \)-serine dehydratase. 
It should be pointed out, however, that the rates of resolution 
appear to be independent of the stability of the thiazolidine.
Although an analysis of the kinetics of resolution was not un-
taken, \( d \)-penicillamine was found to resolve \( \alpha \)-serine dehydratase 
at a rate comparable to \( L \)-cysteine which in turn resolves the 
enzyme at a rate roughly 20 times slower than \( L \)-cysteine.4

An increased rate of resolution of \( \alpha \)-serine dehydratase by \( L-

4 An increased rate of resolution of \( \alpha \)-serine dehydratase by \( L-

5 The complicated nature of the resolution pathway makes it 
difficult to assign accurate values to the relative rates of resolution 
by aminothiols without a detailed kinetic analysis.
where \( f' \) is the ratio of original activity recovered after denaturation of apoenzyme to a functional protein which has a time in the dilution experiment. Such a situation would cause the reduced affinity for pyridoxal-P occurs during the long incubation for the equilibrium constant \( K_P \). Equation 7 rests on the assumption that enzyme which cannot be regenerated does not bind pyridoxal-P.

\[
K_P' = \frac{(1 - f''_a)}{f''_a} \frac{[E]}{[T]} \frac{(1 - f'')}{f''_a} \frac{[E]}{[T]} \tag{7}
\]

where \( f_a \) is the fraction of original activity recovered after regeneration, and it is obtained from the ratio of the activity after regeneration to the activity prior to resolution. The time dependence of the right-hand side of Equation 6 (circles) and 7 (squares) is illustrated in Fig. 4. A value of 14 nhr was estimated for the equilibrium constant \( K_P \) from the limiting value of \( K_P' \) in Fig. 4.

This value for \( K_P \) is significantly higher than the value of 7.0 \( \mu \) determined by resolution with cysteine. Perhaps partial denaturation of apoenzyme to a functional protein which has a reduced affinity for pyridoxal-P occurs during the long incubation time in the dilution experiment. Such a situation would cause the dilution method for determining \( K_P \) to yield an overestimate of the true value of \( K_P \). The value of 35 \( \mu \) for \( K_P \) previously determined under similar conditions by Dowhan and Snell (14), from the activity of dilute solutions of previously isolated apoenzyme and cofactor, is also larger than the value of \( K_P \) determined here by resolution with cysteine. Although further work is required to resolve this discrepancy, the problems associated with making equilibrium measurements in dilute solutions of cofactor and apoenzyme may be at least partly responsible for the difference between the values of \( K_P \) determined by dilution and resolution by cysteine. Because equilibrium is reached in minutes or hours when the enzyme is resolved with aminothiol, instead of days when the enzyme is resolved by high dilution, resolution with an amnithiol is the method of choice for determination of the dissociation constant for d-side dehydratase, and very likely for other pyridoxal-P-requiring enzymes which release cofactor slowly.

**APPENDIX**

Derivation of Equations 1 and 2 for Scheme 4 of Table I.

\[
[A] = [T] + [PLP]
\]

From the equilibrium \( PLP + Cys \xrightarrow{K_P} T \) it follows

\[
[A] = [T] (1 + \frac{1}{K_P[Cys]})
\]

for all the resolution experiments

\[
\frac{1}{K_T[Cys]} << 1
\]

\[
(K_T = 1.56 \times 10^5 M^{-1} \text{ at pH 7.80} \tag{18})
\]

so \([A] = [T]\).

A material balance for total enzyme, \( E_t \), yields

\[
[E_t] = [E] + [ECys_2] + [K_3] + [A]
\]

\[
= [E] \left( 1 + K_4[Cys] + K_4K_5[Cys] + \frac{K_4K_5[Cys]}{[E]} \right)^{0.5}
\]

since

\[
f_a = \frac{[E]}{[E_t]} \quad \text{and} \quad f_a' = \frac{[E] + [ECys]}{[E_t]}
\]

it follows that (Equation 1)

\[
\frac{1 - f'_a}{f'_a[Cys]} = \left( \frac{K_4K_5}{[E]_t[Cys]} \right)^{0.5} + K_4(1 + K_2)
\]

and (Equation 2)

\[
\frac{1 - f'_a}{f'_a[Cys]} = \left( \frac{K_4K_5}{[E]_t[Cys]} \right)^{0.5} + K_4 \frac{1}{1 + K_4[Cys]}
\]

**REFERENCES**

1. Snell, E. (1958) *Vitam. Horm.* 15, 77-125
2. Braunstein, A. E. (1960) in *The Enzymes* (Boyer, P. D., Lardy, H., and Myrback, K., eds) 2nd ed., Vol. 2, pp. 113-134, Academic Press, New York
3. Guillard, B., and Snell, E. E. (1964) in *Comprehensive Biochemistry* (Florkin, M., and Stotz, E. H., eds) Vol. 15, p. 138-199, American Elsevier, New York
4. Snell, E. E. (1970) in *Proceedings of the International Congress of Pharmacology* (Eigemtan, E., ed) Vol. 5, p. 210-220, Schwabe, Basel, Switzerland
5. Gopinathan, K. P., and Demoss, R. D. (1968) *Biochemistry* 7, 1685-1691
6. Siegmund, P., Hasebank, G., and Koerber, F. (1968) *Hoppe-Seyler's Z. Physiol. Chem.* 349, 1062-1070
7. Hasebank, G., Koerber, F., and Siegmund, P. (1968) *Hoppe-Seyler's Z. Physiol. Chem.* 349, 310-316
8. Festana, A., Sandova1, I. V., and Solis, A. (1971) Arch. Biochem. Biophys. 146, 373-379
9. Schirch, L., and Mason, M. (1962) *J. Biol. Chem.* 237, 2575-2581
10. Dimitr6, I. F., Iordachescu, D., and Niculescu, G. (1972) *Enzymologia* 42, 75-85
11. Kumasaki, H., Nagata, T., Yoshida, H., and Yamada, H. (1972) *Biochem. Biophys. Acta* 255, 779-790
12. Hedrick, J. L. (1972) *Adv. Biochem. Psychopharmacol.* 4, 25-37
13. Dupouy, D., Newton, W. A., and Snell, E. E. (1966) *J. Biol. Chem.* 241, 1233-1238
14. Dowhan, W., Jr., and Snell, E. E. (1970) *J. Biol. Chem.* 245, 4629-4635
15. Shaltiel, S., Hedrick, J. L., and Fischer, E. H. (1969) *Biochemistry* 8, 2409-2436
16. Shaltiel, S., Hedrick, J. L., and Fischer, E. H. (1966) *Biochemistry* 5, 2108-2116
17. Hedrick, J. L., Shaltiel, S., and Fischer, E. H. (1969) *Biochemistry* 8, 2429-2432
18. Schonbeck, N. D., Skalski, M., and Shafer, J. A. (1975) *J. Biol. Chem.* 250, 5343-5351
19. McFall, E. (1964) *J. Biol. Chem.* 241, 746-753
20. Labow, R., and Robinson, W. G. (1966) *J. Biol. Chem.* 241, 1209-1213
21. Layne, E. (1957) *Methods Enzymol.* 3, 447-454
22. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) *J. Biol. Chem.* 193, 265-275
23. Fraser, D., and Jerrell, E. A. (1953) *J. Biol. Chem.* 205, 291-295
24. Dowhan, W., Jr., and Snell, E. E. (1970) *J. Biol. Chem.* 245, 4618-4628
25. Dug, L. (1965) *Anal. Biochem.* 12, 26-30
26. Estrella, M., and Greenstein, J. P. (1947) *Arch. Biochem. Biophys.* 16, 445-448
27. Drew, J. M., and Ashworth, R. B. (1909) *J. Chem. Ed.* 46, 41-45
28. Schonbeck, N. D. (1973) Ph.D. thesis, University of Michigan, Ann Arbor, Michigan
29. Young, H. O. (1962) *Statistical Treatment of Experimental Data*, p. 75, McGraw-Hill, New York
30. Schonbeck, N. D., Skalski, M., and Shafer, J. A. (1975) *J. Biol. Chem.* 250, 5359-5363
Resolution of D-serine dehydratase by cysteine. An analytical treatment.
N D Schonbeck, M Skalski and J A Shafer

J. Biol. Chem. 1975, 250:5352-5358.

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