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PROPERTIES OF HOMOSERINE DEHYDROGENASE IN A THERMOPHILIC BACTERIUM

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SUMMARY

A highly thermostable homoserine dehydrogenase (L-homoserine $NADP^+$ oxidoreductase, EC 1.1.1.3) from a thermophilic organism is described. Maximal activity was obtained at 70 °C and at a pH of 9.8; K^+ was essential for activity. The enzyme was feedback inhibited by L-threonine, the inhibition being of a competitive type. The feedback inhibition was not reversed by isoleucine or methionine, nor was it affected by heat (up to 70 °C). Addition of either L-threonine or L-methionine repressed the synthesis of the enzyme and a stronger effect was obtained by a combination of both amino acids.

INTRODUCTION

L-Homoserine is the branch point precursor in the synthesis of methionine, threonine and isoleucine[1]. Thus, the enzyme homoserine dehydrogenase (L-homoserine $NADP^+$ oxidoreductase, EC 1.1.1.3), which catalyzes the interconversion of L-aspartyl-β-semialdehyde to homoserine, in the presence of pyridine nucleotide coenzymes[2,3], might be subjected to regulatory control by the several end-products made from homoserine. In the various microorganisms examined thus far, L-threonine is a potent feedback inhibitor of the enzymatic activity. However, while in *Rhodospirillum rubrum*[2] the inhibition is competitively overcome by homoserine, in *Escherichia coli*[4] it is non-competitive. On the other hand, in the yeast *Saccharomyces cerevisiae* L-methionine feedback inhibits homoserine dehydrogenase activity[5], while in various bacteria no such effect was seen. In the preceding paper[6], the regulatory properties of the aspartokinase from a thermophilic bacterium was demonstrated. This report deals with the properties of L-homoserine dehydrogenase, another key enzyme of the aspartate pathway, obtained from the thermophilic microorganism.

METHODS

Cells were grown, harvested and the cell-free extracts prepared, as described previously[6].

Enzyme purification

To the cell-free extract 10% streptomycin was added and the precipitate formed was removed by centrifugation at 30 000 × *g* for 20 min. Solid $(NH_4)_2SO_4$ was added to the supernatant fluid to achieve 50% saturation. After stirring for 30 min, the suspension was left for additional 30 min without stirring and the precipitate formed was removed at 30 000 × *g* for 20 min. More solid $(NH_4)_2SO_4$ was added to the supernatant to obtain 85% saturation and the suspension was centrifuged, as above. The precipitate, which contained most of the activity, was dissolved in a small volume of 10 mM Tris buffer (pH 8.0) containing 10 mM mercaptoethanol; it was dialyzed at 4 °C for 18 h against 500 vol. of the same buffer. The dialyzed solution was applied to a DEAE-cellulose column equilibrated with 10 mM Tris buffer (pH 8.0) containing 10 mM mercaptoethanol. The activity was eluted from the column with a linear gradient of KCl from 0.2 to 0.6 M in the above buffer; 4.5-ml fractions were collected.

Enzyme assay

The homoserine dehydrogenase activity was assayed by measuring spectrophotometrically at 340 nm the NADPH formed according to the following equation: homoserine + $NADP^+$ → aspartyl-β-semialdehyde + NADPH. The reaction mixture consisted of 100 mM Tris–HCl buffer (pH 9.0), 10 mM $MgCl_2$, 80 mM KCl, 0.4 mM $NADP^+$, 20 mM L-homoserine, and rate limiting concentrations of the enzyme; the final volume of the reaction mixture was 1.0 ml. Incubation at 55 °C unless otherwise stated.

Aspartokinase activity was measured as described previously[6].

Protein was determined by the method of Lowry *et al.*[7].

RESULTS

Properties of homoserine dehydrogenase

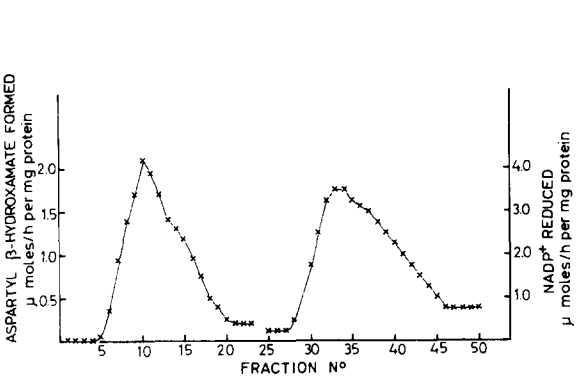
Since homoserine dehydrogenase and aspartokinase activities are sometimes carried by the same protein[4,8] it was of interest to find out about the properties of the homoserine dehydrogenase from the thermophilic organism. The results of Table I show that short exposure to trypsin destroyed all homoserine dehydrogenase activity, while aspartokinase activity remained intact. Thus, indirect evidence for separate enzymatic activities could be postulated. This assumption was indeed con-

TABLE I

EFFECT OF TRYPSIN ON HOMOSERINE DEHYDROGENASE AND ASPARTOKINASE ACTIVITIES

The crude extract was incubated with trypsin (0.5 mg/mg protein) for 5 min at 37 °C, then trypsin inhibitor (1 mg/mg protein) was added. In the control (0 time), the trypsin inhibitor was added prior to the addition of trypsin.

Trypsin treatment (min)	*Aspartokinase activity (Aspartyl-β-hydroxamate formed, μmoles/mg protein per h)*	*Homoserine dehydrogenase activity (NADPH formed, μmoles/mg protein per h)*
0	2.5	1.8
5	2.5	0



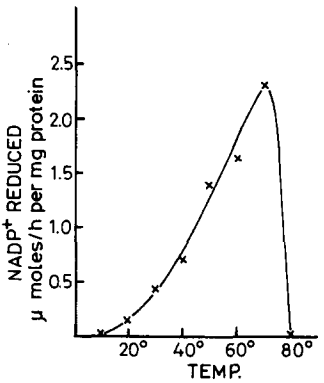


Fig. 1. Separation between aspartokinase and homoserine dehydrogenase activities on a DEAE-cellulose column. A 15 ml sample of the fraction precipitated with $(NH_4)_2SO_4$ (between 50–85% saturation) was applied to a DEAE-cellulose column, previously equilibrated with 10 mM Tris buffer (pH 8.0) containing 1 mM β-mercaptoethanol. The activities were eluted from the column with a linear gradient of KCl (from 0.2 to 0.6 M) in the above buffer. A total of 67 fractions, 4.5 ml each, were collected. Aspartokinase and homoserine dehydrogenase activities were measured as described under Methods.

Fig. 2. Effect of temperature on homoserine dehydrogenase activity. The reaction mixture was equilibrated for 3 min at the temperature indicated, $NADP^+$ was added and formation of NADPH was followed for additional 5 min.

firmed, as the homoserine dehydrogenase activity could be separated from the aspartokinase activity by fractionation on DEAE-cellulose; Fig. 1 shows the elution profile of the two enzymatic activities.

Temperature optimum

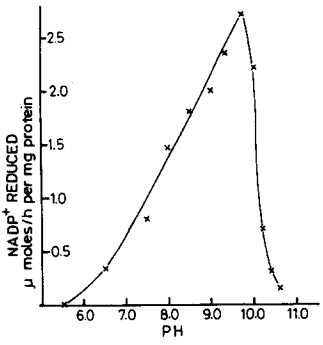
The homoserine dehydrogenase shows a rather interesting temperature relationship. The activity increased sharply by elevating the temperature from 20 to 70 °C at which temperature maximal activity was achieved; it then dropped very sharply to zero at 80 °C (Fig. 2). Like homoserine dehydrogenase isolated from other bacterial sources, this enzyme was found to be sensitive to feedback inhibition by L-threonine. However, in contrast to enzymes from other sources, feedback inhibition of this enzyme was not affected by the high temperature.

Effect of pH

The enzyme was active in the alkaline range of pH with a maximum at 9.8. The activity at pH 9.8 was about 8 times higher than that obtained at pH 6.7 (Fig. 3). However, when the pH was further increased to 10.2 a sharp drop in activity was found and almost none was left at pH 10.6. The lack of activity at pH 10.6 was not due to protein denaturation, since preincubation at this high pH for 5 min at 55 °C followed by readjustment of the pH to 9.0 yielded the same activity as found in the untreated extract at the same pH.

Potassium requirement and specificity of cations

Like the homoserine dehydrogenase from *E. coli* K-12[4] or from *Pseudomonas fluorescens*[9], the enzyme from the thermophilic bacterium required K^+ for activity.



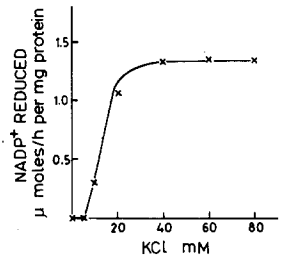


Fig. 3. Influence of pH on the homoserine dehydrogenase activity. The buffers used: Tris–maleate (between pH 5.5 and 7.5), Tris–HCl (between pH 7.5 and 9.0) and glycine (between pH 9.0 and 10.6).

Fig. 4. Effect of KCl on homoserine dehydrogenase activity.

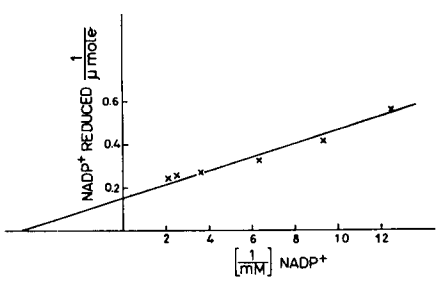
However, unlike the former enzymes the thermophilic enzyme showed no activity at K^+ levels below 5 mM, and a 40 mM concentration was required for maximal activity (Fig. 4). Several monovalent cations could substitute for K^+, but they were considerably less effective than the former (see Table II). Increasing the concentration of cations other than K^+ to 80 mM did not further increase enzymatic activity.

Specificity of pyridine nucleotides

Although less active, NAD^+ replaced $NADP^+$ in the reaction. The results are given in Table III. The apparent K_m for $NADP^+$ calculated from the Lineweaver–Burk plot (Fig. 5) was found to be $1.8 \cdot 10^{-4}$ M.

Feedback inhibition

The various homoserine dehydrogenases studied are sensitive to feedback inhibition by L-threonine. The kinetic data, presented in Fig. 6, show that inhibition of enzymatic activity by L-threonine was competitive with respect to L-homoserine. The apparent K_m for homoserine, as calculated from the double reciprocal plot (Fig. 6) was found to be $3.8 \cdot 10^{-3}$ M.



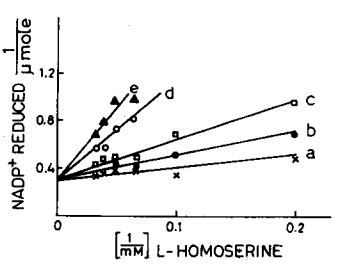


Fig. 5. Enzyme activity as a function of the concentration of $NADP^+$.

Fig. 6. Effect of substrate concentration on homoserine dehydrogenase activity in presence of increasing concentrations of L-threonine (mM): a, without threonine; b, 0.25; c, 0.5; d, 0.75 and e, 1.0 mM threonine.

TABLE II

EFFECT OF VARIOUS CATIONS ON HOMOSERINE DEHYDROGENASE ACTIVITY

Reaction mixture as described under Methods except for the cations tested, which were added as chlorides.

Cation added (40 mM)	*NADPH formed (μmoles/mg protein per h)*
None	0
K^+	1.7
Li^+	1.1
Na^+	0.7
Rb^+	0.5
NH_4^+	0.4
Cs^+	0

TABLE III

COMPARISON BETWEEN NAD^+ AND $NADP^+$ AS COENZYMES FOR HOMOSERINE DEHYDROGENASE ACTIVITY

Reaction mixture as described under Methods.

Nicotinamide nucleotide added (mM)	*NADH or NADPH formed (μmoles/mg protein per h)*
$NADP^+$ (0.4)	2.0
NAD^+ (0.4)	0.5
NAD^+ (0.8)	1.0
NAD^+ (1.6)	1.2
NAD^+ (2.0)	1.2
NAD^+ (2.4)	1.2

TABLE IV

INHIBITION OF HOMOSERINE DEHYDROGENASE BY THREONINE DERIVATIVES AND CYSTEINE

Additions	*Concentration (mM)*	*Homoserine dehydrogenase activity (NADPH formed, μmoles/mg protein per h)*	*Inhibition (%)*
None		1.5	0
L-Threonine	0.5	1.1	27
L-Threonine	1.0	0.7	53
L-Threonine	1.5	0.3	80
DL-*allo*-threonine	2.5	1.15	23
DL-*allo*-threonine	5.0	0.55	63
DL-*allo*-threonine	10.0	0.0	100
D-Threonine	10.0	1.5	0
D-Threonine	25.0	1.1	27
D-Threonine	50.0	0.9	40
D-Threonine	100.0	0.3	80
L-Cysteine	1.0	1.4	7
L-Cysteine	2.5	1.1	27
L-Cysteine	5.0	1.05	30
L-Cysteine	10.0	1.0	34
L-Cysteine	20.0	1.0	34

TABLE V

EFFECT OF METHIONINE AND ISOLEUCINE ON THE L-THREONINE INDUCED INHIBITION OF HOMOSERINE DEHYDROGENASE ACTIVITY

Amino acids added	*Concentration (mM)*	*Homoserine dehydrogenase activity (NADPH formed, μmoles/mg protein per h)*
None		1.8
L-Methionine	40	1.8
L-Isoleucine	40	1.7
L-Threonine	1.5	0.6
L-Threonine	1.5+	
L-Methionine	40	0.55
L-Threonine	1.5+	
L-Isoleucine	40	0.5

Amino acids related to L-threonine were tested for their ability to influence homoserine dehydrogenase activity. As can be seen from Table IV, D-threonine inhibited slightly (about 2%) the enzymatic activity in comparison with the natural isomer. On the other hand, DL-*allo* threonine proved to be a strong inhibitor, being only 2–3 times less active than L-threonine. No other amino acids of the aspartate pathway showed any effect on the activity of the enzyme.

L-Cysteine was found to inhibit homoserine dehydrogenase activity from various microorganisms as strongly as L-threonine[10–12]. However, the enzyme from the thermophilic organism was much less affected by cysteine; the highest inhibition obtained was about 35% (Table IV).

No reversal of the feedback inhibition by threonine was obtained by the addition of either methionine or isoleucine, as shown in Table V. This result is similar to that obtained with *P. fluorescens*[9] and in contrast to the enzyme from *R. rubrum*[3].

TABLE VI

REPRESSION OF HOMOSERINE DEHYDROGENASE SYNTHESIS BY THREONINE AND METHIONINE

Amino acids, as indicated, were added to log grown cells at 55 °C. Cells were harvested before the culture reached stationary phase and extracts were prepared as described under Methods.

Amino acids added to the growth medium	*Concn (mM)*	*Homoserine dehydrogenase activity (NADPH formed, μmoles/mg protein per h)*	*Repression (%)*
None		1.5	0
L-Threonine	1	0.9	40
L-Threonine	2	0.8	47
L-Threonine	5	0.75	50
L-Methionine	1	1.0	33
L-Methionine	2	0.9	40
L-Methionine	5	0.7	53
L-Threonine	1+		
L-Methionine	1	0.8	47
L-Threonine	2+		
L-Methionine	2	0.6	60
L-Threonine	5+		
L-Methionine	5	0.3	80

Repression of homoserine dehydrogenase and aspartokinase

It was reported previously[6] that L-threonine inhibited the growth of the thermophilic bacterium, and that methionine reversed the inhibition. It was therefore of interest to test the effect of addition of threonine or methionine during growth on the synthesis of homoserine dehydrogenase and of aspartokinase, the first enzyme in this pathway. Cells were grown in the presence of different concentrations of L-threonine, L-methionine or both and the homoserine dehydrogenase and aspartokinase activities formed by the cells were assayed on cell-free extracts prepared from them. As can be seen from Table VI, L-threonine and L-methionine repressed to almost a similar extent the synthesis of homoserine dehydrogenase. Repression of enzyme synthesis in cells which grew in the presence of both L-threonine and L-methionine was stronger (up to 80%) than in the cells which grew in presence of each of these amino acids alone (50 and 53%, respectively). On the other hand, no repression of aspartokinase synthesis was observed when the cells were grown in presence of threonine, lysine or with both amino acids (Table VII). The aspartokinase

TABLE VII

EFFECT OF ADDITION OF LYSINE AND/OR THREONINE TO THE GROWTH MEDIUM ON ASPARTOKINASE SYNTHESIS AND ON THE SENSITIVITY OF THE ENZYME TO FEEDBACK INHIBITION

Amino acids were added to log grown cells at 55 °C, as indicated. Cells were harvested before the cultures reached stationary phase and cell-free extracts were prepared as described under Methods. Aspartokinase activity was determined in the absence and in presence of various concentrations of L-threonine or L-lysine. Percent inhibition was plotted against increasing concentrations of threonine or lysine and from the plots, the concentration required for 50% inhibition was calculated.

Amino acids added to the growth medium	*Concn (mM)*	*Aspartyl-β-hydroxamate formed (μmoles/mg protein per h)*	*Concentration required to inhibit 50% aspartokinase activity*	
			L-*Threonine (mM)*	L-*Lysine (mM)*
None		1.9	1.4	3.2
L-Threonine	1	1.9	1.4	3.1
L-Lysine	5	1.9	1.4	2.9
L-Threonine	1 +			
L-Lysine	5	1.75	1.3	3.3

formed during growth in the presence of threonine, methionine or lysine exhibited the same sensitivity to feedback inhibition by threonine or lysine as the enzyme from control cells.

DISCUSSION

Homoserine dehydrogenase from the thermophilic organism exhibits certain similarities to the enzyme from other microorganisms with regard to catalytic and regulatory properties. Unlike the enzyme from *E. coli* K-12[8] and similar to that of *P. fluorescens*[9] and of *P. putida*[13] the homoserine dehydrogenase from the thermophile is a distinct protein which can be separated from aspartokinase by chromatography on DEAE-cellulose column. Homoserine dehydrogenase activity from the thermophile was optimal at about 70 °C and no desensitization to feedback inhibition

occurred at this high temperature, as found with enzymes from mesophiles. The homoserine dehydrogenase from the thermophilic organism shows a very alkaline pH optimum (pH 9.8) in contrast to that shown by this enzyme from other sources[14]. It was recently reported by Brock *et al.*[15] that maximal radioactivity uptake by sulfur bacteria from Boulder Spring was at a pH of 9.2 and at a temperature of 90 °C. Whether biochemical activity at high pH values is a unique property of thermophilic microorganisms remains to be seen.

The homoserine dehydrogenase from the thermophilic bacterium like that from *E. coli* K-12 showed a requirement for K^+. However, while the thermophilic enzyme showed no activity in absence of the cation (Fig. 4), the activity of the *E. coli* enzyme was only augmented by K^+ (ref. 4).

Unlike the homoserine dehydrogenases from *E. coli*[4] and *P. putida*[13] the feedback inhibition by L-threonine was competitive with respect to L-homoserine. In contrast to the finding with *R. rubrum*[3], neither isoleucine nor methionine reversed the inhibition caused by L-threonine. Still another difference is the relative ineffectiveness of L-cysteine as feedback inhibitor[14] as compared to its effect on the enzyme from other microorganisms[10]. It was reported by us[6] that addition of L-threonine to the growth medium arrested bacterial growth, an effect which could be overcome by addition of homoserine or methionine. We assumed that addition of L-threonine to the growth medium inhibited aspartokinase activity, which led to depletion of homoserine and methionine from the cells. On the other hand, no repression of aspartokinase synthesis was caused by the addition of threonine (Table VII) while the formation of homoserine dehydrogenase was repressed to about 50% (Table VI). A much stronger repression (80%) of homoserine dehydrogenase synthesis was obtained when both threonine and methionine were present during growth. Still it is not known what is the effect, if any, of threonine on aspartyl-β-phosphate dehydrogenase, the second enzyme in the aspartate–homoserine pathway.

ACKNOWLEDGMENTS

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