Biochimica et Biophysica Acta, 403 (1975) 208—220

BBA 67567

STUDIES OF HOMOGENEOUS "BIOSYNTHETIC" L-THREONINE DEHYDRATASE FROM *ESCHERIA COLI* K-12

SOME KINETIC PROPERTIES AND MOLECULAR MULTIPLICITY

Z.S. KAGAN, A.I. DOROZHKO, S.V. KOVALEVA and L.I. YAKOVLEVA
The All-Union Vitamin Research Institute, Moscow (U.S.S.R.)
(Received January 10th, 1975)

Summary

"Biosynthetic" L-threonine dehydratase (EC 4.2.1.16) was purified to a homogeneous state with 29% yield of total activity from *Escherichia coli* K-12. The homogeneity of the enzyme was shown by polyacrylamide gel disc electrophoresis in the presence of dodecyl sulphate. The enzyme consisted of equal subunits having a molecular weight of about 57 000. The polyacrylamide gel disc electrophoresis has shown that the native enzyme consisted of a set of oligomeric forms.

The multiplicity of molecular organization of the enzyme was reflected in complicated kinetic behaviour: at pH >9 on the plots of initial reaction rate (v) versus initial substrate concentration ($[S]_0$) there were four inflexion points (two intermediate plateaux), the position and deepness of which depended on enzyme concentration.

At pH 8.3 on the v versus $[S]_0$ plots appeared two inflexion points (one intermediate plateau), the position of which practically did not depend on enzyme concentration in the reaction mixture, but strongly depended on the enzyme concentration in the stock solution.

Repeated polyacrylamide gel disc electrophoresis of several oligomeric forms, isolated by the first electrophoresis, has shown that the oligomeric forms underwent a slow polymerization.

It was suggested that "biosynthetic" L-threonine dehydratase from *E. coli* K-12 is a set of multiple oligomeric forms, having different kinetic parameters. Probably, each form of the enzyme has a "simple" kinetics characterized by hyperbolic or sigmoidal shape of v versus $[S]_0$ plots. The rate of equilibrium installation between the oligomeric forms was small in comparison with the enzyme reaction velocity, that lead to the complex kinetic curves, appearing as a result of summing up of the kinetics inherent to the individual forms.

Introduction

In previous work [1] it has been shown that "biosynthetic" L-threonine dehydratase (EC 4.2.1.16) in the crude extract of *Escherichia coli* K-12 is characterized by a very complicated reaction kinetics, namely, at pH >9 having four inflection points (two intermediate plateaux) on the plots of initial reaction rate (v) versus initial substrate (L-threonine) concentration ($[S]_0$). It was shown that these kinetic properties of the enzyme strongly depended on protein concentration in the reaction mixture and can not be explained by a set of isoenzymes with different kinetic characteristics. It was suggested that the obtained data may be explained by the possibility of two types conformation transitions: in the enzyme oligomer itself and reversible dissociation of the oligomer.

The detailed analysis of kinetic and allosteric mechanisms of enzyme function and of the relationship between its quaternary structure and the kinetic demonstrations of allosteric interactions was possible, however, only by using enzyme of high purity. The purpose of this work was the development of a method to purify "biosynthetic" L-threonine dehydratase from *E. coli* K-12 to an homogeneous state and the examination of some kinetic properties of the enzyme.

Materials and Methods

E. coli K-12 was cultivated under aerobic conditions in mineral medium with glucose; for the enzyme isolation, the cells were disrupted ultrasonically, and the enzymatic activity was determined as described previously [1].

Enzymatic activity during purification procedures was measured in 0.25 M K_3PO_4 buffer (pH 8.2) using a saturating concentration of L-threonine ($5 \cdot 10^{-2}$ M). The buffer ingredients and the conditions of activity measurement in the kinetic studies will be specifically indicated.

The unit of enzymatic activity was defined as the amount of protein which catalyzed the formation of 1 μmole of 2-oxobutyrate per min at 26° C. Specific activity was defined as the amount of the unit per mg of protein determined by the method as described by Lowry et al. [2].

Disc electrophoresis on polyacrylamide gel were carried out by Davis [3] and Maurer's [4] methods. The protein bands on electrophoregrams were stained with Coomassie blue. For the demonstration of enzymatic activity gel proteins were stained by the method described by Feldberg and Datta [5]. Catalytically active proteins were developed as green zones. On incubation of the stained gel for 24 h in the light, broad green zones divided into several distinct bands and became red. It must be emphasized that the primary green colouring on zymogram truly manifested the localization of the L-threonine dehydratase. The red colouring developing later, which enveloped a larger number of protein bands, is possibly inherent to pyridoxal phosphate-containing proteins and caused, perhaps, by the formation of an aldimine link between the aldehyde group of pyridoxal 5′-phosphate and ϵ-NH_2-group of lysine residue in protein. Indeed, electrophoresis under similar conditions of the homogeneous preparations of aspartate transaminase from hog heart muscle (cytoplasmic

isoenzyme) and phosphorylase *b* from rabbit skeletal muscle produced red coloured bands after the gel was exposed to light without developing the primary green colour.

Disc electrophoresis on polyacrylamide gel in the presence of sodium dodecyl sulphate was carried out by the method described [6]. The proteins were stained with Coomassie blue for 24 h. Before applying proteins (30—70 μg) on the gel, the former were incubated for 3 h at 37°C in 0.2—0.3 ml solution of 1% sodium dodecyl sulphate containing 1% of 2-mercaptoethanol, and dialyzed for 16—18 hours at room temperature against 0.1% sodium dodecyl sulphate solution containing 0.1% of 2-mercaptoethanol.

Results

Enzyme purification

Precipitation of nucleic acids and nucleoproteins with protamine sulphate and fractionation of proteins with $(NH_4)_2SO_4$. For enzyme purification, cells of *E. coli* K-12 were collected by centrifugation, washed with $1 \cdot 10^{-3}$ M potassium phosphate buffer (pH 8.2) and then resuspended (1 : 10 by weight) in 0.05 M potassium phosphate buffer, containing $1.2 \cdot 10^{-4}$ M pyridoxal 5′-phosphate, $3.9 \cdot 10^{-4}$ M reduced glutathione (GSH) and $1 \cdot 10^{-3}$ M L-isoleucine; the final pH was 7.7. Hereafter this buffer will be called the stabilizing buffer.

The homogeneous cell suspension could be stored in a frozen state at —10 to 15°C without noticeable loss of activity at least for two months. The cells were thawed and disrupted ultrasonically before use.

For separation of nucleic acids and nucleoproteins from supernatant (18 000 × *g*; 15 min; 0—4°C), protamine sulphate solution (20 mg/ml) was added, the quantity calculated as 1.4 mg per ml of the extract. The precipitate after 30 min at 0—2°C was discarded by centrifugation.

The proteins were fractionated with a saturated solution of $(NH_4)_2SO_4$; the pH value of 8.2 was reached by addition of 0.25 M NH_4OH; the $(NH_4)_2SO_4$-saturated solution contained L-isoleucine ($1 \cdot 10^{-3}$ M), GSH ($3.9 \cdot 10^{-4}$ M), EDTA ($5.4 \cdot 10^{-5}$ M) and 0.05 M potassium phosphate buffer (pH 8.2). The protein precipites were diluted in the stabilizing buffer containing 0.23 M KCl.

Desalting on Sephadex G-25. For desalting of the 40—60% protein fraction, a Sephadex G-25 (crude) column (1.8 × 24.5 cm) equilibrated with the stabilizing buffer containing 0.23 M KCl was used. The elution rate was 0.9 ml/min.; the fractions were collected on 3.2—3.5 ml. The first and the fifth fractions had lower specific activities than the protein placed on the column, and therefore the combined 2—4 fractions were used for further purification.

Chromatography on DEAE-cellulose. The DEAE-cellulose (DE-23; purchased from Whatman, U.K.) column (1.4 × 40 cm) equilibrated with the stabilizing buffer (pH 7.7 measured at 20°C) with 0.23 M KCl addition was used. All procedures were carried out at 0—4°C; the elution rate was 0.8—0.9 ml/min. Unabsorbed proteins on the column were totally eluted with the same buffer (pH 7.7), then the elution was continued with the same buffer having a pH of 6.7 (measured at 20°C). In this case, the protein was eluted by the

asymmetric peak having a maximal specific activity. The eluate of the fraction had a pH value of 7.0—7.1.

By this method, the active protein was purified 56-fold (as calculated from the specific activity on the enzyme placed on the column) and had a specific activity of 36.5 μmol(mg of protein · min)$^{-1}$. This fraction had 45% of the total activity of protein placed on the DEAE-cellulose column and contained 0.78% of total proteins of the same fraction.

Concentration of the enzyme solution. This was carried out by the following procedure: pH value of 7.7 of the active fraction was achieved by addition of KOH solution, ionic strength was lowered to 0.17 M (as calculated by KCl) by addition of the stabilizing buffer, protein was absorbed on DEAE · cellulose and the enzyme was eluted with a small volume of the stabilizing buffer containing 1 M KCl.

All the later experiments were carried out with fresh enzyme preparations.

The purification results are presented in Table I. This data shows that following stages of purification, namely, the precipitation of nucleic acid and nucleoproteins with protamine sulphate from cell-free extract; the fractionation of proteins with $(NH_4)_2SO_4$, and chromatography on DEAE · cellulose, lead to 270-fold enzyme purification. The enzyme was obtained with a yield of 29% of total activity and 0.1% of proteins in a cell-free extract and had a specific activity of 36.5 μmol(mg of protein · min)$^{-1}$ under standard measurement conditions (0.25 M potassium phosphate buffer, pH 8.2, L-threonine concentration was $5 \cdot 10^{-2}$ M; 26°C). If the enzyme activity was measured at the pH optimum (pH 9.5) the enzyme specific activity was about 60 μmol(mg of protein · min)$^{-1}$.

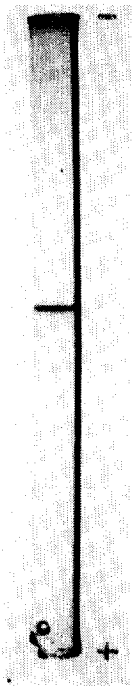
The enzyme purity and molecular weight of its subunit. For the purpose of enzyme purity determination disc electrophoresis on polyacrylamide gel in the presence of sodium dodecyl sulphate was carrie; out. Fig. 1 shows that the enzyme preparation was homogeneous and consisted, probably, of identical subunits. Indeed, only one protein band was demonstrated with 30 to 70 μg of protein in the tube. The subunit has a molecular weight of about 57 000 (the average value from three measurements) as found by the method as described by Laemmli [6] (see Fig. 2).

However, we can not exclude the possibility that the subunit contains

TABLE I

PURIFICATION OF "BIOSYNTHETIC" L-THREONINE DEHYDRATASE FROM *E. COLI* K-12

Steps		Total proteins (mg)	Spec. act. *E*(μmol (mg protein · min)$^{-1}$)	Total activity (*E* · total proteins)	Purification (-fold)	Yield (%)	
						Of proteins	Of total activity
1	Crude extract	672.0	0.135	93.0	1	100	100
2	Protamine sulphate	590.0	0.145	86.1	1.1	87.8	92.6
3	40—60% saturation with $(NH_4)_2SO_4$	118.0	0.620	73.2	4.6	17.6	78.7
4	Sephadex G-25	94.5	0.650	61.2	4.8	14.1	65.8
5	DEAE · cellulose	0.74	36.500	27.0	270.0	0.1	29.0



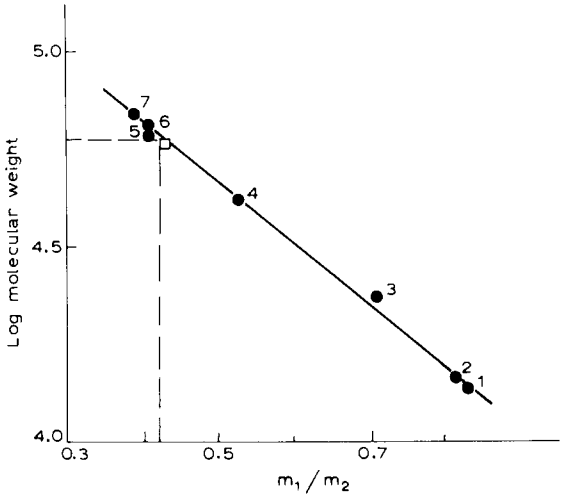


Fig. 1. Disc electrophoresis on polyacrylamide gel in the presence of sodium dodecyl sulphate highly purified "biosynthetic" L-threonine dehydratase from *E. coli* K-12. 26 μg protein was placed in the tube.

Fig. 2. Determination of molecular weight of subunit of highly purified "biosynthetic" L-threonine dehydratase from *E. coli* K-12. The following proteins were used as markers: 1, cytochrome *c* (M_r, 14 000); 2, lysozyme (M_r, 14 390); 3, trypsin (M_r, 23 000); 4, pepsinogen (M_r, 41 000); 5, catalase (M_r, 60 000); 6, human serum albumin (M_r, 65 000); 7, ox serum albumin (M_r, 69 000); the relative electrophoretic mobility of enzyme under study is denoted by a square.

several polypeptides which are so strongly linked that they do not separate by treatment of the enzyme with dodecyl sulphate in the presence of 2-mercaptoethanol (2% dodecyl sulphate + 1% 2-mercaptoethanol, 37°C, 3 h). It should be emphasized that before such treatment of the enzyme preparation containing the stabilizing buffer, the former should be dialyzed against water. (Dialysis against 0.67% 2-mercaptoethanol solution led to the same result.) On the other hand, if the undialyzed enzyme preparation was treated with dodecyl sulphate in the presence of 2-mercaptoethanol there appeared 2—3 protein bands on the electrophorograms.

Molecular multiplicity

As seen in Fig. 3, the preparation of "biosynthetic" L-threonine dehydratase from *E. coli* K-12 purified by the abovementioned method during disc electrophoresis on 7% polyacrylamide gel pH 7.5, separated at least into 11 bands, if the bands were stained for protein. (In this case about 300 μg of protein was placed on the tube; when we used the lower amount of protein, the number of the protein bands was fewer but was still rather large). The number of the protein bands as well as their relative intensity was changed if electro-

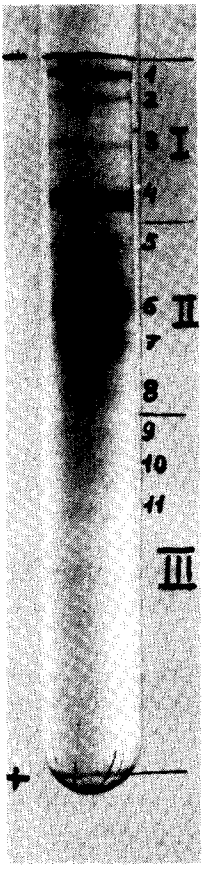


Fig. 3. Disc electrophoresis on polyacrylamide gel of highly purified "biosynthetic" L-threonine dehydratase from *E. coli* K-12. Tris-veronal buffer (pH 7) was used as electrode buffer; the separation was carried out at pH 7.5. 300 μg of protein was placed on the tube.

phoresis was carried out at pH 8.9. This indicates that these molecular enzyme forms are interconvertible.

Previously determined values for the molecular weight of different molecular enzyme forms by electrophoresis on gradient polyacrylamide gel have shown that the first band was a polymer with molecular weight above 10^6. Previous study using the ultracentrifuge confirmed the formation of polymer with a high molecular weight at a protein concentration of about 0.5 mg/ml.

The green zone in the process of gel staining for the catalytical activity overlapped the 5th to the 8th bands (see Fig. 3); the first band was also richly coloured. After light exposure of the treated gel, all electrophorogram bands which may be developed as a result of protein staining were red. Hence, all the protein bands which divided during disc electrophoresis on polyacrylamide gel contained pyridoxal 5′-phosphate but not all of them had catalytical activity. (It was possible, however, that the activity of some protein bands was so small that it could not be seen because the method was not sufficiently sensitive or was possibly caused by inactivation of some enzyme subforms in the process of purification.)

It can be deduced from examination of the data obtained by us from disc electrophoresis on polyacrylamide gel untreated and treated with dodecyl sulphate enzyme preparations, that the enzyme under study consists of a set of multiple forms. As qualitative features during electrophoresis on gradient polyacrylamide gel or standard 7% gel did not differ from each other, it may be concluded that the enzyme multiplicity is determined by degree of polymerization.

It should be mentioned that enzyme multiplicity may not result from partial proteolosis of the enzyme in the process of purification. Indeed, proteolosis must inevitably lead to the formation of several polypeptides which in the process of electrophoresis in the presence of dodecyl sulphate can be seen as separate bands. Moreover, the same molecular multiplicity was observed as a result of disc electrophoresis of crude cell extracts and also enzyme preparations on all the abovementioned purification steps (i.e. the zones which were stained green).

pH-Optimum and substrate specificity

The purified enzyme had an optimum pH range of 9.4—9.6 (determined at saturating substrate concentrations); the same pH optimum was found for the enzyme in crude extracts [1].

The enzyme catalyzed dehydration not only of L-threonine but also of L-serine; this is inherent for all the studied L-threonine dehydratases (for "biosynthetic" as well as for "biodegradative" isoenzymes) [7]. The enzymatic activity with L-serine as substrate was 50—100-fold lower than with L-threonine. On the basis of literature analysis [7] we may assume that the chemical mechanism of α,β-elimination reaction will cause at least a slow L-serine dehydratase reaction for any L-threonine dehydratase; just as any L-serine dehydratase must have L-threonine dehydratase activity which may be high or low. The study of allosteric regulation of the enzyme activity or the regulation of its synthesis or degradation in the cell may be a criterium for relating the activity of the dehydratation of one of the two α-amino-β-hydroxy acids to any specified enzyme. Indeed, by this method it has been shown [29] that in *E. coli* K-12 there are individual L-serine and L-threonine dehydratases.

Kinetic properties

Previous studies [1] on the crude extract of *E. coli* K-12 cells have shown that the initial reaction rate (v) versus initial substrate concentration ($[S]_0$) plots have a very complicated shape (two intermediate plateaux, i.e. four inflexion points), which were determined by the pH-value of the reaction mixture and the enzyme protein concentration. It was also shown [1] that such a type of reaction kinetics is not determined by the presence in the crude extracts of "biosynthetic" L-threonine dehydratase isoenzymes which could have different kinetic properties.

As seen in Fig. 4, kinetic plots at pH 9.3 did not qualitatively differ from the ones obtained by using of the crude extracts. At a protein concentration of 2.5 μg/ml (Curve 4) there were 4 inflexion points (two intermediate plateaux). The increase of the enzyme concentration to 6.0 μg/ml (Curve 3) and to 15.5 μg/ml (Curve 2) led to the disappearance of the first intermediate plateau,

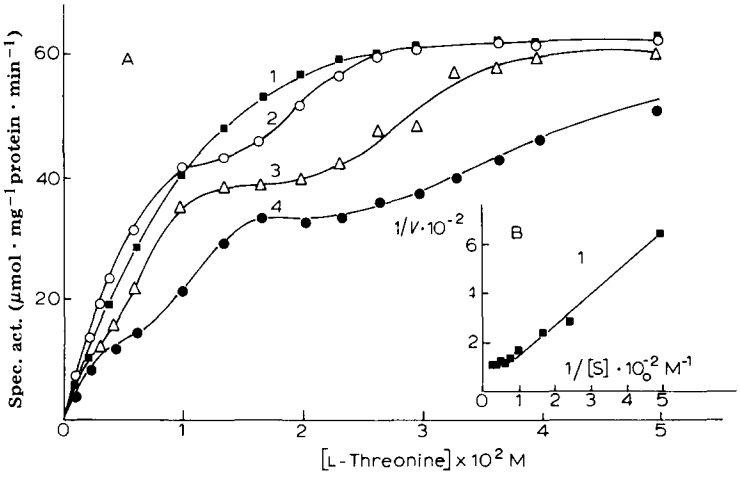


Fig. 4. Dependence of spec. act. of highly purified "biosynthetic" L-threonine dehydratase from *E. coli* K-12 on L-threonine concentration at pH 9.3 and different enzyme protein concentrations. A, data represented in the coordinates of v versus $[S]_0$; B, the same in the double reciprocal coordinates. 1 and 2, the protein concentration was 15.5 μg/ml; 3-6 μg/ml; 4-2.5 μg/ml; 1, in the presence of L-isoleucine ($2.6 \cdot 10^{-4}$ M), 2, without L-isoleucine.

manifested at low substrate concentrations, and clearly demonstrated the second intermediate plateau which shifted to the side of higher substrate concentrations.

From Fig. 4 it can be seen that L-isoleucine ($2.6 \cdot 10^{-4}$ M) at the enzyme concentration of 15.5 μg/ml abolished the intermediate plateau, activating the enzyme at substrate concentrations at which the intermediate plateau became visible, but did not change the value of V and did not lead to "normalization" of the kinetic plot; this is distinctly seen from the data represented in the double reciprocal coordinates. The calculation of Hill's coefficient values by a graphical method has shown that the plot was not hyperbolic, and did not coincide with Hill's equation. Hence, in this case as well, there was no noticeable difference between the effect of L-isoleucine on purified enzyme and on the enzyme in the crude cell extracts.

It should be emphasized that the ionic strength of the buffer in which the enzyme was dissolved strongly influenced the shape of the kinetic plots of product accumulation versus reaction time. When the enzyme dissolved in the stabilizing buffer at high ionic strength (1 M potassium phosphate plus 1 M KCl) the plots of time-dependent product accumulation had a linear shape. Thus, in the above experiments, the values for specific activity were calculated from initial reaction rates. On the other hand, if the enzyme dissolved in the stabilizing buffer containing 0.23 M KCl (i.e. at lower ionic strength) was used, the shape of the plots of product accumulation versus reaction time differed from linear one (the lag period was observed).

For homogeneous enzyme the plots of v versus $[S]_0$ at pH 7.4, as well as for the enzyme in the crude extracts from *E. coli* K-12, had a typical sigmoidal shape; L-isoleucine ($3 \cdot 10^{-3}$ M) shifted the sigmoidal part of this plot towards higher substrate concentrations but did not necessarily change the value of V.

In a theoretical analysis [9,10] for allosteric enzymic systems for which equilibrium between oligomeric forms occurs, this is slower than the enzymatic or comparable reaction, and the allosteric transition in the oligomer itself is at high velocity, showing that in such systems the kinetics of allosteric interactions may be expressed in a different way, possibly by v versus $[S]_0$ plots of a number of inflexion points (one or two intermediate plateaux). If oligomeric systems (for which a rate of achievement of equilibrium between different enzymic forms is slower than the rate of enzymatic reaction [9]), are analysed, it can be seen that the equilibrium between the enzyme forms for practical purposes does not change during a time essential for initial reaction rate determination. Similar systems may be expressed formally as heterogeneous ones, i.e. complicated shapes for v versus $[S]_0$ plots may result from the combination of more simple kinetic characteristics (a hyperbola, a sigmoid) inherent to individual components which differ from one another by kinetic parameters. However, in spite of enzyme preparations containing non-interconvertible enzymic forms, e.g. isoenzymes, for a slowly dissociating enzyme system, a ratio between the oligomeric forms and features of kinetic plots depends on the "prehistory" of the system, i.e. on the experimental conditions at which it was restored.

As seen in Fig. 5, practically the shape of the v versus $[S]_0$ plot at pH 8.3 did not depend on the used enzyme concentration in the reaction mixture (the enzyme stock solution concentration was 0.075 mg/ml); at this pH value only one intermediate plateau was demonstrated on the kinetic curve.

If the enzyme stock solution was initially diluted the intermediate plateau on the v versus $[S]_0$ plot disappeared, and the value of V decreased to some extent (see Fig. 6). An analysis of these kinetic curves in the Lineweaver-Burke's coordinates had shown that in the second case (Plot 2) when the enzyme stock solution was stored in a diluted state before the measurement of enzymatic activity, the v versus $[S]_0$ plot had a tendency to kinetically revealed "negative" cooperativity [11].

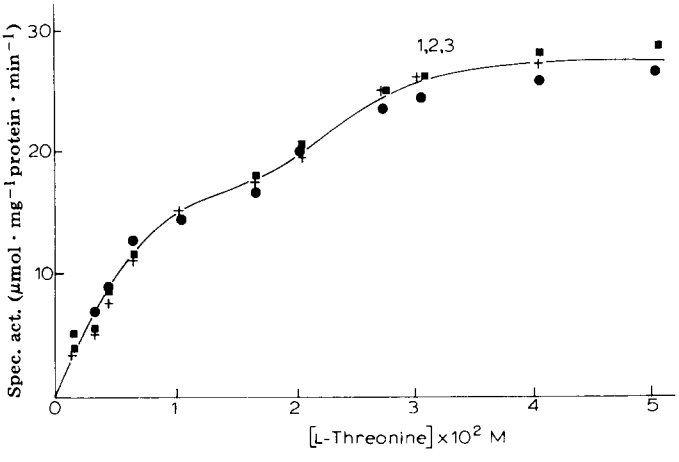


Fig. 5. Dependence of spec. act. of highly purified "biosynthetic" L-threonine dehydratase from *E. coli* K-12 on L-threonine concentration at pH 8.3 and at different enzyme protein concentrations in the reaction mixture. 1, 1.1 μg/ml; 2, 2.7 μg/ml; 3, 7.0 μg/ml.

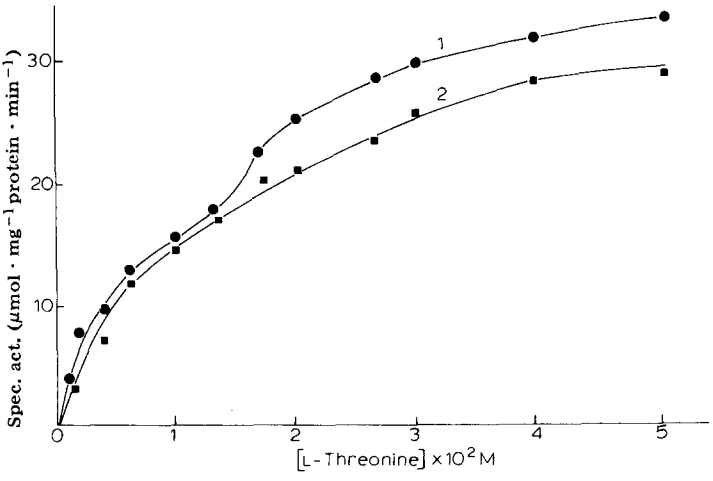


Fig. 6. Dependece of specific activity of highly purified "biosynthetic" L-threonine dehydratase from *E. coli* K-12 on L-threonine concentration at pH 8.1 and at different methods of enzyme dilution from stock solution. 1, Enzyme concentration in stock solution was 0.19 mg/ml; enzyme protein concentration in the reaction mixture was 1.5 μg/ml; 2, the enzyme stock solution (0.19 mg/ml) was 6-fold dilution (0.031 mg/ml) and stored for 12 h at 0—4°C. Enzyme protein concentration in the reaction mixture was 1.0 μg/ml.

The comparison of the data represented in Fig. 5 and Fig. 6 gave additionally confirmation of the idea that the enzyme under study was a set of oligomeric forms differing by the degree of polymerization and by kinetic properties, and that the rate of equilibrium installation between these forms was distinctly lower than the rate of the catalytic reaction.

In order to prove that the process of equilibrium installation was slow, the following experiment was carried out. Two samples of homogeneous enzyme were subjected to disc electrophoresis on 7% polyacrylamide gel. One of the gels was protein-stained (see Fig. 3), and from the second gel zones corresponding to the bands 1 + 2 + 3 + 4 (the zone I), to the bands 5 + 6 + 7 + 8 (the zone II) and to the bands 9 + 10 + 11 (the zone III) were cut out. The enzymes from each zones were by grinding and twice extracting with the stabilizing buffer (pH 7.7); the gels were separated by centrifugation, and after incubation for 30 min or 14—15 h (at 0—4°C) the enzymes were once more subjected to disc electrophoresis under the same conditions. It was shown that for the enzyme solution stored during 30 min the protein bands in zones I, II and III after the second electrophoresis had the same electrophoretical mobilities. On the other hand, storage of the enzyme isolated from Zone II for 14—15 h led to the appearance of band 2 on the protein stained gel; this band is a characteristic feature of zone I; the other bands were characteristic features of zone II. Thus, the enzyme underwent a process of slow polymerization.

It was very interesting to study the kinetics of the reaction catalyzed by individual protein bands. Unfortunately, we could not isolate the protein from individual bands because the difference between the values of electrophoretical mobilities of certain protein bands was small. Fig. 7 shows the kinetics of the reaction catalyzed by proteins isolated from zones I and II. (The proteins from gels were extracted with buffer, pH 8.0, which was used for kinetic study). As

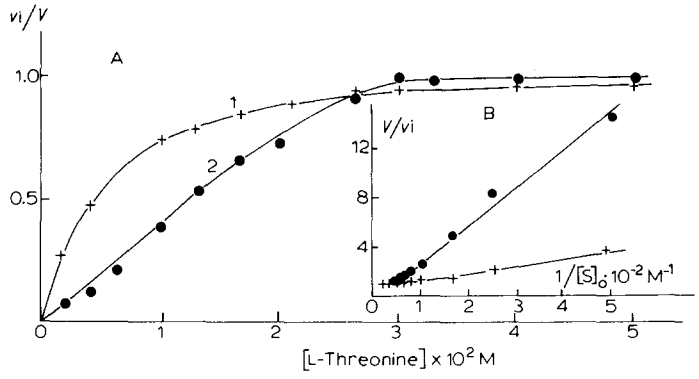


Fig. 7. Dependence of relative enzyme activity of different subforms of "biosynthetic" L-threonine dehydratase from *E. coli* K-12 on L-threonine concentration pH 8.3. A, data represented in the coordinates of v versus $[S]_0$; B, the same in double reciprocal coordinates. 1, Zone II (bands 5 + 6 + 7 + 8, see Fig. 3), 2, Zone I (bands 1 + 2 + 3 + 4, see Fig. 3).

seen in Fig. 7 in these cases the intermediate plateau on v versus $[S]_0$ plots was absent; Curve 1 (for proteins from zone II) had a typical hyperbolic shape; $K_m = 5.7 \cdot 10^{-3}$ M (see Fig. 7B), and Curve 2 (for proteins from zone I), not being sigmoidal at the same time, was not hyperbolic (the value of $[S]_{0.5} = 1.2 \cdot 10^{-2}$ M). These data suggested that in a complete system an appearance of one intermediate plateau on the v versus $[S]_0$ plot at pH 8.3 may reflect the molecular heterogeneity of the enzyme system, the components of which (different oligomeric enzyme forms) have different catalytical parameters, and the rate of equilibrium installation between them being small in comparison with the rate of catalytical reaction.

It should be noted that the results represented in Fig. 7 should be considered as preliminary, since in the process of cutting out protein bands from polyacrylamide gels it was impossible to isolate individual proteins and since after isolation from the gels, the protein concentration in the stock solutions, and hence in the reaction mixtures, was very low in comparison with the enzyme concentration used while studying the dependence of v on $[S]_0$ for the complete system.

Discussion

As follows from the experimental data, "biosynthetic" L-threonine dehydratase from *E. coli* K-12 is a system of molecular forms where each individual form possibly differs by the degree of oligomerization and kinetic parameters.

A multiplicity of microbial L-threonine dehydratase had already been shown. It had been well documented for "biodegradative" isoenzyme from *E. coli* [12] and from *Clostridium tetanomorphum* [13] and also for "biosynthetic" L-threonine dehydratase from *Azotobacter vinelandii* [14]. However, for "biosynthetic" isoenzyme from *E. coli* strain which differs from the strain used in this study, it was shown that purified enzyme consisted of a dimer ⇋ tetramer system with a high equilibrium attainment rate [15] or a system of two other oligomeric forms [16]; for analogous enzyme from *R. rubrum* [17],

it was observed that there exists not only dimer and tetramer forms but also an octamer form.

The most interesting feature of the system is the slowness at which equilibrium is achieved between the oligomeric forms; the rate of the process depends, first of all, on the pH value. The appearance of two intermediate plateaux on the kinetic curves at pH > 9 and dependence of their position and the degree of expression on the concentration of enzyme protein in the reaction mixture is, perhaps, due to an increase in the equilibrium installation rate between certain oligomeric forms with increasing pH values. On the other hand, it may be due to the increase in the number of oligomeric and/or change of their kinetical parameters.

The nature of the buffer may also influence the shape of the v versus $[S]_0$ plots as was distinctly demonstrated for "biodegradative" L-threonine dehydratase from the same strain of *E. coli* [18]: in the phosphate buffer these plots had a complicated non-hyperbolic shape, without intermediate plateaux, but in phosphate-carbonate buffer with the same ionic strength and pH value these plots had one distinct intermediate plateau.

Considering that the equilibrium installation between different oligomeric forms of "biosynthetic" L-threonine dehydratase from *E. coli* K-12 is slow, it may be supposed that in the proces of enzyme purification it would be possible to isolate enough stabile oligomeric forms in individual state. The different number of molecular forms of the enzyme obtained by us and other authors at the present moment can not be explained. However, it is possible that the abovementioned authors used such purification methods that led to the isolation of individual oligomeric forms or to a system with a fewer number of those forms.

Perhaps, each form is featured by "simple" kinetics, i.e. a hyperbolic or sigmoidal shape of the v versus $[S]_0$ plot, and the complicated shape of the resulting curve is reflected by the summing up of kinetic parameters for each of the forms. The phenomenon of slow equilibrium installation between oligomeric forms having different kinetic parameters is sufficient in itself to explain the highly complicated shape of v versus $[S]_0$ plots, i.e. the appearance on these plots of two intermediate plateaux at pH > 9, which was observed in the experiments with homogeneous enzyme as well as in the experiments with the enzyme in the crude cell extracts [1]. A theoretical consideration of allosteric enzyme systems with a slow equilibrium installation process between catalytically active forms shows [9,10,19] that in such systems at certain values of kinetic parameters a complicated shape of curves of v versus $[S]_0$ or v versus initial allosteric effector concentration ($[F]_0$) may be observed: plots with sigmoidality, plots with maximum and minimum simultaneously etc. For such systems a "hysteretic" reaction catalysed by them is postulated which is possible to demonstrate at certain experimental conditions, i.e. a deviation of plots of time-dependent product accumulation from linearity [10].

The deviation of kinetic plots of product accumulation versus reaction time from linear shape in the presence of L-isoleucine was shown for "biosynthetic" L-threonine dehydratase from *E. coli* [20], *B. subtilis* [21] and *Rhodopseudomonas spheroides* [22], which indicates hysteretic feature [23] of the reaction catalyzed by the enzyme.

Acknowledgement

We thank Dr B.I. Kurganov (Laboratory of Biochemistry, the All-Union Vitamin Research Institute, Moscow) for helpful discussion of the manuscript.

References

1 Kagan, Z.S. and Dorozhko, A.I. (1973) Biochim. et Biophys. Acta 302, 110—128
2 Lowry, O.H., Rosebrough, N.S., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265—275
3 Davis, B.J. (1964) Ann. N.Y. Acad. Sci. 121, 404—427
4 Maurer, H.R. (1968) Disk-elektrophorese, parts 2,3, Walter de Gruyter and Co., Berlin
5 Feldberg, R.S. and Datta, P. (1970) Science 170, 1414—1416
6 Laemmli, U.K. (1970) Nature 227, 680—685
7 Kagan, Z.S. and Tomova, V.S. (1968) Uspekhi Sovremennoy Biologii 66, 315—338
8 Isenberg, S. and Newmann, E.D. (1974) J. Bacteriol. 118, 53—58
9 Kurganov, B.I., Dorozhko, A.I., Kagan, Z.S. and Yakovlev, V.A. (1975) J. Theoret. Biol. (Part II) in the press
10 Kurganov, B.I., Dorozhko, A.I., Kagan, Z.S. and Yakovlev, V.A. (1975) J. Theoret, Biol. (Part III), in the press
11 Kurganov, B.I., Kagan, Z.S., Dorozhko, A.I. and Yakovlev, V.A. (1974) J. Theoret. Biol. 47, 1—41
12 Phillips, A.T., Wanger, P.D., Rabinovitz, K.B., Shada, J.D., Wood, W.A. (1968) in Chemistry and Biology of Pyridoxal Catalysis, pp. 331—345, Nauka, Moscow
13 Whiteley, H.R. (1966) J. Biol. Chem. 241, 4890—4897
14 Kretovich, W.L. and Loseva, L.P. (1973) Mikrobiologiya 42, 599—607
15 Galhoun, D.H., Rimerman, R.A. and Hatfield, G.W. (1973) J. Biol. Chem. 248, 3511—3516
16 Grimminger, H., Rahimi-Laridjani, I., Koerner, K. and Lingens, F. (1973) FEBS Lett. 35, 273—275
17 Feldberg, R.S. and Datta, P. (1971) Eur. J. Biochem. 21, 438—446
18 Sinelnikova, E.M., Dvorezkova, T.V. and Kagan, Z.S. (1975) Biokhimiya, 40, 645—651
19 Kurganov, B.I., Dorozhko, A.I., Kagan, Z.S. and Yakovlev, V.A. (1975) J. Theoret. Biol. (Part I), in the press
20 Hatfield, G.W. (1971) Biochem. and Biophys. Res. Commun. 44, 464—470
21 Hatfield, G.W. and Umbarger, E.H. (1970) J. Biol. Chem. 245, 1748—1754
22 Barrit, G.E. and Morrison, J.F. (1972) Biochim. et Biophys. Acta 284, 508—520
23 Frieden, C. (1970) J. Biol. Chem. 245, 5788—5799