Homoserine Dehydrogenase of *Rhodospirillum rubrum*. Conformational Changes in the Presence of Substrates and Modifiers*

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ABSTRACT: Homoserine dehydrogenase of *Rhodospirillum rubrum* can exist in several different conformational states depending on the salt concentration, and on the interaction of certain substrates and feedback modifiers to the protein. On a Sephadex G-200 column equilibrated in 25 mM potassium phosphate, the enzyme exhibits a molecular weight of approximately 138,000; in the same buffer supplemented with 200 mM KCl, the apparent molecular weight is 210,000. Under the latter conditions, the enzyme shows enhanced stimulation of activity by L-isoleucine and a decreased sensitivity to L-threonine inhibition. In low salt (2 mM potassium phosphate or less), on the other hand, the enzyme dissociates into a species with an apparent molecular weight of 76,000 daltons. The dissociated form, although fully active, is completely desensitized with respect to the feedback modifiers; addition of KCl resensitizes the enzyme. The apparent K_m values for homoserine were essentially unchanged in the presence or absence of the salt. Titration of sulfhydryl groups with 5,5′-dithiobis-2-nitrobenzoic acid (DTNB) in the native enzyme reveals 1 SH group/138,000 g with no significant loss in enzyme activity; upon addition of L-threonine, an additional half-cystine residue reacts with DTNB with concomitant loss of catalytic activity. These results indicate a new state of the protein where a buried SH group, which is essential for enzyme activity, is exposed by the amino acid L-threonine. Since $NADP^+$ or homoserine protects the enzyme against inactivation by the thiol reagents, it is likely that the substrates prevent the threonine-induced conformational changes in the protein. In 200 mM KCl, approximately 2 moles of SH/138,000 g can be titrated with DTNB, however, under these conditions, the loss of enzyme activity is minimal.

The pyridine nucleotide coenzymes, but not the amino acid substrates, are also strong protecting ligands against inactivation of the enzyme by trypsin. The feedback modifiers, L-isoleucine and L-threonine, do not protect against trypsin inactivation.

We conclude from these studies that the *R. rubrum* homoserine dehydrogenase can assume various conformational states in the presence of bound ligands.

One of the more prominent features of regulatory enzymes is the allosteric sites which bind regulatory modifiers, and which are physically distinct from the substrate binding sites (Gerhart and Pardee, 1962; Changeux, 1963; Monod *et al.*, 1963). Thus, no direct interactions may occur between the substrate and the modifier molecules, and the effects may be translated through reversible conformational alterations induced in the protein when it binds the small molecules (Monod *et al.*, 1965; Koshland *et al.*, 1966). Homoserine dehydrogenase (L-homoserine:$NADP^+$ oxidoreductase, EC 1.1.1.3) of *Rhodospirillum rubrum* is an enzyme which catalyzes the reversible transformation of aspartate β-semialdehyde and homoserine in the presence of pyridine nucleotide coenzymes (Sturani *et al.*, 1963; Datta and Gest, 1965). The catalytic properties of the enzyme are significantly influenced by three amino acid modifiers: L-threonine, L-isoleucine, and L-cysteine (Sturani *et al.*, 1963; Datta and Gest, 1965; Datta, 1967). Furthermore, studies with partially purified enzyme have revealed that the feedback inhibitor L-threonine causes aggregation of the enzyme presumably to a dimer (Datta *et al.*, 1964). Recently, purified enzyme from *R. rubrum* became available (Datta, 1970) to study the effects of substrates and modifiers on the conformational changes in the protein. The results, reported here, show that the enzyme can exist in several distinct conformational states in the presence of salt, substrates, and certain feedback modifiers.

Experimental Section

Materials and Methods

Reagents. NAD^+, $NADP^+$, and the reduced coenzymes were obtained from P-L Laboratories or from Sigma Chemical Co. DTNB[1] and iodoacetamide were bought from Calbiochem. *N*-Ethylmaleimide and *p*-mercuribenzoate were obtained from Mann Research Chemicals. Purified yeast alcohol dehydrogenase and beef liver catalase were purchased from Worthington. Pig heart fumarase and horseradish peroxidase were kindly supplied by Drs. V. Massey and D. Hultquist, respectively. Trypsin, twice crystallized (pancreatic type I), and soybean trypsin inhibitor were supplied by Sigma. L-Homoserine and allo-free L-threonine and L-isoleucine were bought from Calbiochem, Nutritional Biochemical Corp., or Mann Research Chemicals. DL-Aspartate β-semialdehyde was prepared according to the method described by Black (1963). All other chemicals were of reagent grade.

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[1] Abbreviations used are: DTNB, 5,5′-dithiobis 2-nitrobenzoic acid; DTT, dithiothreitol; SDS, sodium deodecyl sulfate; Hse, L-homoserine; ASA, DL-aspartate β-semialdehyde.

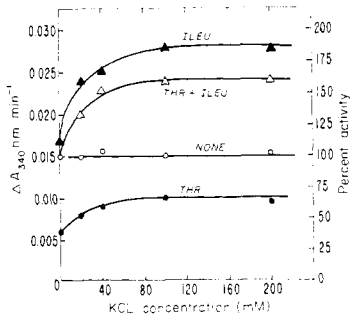

FIGURE 1: Effects of increasing concentrations of KCl in the assay mixtures on the activity of homoserine dehydrogenase in the presence and absence of various feedback modifiers. Enzyme activities (2 μg of protein) were assayed at pH 8.4 in the reverse direction (L-homoserine + $NADP^+$ → L-aspartate β-semialdehyde + NADPH) according to the procedure described (Datta, 1970), and are expressed as ΔA_{340} nm min^{-1}. Concentrations of amino acid modifiers were 10 mM each. Abbreviations are: Ile, L-isoleucine; Thr, L-threonine.

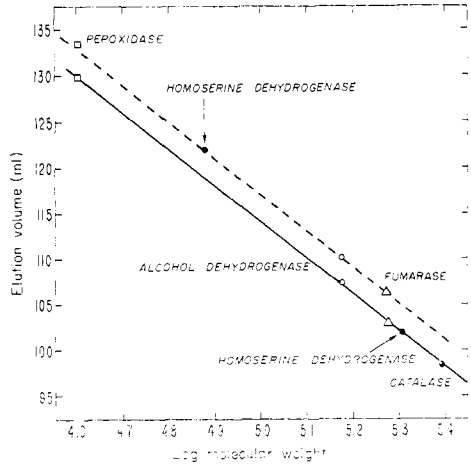

FIGURE 2: Gel filtration on Sephadex G-200 in the presence of high and low salt. Aliquots (1 ml) of a solution containing 0.3 mg of purified homoserine dehydrogenase and the reference proteins were layered on top of Sephadex G-200 columns equilibrated at 25°, either in 25 mM potassium phosphate buffer (pH 7.5), containing 1 mM each of EDTA and DTT, and 200 mM KCl (column 60 × 1.5 cm, solid line), or in 2 mM potassium phosphate buffer (pH 6.9), containing 1 mM EDTA and 0.5 mM DTT (column 62 × 1.5 cm, dashed line). The columns were eluted with the respective buffers and 1-ml fractions were collected. Activities of homoserine dehydrogenase as well as the other enzymes were assayed as described previously (Datta, 1970). The data are plotted according to Andrews (1964).

Enzyme Purification and Assay. Homoserine dehydrogenase was purified from the photosynthetic bacterium *Rhodospirillum rubrum* strain Hughes according tot he method described elsewhere (Datta, 1970). The specific activity of the purified enzyme was 58 μmoles of NADPH oxidized per min per mg of protein when assayed at 25° with DL-aspartate β-semialdehyde in 100 mM potassium phosphate buffer (pH 6.8). The detailed procedures for assays of homoserine dehydrogenase activity and other enzymes have been reported (Datta and Gest, 1965; Datta, 1970).

Other Methods. Protein was determined by the method of Lowry *et al.* (1951). The methods for Sephadex gel filtration and for titration of SH groups by DTNB have been described (Datta *et al.*, 1964; Datta, 1970).

Results

Effects of High-Salt Concentration. Unlike the enzyme isolated from *Escherichia coli* K12 (Patte *et al.*, 1963; Wampler and Westhead, 1968; Ogilvie *et al.*, 1969), the homoserine dehydrogenase of *R. rubrum* has no obligatory requirement for K^+ for activity; however, the stability of this enzyme is greatly influenced by several salts including KCl. In the standard mixture for the reverse direction assay (L-homoserine + $NADP^+$ → L-aspartate β-semialdehyde + NADPH), increasing concentrations of KCl up to 200 mM had no significant effect on the catalytic activity (Figure 1), however, these concentrations of KCl drastically increased the stimulation of activity by L-isoleucine; a maximum stimulation of about 80% was obtained at a KCl concentration of about 100 mM. The effect of increasing salt concentration on the extent of L-threonine inhibition was less dramatic; the inhibition due to L-threonine was reduced from 60% to about 35%. When both L-threonine and L-isoleucine were present simultaneously, the activity curve with increasing KCl concentrations was parallel to that found for L-isoleucine alone (Figure 1). One explanation for the effect of KCl on the modifier sensitivity is that the enzyme shows a different conformation in the presence of the salt.

In 25 mM potassium phosphate buffer (pH 7.5), containing 1 mM EDTA and 1 mM DTT, the peak of *R. rubrum* homoserine dehydrogenase activity was eluted from a Sephadex G-200 column slightly after yeast alcohol dehydrogenase; the calculated molecular weight from the gel filtration experiment is identical with the value of 138,000 daltons obtained from the sedimentation velocity centrifugation (Datta, 1970). However, the results presented in Figure 2 (solid line) show that the elution pattern of the dehydrogenase was significantly altered if the buffer for the gel filtration step was supplemented with 200 mM KCl; under these conditions the peak of the enzyme activity appeared slightly earlier than the peak of fumarase activity, an enzyme of known molecular weight of 190,000. From the relationships of V_e/V_0 *vs.* log molecular weight (Andrews, 1964), the apparent molecular weight of the KCl-treated enzyme was estimated to be about 210,000. Since the elution sequence of proteins during gel filtration through Sephadex G-200 is more strictly proportional to their Stokes' radii rather than on their molecular size (Siegel and Monty, 1966), it is likely that the apparent increase in the molecular weight of the homoserine dehydrogenase may simply reflect a change in the protein conformation resulting in an increased Stokes' radius. Alternatively, the displacement in the elution of the enzyme in buffer solution containing 200 mM KCl, may result from a rapid equilibrium established in solution between more than one molecular forms present in the high-salt buffer. It was reported earlier (Datta *et al.*, 1964) that the sedimentation rate of the enzyme in the presence of L-isoleucine through a linear sucrose gradient was faster than the sedimentation rate of the native enzyme in the absence of isoleucine; the elution profile through Sephadex G-200 was also displaced toward the exclusion volume by L-isoleucine. Furthermore, the dehydrogenase showed association–dissociation behavior in the presence of the feedback inhibitor, L-threonine (Datta *et al.*, 1964; Datta, 1970). These results, taken collectively, strongly suggest that the *R. rubrum* homoserine dehydrogenase can undergo drastic conformational changes, or reveal aggregation characteristics, when certain ligand molecules are bound on the protein. It is interesting that 100 mM KCl or 10 mM L-isoleucine,

TABLE I: Effects of High and Low Salt on the Apparent K_m Values for L-Homoserine.[a]

Enzyme	App K_m^H (mM)	App K_m^L (mM)
Native enzyme	3.3	0.2
Native enzyme plus 200 mM KCl	4.0	0.1
Desensitized enzyme	4.3	0.2
Desensitized enzyme plus 5 mM L-threonine	4.0	0.3

[a] Enzyme activities were measured as described previously (Datta, 1970) and the apparent K_m values, K_m^H and K_m^L (see Datta and Gest, 1965) were calculated from the double reciprocal plots of initial velocities and L-homoserine concentrations at saturating levels of $NADP^+$. Low-salt desensitized enzyme preparation was obtained by dialyzing the native enzyme (in 50 mM potassium phosphate buffer, pH 7.5) for 16 hr at 25° against 1000 volumes of 2 mM potassium phosphate buffer (pH 7.5), containing 1 mM each of EDTA and DTT. The densesitized enzyme was completely insensitive to 10 mM L-threonine in the standard assay mixture.

separately, did not significantly affect the enzyme activity, however, the presence of both ligands in the assay mixture showed a synergistic effect in stimulating enzyme activity (Figure 1).

Effects of Low-Salt Concentration. We have previously reported that the sensitivity of homoserine dehydrogenase of *R. rubrum* to modifiers is extremely dependent on the salt concentration (Datta and Gest, 1965). Enzyme solution in 1 mM potassium phosphate buffer (pH 7.5), containing 1 mM EDTA and 1 mM DTT was insensitive to L-threonine inhibition, and the activity was not stimulated by L-isoluecine; addition of 50 mM KCl restored modifier sensitivity (Datta and Gest, 1965). This reversible desensitization phenomenon appeared to be time dependent; several monovalent and divalent cations and anions significantly enhanced the rate and extent of resensitization of the modifier insensitive form (Mankovitz and Segal, 1969). From the kinetic data obtained with partially purified enzyme of *R. rubrum*, Mankovitz and Segal (1969) have postulated that the enzyme, desensitized in low salt concentrations, exists as half-molecules. Results of the gel filtration experiment with purified *R. rubrum* dehydrogenase, shown in Figure 2 (dashed line), provide the physical evidence that in 2 mM potassium phosphate-1 mM EDTA–0.5 mM DTT (pH 6.9) the enzyme exists as a smaller molecular weight species. A molecular weight of about 76,000 was estimated from the elution pattern. Since the native enzyme appears to have a molecular weight of approximately 138,000 as determined by the gel filtration on Sephadex G-200 and from the sedimentation velocity centrifugation experiments (Datta, 1970), a value of 76,000 for the low-salt desensitized enzyme is consistent with the interpretation that the modifier insensitive form is about one-half the molecular size of the native enzyme.

The result of the gel filtration experiment (see Figure 2) also indicated that the dissociated form of the homoserine dehydrogenase was enzymatically active. In this experiment the elution pattern of the dehydrogenase was located by assaying for enzyme activities of various fractions eluted from the Sephadex G-200 column in Tris-HCl buffer (pH 8.4) in reverse direction assay. Since the resensitization process was of the second order with respect to protein concentration and showed time dependency, and since the substrates homoserine and $NADP^+$ had no significant effect on resensitization (Mankovitz and Segal, 1969), the enzyme activity of the fractions corresponding to small molecular weight species cannot be attributed to reassociation of the half-molecules to form the native species. Furthermore, when the fractions were preincubated with L-threonine for various lengths of time and assayed in the presence of L-threonine which favors association (Mankovitz and Segal, 1969), a considerable lag was observed before the enzyme activity could be inhibited by the feedback inhibitor.

The enzyme which had been desensitized in buffer solutions of low ionic strength, especially with respect to K^+, showed loss of stability under certain conditions. Rapid inactivation of the enzyme occurred when incubated at 4° in 1 mM potassium phosphate buffer (pH 7.5), containing 1 mM EDTA and 1 mM DTT; the same enzyme solution retained much of its activity when stored at 25° (Datta and Gest, 1965). None of the substrates or amino acid modifiers had any protecting effect against cold inactivation of the enzyme in buffer solutions containing low concentrations of salt. We can conclude, therefore, that the dissociated molecules, which are catalytically active, may undergo further dissociation to give rise to enzymatically inactive subunits, or alternatively assume a conformation devoid of biological activity.

The substrate saturation kinetics of the low-salt desensitized enzyme preparations were not significantly different from that observed with the native enzyme. Table I summarizes the data obtained on the apparent K_m of homoserine at saturating concentrations of $NADP^+$. The same two K_m values (K_m^H and K_m^L; see, Datta and Gest, 1965) were comparable for both the native and desensitized enzyme preparations; addition of 5 mM L-threonine to the desensitized enzyme did not alter the two apparent K_m values for L-homoserine. No significant change in the K_m values of homoserine could be detected if the native enzyme was supplemented with 200 mM KCl. It must be emphasized that under the assay conditions, *i.e.*, no prior incubation, low enzyme concentration, and absence of salts which resensitize the desensitized enzyme (Mankovitz and Segal, 1969), it is highly unlikely that the enzyme in low-salt buffer reassociates during the short-term spectrophotometric assay.

Reactivity of Sulfhydryl Groups. Titration of sulfhydryl groups of the *R. rubrum* homoserine dehydrogenase with DTNB showed 1 reactive sulfhydryl group/138,000 g; in the presence of SDS, between 3 and 4 sulfhydryl groups per 138,000 g were reacted (Figure 3; also see Datta, 1970). Figure 3 shows the kinetics of DTNB titration of the native enzyme in 100 mM Tris-HCl buffer (pH 8.0) in the presence or absence of 1 mM $NADP^+$ or 5 mM L-threonine. In the presence of L-threonine, approximately 2 moles of SH/138,000 g was titrated indicating that the amino acid caused a sufficient change in the protein conformation to expose one of the "buried" half-cystine residue not available for titration in the native enzyme. Since L-threonine caused extensive irreversible polymerization of the dehydrogenase under conditions when oxidation of free sulfhydryl groups can occur (Datta, 1970), it is possible that the threonine-induced aggregation is due to interchain disulfide linkages of native protein molecules. In contrast to the *R. rubrum* enzyme, the purified homoserine dehydrogenase I–aspartokinase I complex of *E. coli* behaves differently in the presence

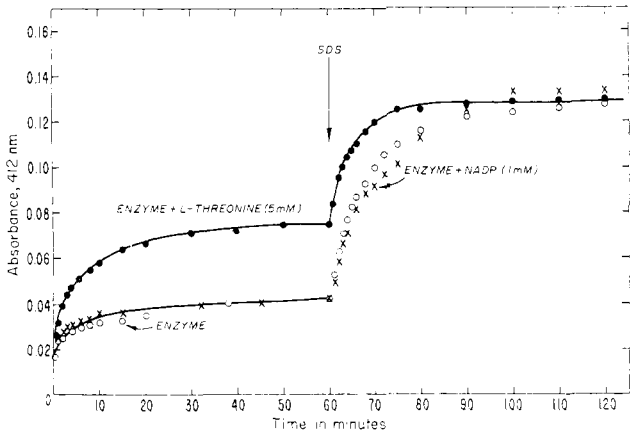

FIGURE 3: Titration of sulfhydryl groups with DTNB in the presence of various ligands. Samples of purified protein were added to a buffered solution, in a final volume of 1 ml, containing 100 μmoles of Tris-HCl buffer (pH 8.0), 1 μmole of EDTA, and $NADP^+$ and L-threonine as specified. The reaction was started with 0.05 ml of a freshly prepared solution of 1 mM DTNB in 10 mM potassium phosphate buffer (pH 7.2), containing 0.1 mM EDTA. The absorbances of the mixtures at 412 nm were recorded with time using a Zeiss PMQII spectrophotometer. A reagent blank was always included for each experiment which contained all components except protein. At 60 min, enough SDS in the Tris-HCl buffer was added to each mixture to bring the final concentration of SDS to 0.15%. Absorbances at 412 nm are plotted against time (in minutes) after correcting for the absorbances due to reagent blanks, and also for any dilution due to addition of SDS. The number of SH groups titrated was calculated according to the method of Ellman (1959), assuming a molecular weight of 138,000 g for the *R. rubrum* homoserine dehydrogenase (Datta, 1970). Protein concentration was 390 μg (2.8 mμmoles) for each experiment.

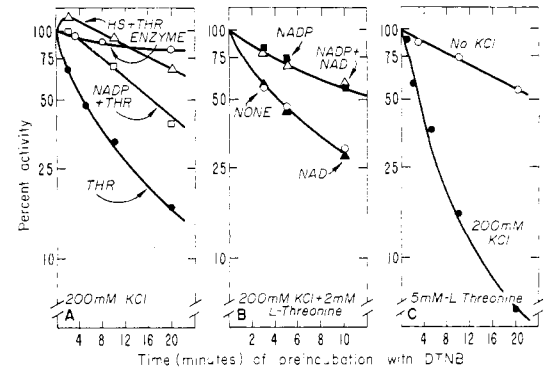

FIGURE 4: Effects of KCl, substrates, and the feedback modifier L-threonine, on the loss of homoserine dehydrogenase activity preincubated with DTNB. Samples of purified enzyme (8 μg of protein) were incubated with 20 μM (A,B) or 50 μM (C) DTNB in the buffered solution containing, in a final volume of 1 ml, the following components in micromoles: Tris-HCl, pH 8.4, 100; EDTA, 1; and wherever specified KCl, 200; $NADP^+$, 0.4; NAD^+, 0.4; L-homoserine, 10; L-threonine 2 (A,B) or 5 (C). At times indicated enzyme activities were assayed in the reverse direction assay (L-homoserine + $NADP^+$ $\rightarrow$ aspartate β-semialdehyde + NADPH) using the method described (Datta, 1970). Activities are expressed as per cent of zero-time control. All assays were linear at least for 2 min. Abbreviations are: HS, L-homoserine; Thr, L-threonine.

of L-threonine; with this protein, L-threonine protects the SH groups against DTNB titration that are otherwise available for reaction in the native form (Truffa-Bachi *et al.*, 1968, 1969).

The data presented in Figure 3 also show that $NADP^+$ had no effect on the DTNB titration. Addition of 0.15% SDS resulted in further reaction with DTNB in all three reaction mixtures, and in each case almost four sulfhydryl groups were titrated.

In a separate set of experiments when the enzyme solution was reacted with DTNB in 100 mM Tris-HCl buffer (pH 8.0), containing 1 mM EDTA and 200 mM KCl, 1.73 moles of DTNB was reacted per 138,000 g. It may be recalled that this concentration of KCl also caused the displacement of the elution profile of the enzyme toward the exclusion volume upon gel filtration on Sephadex G-200 (see Figure 2). This finding supports the general conclusion that the effect of high salt on the enzyme molecule is to provoke a conformational change or to cause association to a higher molecular weight species; under these conditions one more half-cystine residue becomes accessible for reaction with DTNB.

Inactivation of homoserine dehydrogenase upon treatment with DTNB, and several other sulfhydryl reagents, was examined by preincubating enzyme solutions at 25° with excess DTNB in 100 mM Tris-HCl buffer (pH 8.4), containing 1 mM EDTA. Wherever specified the incubation mixtures were supplemented with KCl, $NADP^+$, homoserine, and the amino acids at the concentrations indicated. Figure 4 shows the kinetics of enzyme inactivation under a variety of experimental conditions. In the presence of 2 mM L-threonine and 20 μM DTNB in Tris-HCl buffer containing 200 mM KCl, enzyme inactivation proceeded rapidly with a half-life of about 5 min, whereas, in the absence of threonine, 85% of the enzyme activity was retained after 20-min preincubation at 25° (Figure 4A). Homoserine and $NADP^+$, the substrates for the reverse direction assay, afforded significant protection against threonine-dependent DTNB inactivation of the enzyme. The data presented in Figure 4B clearly demonstrate that NAD^+, at a concentration equal to that of $NADP^+$, offered no protection from such loss of activity and did not interfere with $NADP^+$ in its stabilizing effect. Figure 4C shows the effect of 200 mM KCl on the loss of enzyme activity when the preincubation mixtures contained 5 mM L-threonine and 50 μM DTNB; the loss of enzyme activity was more rapid when KCl was present along with L-threonine.

The effects of *p*-mercuribenzoate, *N*-ethylmaleimide, and iodoacetamide on the enzyme inactivation are summarized in Table II. These results are consistent with the interpretation that threonine induces a change in the conformation of the enzyme which is more susceptible to inactivation by the sulfhydryl reagents. Furthermore, both homoserine and $NADP^+$ protect the enzyme against inactivation by the thiol reagents, presumably by stabilizing the native state of the protein. In view of the fact that two half-cystine residues are reacted with DTNB in the presence of L-threonine, as compared to approximately 1 mole of SH/mole of protein in the native enzyme (see Figure 3), and since the native enzyme is not significantly inactivated by the sulfhydryl reagents in the absence of threonine (Figure 4A; Table II), it is reasonable to conclude that one of the "buried" half-cystine residues plays a crucial part in the catalytic activity of the enzyme. Further experiments are required to establish the identity of the half-cystine residue located at the active center.

Susceptibility to Trypsin Digestion. One of several techniques which has been used frequently to study conformational changes in proteins exploits the differences observed in the susceptibility of the enzyme to limited proteolytic digestion in the presence and absence of ligands suspected

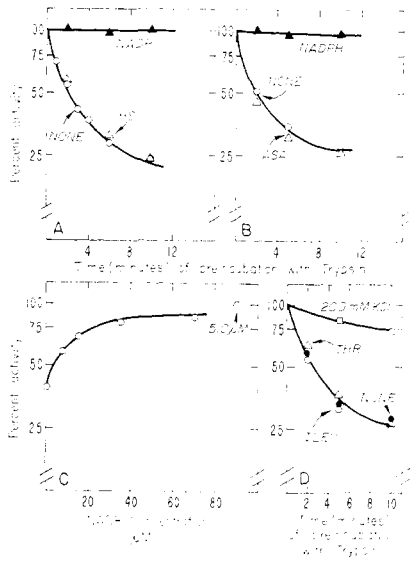

FIGURE 5: Effects of various ligands on the trypsin inactivation of homoserine dehydrogenase. Samples of purified enzyme (8 μg of protein) were incubated at 25° with 0.5 μg of trypsin in 0.8 ml of 100 mM Tris-HCl buffer (pH 8.4), supplemented with various ligands as indicated. At times specified by the symbols, a 200-fold excess (100 μg) of soybean trypsin inhibitor was added to each mixture, and the activities were determined as described previously (Datta, 1970). All assays were linear at least for 2 min and the activities are expressed as per cent of zero-time control. Control experiments revealed that simultaneous addition of trypsin and trypsin inhibitor did not influence the homoserine dehydrogenase activity. Furthermore, addition of various ligands at the concentrations specified did not have any effect on the hydrolytic property of the trypsin as judged from its ability to digest denatured bovine serum albumin. The concentrations of various components in the incubation mixtures were: $NADP^+$, 0.51 mM; L-homoserine, 10 mM; NADPH, 0.51 mM; DL-aspartate β-semialdehyde, 0.4 mM; L-threonine, 5 mM; L-isoleucine, 5 mM. In C, the preincubation mixtures were supplemented with increasing concentrations of $NADP^+$, and the trypsin digestion was continued for 3 min in all cases. Abbreviations are: HS, L-homoserine; ASA, DL-aspartate β-semialdehyde; Thr, L-threonine; Ileu, L-isoleucine.

TABLE II: Effects of Various Thiol Reagents on Homoserine Dehydrogenase Activity with and without L-Threonine.[a]

Preincubation Mixture	% Inactivation: Enzyme	% Inactivation: Enzyme + 2 mM L-Threonine
p-Mercuribenzoate	21	83
p-Mercuribenzoate plus $NADP^+$ (0.5 mM)		50
p-Mercuribenzoate plus L-homoserine (10 mM)		25
N-Ethylmaleimide	0	30
Iodoacetamide	5	35

[a] Enzyme solutions (8 μg of protein) in 100 mM Tris-HCl buffer (pH 8.4), containing 1 mM EDTA and 200 mM KCl, were incubated with the thiol reagents (see below) at 25° in the presence or absence of 2 mM L-threonine. In some cases the preincubation mixtures also contained $NADP^+$ or L-homoserine at the concentrations specified. Enzyme activities of the mixtures were assayed by the procedure described (Datta, 1970) and are expressed as per cent of zero-time control. The initial velocities were linear for at least 2 min. Preincubation conditions: *p*-mercuribenzoate, 100 μM, 8 min; *N*-ethylmaleimide, 20 μM, 60 min; iodoacetamide, 20 μM, 60 min.

to cause conformational changes (Rupley and Scheraga, 1963; Zito *et al.*, 1964; McClintock and Markus, 1968; Gerhart and Schachman, 1968). One advantage of this method is that partially purified enzyme may be used for these studies if the biological activity of the protein can be easily determined. In the present study, the effect of trypsin on the purified *R. rubrum* homoserine dehydrogenase was examined under a variety of experimental conditions. Figure 5A,B shows the kinetics of trypsin digestion in the presence and absence of $NADP^+$ and homoserine in the reverse direction assay, and with or without NADPH and aspartate β-semialdehyde in the forward direction assay. The data clearly show that, in both sets of assays, the pyridine nucleotide coenzymes were very strong protecting ligands against trypsin digestion; no protection was afforded by the amino acid substrate aspartate β-semialdehyde and homoserine. The degree of protection provided by $NADP^+$ against trypsin inactivation of the dehydrogenase can be estimated from the data given in Figure 5C. In the absence of $NADP^+$, the enzyme lost 60% of its activity when preincubated with trypsin for 3 min at 25° in 100 mM Tris-HCl buffer (pH 8.4), containing 1 mM EDTA; addition of increasing concentrations of $NADP^+$ in the preincubation mixture afforded increased protection, and at about 70 μM $NADP^+$ only 10% of the activity was lost due to trypsin digestion. Figure 5D shows that either L-threonine or L-isoleucine, the two feedback modifiers, failed to counteract trypsin digestion, however, 200 mM KCl had a significant effect on the trypsin sensitivity of the homoserine dehydrogenase. Control experiments, not documented here, revealed that the coenzymes, amino acid substrates, and high salt concentrations did not affect the hydrolytic property of the trypsin as judged from its ability to digest denatured bovine serum albumin under identical experimental conditions.

Discussion

The catalytic activity of an enzyme largely depends on the unique conformation it assumes during chemical transformation of the substrate to the product. A large body of information has been compiled on the conformational changes in enzymes due to specific interactions between substrates and modifiers from binding of these molecules on the protein (Datta *et al.*, 1964; Monod *et al.*, 1965; Koshland *et al.*, 1966; Gerhart and Schachman, 1968; McClintock and Markus, 1968; Koshland and Neet, 1968; Cohen, 1969; Karni-Katsadimas *et al.*, 1969). These data are particularly pertinent for those biosynthetic enzymes where the catalytic potential is greatly modulated by allosteric modifiers which are usually bound on the enzyme at different sites than that occupied by the substrates. Homoserine dehydrogenase is a key branch-point enzyme for the biosynthesis of methionine, threonine, and isoleucine, and appears to be an important control step for the control of biosynthesis of these amino acids (Datta, 1969). The enzyme from the photosynthetic bacterium *R. rubrum* shares many of the overall catalytic and regulatory characteristics with the dehydrogenases isolated from other microorganisms.

However, the physicochemical properties of the *R. rubrum* enzyme (Datta, 1970) is distinct from that reported for the two purified homoserine dehydrogenase–aspartokinase complexes isolated from *E. coli* (Cohen, 1969). It is, therefore, not surprising that large differences would exist insofar as the structure–function relationships are concerned.

Previous studies (Datta, 1970) have shown that in the native state the enzyme appears to have a molecular weight of about 138,000. In SDS, the protein dissociates into subunits each having a molecular weight of approximately 48,000. Between 3 and 4 half-cystine residues per enzyme molecule are reacted with DTNB in the presence of SDS, only one of which is accessible to the sulfhydryl reagent in the absence of this denaturing agent. The results presented here demonstrated several significant alterations in the protein structure in low or high salt concentration. In 200 mM KCl, although the catalytic activity remained essentially unaltered, the enzyme appeared to exist in a different form as compared to the native enzyme; the protein was slightly retarded on Sephadex G-200 (Figure 2) indicating either a large increase in the Stokes' radius or an association of the native enzyme to alter the molecular size. In the presence of the salt the enzyme showed enhanced stimulation in activity by L-isoleucine, and a decreased sensitivity to L-threonine inhibition (Figure 1). Further, an additional half-cystine residue was titratable in 200 mM KCl. At low salt concentration (2 mM potassium phosphate), on the other hand, the *R. rubrum* dehydrogenase dissociated into smaller molecular weight species of about 76,000 (Figure 2). The catalytic activity of this form was not changed drastically but the enzyme became cold sensitive. The dissociated form of the enzyme was also completely desensitized with respect to feedback modifiers; addition of KCl resensitized the enzyme (also *cf.* Datta and Gest, 1965). The latter results strongly suggest that the binding of the amino acid modifiers on the enzyme in its native form requires physical association of the dissociated species, a prediction made by Mankovitz and Segal (1969) from their data on the kinetics of reversible desensitization of the enzyme in the presence and absence of various salts.

Considering the changes in the association–dissociation states of the enzyme in high or low salt concentration, respectively, it is surprising that the apparent K_m values for homoserine remained essentially unaltered with various levels of KCl in the reaction mixture (Table I). Addition of L-threonine to the desensitized enzyme also had no effect on the apparent K_m for homoserine. Although we cannot unambiguously predict whether the two K_m values reflect two active sites per enzyme molecule, or one active and one activating site per molecule (Datta and Gest, 1965), it is obvious that upon dissociation of the native enzyme in low salt, or upon association of the enzyme in high salt, the two K_m components are still observed. In other words, salt-induced changes in the physical state of the enzyme generally do not interfere with the negative homotropic cooperativity (Levitzki and Koshland, 1969) between homoserine molecules; the presence or absence of heterotropic interactions between the substrate and the feedback modifiers, however, depends on the particular state of the protein stabilized in the high-salt or in the low-salt environment.

The experiments on the reactivity of free SH groups on the enzyme by various thiol reagents, especially DTNB, in the presence and absence of substrates and modifiers, clearly demonstrated some of the changes occurring in the protein due to binding of these small molecules. In the native enzyme only one SH group per molecule was titratable with DTNB, and the enzyme retained a large part of its activity after reacting with the sulfhydryl reagent (Figure 3 and Table II). Upon addition of L-threonine, an additional half-cystine residue reacted with the thiol reagents with concomitant loss of a large part of the catalytic activity; the results indicate a new state of the protein in which a "buried" sulfhydryl group, which is essential for enzymatic activity, is exposed. Since $NADP^+$ and homoserine, individually, protected the enzyme from the threonine-dependent inactivation by the thiol reagents, these molecules may counteract the threonine-induced conformational changes in the protein. In the presence of 200 mM KCl during titration of free SH groups, approximately 2 moles of SH/138,000 g was reacted with DTNB. However, loss of enzyme activity during 20-min preincubation with DTNB in the presence of 200 mM KCl was only 20% as compared to an 85% inactivation in the presence of KCl *and* L-threonine (Figure 4A). These data lead us to conclude that, although the *same number* of free SH are available for titration either with KCl or with L-threonine, these two ligands expose two *different* half-cystine residues on the enzyme molecule. The data plotted in Figure 4C show that the enzyme activity was lost more rapidly when both L-threonine and KCl were present during preincubation than when the preincubation mixture contained only L-threonine, indicating a synergistic effect of these ligands.

The protective effects of the pyridine nucleotide substrates and high-salt concentration against trypsin digestion (Figure 5) also support the notion that the homoserine dehydrogenase of *R. rubrum* exhibits conformational changes in the presence of these molecules. There is no *a priori* reason to believe that the changes in the protein structure due to binding of the amino acid modifiers are of the same nature as seen when pyridine nucleotides were present; in fact the experimental evidence indicate that they are not. The amino acids, L-threonine and L-isoleucine, did not protect the enzyme against trypsin inactivation (Figure 5D) whereas, L-threonine modified the protein structure sufficiently to expose a "buried" half-cystine residue (Figure 3). On the other hand, $NADP^+$ did not influence the kinetics nor the extent of DTNB titration of the enzyme; nevertheless, at very low concentrations, $NADP^+$ protected the enzyme from trypsin attack (Figure 5C). Since the two analytical methods, namely, titration of sulfhydryl groups with DTNB and the determination of the susceptibility to proteolytic digestion, demand significantly *different specificities* insofar as the protein structure is concerned, we can conclude that the new conformation states of the enzyme promoted by the interaction of the substrate molecules and by the amino acid modifiers are not due to *similar* structural alterations in the protein. A quantitative description of these changes in protein conformation will require further studies using sophisticated physical and chemical techniques.

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Inhibition of Tyrosine Aminotransferase Activity by L-3,4-Dihydroxyphenylalanine*

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ABSTRACT: Purified liver cytosol tyrosine aminotransferase was shown to be capable of transaminating 3,4-dihydroxyphenylalanine (DOPA). The ratios of activities of the enzyme to both tyrosine and DOPA remained constant throughout the purification procedure. A study of DOPA as an inhibitor of the enzyme was undertaken. DOPA, *m*-tyrosine, and other *m*-hydroxyphenylethylamines behave as noncompetitive inhibitors of tyrosine aminotransferase activity. Thus DOPA, *m*-tyrosine, and norepinephrine were noncompetitive inhibitors with respect to tyrosine concentration. The inhibitory activity could be ascribed to the formation of an inhibitor, the corresponding isoquinoline derivative of pyridoxal 5-phosphate. A number of aromatic amino acid derivatives were studied as inhibitors of tyrosine aminotransferase. The implications of these findings in patients treated with L-DOPA for the relief of Parkinsonism are discussed.

Analysis of brain tissue obtained at autopsy from patients afflicted with Parkinsonism revealed a marked decrease of dopamine content in the basal ganglia (Ehringer and Hornykiewicz, 1960; Hornykiewicz, 1962). The tentative hypothesis that defects in catecholamine biosynthesis is responsible for the symptoms associated with this disease is supported by a number of ancillary observations including the provocation of tremor by agents which decrease central nervous system catecholamine content (Hornykiewicz, 1966). Although no clear animal model exists which corresponds precisely to the human condition, lesions in certain areas of midbrain of monkeys lead to a decrease in dopamine content in the striatum and pallidum, and to the appearance of extrapyramidal disfunction (Poirier and Sourkes, 1965; Sourkes and Poirier, 1966). These studies have encouraged a rational therapeutic approach to the treatment of Parkinsonism based upon replacement of brain dopamine with the precursor amino acid DOPA.[1] While early reports of therapeutic attempts utilizing DOPA were conflicting (Birkmayer and Hornykiewicz, 1961; Barbeau, 1962; Gerstenbrand *et al.*, 1963; Fehling, 1966), more recently success has been claimed in controlling the disability utilizing carefully regulated dosages

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[1] The following abbreviations were used: DOPA, 3,4-dihydroxyphenylalanine; tyrosine aminotransferase, L-tyrosine:2-oxoglutarate aminotransferase (EC 2.6.1.5); *m*-tyrosine, 3-hydroxyphenylalanine.