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HOMOSERINE DEHYDROGENASE OF *PSEUDOMONAS PUTIDA* PROPERTIES AND REGULATION

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SUMMARY

A single homoserine dehydrogenase (L-homoserine:NADP$^+$ oxidoreductase, EC 1.1.1.3) has been partially purified from *Pseudomonas putida*. The general properties of the enzyme are described.

Homoserine dehydrogenase of *P. putida* is regulated by feedback inhibition with L-threonine.

Its synthesis is partially repressed by L-methionine and by L-threonine.

The enzyme synthesized in the presence of the endproducts has an altered sensitivity to L-threonine.

INTRODUCTION

We reported previously on the results of investigations of β-aspartate kinase, the first enzyme of the branched biosynthetic pathway of the aspartate family in *Pseudomonas putida*.

The third reaction of the same pathway catalyzed by homoserine dehydrogenase is common to the biosynthesis of threonine, of isoleucine and of methionine[2]. This enzyme catalyzes the conversion of aspartate β-semialdehyde to homoserine, according to the equation[3]:

$$^-OOC—\underset{\displaystyle NH_3^+}{\underset{|}{CH}}—CH_2—CHO + NADPH + H^+ \rightleftharpoons {}^-OOC—\underset{\displaystyle NH_3^+}{\underset{|}{CH}}—CH_2—CH_2OH + NADP^+$$

Thus, the reaction involves the reversible reduction of an aldehyde group to a primary hydroxyl. The product of the reaction, homoserine, is the branch point for the synthesis of the end products of the pathway mentioned above.

In *Escherichia coli* K12 and other Enterobacteriaceae there are two separable and individually regulated isofunctional homoserine dehydrogenases[4–8]. It has also been shown that in *E. coli* aspartate kinase and homoserine dehydrogenase are carried by the same protein[7,8].

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In several other organisms, including *P. putida*, only one homoserine dehydrogenase was found[5,9–15]. In *P. putida* the enzyme can be separated from the aspartate kinase by physical methods[1].

A wide variation in the inhibition and repression patterns of this enzyme was observed in different organisms[9–15]. Our purpose was to investigate whether the homoserine dehydrogenase of *P. putida* shares several characteristics with other microbial homoserine dehydrogenase, with respect to kinetic properties and regulation.

MATERIAL AND METHODS

The strain, buffer and culture conditions for normal growth and for repression experiments, as well as the methods used for protein determination, are indicated in our previous paper[1].

Chemicals

In addition to chemicals used in our previous work, the following were used: NADPH (Sigma Chemical Co.), (in former experiments it was observed that homoserine dehydrogenase of *P. putida* is more active with NADPH as cofactor than with NADH[5]); allyl-DL-glycine (Nutritional Biochemicals Corp.); DL-aspartic semialdehyde was prepared by ozonolysis of allyl-DL-glycine following the method of BLACK AND WRIGHT[16].

Ensyme assay

The activity of homoserine dehydrogenase was measured in the direction: aspartic β-semialdehyde $\rightarrow$ homoserine, as described by COHEN *et. al.*[17]. We used an Unicam SP-800 recording spectrophotometer. One unit of enzyme is defined as the amount which catalyzes the oxidation of 1 nmole of NADPH per min at room temperature under the assay conditions.

Specific activities were expressed as units/mg protein.

RESULTS

The conditions of purification and the successive steps of purification were described in our previous paper[1].

Homoserine dehydrogenase was slightly more stable at 21° than at 4°. All the

TABLE I

EFFECT OF DIFFERENT PROTECTING AGENTS ON THE CATALYTIC ACTIVITY OF HOMOSERINE DEHYDROGENASE

Protecting agent added before sonication	*Concn. (mM)*	*Specific activity (nmoles NADPH per min per mg protein)*
None	—	90
L-Lysine + L-threonine	0.5 each	135
L-Aspartate	5	125
β-Mercaptoethanol	10	130

TABLE II

PARTIAL PURIFICATION OF HOMOSERINE DEHYDROGENASE FROM *P. putida*

Fraction No.	*Total units*	*Specific activity*	*Purification*	*Yield (%)*
I. Crude extract*	490 000	122	1.0	100
II. Streptomycin supernatant	490 000	129	1.06	100
III. 25–48% $(NH_4)_2SO_4$ fraction	450 000	178	1.46	92
IV. DEAE-Sephadex	400 000	2670	22	81.5

* 30 g of wet weight cells.

substances used for maintaining the catalytic activity of the enzyme had approximately the same protective effect, as shown in Table I.

We wanted to isolate aspartate kinase and homoserine dehydrogenase from the same cell-free extract, so we chose purification conditions suitable for both enzymes. A typical purification procedure of homoserine dehydrogenase is presented in Table II. The two enzymes, aspartate kinase and homoserine dehydrogenase, were separated by DEAE-Sephadex chromatography as described earlier[1].

The apparent molecular weight of homoserine dehydrogenase, as determined by gel filtration on Sephadex G-200, is about 126 000 (ref. 1).

Properties of the enzyme

The relationship between enzyme activity and protein concentration was linear at least up to a protein concentration of 120 μg/assay. The optimal pH for enzyme activity was between 6.2 and 6.7.

Enzyme activity during growth

The specific activity and sensitivity of homoserine dehydrogenase to L-threonine was determined in cells harvested at various stages of growth (Table III). This enzyme, like aspartate kinase, is most active when cells are harvested at about $\frac{2}{3}$ of the exponential growth phase. Sensitivity to the inhibitor decreases slightly at the beginning of the stationary phase.

TABLE III

CATALYTIC ACTIVITY AND FEEDBACK INHIBITION BY THREONINE OF HOMOSERINE DEHYDROGENASE IN CRUDE EXTRACTS (FRACTION I) DURING GROWTH

A_{660} *nm*	*Specific activity*	*%Inhibition by 500 μM L-threonine*
0.48	90	55
0.54	100	55
0.58	125	55
0.68	125	50
0.90	95	50
1.18	49	45
1.68	42	48

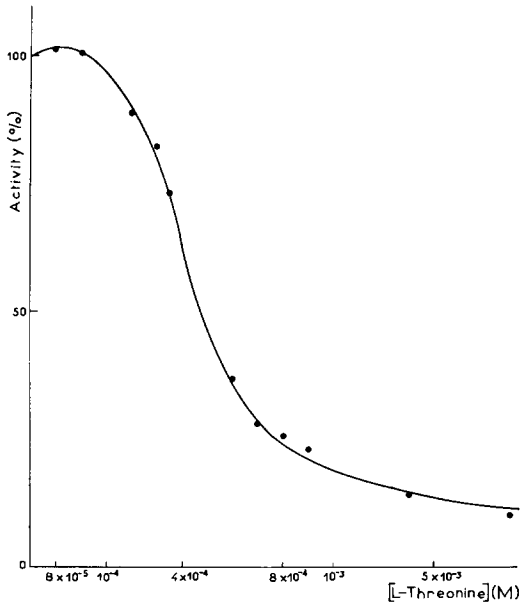


Fig. 1. Inhibition of homoserine dehydrogenase activity with increasing concentrations of L-threonine. (Enzyme is from Fraction III, see Table II.)

Allosteric inhibition of homoserine dehydrogenase by L-*threonine*

The inhibition of homoserine dehydrogenase activity with increasing concentrations of L-threonine at a constant substrate level is shown in Fig. 1. The sigmoid shape of the curve indicates co-operativity in the effect of the inhibitor. 470 μM L-threonine cause half-maximal inhibition of the enzyme.

The other end products of the biosynthesis, namely, L-methionine, L-lysine and L-isoleucine, did not inhibit the enzyme activity at a concentration of 10 mM each, neither alone nor in combination with L-threonine.

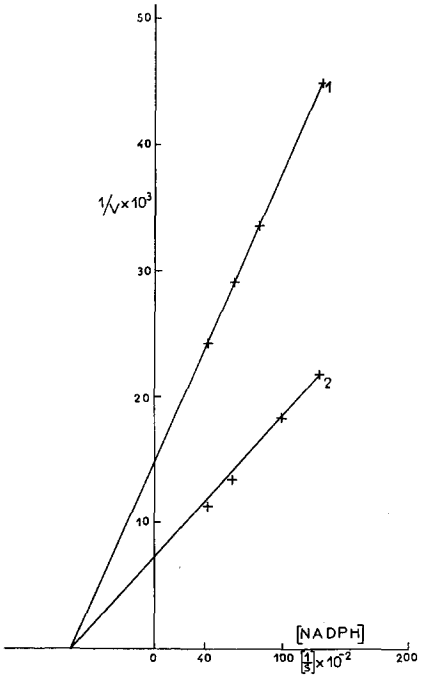
Kinetic constants

K_m determinations for NADPH and for aspartate β-semialdehyde are shown in Figs. 2 and 3. K_m for aspartate β-semialdehyde is $1.3 \cdot 10^{-4}$M; for NADPH the value is $1.4 \cdot 10^{-4}$M.

Inhibition of the enzyme by threonine is noncompetitive with respect to both substrates.

Effect of structural analogues of L-*threonine on enzyme activity*

As is shown in Table IV, L-α- and L-γ-amino butyric acid have no effect on the catalytic activity of the enzyme. As 1mM DL-threonine and 0.5 mM L-threonine produce the same inhibition, we can conclude that D-threonine is not an inhibitor of this homoserine dehydrogenase. L-Serine and DL-*allo*threonine in a concentration 20 times higher than L-threonine do produce less inhibition than 0.5 mM L-threonine. The inhibition by these structural analogues was not increased in a concerted manner when they were added together with L-threonine to the enzyme.



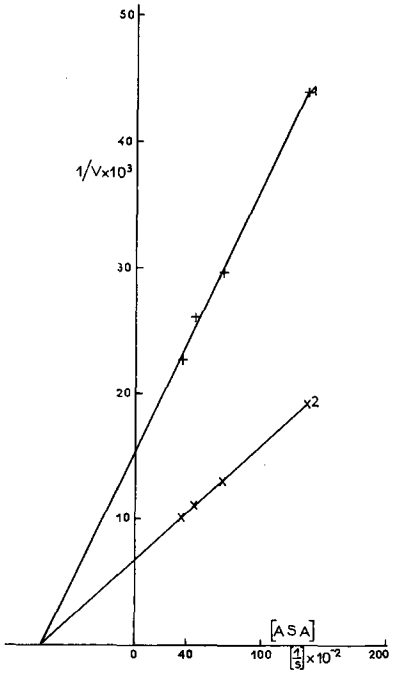


Fig. 2. Double reciprocal plots of enzyme activity and coenzyme concentration (NADPH) without inhibitor (Curve 2) and in the presence of 500 μM L-threonine (Curve 1). Enzyme used was from Fraction III (see Table II).

Fig. 3. Double reciprocal plots of initial velocity and aspartate β-semialdehyde (ASA) concentration without inhibitor (Curve 2) and in the presence of 500 μM L-threonine (Curve 1). Enzyme was from Fraction III (see Table II).

Effect of L-*threonine and KCl on the heat stability of the enzyme*

Homoserine dehydrogenase loses its activity after 40 min of heating at 60°, as shown in Table V. 10 mM of L-threonine or buffer containing 1.5 M KCl preserves the catalytic activity from heat destruction under the same conditions.

TABLE IV

EFFECT OF STRUCTURAL ANALOGUES OF L-THREONINE ON THE CATALYTIC ACTIVITY OF HOMOSERINE DEHYDROGENASE*

Analogue	*Concn. (mM)*	*Inhibition (%)*
DL-Threonine	1	55
L-Threonine	0.5	55
L-Serine	10	27
DL-*Allo*threonine	10	30
L-α-Aminobutyric acid	10	2
L-γ-Aminobutyric acid	10	2

* Enzyme used in this experiment was from Fraction IV (See Table II).

TABLE V

EFFECT OF HEATING AT 60° ON ENZYME ACTIVITY AND PROTECTION BY THREONINE AND KCl

The incubation mixture contained 1 mg of protein from Fraction III in 0.2 ml buffer solution containing 135 mM KCl, in the presence or absence of 10 mM L-threonine or KCl as indicated. After heating, 0.01 ml of mixture containing 50 μg of protein was taken and immediately cooled in ice.

Protecting agent added	*Concn. during heating (mM)*	*Concn. during assay (μM)*	*Activity (%) at incubation times (min)* 0	2	5	10	20	30	40
None	None	None	100	83	41.4	34.5	24.1	14	0
L-threonine	10	100 (67 μM KCl)	100	100	100	100	100	90	90
KCl	1.5	750	100	100	100	100	100	100	100
KCl	135	67	100	80	40	35	23	15	0

Regulation by repression of enzyme synthesis

As shown in Table VI, all the end products prolong the generation time when added to the culture. Repression was not very high, ranging between 20 and 25%, with the exception of L-lysine, which did not significantly repress enzyme synthesis.

The end products (except threonine), when added during growth, decrease the threonine sensitivity of homoserine dehydrogenase. When the enzyme was synthesized in the presence of L-threonine, it became more sensitive to this inhibitor than the enzyme produced in the absence of this amino acid.

TABLE VI

REPRESSION AND FEEDBACK INHIBITION OF HOMOSERINE DEHYDROGENASE OF *P. putida* UNDER DIFFERENT GROWTH CONDITIONS

Growth supplement concn. (= 5 mM)	*Generation time*	*Specific activity in crude extract*	*Repression (%)*	*% Inhibition by 500 μM L-threonine*
None	1 h 12 min	122	0	55
L-Lysine	4 h 12 min	112	8	22
L-Threonine	2 h 42 min	98	20	72
L-Methionine	3 h 30 min	91	25	30

DISCUSSION

Homoserine dehydrogenase catalyzes the interconversion of L-aspartate β-semialdehyde and homoserine. The product of the reaction, homoserine, is at the branch point in the biosynthesis of methionine, threonine and isoleucine. The enzymatic reaction is under regulatory control in several organisms [4–15, 18] by feedback inhibition of its activity and by repression of its synthesis.

There exist two major mechanisms for the regulation of homoserine dehydrogenase activity:

(1) *Via* isofunctional enzymes individually regulated by the end products (*E. coli* and other Enterobacteriaceae[4,7,8]).

(2) *Via* a single enzyme, regulated by one or more effectors[5,9–15].

As *P.putida* belongs to this second group, we discuss only this mechanism of regulation.

The only common feature in the regulation of homoserine dehydrogenase of this category is its feedback inhibition by threonine. Otherwise, every species has its own regulatory system. For instance in *Saccharomyces cerevisiae*[11], *Brevibacterium flavum*[14] and *Zea mays*[15] the enzyme is also inhibited by amino acids other than threonine. In *Rhodospirillum rubrum* the inhibition of homoserine dehydrogenase by threonine is reversed by isoleucine or methionine[12]. In *Micrococcus glutamicus*[9] methionine represses enzyme synthesis by 75%, and in *Rhodopseudomonas spheroides* the enzyme does not appear to be repressible[10].

The homoserine dehydrogenase of *P. putida* is very sensitive to threonine inhibition. The catalytic activity of the enzyme was inactivated by heat but could be protected by threonine or by high concentrations of KCl.

The observation that the enzyme was noncompetitively inhibited by threonine suggests that there is an inhibition site on the enzyme independent of the substrate site. The synthesis of homoserine dehydrogenase in *P. putida* is less sensitive to repression by end products than is aspartate kinase, the first enzyme on the pathway[1]. Nevertheless, when bacteria are growing in the presence of end products, an alteration in the sensitivity of the enzyme to the inhibitor is produced. We observed the same phenomenon with aspartate kinase. One can suppose that endproducts indirectly cause a change in the structure of the protein, resulting in altered inhibitor sensitivity.

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