MULTIPLE INTERACTIONS OF THREONINE WITH AN ASPARTOKINASE-HOMOSERINE DEHYDROGENASE COMPLEX

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It has been proposed that the activities of threonine-sensitive aspartokinase and homoserine dehydrogenase reside in one protein of Escherichia coli K-12 because both activities are fractionated together during multiple stages of purification and are stabilized by threonine and control of both activities by threonine inhibition was modified by a single mutation (1). Furthermore, the aspartokinase from a mutant lacking homoserine dehydrogenase activity was found to dissociate in the absence of threonine to a lower molecular weight unit with the loss of threonine control (2).

The present investigation concerns the effects of threonine upon an aspartokinase and homoserine dehydrogenase complex which is formed as a result of lysine supplementation of E. coli 9723 (3). Threonine has been found to cause first, at low concentrations, a reversible association of the complex into a higher molecular weight form and then to inhibit at higher concentrations each of the activities of the complex. Inhibition of these activities requires threonine concentrations 50 to 100 times greater than that needed for reversible association to a higher molecular

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weight form. In addition differences in the nature of the inhibition of the two activities have been observed which suggests multiple interactions of threonine with the enzyme complex.

Experimental Methods.--Cells of E. coli 9723 were cultured in salts-glucose medium (4) supplemented with L-lysine and L-methionine (0.5 mM each), until the optical density was 0.18 - 0.22 at 660 mμ (about 10 hours incubation at 30°). Cells were harvested, washed twice in 0.02 M potassium phosphate buffer (pH 6.8) containing 2 mM L-threonine and 0.03 M β-mercaptoethanol, and then resuspended in fresh buffer (20 to 30 mg of cell dry weight per ml) for disruption by sonic oscillation at 10 kc. After centrifugation of the crude cell extract for one hour at 150,000 x g, the supernatant liquid was fractionated by precipitation with ammonium sulfate between 35 and 48% saturation. The protein from this fraction was recovered by centrifugation and taken up in 0.02 M potassium phosphate buffer (pH 6.8) containing 0.03 M β-mercaptoethanol and 5 mM threonine, divided into 10 ml fractions and heated for 5 minutes at 55°. The denatured protein was removed by centrifugation, and ammonium sulfate was added to the supernatant to give a concentration of 50%. This fraction was then stored at 4° C. Just prior to use, the protein from this fraction was recovered by centrifugation and dissolved in buffer (0.02 M potassium phosphate, 0.03 M β-mercaptoethanol plus additives as will be indicated).

Gel filtration with a Sephadex G-100 was performed by the method of Andrews (5). Sucrose density gradient centrifugation was performed by the method of Martin and Ames (6) with a 5-20% sucrose gradient (in the above described buffers) for 210 minutes at 65,000 rpm in a Beckman Model L-2 with a 65 K rotor. Samples from the gradient were collected using an ISCO Density-Gradient Fractionator. All procedures were carried out at 4° C.

Aspartokinase was assayed as described by Lee et al. (7). Homoserine

dehydrogenase was assayed by measuring reduction of $NADP^+$ by changes in absorption at 340 mμ for a period of 1.5 minutes in a 1.0 ml mixture (pH 8.8) containing 100 mM Tris, 80 mM KCl, 50 mM L-homoserine and 0.4 mM $NADP^+$.

Results and Discussion.--The first observed effect with the lowest concentrations of threonine upon the threonine-sensitive aspartokinase and homoserine dehydrogenase complex from E. coli 9723 is a reversible association to form a higher molecular weight complex. As shown in Figure 1, the retention of the complex on Sephadex G-100 is decreased considerably in the presence of 20 μM threonine in the eluting buffer. The retention pattern in the absence of threonine corresponds to the molecular weight range of 80,000 - 100,000 whereas in the presence of threonine the enzyme complex is not appreciably retarded by the gel.

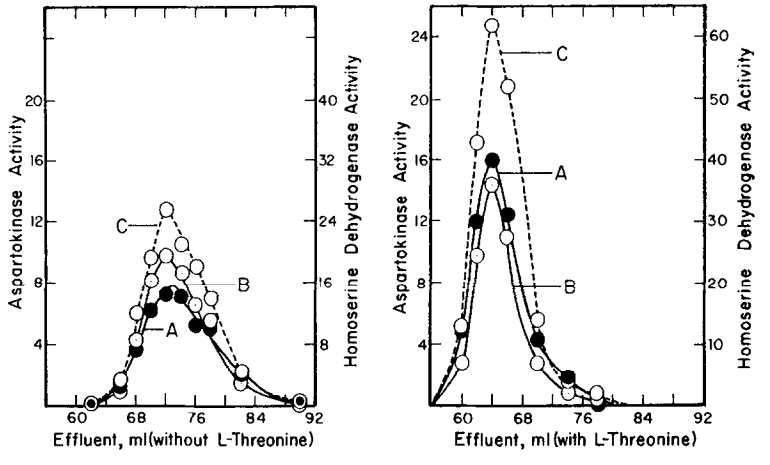

FIG. 1. Elution pattern of threonine sensitive aspartokinase and homoserine dehydrogenase complex on Sephadex G-100 (2.0 x 60 cm flow rate 20-25 ml/hr) in the absence of and in the presence of threonine 20 μM. A - Threonine sensitive aspartokinase activity, B - Threonine sensitive homoserine dehydrogenase activity, C - Threonine sensitive homoserine dehydrogenase activity in the presence of the substrates of the aspartokinase (aspartic acid, 10 mM, and ATP, 10 mM with Mg^{++}, 1.6 mM). All columns were run in 0.02 M PO_4 buffer containing 0.03 M β-mercaptoethanol plus additives as indicated. Aspartokinase activity is expressed as micromoles of β-aspartylhydroxamate produced per ml during a 20 minute incubation. Homoserine dehydrogenase activity is expressed as millimicromoles of NADPH produced per ml per minute.

The mobilities of the enzyme complex in a sucrose gradient were compared to that of well characterized enzymes in order to obtain an estimate of the sedimentation coefficient ($S_{20,w}$) under each condition. Tracings of the A_{280} of the sucrose gradient revealed a small peak which corresponded to the position of the enzyme complex as determined by analysis for enzymatic activity. This method gave $S_{20,w}$ values of 6.3 without threonine and 9.7 with 20 μM threonine. These $S_{20,w}$ values correspond to an approximate molecular weight of 100,000 - 120,000 and 195,000 - 220,000, thus indicating an apparent doubling of molecular weight although it is remotely possible that drastic changes in the shape of the enzyme complex could result in the observed changes in the mobilities noted above. The reversibility of the association was demonstrated by removal of threonine from the higher molecular weight form which resulted in the appearance of the lower molecular weight form as indicated by Sephadex G-100 filtration. Also, at a concentration of 2 μM threonine, an elution pattern intermediate between that of the higher and lower molecular weight forms with a broader elution volume was obtained.

Appreciable activation of homoserine dehydrogenase by the substrates of aspartokinase (aspartic acid and ATP with Mg^{++} as shown in Fig. 1) indicates that the low molecular weight form as well as the higher molecular weight form contains both activities. Either substrate alone has an appreciable activating effect. Apparently changes in conformation of the aspartokinase as a result of binding with its substrates causes changes in the homoserine dehydrogenase unit resulting in enhanced activity.

Threonine at considerably higher concentrations than necessary for the association reaction inhibits the aspartokinase and the homoserine dehydrogenase activities (Fig. 2); however, there is a difference in the type of inhibition curve obtained with increasing concentrations of threonine for each activity. The data for inhibition of homoserine dehydrogenase by threonine is consistent with independent interaction

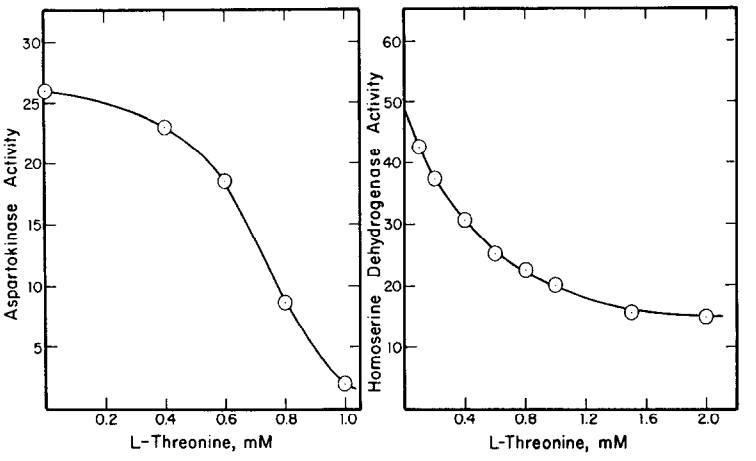

FIG. 2. Effect of threonine on the activity of the threonine sensitive aspartokinase and homoserine dehydrogenase. Activities expressed as described in Fig. 1.

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of an inhibitor at a single regulatory site; however, the inhibition of the aspartokinase activity appears to be more complex, with some interdependence of inhibitor sites. The initial binding of the inhibitor with the complex could cause an enhancement of the binding at a subsequent site or alternatively, the initial binding could have little inhibitory effect upon activity until a subsequent site is also bound to the inhibitor. The conformational change resulting from initial binding of the inhibitor could be expected to facilitate analogous conformational changes in the other catalytically active portion of the associated complex under appropriate conditions. This could be expected if these binding sites lie between points of association of the complex within moieties with preferred conformations so that changes in the distances between points of association of one part with initial inhibitor binding would facilitate similar changes upon its symmetrical part. Alternatively, binding at more than one site may be essential for effective inhibition with the initial binding exerting little effect.

Kinetic studies[1] indicated that not only aspartic acid as previously reported (8) but also magnesium ion reverses the threonine inhibition of aspartokinase activity in a competitive manner. However, aspartic acid (10 mM) and magnesium ion (3.3 mM) do not cause or prevent the formation of the higher molecular weight complex which is formed in the presence of threonine (0.02 mM or 0.4 mM) as determined by gel filtration. In addition, the inhibition by threonine of the homoserine dehydrogenase activity is not reversed by aspartic acid and magnesium ion. Thus, the association and either of the two inhibitions can each be caused to occur at different concentrations of threonine, and the aspartokinase inhibition appears to involve a two stage interaction.

In summary, it appears that there are four distinguishable interactions of threonine with the enzyme complex: (1) reversible increase in molecular weight due to interaction of threonine with a lower molecular weight unit of the enzyme, (2) and (3) cooperative inhibition of aspartokinase due to interaction of threonine at two or more sites, and (4) inhibition of homoserine dehydrogenase. The inhibition of the aspartokinase can be reversed in a competitive manner by either Mg^{++} or aspartic acid; however, neither the inhibition of homoserine dehydrogenase nor the increase in molecular weight is reversed by aspartic acid or Mg^{++}.

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