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Homoserine Dehydrogenase of *Rhodospirillum rubrum*

Physical and Chemical Characterization

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A detailed physicochemical characterization of purified homoserine dehydrogenase of *Rhodospirillum rubrum* is presented. The enzyme has a molecular weight of 110000 and consists of two subunits of identical molecular weight of 55000. Depending on the ionic strength and protein concentration it is possible for the native enzyme to dimerize to produce an enzymatically active species of molecular weight 220000.

Titrations of the native and detergent-treated enzyme with a variety of sulfhydryl reagents show 2 mol free –SH groups per 110000 g, one of which is buried in the protein interior. L-Threonine and/or high concentrations of salt can expose the buried –SH group, and this –SH group is essential for the catalytic activity of the enzyme. Two independent lines of evidence show that extensive polymerization of the enzyme caused by L-threonine and/or high concentrations of salt does not involve the formation of intermolecular disulfide bonds.

Since its discovery in 1953 [1] the enzyme homoserine dehydrogenase, which catalyzes the pyridine-nucleotide-dependent interconversion of aspartate β-semialdehyde and homoserine, has been purified to homogeneity from *Escherichia coli* [2] and from the photosynthetic bacterium *Rhodospirillum rubrum* [3]. A preliminary characterization of some of the properties of the *R. rubrum* enzyme [3] and a comparison of these properties with the threonine-sensitive homoserine dehydrogenase I-/aspartokinase I from *E. coli* show that the two proteins are structurally quite dissimilar. This report is concerned with the physical and chemical characterization of the homoserine dehydrogenase from *R. rubrum* with special reference to molecular weight, subunit structure, and the role of sulfhydryl groups in the catalytic function as well as in the formation of enzyme aggregates.

MATERIALS AND METHODS

Materials

L-Homoserine and allo-free L-threonine were obtained from Calbiochem. DL-Aspartate β-semialdehyde was prepared according to the method of Black [4]. Ammonium sulfate (ultrapure) and *p*-chloromercuribenzoate were from Schwartz-Mann. The following reagents were purchased from Sigma Chemical Company: NAD^+, $NADP^+$ and their reduced derivatives, triethanolamine hydrochloride (99% purity), dithiothreitol, nitro blue tetrazolium, sodium dodecyl sulfate, glutaraldehyde and Triton X-100. The reagents for polyacrylamide gel electrophoresis as well as recrystallized guanidine hydrochloride were from Eastman Organic Chemicals. Dimethylsuberimidate was a product of Pierce Chemical Company. Phenazine methosulfate and 5,5′-dithio-bis(2-nitrobenzoate) were purchased from Calbiochem. Sephadex beads were products of Pharmacia, and ampholyne solution was obtained from LKB Instruments. Yeast alcohol dehydrogenase, bovine liver catalase and horseradish peroxidase were bought from Worthington, while ovalbumin (twice crystallized) was obtained from Schwartz-Mann. β-Galactosidase (*E. coli*) was a gift from Dr J. Distler; chymotrypsinogen A and cytochrome *c* (muscle) were from Dr L. DeFilippi. Crystalline bovine serum albumin was from Sigma. Iodo[1-^{14}C]acetate and *p*-chloromercuri[^{14}C]benzoate were brought from New England Nuclear and ICN Radiochemicals, respectively. All other chemicals were of reagent grade.

Enzyme Purification

Homoserine dehydrogenase was purified from *R. rubrum* strain SIH (ATCC 25903) essentially by the method described [3]. Since enzyme purified by

Abbreviation. Nbs_2, 5,5′-dithio-bis(2-nitrobenzoate).

Enzymes. Homoserine dehydrogenase (EC 1.1.1.3); yeast alcohol dehydrogenase (EC 1.1.1.1); bovine liver catalase (EC 1.11.1.6); horseradish peroxidase (EC 1.11.1.7); *E. coli* β-galactosidase (EC 3.2.1.23).

this procedure contained some aggregated material [3], the pooled fraction from step VI (see [3]) was passed through a Sephadex G-200 column equilibrated in 0.05 M potassium phosphate buffer, pH 7.5, containing 0.05 M KCl, 0.001 M EDTA, 0.007 M 2-mercaptoethanol and 0.001 M dithiothreitol. Removal of the aggregated protein conferred greater stability to the enzyme during storage at 4 °C. Purity of enzyme preparations was checked routinely by polyacrylamide gel electrophoresis (see Fig. 2).

Enzyme Assay

Homoserine dehydrogenase activity was measured spectrophotometrically both in forward and reverse directions as described earlier [3]. Enzyme activity on polyacrylamide gels was detected by the nitro blue tetrazolium assay [3].

Determination of Molecular Weights

The sedimentation equilibrium centrifugation, based on the meniscus depletion method of Yphantis [5], was carried out according to the manual of Chervanka [6]. A Beckmann model E analytical centrifuge equipped with Rayleigh interference optics, a top-loading Yphantis cell with a 12-mm light path and an AN-E rotor was used. Centrifugations were conducted at a rotor speed of 15000 rev/min at 20 °C and fringe patterns on photographic plates were read with a Nikon profile projector model 6C. A Hewlett-Packard 9810A calculator and a 9862A plotter were employed to analyze data using the Hewlett-Packard family regression package.

The molecular weight and Stokes' radius of homoserine dehydrogenase were determined on calibrated Sephadex G-200 columns according to the method of Siegel and Monty [7]; reference proteins were detected by their activities as described elsewhere [3,8].

The molecular weights of the two active species of homoserine dehydrogenase were established by the method of Hedrick and Smith [9] using 4–10% acrylamide gels in the standard buffer system of Brewer and Ashworth [10]; a calibration curve was constructed from reference proteins to determine the slopes.

Estimation of the size of the subunits of native, reduced, and alkylated enzymes was done by the method of Weber *et al.* [11] in sodium dodecyl sulfate/polyacrylamide gels. Alkylation by iodoacetate in guanidine hydrochloride was carried out as described in method 2 of Weber *et al.* [11]. For cross-linking of subunits, the enzyme was treated with varying concentrations of glutaraldehyde or dimethylsuberimidate as described by Richaud *et al.* [12] or Davies and Stark [13], respectively. Electrophoresis of cross-linked subunits was carried out in 5% sodium dodecyl sulfate/polyacrylamide gels in the borate/acetate buffer system [13]. Proteins were stained with Coomassie blue by the method of Fairbanks *et al.* [14].

Amino Acid Analysis and Sulfhydryl Titrations

Salt-free lyophilized enzyme, prepared by the method of Blackburn [15], was dissolved in glacial acetic acid, hydrolyzed in constant-boiling 6 M HCl at 110 °C for various lengths of time and analyzed on a Durrum amino acid analyzer. Duplicate 24-h acid hydrolyses were also performed on performic-acid-oxidized material. The amounts of tyrosine and tryptophan were determined by the spectrophotometric method of Bencze and Schmid [16].

Three independent methods were employed for the titration of sulfhydryl groups on the protein. Freshly purified and exhaustively dialyzed enzyme was reacted with Nbs_2 at 25 °C by the Ellman procedure [17] as described by Datta [3]. In a different method [18] enzyme solution was incubated with a 20–50-fold molar excess of *p*-chloromercuri[^{14}C]benzoate and the inactive enzyme was passed through a Sephadex G-50 column (56 × 0.8 cm). Inactive enzyme was located by assaying for enzyme activity with 0.0025 M dithiothreitol; peak tubes were pooled for protein and radioactivity determinations. For titration with iodoacetate the enzyme was reacted with iodo[^{14}C]acetate in 0.1 M Tris-HCl, pH 8.0, containing 0.001 M EDTA and either 2% dodecylsulfate or 5.5 M guanidine hydrochloride under anaerobic condition in the dark as recommended by Jones [19]. The samples were dialyzed exhaustively to remove unbound radioactivity, and duplicate samples were analyzed for protein and radioactivity. A molecular weight of 110000 was assumed to calculate the number of free sulfhydryl groups per molecule.

Isoelectric Focusing

Microisoelectric focusing in 7.5% polyacrylamide gels was performed by the method of Wrigley [20]. A pH-3–10 ampholyte was used and the buffers at the anode and cathode were 0.2% phosphoric acid and 0.4% ethylenediamine, respectively. Electrofocusing was carried out at 4 °C for 2.5 h at 340 V; gels were stained with 1% amido black. The pH gradient was determined by slicing duplicate gels (without protein) in 2-mm sections, and suspending them in H_2O for 6 h at 4 °C prior to pH measurement.

Other Methods

Protein was determined by the method of Lowry *et al.* [21] using bovine serum albumin as the standard.

The nitrogen content of the enzyme was determined by digesting a known amount of enzyme pro-

tein in 3.5 M H_2SO_4 and 30% H_2O_2 by the procedure of So and Goldstein [22] and estimating the amount of nitrogen with the ninhydrin reagent of Rosen [23].

An estimation of the dry weight of homoserine dehydrogenase was made by the modified biuret reaction as described by Janatova *et. al.* [24]. A calibration curve obtained with bovine serum albumin relating dry weights and the differences in absorbance at 390 nm and 290 nm (provided by Dr M.J. Hunter) was used to determine the dry weight of the sample.

Radioactivity was measured using a cocktail of Omnifluor (4 g/l of toluene) and Triton X-100 (7/3,v/v) in a Packard Tricarb spectrometer. A [^{14}C]toluene standard was used to determine counting efficiency.

RESULTS AND DISCUSSION

Molecular Weight and Subunit Composition

The calculated molecular weight of the *R. rubrum* homoserine dehydrogenase has been known to vary with ionic strength in gel filtration experiments [3,8,25] and the true molecular size could not be accurately determined from the sedimentation coefficient and diffusion constant [3] due to the presence of aggregated high-molecular-weight species in solution. In view of this, the enzyme was subjected to sedimentation equilibrium centrifugation by the meniscus depletion method of Yphantis [5]. At protein concentrations ranging from 78 to 234 μg/ml (in phosphate buffer, pH 7.5, $I = 0.138$ M), curved plots of $\gamma_b^2/2$ versus ln Δj were obtained which revealed molecular heterogeneity. An example of such a plot at a protein concentration of 78 μg/ml is shown in Fig. 1. The calculated molecular weights obtained from overlapping sections of the $\gamma_b^2/2$ versus ln Δj plot (Fig. 1) varied between 120000 and 216000 indicating a rapid equilibrium between more than one form of the enzyme in solution. The results of the gel filtration experiments in identical buffer composition but at lower protein concentrations confirmed this notion; as the initial protein concentration applied to the gel increased from 22 to 112 μg/ml, the Stokes' radius of the enzyme increased from 4 nm to 4.45 nm corresponding to approximate molecular weights of 115000 and 150000, respectively (Table 1). We may recall that a Stokes' radius of 4.2 nm was obtained during gel filtration in buffer of lower ionic strength ($I = 0.006$ M) at a protein concentration of 100 μg/ml [25].

Polyacrylamide gel electrophoresis of the purified enzyme in the pH-9.3 Tris/glycine buffer system [10] showed one major and one minor active species (Fig. 2). A plot of relative mobilities as a function of acrylamide concentrations by the procedure of Hedrick and Smith [9] revealed that the major and minor active bands had slopes of −7.3 and −11.3, respectively, corresponding

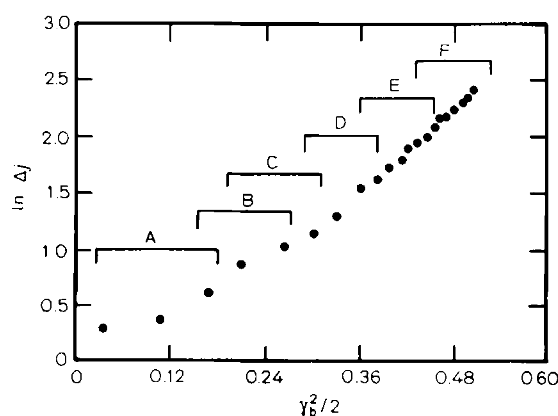


Fig. 1. *Sedimentation equilibrium centrifugation of homoserine dehydrogenase.* Purified enzyme dialyzed against 0.025 M potassium phosphate buffer, pH 7.5, containing 0.001 M EDTA, 0.001 M dithiothreitol and 0.05 M KCl ($I = 0.138$ M) was centrifuged at a rotor speed of 15000 rev./min at 20 °C. Protein concentration was 78 μg/ml. For other details, see Materials and Methods and [6]. Calculated molecular weights for the overlapping portions of the curve are as follows. (A) 120900, (B) 146600, (C) 159600, (D) 198400, (E) 207000, (F) 216000

Table 1. *Effect of protein concentration on the Stokes' radius and apparent molecular weight*

Enzyme solutions dialyzed against 0.025 M potassium phosphate buffer, pH 7.5, containing 0.05 M KCl, 0.001 M EDTA and 0.001 M dithiothreitol were applied on separate Sephadex G-200 columns (78 × 1.4 cm) equilibrated at 4 °C with the same buffer and previously calibrated with reference proteins. 1-ml fractions were collected at flow rates of about 5 ml/h. Fractions were assayed for enzyme activity in the forward direction assay. Calculation of Stokes' radius and molecular weights were done by the method of Siegel and Monty [7]

Initial protein concn	Stokes' radius	Apparent molecular weight
μg/ml	nm (Å)	
22	4.00 (40.0)	115000
30	4.10 (41.0)	120000
112	4.45 (44.5)	150000

to the molecular weight values of 106000 and 222000 as determined from a standard curve with several reference proteins. When the major active band was sliced off, homogenized, and subjected to re-electrophoresis, both bands were again observed. These findings strongly suggest that the molecular weight of the native homoserine dehydrogenase is about 110000; the higher value of 140000 estimated earlier [3,8] most likely reflected the average molecular weight of the monomer and dimeric form of the protein due to a rapid equilibrium between these species in solution. The results on the number and molecular weight of the enzyme subunits presented below also strengthens this conclusion.

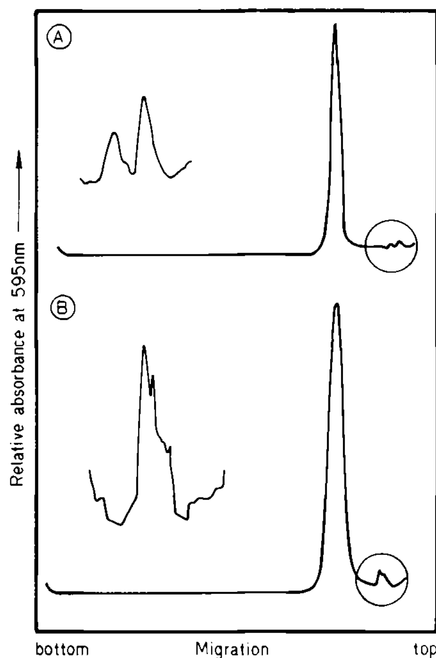
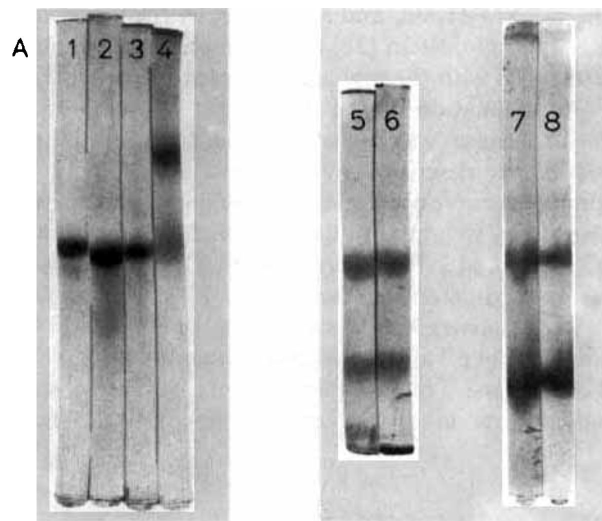


Fig. 2. *Densitometric scans of polyacrylamide gels.* Purified enzyme (25 µg each) was electrophoresed on two 7.5% polyacrylamide gels according to Brewer and Ashworth [10]. One gel was stained with Coomassie blue, and the other was stained for homoserine dehydrogenase activity as described elsewhere [3]. Densitometric scans at 595 nm of the gels stained for protein (A) and for enzyme activity (B) are shown. The inset in A shows the minor peaks (enclosed in circle) after 5-fold magnification. The inset in B shows the activity stain of one of the minor peaks (in circle) after 6-fold magnification



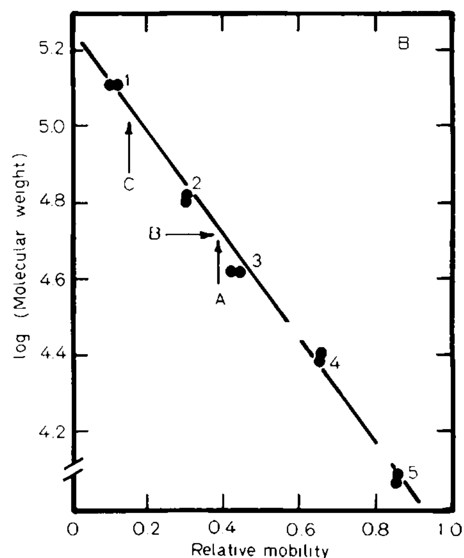


Fig. 3. *Sodium dodecyl sulfate polyacrylamide gel patterns of native and cross-linked proteins and estimation of their molecular size.* Purified homoserine dehydrogenase was subjected to various treatments and electrophoresed as described in Materials and Methods. (A) Photograph of gel patterns. Gel 1: 20 µg of reduced protein boiled for 5 min at 100 °C. Gel 2: 20 µg of native protein not subjected to reduction with 2-mercaptoethanol. Gel 3: 23 µg of reduced and alkylated protein (see [11]). Gel 4: 36 µg of protein cross-linked with 2% glutaraldehyde. Gels 5 and 6: each gel contained 40 µg of glutaraldehyde-cross-linked protein. Gels 7 and 8: 31 µg and 39 µg of protein, respectively, cross-linked with 2% dimethylsuberimidate. Enzyme samples applied on gels 5 and 7 were cross-linked under conditions favoring monomeric enzyme, while samples applied on gels 6 and 8 were cross-linked under conditions favoring polymeric enzyme (see text and [8]). (B) Determination of molecular weight on calibrated dodecylsulfate/polyacrylamide gel. 25 µg of standard proteins and one sample each of reduced (25 µg) and glutaraldehyde-cross-linked (40 µg) homoserine dehydrogenase were electrophoresed according to the procedure given in Materials and Methods. The data are plotted as log (molecular weight of the polypeptide chain) versus relative mobility. Standard proteins are: (1) β-galactosidase, (2) bovine serum albumin, (3) ovalbumin, (4) chymotrypsinogen A, (5) cytochrome *c*. The arrow labeled A represents the position of reduced enzyme. The arrows labeled B and C represent two forms of cross-linked enzyme, intra-subunit (or unreacted) and inter-subunit cross-linked species, respectively

The photograph in Fig. 3A shows that the native enzyme with and without reduction with 2-mercaptoethanol (gels 1 and 2, respectively), and the protein reduced and alkylated with iodoacetate (gel 3) migrated as single bands having identical relative mobilities; from a plot of several reference proteins (Fig. 3B), a subunit molecular weight of 53000 ± 5000 was calculated, which is a little higher than that of 48000 ± 5000 seen before [3].

Evidence for the number of subunits was sought by cross-linking enzyme subunits with glutaraldehyde and dimethylsuberimidate. As seen in gel 4 of Fig. 3A, cross-linking with glutaraldehyde yielded two bands; the molecular weights of these species were approximately 53000 and 106000 as calculated from the calibration curve shown in Fig. 3B. The gel patterns of enzyme treated with glutaraldehyde and dimethylsuberimidate under conditions favoring both monomer and polymeric forms of the enzyme [8] are shown in gels 5–8 (Fig. 3A). In all cases, only two protein species were observed; no estimation of molecular weights were made for the species obtained after treatment with dimethylsuberimidate. From the cumulative data presented thus far we conclude that the homoserine dehydrogenase of *R. rubrum* is a protein of molecular

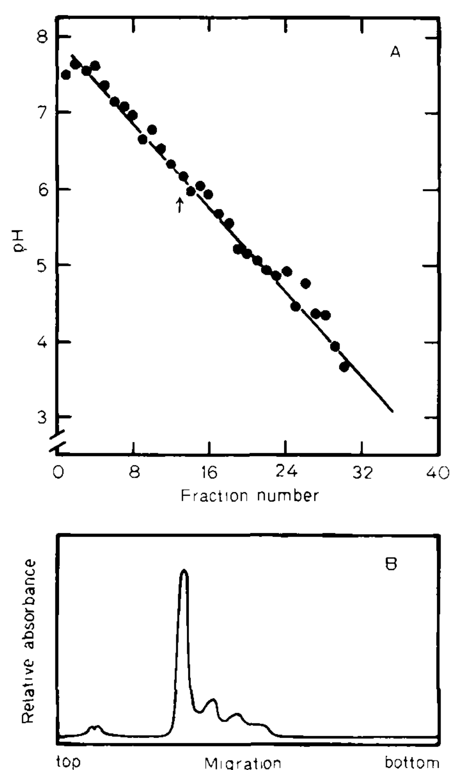


Fig. 4. *Determination of isoelectric point of homoserine dehydrogenase.* 25 μg of purified enzyme were subjected to electrofocusing on polyacrylamide gel as described in Materials and Methods. (A) A plot of pH versus fraction number (2-mm slice); the arrow indicates the position of homoserine dehydrogenase. (B) A densitometric scan of protein bands seen after electrofocusing and stained with 1% amido black

weight approximately 110000 and consists of two subunits of equal size having a molecular weight of 55000. Further, depending on the ionic strength and protein concentration, the native enzyme can dimerize to produce an active species with molecular weight 220000 (see Fig. 2).

Isoelectric Point

As determined on polyacrylamide gel, the isoelectric point of the *R. rubrum* enzyme is pH 6.1 (Fig. 4). The isoelectric points of three minor components are 5.7, 5.3 and 5.0. These species may have arisen from deamidation of asparagine and/or glutamine residues during electrofocusing, or alternately, due to aggregation of the enzyme at lower pH values [8] which led to altered pK_a values for the various amino acid side chains.

Amino Acid Composition

The amino acid composition of homoserine dehydrogenase is presented in Table 2. The number of residues were calculated from the average of duplicate 24-h and 72-h hydrolyses with the following exceptions: the values for valine and isoleucine were obtained from duplicate 72-h hydrolyses, whereas, those for threonine and serine were calculated from extrapolation to zero time of hydrolysis. The number of half-cystines were determined by performic acid oxidation of the protein [15] prior to acid hydrolysis. The number of tryptophan residues were obtained by the spectral method of Bencze and Schmid [16]; the ratio of tyrosine to tryptophan was 1.2. All calculations were based on the dry weight of the protein, and the recoveries during column chromatography were normalized with respect to norleucine added as internal standard during acid hydrolysis.

Table 2. *Amino acid composition of homoserine dehydrogenase*

Amino acid	Residues per 110000 g
Aspartic acid	83.0
Threonine	35.5
Serine	43.3
Glutamic acid	71.2
Proline	46.0
Glycine	95.2
Alanine	158.5
Valine	108.8
Methionine	19.5
Isoleucine	40.6
Leucine	91.0
Tyrosine	14.6
Phenylalanine	25.8
Histidine	21.9
Lysine	22.0
Arginine	72.6
Tryptophan	12.2
Half-cystine	6.7

The partial specific volume of the protein calculated from the amino acid composition according to the method of Schachman [26] was 0.734 cm^3/g; the mean residue weight was 107 as estimated by the method of Adler *et. al.* [27].

Titration of Sulfhydryl Groups

Previous experiments [3,25] on the number of −SH groups in the native enzyme indicated 1 mol −SH per 140000 g, whereas, in the buffer containing sodium dodecyl sulfate between 3 and 4 mol −SH per 140000 g were titrated; surprisingly, however, only 2 mol −SH per 140000 g were reacted in 5.4 M guanidine hydrochloride [3]. In view of the new data on the molecular weight of the native enzyme, we have carefully reexamined the number of −SH groups on the protein under a variety of experimental conditions. The data presented in Table 3 show that using Nbs_2 as the sulfhydryl reagent only 1 mol −SH per 110000 g is reacted in the native enzyme, and with both Nbs_2 and iodo[^{14}C]acetate a maximum of 2 mol − SH

Table 3. *Titration of sulfhydryl groups with Nbs_2 and Iodoacetate*
Freshly prepared enzyme dialyzed against 0.1 M Tris-HCl buffer, pH 8.0, containing 0.001 M EDTA was titrated at 25 °C with Nbs_2 [17] in the presence or absence of sodium dodecyl sulfate. Alternately, the enzyme was reacted with a 40-fold molar excess of iodo[^{14}C]acetate (specific activity, 12.6 Ci/mol) in the Tris buffer at 25 °C for 20 h under anaerobic condition in the dark with sodium dodecyl sulfate or guanidine hydrochloride. Protein concentrations ranged from 0.133 to 0.596 mg/ml per experiment. For experimental detail, see Materials and Methods

Sulfhydryl reagent	Denaturant	–SH/ 110000 g
		mol
	None	0.70
5,5′-Dithio-bis(2-nitrobenzoate)	0.5% dodecylsulfate	2.40
	2.0% dodecylsulfate	2.03
Iodoacetate	2.0% dodecylsulfate	2.06
	5.5 M guanidine · HCl	1.69

Table 4. *Titration of sulfhydryl groups in the presence of L-threonine and/or in high concentrations of salt*
Freshly prepared enzyme solutions were dialyzed against either 0.025 M potassium phosphate buffer, pH 7.5, containing 0.001 M EDTA or 0.1 M Tris-HCl buffer, pH 8.0, containing 0.001 M EDTA. Titrations with Nbs_2 in the above buffers with supplements as indicated were carried out as described in Materials and Methods. Enzyme concentrations varied from 0.07 to 0.45 mg/ml per experiment. Polymerized enzymes were prepared by passing native enzyme through Sephadex G-200 columns equilibrated with 0.1 M Tris-HCl buffer, 0.001 M EDTA, pH 7.5, supplemented with either 0.02 M L-threonine or 0.5 M NaCl and pooling the material eluted at the exclusion volume (see [8])

Enzyme	Supplements during titrations	–SH/110000 g in phosphate buffer	–SH/110000 g in Tris-HCl buffer
		mol	
	None	0.68	0.70
Native	L-threonine, 0.002 M	1.40	1.44, 1.50
	KCl, 0.2 M	1.45	1.39, 1.49
	L-threonine, 0.002 M + KCl, 0.2 M	1.64	1.43
Polymer	L-threonine, 0.002 M	–	0.55
	NaCl, 0.5 M	–	1.11

per 110000 g are seen under two different denaturing conditions.

Titrations of –SH groups with Nbs_2 in the presence of L-threonine or KCl, or both, show 1.4–1.6 mol –SH per 110000 g (Table 4) indicating that threonine and KCl exposed 'buried' –SH group that was not accessible in the native enzyme (*cf.* [25]). Significant variations in the reactivity of the sulfhydryl groups and a less than the theoretical yield of 2 mol –SH per 110000 g under these conditions may be explained by the fact that threonine and/or high salt

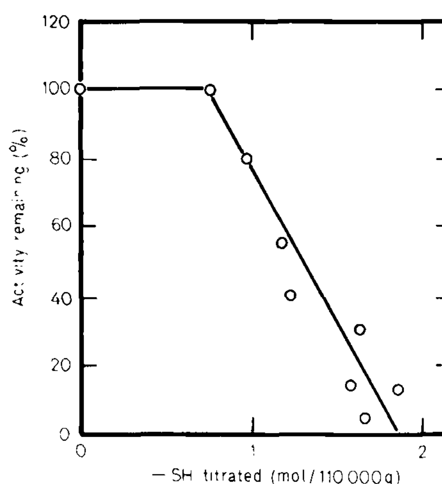


Fig. 5. *Residual enzyme activity as a function of the number of sulfhydryl groups titrated.* Aliquots of purified enzyme (0.85 μM) were incubated with increasing concentrations of Nbs_2 (0–10 μM, 0 to 5.9 molar equivalents assuming 2 mol –SH per 110000 g) at 25 °C for 2 h in 0.1 M Tris-HCl buffer, pH 8.0, containing 0.001 M EDTA, 0.002 M L-threonine and 0.2 M KCl. Following incubation, the numbers of –SH groups titrated were calculated from the absorbances at 412 nm (after correting for blanks), and the residual enzyme activities were determined. The data are plotted as residual enzyme activity as a function of the moles of –SH groups titrated per 110000 g of enzyme

concentration are known to cause extensive aggregation of the *R. rubrum* homoserine dehydrogenase [3,8,25] and, as shown in Table 4, that in the highly aggregated form, free –SH groups are not completely accessible to Nbs_2.

Additional evidence for the existence of 2 mol –SH /110000 g was obtained by titrating enzyme solution with increasing molar equivalents of Nbs_2. The data presented in Fig. 5 reveal that with increased molar equivalents of Nbs_2 in the presence of L-threonine and KCl, approximately 0.8 mol of –SH per mol of enzyme was titrated without any loss of catalytic activity; titration of about 2 mol –SH per 110000 g completely abolished enzyme activity. No further –SH group was titrated even at 6-fold molar excess of Nbs_2. These results are consistent with the previous observation [25] that only one of the two –SH groups is essential for catalytic activity of the enzyme.

The kinetics of enzyme inactivation by *p*-chloromercuribenzoate in the presence or absence of threonine and/or KCl are shown in Fig. 6. With 25-fold molar excess of *p*-chloromercuribenzoate, the native enzyme in phosphate buffer lost 50% of its activity in 3 h. L-Threonine increased the rate of inactivation by 25-fold, and with KCl a 6-fold increase in the inactivation rate was observed; a 50% loss in enzyme activity was observed in 3 min, when threonine and KCl were added together. Since threonine and/or high concentrations of salt exposed the –SH group normally unaccessible in the native enzyme, rapid loss

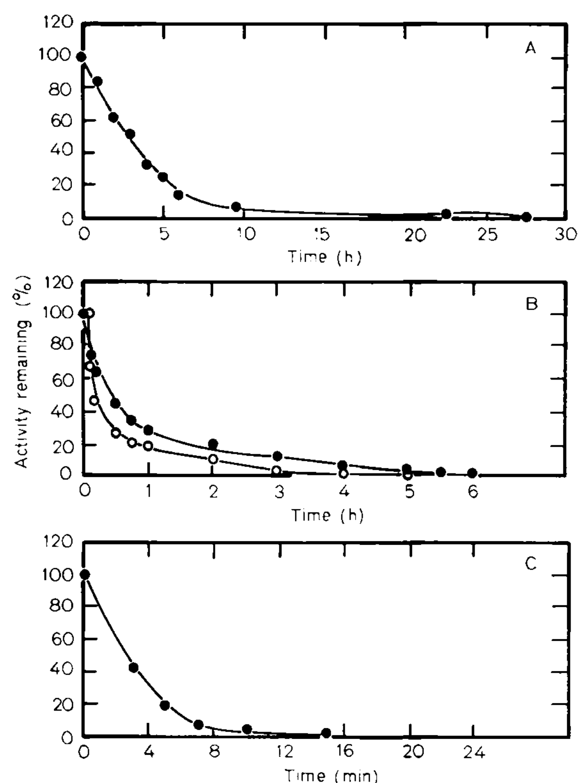


Fig. 6. *Kinetics of enzyme inactivation by* p-*chloromercuribenzoate.* Aliquots of purified enzyme (0.448 mg/ml) were incubated at 25 °C with 25-fold molar excess of *p*-chloromercuribenzoate in 0.025 M potassium phosphate buffer, pH 7.5, containing 0.001 M EDTA and other supplements as indicated. At specified intervals, samples were assayed for enzyme activity after appropriate dilution to reduce the concentration of *p*-chloromercuribenzoate below that which is inhibitory. The data are plotted as the percentage of enzyme activity (relative to zero time) remaining as a function of incubation time. (A) Incubated in buffer only. (B) Incubated in buffer supplemented with either 0.002 M L-threonine (-O O-) or 0.2 M KCl (●——●). (C) Incubated in buffer supplemented with 0.002 M L-threonine and 0.2 M KCl

of catalytic activity with threonine and/or KCl suggests that the 'buried' –SH group is necessary for enzyme function.

In a separate experiment, we analyzed the number of –SH groups titrated when more than 99% of the enzyme activity was lost in the absence or presence of threonine and/or KCl. Although the time required for 99% inactivation varied widely depending on the composition of the incubation mixtures (*cf.* Fig. 6), the amounts of mercuri[^{14}C]benzoate bound per mol of enzyme were 2.19, 2.23, 2.43 and 2.64 mol for the enzyme in buffer alone, buffer plus 0.002 M L-threonine, buffer plus 0.2 M KCl, and buffer plus 0.002 M L-threonine and 0.2 M KCl, respectively. Values higher than 2 mol of mercuri[^{14}C]benzoate bound per mol of enzyme, especially under conditions favoring enzyme aggregation, may be due to trapping of mercuribenzoate or by non-specific electrostatic interactions between the enzyme and positively charged mercury and the negatively charged carboxyl group of mercuribenzoate, see [18].

On the basis of short-term incubations lasting 20 min or less we had concluded earlier [25] that the native enzyme in phosphate buffer was resistant to inactivation by mercurials. However, the results presented above clearly indicate that, given enough time, once mercuribenzoate is bound to the 'surface' sulfhydryl group, the enzyme in buffer alone undergoes a slow conformational change exposing the 'buried' –SH group which is then able to react with the mercurial causing enzyme inactivation. This notion is supported by the observation [18] that drastic conformational changes occur due to binding of mercuribenzoate on the 'surface' –SH of the native protein as seen from the circular dichroism spectrum.

Enzyme Aggregation

The homoserine dehydrogenase from *R. rubrum* can polymerize extensively under a variety of experimental conditions [3,8,25,28]. Two observations which led to the speculation that enzyme aggregates may be covalently linked together through interchain disulfide bonds are: (a) both threonine and KCl exposed 'buried' –SH groups and caused aggregation of the enzyme, and (b) dithiothreitol appeared to decrease somewhat the extent of non-specific aggregation by either threonine or high concentrations of salt. Two types of experiments were undertaken to test this hypothesis by blocking the free –SH groups with sulfhydryl reagents prior to incubation of the enzyme with either threonine or KCl, and titration of free –SH groups of the aggregated enzyme.

Purified enzyme was incubated under anaerobic condition with 135-fold molar excess of iodo[^{14}C]-acetate in buffer containing 0.002 M L-threonine and 0.2 M KCl; under this condition the enzyme was completely inactivated indicating that both sulfhydryl groups were reacted with iodoacetate. Equal aliquots of the inactive enzyme were applied to separate Sephadex G-200 columns equilibrated in buffer alone, buffer plus 0.002 M L-threonine, or buffer containing 0.5 M KCl. The results depicted in Fig. 7 show that modification of the free –SH groups did not prevent enzyme aggregation by either threonine or KCl. Similar results were obtained when *p*-chloromercuribenzoate was used to block the two free –SH groups prior to treatment with threonine or KCl (not shown).

The above conclusion was confirmed independently by titration of free –SH groups of the enzyme polymerized by threonine or in high concentration of salt. For this experiment, polymeric forms of the enzyme were isolated by passing the native enzyme through Sephadex G-200 columns equilibrated with either 0.02 M L-threonine or 0.5 M NaCl and pooling the material eluted at the exclusion volume (see [8]).

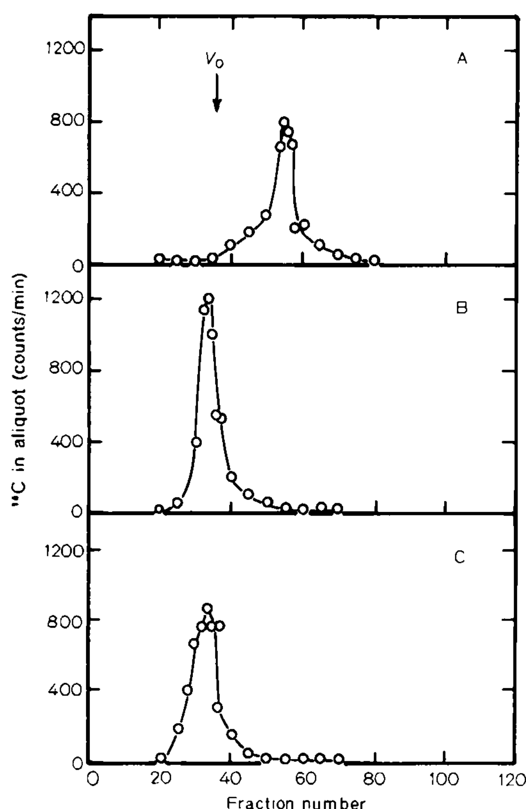


Fig 7. *Polymerization of enzyme by threonine and in high concentration of salt pretreated with iodoacetate to modify sulfhydryl groups.* Purified homoserine dehydrogenase (1.05 mg) was inactivated with iodo[^{14}C]acetate as described in the text. Aliquots of inactivated enzyme (229 µg) were applied to separate Sephadex G-200 columns (65 × 1.4 cm) equilibrated with 0.025 M potassium phosphate buffer, pH 7.5, containing 0.001 M EDTA (A) and supplemented with 0.002 M L-threonine (B) or 0.5 M KCl (C). The columns were eluted at 4 °C at 5 ml/h with the same buffers used for equilibration and 1-ml fractions were collected. 200-µl aliquots were counted for radioactivity to locate the protein peaks. The exclusion volume of the column was determined by blue dextran during equilibration and is marked with an arrow

Titration of the polymerized enzymes with Nbs_2 in the absence of a denaturant show between 0.6 and 1.1 mol of –SH per 110000 g (see Table 4, bottom lines) suggesting that only one of the two free –SH groups was accessible to Nbs_2. However, Nbs_2 titration in the presence of 0.5% sodium dodecyl sulfate revealed 1.99 and 2.49 mol of –SH per 110000 g for enzyme polymerized by threonine and NaCl, respectively. These data clearly show that both –SH groups were indeed free in the polymeric forms of the enzyme, and that enzyme aggregation does not involve the formation of disulfide bonds.

The results presented here on the physical and chemical properties of the homoserine dehydrogenase of *R. rubrum* clearly indicate that the enzyme is quite distinct from the two isoenzymes isolated from *E. coli*. The *R. rubrum* enzyme is also different from the *E. coli* enzymes insofar as the regulation of enzyme activity by the end-product amino acids threonine, methionine and isoleucine is concerned [29,30].

We may ask why is it that the enzymes from *R. rubrum* and *E. coli*, which exhibit the same biochemical and physiological functions, are structurally so divergent? It would be interesting to compare the amino acid sequence of the *R. rubrum* enzyme with that of the threonine-sensitive homoserine dehydrogenase I/aspartokinase I of *E. coli*, especially with the 55000-M_r piece from the carboxy terminus end of the bifunctional protein which posesses the homoserine dehydrogenase activity [31,32]. Since the subunit molecular weight of the *R. rubrum* enzyme is also 55000, we would not be surprised if a great deal of sequence homology does exist between these protein molecules indicating a common evolutionary origin.

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