# Homoserine Dehydrogenase of *Rhodospirillum rubrum*. Enzyme Polymerization in the Presence and Absence of Threonine†

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ABSTRACT: Depending on the pH and ionic strength, homoserine dehydrogenase of *Rhodospirillum rubrum* can polymerize in the presence or absence of the feedback modifier L-threonine. In the absence of threonine, significant alterations in the protein structure were detected when the standard buffer solution (25 mM potassium phosphate containing 1 mM EDTA and 2 mM dithiothreitol) was supplemented with increasing concentrations of KCl. At pH 7.1 and an ionic strength of 0.075, the Stokes' radius of the enzyme was about 47 Å; a value of 58 Å was calculated at an ionic strength of 0.275. Since the sedimentation coefficient of the enzyme was unchanged under these conditions, the increase in Stokes' radius presumably reflects a slight unfolding of the molecule rather than polymerization. At a still higher ionic strength of 0.575, however, a fraction of the enzyme became aggregated at pH 7.1, and the extent of aggregation was greater when the pH was lowered to pH 6.3. In the standard buffer solution with 1 mM L-threonine, the extent of enzyme aggregation was also dependent on pH and ionic strength. At an ionic strength of 0.075, the amount of polymeric species varied from 80% at pH 6.3 to about 20% at pH 7.1; aggregation was barely detectable at pH 7.5. When the ionic strength of the buffer was increased to 0.13, almost 90% of the enzyme was aggregated at pH 7.1. We conclude from these data that at low threonine concentrations salt and/or pH-dependent conformational changes in the protein structure may be required for the threonine-mediated polymerization of the enzyme.

In the past few years, considerable attention has been focused on the ligand-mediated association–dissociation of various allosteric enzymes for two reasons. First, in the context of metabolic control, polymerization or depolymerization reactions are thought to be highly significant in modulating the rates of enzyme reactions *in vivo*. Second, in terms of protein structure, these enzymes serve as models to study the mechanisms of intramolecular and intermolecular subunit interactions. Frieden (1971) has recently compiled a list of some 30 enzymes, most of which are involved in polymerization reactions. Included in that list is the homoserine dehydrogenase (L-homoserine:$NADP^+$ oxidoreductase, EC 1.1.1.3) from *Rhodospirillum rubrum* which catalyzes the reversible conversion of aspartate β-semialdehyde and homoserine in the presence of NADPH. This enzyme, having a mol wt of ~140,000, exhibits association of the native monomer[1] to form enzyme polymers in the presence of L-threonine, the feedback inhibitor (Datta *et al.*, 1964; Datta, 1970); the molecular weights of the aggregates seem to vary from 300,000 to $2 \times 10^6$ daltons (Datta, 1970). In the absence of L-threonine and in buffers of low ionic strength, the enzyme appears to have a smaller molecular size than the monomeric species and is catalytically active but completely densitized to L-threonine inhibition (Datta and Gest, 1965; Mankovitz and Segal, 1969; Datta, 1971). In the presence of high salt the apparent molecular weight of the monomer was increased to 210,000 and this form of the enzyme showed enhanced stimulation of enzyme activity by L-isoleucine and a slightly decreased sensitivity to L-threonine inhibition (Datta, 1971). Furthermore, since in the presence of high salt a buried SH group was titrated with $Nbs_2$,[2] it was concluded that the *R. rubrum* homoserine dehydrogenase could assume various conformational states in the presence of these bound ligands (Datta, 1971).

This communication reports that depending on the pH and ionic strength, the enzyme can polymerize in the presence or absence of the feedback modifier, L-threonine. The data suggest that at low threonine concentrations a salt and/or pH-dependent conformational change in the protein structure is required for the threonine-mediated polymerization of the enzyme.

## Experimental Section

### *Materials and Methods*

*Reagents.* L-Homoserine and allo-free L-threonine were obtained from Calbiochem or Sigma Chemical Co. DL-Aspartate semialdehyde was prepared according to the method described by Black (1963). $NADP^+$, NADPH, and NADH were purchased from P-L Laboratories or from Sigma. Dithiothreitol was bought from Calbiochem. Phosphoenolpyruvate and ADP were purchased from Sigma, and Sephadex and blue dextran were obtained from Pharmacia Fine Chemicals. Horseradish peroxidase and yeast alcohol dehydrogenase were obtained from Worthington, bovine serum albumin was from Miles Laboratories, and rabbit muscle pyruvate kinase was bought from Sigma. Beef heart lactate dehydrogenase

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[1] Throughout this article, the term "monomer" is used to designate the smallest physical unit that is catalytically active; the polymeric forms are those composed of two or more of these monomeric units (see Frieden, 1971). According to this usage, monomers may contain one or more enzymatically inactive polypeptide chains (subunit(s)) that may or may not be identical in their primary sequence.

[2] Abbreviation used is: $Nbs_2$, 5,5′-dithiobis(2-nitrobenzoic acid).

was a product of Calbiochem. All other chemicals were of reagent grade.

*Enzyme Purification and Assay.* Homoserine dehydrogenase was purified from the photosynthetic bacterium *Rhodospirillum rubrum* SIH (ATCC 25903) as described earlier (Datta, 1970). The detailed procedures for measuring enzyme activity in the forward and reverse direction assays have been described (Datta and Gest, 1970; Datta, 1970). The standard assay methods for the other enzymes such as alcohol dehydrogenase, pyruvate kinase, and peroxidase have been reported elsewhere (Feldberg and Datta, 1971).

*Stokes' Radius Determination.* Stokes' radius of the enzyme molecule was determined from the equations relating elution volume and molecular radius using calibrated Sephadex G-200 as described by Ackers (1964) and by Siegel and Monty (1966).

*Analysis of Sephadex Gel Filtration Data.* Gel filtration through calibrated Sephadex G-200 columns (85 × 1.5 cm) was used exclusively to determine the extent of polymerization under various experimental conditions. The per cent of monomer present in a given gel filtration experiment was estimated from the amount of monomeric species (obtained by integrating the area under the peak eluting slightly ahead or at the same place with yeast alcohol dehydrogenase) divided by the total area under all peaks and multiplied by 100. There were two other distinct advantages in using this method. First, the concentration of homoserine dehydrogenase was kept low to avoid any complication arising from higher protein concentrations; second, the Stokes' radius of the various enzyme species could be calculated from the data. In addition, approximate estimations of molecular weights of the various forms were possible. All experiments were carried out at 25°, and the amount of enzyme activity recovered after column runs varied from 80 to 100%.

*Other Methods.* Protein was determined by the method of Lowry *et al.* (1951). Sucrose density gradient centrifugations were carried out according to Martin and Ames (1961) except that a linear gradient from 2.5 to 15% sucrose in appropriate buffers was used.

## Results

*Gel Filtration of the Native Enzyme.* In 25 mM potassium phosphate buffer (pH 7.1) containing 1 mM EDTA and 2 mM dithiothreitol (buffer P) the *R. rubrum* homoserine dehydrogenase eluted slightly ahead of yeast alcohol dehydrogenase, an enzyme of mol wt 140,000 (Figure 1A). The elution peak was fairly symmetrical, and estimates of the Stokes' radius and the molecular weight from the equation relating $K_D$ to these parameters (Ackers, 1964; Siegel and Monty, 1966) gave values of 47 Å and 150,000 daltons, respectively. These values were slightly higher than 45 Å and 138,000 reported earlier in 25 mM potassium phosphate buffer (pH 7.5) containing 1 mM EDTA and 1 mM dithiothreitol (Datta, 1970).

The data in Figure 1 show that, with increasing KCl concentrations in buffer P, first there was a shift of the enzyme elution profile from a position slightly before yeast alcohol dehydrogenase (mol wt 140,000) to the fractions almost coincident with the elution profile of rabbit muscle pyruvate kinase of mol wt 237,000 (Figures 1A–C). From the plots of Stokes' radii of various marker enzymes as a function of the distribution coefficients we calculated the molecular radius of homoserine dehydrogenase that existed in buffers of different ionic strength. These results are summarized in Figure 2. The Stokes' radius of the enzyme increased from 47 to about 58 Å by increasing the total ionic strength of the gel filtration buffer from 0.075 to 0.275. The peak of enzyme activity eluting in fraction 82 at an ionic strength of 0.575 (Figure 1D) corresponded to a form with a Stokes' radius of 59.4 Å; at the same ionic strength a fraction of the enzyme also became aggregated.

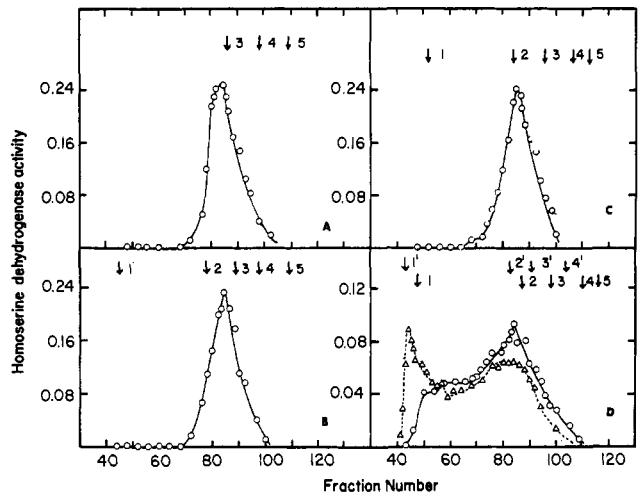


FIGURE 1: Elution profiles of homoserine dehydrogenase from Sephadex G-200 columns in buffers of increasing KCl concentrations in the absence of threonine. The basic buffer was 25 mM potassium phosphate (pH 7.1) containing 1 mM EDTA, 2 mM dithiothreitol, and the salt concentrations as follows: (A) no KCl; (B) 50 mM KCl; (C) 200 mM KCl; and (D) open circles, 500 mM KCl. The results shown by open triangles in (D) were obtained by lowering the pH of the buffer solution containing 500 mM KCl at pH 6.3. In each case, the column was equilibrated at 25° with the appropriate buffer. Purified homoserine dehydrogenase (100 μg) was mixed with blue dextran, pyruvate kinase, yeast alcohol dehydrogenase, bovine serum albumin, and horseradish peroxidase in a final volume of 1 ml in the column buffer, and applied to the column. The column was eluted with the same buffer at a flow rate of about 5 ml/hr and 16-drop fractions (0.75 ml) were collected. Blue dextran and bovine serum albumin were located by their absorbances at 600 and 280 nm, respectively. Enzyme activities were assayed as described in the Experimental Section. Vertical arrows with numbers next to them show the locations of standard markers as follows: (1) blue dextran; (2) pyruvate kinase; (3) yeast alcohol dehydrogenase; (4) bovine serum albumin; (5) peroxidase.

An increase in the Stokes' radius of the native enzyme from 47 to 59 Å due to an increase in the ionic strength of the elution buffer may indicate either unfolding of the enzyme mole-

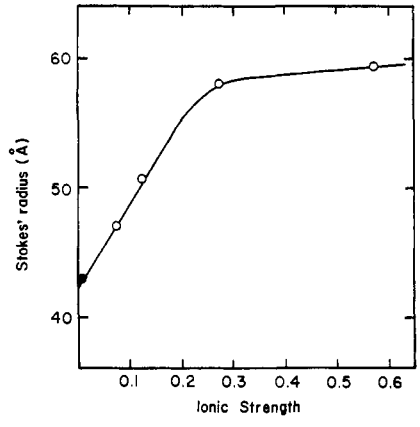


FIGURE 2: A plot of Stokes' radius *vs.* ionic strength of the eluting buffer during Sephadex G-200 gel filtration. For each data point, the Stokes' radius of the enzyme was calculated from the results given in Figure 1. The closed circle represents an independent measurement of the Stokes' radius in the standard potassium phosphate buffer of a total ionic strength of 0.006.

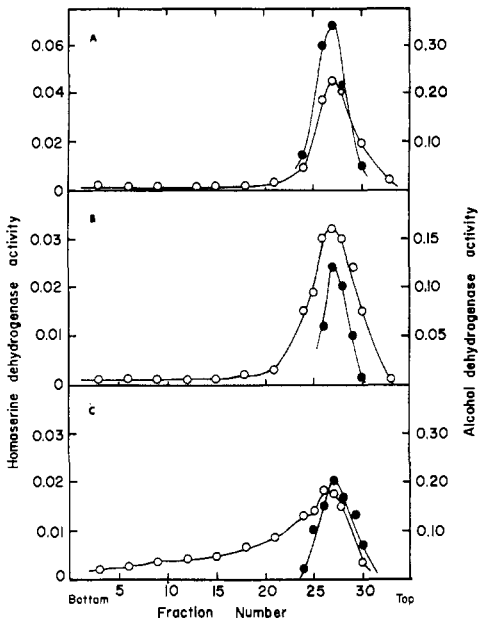


FIGURE 3: Sucrose density gradient centrifugation of homoserine dehydrogenase in the presence and absence of KCl. The basic buffer was 25 mM potassium phosphate buffer (pH 7.1) containing 1 mM EDTA, 2 mM dithiothreitol, and KCl concentrations as follows: top, no KCl; middle, 100 mM KCl; and bottom, 500 mM KCl; open circles, homoserine dehydrogenase activity; closed circles, yeast alcohol dehydrogenase activity. The gradients were centrifuged at 24,000 rpm for 20 hr at 20°. For other experimental details, see Martin and Ames (1961).

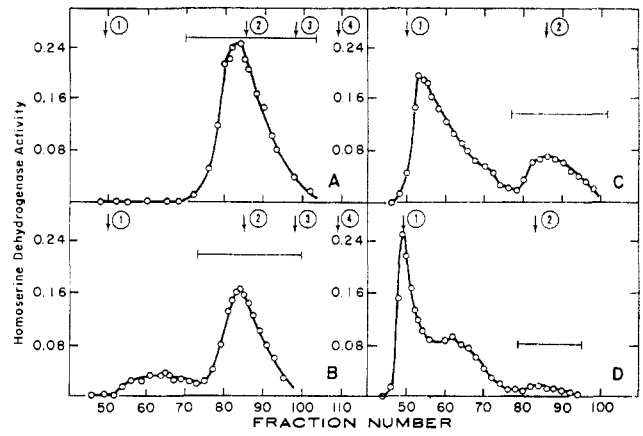


FIGURE 4: Elution profiles of homoserine dehydrogenase from Sephadex G-200 in the presence of increasing concentrations of L-threonine. The basic buffer was 25 mM potassium phosphate buffer (pH 7.1) containing 1 mM EDTA and 2 mM dithiothreitol, and the concentrations of L-threonine are as follows: (A) no threonine; (B) 1 mM L-threonine; (C) 5 mM L-threonine; and (D) 20 mM L-threonine. The locations of standard markers are: (1) blue dextran; (2) alcohol dehydrogenase; (3) bovine serum albumin; and (4) peroxidase. The horizontal bars indicate the areas under the peaks integrated to estimate the amount of the monomeric species (see Experimental Section). For other details, see the legend to Figure 1.

cule or formation of a protein dimer. For example, dimerization of a spherical molecule of radius $R_1$ would result in an increase of Stokes' radius $R_2 = 2^{1/3}R_1 = 1.26R_1$ (Waldmann-Meyer, 1972); therefore, the homoserine dehydrogenase molecule of about 59 Å may represent a dimer of a monomeric unit having a molecular radius of 47 Å. If this were the case, the sedimentation coefficient of the enzyme in the presence of high salt would be expected to be larger than the $s$ value of the monomer in low salt. We had reported earlier (Datta, 1970) that using Beckman Model E ultracentrifuge the enzyme (3 mg/ml) in potassium phosphate buffer (pH 7.5) and at an ionic strength of 0.20 showed an $s_{20,w}$ value of 6.9. The results of sucrose density gradient centrifugation studies reported here revealed that the sedimentation coefficient of homoserine dehydrogenase was about 7.6 S (with reference to yeast alcohol dehydrogenase) in buffers of ionic strength of 0.075 and 0.175 (Figure 3, top and middle frames). At an ionic strength of 0.575, a fraction of the enzyme molecules also sedimented with an $s$ value very similar to the enzyme in low ionic strength buffers (Figure 3, lower frame); this species was presumably similar to that exhibiting a Stokes' radius of 59 Å in the Sephadex experiment. The cumulative data from Sephadex gel filtration and sucrose density gradient centrifugation experiments show, therefore, that the increase in Stokes' radius to 59 Å at high salt concentration was the result of a slight unfolding of the enzyme molecule and not due to dimer formation.

As stated above, upon gel filtration in the presence of high salt, a fraction of the enzyme eluted in a fashion to indicate protein aggregation (see Figure 1D, open circles); when the pH of the same buffer solution was lowered to pH 6.3, further polymerization occurred and a considerable amount of the enzyme of high molecular weight was excluded from Sephadex (Figure 1D, open triangles). The results of sucrose density gradient centrifugation studies (Figure 3C) also showed that the $s$ values of the heterogeneous polymeric species were higher as evidenced by the enzyme activity detected in fractions beginning from fraction 27 to fraction 3 at the bottom of the gradient. Therefore, we conclude that a significant fraction of the enzyme showed a high degree of polymerization in high salt but in the absence of L-threonine.[3]

An interesting aspect of the protein structure is revealed from the plot of Stokes' radius *vs.* ionic strength as depicted in Figure 2. Extrapolation of the curve drawn through the experimentally determined Stokes' radii at various salt concentrations to zero ionic strength yielded a value of about 42 Å; an independent measurement of the Stokes' radius at an ionic strength of 0.006 (shown by the solid circle in Figure 2) confirmed the finding. In view of these results, it is difficult, therefore, to assign a definite molecular weight value to the monomeric species; depending on the ionic strength of the buffer solution, the *apparent molecular weight* may vary from 100,000 to 150,000 as determined from the relationship of $K_D$ and molecular weight suggested by Laurent and Killander (1964).

*Enzyme Polymerization in the Presence of* L-*Threonine.* Figure 4A shows the elution profile of the native enzyme in 25 mM potassium phosphate buffer (pH 7.1) containing 1 mM EDTA and 2 mM dithiothreitol (buffer P). When the same buffer system was supplemented with increasing amounts of L-threonine the elution profiles changed drastically (Figure 4B–D). Three major points should be mentioned in this

[3] Extensive aggregation of the enzyme was also observed during enzyme purification. When the dialyzed enzyme solution obtained from the ammonium sulfate precipitation step (Datta, 1970) was centrifuged at 37,000*g* for 30 min, all the enzyme activity could be recovered in the pellet; however, upon gel filtration on Sephadex G-200 in 0.05 M potassium phosphate buffer (pH 7.5) containing 0.001 M EDTA, 0.001 M dithiothreitol, and 0.007 M $\beta$-mercaptoethanol, greater than 70% of the enzyme activity was eluted at a position corresponding to a species with a mol wt of about 140,000. These results indicate depolymerization of the salt-induced aggregated forms during gel filtration.

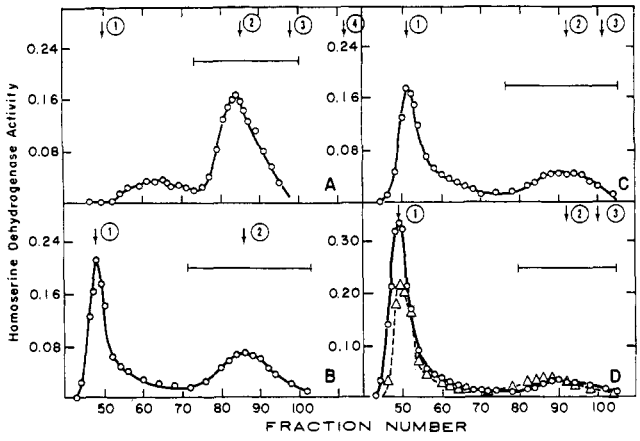


FIGURE 5: Elution profiles of homoserine dehydrogenase from Sephadex G-200 columns in the presence of increasing concentrations of KCl. The basic column buffer was 25 mM potassium phosphate buffer (pH 7.1) containing 1 mM EDTA, 2 mM dithiothreitol, 1 mM L-threonine, and the concentrations of KCl as follows: (A) no KCl; (B) 10 mM KCl; (C) 25 mM KCl; and (D) open circles, 50 mM KCl; open triangles, 50 mM NaCl. The locations of standard markers are: (1) blue dextran; (2) alcohol dehydrogenase; (3) bovine serum albumin; and (4) peroxidase. The horizontal bars indicate the areas under the peaks integrated to estimate the amount of the monomeric species (see Experimental Section). For other details, see the legend to Figure 1.

context. (1) With increasing L-threonine concentrations, the amount of enzyme found in the monomeric form decreased, and at 20 mM L-threonine almost complete conversion to polymeric species was seen. (2) The degree of polymerization and the extent of heterogeneity within the various polymeric forms were dependent on the amount of L-threonine added during gel filtration. With intermediate levels of L-threonine (1 and 5 mM) the largest polymeric species were not excluded by Sephadex G-200; as the threonine concentration was increased, more and more of the higher polymers was detected, and at the highest L-threonine concentration used, a large fraction of the polymers was excluded from the gel. (3) Although the amount of monomer decreased with increasing

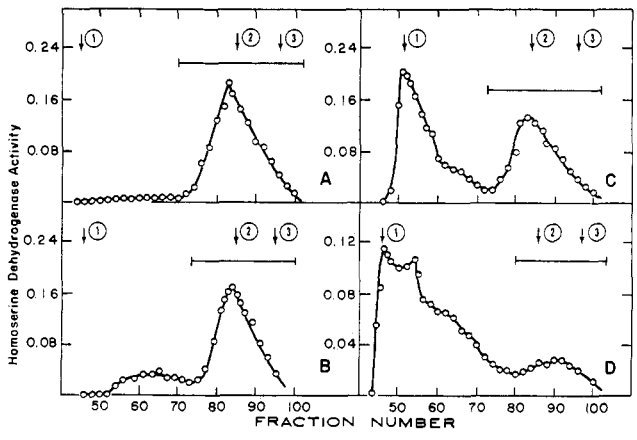


FIGURE 6: Elution profiles of homoserine dehydrogenase from Sephadex G-200 columns in buffers of decreasing pH values. The basic column buffer was 25 mM potassium phosphate buffer containing 1 mM EDTA, 2 mM dithiothreitol, and 1 mM L-threonine adjusted to pH values as follows: (A) 7.5; (B) 7.1; (C) 6.7; and (D) 6.3. The location of standard markers are: (1) blue dextran; (2) alcohol dehydrogenase; and (3) bovine serum albumin. The horizontal bars indicate the areas under the peaks integrated to estimate the amount of monomeric species (see Experimental Section). For other details, see the legend to Figure 1.

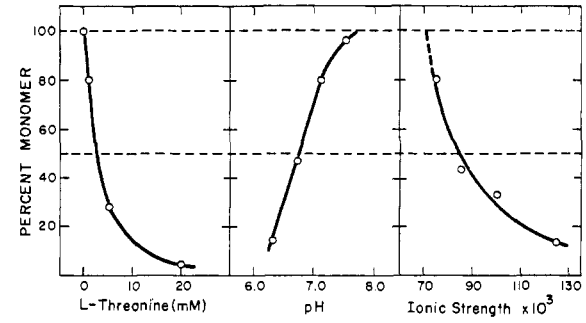


FIGURE 7: Estimation of the amount of homoserine dehydrogenase in monomeric form during Sephadex gel filtration in various buffers. The data are replotted from the results shown in Figures 4–6. The percentage monomer was calculated from the amount of monomeric species (obtained by integrating the area under the horizontal bar) divided by the total area under all peaks and multiplied by 100 (see Experimental Section).

L-threonine concentrations, the fraction of the enzyme that remained as monomer always eluted at the same position that is expected of a molecule having the native monomeric structure. These data suggest that different polymeric species are in rapid equilibrium with respect to each other and higher threonine concentrations shift the equilibrium in favor of large molecular weight aggregates (see Figure 4). This notion is consistent with the finding (not shown) that the aggregation phenomenon was protein concentration dependent (also *cf.* Datta, 1970).

*Effects of Ionic Strength and pH on the Threonine-Dependent Polymerization.* Since the "state" of the native enzyme was drastically altered by ionic strength and pH during gel filtration (*cf.* Figure 1 and 2), we examined the threonine-dependent aggregation of the protein in various buffer systems. The data shown in Figure 5 reveal the effect of *low* concentrations of KCl on the enzyme polymerization by threonine. In buffer P without KCl, but in the presence of 1 mM L-threonine, about 80% of the enzyme was present in the monomer state (Figure 5A); 10 mM KCl in the above buffer brought about 50% polymerization (Figure 5B) and, with 50 mM KCl, almost 90% of the enzyme was found as polymers (Figure 5D). An important aspect of the KCl-augmented threonine-mediated polymerization reaction was that almost all of the polymeric forms were excluded from the Sephadex matrix indicating very large species, and that very little of the intermediate size polymers were visible (*cf.* Figure 4C and D). Again, as seen in Figure 4, the unaggregated material eluted at about the same position that is typical for the monomer. The results given in Figure 5D also show that NaCl replaced KCl equally well indicating that the total ionic strength rather than the specific ion effect was the contributing factor.

Figure 6 reveals that the pH values of the enzyme solution and of the gel filtration buffer were also determining factors for the extent of enzyme polymerization. While 1 mM L-threonine in buffer P produced very little, if any, aggregation at pH 7.5 (Figure 6A), progressive lowering of the pH of the same buffer system to a value of 6.3 increased the amount of polymeric forms to greater than 80%. In contrast to the effect of ionic strength mentioned above, polymers of intermediate size that were not excluded by gel matrix were found under these conditions. Although the unaggregated enzyme fraction retained the molecular size of the monomer between pH 7.5 and 6.7 (Figure 6A–C), at pH 6.3 this material appeared to have a slightly smaller size (eluting between yeast alcohol dehydrogenase and bovine serum albumin; see Figure 6D).

Figure 7 summarizes the data presented with respect to pH, ionic strength, and L-threonine concentrations. The fraction of the enzyme detected in monomeric form was calculated by integrating the area under the peak eluting slightly ahead or at the same place with the yeast alcohol dehydrogenase. It is clear that 50% polymerization occurred at pH 6.8 in buffer P in the presence of 1 mM L-threonine, whereas 2.5 mM L-threonine was required at pH 7.1. At pH 7.1 and at an L-threonine concentration of a 1 mM, one-half of the homoserine dehydrogenase molecules were aggregated at an ionic strength of about 0.085.

## Discussion

The experiments described above indicate clearly that the homoserine dehydrogenase of *R. rubrum* show complex association–dissociation behavior in the presence of the feedback modifier, L-threonine. In the absence of this amino acid, the protein also exhibits a reversible aggregation–deaggregation phenomenon under certain experimental conditions.[3]

For example, in low ionic strength buffer, the Stokes' radius of the enzyme was 42 Å (see Figure 2) with an apparent mol wt of 105,000. In low salt the enzyme was also desensitized with respect to threonine inhibition (Datta and Gest, 1965; Mankovitz and Segal, 1969; Datta, 1971). With increasing ionic strength (from 0.075 to 0.275) the Stokes' radius of the enzyme increased significantly and reached a value of 58 Å indicating unfolding of the enzyme molecule. At a still higher ionic strength a fraction of the enzyme became aggregated at pH 7.1, and the extent of aggregation was greater when the pH was lowered to pH 6.3.

At an ionic strength of 0.075, low concentrations of L-threonine caused aggregation of the enzyme under certain conditions. The amount of polymeric species varied from 80% at pH 6.3 to about 20% at pH 7.1; at pH 7.5, aggregation by threonine was barely detectable (see Figure 6A). However, when the ionic strength of the same buffer was increased to 0.13 almost 90% of the enzyme was aggregated at pH 7.1.

These results clearly imply that threonine-dependent structural alterations are intimately related to the "state" of the homoserine dehydrogenase. The ionic strength and pH of the enzyme solution critically alter the native conformation, and these various conformations are subject to further modifications in the presence of the feedback inhibitor. A more compact form of the enzyme is less susceptible to polymerization, and a slight unfolding of the molecule is a prerequisite for the polymerization reaction to occur. This notion is strongly supported by the fact that a combination of low salt plus threonine or high salt without threonine can cause enzyme aggregation (Figures 1 and 5).

An important conclusion from this study is the apparent lack of correlation observed between threonine-dependent polymerization of the protein and the regulation of enzyme activity by the amino acid modifier. In the forward direction assay (aspartate $\beta$-semialdehyde + NADPH $\rightarrow$ homoserine + $NADP^+$) the $K_i$ value of threonine remained essentially unaltered at 0.5 mM in the pH range of 6.3–7.2 with and without 200 mM KCl (Datta, unpublished observations); the apparent $K_m$ values for homoserine in the reverse direction assay were also unaffected for the native as well as desensitized enzyme preparations in the presence or absence of high salt (Datta, 1971). The results summarized in Figure 7 show that the extent of aggregation in low threonine concentration may vary as much as tenfold depending on the exact pH and ionic strength of the enzyme solutions. We interpret these data to mean that the binding of threonine *per se* is not the ultimate event leading to the formation of enzyme polymers. In other words, in the presence of *low* threonine, the monomeric form of the enzyme can aggregate to form polymers *only* when other conditions such as pH and/or ionic strength are favorable for the aggregation process. For example, greater than 90% polymerization was seen at pH 7.1 when the ionic strength was increased to 0.13; at 0.08 ionic strength only 20% polymerization was detected. In the absence of threonine, enzyme polymerization is barely detectable under the above conditions. It is, therefore, highly likely that the primary event in the regulation of enzyme activity is an intramolecular conformational change, and that intermolecular interactions leading to enzyme aggregation follow this conformational alteration in the protein molecule. Recently Wampler (1972) has shown that the threonine-mediated aggregation state of the *Escherichia coli* aspartokinase I–homoserine dehydrogenase I is also unlikely to play a role in the metabolic regulation of this enzyme.

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