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Homoserine Kinase from *Escherichia coli* K12

Benjamin BURR, John WALKER, Paolo TRUFFA-BACHI, and Georges N. COHEN
Service de Biochimie Cellulaire et Service de Chimie des Protéines, Institut Pasteur, Paris

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Homoserine kinase was purified to apparent homogeneity from a derepressed strain of *Escherichia coli* K12, using standard fractionation techniques. It is a dimer (M_r = 60000) composed of apparently identical polypeptide chains (M_r = 29000). Its amino acid composition and N-terminal sequence have been determined. L-Threonine is a competitive inhibitor of the substrate L-homoserine; this inhibition is straightforward and shows no sign of co-operativity. Evidence is presented that homoserine and threonine bind to the same site of this non-allosteric enzyme. The binding of homoserine and threonine can also be studied by difference spectroscopy; the latter studies reveal an unexpected effect of magnesium ions, which might be the basis for the unusual high Mg^{2+} requirement for optimal enzyme reaction.

Homoserine kinase catalyzes the reaction: L-homoserine + ATP → homoserine phosphate + ADP. It occupies a key position in the bacterial threonine biosynthetic pathway as it performs the first reaction after the branch-point to methionine. Wormser and Pardee [1] first showed that one of the ways threonine could regulate its own synthesis was by inhibiting this step. We were interested in the characterization of this enzyme and in the mechanism of its inhibition by threonine. In addition, more information on the physical nature of the enzyme was needed to complement genetic studies on the threonine operon [2] and an investigation of the evolutionary relationships of the enzymes in the threonine biosynthetic pathway [3].

MATERIALS AND METHODS

Materials

All chemical compounds used were of the highest grade available and were purchased from Calbiochem, Sigma, Cyclo Chemicals or Merck (Darmstadt) except disodium ATP, which was from Fould-Springer. The enzymes used as molecular weight markers or as auxiliary enzymes for coupled assays were obtained from Sigma and Boehringer, except diisopropylphosphorofluoridate-treated chymotrypsinogen, which was a gift from Gilbert Orsini. Homoserine phosphate was prepared from DL-homoserine, ATP and purified *Escherichia coli* homoserine kinase under our assay conditions at 37 °C; it was purified essentially according to Wormser and Pardee [1]. Homocystine was converted to homocysteine by 10 mM dithiothreitol, a concentration of reducing agent which had no effect upon the activity of homoserine kinase or on the enzymes used to test the appearance of ADP. Iodoacetic acid was recrystallized from petroleum ether.

Sequencer chemicals were obtained from Beckman Instruments (Palo Alto, California, U.S.A.).

Buffer A contained 20 mM potassium phosphate, pH 7.2, 150 mM KCl, 2 mM magnesium Titriplex, and was supplemented with 4 mM DL-homoserine unless otherwise noted.

Strain Tir 8 of *E. coli* K12, derepressed for the enzymes of the threonine operon [2], was obtained from Dr H. E. Umbarger [4].

Enzyme Assay

The assay of homoserine kinase was adapted from Thèze *et al.* [5]. The reactants in a final volume of 1 ml were 100 mM Hepes, 250 mM KCl, 30 mM $MgSO_4$, 0.31 mM phospho*enol*pyruvate and 33 μg each pyruvate kinase and lactate dehydrogenase. The pH was 7.8 (Hepes having been neutralized with KOH). Unless otherwise stated, maximum velocity

This paper is dedicated to the memory of Dr Huguette Szulmajster.

Abbreviations. Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid; quadrol, *NNN'N'*-tetrakis(2-hydroxypropyl)ethylenediamine.

Enzymes. Homoserine kinase or ATP: L-homoserine *O*-phosphotransferase (EC 2.7.1.39); aspartokinase or ATP: L-aspartate 4-phosphotransferase (EC 2.7.2.4); homoserine dehydrogenase or L-homoserine: $NADP^+$ oxidoreductase (EC 1.1.1.3); malate dehydrogenase or L-malate: NAD^+ oxidoreductase (EC 1.1.1.37); creatine kinase or ATP: creatine *N*-phosphotransferase (EC 2.7.3.2); pyruvate kinase or ATP: pyruvate 2-*O*-phosphotransferase (EC 2.7.1.40); lactate dehydrogenase or L-lactate: NAD^+ oxidoreductase (EC 1.1.1.27); hexokinase or ATP: D-hexose 6-phosphotransferase (EC 2.7.1.1); glucose-6-phosphate dehydrogenase or D-glucose-6-phosphate: $NADP^+$ 1-oxidoreductase (EC 1.1.1.49).

was measured with 5 mM ATP and 2 mM L-homoserine, and was followed by the linear rate of absorbance decrease at 340 nm. The units are expressed in μmol NADH oxidized/min, equivalent to μmol ADP formed/min under the action of homoserine kinase. Specific activities are expressed in units/mg

Determination of Molecular Weights

A 1.5 × 100-cm Sephadex G-100 column was equilibrated with buffer A and 4 mM DL-homoserine at 4 °C. A typical experiment was performed with 1.2 mg diisopropylphosphorofluoridate-treated chymotrypsinogen, 0.2 mg pig heart malate dehydrogenase, 2.5 mg rabbit muscle creatine kinase, 0.7 mg ovalbumin and 0.03 mg pure homoserine kinase. The elution volumes of the enzymes were determined by standard enzymic assay procedures and those of ovalbumin and chymotrypsinogen by absorption at 280 nm. The logarithms of the molecular weights of the known proteins were plotted against the elution volume; a straight line was fitted to the data by the method of least squares.

Sodium dodecylsulfate gels, 5.6% in acrylamide, were prepared and run according to Fairbanks *et al.* [6]; except for malate dehydrogenase, the same molecular weight markers were used as in the gel filtration experiments.

Amino Acid Analysis, Tryptic Peptide Map and N-Terminal Sequence

Homoserine kinase was reduced with 50 mM dithiothreitol in 8 M urea, 0.5 M Tris-HCl, pH 8.0, and alkylated with iodoacetic acid, in a 1.1 molar excess over the total −SH groups. The carboxymethylated protein was dialyzed extensively against 0.01 M HCl. Acid and base spectra were read; 1.5-mg aliquots were individually lyophilized for acid hydrolysis. Larger aliquots were lyophilized for automatic sequencing.

One sample was hydrolyzed for 24 h with toluene sulfonic acid [7] and three others were hydrolyzed for 24 h, 48 h and 72 h with 5.7 N HCl at 110 °C. The toluene sulfonic acid was neutralized with NaOH and the HCl was removed by evaporation. Before acid hydrolysis, 50 nmol each of norleucine and 2-amino-3-guanidopropionic acid were added to each sample; amino acids were determined in duplicate runs on a JEOL automatic amino acid analyzer.

The carboxymethylation of the protein for peptide maps was performed as follows: the protein (4.5 mg, *i.e.* 0.15 mmol) was denatured in 1 ml 6 M guanidinium chloride containing 0.2 M Tris-HCl, pH 8.3, and 1 mg Mg-Titriplex. A concentration of dithiothreitol, in a three-fold excess over the concentration of cysteines, was added to the denatured protein, which was then incubated for 90 min at room temperature. Iodo[2-^{14}C]acetic acid (The Radiochemical Center, Amersham; specific activity 2 mCi/mmol) was added in a 1.5 excess over the total −SH group concentration; the reaction was stopped by addition of a large excess of dithiothreitol after 2 h. The carboxymethylated protein was then extensively dialyzed against 0.1 M ammonium bicarbonate. The tryptic hydrolysis, the fingerprint, the detection of the radioactive peptides and of the tryptophan-containing peptides was carried out as previously described [8].

The amino-terminal sequence analysis was carried out by automated Edman degradation in a Beckman 890C sequencer using a quadrol double-cleavage programme (Beckman programme 072172C). After conversion of the anilinothiazolinones in the presence of 0.1 N hydrochloric acid at 80 °C for 10 min and subsequent extraction with ethyl acetate the phenylthiohydantoin amino acids were identified by gas chromatography [9], thin-layer chromatography [10] and by amino acid analysis following back-hydrolysis with hydriodic acid [11].

Spectroscopic Changes upon Ligand Binding

Homoserine kinase stored as a precipitate in 50% saturated ammonium sulfate at 4 °C was resuspended in buffer A with homoserine and desalted by passage through a Sephadex G-25 column equilibrated with 0.1 M Hepes, 250 mM KCl with or without 30 mM $MgSO_4$, pH 7.8. The ultraviolet difference spectra were recorded on a Cary Model 17 spectrophotometer with a full scale of 0.1 absorbance unit, using an enzyme solution containing approximately 1 mg/ml. Aliquots of L-homoserine or L-threonine were added to the test sample and the same volumes of water were added to the blank. Differences at 294 nm were corrected for dilution and were then plotted against ligand concentration.

RESULTS

Homoserine kinase was routinely prepared during the purification of aspartokinase I-homoserine dehydrogenase I. Thus, preliminary steps were performed according to Truffa-Bachi and Cohen [12]. The two activities were copurified until the DEAE-Sephadex step, when homoserine kinase was eluted with the starting buffer just after the void volume peak, whereas aspartokinase I-homoserine dehydrogenase I was eluted only after a salt gradient had been applied to the column. All subsequent steps were performed in buffer A. The proteins in the peak of homoserine kinase activity were pooled, precipitated with 50% saturated ammonium sulfate, and dialysed extensively against buffer A. They were then placed on a 7 × 50-cm column of DEAE-Sephadex (A-50)

Table 1. *Purification of homoserine kinase from approximately 4 kg* E. coli *K12, strain Tir 8*
Activity in the crude extract ranges from 0.1–0.3 μmol min^{-1} mg^{-1}

Purification step	Protein	Activity	Specific activity	Recovery
	g	kU	U/mg	%
24–40% $(NH_4)_2SO_4$ after dialysis	49	43	0.9	100
DEAE-Sephadex I	12.3	38	3.1	85
50% $(NH_4)_2SO_4$ after dialysis	–	24	–	56
DEAE-Sephadex II	0.7	17	24	40
Heat treatment	0.388	16	47	37
G-100	0.159	9.25	58	22

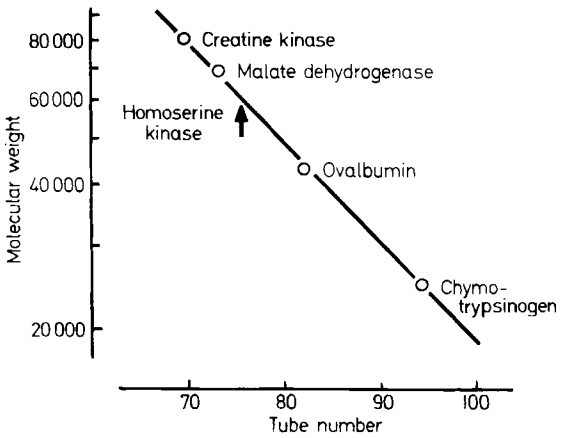

Fig. 1. *Elution of homoserine kinase and molecular weight markers from a Sephadex G-100 column.* See conditions in the text

equilibrated and washed with the same buffer. The activity was eluted with the last peak of protein in the wash. The pooled contents of the peak were made 20 mM in DL-homoserine, heated at 55–57 °C for 10 mn and centrifuged. The supernatant was concentrated in the cold by ultrafiltration (Amicon membrane PM 10) and loaded on a 2.5 × 100-cm Sephadex G-100 column. The activity peak corresponded to the major peak of protein. It was precipitated with 50% saturated ammonium sulfate and stored at 4 °C without further loss of activity.

The evidence presented later that fairly high concentrations of magnesium ions are necessary for the binding of homoserine to the enzyme, as well as a recent purification, suggest that higher yields may be obtained if 10 mM $MgCl_2$ is substituted for magnesium Titriplex in buffer A.

Table 1 summarizes the above purification.

Criteria of Homogeneity, Molecular Weight, and Amino Acid Analysis

The purified enzyme migrates as a single band upon polyacrylamide gel electrophoresis. The N-terminal sequence determined in the automatic sequencer shows no sign of heterogeneity (see below).

Table 2. *Amino acid composition of homoserine kinase*
M_r polypeptide chain = 29000

Amino acids	Yield ± S.D.
	mol/chain
Tryptophan	4
Lysine	10.7 ± 0.3
Histidine	5.9 ± 0.3
Arginine	16.6 ± 0.6
Carboxymethylcysteine	7.9 ± 0.2
Aspartate	21.2 ± 0.5
Threonine	8.2 ± 0.3
Serine	13.0 ± 0.9
Glutamate	28.9 ± 0.7
Proline	13.1 ± 0.3
Glycine	22.5 ± 0.9
Alanine	33.0 ± 1.3
Valine	19.3 ± 0.6
Methionine	6.8 ± 0.1
Isoleucine	13.6 ± 0.3
Leucine	27.2 ± 0.4
Tyrosine	7.0 ± 0.2
Phenylalanine	9.0 ± 0.2

Three filtrations on Sephadex G-100 using buffer A (Fig. 1) gave a value of 60000 for the molecular weight of homoserine kinase.

The amino acid composition is given in Table 2. Most of the values are calculated from the zero-time intercept as determined by linear regression. Isoleucine reached a plateau and the average for 48 h and 72 h was taken. Methionine, leucine, phenylalanine and valine increased linearly with hydrolysis time and the 100-h intercept was arbitrarily taken. Tryptophan was determined by hydrolysis by toluene sulfonic acid and spectral analysis [13] and gave respectively 7.4 and 9 equiv./mol of native enzyme. An absorption coefficient, $A_{280}^{0.1\%} = 1.1$ was calculated, based on the amino acid analysis.

Tryptic Maps and Amino-Terminal Sequence

The fingerprint (Fig. 2) shows 25 peptides revealed by ninhydrin plus two cysteine-containing (CmCys 3 and CmCys 6) and one tryptophan-containing (Trp 3)

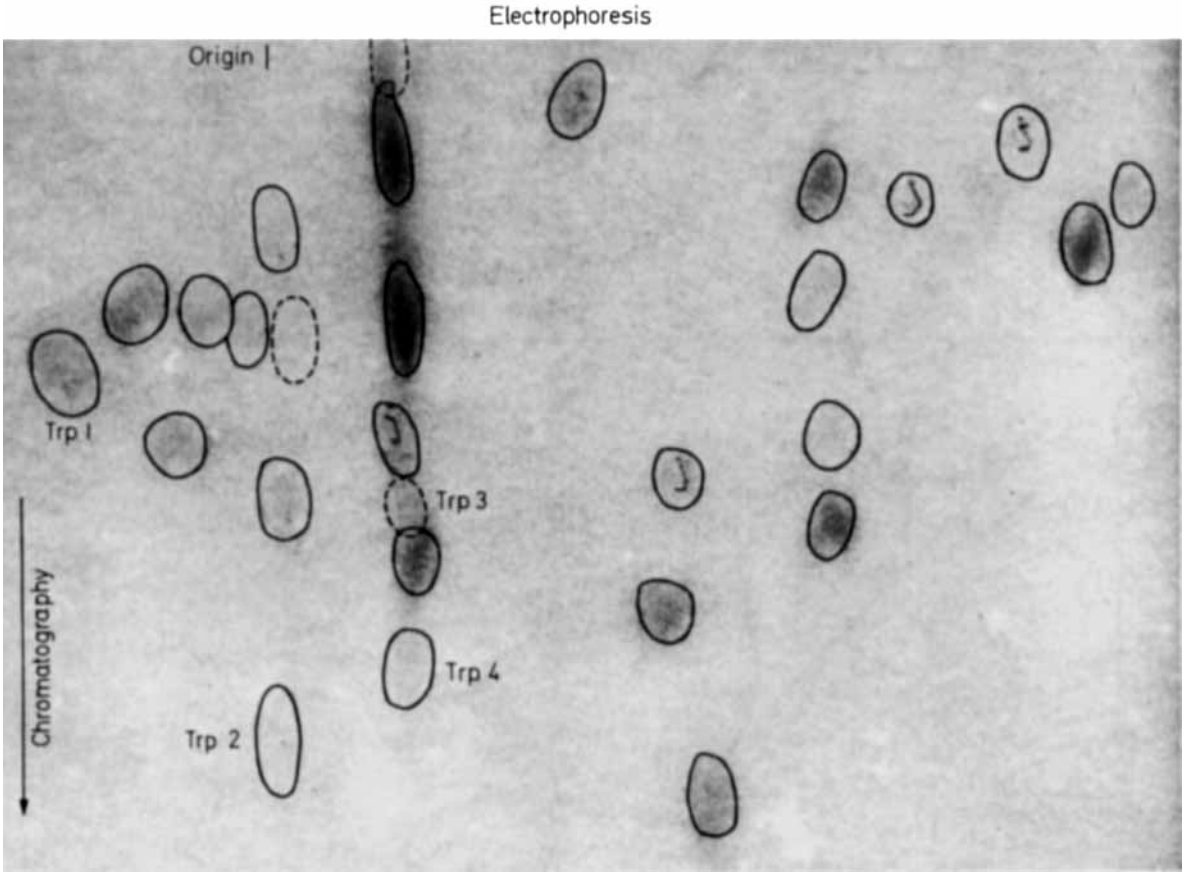

Fig. 2. *Fingerprint of the tryptic digest of radioactive carboxymethylated homoserine kinase.* The three peptides that were not stained by ninhydrin and which have been revealed by either their radioactivity or tryptophan content have been surrounded by dots

peptides not stainable by ninhydrin giving a total number of 28 peptides. No core was observed. The number of peptides expected from the number of lysine and arginine residues is 28–29; the experimental data are thus in good agreement with a protein composed of two identical subunits (see below).

Four peptides containing tryptophan are found, a value in agreement with the number determined by chemical analyses; however, one of these peptides (Trp 3) is much more stained than Trp 1 and Trp 2 by Ehrlich's reagent while Trp 4 is poorly stained.

Eight radioactive peptides are found (Fig. 3), one of them containing two cysteine residues (Table 3). It should be noted that whereas the tryptic maps are interpreted as indicating nine unique cysteine sequences, the amino acid analysis yields only eight.

It should also be noted that two tryptic peptides contain both tryptophan and carboxymethylcysteine. They are respectively Trp 1 (= CmCys 1) and Trp 2 (= CmCys 5).

The amino acid sequence of the amino terminal region of homoserine kinase was determined as Val-Lys-Val-Tyr-Ala[5]. Pro-Ala-Asn[8] ... The yield of phenylthiohydantoin valine at step 1 was 93%. The first seven residues were obtained in good yield, but at cycle 8 a very low recovery of asparagine was observed and no further unequivocal sequence information was obtained.

Reduction of the duration of the acid cleavage at cycle 7 did not overcome this problem [14].

Subunit Composition

The subunit molecular weight was estimated on sodium dodecylsulfate polyacrylamide gels from the electrophoretic migration relative to proteins of known molecular weight (Fig. 4). Two separate experiments gave values of 29000. From the molecular weight of the native enzyme, the unique N-terminal sequence, the number of tryptic peptides, we deduce that the enzyme is a dimer composed of identical subunits.

Kinetics

The enzyme displays normal Michaelian kinetics when the concentration of either of its substrates varied: Michaelis constants of 0.55 mM for ATP and 0.24 mM for L-homoserine were calculated.

An attempt was made to see if the reverse reaction could be measured with the pH 7.8 assay buffer at

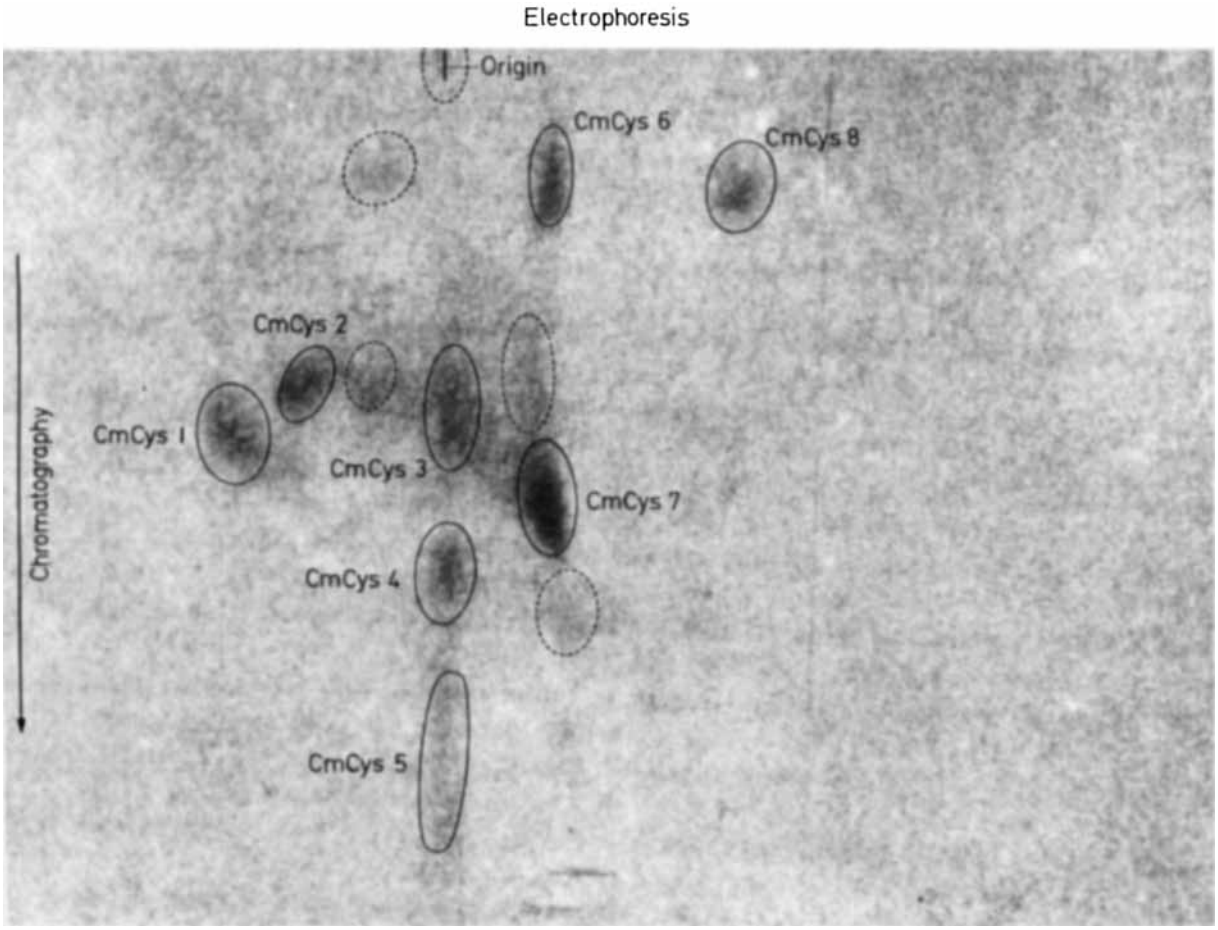

Fig. 3. *Radioautography of the fingerprint shown in Fig. 2.* The five weak radioactive spots numbered 9–13 are surrounded by dots

Table 3. *Radioactivity present in the individual peptides of the autoradiography of Fig. 3*
Assuming the maximum value (CmCys 2 and 4) of 71000 counts/min for each CmCys residue, we must consider the weak radioactivity present in spots 9–13 as resulting from non-specific tryptic cleavage

Peptides	Radioactivity	Number of CmCys residues
	counts/min	
CmCys 1	62000	0.87
CmCys 2	72000	1.01
CmCys 3	59000	0.83
CmCys 4	70000	0.99
CmCys 5	65000	0.92
CmCys 6	49000	0.69
CmCys 7	125000	1.76
CmCys 8	45000	0.63
9	17000	0.24
10	24000	0.34
11	19000	0.27
12	16000	0.23
13	17000	0.24
Total	640000	9.02

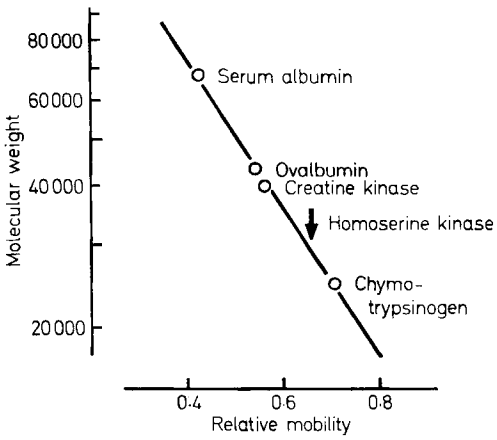

Fig. 4. *Sodium dodecylsulfate gel electrophoresis of homoserine kinase and molecular weight markers*

25 °C using hexokinase, glucose-6-phosphate dehydrogenase and the appropriate substrates to detect the formation of ATP. No reaction could be detected when ADP was varied from 0.5 mM to 5 mM and homoserine phosphate was varied from 1.8 mM to

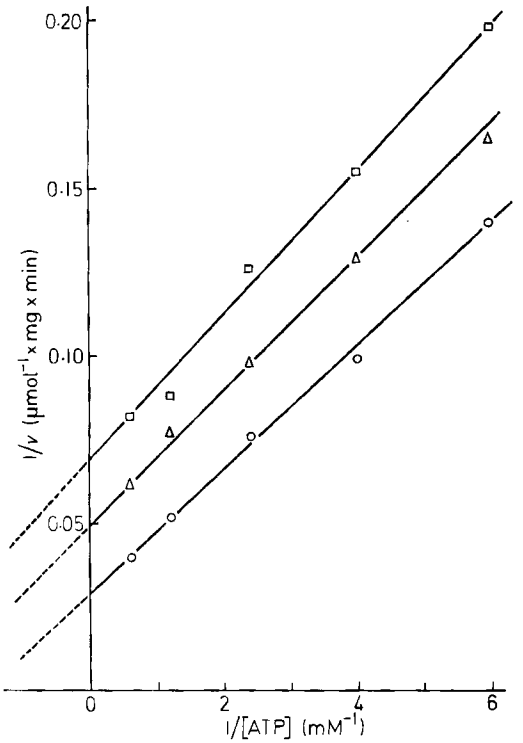

Fig. 5. *Double-reciprocal plot of inital velocity of homoserine kinase with varying concentrations of ATP and 0.5 mM* L-*homoserine showing inhibition by* L-*threonine.* (○) No L-threonine; (△) 1 mM L-threonine; (□) 2 mM L-threonine. Enzyme concentration was 0.83 μg/ml

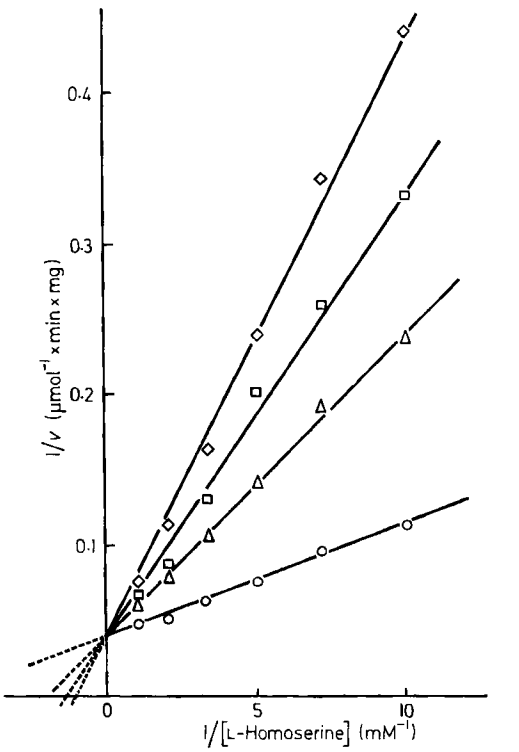

Fig. 6. *Double-reciprocal plot of initial velocity of homoserine kinase with varying concentrations of* L-*homoserine and 0.83 mM ATP showing inhibition by* L-*threonine.* (○) No L-threonine; (△) 1 mM L-threonine; (□) 2 mM L-threonine; (◇) 3 mM L-threonine. Enzyme concentration was 0.83 μg/ml

18 mM. No inhibition of the forward reaction by 16 mM homoserine phosphate was observed, even when L-homoserine and ATP were each reduced to 0.05 mM.

Inhibition by L-*Threonine*

When ATP is the variable substrate, L-threonine is not a competitive inhibitor of the enzyme (Fig. 5). Although the slopes appear parallel, they actually converge below the abscissa. Conversely, L-threonine is a competitive inhibitor of L-homoserine (Fig. 6).

Since it has been reported that L-threonine may be an allosteric inhibitor of *E. coli* homoserine kinase [5], a series of experiments was undertaken to determine the Hill coefficient of threonine inhibition. From eight separate experiments where ATP ranged from 1 mM to 0.05 mM and L-homoserine from 0.5 mM to 0.05 mM (in each experiment only L-threonine was varied), an average $n = 1.05 \pm 0.11$ was calculated.

Substrate and Inhibition Specificities

L-Threonine, L-serine, L-aspartate and GTP are not substrates of the enzyme. A number of threonine analogues were tested for their inhibitory power:

Table 4. *Threonine analogues which are inhibitors of homoserine kinase*

Inhibitors	Inhibition constant
	mM
L-Threonine	0.6
L-Cysteine	1.0
L-2-Aminobutyrate	0.4
L-Homocysteine	4.0
L-Isoleucine	4.5
L-Serine	27
2-Chloro-L-alanine	15
L-Methionine	35
L-Valine	10

the following, used at a 10 mM concentration, did not inhibit the enzyme in the presence of 0.5 mM L-homoserine and 1 mM ATP: D-threonine, glycyl-L-threonine, *O*-methyl threonine, 3 and 4-hydroxybutyric acids, 3-aminobutyric acid. The following compounds were non-competitive inhibitors of ATP and linear competitive inhibitors of L-homoserine: L-homocysteine, L-cysteine, 2-chloro-L-alanine and L-2-aminobutyrate. The dissociation constants of these compounds are shown in Table 4. Since homo-

serine kinase is not inhibited by 100 mM mercaptoethanol or 10 mM dithiothreitol, it appears unlikely that the inhibition by cysteine or homocysteine occurs through an effect on disulfide bonds that may be present in the enzyme.

Difference Spectrum upon Binding of Homoserine or Threonine to the Enzyme

There is a marked change of the ultraviolet spectrum of homoserine kinase in the presence of homoserine (Fig. 7). Interestingly, a similar spectral change, although of a different amplitude, is induced by L-threonine, but not by D-threonine. The decrease in absorbance at 294.4 nm in response to added ligand was chosen to measure binding constants. In the presence of non-saturating amounts of L-homoserine there is a pronounced effect of Mg^{2+} on the spectral change. The increase in deflection with respect to Mg^{2+} concentration does not appear to follow a simple adsorption isotherm: rather, a straight line is obtained when the change in absorbance at 294.4 nm is plotted against the square of magnesium concentration. No simple interpretation for this result can be given at present. Fig. 8 A and B show the effect of Mg^{2+} on the interaction of L-homoserine with the enzyme. In the absence of $MgSO_4$, the binding constant derived from these studies is 11 mM, while it is only 1.75 mM in the presence of 20 mM $MgSO_4$. Similarly, the values derived for the threonine binding constants are respectively 0.5 M and 20 mM. There is no indication of co-operativity in the binding of L-homoserine: the Hill numbers determined in the absence and presence of $MgSO_4$ were 1.08 and 0.91 respectively.

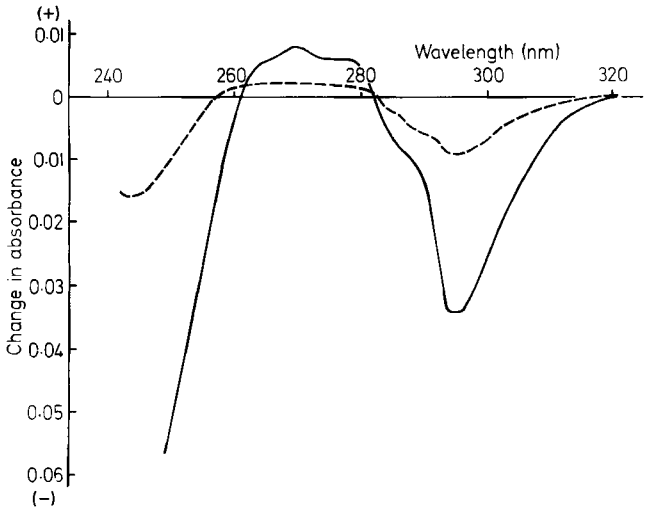

Fig. 7. *Ultraviolet difference spectra of 1.36 mg/ml homoserine kinase in the absence of Mg^{2+} with either 7 mM L-homoserine (——) or 5 mM L-threonine (– – –)*

DISCUSSION

Homoserine kinase has been purified to apparent homogeneity. Based on the facts that the molecular weight determined by electrophoresis in dissociating gels is half that of the native enzyme, that the tryptic maps yields a number of peptides of the order expected for a homodimer, that a unique N-terminal sequence has been determined on the native enzyme, and that the structure of the enzyme is coded by a single cistron [2], we conclude that homoserine kinase

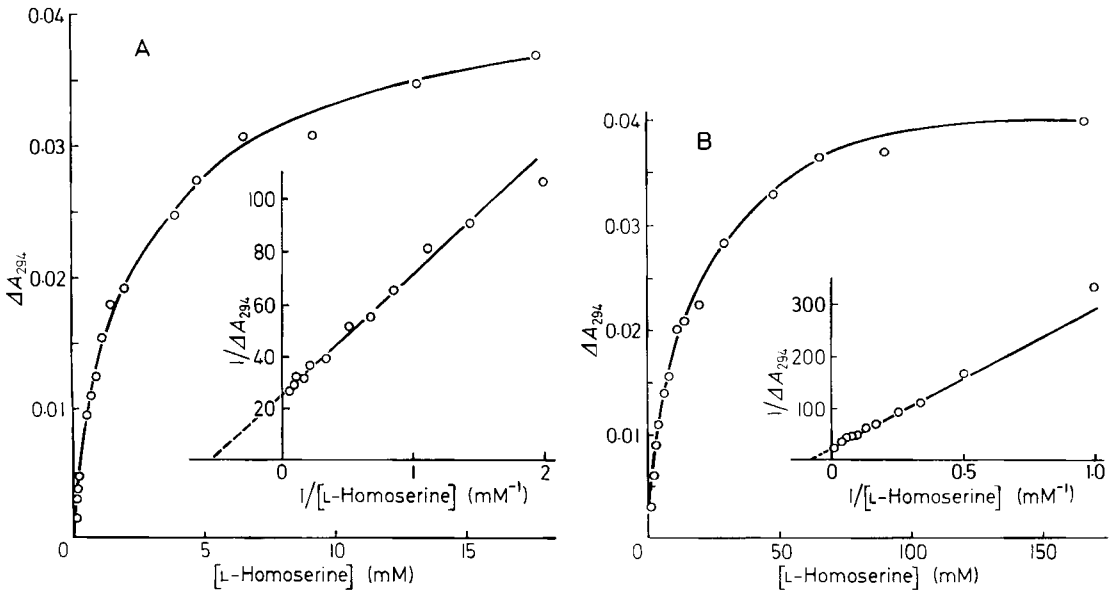

Fig. 8. *Decrease in absorbance at 294 nm plotted against L-homoserine concentration.* (A) 1.6 mg/ml homoserine kinase in the presence of 20 mM $MgCl_2$; (B) 1.36 mg/ml homoserine kinase, no $MgCl_2$

is a dimer composed of identical subunits of 29000 molecular weight.

Homoserine and threonine probably compete for the same binding site. First of all, threonine is a linear competitive inhibitor of homoserine and in reducing the binding of homoserine acts as a non-competitive inhibitor of ATP. Secondly, a number of amino acids and analogues structurally similar to homoserine and threonine are also inhibitors of the enzyme. In fact, as shown by the inspection of the results of Table 2 and of Wormser and Pardee on the extracts of *E. coli* B [1] the inhibition is rather non-specific and can be obtained by many amino acids (at higher concentration though than with L-threonine), which meet the necessary space filling and perhaps steric and charge requirements of the binding site. Finally there is no evidence of co-operativity either in the binding of homoserine or in the inhibition of activity by threonine. We have obtained identical results using the enzyme prepared without a heat step, thus ruling out a possible loss of sensitivity of an allosteric site.

Homoserine is an intermediate in the biosynthetic pathway leading to methionine, threonine and isoleucine and its concentration in the amino acid pool of wild-type *E. coli* is exceedingly low, actually below detection [15]. Thèze *et al.* [5] have already pointed out that, as a consequence, physiological concentrations of threonine can readily regulate the activity of homoserine kinase by competitive inhibition. We propose that, in the absence of a selective pressure, there was no need for *E. coli* to evolve an allosteric binding site on this enzyme.

Homoserine kinase requires between 20 mM and 30 mM Mg^{2+} for maximal activity [5]. This is much more than the approximately 0.1 mM excess required to form a Mg-ATP complex at the highest substrate concentrations (and at $I = 0.1$ M). We were fortunate to be able to use the ultraviolet spectral changes to study the binding of homoserine. In the absence of $MgSO_4$ the apparent affinity for homoserine was much lower than in its presence. This effect cannot be ascribed to the complexing of metal ions by the buffer since both Tris and Hepes have a negligible association with Mg^{2+} [16]. The present study does not permit us to say whether Mg^{2+} interacts with the enzyme, the substrate, or both. However, the effect of Mg^{2+} on ligand binding shows that the previous results were not artifactual.

Except for the fact that *E. coli* homoserine kinase appears to have an unmeasurably low affinity for its product, homoserine phosphate, the enzyme appears to be generally similar to the one purified from *Brevibacterium flavum* [17].

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B. Burr, Oak Ridge National Laboratory, P.O. Box Y, Oak Ridge, Tennessee, U.S.A. 37830

J. Walker, M.R.C. Laboratory of Molecular Biology, Postgraduate Medical School, University of Cambridge, Hills Road, Cambridge, Great Britain CB2 2QH

P. Truffa-Bachi and G. N. Cohen, Service de Biochimie Cellulaire, Institut Pasteur, 28 Rue du Docteur-Roux, F-75724 Paris-Cedex-15, France