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EFFECTS OF K^+ ON THE CATALYTIC AND REGULATORY PROPERTIES OF HOMOSERINE DEHYDROGENASE OF *PSEUDOMONAS FLUORESCENS*

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SUMMARY

Partially purified homoserine dehydrogenase (L-homoserine:$NADP^+$ oxidoreductase, EC 1.1.1.3) isolated from *Pseudomonas fluorescens* requires K^+ for its stability. At 1 mM K^+, the apparent first-order rate constant for enzyme inactivation at 25° was 25-fold higher than that observed at 20 mM K^+. A dissociation constant, K_D, of 3 mM of the ion–enzyme complex was calculated.

Following inactivation of the enzyme by depletion of K^+, the activity could be regenerated to a large extent by incubation with KCl and $NADP^+$; no other cation was nearly as effective as K^+.

Binding of K^+ on the enzyme molecule also plays an important part in the regulation of enzyme activity by the allosteric modifier L-threonine. At 1 mM K^+, the catalytic activity was strongly inhibited by 10 mM L-threonine, whereas, at 10 mM K^+ concentration in the assay mixture, L-threonine inhibition was almost completely abolished; threonine and K^+ were kinetically competitive. It is proposed that K^+ can induce specific conformational changes in the protein molecule that are either sensitive or insensitive to feedback inhibition control; in addition, the cation is necessary for the maintenance of the protein structure to ensure catalytic activity.

INTRODUCTION

The amino acid homoserine is the branch-point precursor for the synthesis of methionine, threonine, and isoleucine[1]. Thus the enzyme, homoserine dehydrogenase (L-homoserine:$NADP^+$ oxidoreductase, EC 1.1.1.3), which catalyzes the synthesis of homoserine from aspartate β-semialdehyde, is an important control point for the regulation of biosynthesis of these amino acid end products. In bacteria, two general mechanisms, repression of enzyme synthesis and feedback control of enzyme activity, are known to control this enzymatic reaction, although considerable variations of these basic themes have been uncovered in a wide variety of bacterial species[2,3]. Nevertheless, a common feature of all homoserine dehydrogenases tested thus far is

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that both L-threonine and L-cysteine are potent inhibitors of the activity of this enzyme[2-4]. The roles of these feedback modifiers in the overall control patterns of this biosynthetic pathway have been discussed[2,3,5]. In this communication we report the requirement of monovalent cations, especially K^+, for the maintenance of catalytic activity of the homoserine dehydrogenase from *Pseudomonas fluorescens*. In addition, we present experimental evidence to show that the regulatory effects of L-threonine on the enzyme activity can be greatly influenced by the concentrations of K^+ in the assay mixture. At low K^+ concentrations the enzyme remains highly sensitive to feedback inhibition by L-threonine, whereas, at high K^+ levels the inhibitory effect is completely abolished. The relevance of these observations in the context of biological control mechanisms, and a comparison of these effects with the other homoserine dehydrogenases are included in the discussion.

MATERIALS AND METHODS

Bacterial strain and growth conditions

P. fluorescens, Strain 14, was kindly donated by Dr. R. H. Olsen and was maintained on agar slants. Cells were cultivated aerobically at 25° in the minimal medium of VOGEL AND BONNER[6] containing 0.2% glucose as the sole carbon source. A 250-l fermentor (Fermacell, New Brunswick Instrument Company) was used for large-scale production of bacteria. Cells were harvested at the end of the logarithmic growth phase, and were washed once in 50 mM potassium phosphate buffer (pH 7.5) containing 1 mM β-mercaptoethanol. If not used immediately the cell-paste, with an overlayer of buffer, was stored at −15°.

Chemicals

All amino acids were purchased from Calbiochem, Mann Research Chemicals or Sigma Chemical Company. Sodium salts of $NADP^+$ and NADPH were obtained from Sigma. Analytical grade KCl was bought from the J. T. Baker Company. All other chemicals were of reagent grade. The pH of all solutions was adjusted either with Tris base or with phosphoric acid.

Enzyme assay

The homoserine dehydrogenase activity was measured spectrophotometrically[7] using a Gilford 2000 recording spectrophotometer at a full-scale deflection of 0.1 or 0.2 absorbance unit. The temperature was 25 ± 1°. All assays were carried out in the direction of homoserine + $NADP^+$ → aspartate β-semialdehyde + NADPH. The reaction mixture contained, in a final volume of 1 ml, the following components in μmoles: Tris–HCl buffer (pH 8.4), 100; EDTA, 1; $NADP^+$, 0.4; DL-homoserine, 20; and rate-limiting concentration of enzyme. All assays were linear for at least 5 min. Since D-homoserine is not a substrate of this enzyme all calculations are based on the L-isomer concentration. Enzyme activity is expressed as $\Delta A_{340\ nm} \cdot min^{-1}$. Specific activities are expressed as units/mg protein where the concentration of protein was estimated by the spectrophotometric method of WARBURG AND CHRISTIAN[8].

Enzyme preparation

Unless otherwise specified all operations were carried out between 0 and 4°.

50 g of cells, wet weight, suspended in 150 ml of 50 mM potassium phosphate buffer (pH 7.5) containing 1 mM β-mercaptoethanol were sonically disrupted for 15 min in 1-min pulses and the extract was centrifuged at 45 000 × *g* for 70 min. To the supernatant fluid a 2% solution of protamine sulfate in the above buffer was added slowly until a protamine sulfate to protein ratio of 0.15 mg/mg was attained. The precipitate formed was removed by centrifugation at 45 000 × *g* for 20 min. Solid $(NH_4)_2SO_4$ was added to the supernatant fluid to achieve 30% saturation. After stirring for an additional 30-min period, the suspension was centrifuged at 45 000 × *g* for 20 min and the precipitate was discarded. The supernatant solution was brought to 55% saturation with solid $(NH_4)_2SO_4$ and was centrifuged as above. The precipitate containing most of the enzyme activity was dissolved in a small volume of 50 mM potassium phosphate–1 mM β-mercaptoethanol (pH 7.5) and was dialyzed at 25° for 12 h against 100 vol. of the phosphate buffer.

The dialyzed solution was applied on a 40 cm × 2 cm DEAE-cellulose column equilibrated at 25° with 50 mM potassium phosphate buffer (pH 7.5) with 1 mM β-mercaptoethanol. The column was eluted with a 400-ml linear gradient of KCl from 0 to 0.4 M in the above buffer at a flow rate of 50 ml/h, and 5-ml fractions were collected. Fractions containing maximum specific activities were pooled.

The enzyme solution was concentrated to a small volume in the Schleicher and Schuell collodion bag apparatus under reduced pressure, and was applied on top of a 50 cm × 1.4 cm Sephadex G-150 column equilibrated at 0° in 50 mM potassium phosphate buffer (pH 7.5) containing 1 mM β-mercaptoethanol. The column was eluted with the same buffer at a flow rate of 10 ml/h, and 5-ml fractions were collected. Fractions containing highest specific activities were pooled. This preparation had a specific activity of 1.43 $\Delta A_{340\ nm} \cdot min^{-1} \cdot mg^{-1}$ representing a 95-fold purification over the crude extract. The overall yield was 70%. Electrophoresis on polyacrylamide gel according to the method of DAVIS[9] and comparison of specific activities to those of other homoserine dehydrogenases obtained in pure form[7,10], indicated that the *P. fluorescens* enzyme was about 15% pure. The enzyme was free of aspartate β-semialdehyde dehydrogenase activity. The final preparation (1.68 mg protein per ml) was divided up in small aliquots and was stored frozen in the presence of 20% glycerol. Unless otherwise mentioned this preparation was used in all experiments reported here.

RESULTS

Properties of the enzyme

In contrast to *Escherichia coli* K12, where the homoserine dehydrogenase and aspartokinase activities are associated with a single protein species[10], the dehydrogenase of *P. fluorescens* can be separated from the aspartokinase activity during the purification procedures. Fig. 1 shows the elution profile of the two enzyme activities upon fractionation on a DEAE-cellulose column. Similar results have also been reported for the enzymes from *Pseudomonas putida* by ROBERT-GERO *et al.*[12]. From the regulatory point of view, the Pseudomonas enzymes are, therefore, quite distinct from the multifunctional isoenzymes observed in a wide variety of bacteria belonging to the coliaerogenes group[3].

The partially purified homoserine dehydrogenase of *P. fluorescens* revealed

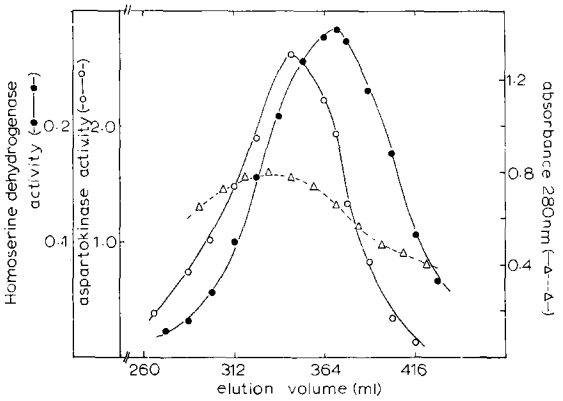


Fig. 1. Separation of aspartokinase and homoserine dehydrogenase activities from *P. fluorescens* by DEAE-cellulose chromatography. A 55-ml sample of crude extract was applied to a DEAE-cellulose column (35.3 cm × 2.2 cm) previously equilibrated with 50 mM potassium phosphate buffer (pH 7.5) containing 1 mM β-mercaptoethanol. The column was eluted with a linear gradient from 0 to 400 mM KCl in the above buffer. The total gradient volume was 600 ml and 6.5-ml fractions were collected at a flow rate of 35 ml/h. Homoserine dehydrogenase activities of 0.1-ml aliquots were assayed by the routine method described in the text; the activities are expressed as $\Delta A_{340\ nm} \cdot min^{-1} \cdot ml^{-1}$. For aspartokinase, activities of 0.1-ml aliquots were measured by the hydroxamate assay reported previously[11]. The activities are expressed as $\Delta A_{540\ nm} \cdot ml^{-1}$ per 30 min. Absorbance at 280 nm for protein was recorded after an 11-fold dilution of the fractions in the phosphate buffer.

some interesting properties insofar as its stability is concerned. The enzyme was relatively more stable at 25° than at 4°. Addition of excess reducing agents such as β-mercaptoethanol or dithiothreitol, or the amino acids lysine and threonine, did not protect the enzyme from inactivation at 4°. However, the enzyme could be stored in 50 mM potassium phosphate buffer (pH 7.5) containing 1 mM β-mercaptoethanol

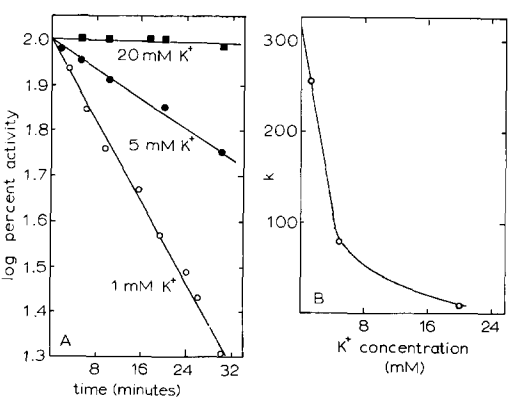


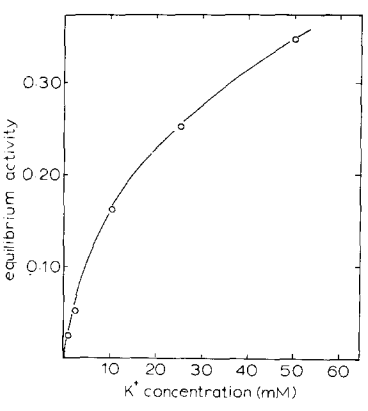
Fig. 2. Progressive inactivation of homoserine dehydrogenase at 25° in buffer solutions containing varying concentrations of K^+. Stock enzyme solution (1.68 mg/ml) was diluted 80-fold in 100 mM Tris–HCl buffer (pH 8.4) with 1 mM EDTA containing K^+ concentrations as specified. At times indicated, aliquots were withdrawn and enzyme activities were measured as described in MATERIALS AND METHODS. In 2A, the data are plotted as log percent activity *vs.* time of incubation at 25°. In 2B, the calculated first-order rate constants ($k \times 10^4\ min^{-1}$) for enzyme inactivation, obtained from the data given in 2A, are plotted against K^+ concentrations.

and 20% (v/v) glycerol either at 4° or kept frozen for several months without any significant loss of activity.

Effects of cations on the catalytic properties of the enzyme

The results presented in Fig. 2 show that following large dilutions (80-fold) of the stock enzyme solution in 100 mM Tris–HCl buffer (pH 8.4) with 1 mM EDTA, which resulted in the reduction in K^+ concentrations, a progressive loss of enzyme activity was observed. At 1 mM K^+, 75% of the activity was lost in 30 min at 25°, whereas, significantly less loss of activity was detected over the same period if the dilution buffer was supplemented with 20 mM K^+ (Fig. 2A). The kinetics of inactivation appears to be of the first-order with respect to the active enzyme. The calculated first-order rate constants for enzyme inactivation plotted against K^+ concentrations (Fig. 2B) show that, at 3 mM K^+, the rate of inactivation was reduced by one-half. Assuming that only one K^+ is bound per enzyme molecule and that the free enzyme is inactive, the K^+ concentration giving half-maximal inactivation can be taken as equivalent to the dissociation constant, K_D, of the ion–enzyme complex[13].

Since the concentration of K^+ was critical not only for the initial rate of enzyme inactivation but also for the extent of inactivation, it was desirable to estimate the true dependence of the enzyme for its K^+ requirements. Several aliquots of the stock enzyme solution were diluted in 100 mM Tris–HCl buffer (pH 8.4) *plus* 1 mM each of β-mercaptoethanol and EDTA containing various concentrations of KCl ranging from 1 to 50 mM. The mixtures were incubated at 25° for 3 h and the enzyme activities were measured at the end of this incubation period using the standard assay con-



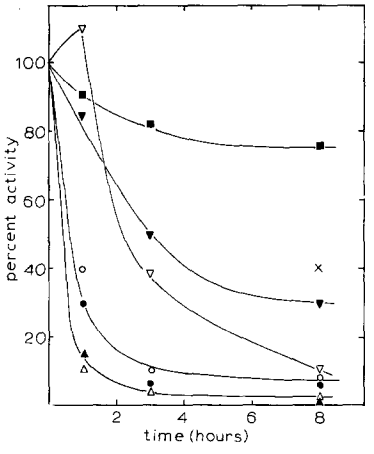


Fig. 3. Dependence of K^+ concentrations on the extent of inactivation of homoserine dehydrogenase. The experimental details are given in the text. The enzyme activities were assayed as described under MATERIALS AND METHODS. The activities remaining after the 3-h incubation period (equilibrium activity), expressed as $\Delta A_{340\text{ nm}} \cdot \text{min}^{-1} \cdot \text{ml}^{-1}$, were plotted against K^+ concentrations.

Fig. 4. Effects of various cations on the stability of homoserine dehydrogenase. The experimental details are given in the text. The enzyme activities were assayed as described under MATERIALS AND METHODS. ■—■, KCl; ▽—▽, LiCl; ▼—▼, NH_4Cl; ○—○, NaCl; ●—●, CsCl; ▲—▲, Tris–HCl; △—△, tetramethylammonium chloride. A single point, denoted by ×, was obtained by incubating enzyme in 125 mM RbCl for 8 h.

ditions. The 3-h incubation period was found to be sufficient to achieve the maximum inactivation at low K^+ concentrations employed. At 50 mM K^+, however, a further inactivation no more than 10%, was observed beyond the 3-h incubation period; nevertheless, the equilibrium activities obtained at the end of 3 h were used to plot the data shown in Fig. 3. The equilibrium activity *versus* K^+ concentration curve shows that even at 50 mM K^+ concentration, maximum protection against enzyme inactivation was not attained. In fact, a 25% loss in enzyme activity was observed in 8 h at 25° when the buffer solution contained 125 mM KCl (see Fig. 4).

In a separate series of experiments, the specific requirement of K^+, as opposed to general cation effect, for the maintenance of enzyme activity was examined. Aliquots of enzyme solution were diluted 80-fold at 25° to reduce the K^+ concentration to 0.6 mM in 100 mM Tris–HCl buffer (pH 8.4) containing 1 mM each of β-mercaptoethanol and EDTA, and were supplemented with various cations as the chloride salts at 125 mM each. The dehydrogenase activities of various aliquots were assayed at different time intervals up to a period of 8 h. The results are given in Fig. 4. Greater than 90% loss of enzyme activity was observed when the enzyme solutions were incubated at 25° for 8 h in the presence of $Tris^+$ (cationic species of Tris), tetramethylammonium ion, Cs^+, Na^+ or Li^+. Between 60 and 70% inactivation was recorded for the Rb^+ and NH_4^+, whereas, K^+ afforded the maximum protection against enzyme inactivation. These results suggest a relatively high degree of specificity for the latter cation for the stability of the dehydrogenase from *P. fluorescens*.

A number of preliminary experiments revealed that inactivation of the enzyme following a reduction in the K^+ concentration could be reversed to a significant extent by addition of KCl; the reactivation process was time dependent and the degree of reactivation was proportional to the concentration of KCl added. The kinetics of reactivation is shown in Fig. 5. In this experiment the stock enzyme solution was diluted to contain 2 mM K^+ in 100 mM Tris–HCl–1 mM EDTA buffer (pH 8.4) and was allowed to inactivate at 25° for 30 min. To three separate aliquots of this partially inactivated enzyme were added KCl, $NADP^+$, or both, and the reappearance of enzyme activities was followed with time. In the presence of 100 mM

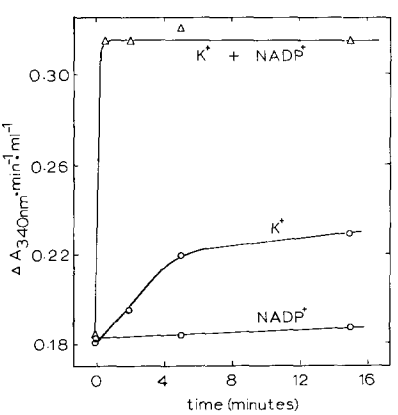


Fig. 5. Kinetics of reactivation by K^+ and $NADP^+$ of the inactive homoserine dehydrogenase. The experimental details are given in the text.

TABLE I

PROGRESSIVE INACTIVATION AND REACTIVATION OF HOMOSERINE DEHYDROGENASE OF *P. fluorescens*

Enzyme solution was diluted at 25° in 100 mM Tris–HCl buffer (pH 8.4) containing 1 mM EDTA, and 1 mM β-mercaptoethanol and 1.5 mM KCl. Two aliquots were taken out at the times specified: using one aliquot the enzyme activity was assayed according to the method described. To the second aliquot sufficient KCl and $NADP^+$ were added to bring the final concentration to 100 and 0.4 mM, respectively, and the activity was measured following a 30-min incubation at 25°. Activities are expressed as percent of zero time control.

Time (min)	*Enzyme activity*		
	(A) No preincubation	*(B) Preincubation with KCl + NADP*	*B/A*
0	100	100	1.0
10	63	72	1.14
20	44	63	1.44
40	23	58	2.50

KCl, a gradual reactivation occurred reaching a low equilibrium value after about 8 min at 25°. With 0.4 mM $NADP^+$, no significant reactivation could be detected. When both KCl and $NADP^+$ were present, a rapid appearance of enzyme activity was observed; the equilibrium activity was about 3-fold higher than that observed with KCl alone, and the maximum reactivation (50% of the initial activity) was complete in 30 sec after mixing the inactive enzyme with KCl and $NADP^+$. No adequate explanation is available for the synergystic effect of K^+ and $NADP^+$ in the reactivation of the enzyme.

Although the kinetics of reactivation was rapid in the presence of KCl and the coenzyme substrate, nevertheless, a complete reactivation of the inactive enzyme

TABLE II

REACTIVATION OF *P. fluorescens* HOMOSERINE DEHYDROGENASE BY VARIOUS CATIONS

Enzyme solution (1.68 mg/ml) was inactivated by dialyzing at 25° for 20 h against 1000 vol. of 50 mM Tris–HCl buffer (pH 7.5) containing 1 mM β-mercaptoethanol. 20-μl aliquots of the inactive enzyme were incubated for 60 min at 25° in a final volume of 0.9 ml containing the following components in μmoles: Tris–HCl (pH 8.4), 100; EDTA, 1; $NADP^+$, 0.4; and the cations as specified (as the chloride salts), 100 μmoles. Following incubation, the reaction was initiated by adding 20 μmoles of DL-homoserine in a volume of 0.1 ml. Reduction of $NADP^+$ was followed at 340 nm as described in the MATERIALS AND METHODS. All values are average of duplicate assays. Crystal radii of various ions, shown in the middle column, was taken from PAULING[14].

Cation added	*Radius* (Å)*	$\Delta A_{340\ nm} \cdot min^{-1} \cdot ml^{-1}$
$Tris^+$	—	0.010
K^+	1.33	0.193
NH^+_4	1.49	0.098
Rb^+	1.48	0.092
Na^+	0.95	0.079
Li^+	0.60	0.067
Cs^+	1.69	0.045
TMA^{+**}	—	0.022

* Crystal radii.
** TMA^+, tetramethylammonium ion.

was never observed. The data in Table I show that with progressive inactivation of the enzyme in low K^+, the absolute level of reactivation in the presence of KCl and $NADP^+$ was also decreased; the degree of reactivation (Column B/A in Table I) however was higher as the inactivation progressed with time.

To determine the cation specificity of the reactivation process an aliquot of the enzyme solution was dialyzed at 25° for 20 h against 1000-vol. of 50 mM Tris–HCl buffer (pH 7.5) with 1 mM β-mercaptoethanol to achieve almost complete inactivation. Aliquots of the inactive enzyme were allowed to reactivate by incubating for 60 min at 25° in 100 mM Tris–HCl buffer (pH 8.4) containing 1 mM EDTA, 0.4 mM $NADP^+$ and 100 mM each of the various cations. After the incubation period, enzyme activities were measured by the standard assay method. The results given in Table II indicate that K^+ was by far the most effective cation for the reactivation process; NH_4^+ and Rb^+ were moderately effective, whereas, small but significant reactivation was observed with each of the remaining cations except tetramethylammonium ion. It may be recalled that almost the same order of specificity among various cations was found to be most effective for the protection against enzyme inactivation of the K^+-depleted enzyme (*cf.* Fig. 4). It should be emphasized that the data presented in Table II only show the extent of reactivation at equilibrium, and not the rate of reactivation.

TABLE III

EFFECTS OF VARIOUS AMINO ACIDS ON THE HOMOSERINE DEHYDROGENASE ACTIVITY FROM *P. fluorescens*

Enzyme activities were measured as described under MATERIALS AND METHODS. Amino acid concentrations were 10 mM and the assay mixtures contained 2 mM K^+.

Addition	*% Enzyme activity*
None	100
L-Lysine	103
L-Valine	101
L-Methionine	102
L-Aspartate	87
L-Isoleucine	97
L-Cysteine	36
L-Threonine	31
L-Isoleucine + L-threonine	33

Effects of cations on the regulatory properties of the enzyme

With the single exception of the methionine-repressible homoserine dehydrogenase II–aspartokinase II complex from *E. coli* K12 (see ref. 10), the common property of all homoserine dehydrogenases tested thus far is their sensitivity to feedback inhibition by L-threonine. The data presented in Table III show that the *P. fluorescens* dehydrogenase is inhibited by L-threonine; L-cysteine is also a strong inhibitor of enzyme activity (also *cf.* ref. 4). No other amino acid related to the aspartate pathway influences the activity of this enzyme. In contrast to the enzyme from *Rhodospirillum rubrum*, where the inhibition of enzyme activity by L-threonine is reversed by L-isoleucine[15], the homoserine dehydrogenase from *P. fluorescens* is

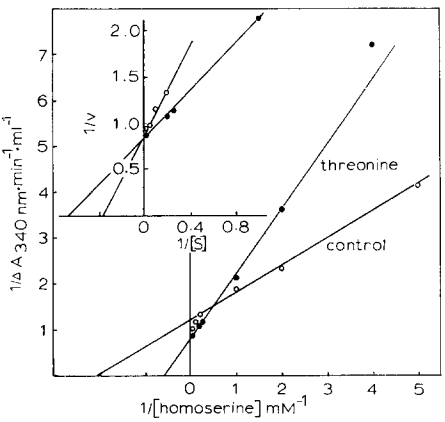


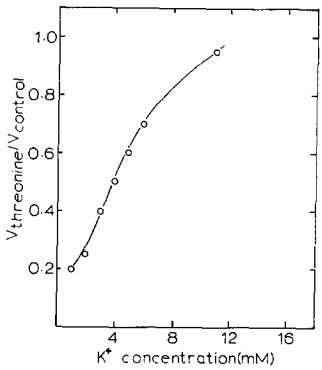
Fig. 6. Kinetics of inhibition by L-threonine of the homoserine dehydrogenase. Double reciprocal plots of initial velocity and homoserine concentration. Enzyme activities were assayed as described under MATERIALS AND METHODS. K^+ concentration was 2 mM during assay.

similar to the other bacterial enzymes in that no reversal of the threonine inhibition by isoleucine is observed (Table III).

The double reciprocal plots of initial velocities *versus* homoserine concentrations in the presence and absence of L-threonine reveal complex kinetic behavior (Fig. 6). With no threonine, the biphasic homoserine saturation curve showing two K_m components, K_m^L and K_m^H, was similar to that observed with two other homoserine dehydrogenases isolated from *R. rubrum*[15] and *Zea mays*[16]. When the enzyme activities were measured with 0.4 mM L-threonine, the double reciprocal plot was linear and showed only one K_m component (K_m^H). An apparently "competitive" kinetic relationship between L-threonine and L-homoserine was noted in which the v_{max} was unaffected (see inset of Fig. 6). In the presence of the inhibitor, however, the K_m^H for homoserine was decreased from 2.9 mM to approx. 1.5 mM; in other words, at high concentrations of homoserine, low levels of threonine appear to behave like an activating modifier, whereas, at low homoserine concentrations the amino acid was inhibitory. With the *P. putida* homoserine dehydrogenase, non-competitive kinetic relationships between threonine on one hand, and homoserine and aspartate β-semialdehyde on the other, have been reported by ROBERT-GERO *et al.*[12].

The sensitivity of the *P. fluorescens* dehydrogenase to threonine inhibition is critically influenced by the concentrations of K^+ present in the assay mixture. When the concentration of K^+ was 1 mM, an 80% inhibition of enzyme activity was observed at 10 mM L-threonine when assayed in the presence of 10 mM L-homoserine. With increasing concentrations of the cation, the inhibitory effect was progressively decreased; at 10 mM K^+, the threonine inhibition was almost completely abolished (Fig. 7). These results strongly suggest some competition between the cation and the amino acid modifier on the enzyme molecule. Indeed the Lineweaver–Burk plots shown in Fig. 8 clearly demonstrate that threonine and K^+ are kinetically competitive.

Since kinetic interactions were also observed between threonine and homoserine (*cf.* Fig. 6), it is expected that K^+ would also affect the enzyme activity by influencing the binding of substrate molecules on the enzyme. In fact, at 20 mM K^+



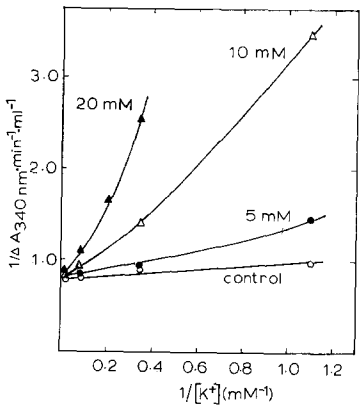


Fig. 7. Reversal of L-threonine inhibition by K^+. Enzyme activities were assayed in the presence or absence of 10 mM L-threonine as described under MATERIALS AND METHODS. Concentrations of K^+ were varied as indicated.

Fig. 8. Effect of increasing concentrations of K^+ on the kinetics of L-threonine inhibition. Double reciprocal plots of initial velocity and K^+ concentration. Enzyme activities were assayed in the presence or absence of several concentrations of L-threonine as specified.

the apparent K_m^L for homoserine was significantly less than that found when the K^+ concentration was 1 mM. With the limited data available, it is not possible at present to conclude whether the amino acid inhibitor and the cation occupy the same or overlapping binding sites on the enzyme molecules, or that they are indeed bound to different sites on the enzyme and interact indirectly through "allosteric transition" (see ref. 17). In the latter case, indirect interactions between homoserine, threonine, and K^+ would have to be invoked to explain the observed experimental data. It should be pointed out that K^+ does not alter the apparent K_m for $NADP^+$, at saturating concentrations of L-homoserine.

In view of the fact that, among the various monovalent cations, only K^+ was highly specific in preventing enzyme inactivation (*cf.* Fig. 4), and since K^+ was also

TABLE IV

EFFECTS OF VARIOUS CATIONS ON THE REVERSAL OF L-THREONINE INHIBITION OF HOMOSERINE DEHYDROGENASE FROM *P. fluorescens*

Enzyme activities, with or without 10 mM L-threonine, were assayed in the presence of 100 mM concentration of the cations (as the chloride salts) as specified. For details of assay see MATERIALS AND METHODS.

Addition	*Activity with threonine* / *Activity without threonine*
$Tris^+$	0.23
K^+	0.85
Rb^+	0.92
NH^+_4	0.30
Na^+	0.17
Li^+	0.20
Cs^+	0.16

by far the most effective cation in the reactivation of the inactive enzyme (*cf.* Table II), it was desirable to test if this were also true for the reversal of threonine inhibition of the dehydrogenase activity from *P. fluorescens*. Table IV summarizes these results. In these experiments enzyme activities were assayed in the presence of 100 mM concentrations of various cations, with and without 10 mM L-threonine. The data show only K^+ and Rb^+ were able to counteract the inhibitory effect of L-threonine on the activity of homoserine dehydrogenase.

DISCUSSION

There are numerous examples in the literature where certain monovalent cations have been shown to influence a variety of enzyme-catalyzed reactions. SUELTER[18] has recently compiled a list of more than sixty such enzymes where the mechanisms of activation appear to involve a functional ternary complex between the substrate, enzyme, and the monovalent cation in question. Although direct participation of the cations in the catalytic process may accompany some conformational changes in the protein structure, it seems likely that the cations may also play an important part by maintaining a specific conformational state of the protein necessary for maximum stability as well as for optimal catalytic potential[19]. Studies with the homoserine dehydrogenases from *R. rubrum*[7,15,20], *E. coli*[10,21–23], and the results obtained with the *P. fluorescens* enzyme presented here, clearly demonstrate that, in the absence of catalytic turnover of the substrate to product, the monovalent cations are able to combine with the protein and alter the physical state to influence both catalytic and regulatory behaviors.

It has been reported[7,20] that the homoserine dehydrogenase of *R. rubrum* does not have an obligatory requirement for K^+ for its activity, however, two different conformational states of the protein can be easily identified when the enzyme is subject to various treatments in the presence of either low K^+ (2 mM) or high K^+ (200 mM) concentration. In buffer containing low K^+, the *R. rubrum* dehydrogenase dissociates into fully active low-molecular-weight species; under these conditions the enzyme becomes susceptible to cold-inactivation. When the K^+ concentration of the enzyme solution is increased to 200 mM on the other hand, the enzyme appears to have a molecular weight of about 210 000 by gel filtration on Sephadex G-200, in contrast to a molecular weight 138 000 for the native enzyme in 50 mM K^+. Since the elution from Sephadex is more strictly proportional to the Stokes' radius of the molecule rather than on the actual molecular size[24], the apparent increase in the molecular weight of the homoserine dehydrogenase in high K^+ environment may be due to some "loosening" of the protein structure. This notion is also consistent with the other observation that, in 200 ml KCl, an additional free sulfhydryl group can be titrated with 5,5'-dithiobis-2-nitrobenzoic acid[20]. This latter observation indicates a relatively large change in the protein structure which results in the availability of a second reactive –SH group otherwise buried in the protein molecule in the absence of KCl.

With the *P. fluorescens* enzyme we have observed that the dehydrogenase has a strict requirement for K^+ to maintain its catalytic function. The enzyme is rapidly inactivated at 25° in Tris–HCl buffer containing 1 mM K^+ (Fig. 2), whereas, 20 mM K^+ protects the enzyme from significant inactivation. At low K^+, the first-order rate

constant for enzyme inactivation is approx. 25-fold higher than that observed at 20 mM K^+. Furthermore, if the enzyme is inactivated upon dilution of the cation, the activity can be regained to a large extent by adding KCl and $NADP^+$ in the buffer solutions (Fig. 5); no other cation is nearly as effective as K^+. The data given in Table I show that with progressive inactivation of the enzyme due to depletion of K^+, the maximum reactivation is also progressively decreased. Similar observations have been made by COHEN *et al.*[21], and by WAMPLER AND WESTHEAD[22] using the threonine-sensitive *E. coli* homoserine dehydrogenase. Collectively these results indicate that once the enzyme is freed of the bound cation, there is a time-dependent irreversible inactivation of the enzyme presumably due to loss of conformational stability. This notion is also supported by the other findings that, in contrast to $Tris^+$ and tetramethylammonium ion, Rb^+ and NH_4^+ are able to protect the Pseudomonas enzyme from inactivation to a significant extent, especially during prolonged incubation period (Fig. 4). Since the crystal radii of Rb^+ and NH_4^+ are not too different than that of K^+ (Table II) it is not surprising that these two cations may be able to afford a reasonable degree of protection against conformational changes that lead to loss of enzyme activity. The unusually small or large crystal radii of the other cations on the other hand, may preclude any such interaction with the enzyme molecule.

Apart from the protection against enzyme inactivation, the effect of K^+ on the ability of L-threonine to inhibit enzyme activity has also been elucidated in *R. rubrum*, *E. coli*, and in this report, with *P. fluorescens*. The homoserine dehydrogenase from *R. rubrum* is completely desensitized in buffer containing low K^+ concentration; addition of K^+ resensitizes the enzyme towards feedback inhibition by L-threonine and the reversal of threonine inhibition by L-isoleucine[15,20,25]. In 200 mM K^+, the inhibitory effect of threonine is reduced, whereas, stimulation by isoleucine is significantly enhanced[20]. With the *E. coli* dehydrogenase, a 2–3 fold higher L-threonine concentration is required for 50% inhibition of enzyme activity when the standard assay mixture is supplemented with 500 mM KCl[22]. Using equilibrium dialysis technique with [^{14}C]threonine, COHEN[10] has reported that at saturating concentrations of threonine, a maximum of six moles of threonine is bound per mole of enzyme; however, at unsaturating concentrations of the amino acid modifier, the binding of threonine is gradually eliminated with increasing concentrations of KCl ranging from 500 to 1000 mM present during the binding assay. Our results using the enzyme from *P. fluorescens* also show a competitive kinetic relationship between K^+ and the feedback inhibitor (Figs. 7 and 8). These experimental observations with three separate homoserine dehydrogenases clearly demonstrate that the binding of K^+ on the enzyme molecules plays a crucial part in the regulation of enzyme activity by the small-molecular-weight end product, L-threonine, Since the feedback inhibitor occupies an allosteric site on the *P. fluorescens* enzyme as is the case with the other two enzymes from *R. rubrum*[15,20] and *E. coli*[10], it is reasonable to conclude that K^+ can induce specific conformational changes in the protein molecule that are either sensitive or insensitive to feedback inhibition control.

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