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# Dissociation-Produced Loss of Regulatory Control of Homoserine Dehydrogenase of *Rhodospirillum rubrum*. I. Factors Which Affect the Interconversion of the Regulatable and Nonregulatable States*

Ralph Mankovitz† and Harold L. Segal

ABSTRACT: Homoserine dehydrogenase of *Rhodospirillum rubrum* undergoes a reversible dissociation with a half-time of 1–2 min into a form which is insensitive to inhibition by threonine and appears not to be bound by threonine. The association–dissociation curve fits closely that for a unimolecular–bimolecular equilibrium, so it is concluded that the threonine-sensitive form is a dimer of the insensitive form. The equilibrium is shifted toward the aggregated, theonine-sensitive form by divalent cations ($Mg^{+2}$, $Mn^{+2}$, and $Ca^{+2}$) and monovalent anions ($Cl^-$ and acetate), and by threonine and other allosteric ligands (isoleucine, methionine, norleucine, and β-hydroxynorvaline). Homoserine, serine, asparate, and adenosine triphosphate shift the equilibrium slightly toward the nonaggregated, theonine-insensitive form while the other substrates are without effect. Threonine and β-hydroxynorvaline, in addition, induce a slower formation of what appears to be a hyperaggregated form on the basis of the Hill coefficient for the reaction and earlier sedimentation and gel filtration data. A model consistent with the results is proposed. A method for aspartic semialdehyde purification is presented.

A level of sophistication not previously appreciated in the capabilities of enzymes has been revealed with the formulation of the concepts of allosteric modulator binding sites and the underlying structural feature of protein subunit interaction. The theory proposes that modulator binding effects the equilibrium between conformational states of peptide chains (Monod *et al.*, 1965) (or induces new conformational states (Koshland and Neet, 1968)), thus leading to shifts in their interaction with one another and modification of the catalytic site and properties. The most readily observable sort of shift in the state of interaction between protein subunits is that leading to outright dissociation or association.

Aggregation of disaggregation under the influence of modifiers or substrates has been reported for a number of enzymes subject to modulator control (glutamic dehydrogenase, Frieden, 1959a,b; Tomkins and Yielding, 1961; hemoglobin, Briehl, 1963; threonine deaminase, Changeux, 1963; acetyl-CoA carboxylase, Vagelos *et al.*, 1963; homoserine dehydrogenase, Datta *et al.*, 1964; dCMP deaminase, Maley and Maley, 1964; fructose diphosphatase, Rosen *et al.*, 1966; phosphorylase, Metzger *et al.*, 1967; formyltetrahydrofolate synthetase, Scott and Rabinowitz, 1967; aspartokinase, Wampler and Westhead, 1968; and glutaminase, Katsunuma *et al.*, 1968). In the experiments of Datta *et al.* (1964) with homoserine dehydrogenase of *Rhodospirillum rubrum*, enzyme

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† Present address: Department of Medical Physics, University of Toronto, Can.

TABLE I: Preparation of Homoserine Dehydrogenase.

| Fraction | Enzyme Act. (units/ml) | | Protein (mg/ml) | Sp Act. (mg/ml) | Total Units |
|---|---|---|---|---|---|
| | Control | +Threonine[a] | | | |
| Crude extract | 1.1 | 0.32 | 18.0 | 0.061 | 385 |
| 35,000*g* fraction | 0.80 | 0.31 | 7.1 | 0.11 | 250 |
| Protamine sulfate | 0.70 | 0.33 | 6.7 | 0.10 | 230 |
| Ammonium sulfate | 14.0 | 1.9 | 14.0 | 1.0 | 94 |

[a] The assay medium contained 0.48 mM L-threonine in addition to the usual components.

species of distinct sedimentation coefficients and migration rates on gels were found in the presence of modulators of activity of the enzyme. However, no experiments relating kinetic properties to molecular size were undertaken. In this paper we report a dissociation-induced loss of threonine inhibitability of this enzyme and a description of factors which influence this process. The time and concentration parameters make this case a particularly favorable one for correlating changes in the state of aggregation with catalytic function. An accompanying paper compares a number of the properties of the aggregated and disaggregated enzyme (Mankovitz and Segal, 1969b).

## Experimental Section

*Materials.* Protamine sulfate, DL-*C*-allylglycine, L-threonine, L- and DL-homoserine, DL-β-hydroxynorvaline, TPNH, and $TPN^+$ were purchased from Sigma Chemical Co. (St. Louis, Mo.) DL-Norleucine was from Calbiochem (Los Angeles, Calif.). Enzyme grade ammonium sulfate was purchased from Mann Research Laboratories (New York, N. Y.). Dowex AS 50W-X8 (200–400 mesh) in the hydrogen form was purchased from Bio-Rad Laboratories (Richmond, Calif.).

*Preparation of Aspartic β-Semialdehyde.* DL-Aspartic β-semialdehyde was prepared from DL-allylglycine by the procedure of Black and Wright (1955), with an improvement in the final purification step as described below. The procedure of Black and Wright is based on the ozonolysis of an acid solution of allylglycine. The change in the chromatographic step was made in order to remove aspartic acid which arises presumably as a result of overoxidation.

Dowex AG 50W-X8 in the hydrogen form, washed with glass-distilled water, was packed in a column 142 × 2 cm at room temperature. Eluates were assayed for ninhydrin-positive material by spotting the material on Whatman No. 3MM paper, air drying the paper, and dipping it in 0.25% ninhydrin in acetone.

The ozonized solution (20–40 ml) was applied to the column at a flow rate of about 20 ml/hr. The column was then washed at a flow rate of about 60 ml/hr, first with 4–8 l. of water followed by 8 l. of 0.03 N HCl. No ninhydrin-positive material was eluted in these washes.

The column was then eluted with 0.6 N HCl at a flow rate of 60 ml/hr. The first ninhydrin-positive material was detected after about 6 l. of the second HCl solution had passed through the column. Aliquots from each fraction (30 ml) thereafter were spotted on Whatman No. 3MM paper, previously dampened with pyridine–acetic acid buffer (pH 4.7) and subjected to paper ionophoresis for 0.5–0.75 hr at a 4.5-kV potential as described by Atfield and Morris (1961). After ionophoresis the paper was air dried and ninhydrin treated as described above. Since no more than 10 μl of material is needed for a spot and since the aspartic acid contaminant moves no more than about 5 cm from the origin under the given conditions, a large number of samples could be ionophoresed at one time. Aspartic β-semialdehyde remains at the orgin at this pH.

The first few tubes which contained ninhydrin-positive material gave a single blue ninhydrin spot (aspartic acid) displaced from the origin. As elution continued, an additional brown ninhydrin spot (aspartic β-semialdehyde) appeared at the origin. With further elution, the size and density of the aspartic acid spot increased through a maximum which preceded the maximum of the aspartic β-semialdehyde spot. At the point where no more aspartic acid appeared, the eluent was replaced with 4 N HCl. The remainder of the aspartic β-semialdehyde solution on the column was eluted in the next few (four to six) collection tubes. When all the tubes corresponding to single aspartic β-semialdehyde spots were pooled and reionophoresed, only a single brown ninhydrin spot appeared at the origin. The final product was 118 ml of a 121 mM solution (as L-aspartic β-semialdehyde, assayed with homoserine dehydrogenase and TPNH) in 2 N HCl, representing about 25–50% yield in several preparations.

*Buffers.* Buffer A was 30 mM potassium phosphate–1 mM $K_3$-EDTA–0.1 M KCl (pH 7.2, $\mu$ = 175 mM). Buffer B was 3 mM potassium phosphate–1 mM $K_3$EDTA (pH 7.2, $\mu$ = 12.2 mM).

*Enzyme and Protein Assays.* The standard assay system contained 15 μM TPNH and 58 μM L-aspartic β-semialdehyde in 3.1 ml of buffer A. Assays were carried out at 23° in a Cary Model 11 spectrophotometer using a 0–0.1 slide wire and a 1-cm light path. Rates were linear for at least 1 min. The stock aspartic β-semialdehyde solution was 24 mM DL-aspartic β-semialdehyde in 0.2 N HCl. Aliquots were neutralized with solid $KHCO_3$, kept at 0°, and used within 20 min of neutralization. One unit of enzyme is defined as the amount catalyzing the oxidation of 1 μmole of TPNH/min under these conditions. In some experiments, where indicated, buffer B instead of buffer A was used in the assays. This change had no significant effect on initial velocities, but was employed where appropriate (Figure 4A) to prevent too rapid a resensitization in the assay cuvet. Protein was determined by the method of Lowry *et al.* (1951).

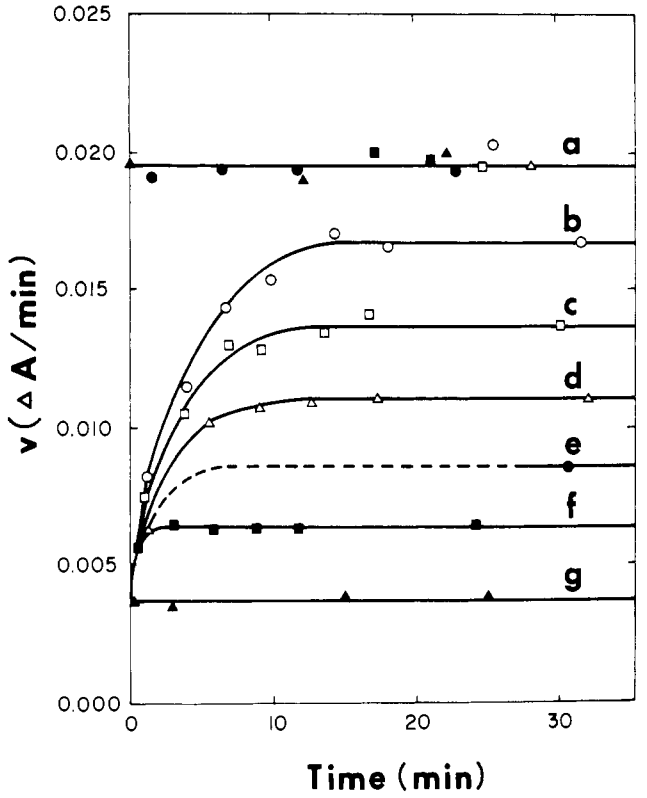


FIGURE 1: Desensitization of homoserine dehydrogenase to threonine inhibition by enzyme dilution. Enzyme solution (9.5 units/ml) was diluted with buffer A as indicated below and incubated at 23°. Curve a is the activity in the absence of threonine, and curves b through g are the activities in the presence of 0.48 mM L-threonine of enzyme diluted 1000-fold, 300-fold, 100-fold, 30-fold, 10-fold, and undiluted, respectively; 1 μl of the undiluted enzyme was used for assay and correspondingly larger aliquots of diluted enzyme so that the total enzyme in the assay cuvet was constant. The symbols in curve a refer to the dilutions in curves b through g with corresponding symbols. The abscissa is time after dilution.

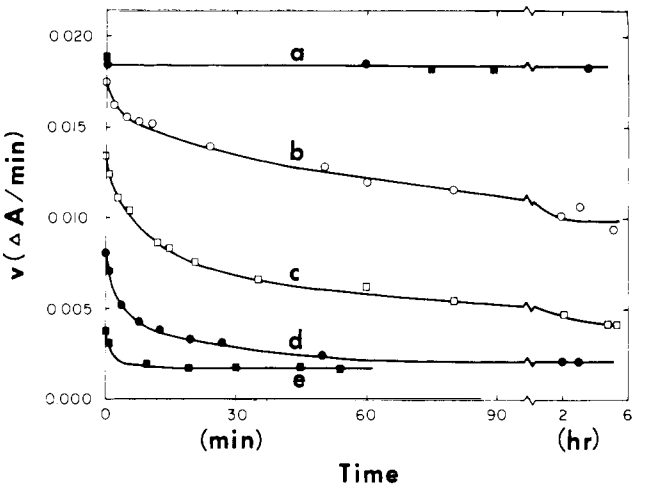


FIGURE 2: Resensitization by threonine of homoserine dehydrogenase previously desensitized by enzyme dilution. Enzyme (9.5 units/ml) was desensitized by dilution (see Figure 1). L-Threonine solution was then added to produce a final concentration of 0.5 mM and the solution was further incubated at 23°. Quantities of enzyme taken for assay were as in Figure 1. Curve a is the activity in the absence of added threonine in the assay cuvet. Curves b through e are the activities in the presence of 0.48 mM L-threonine in the assay cuvet of enzyme diluted 3000-fold, 300-fold, 30-fold, and undiluted, respectively. Zero time points were assays after desensitization but before addition of threonine. The symbols in curve a refer to the dilutions in curves b through e with corresponding symbols.

*Growth of Bacteria and Harvesting of Cells. R. rubrum* (a gift of Dr. H. Gest) was grown photosynthetically under anaerobic conditions in 5.5-l. batches in Povitsky bottles in a synthetic malate medium identical with that described by Datta and Gest (1965).

The cells were harvested after 48–60-hr growth by centrifugation at 16,000*g* in a refrigerated centrifuge. The harvested cells were washed once with cold buffer A. The cell paste with an overlayer of buffer was stored at −15°.

*Preparation of Homoserine Dehydrogenase.* The cells were thawed and centrifuged at 16,000*g* for 10 min. The packed cells (110 g wet weight) were resuspended in 300 ml of cold buffer A containing 15 mM L-homoserine and sonicated in ten 30-ml portions for 0.5 min each using a Branson "Sonifier" (Branson Instruments, Inc., Stamford, Conn.). During the sonication and throughout the purification procedure, except where stated, the temperature was maintained as close to 0° as possible. The pooled sonicated suspension was centrifuged successively at 27,000*g* for 20 min (crude extract) and 35,000*g* for 10.5 hr, and the pellets were discarded.

The 35,000*g* supernatant solution was treated with protamine sulfate, substantially as described by Datta and Gest (1965). A 2% solution of protamine sulfate, adjusted to pH 7.7 with 1 M ammonium hydroxide, was added dropwise with continuous stirring until a protamine sulfate to protein ratio of 0.2 mg/mg was attained. The suspension was stirred for an additional 10 min and then centrifuged at 27,000*g* for 20 min.

To the supernatant solution solid ammonium sulfate (200 mg/ml) was added. The mixture was stirred for 0.5 hr, then centrifuged. The pellet was resuspended in buffer A containing 10 mM L-homoserine and clarified by centrifugation at 27,000*g* for 20 min.

The solution was dialyzed against buffer A containing 10 mM L-homoserine and then against buffer A without homoserine. The dialyzed preparation was frozen at this point.

Several days later, the preparation was thawed and centrifuged at room temperature yielding a clear solution. The purification results are presented in Table I.

Enzyme from the final step in the preparation procedure was used in all the experiments described here.

## Results

*Reversible Desensitization of Homoserine Dehydrogenase to Threonine Inhibition by Enzyme Dilution.* Dilution of the enzyme solution produced a time-dependent decrease in its sensitivity to threonine inhibition (Figure 1). Dilution was with buffer A so that there was no change in salt composition or concentration. It may be noted that the initial relative rate of desensitization was independent of enzyme concentration, indicating a first-order reaction.

Resensitization could be achieved by preincubation of the desensitized enzyme with threonine (Figure 2). The resensitization reaction was biphasic, and the initial rate increased with increasing enzyme concentration. It may be noted by com-

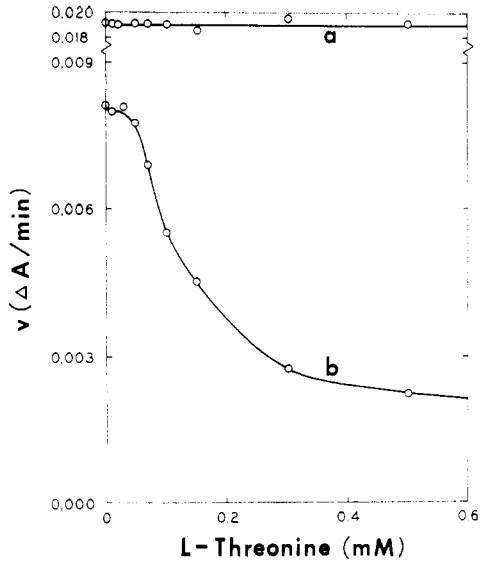


FIGURE 3: Resensitization of homoserine dehydrogenase as a function of threonine concentration. Enzyme (9.5 units/ml) was diluted 30-fold with buffer A and desensitized as in Figure 1. L-Threonine was then added to give the final concentrations shown, and the solutions were further incubated at 23° for 2 hr. Aliquots (30 μl) were removed for assay in the absence (curve a) and presence (curve b) of 0.48 mM L-threonine. Each point on the shoulder of curve b is an average of several measurements.

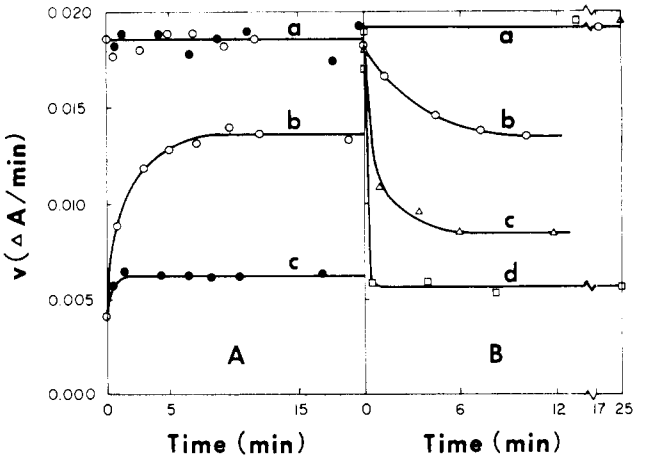


FIGURE 4: (A) Desensitization of homoserine dehydrogenase to threonine inhibition by salt dilution. One volume of enzyme solution in buffer A (9.5 units/ml) was diluted with ten volumes of buffer B. The ionic strength was thereby reduced from 175 to 26.7 mM. The solution was held at 23° and aliquots (normalized to 11 μl) were removed periodically for standard assay (curve a) and assay in the presence of 0.48 mM L-threonine (curve b) with buffer B in the assay cuvet. As a control for the dilution of the enzyme *per se*, a similar dilution of the enzyme was made with buffer A. Corresponding aliquots were removed for assay in the absence (curve a) and presence (curve c) of threonine. Zero time points were obtained with 1 μl of undiluted enzyme. The symbols in curve a refer to the dilutions in curves b and c with corresponding symbols. (B) Resensitization by salt addition of homoserine dehydrogenase previously desensitized by salt solution. Enzyme (9.5 units/ml) was transferred to buffer B by passage through Sephadex G-25 previously equilibrated with this buffer. This caused a dilution of about 2-fold. Velocities are normalized by this factor. An aliquot of the solution from the column (squares) and aliquots further diluted 10-fold (triangles) and 100-fold (circles) with the same buffer were incubated at 23° to obtain desensitized enzyme. Then KCl was added to a final concentration of 0.1 M, and the solutions were further incubated at 23°. Identical quantities of enzyme were taken from each tube for periodic assay with and without threonine. Curve a is the uninhibited activity and curves b through d the activity in the presence of 0.48 mM L-threonine for the respective dilutions. Zero time points were assays after desensitization but before addition of KCl.

parison with curve g of Figure 1 that in curves d and e of Figure 2 the enzyme was sensitized beyond the original level. This is referred to further below.

The degree of resensitization as a function of the concentration of L-threonine in the preincubation mixture is shown in Figure 3. A distinct sigmoidicity is apparant. The Hill coefficient, $n$, calculated from these data is 2.2 (see Appendix, A).

*Reversible Desensitization of Homoserine Dehydrogenase to Threonine Inhibition by Salt Dilution.* Reduction of the salt concentration of the enzyme solution produced a time-dependent decrease in the sensitivity of the enzyme to threonine inhibition (Figure 4A). The addition of salt produced a time-dependent resensitization (Figure 4B). The initial rate of resensitization again increased with increasing enzyme concentration.

The degree of resensitization at a given ionic strength depended upon the ions present (Figure 5). The divalent cations ($Mg^{2+}$, $Mn^{2+}$, and $Ca^{2+}$) were more effective than the monovalent cations ($Na^+$ and $K^+$), while the monovalent anions ($Cl^-$ and acetate) were more effective than $SO_4^{2-}$. In fact from curve a it appears that $K^+$, $Na^+$, and $SO_4^{2-}$ are either virtually totally without effect or counteract one another.

*Sensitivity of Homoserine Dehydrogenase to Threonine Inhibition As a Function of Enzyme Concentration.* In order to obtain a clearer impression of threonine sensitivity as a function of enzyme concentration under various conditions, these parameters were compared systematically as shown in Figure 6. The sensitivity of the enzyme to threonine inhibition is defined as $\sigma = (1 - v_i/v_o)$, where $v_o$ and $v_i$ are the activities in the absence and presence of 0.48 mM L-threonine, respectively. Curves a and b represent enzyme at various concentrations in buffers A and B, respectively. Curve c demonstrates the increased sensitivity obtained by preincubation of enzyme solution from curve a with threonine (*cf.* Figure 2), and curve d demonstrates the increased sensitivity obtained by preincubation of enzyme solutions from curve b with KCl (*cf.* Figure 4B). The plateau value in curve c is the maximum sensitivity when the enzyme is fully in the sensitive form. These experiments make it apparant that the enzyme at the concentration and in the medium in which it is present at the end of the preparation procedure (Table I) is predominantly in the sensitive form. For further confirmation of the existence of the fully sensitized state, KCl was added to an enzyme sample corresponding to the 0.095-unit/ml point on curve a. With increasing concentrations of KCl in the preincubation mixture, the value of $\sigma$ increased to that for full sensitization (vertical dotted line).[1] Increasing pH shifted the curves in the direction of greater sensitivity (Mankovitz, 1968).

*Effects of Other Ligands on Homoserine Dehydrogenase Sensitivity to Threonine Inhibition.* Isoleucine, methionine, and

[1] With enzyme brought to full sensitivity with threonine or KCl, a brief lag in the $v_i$ assay curves was noted. The values of $v_i$ were obtained from the linear portions of the curves.

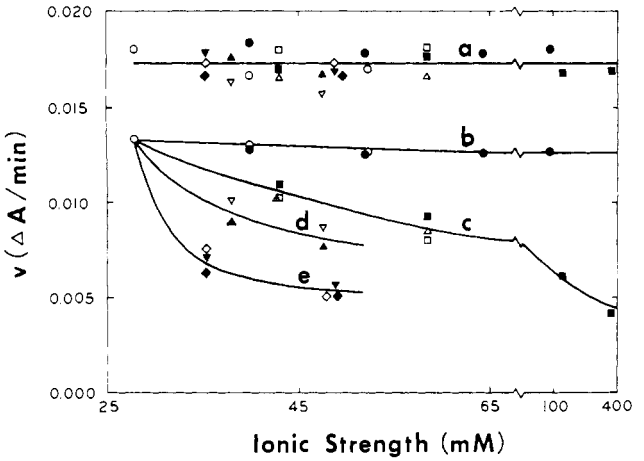


FIGURE 5: Resensitization of homoserine dehydrogenase as a function of ionic strength and ionic species. Enzyme (9.5 units/ml) was desensitized by salt dilution as in Figure 4A and assayed with and without threonine (points at $\mu$ = 28.1). To aliquots of the desensitized enzyme solution the appropriate salt was added and resensitization was allowed to occur at 23°, followed by assay with and without threonine. Curve a is the uninhibited activity and curves b through e the activity in the presence of threonine after preincubation in salt. The key is: $Na_2SO_4$, open circles; $K_2SO_4$, filled circles; NaCl, open squares; KCl, filled squares; NaAc, open upright triangles; $MgSO_4$, filled upright triangles; $MnSO_4$, open inverted triangles; $MgCl_2$, filled inverted triangles; $Mg(Ac)_2$, open diamonds; $CaCl_2$, filled diamonds. Ionic strength shown includes that of the buffer with appropriate correction for reaction of an equivalent of divalent cation with the EDTA present.

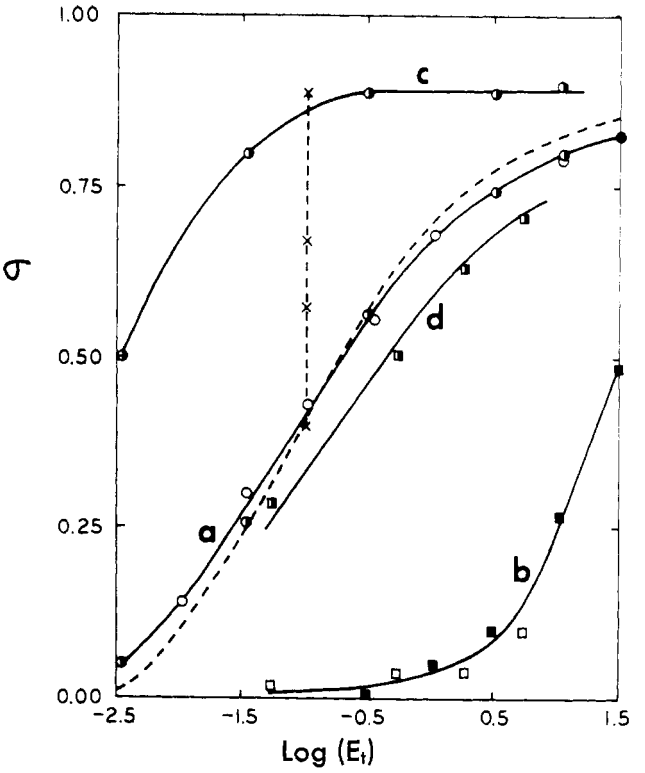


FIGURE 6: Sensitivity of homoserine dehydrogenase to threonine inhibition as a function of enzyme concentration under various conditions. $\sigma$ is sensitivity to threonine as defined in the text, and was measured after equilibration following dilution and threonine or salt addition. Total concentration of enzyme, ($E_t$), is in units per milliliter. Curves a and b represent enzyme in buffers A and B, respectively. Curve c represents enzyme from curve a resensitized by incubation with 0.55 mM L-threonine (*cf.* Figure 2). Curve d represents enzyme from curve b resensitized with 0.1 M KCl (*cf.* Figure 4B). Enzyme at a concentration of 9.5 units/ml on curve a was that yielded by the preparation procedure described in Table I. Lower concentrations were obtained by dilution with buffer A. Open circles and half-filled circles on curve a represent separate experiments. Enzyme at a concentration of 4.8 units/ml on curve a was that yielded by elution from Sephadex G-25 in buffer B. Lower concentrations (open squares) were obtained by dilution with buffer B. The concentrations of 28 and 27 units per ml on curves a and b, respectively (filled symbols), were obtained by ammonium sulfate precipitation of enzyme of lower concentration, followed by dissolution in a small volume and dialysis against buffer A and buffer B, respectively. Dilutions of the 27 units/ml of solution on curve b are shown in filled squares. The dotted line overlapping curve b is a theoretical curve assuming the enzyme dissociates into two subunits (see Appendix, B). In a separate experiment enzyme was diluted with buffer A to 0.0095 units/ml for desensitization, then to separate aliquots solid KCl was added to the equivalent of 0.5, 1.0, or 4.0 mmoles per ml of enzyme solution. Sensitivity (X's) increased progressively with increasing KCl concentration (vertical dotted line).

norleucine are activators of homoserine dehydrogenase and/or liberators of threonine inhibition (Sturani *et al.*, 1963; Datta and Gest, 1965). $\beta$-Hydroxynorvaline is an inhibitor of homoserine dehydrogenase analogous to threonine (Mankovitz and Segal, 1969b). All of these ligands produced a resensitization of desensitized enzyme analogous to the effect of threonine. The time course of resensitization is shown for isoleucine (Figure 7A), and the degree of resensitization as a function of ligand concentration is shown in Figure 7B for all four amino acids. The time course of resensitization with $\beta$-hydroxynorvaline (not shown) was biphasic, as in the case with threonine (Figure 2), and almost superimposable on the threonine sensitization curve.

Substrates and a number of other amino acids were tested for their effect on partially desensitized enzyme (Table II). Homoserine, serine, aspartate, and ATP further desensitized the enzyme. The effects were small but reproducible. $TPN^+$, TPNH, and aspartic $\beta$-semialdehyde, as well as asparagine and cysteine, had no significant effect.

## Discussion

A simple model can be proposed to synthesize the results presented in this paper, based upon a reversible dissociation of the enzyme to a form insensitive to threonine but fully active

$$\begin{array}{ccc} E_n & \overset{K_1}{\rightleftharpoons} & nE \\ {\scriptstyle K_2}\uparrow\downarrow{\scriptstyle n\theta} & & \\ E_n\theta_n & & \end{array} \qquad (1)$$

where $\theta$ represents threonine and the $K$'s are equilibrium constants. The value of $n$ appears to be 2 from the reasonably close fit of the dissociation curve (curve b in Figure 6) to a unimolecular–biomolecular equilibrium (see Appendix, B). A value for $K_1$ of about 0.15 unit/ml was obtained graphically from the points on the middle half of this curve (see Appendix, B). If we assume, as rough approximations, that the specific activity of pure enzyme is 100 units/mg (data of Datta and Gest, 1965)

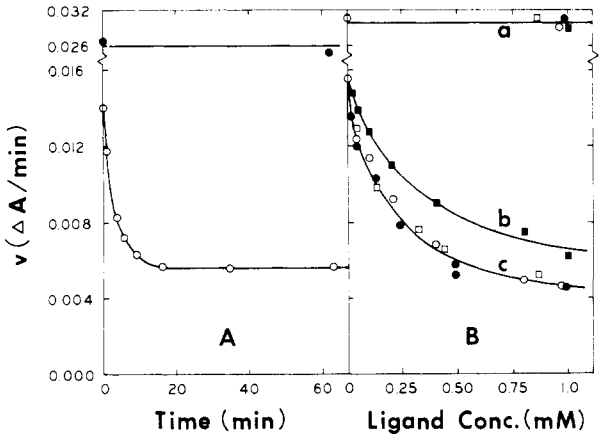


FIGURE 7: Resensitization by isoleucine, methionine, β-hydroxynorvaline, and norleucine of enzyme previously desensitized by enzyme dilution. Enzyme (14 units/ml) was diluted 100-fold with buffer A for desensitization. (A) L-Isoleucine was added to give a final concentration of 0.42 mM and the solution further incubated at 23°. Aliquots (0.1 ml) were removed periodically for assay in the absence (filled circles) and presence (open circles) of 0.48 mM L-threonine. Zero time points were assays after desensitization but before addition of isoleucine. (B) Either L-isoleucine (open squares), L-methionine (filled squares), DL-β-hydroxynorvaline (open circles), or DL-norleucine (filled circles) was added to the final concentration shown (of the L form). After a time sufficient for equilibration at 23°, aliquots (normalized to 0.1 ml) were removed for assay in the absence (curve a) and presence (curves b and c) of 0.48 mM L-threonine.

TABLE II: Effect of Preincubation with Substrates and Amino Acids on the Sensitivity of Homoserine Dehydrogenase to Threonine Inhibition.[a]

| Compound | Concn (mM) | Homoserine Dehydrogenase Act. (ΔA/min) Control | +Threonine |
|---|---|---|---|
| None | | 0.0031 | 0.0016 |
| L-Homoserine | 1 | | 0.0020 |
| L-Homoserine | 2 | | 0.0023 |
| L-Homoserine | 10 | 0.0032 | 0.0024 |
| DL-Aspartic β-semialdehyde | 0.2 | 0.0033 | 0.0016 |
| DL-Aspartic β-semialdehyde | 3.8 | 0.0033 | 0.0015 |
| $TPN^+$ | 0.8 | 0.0030 | 0.0016 |
| TPNH | 0.1 | 0.0032 | 0.0016 |
| TPNH | 0.9 | 0.0030 | 0.0017 |
| L-Aspartate | 2 | 0.0031 | 0.0025 |
| L-Serine | 2 | 0.0032 | 0.0020 |
| DL-Asparagine | 4.2 | 0.0031 | 0.0016 |
| L-Cysteine | 1.8 | 0.0031 | 0.0017 |
| ATP | 4.4 | 0.0031 | 0.0023 |

[a] Homoserine dehydrogenase (14 units/ml) was diluted 100-fold with buffer A to obtain partially desensitized enzyme (*cf.* Figure 7). Test compounds were added in small volumes to give the final concentrations shown, and the solutions incubated at 24° for 1–5 hr. Aliquots (10 μl) were removed for assay in the absence and presence of 0.48 mM L-threonine.

in our units and the molecular weight is 100,000 (Datta *et al.*, 1964), $K_I$ can be converted into approximately $10^{-8}$ M with a corresponding standard free energy for dissociation of about 10 kcal, which could be provided by three or four hydrogen bonds or salt linkages.

The dissociated form, E, is totally insensitive to threonine (see curve b, Figure 6). This, together with the ability of threonine to shift the equilibrium fully to the aggregated form (curve d, Figure 6) indicates that threonine does not bind to dissociated forms, even in a noninhibitory manner. The latter consideration appears to rule out a dissociation into distinct active and regulatory subunits, as in the case of *p*-mercuribenzoate-treated aspartic transcarbamylase (Gerhart and Schachman, 1965).

The invariance of activity with dissociation indicates that the number of active sites is independent of the state of aggregation of the enzyme. The regulatory binding sites, however, exist only when the subunits are associated. Two alternatives suggest themselves to account for the lack of threonine binding to the dissociated form. One is that the site is composed of elements from two subunits. In a manner of speaking, it exists on a "seam." The other is that the regulatory site is wholly within a single subunit but becomes unrecognizably distorted in the dissociated state. The latter implies the following sequence

$$2E \overset{1}{\rightleftharpoons} 2E' \overset{2}{\rightleftharpoons} E_2 \qquad (2)$$

where E′ is a conformer of E, still containing a recognizable regulatory site, and therefore threonine sensitive. This alternative seems to us to be ruled out by the following considerations. The rate of resensitization is higher than first order in enzyme concentration (see Figures 2 and 4B). Therefore, in eq 2, step 2 is rate limiting. The threonine-induced shift toward the sensitive state occurs *via* the rate-limiting reaction and is higher than first order in enzyme concentration, whereas binding of threonine to E′ would shift the equilibrium toward sensitive forms *via* a fast reaction first order in enzyme concentration. Thus a form with the postulated properties of E′ does not exist. Therefore, we propose that the regulatory site exists only when the enzyme is in the associated state and is composed of elements from separate subunits. If this is correct it suggests a sequence of events in the molecular evolution of the enzyme. Presumably the primitive form was a nonassociable, nonregulatable monomer which mutated into a species with two partially complementary sites for threonine. Such a species would be associable in the presence of threonine. Further mutation would produce binding sites capable of leading to association even in the absence of threonine.

β-Hydroxynorvaline, which is analogous to threonine in its inhibitory properties (Mankovitz and Segal, 1969b), as well as the liberators of threonine inhibition, methionine, isoleucine, and norleucine (Sturani *et al.*, 1963; Datta and Gest, 1965), also shift the equilibrium in eq 1 toward the sensitive (aggregated) form (Figure 7B). Since these modulators, like threonine, fail to affect the dissociated form kinetically (Manko-

vitz and Segal, 1969b), their binding sites have properties in these respects identical with the threonine-binding site.

The ability of homoserine to shift the equilibrium between aggregated and disaggregated forms slightly toward the latter (Table II) suggests a bias in the affinity of homoserine in favor of this form. We have not yet tested this possibility kinetically. Serine behaves analogously, consistent with the evidence that serine is an isosteric inhibitor (Mankovitz and Segal, 1969b). The similar effect with aspartate and ATP is intriguing in view of the evidence that aspartokinase and homoserine dehydrogenase activity exist within the same molecular complex in *E. coli* and that the substrates of each reaction inhibit the velocity of the other (Patte *et al.*, 1966). We have tested our preparations of homoserine dehydrogenase for aspartokinase activity but have found none. On the other hand, ATP and aspartate inhibit *R. rubrum* homoserine dehydrogenase (Mankovitz, 1968). It is not inconceivable that the *R. rubrum* enzyme represents either a stage in the evolution toward the *E. coli* type complex or a vestige of the latter type.

Divalent cations and monovalent anions shift the equilibrium toward the sensitive (aggregated) state. Since this is not a nonspecific ionic strength effect, we conclude that it reflects a bias in the binding of these substances as well, in this case in favor of the aggregated form.

Further light can be expected to be shed on some of these questions by an investigation of the homogeneity of the subunits and by ligand binding experiments with a more purified preparation.

Three independent considerations reveal an additional complexity in the interactions of homoserine dehydrogenase subunits. Firstly, resensitization by threonine and β-hydroxynorvaline is biphasic (Figure 2) with a rapid phase analogous to that seen with salt and isoleucine resensitization (Figures 4B and 7A), followed by a slow phase. Secondly, the Hill coefficient calculated from the experiment in Figure 3 is 2.2. Thirdly, earlier sucrose density gradient centrifugation and gel filtration experiments (Datta *et al.*, 1964) indicated that threonine produced a species of higher sedimentation coefficient than did isoleucine or methionine. These observations can be explained by an expansion of the model to include the formation of a higher state of aggregation (we assume a tetramer for the purposes of the diagram)

$$\begin{array}{ccccccc} 4E & \overset{1}{\rightleftharpoons} & 2E_2 & \overset{2}{\rightleftharpoons} & 2E_2\theta_2 & \overset{3}{\rightleftharpoons} & E_4\theta_4 \\ & & 4\uparrow\downarrow 4L & & & & \\ & & 2E_2L_2 & & & & \end{array} \qquad (3)$$

where $\theta$ represents threonine (and β-hydroxynorvaline) and L represents the liberators and/or activators, isoleucine, methionine, and norleucine. From inspection of Figures 1 and 4A a half-time for dissociation into monomers of 1–2 min is obtained. The equilibrium constant for eq 3 appears to be approximately the same as that for eq 1 (Figure 2).

To summarize, the model proposes that there is a spontaneous, moderately slow monomer–dimer equilibrium; that allosteric ligands do not bind to the monomer; and that the threonine-bound dimer undergoes a dimer–tetramer equilibrium involving bonding forces of similar strength (and thus presumably the same groups) as in the monomer–dimer reaction.

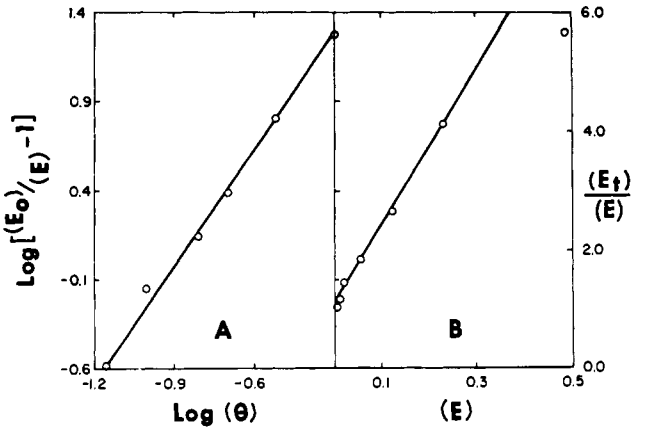


FIGURE 8: Statistical studies. (A) Hill plot for threonine sensitization Symbols are explained in the text. Data are taken from Figure 3. (B) Plot for the determination of $K_1$. Symbols are explained in the text. Data are taken from curve c, Figure 6.

## Appendix

*A. Calculation of Hill Coefficient for Sensitization by Threonine (See Figure 3).* We write the reaction for threonine-induced conversion into threonine-sensitive forms as

$$E + n\theta \overset{K}{\rightleftharpoons} E\theta_n \qquad K = \frac{(E)(\theta)^n}{(E\theta)_n}$$

where E represents nonsensitive forms and $E\theta_n$ represents sensitive forms.

Let $(E_0)$ be the total concentration of nonsensitive enzyme at $(\theta) = 0$. Then

$$K = \frac{(E)(\theta)^n}{(E_0) - (E)}$$

which can be rearranged to

$$(E_0)/(E) = 1 + \frac{(\theta)^n}{K}$$

and

$$\log [(E_0)/(E) - 1] = n \log \theta - \log K$$

The values of (E) at any concentration of threonine $(\theta)$ can be obtained from the corresponding velocity values as described in Appendix B (eq 7).

From the plot shown in Figure 8A of the data in Figure 3, a value for the Hill coefficient, $n$, of 2.2 is obtained.

*B. Calculation of Monomer–Dimer Equilibrium Constant (See Discussion, Eq 1).* For the reaction

$$E_2 \overset{K_1}{\rightleftharpoons} 2E$$

we write the equilibrium constant

$$K_1 = \frac{(E)^2}{(E_2)}$$

and the conservation of mass equation

$$(E_t) = 2(E_2) + (E) \quad (1)$$

where $(E_t)$ is the total concentration of subunits. Therefore

$$K_1 = \frac{2(E)^2}{(E_t) - (E)} \quad (2)$$

and

$$(E_t)/(E) = 1 + \frac{2(E)}{K_1} \quad (3)$$

It is necessary now to obtain expressions for $(E_t)$ and (E) in terms of measured velocities. The uninhibited velocity is proportional to the total number of subunits.

$$v_o = k(E_t)$$

By defining E concentration in the same units as $v$'s (*i.e.*, units/ml) we can drop the proportionality constant and write simply

$$(E_t) = v_o \quad (4)$$

The velocity in the presence of inhibitor is proportional to the number of dissociated (nonsensitive) subunits, E, plus the number of associated subunits times 0.1 (since in a state of full association the velocity in the presence of inhibitor is not 0 but 10% of the uninhibited velocity (see curve c, Figure 6)) Therefore

$$v_i = [(E) + 0.1 \times 2(E_2)]$$

$$= [0.2(E_2) + (E)] \quad (5)$$

$(E_2)$ can be eliminated from eq 5 by utilizing eq 1, giving

$$v_i = \left[\frac{(E_t) - (E)}{10} + (E)\right] = \frac{(E_t) + 9(E)}{10} \quad (6)$$

We replace $(E_t)$ with $v_o$ (eq 4) and solve for (E), obtaining

$$(E) = \frac{10v_i - v_o}{9} \quad (7)$$

Corresponding values of $(E_t)$ and (E) are obtained thusly from the velocity data in Figure 6 and plotted as shown in Figure 8B. As seen from eq 3, the slope is $2/K_1$. The value of $K_1$ obtained is 0.15 unit/ml.

The theoretical curve of $\sigma$ *vs.* log $(E_t)$ shown in Figure 6 for a monomer–dimer equilibrium, where $\sigma = (1 - v_i/v_o)$, was calculated as follows.

At each value of $(E_t)$, $v_o = (E_t)$ (eq 4).

Knowing $K_1$ from Figure 8B, values of (E) at each value of $(E_t)$ can be obtained from eq 2. For each set of $(E_t)$ and (E) values, $v_i$ is calculated from eq 6.

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