JOURNAL OF BACTERIOLOGY, May 1974, p. 577-581
Vol. 118, No. 2

Homoserine Kinase from *Escherichia coli* K-12: Properties, Inhibition by L-Threonine, and Regulation of Biosynthesis

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Received for publication 19 November 1973

We have partially purified homoserine kinase from a genetically derepressed strain of *Escherichia coli* K-12. The optimum pH of the enzyme-substrate reaction was 7.8 and the K_m values for L-homoserine and adenosine 5'-triphosphate were both 3×10^{-4} M. K^+ (or NH_4^+) as well as Mg^{2+} were required for its activity. The sedimentation coefficient determined by ultracentrifugation in a sucrose density gradient was 5.0 ± 0.25*S*. L-Homoserine was an excellent protector against heat inactivation of homoserine kinase. L-Threonine was a competitive inhibitor of homoserine kinase, suggesting that end-product inhibition of this enzyme plays a role in vivo in the overall regulation of threonine biosynthesis. The specific activity of aspartokinase I-homoserine dehydrogenase I and of homoserine kinase showed a strong positive correlation in extracts from strains under widely varying conditions of genetic or physiological derepression; it was concluded that these two enzymes are coordinately regulated in *E. coli* K-12.

Homoserine kinase (EC 2.7.1.39.), which catalyzes the reaction L-homoserine + ATP → L-homoserine phosphate + ADP, is the first of the two enzymes leading to the biosynthesis of L-threonine from L-homoserine. Many years ago, Wormser and Pardee (19) showed that the homoserine kinase (HSK) from *Escherichia coli* B was inhibited by L-threonine. The present paper reports and analyzes the inhibition by L-threonine of homoserine kinase from *E. coli* K-12 and describes some of its properties: optimal conditions for activity, kinetic constants, stability, and molecular weight.

Aspartokinase I (EC 2.7.2.4.)-homoserine dehydrogenase I (EC 1.1.1.3.) is the key enzyme in the regulation of threonine biosynthesis (1, 6). It was recently established that the genes coding for aspartokinase I-homoserine dehydrogenase I (AK I-HDH I) and HSK are localized in the same region of the *E. coli* K-12 chromosome (15, 16). We now present evidence that AK I-HDH I and HSK are coordinately regulated and discuss the role of homoserine kinase in the overall regulation of threonine biosynthesis.

MATERIALS AND METHODS

Strains. Table 1 provides a list of strains used in this work.

Enzymes and chemicals. Lactate dehydrogenase (EC 1.1.1.27.) and pyruvate kinase from rabbit muscle (EC 2.7.1.40) as well as alcohol dehydrogenase (EC 1.1.1.1.) from yeast and phosphoenol pyruvate (tricyclohexyl ammonium salt) were obtained from Boehringer. Reduced nicotinamide adenine dinucleotide (NADH), adenosine 5'-triphosphate (ATP), dithiothreitol (DTT), HEPES (*N*-2-hydroxyethyl piperazine-*N'*-2 ethanesulfonate), L-homoserine, L-isoleucine, and L-threonine were purchased from Sigma. L-α-Aminobutyric acid, α-amino-β-hydroxy valeric acid, and *O*-methyl-L-threonine were obtained from Calbiochem. Borrelidin was a generous gift of the Boots Company Ltd. Inorganic compounds and magnesium titriplex (dipotassium magnesium ethylenedinitrilotetraacetic acid, dihydrate) were purchased from Merck.

Buffer solutions. Buffer A consisted of 0.02 M potassium phosphate (pH 7.2) containing 0.15 M KCl, 2×10^{-3} M magnesium titriplex, and 10^{-3} M DTT. Buffer AT contains, in addition, 2×10^{-3} M L-threonine.

Growth of the cells. Cells were grown aerobically at 37 C in a minimal salts medium (3) supplemented with 4% glucose and 1 μg of thiamine per ml. For nonauxotrophic strains, an overnight culture was diluted to 10^8 bacteria per ml and was allowed to grow until it reached 10^9 bacteria per ml.

For the threonine auxotroph GT143, in order to avoid a complete repression of AK I-HDH I (2, 7) and HSK, an overnight culture was diluted in a medium containing a limited concentration of L-threonine (1.5×10^{-4} M). The auxotrophic bacteria were harvested 1 h after they had reached the plateau due to complete threonine starvation.

When bacteria were grown in the presence of borrelidin, 7 μg of the antibiotic per ml was added to an exponentially growing culture and the cells were allowed to grow for at least three generations (11).

TABLE 1. *List of the strains used in this work*

Strain no.	Genotype
Tir 8[a]	Thiaisoleucine resistant
GT 100[b]	*metLM1005, lysC1004, lacZU239*
GT 200[b]	*metLM1005, lysC1004, lacZU239*, thiaisoleucine resistant
GT 143[b]	*thrC1019, metLM1005, lysC1004, lacZU239*

[a] From Umbarger (5, 14).

[b] These strains are described in J. Thèze and I. Saint Girons (J. Bacteriol., in press). All these strains had lost aspartokinase II-homoserine dehydrogenase II and aspartokinase III activities coded by *metLM* and *lysC* genes, respectively (16). Strain GT143 has lost threonine synthetase activity coded by *thrC* gene (16).

Preparation of crude extracts. The bacteria were harvested by centrifugation at 4 C, washed twice in minimal medium, suspended in AT buffer (2 ml for 1 g of bacteria), and sonically disrupted. The bacterial debris was removed by centrifugation (2 h at 105,000 × *g*), reducing by 50% the nonspecific substrate transformation in the assay of HSK (see below). The specific activity of HSK and AK I of the different strains was measured in the supernatant obtained.

Assay of HSK: method A. Enzyme activity was usually measured in a coupled assay using pyruvate kinase and lactate dehydrogenase. The decrease in absorption of NADH was followed at 340 nm. Assays were performed at 27 C, using the following reaction mixture in 1 ml final volume: 0.25 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride, pH 7.8; 10^{-2} M $MgSO_4$; 0.5 M KCl; 3.75×10^{-4} M NADH; 3.2×10^{-4} M phosphoenol pyruvate; 2×10^{-3} M ATP; 20 units of lactate dehydrogenase; and 10 units of pyruvate kinase (units as defined by the supplier). The homoserine kinase was added, and after 2 min of incubation at 27 C, the reaction was initiated by adding 10^{-3} M L-homoserine. A blank mixture without homoserine was always done when enzyme activity was measured in crude extracts where adenosine triphosphatase and NADH oxidase activities are present.

Method B. For determination of optimum pH and salt requirements under assay conditions which could affect the activity of the auxiliary enzymes, the following procedure was used. In a first step the reaction was allowed to proceed for 5 min in a mixture containing ATP and homoserine in the same concentration as above. The reaction was stopped by immersing the mixture for 30 s in a boiling-water bath. The quantity of adenosine diphosphate (ADP) formed was then estimated in a sample using lactate dehydrogenase and pyruvate kinase. A blank was made without homoserine.

Specific activity is expressed in micromoles of ADP formed per minute per milligram of protein.

Assay of AK I. AK I was measured with the same assay used for HSK except that L-homoserine was replaced by 10^{-2} M L-aspartate (18). L-Lysine (10^{-2} M) was added in order to inhibit aspartokinase III (13).

Estimation of protein concentration. The concentration of protein was determined by the biuret method (8).

Partial purification. Streptomycin sulfate (30 mg/ml) was added to the crude extract prepared from the strain Tir 8. After 1 h of stirring at 4 C, the precipitate was removed by centrifugation. Ammonium sulfate was added to bring the supernatant to 25% saturation. After stirring at 4 C for 30 min, the precipitate was removed by centrifugation, and the supernatant was brought to 40% $(NH_4)_2SO_4$ saturation. The resulting precipitate was redissolved, dialyzed against AT buffer, and stored at 4 C. Under these conditions no detectable loss of activity over a period of 2 months was observed. The sensitivity of the enzyme to threonine was not changed after purification and storage at 4 C.

The HSK activity was purified fivefold by this process, and the yield of purification was 97%.

Adenosine triphosphatase and NADH oxidase activities represented 2% of the total activity in the purified extract whereas they accounted for 10% of the total activity in the crude extract.

Heat inactivation. To test the effect of heat on the HSK activity, the enzyme was added to buffer A maintained in a constant-temperature bath and rapidly mixed. When indicated, buffer A contained 10^{-2} M L-threonine or L-homoserine. Portions were taken at various intervals, and the reaction was stopped by rapid chilling.

RESULTS

General properties of HSK. The following kinetic studies were performed with the partially purified enzyme. The optimum pH for the reaction was found to be 7.8. The following buffers were used: 0.25 M HEPES (HCl) from pH 6.5 to 8.5 and 0.25 M Tris-hydrochloride from pH 8.0 to pH 9.5. HSK had an absolute requirement for Mg^{2+}, optimal activity being observed between 10^{-2} M to 2×10^{-2} M. Potassium was a strong activator of HSK and gave maximal activity between 0.1 and 0.5 M. Ammonium ions could replace K^+, but even at its optimal concentration (5×10^{-2} M) it gave only 30% of the activity obtained with 0.5 M potassium ions.

The double reciprocal plots of HSK activity as a function of L-homoserine and ATP concentration were linear. A slight inhibition was, however, observed for homoserine concentration above 2×10^{-3} M. The K_m values for homoserine and ATP were both 3×10^{-4} M. L-Homoserine (10^{-2} M) and L-threonine (10^{-2} M) did not affect the activity of lactic dehydrogenase and pyruvate kinase used in assay A.

A small cooperativity with respect to homoserine has been reported (J. C. Patte, Thèse

de doctorat de l'Universite de Paris. 1967). From our data we obtained a Hill coefficient of $n = 1.4$, in good agreement with the value previously found. This Hill coefficient is probably too low to be interpretable in terms of a cooperative interaction. For ATP we have found an n value of 1.

Ultracentrifugation in a linear sucrose gradient showed that the enzymatic activity migrated as a single peak (see Fig. 1). The sedimentation coefficient measured by Martin and Ames (10) was 5.0 ± 0.25*S*, using alcohol dehydrogenase and AKI-HDH I as internal standards. A molecular weight of 60,000 was found by gel filtration using Sephadex G100 (B. Burr, personal communication).

The enzyme was stable up to 50 C in the absence of any ligands. The kinetics of denaturation of homoserine kinase at 57 C is shown in Fig. 2. In the absence of L-threonine or L-homoserine the half-life of homoserine kinase was 10 min. L-Homoserine (10^{-2} M) afforded excellent protection against heat denaturation whereas at the same concentration, L-threonine was not as good a protector. No desensitization of HSK to inhibition by threonine was observed under any of these conditions.

Inhibition by threonine. As shown in Table 2, HSK activity was inhibited by L-threonine. An inhibition of 75% was reached at approximatively 2×10^{-2} M L-threonine, and the concentration required for half maximal inhibition was 2×10^{-3} M. Among the threonine analogues tested, L-α-aminobutyric acid and α-amino-β-hydroxy valeric acid were also inhibitors, whereas *O*-methyl L-threonine did not inhibit HSK activity. The inhibition found with L-isoleucine was not additive to the inhibitory effect of threonine.

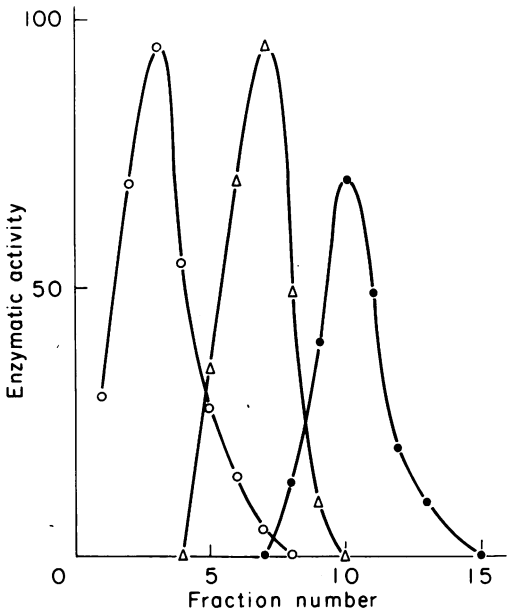

FIG. 1. *Determination of the sedimentation coefficient of homoserine kinase. Protein (500 μg) from a partially purified preparation, 5 μg of aspartokinase I-homoserine dehydrogenase I, and 10 μg of yeast alcohol dehydrogenase were layered on the top of 5-ml sucrose gradient. After 5 h at 65,000 rpm, 15 fractions were collected. The activities of aspartokinase I, alcohol dehydrogenase, and homoserine kinase were measured in each fraction, and expressed in arbitrary units. Symbols: ○, aspartokinase I; △, alcohol dehydrogenase; ●, homoserine kinase. We have used a sedimentation coefficient of 11 for aspartokinase I-homoserine dehydrogenase I (17), and 7.6 for yeast alcohol dehydrogenase (Handbook of Biochemistry, the Chemical Rubber Co. [ed.]).*

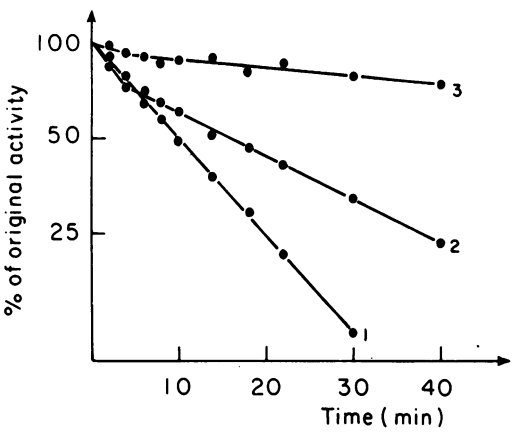

FIG. 2. *Kinetics of heat inactivation of homoserine kinase at 57 C. Curve 1, buffer A; curve 2, buffer A plus 10^{-2} M L-threonine; curve 3, buffer A plus 10^{-2} M L-homoserine.*

TABLE 2. *Inhibition of homoserine kinase activity*[a]

Inhibitors	Inhibition (%)
L-Threonine (2.10^{-3} M)	50
L-Threonine (2.10^{-2} M)	75
L-Isoleucine (2.10^{-2} M)	39
L-Threonine (2.10^{-2} M plus L-isoleucine [2.10^{-2} M])	75
L-α-aminobutyric acid (4.10^{-2} M)	50
O-methyl L-threonine (10^{-2} M)	0
α-Amino-β-hydroxy valeric acid (mixture of the 4 diastereoisomers) (3.10^{-2} M)	50

[a] The assay mixture contained 10^{-3} M L-homoserine.

The variation of HSK activity, as a function of L-homoserine concentration in the presence of various concentrations of L-threonine, clearly showed that this inhibition was competitive (Fig. 3). The K_i for L-threonine was 10^{-3} M as estimated by the method of Dixon (4).

Inhibition of HSK by L-threonine in the presence of 10^{-3} M L-homoserine was cooperative (n = 1.4). Here again, the low value of the Hill coefficient does not allow one to conclude that inhibitor sites interact cooperatively.

Regulation of HSK biosynthesis. To test the possibility that AK I-HDH I and HSK are coordinately expressed, we have measured the specific activity of these two enzymes in crude extracts of different strains under widely different physiological or genetic states of derepression.

Except for Tir 8, the strains analyzed are derived from GT100 and have lost aspartokinase II-homoserine dehydrogenase II and aspartokinase III. GT200 is a thiaisoleucine-resistant derivative of GT100 isolated as described by Szentirmai et al. (14); GT143 is a threonine auxotroph of GT100 lacking threonine synthetase activity (Table 1). The results are summarized in Table 3. The enzymatic activities of AK I and HSK of strain GT100 grown in minimal medium are taken as references. Strain GT200 is genetically derepressed for AK I and HSK as is the strain Tir 8. Strain GT100 grown in the presence of borrelidin is phenotypically derepressed for these two activities. This antibiotic is known to inhibit threonyl-transfer RNA synthetase (9) and to derepress AK I-HDH I (11). On the other hand, the specific activities of AK I and HSK of the threonine auxotroph strain GT143 are lower than those of GT100. This is due to the presence of threonine in the growth medium which, even at low concentration (1.5×10^{-4} M), appears to repress these two activities. At higher concentrations of threonine the repression is more efficient and the two activities studied become too low, with respect to the blanks, to be measured precisely (see legend of Table 3).

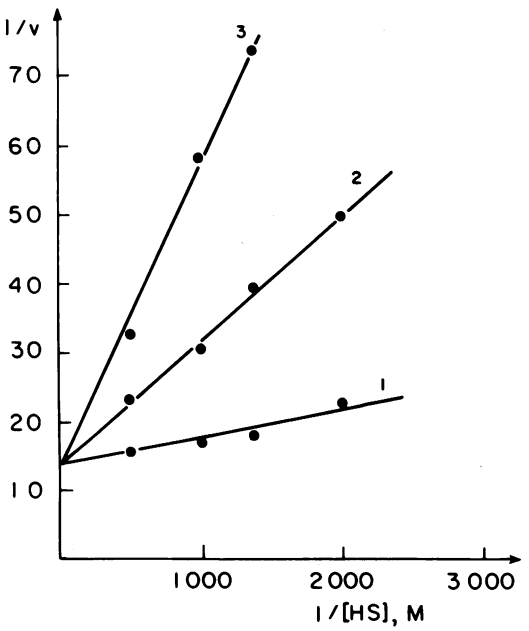

FIG. 3. *Double reciprocal plot of the inhibition of homoserine kinase activity by L-threonine at different concentrations of L-homoserine. Curve 1, no threonine; curve 2, 4 × 10^{-3} M L-threonine; curve 3, 10^{-2} M L-threonine. Velocity is expressed in arbitrary units.*

TABLE 3. *Specific activities of aspartokinase I and homoserine kinase in various strains*[a]

Strain	Sp act		Derepression factor	
	Aspartokinase I	Homoserine kinase	Aspartokinase I	Homoserine kinase
GT100*	0.033	0.053	1	1
GT100	0.155	0.222	4.6	4.1
GT200	0.180	0.320	5.4	6.0
TIR8	0.276	0.330	8.3	6.2
GT143	0.028	0.029	0.8	0.5

[a] The derepression factor is expressed as a function of the specific activity of GT100. In the crude extracts of strains GT100*, GT200 and Tir 8 derepressed for homoserine kinase; the adenosine triphosphatase and NADH oxidase activities represented about 10% of the total activity. In strains GT100 and GT143, these activities measured without homoserine represented about 50% of the total activity. Asterisk indicates this strain was grown in presence of borrelidin as defined in Materials and Methods.

As shown in Table 3, the levels of AK I-HDH I and HSK in repressed or derepressed conditions are comparable and the range of variation is similar. This supports the hypothesis of a coordinate expression of these two enzymes.

DISCUSSION

The results reported in this paper show that L-threonine is a competitive inhibitor of the homoserine kinase of *E. coli* K-12, as was found in *E. coli* B (19). As L-homoserine and L-threonine are structurally similar, the inhibition of HSK could be explained by a strict competition at the catalytic site. However, we have found limited cooperativity with respect to substrate (n = 1.4) and inhibitor (n = 1.4), but since these

values are low their interpretation as indicating genuine cooperative interaction of substrate and inhibitor sites is dubious. This small cooperativity could be also explained by a possible monomer → oligomer interconversion where the binding constants differ for each molecular form (20).

Whatever the mechanism of inhibition by threonine is, its biological significance is clear if one considers that homoserine, the substrate of this enzyme, lies at the branching point of L-methionine and L-threonine biosynthesis. L-Threonine is not a strong inhibitor of HSK, but since the inhibition is competitive, it becomes quantitatively stronger when homoserine concentration diminishes; for example, 7×10^{-4} M L-threonine is required for half-maximal inhibition in the presence of 10^{-4} M L-homoserine whereas 2×10^{-3} M L-threonine is needed, for the same extent of inhibition, when homoserine reaches 10^{-3} M. As the amount of L-homoserine in the amino acid pool of *E. coli* is negligible (12), L-threonine is probably, in vivo, a strong inhibitor of HSK. It is also worth noting that three activities concerned with L-threonine biosynthesis, AK I, HDH I, and HSK, are inhibited in the same range of concentration (1), by their common end product L-threonine.

It is well known that threonine and isoleucine participate in the regulation of the synthesis of aspartokinase I-homoserine dehydrogenase I (2, 7). Thus, in addition to controlling homoserine kinase activity by end product inhibition, threonine participates in the regulation of the synthesis of this enzyme as shown by its coordinate expression with AK I-HDH I. These results are in complete agreement with our genetic data which suggest that the genes coding for AK I-HDH I, HSK, and threonine synthetase belong to the same operon (J. Thèze and I. Saint Girons, manuscript in preparation).

ACKNOWLEDGMENTS

The work reported in this paper has been supported by grants from the Délégation Générale à la Recherche Scientifique et Technique, the Centre National de la Recherche Scientifique (E.R. No. 85), and the National Institutes of Health.

A. Bromont performed the experiments reported here for determining the effects of Mg^{2+} and K^+ on enzyme activity. We would also like to acknowledge helpful comments and discussions with G. N. Cohen, P. Truffa-Bachi, and B. Burr.

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