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PURIFICATION AND PROPERTIES OF HOMOSERINE DEHYDROGENASE FROM *NEUROSPORA CRASSA*

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SUMMARY

1. Homoserine dehydrogenase (L-homoserine:$NADP^+$ oxidoreductase, EC 1.1.1.3) was purified a minimum of 800-fold from wild-type *Neurospora crassa*.

2. This enzyme is dependent on the presence of relatively high concentrations of certain ionic compounds for stability and for maximal activity.

3. The enzyme possesses simple Michaelis-type kinetics and is insensitive at any stage of purification to pathway end products threonine, methionine and isoleucine.

4. Growth of wild-type Neurospora on excess methionine and (or) threonine does not repress the synthesis of homoserine dehydrogenase. Growth of homoserine-less mutants (mutationally affected at the aspartokinase step in the pathway) on limiting supplement does not derepress the synthesis of this enzyme.

5. The enzyme has a molecular weight of 68 000 as determined by gel filtration.

INTRODUCTION

Homoserine dehydrogenase (L-homoserine:$NADP^+$ oxidoreductase, EC 1.1.1.3), the third enzyme in the conversion of aspartic acid to homoserine and thence into end products threonine, isoleucine and methionine, has been studied in a number of organisms, chiefly with emphasis on its regulatory role in the pathway. The present communication describes the purification and properties of this enzyme from the fungus *Neurospora crassa*, notably the absence of feedback inhibition of enzyme activity and repression of enzyme synthesis.

MATERIALS AND METHODS

Enzyme assay

The assays for both forward and reverse reactions consisted of measuring changes in absorbance at 340 nm. The standard forward reaction mixture contained 0.23 μmoles NADPH, 1 μmole L-aspartate β-semialdehyde (freshly neutralized with NH_4OH) and enzyme, and was run in 0.05 M potassium phosphate buffer (pH 6.8).

The standard reverse reaction mixture, run in 0.05 M 2-amino-2-(hydroxymethyl)-1,3-propanediol buffer (pH 9.1), contained 1.8 μmoles $NADP^+$, 21 μmoles L-homoserine, 0.67 mmole KCl and enzyme. Assay volumes were 1.0 ml and absorbance readings were performed using 1-ml cuvettes (1-cm light path) and a Gilford Model 240 or Beckman DBG spectrophotometer and a Beckman 10 inch recorder. After addition of enzyme the cuvettes were rapidly covered with a piece of Parafilm and inverted twice before placing them in the light path and activating the phototube. This process required approx. 10 sec. Rates of the reactions were linear except at high enzyme concentrations. Activities are expressed as change in absorbance per 0–5 min time interval. Specific activity is expressed as change in $A_{340\ nm}$ per min per mg protein.

Preparation of L-aspartate β-semialdehyde

L-Aspartate β-semialdehyde was synthesized by ozonolysis of L-allylglycine[1] and partially purified by chromatography on a Dowex-50 ion-exchange column. The concentration of the semialdehyde in the stock solution was determined with a Beckman Spinco amino acid analyzer and was found to be 64 μmoles/ml. Aspartic acid, also an ozonolysis product of allylglycine, was present in the stock solution at approximately the same concentration.

Disc electrophoresis

Polyacrylamide electrophoresis was carried out essentially as described by ORNSTEIN AND DAVIS[2] using a Canalco apparatus. Samples, in a maximum volume of 0.05 ml and containing sucrose to increase the density, were applied directly to the top of the 4 mm × 40 mm separating gel. The gels were electrophoresed for 45 min to remove excess persulfate before introduction of the sample. After addition of sample, the current was adjusted to 2.5 mA per column for 10 min and then 5 mA per column for 50 min. Staining for homoserine dehydrogenase activity in gels was done by incubating the gel for 1–5 min in the standard assay reaction mixture containing in addition 0.5 mg nitroblue tetrazolium per ml and then adding 0.1 mg phenazine methosulphate per ml. The reaction was stopped and the color fixed by removing the assay buffer and suspending the gels in 7% acetic acid.

Protein determination

Protein concentration was determined using the method of LOWRY *et al.*[3], or, in the presumed absence of interfering material, by measuring the absorbance at 280 nm.

Isolation of mutants

Homoserineless mutants were isolated from ultraviolet-irradiated conidia of *N. crassa* strain 74A by the filtration enrichment technique of WOODWARD *et al.*[4]. All such mutants used were found to possess greatly reduced (if not abolished) aspartokinase activity and to map in the first linkage group of the Neurospora genome.

Enzyme purification

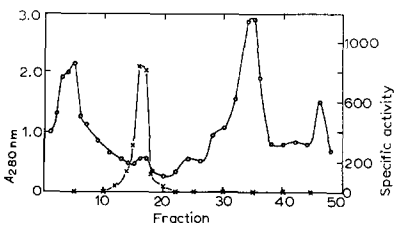
N. crassa strain 74A was grown in 20-l carboys in Vogel's medium[5]. A conidial suspension from a slant culture was used to inoculate 16 l of medium in each carboy, and after 48 h at 25° with vigorous aeration the mycelia were filtered with cheesecloth and washed quickly by suspending in a large volume of distilled water. Washed

mycelia were wrung free of excess water and frozen for storage. The yield of frozen mycelia was about 250 g per 16 l culture. All subsequent preparation was carried out at 0–5° unless noted. The pH values of all buffers are those at room temperature.

Step I—crude extract. The frozen mycelia were macerated in a mortar and pestle, mixed with 4 vol. (4 times the weight of mycelia) of 0.05 M potassium phosphate buffer (pH 6.8) containing 0.5 mM dithiothreitol and treated in a Waring blendor at top speed for 5 min. The resulting slurry was milled for 4 h in a ball mill with a volume of 3-mm glass beads equal to 4 times the weight of mycelium used. The mixture was vacuum filtered through cheesecloth and the resulting filtrate was centrifuged at 30 000 × *g* for 30 min to produce crude extract.

Step II—$(NH_4)_2SO_4$ treatment. To each liter of crude extract were slowly added 368 g solid $(NH_4)_2SO_4$ with stirring. The solution was immediately centrifuged at 10 000 × *g* for 10 min, and to each liter of supernatant were added 132 g $(NH_4)_2SO_4$. The solution was again centrifuged as before and the pellet, after rinsing with distilled water, was dissolved in 0.1 M Tris–HCl buffer (pH 7.2) containing 0.5 mM dithiothreitol. This fraction is referred to as the 60–80% $(NH_4)_2SO_4$ fraction. For application to DEAE-Sephadex this fraction was dialyzed 4 h against 40 vol. of column buffer.

Step III—DEAE-Sephadex. DEAE-Sephadex A-50 beads (Sigma Chemical Co.), particle size 40–120 μ, were equilibrated with 0.1 M Tris–HCl buffer (pH 7.2), containing 0.5 mM dithiothreitol. After several buffer changes the pH remained constant at pH 7.2. Gel columns 3.7 cm × 39 cm were prepared and 75 ml of the dialyzed $(NH_4)_2SO_4$ fraction were diluted with 2 vol. of buffer and applied to the column. Elution of enzyme was accomplished using an 800-ml linear KCl gradient, 0–0.13 M in the above buffer, at a flow rate of 4 ml/cm² per h. 10-ml fractions were collected, and the enzyme peak occurred at Fraction 55. No activity was detected outside this peak and elution of the remaining protein with 0.2 M KCl produced no activity. For application to the electrofocusing apparatus the three peak fractions



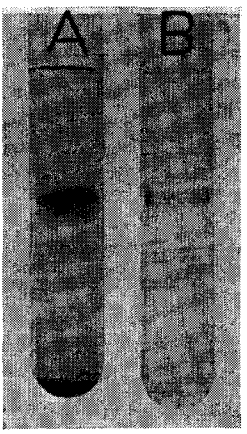


Fig. 1. Protein (○—○) and enzyme activity (×—×) obtained with electrofocusing (Step IV, *Enzyme purification*).

Fig. 2. Disc electrophoresis of material in electrofocusing Fraction 17. A. Protein. B. Enzyme activity. Direction of migration is from top to bottom. Gels were prepared as in MATERIALS AND METHODS. No enzyme activity band was present in a control gel run without homoserine.

from each column having the highest specific activity were salted out with 80% $(NH_4)_2SO_4$ and dissolved in a smaller volume of the above buffer.

Step IV—electrofocusing. Electrofocusing was performed using an LKB 110-ml electrofocusing column and a solution of LKB ampholytes. Techniques generally followed the LKB instrument manual. A pH gradient of pH 4.75–5.25 (1% ampholyte) was prepared from stock pH 4–6 ampholyte. All pH gradients were prepared containing a 0–50% (w/v) sucrose gradient and included 0.5 mM dithiothreitol. Gradients were prepared using a peristaltic pump. The enzyme peak fractions from three DEAE-Sephadex columns (precipitated with $(NH_4)_2SO_4$ as described above) were mixed with the light ampholyte solution and introduced into the electrofocusing column along with the gradient. The voltage from a constant-voltage power supply was increased stepwise from 200 to 750 V during the first 24 h and held at 750 V for an additional 30 h. Cooling solution, thermostated at 5°, was passed through the column cooling jacket. After the run the column was emptied at approx. 1.6 ml/min, and 1.6-ml fractions were collected. Fig. 1 is a profile of protein and enzyme activity obtained with electrofocusing. Fig. 2 gives an indication of the purity of the fractions of highest activity.

RESULTS

Effect of pH on reaction rates

The effects of varying pH on the forward and reverse reaction rates are depicted

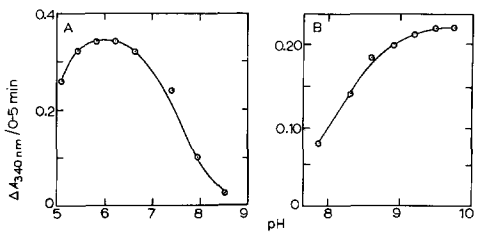


Fig. 3. Effect of pH on forward reaction (A) and reverse reaction (B). Reaction final pH values were determined with a glass combination electrode. Forward reactions were run in 0.1 M acetate buffer (pH 5.0–5.8), 0.1 M phosphate buffer (pH 5.8–7.0) and 0.1 M Tris buffer (pH 7.0–8.5). Reverse reactions were run in 0.1 M 2-amino-2-(hydroxymethyl)-1,3-propanediol buffer.

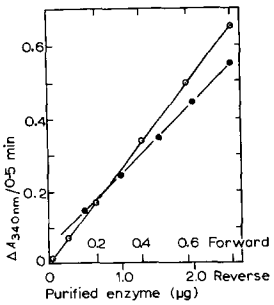
in Fig. 3. Buffer effects in the reverse reaction appear negligible as judged by the smooth curve obtained.

Effect of enzyme concentration

The effect of enzyme concentration on the reaction rates is shown in Fig. 4. At relatively high rates the reaction was not linear with time, and thus these higher rates were obtained by extrapolation of the nearly linear rate obtained during the first minute of reaction.

Effects of salt, β-mercaptoethanol

The reverse reaction was markedly enhanced by the presence of KCl as shown



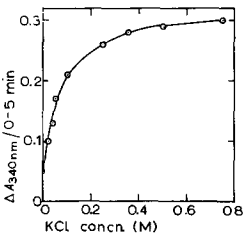


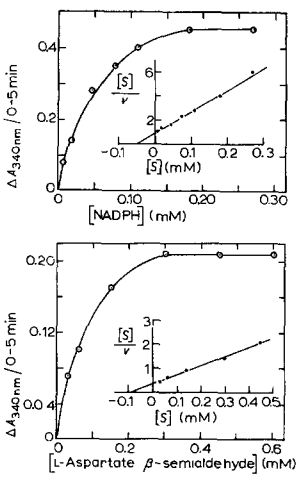
Fig. 4. Dependence of forward (●) and reverse (○) reaction rates on protein concentration. For reaction mixtures see MATERIALS AND METHODS.

Fig. 5. Effect of KCl on reverse reaction. The standard reaction mixture (see MATERIALS AND METHODS) was used with varying KCl.

in Fig. 5. Other salts, NaCl, NH_4Cl and $(NH_4)_2SO_4$ gave approximately the same effect of increasing the reaction rate 5-fold at 1 M concentration, whereas increasing the reaction buffer concentration to 0.5 M caused only a doubling of the rate. When the reaction was run in 0.1 M glycine *plus* 0.1 M buffer no increase in activity was noted.

The forward reaction, run in potassium phosphate buffer, was found to be much less susceptible to stimulation by salts. The standard assay buffer concentration of 0.05 M was optimal; lowering the buffer concentration to 0.01 M caused a 20% decrease in activity, and addition of KCl failed to stimulate the reaction above that observed in 0.05 M phosphate.

β-Mercaptoethanol at 1–7 mM was found not to affect the forward reaction



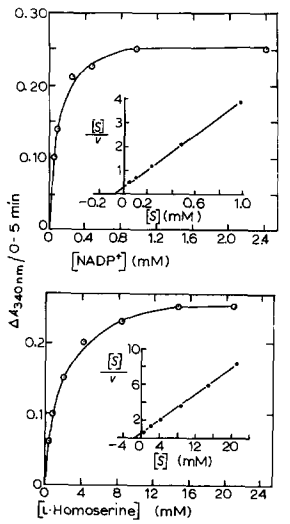


Fig. 6. Dependence of forward reaction rate on substrate concentration.

Fig. 7. Dependence of reverse reaction rate on substrate concentration. Standard reaction mixtures (see MATERIALS AND METHODS) were used except for the component varied.

rate, but 30 mM β-mercaptoethanol inhibited the reaction rate by 17%. At 1 mM, β-mercaptoethanol did not affect the reverse reaction.

Effects of substrate concentrations on reaction rates

The effects of varying substrate and coenzyme concentrations are shown in Figs. 6 and 7. The maximum rates shown were not affected by further addition of substrate or coenzyme. K_m values, determined graphically from these plots were as follows: aspartate semialdehyde, 0.1 mM; NADPH, 0.05 mM; L-homoserine, 1.5 mM; $NADP^+$, 0.07 mM. It is of interest that the Neurospora homoserine dehydrogenase possesses simple Michaelis–Menten kinetics, in contrast with the enzyme from *Rhodospirillum rubrum*[6] and *Zea mays* roots[7].

NAD^+ and NADH were also found to serve as coenzymes. A K_m of 5.5 mM for NAD^+ was observed, and the maximum velocity for NAD^+ was one half that for $NADP^+$. In the forward reaction a rectangular hyperbola was not obtained in plots of velocity *vs.* NADH concentration. The concentration of NADH providing maximum activity was approx. 0.35 mM, and this represented a very sharp optimal concentration; 0.45 mM NADH inhibited the reaction by 40%. Excess NADPH also inhibited the reverse reaction, but here the optimal concentration was much broader. The maximum rates of reaction with NADPH and NADH were approximately equal.

Tests with modifiers on activity

Homoserine dehydrogenase activity from *R. rubrum*[6], *Z. mays*[7], *Escherichia coli* (*e.g.* ref. 8) and yeast[9] has been shown to be affected by certain amino acids, notably by pathway end products threonine, isoleucine and methionine. The Neurospora enzyme was found to be unaffected at any stage of purity in forward or reverse direction with standard assay mixtures (reverse reaction in the presence or absence of KCl) by up to 15 mM L-threonine, L-methionine or L-isoleucine when these were added singly or in combination. When substrate concentrations were lowered to one half the standard assay values, inhibition by these amino acids was still absent (no detectable inhibition under standard assay conditions).

Stability studies

The enzyme was found to be markedly unstable in the absence of a relatively highly ionic environment, and highest stability was conferred only by certain ionic compounds. This stabilizing effect of various salts or other ionic compounds at relatively high concentration is shown in Table II. Fig. 8 details the effect of KCl and glycine in the dialysis solution. Tests with highly purified enzyme gave approximately identical results although the small quantity of purified material available precluded as detailed an experiment. As seen in Table II there is no clear pattern of the stabilizing effects of various compounds tested in the dialysis solution: some salts fail to protect the enzyme at high concentration (some increase instability) while some amino acids are less effective than others. Among the amino acids tested, those structurally similar to glycine gave best stability. The presence of dithiothreitol is generally required for maximum stability, but this stability is achieved only in the presence of some ionic compound such as glycine or alanine, NaCl or KCl. Indeed, with high concentrations of KCl (*e.g.* 0.67 M) no dithiothreitol is necessary.

Exhaustive dialysis at any stage of purity against a large volume of Tris buffer

TABLE I

PURIFICATION OF HOMOSERINE DEHYDROGENASE FROM NEUROSPORA
Procedure is described in the text.

Fraction	*Vol. (ml)*	*Protein (mg)*	*Specific activity ($\Delta A_{340\ nm}$ per min per mg protein)*	*Total units*
Crude extract	2760	19 300	1.25	24 100
$(NH_4)_2SO_4$	209	1 880	10.3	18 800
DEAE-Sephadex, peak fractions	90	258	30	7 750
Electrofocus, Fractions 16 and 17	3.2	3	820	2 460

at pH 7–8 or dilute phosphate buffer at lower pH values caused complete loss of activity. The activity on dialysis against 0.1 M Tris–HCl buffer (pH 7.2), containing 0.5 mM dithiothreitol was found to possess a half life of 7 h; after 36 h essentially no activity remained. The loss of activity after dialysis was found to be completely irreversible; once activity was lost, return to high salt concentrations failed to restore it. The same pattern of stability was obtained whether Tris or potassium phosphate buffer was used for the preparation of crude extract or whether the extract was prepared in 2 mM DL-threonine (threonine has been shown to affect the state of aggregation and the stability of homoserine dehydrogenase from some organisms[6,19]).

Passage of freshly prepared $(NH_4)_2SO_4$ fraction through long columns (1.7 cm × 26 cm) of Sephadex G-100 using 0.1 M Tris–HCl buffer (pH 7.2) containing 1 mM dithiothreitol destroyed all activity; the presence of 0.67 M KCl fully protected the activity during this treatment. Passage of the enzyme through short columns (1.7 cm × 5 cm) of Sephadex G-25, while not destroying activity outright, caused the enzyme to become cold labile.

The possibility that during dialysis the enzyme was deprived of some small molecule initially present in low amounts was tested by evaporating the dialysate to a small fraction of its original volume and adding widely varying amounts of this

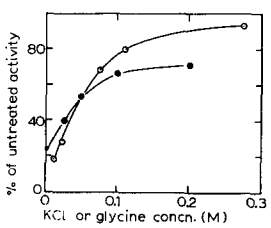


Fig. 8. Effect of KCl (○) and glycine (●) on stability of homoserine dehydrogenase. For the KCl curve a 60–80% satd. $(NH_4)_2SO_4$ fraction was passed through a Sephadex G-100 column (1.7 cm × 26 cm) using 0.1 M Tris–HCl buffer (pH 7.2) containing 0.67 M KCl. Aliquots of the resulting enzyme fraction were dialyzed several hours *vs.* 1000 vol. of standard buffer (see Table II) with external stirring. For the glycine curve aliquots of a 60–80% satd. $(NH_4)_2SO_4$ fraction were dialyzed 12 h *vs.* 1000 vol. standard buffer *plus* varying glycine (reagent grade glycine from Baker Chemical Co.). All sample volumes before and after dialysis were noted and used in correcting activity values for dilution during dialysis.

TABLE II

EFFECT OF DIALYSIS ON HOMOSERINE DEHYDROGENASE ACTIVITY

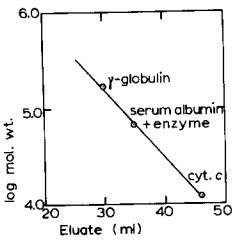
A small sample of 60–80% satd. $(NH_4)_2SO_4$ fraction (*cf. Enzyme purification*), containing 10–15 mg protein/ml was dialyzed 12–14 h at 5° against 1000 vol. of solution with rapid external stirring. The lower percentages of activity remaining after dialysis varied among experiments by about 10%, hence are given as "approx. 25%". Standard buffer: 0.1 M Tris–HCl (pH 7.2) containing 0.5 mM dithiothreitol.

Solution dialyzed against	*% of original activity remaining after dialysis*
Distilled water	0
Distilled water + 0.5 mM dithiothreitol + 0.2 M KCl	75
Distilled water + 0.5 mM dithiothreitol + 0.2 M NaCl	65
Distilled water + 0.5 mM dithiothreitol + 0.2 M glycine	52
0.01 M potassium phosphate buffer (pH 6.8)	25
0.2 M potassium phosphate buffer (pH 6.8)	83
Standard buffer *minus* dithiothreitol	10
Standard buffer *minus* dithiothreitol + 0.67 M KCl	100
Standard buffer	Approx. 25
Standard buffer + 1 mM EDTA	Approx. 25
Standard buffer + 1 mM $CaCl_2$	Approx. 25
Standard buffer + 1 mM $ZnSO_4$	Approx. 25
Standard buffer + 1 mM $MgSO_4$	Approx. 25
Standard buffer + 0.5 mM $NADP^+$	Approx. 25
Standard buffer + 1 mM L-homoserine	Approx. 25
Standard buffer + 0.25 M sucrose	Approx. 25
Standard buffer + 0.13 M $MgCl_2$	0
Standard buffer + 0.67 M KCl	100
Standard buffer + 0.67 M ammonium acetate	100
Standard buffer + 0.15 M $(NH_4)_2SO_4$	46
Standard buffer + 0.13 M glycine	70
Standard buffer + 0.13 M L-alanine	75
Standard buffer + 0.13 M β-alanine	78
Standard buffer + 0.13 M L-methionine	55
Standard buffer + 0.13 M L-isoleucine	33
Standard buffer + 0.1 M L-threonine	50
Standard buffer + 0.1 M L-tryptophan	23

concentrated dialysate to reaction mixtures containing dialysis-inactivated enzyme. No restoration of activity was obtained, therefore if a cofactor had been removed the loss of activity was irreversible. Furthermore, dialysis against cofactor concentrations of various metal ions or nicotinamide coenzyme (Table II) did not prevent loss of activity or restore it. Finally, the preparative steps involving extended treatment of the enzyme with electrofocusing techniques, where the protein was exposed to solutions of charged ampholytes for more than 2 days, yielded approx. 60% recovery of total enzyme units. We interpret these results to mean that highly ionic conditions are necessary for the stability of the enzyme.

Molecular weight determination

The molecular weight of homoserine dehydrogenase from Neurospora was de-



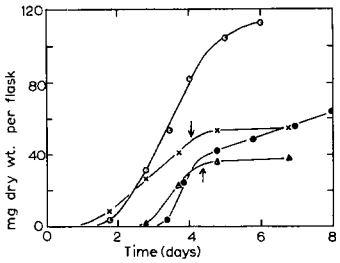


Fig. 9. Determination of molecular weight of Neurospora homoserine dehydrogenase by gel filtration. Sephadex G-200 was equilibrated with 0.1 M Tris–HCl buffer (pH 7.2) containing 0.5 mM dithiothreitol *plus* 0.2 M KCl. The gel column was 1 cm × 56 cm. The sample (0.5 ml in column buffer) consisted of 2 mg horse heart cytochrome *c* (Sigma Chemical Co.), 2 mg bovine serum albumin (Sigma), 2 mg bovine γ-globulin (obtained from Dr. R. Jenness) and purified homoserine dehydrogenase. Various combinations of these sample components gave identical elution patterns in different runs. Elution was performed at a flow rate of 3 ml/h and 1-ml fractions were collected. Approximate molecular weights of standards are cytochrome *c*, 12 400; serum albumin, 68 000; γ-globulin[11], 160 000.

Fig. 10. Growth response of homoserineless strain hs 20 to limiting homoserine, threonine and methionine. Stationary cultures were grown at 25° in 125-ml flasks containing 20 ml Vogel's medium. Each flask was inoculated with 1 drop of a visibly cloudy conidial suspension (approx. 0.05 mg dry wt.). Growth on optimal supplement, also approx. growth of wild type (○), growth on 0.8 mM L-homoserine (●), growth on 2 mM L-threonine *plus* 0.2 mM L-methionine (×), growth on 0.4 mM L-threonine *plus* 0.8 mM L-methionine (△). Arrows mark points at which harvest was made for enzyme level determinations (see Table III).

TABLE III

SPECIFIC ACTIVITY OF HOMOSERINE DEHYDROGENASE FROM WILD TYPE AND HOMOSERINELESS STRAINS GROWN UNDER VARIOUS CONDITIONS

Stationary cultures for enzyme extraction were grown at 25° in 1-l erlenmeyer flasks containing 100 ml of Vogel's medium. Inoculum consisted of 10 drops of a visibly turbid conidial suspension (approx. 0.5 mg dry wt.) per flask. Mycelial pads were harvested by freeing them of excess medium on a Buchner funnel and washed by rapidly suspending in water before a second vacuum filtration. The mycelia were then frozen for storage. Extracts were prepared by sand grinding mycelia in 0.05 M phosphate buffer (pH 6.8) containing 0.5 mM dithiothreitol, filtering through glass wool and centrifuging at 20 000 X *g* for 20 min. The reproducibility of figures is approx. 10% based on duplicate growth and extraction procedures.

Strain	*Time of growth (days)*	*Supplement to minimal medium*	*Specific activity*
74A	2.5	None	1.9
74A	4	None	1.2
74A	2.5	L-Threonine, 12 mM L-Methionine, 4 mM	2.0
hs 1, 10, 11, 20, 22, 25	4	L-Homoserine, 0.4 mM*	1.2–1.6
hs 20	4.1	L-Threonine, 2.0 mM L-Methionine, 0.2 mM*	1.4
hs 20	4.4	L-Threonine, 0.4 mM* L-Methionine, 0.7 mM	1.3

* Limiting concn. (see Fig. 10).

termined by gel filtration[10] as shown in Fig. 9. An approximate molecular weight of 68 000 was found for the enzyme by this method, facilitated by the elution of the enzyme and serum albumin in the same fraction from the gel column.

Enzyme repression studies

Attempts were made to repress and to derepress the synthesis of homoserine dehydrogenase by growing wild type and mutant strains under a variety of conditions which might be expected to alter the enzyme level[12–14] if production of the enzyme were influenced by intracellular end products of the pathway, threonine and (or) methionine and isoleucine. Fig. 10 shows the growth response of one homoserineless isolate, strain hs 20, to limiting concentrations of homoserine, threonine, and methionine. Table III shows that the enzyme level is not changed significantly in cells grown on limiting or excess supplements. The specific activity falls from about 2.0 early in the growth curve to about 1.3 at later times. When homoserineless mutants were grown on low levels of homoserine in aerated culture until the suppiement was depleted the specific activity was again 1.2, identical to wild type. The levels of threonine, methionine and isoleucine in the free amino acid pool of Neurospora have been shown to possess a positive correlation with the level of homoserine in the growth medium[15]. From these results we conclude that the homoserine dehydrogenase level in Neurospora is not subject to control by end products of the pathway, in contrast with the situation in bacteria[8] and yeast[9].

DISCUSSION

The absence in Neurospora of the multiple species of homoserine dehydrogenase which have been found in *E. coli*[8,16] is indicated by (a) the fact that the same single enzyme band appears in polyacrylamide gels of crude and purified preparations and (b) crude preparations are insensitive to feedback inhibition by threonine, methionine and isoleucine. The presence of differentially controlled isozymes at early steps in branched biosynthetic pathways is interpreted as a device for permitting greater flexibility in control over these early steps by pathway end products. In Neurospora such isozymes have thus far been discovered in the aromatic amino acid pathway[17]. From the present studies the homoserine dehydrogenase step in Neurospora seems not to be subject to end product control. This represents a departure from the situation in other organisms[6–9,18] where the enzyme is a focal point in feedback inhibition or repression. The mode of control in Neurospora is thus possibly solely at the first step (aspartokinase) in the biosynthesis of the four-carbon amino acids.

Homoserine dehydrogenase from Neurospora possesses in general somewhat similar features to the enzyme from some other species. There are major differences, however. Thus, K^+ was found to protect the *E. coli* enzyme[19,20] and the *R. rubrum* enzyme[6] from inactivation on standing in buffer. In the case of the Neurospora enzyme a stabilizing effect was found to be produced by various other salts (though KCl is the most effective of those tested) and by some amino acids, especially glycine or structurally similar amino acids. Furthermore, the enzyme from *R. rubrum* showed little activity when cell-free extracts were prepared in Tris buffer. We have found this not to be the case with the Neurospora enzyme, nor does K^+ increase the stability of this enzyme to higher temperatures as reported for the *R. rubrum* enzyme. Threonine

does not produce the degree of stabilization noted with the *E. coli* enzyme[20]. The reverse reaction of the Neurospora enzyme is strongly stimulated not only by KCl but by other salts as well, whereas Na^+ is inhibitory for the *E. coli* enzyme. We interpret these results with the Neurospora enzyme to indicate the presence of relatively non-specific salt effects on this protein, although a continuation of these studies will be necessary to produce a fully adequate explanation of the results presented.

Some of the reported properties of homoserine dehydrogenase from other organisms vary considerably in spite of the general inhibition of activity by threonine. Thus, the presence of K^+ has been demonstrated to be necessary for inhibition of the *R. rubrum* enzyme by threonine whereas the *Z. mays* enzyme was desensitized by incubation with a high concentration of potassium phosphate buffer[7].

No claim for purity can be made regarding the enzyme obtained in the present studies. The protein obviously approaches homogeneity but, as seen in Fig. 2, there are other, enzymatically inactive proteins present. These contaminants (identified by their presence in large amounts in the less pure fractions) appear to constitute about one third of the total protein in the enzymatically purest fraction. This figure is about what one would expect from the specific activity obtained, since biosynthetic enzymes from wild type strains of microorganisms commonly require a purification of 1000–2000-fold before homogeneity is reached. Of interest in this regard is the enormous purification achieved with the electrofocusing technique. For suitable enzymes this technique should prove extremely valuable for purification.

Although the molecular weight of homoserine dehydrogenase from other organisms has not been determined (aside from *E. coli* where it exists as a complex protein in conjunction with aspartokinase[21]) the *R. rubrum* enzyme has been reported[22] to be excluded from Sephadex G-100 and would hence be expected to have a significantly higher molecular weight than the 68 000 found for the Neurospora enzyme.

The evolution of the control over this pathway by end products and other effectors is of considerable interest in view of the diverse characteristics of this and other enzymes of the pathway among different species of organisms. From the collective data presently available from several laboratories we find no predictive power with which to explain why the different types of homoserine dehydrogenase have envolved. Further studies aimed at determining structural and functional comparisons between homoserine dehydrogenases from various organisms should therefore be a feasible undertaking.

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