Active Subunits of the Aspartokinase–Homoserine Dehydrogenase I Complex from *Escherichia coli**

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ABSTRACT: Threonine-sensitive aspartokinase (adenosine triphosphate:1-aspartate-4-phosphotransferase, EC 2.7.2.4) and threonine-sensitive homoserine dehydrogenase (L-homoserine: $NADP^+$ oxidoreductase, EC 1.1.1.3) purify together as a single macromolecular complex. As normally isolated, in phosphate buffer (pH 6.8) containing the feedback inhibitor, L-threonine, the enzyme is a hexamer with a molecular weight of 360,000. When placed in buffers made from *N*-tris(hydroxymethyl)-2-aminoethanesulfonate (TES) or *N*-2-hydroxylpiperazine-*N'*-2-ethanesulfonate (HEPES) the enzyme rapidly dissociates into dimers which exhibit a molecular weight of 122,000. The enzyme is stabilized against subunit formation by the feedback inhibitor and some, but not all, of the aspartokinase and homoserine dehydrogenase substrates. These dimers retain the catalytic activities of native enzyme and show interaction between the two catalytic sites. They appear homogeneous as judged by sedimentation equilibrium and DEAE-Sephadex chromatography. Initially, both activities are sensitive to threonine inhibition. Upon storage in TES or HEPES buffer, homoserine dehydrogenase activity becomes insensitive to threonine inhibition. This desensitized species retains aspartokinase activity which is still 60% inhibited by L-threonine. When the desensitized subunits are returned to phosphate buffer containing potassium chloride and threonine, some of the molecules recover the size and activity of native enzyme.

Cohen has recently published an authoritative review (Cohen, 1969) which describes the control of aspartokinase and homoserine dehydrogenase activities in *Escherichia coli*. *E. coli* has been shown to produce three enzymes which catalyze the phosphorylation of aspartic acid, the first step in the formation of threonine, methionine, and lysine. Each isoenzyme is subject to feedback control by one of these products (Stadtman *et al.*, 1961; Patte *et al.*, 1967). Two of these three aspartokinases also carry homoserine dehydrogenase activity. In this paper we are concerned only with the AK-I[1] and HSDH-I activities, which are inhibited by threonine and carried by a single protein of 360,000 daltons (Truffa-Bachi *et al.*, 1968). Both the aspartokinase activity and the homoserine dehydrogenase activity require potassium ion and there is reciprocal inhibition of one activity by substrates of the other.

Dissociation of the enzyme in guanidine hydrochloride has been shown to produce subunits of 60,000 daltons (Truffa-Bachi *et al.*, 1968). Peptide mapping and end group analysis have led Truffa-Bachi *et al.* (1969) to the conclusion that the six subunits may be identical. Direct binding measurements (Janin *et al.*, 1969) have shown six sites for threonine, but, significantly, only three binding sites for NADPH.

Spontaneous dissociation of the aspartokinase–homoserine dehydrogenase complex into subunits upon removal of threonine has been observed by several investigators. The first report was made by Patte *et al.* (1963), who observed that the sedimentation velocity of homoserine dehydrogenase I activity (in a sucrose gradient) is about twice as fast when the enzyme is protected by potassium chloride or threonine as compared to sodium chloride. Barber and Bright (1968), Cunningham *et al.* (1968), and Wampler and Westhead (1968) have all observed the production of lower molecular weight species when threonine is removed from the buffer.

Related to changes in molecular weight are changes in catalytic properties. Homoserine dehydrogenase activity can be rendered insensitive to threonine inhibition "spontaneously" by removing threonine (Cohen and Patte, 1963; Patte *et al.*, 1963), or chemically by treating with *p*-mercuribenzoate (Truffa-Bachi *et al.*, 1966a), or by putting the enzyme into Tris buffer at pH 9.0 (Truffa-Bachi *et al.*, 1966b). When homoserine dehydrogenase activity is desensitized to threonine the associated aspartokinase activity disappears but remains sensitive to threonine during its disappearance.

The desensitization process leads to the formation of both subunits and aggregates. Cohen *et al.* (1967) reported that desensitized homoserine dehydrogenase I is separated into two peaks by Sephadex G-200 chromatography, one excluded by the gel and the other in a molecular weight region between 150,000 and 180,000. Barber and Bright (1968) reported the production of 7S and 30S forms when the native homoserine dehydrogenase was treated with *p*-mercuribenzoate or stored in the absence of threonine. In all of these studies, subunit formation has been accompanied by loss of aspartokinase activity and a desensitization of homoserine dehydrogenase activity. It had been observed, however (Wampler and Westhead, 1968), that the loss of aspartokinase activity could be partly reversed in the early stages of loss by the addition of threonine or potassium ion.

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[1] Abbreviations used are: AK, aspartokinase; HSDH, homoserine dehydrogenase; ASA, aspartic-β-semialdehyde; TES, *N*-tris(hydroxymethyl)-2-aminoethanesulfonate; HEPES, *N*-2-hydroxylpiperazine-*N'*-2-ethanesulfonate.

We now show that specific ion effects are important in determining the patterns of activity and molecular weight changes that follow the removal of threonine. In HEPES and TES buffers, for example, we find stable dimers (mol wt 122,000) which retain both homoserine dehydrogenase and aspartokinase activities, retain sensitivity to threonine, and retain the normal antagonistic effects between the two catalytic activities. In this paper we also attempt to make deductions about the architecture of the native complex from an analysis of the differences between the hexamer and dimer and the factors influencing their interconversion.

Materials

The buffers used were: buffer P, 20 mM potassium phosphate (pH 6.8), 0.5 mM L-threonine, 2 mM magnesium acetate, 2 mM EDTA, and 0.1 mM dithiothreitol; buffer A, buffer P plus 0.15 M potassium chloride; buffer TES, 20 mM Na TES (pH 8.0) plus 0.1 mM dithiothreitol; buffer HEPES, 20 mM sodium HEPES (pH 8.0), 0.1 mM dithiothreitol, and 0.5 mM NADPH.

TES and HEPES are two of the buffers developed by Good *et al.* (1966), and were purchased from Calbiochem. Aspartic semialdehyde was prepared by ozonolysis of allylglycine as described by Black and Wright (1955). Aspartic acid, which is also formed by this procedure, was removed with a column of Dowex 1 as described by Hirs *et al.* (1954). All other reagents were obtained from commercial sources.

The *E. coli* K12 strain used was a gift from Dr. Martin Freundlich. Cells were grown at the New England Enzyme Center in minimal glucose medium (Davis and Mingioli, 1960), harvested in late-log phase, and sent to us as a frozen cell paste. Aspartokinase–homoserine dehydrogenase I was purified essentially according to the procedure of Truffa-Bachi *et al.* (1968), and stored either as an ammonium sulfate slurry (50% saturation) or as a concentrated solution in buffer A at 4°.

The enzyme, stored in buffer A was homogeneous on disc electrophoresis as well as in the analytical ultracentrifuge and was estimated to be greater than 98% pure. Freshly prepared enzyme had a homoserine dehydrogenase specific activity of 62–65 units/mg with an associated aspartokinase specific activity of 8.8–9.3 units/mg (both assays at 30°). After several months' storage as an ammonium sulfate slurry, the homoserine dehydrogenase activity decreased to 48 units/mg while aspartokinase activity remained unchanged. This "aged" enzyme retains normal sensitivity to threonine (homoserine dehydrogenase activity 80% inhibited and aspartokinase 100% inhibited), has the same sedimentation coefficient, and elutes from a Sephadex G-200 column as a single peak at the same position as fresh enzyme.

Methods

Measurement of Enzyme Activity. One unit of activity is defined as the amount of enzyme required to form 1 μmole of product or remove 1 μmole of substrate per minute under the assay conditions. Specific activity is units of activity per milligram of protein. The protein concentration was estimated from absorbancy at 278 nm, using an extinction coefficient of 0.44 absorbance unit/cm^2 per mg (Truffa-Bachi *et al.*, 1968).

Aspartokinase activity was measured by coupling ADP production to the oxidation of NADH through pyruvate kinase and lactic dehydrogenase, as described previously (Wampler and Westhead, 1968). Homoserine dehydrogenase activity was measured by the oxidation of NADPH as described by Truffa-Bachi *et al.* (1966a) except that the KCl concentration was 0.6 M instead of 0.15 M and the temperature was 30° instead of 27°. Catalase was measured by following the disappearance of H_2O_2 at 240 nm (Beers and Sizer, 1952).

Changing Buffers. Two methods were used to exchange the enzyme from one buffer to the other. Enzyme was either diluted 100-fold into the new buffer or passed through a 0.3 × 25 cm column of Sephadex G-25 which had been equilibrated with the new buffer. When the buffer was changed by dilution, the concentration of all components could be calculated. When we describe results obtained by this method, the enzyme concentrations will be given. The efficiency of threonine removal by gel filtration was examined using L-threonine-^{14}C. In several experiments the L-threonine-^{14}C concentrations in the peak enzyme fraction did not exceed 4×10^{-7} M. In experiments reported below we assume that the threonine concentration in samples passed through the G-25 column is less than 5×10^{-7} M.

Sedimentation Analysis. Sedimentation coefficients were determined with a Model E analytical ultracentrifuge equipped with a photoelectric scanner. The sedimentation boundary was measured by schlerien or scanner techniques or by Cohen's method of sedimenting the enzyme through a homoserine dehydrogenase assay solution and following the oxidation of NADPH (Cohen, 1963). In this last method, NADPH depletion boundaries were followed at 340 nm in a 12-mm double-sector band-forming cell which contained the usual assay constituents with the addition of 1.7% sucrose to minimize convective disturbance.

Sedimentation coefficients were calculated from the equation $S = (\mathrm{d} \ln r/\mathrm{d}t)(1/\omega^2)$, where r = boundary position in centimeters, t = time in seconds, and ω = angular velocity in radians per second. The sedimentation rate, d ln r/dt, was calculated from an unweighted least-squares fit of data collected at 8-min intervals.

Sedimentation equilibrium experiments were performed using a six-channel centerpiece as described by Yphantis (1964) except that the photoelectric scanner was used instead of interference optics. Scans were made at 278 nm with the monochromator exit slit set at 2 mm and the photomultiplier slit set at 0.08 mm. The slowest scanning speed was used and the recorder was set to run at 5 cm/min. These settings resulted in a magnification of the sample height from 0.29 cm in the cell to 20 cm on the recorder chart. Noise suppression was adjusted so that the noise amplitude near the meniscus was not over 1 mm. Pen deflection was measured at 1-cm intervals near the miniscus and at 0.4-cm intervals when the line became more strongly curved. Pen deflection was converted to optical density using a calibration curve constructed from known concentrations of bovine serum albumin, and thence to protein concentration using an extinction coefficient of 0.44 absorbance unit/cm^2 per mg.

Apparent weight-average molecular weights, M_w, were calculated from the equation: $M_w = [2RT/(1 - \bar{v}\rho)\omega^2](\mathrm{d} \ln c/\mathrm{d}r^2)$, where R = the gas constant, T = degrees Kelvin, $\bar{v}$ = partial specific volume, ρ = solvent density, and r = radius. A value of 0.737 cm^3/g was used for $\bar{v}$ (calculated from amino acid composition, Janin *et al.*, 1969).

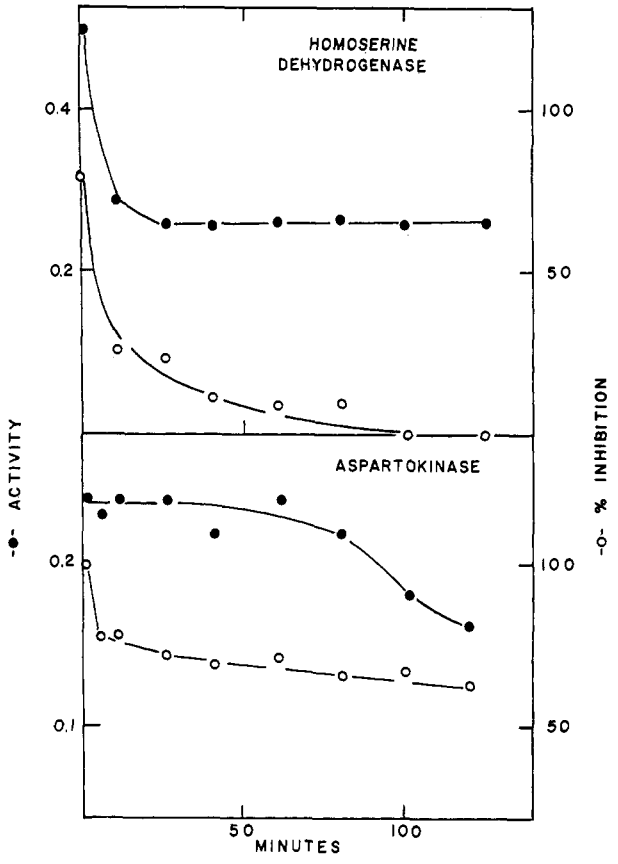

FIGURE 1: Time course of homoserine dehydrogenase and aspartokinase desensitization. Enzyme in buffer P was diluted 100-fold into buffer HEPES (final concentration 0.09 mg/ml). Activity (●) is ΔOD_{340}/min per 10 μl of homoserine dehydrogenase or 25 μl of aspartokinase. The aspartokinase assay contained 50 mM HEPES and 0.1 M KCl instead of the normal 50 mM Tris and 0.6 M KCl. Percent inhibition (○) was calculated from activity in the presence of 8.5 mM L-threonine.

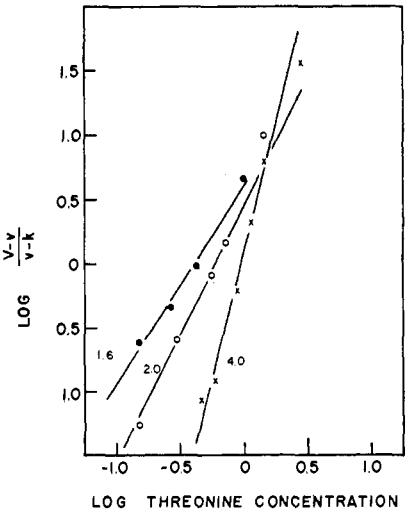

FIGURE 2: The shape of the threonine saturation curve was analyzed before exchange into buffer TES (×), immediately after exchange into buffer TES (○), and 4 hr after exchange into buffer TES (●). The slope of each line (n in eq 1) was calculated from an unweighted least-squares fit of the data and is given beside the line.

Results

Kinetic Properties of Subunits. Diluted into buffer HEPES, homoserine dehydrogenase rapidly loses sensitivity to threonine inhibition. The time course of desensitization is illustrated in Figure 1. Homoserine dehydrogenase loses more than half of the normal sensitivity to L-threonine in 10 min and is completely desensitized in less than 2 hr. Aspartokinase activity loses sensitivity more slowly and even after several days is 55–60% inhibited by L-threonine. Associated with this loss in feedback sensitivity is a loss in specific activity (Figure 1).

Desensitization was slower when the enzyme was exchanged into buffer TES by gel filtration. As will be described later, enzyme which had been in buffer TES for 2 days was not fully desensitized whereas after 2 weeks it was. The reason for this difference in desensitization rate has not yet been fully explored. Exchanged into buffer TES or buffer HEPES, homoserine dehydrogenase activity was never more than 60% inhibited while aspartokinase activity was never less than 50% inhibited by 20 mM threonine.

Exchange into buffer TES or buffer HEPES also causes a reduction in the cooperativity of threonine inhibition. For cases of incomplete inhibition the Hill equation takes the form

$$\log \frac{V - v}{v - k} = n \log (\mathrm{I}) + \log (\mathrm{I}_{0.5}) \qquad (1)$$

where V is the maximum velocity, v is the velocity at inhibitor concentration (I), k is the velocity at saturating inhibitor concentration, ($I_{0.5}$) is the inhibitor concentration which gives half-maximal inhibition, and n is the degree of cooperativity. The value of n is calculated from a plot of log $[(V - v)/(v - k)]$ *vs.* log (I) and is referred to as the Hill coefficient.

As shown in Figure 2, the aspartokinase activity of enzyme stored in buffer A gives a Hill coefficient of 4.0 and is more than 98% inhibited by L-threonine. This value agrees with previous reports (Patte *et al.*, 1966; Wampler and Westhead, 1968). To test threonine saturation of desensitized enzyme, 0.08 mg of aspartokinase–homoserine dehydrogenase I was exchanged into buffer TES by gel filtration (final enzyme concentration 36 μg/ml) and immediately assayed for threonine sensitivity. Within the first hour, inhibition data gave a Hill coefficient of 2.0 and a maximum inhibition of 79% (Figure 2). After four hours the Hill coefficient had dropped to 1.6 and the sample was 69% inhibited at saturating threonine concentrations. Although in some experiments the maximum inhibition did go down as low as 50%, the Hill coefficient never went below 1.5.

Although the interaction of enzyme with feedback inhibitor is markedly changed by exchange into buffer TES or buffer HEPES, the apparent Michaelis constants for NADPH and aspartic semialdehyde remain essentially the same (Table I), and the aspartokinase substrates (aspartate and ATP), continue to inhibit homoserine dehydrogenase activity. Since aspartate inhibition remains noncompetitive with aspartic semialdehyde (unpublished data) and the ($I_{0.5}$) values agree with the K_m values measured in the aspartokinase reaction, it seems likely that aspartate and ATP bind at the aspartokinase site and that the communication between catalytic sites has remained intact.

Molecular Weight Changes. Subunit formation could be tested for rapidly by sedimentation velocity measurements.

TABLE I: Properties of Native and Desensitized HSDH.

	Native	Desensitized
Apparent K_m, K^+	0.13	0.27
Apparent, K_m, NADPH	0.08 mM	0.13 mM
Apparent K_m, ASA	0.1 mM	0.11 mM
% inhibition, 25 mM ATP	28	40
% inhibition, 20 mM aspartate	57	57
($I_{0.5}$) aspartate	1.8 mM	2.0 mM
% inhibition, 20 mM threonine	80	0
($I_{0.5}$) threonine (in AK assay)	1.0 mM	0.53 mM
Ratio HSDH:AK	7	9

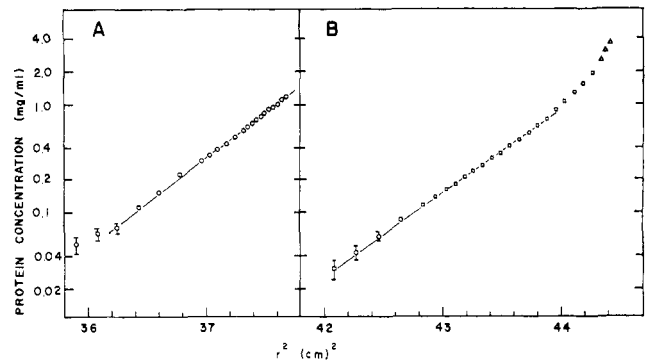

FIGURE 3: Equilibrium sedimentation in buffer TES plus 1.5% sucrose. Scans were taken after 24 hr at 20° and 16,000 rpm as described in Methods. The loading concentration of protein was (A) 0.26 and (B) 0.7 mg per ml. Scans were made at the 0–1 optical density range (○) and at the 0–2 optical density range (△). At medium protein concentrations results of the two optical density ranges varied no more than 7% and were averaged (□). The position of the oil meniscus was 37.80 cm² in part A and 44.66 cm² in part B. The vertical bars indicate the error that would result from a 0.2-mm error in measuring pen deflection.

The results of many experiments indicate that the enzyme exists in two distinct forms, one which sediments at 11 S and another which sediments at 7 S. The native enzyme has a sedimentation coefficient, $s_{20,w}$, between 11 and 11.5 S (Truffa-Bachi *et al.*, 1968; Janin *et al.*, 1969; Table II). Sedimentation at the high dilutions present under assay conditions does not change the apparent sedimentation coefficient (line 2, Table II). In contrast, when enzyme was exchanged into buffer TES and centrifuged in that buffer, or diluted into buffer HEPES and centrifuged in assay medium containing 9 mM L-threonine, sedimentation coefficients of 7.0 and 6.8 S were obtained (lines 3 and 4, Table II).

When the enzyme was subjected to equilibrium centrifugation at 12,000 rpm in buffer A, it exhibited a molecular weight of 361,000 ± 5000 and the plot of ln c *vs.* r^2 was linear throughout the usable range. The molecular weight of the 7S species was determined by exchanging enzyme into buffer TES containing 1.5% sucrose and then subjecting it to high-speed equilibrium sedimentation. Two different samples centrifuged at a loading concentration of 0.7 mg/ml gave weight-average molecular weights of 118,000 and 121,000. At a loading concentration of 0.26 mg/ml the weight-average molecular weight was calculated to be 128,000. Data for the high and low values are given in Figures 3. At the lower loading concentration the preparation appears homogeneous all the way to the bottom of the sample column while at a higher protein concentration there is some evidence of aggregation near the bottom. The straight line portions in Figure 3 extend over a 20-fold range of protein concentration in each case, showing that the molecular weights reported are independent of protein concentration in the range shown. The scanner data are unfortunately not sufficiently precise to give information about the aggregation reaction.

Having established that the native enzyme can form dimeric subunits which have a $s_{20,w}$ of approximately 7.0 S, a study was undertaken to determine what ionic species affected the conversion into dimeric form. Except as noted, the sedimentation data described below were obtained by conventional scanner techniques using the four place, AN-F rotor at 44,000 or 48,000 rpm at protein concentration of 0.4 or 0.5 mg per ml. In each case the enzyme was exchanged into the sedimentation buffer by gel filtration and centrifugation was completed within 5 hr from the time of buffer change.

In either 20 mM potassium or sodium phosphate buffer (which, like all buffers, contained 10^{-4} M dithiothreitol) the enzyme exhibited a sedimentation coefficient of 9.8 S. This form of the enzyme was unstable, however, and after 24 hr in the potassium phosphate buffer, the sedimentation coefficient dropped to 6.8 S. Thus, both phosphate buffer and buffer TES allow the formation of a slower sedimenting species, although the dissociation occurs faster in buffer TES.

We have found that those ions which affect the allosteric equilibrium between inhibited and active forms of the enzyme (see Janin and Cohen, 1969) also affect the equilibrium between dimers and hexamers. The three principle allosteric affectors, potassium ion, threonine, and aspartate, all stabilize the hexameric state while NADPH, which does not affect the allosteric equilibrium, does not stabilize either

TABLE II: Sedimentation Coefficient of AK:HSDH-I in Several Buffers.

Buffer[a]	pH	μg of Protein/ml	$s_{20,w}$ (S)
A	6.8	500	11.1
Assay	7.5	<0.05	11.3
HEPES assay	7.5	<0.02	6.8
TES	8.0	500	7.0

[a] The buffers used are: A = buffer A; assay = 0.05 M Tris (pH 7.5), 0.3 M KCl, 2 mM $MgAc_2$, 2 mM EDTA, 0.015 mM L-threonine, 1 mM ASA, 0.13 mM TPNH, and 1.7% sucrose; HEPES assay = 0.05 M HEPES (pH 7.5), 0.1 M KCl, 2 mM $MgAc_2$, 2 mM EDTA, 0.9 mM threonine, 1 mM ASA, 0.13 mM TPNH, and 1.7% sucrose; TES = buffer TES. Since the enzyme is diluted by diffusion as it sediments through the assay mixture the enzyme concentrations listed in lines two and three are the concentrations which were layered on the assay solution.

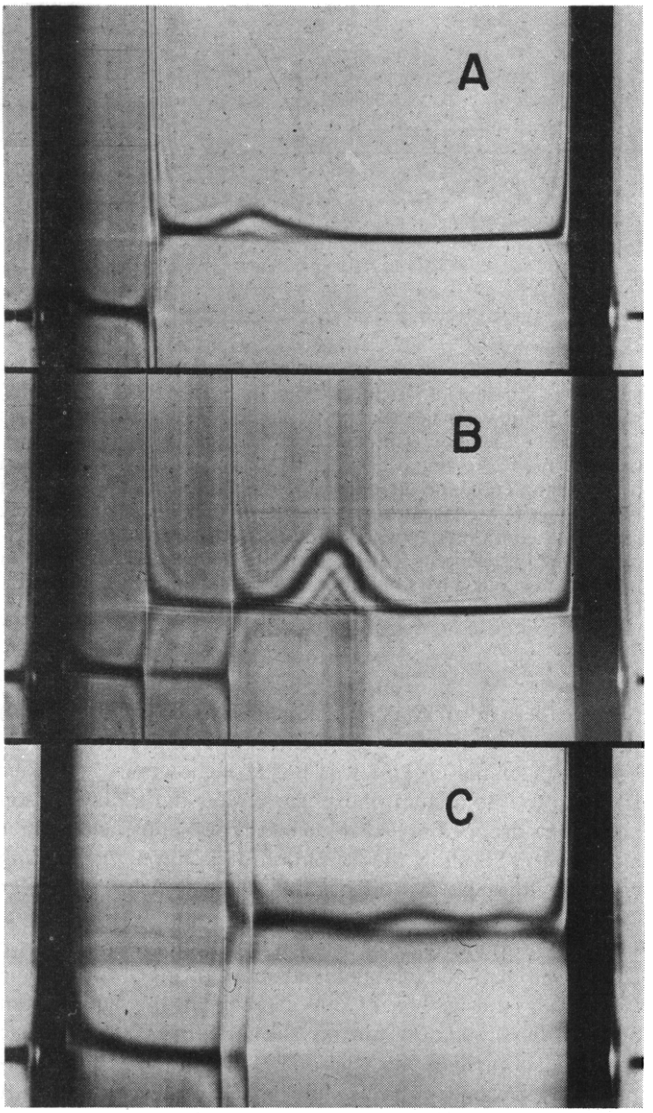

FIGURE 4: Sedimentation velocity of subunits in buffer TES (A and B) and reconstituted enzyme in buffer P (C). The temperature was 23° in parts A and B and 24° in part C. (A) 0.5 mg/ml, 36 min after reaching 44,000 rpm; (B) 3.2 mg/ml, 35 min after reaching 44,000 rpm; and (C) 1 mg/ml, 60 min after reaching 44,000 rpm.

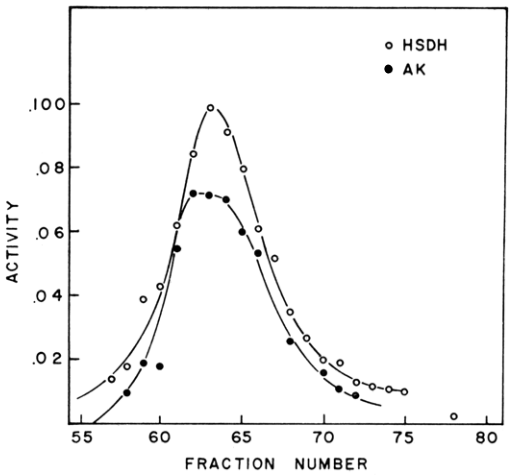

FIGURE 5: DEAE-Sephadex chromatography of desensitized AK–HSDH-I. Enzyme was desensitized by dilution into buffer HEPES and then applied to a 1 × 15 cm column of DEAE-Sephadex and eluted as described in results. 1.5-ml fractions were collected and assayed for aspartokinase (●) and homoserine dehydrogenase (○) activity. Activity is expressed as ΔOD_{340}/min per 25 μl of aspartokinase and ΔOD_{340}/30 sec per 5 μl of homoserine dehydrogenase.

form preferentially. When enzyme was exchanged into buffer TES containing 40 μM threonine or 100 mM potassium chloride or 20 mM aspartate, the sedimentation coefficients (within 4 hr) were 10.6, 9.9, and 8.1 S, respectively. This stabilization of the faster sedimenting species by molecules which affect the allosteric equilibrium is similar to the temporary stabilization by phosphate already mentioned. Enzyme centrifuged in buffer TES containing 10 μM NADPH exhibited a sedimentation coefficient of 6.9 S.

Increasing pH had little effect on sedimenting in TES buffer (7.5 S at pH 7.0, 7.0 S at pH 8.0, and 7.1 S at pH 9.0). Increasing protein concentration at pH 8.0 resulted in an increase in sedimentation coefficient from 6.8 S (at 0.02 mg/ml, measured by the substrate depletion method, Cohen, 1963) to 7.8 S at a concentration at 3.2 mg/ml. These data (at six different concentrations) extrapolate to give an $s^0_{0,w}$ of 6.8 S. This increase is compatible with the apparent aggregation at high protein concentration observed in sedimentation equilibrium (Figure 3). This concentration dependence and the fact that the enzyme sediments as a single peak at both high and low concentrations (Figure 4A,B) suggests that the subunits are in rapid equilibrium with higher molecular weight aggregates at high protein concentrations.

We have previously observed an acceleration of aspartokinase inactivation (and presumably subunit formation) by low temperatures in phosphate buffer (Wampler and Westhead, 1968) and Cohen *et al.* (1965) have reported the cold inactivation of homoserine dehydrogenase in Tris buffer. Desensitization and subunit formation in buffer TES and buffer HEPES, on the other hand, showed no cold effects.

DEAE-Sephadex Chromatography of Subunits. Although the subunits appeared homogeneous by sedimentation velocity and sedimentation equilibrium, an attempt was made to separate homoserine dehydrogenase and aspartokinase activities by DEAE-Sephadex chromatography. Enzyme (3 mg) was exchanged into buffer HEPES and allowed to stand overnight; concentrated NaCl was then added to give a final concentration of 0.15 M. This enzyme solution was then chromatographed on a 1 × 15 cm DEAE-Sephadex column equilibrated in 0.02 M HEPES buffer (pH 8.0) plus 0.15 M NaCl, 1 mM dithiothreitol, 0.1 mM EDTA, and 0.1 mM NADPH. Elution was accomplished with a linear gradient (150 ml) of NaCl from 0.15 to 0.5 M. The results, shown in Figure 5, again indicate that the sample is homogeneous since the ratio of aspartokinase and homoserine dehydrogenase activities was constant throughout the peak.

Reaggregation of Subunits. To test the possibility that subunits could be reconstituted to native enzyme, subunits were exchanged back into buffers containing threonine. In one experiment, L-threonine was added to a desensitized enzyme sample which had been in buffer TES for two weeks (final threonine concentration 10 mM), and the sample was

then dialyzed against buffer P containing 10 mM L-threonine for four days and analyzed for kinetic and sedimentation properties. Enzyme treated in this way is composed of three molecular weight species: (1) a large aggregate which sediments as a small, broad peak, (2) a species with a sedimentation coefficient of 11 S, and (3) a species with a sedimentation coefficient of 6.8 S. The latter two forms account for 80–90% of the protein in solution and could be clearly distinguished either by schlieren (Figure 4C) or absorption optics. These species were partially resolved on a 0.9 × 43 cm column of Sephadex G-200 which had been equilibrated with the dialysis buffer. The first active fractions, which came out in the void volume, contained homoserine dehydrogenase activity which was not inhibited by L-threonine. This was followed by activity which reached a maximum of 45% inhibition and finally by an activity which was only 8% inhibited.

In a second experiment we used freshly prepared subunits which still had threonine-sensitive homoserine dehydrogenase. Enzyme was exchanged into buffer TES (final protein concentration 0.5 mg/ml) and quickly centrifuged to confirm that subunits had been produced ($s_{20,w}$ = 6.8 S, homoserine dehydrogenase 55% inhibited, aspartokinase 87% inhibited). A concentrated mixture of threonine and potassium chloride was then added to both sectors of the centrifuge cell (final threonine concentration 4 mM, final potassium chloride concentration 150 mM) and the sample was immediately recentrifuged. This time the protein sedimented as a broad boundary with the midpoint giving an $s_{20,w}$ of 9.5 S. The sample was stored at 4° overnight and then passed through a 0.9 × 43 cm column of G-200 which had been equilibrated with buffer A. Activity eluted as illustrated in Figure 6. In this case there was considerably less of the uninhibitable aggregate, and better separation of the two peaks which eluted in the region of native enzyme and subunits. Specific activity was not calculated because of the very low protein concentration (if tube 9 had a specific activity of 60, the A_{278} would be less than 0.001).

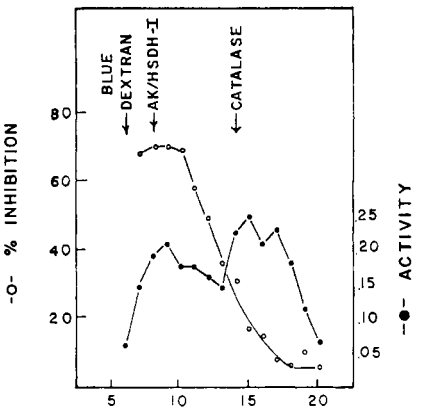

FIGURE 6: Enzyme which had been dissociated by exchanging into buffer TES was "reconstituted" as described in results. The "reconstituted" activity was put thru a 0.9 × 55 cm column of Sephadex G-200 which had been equilibrated with Buffer A containing 10 mM L-threonine. Each 10-drop fraction (about 0.3 ml) was assayed for homoserine dehydrogenase activity (●) and threonine sensitivity (O). Activity is expressed as $\Delta OD_{340}/2$ min per 25 μl. The elution positions of blue dextran, native aspartokinase–homoserine dehydrogenase I and catalase, determined in a parallel experiment, are indicated.

Discussion

Although subunits of the aspartokinase–homoserine dehydrogenase complex have been observed under a variety of conditions, in previous reports they have been found as transient forms with low specific activities or they have been produced by chemical or genetic modification. In this paper we describe a method for preparing a homogeneous solution of aspartokinase-active, threonine-sensitive subunits. Subunit formation is accompanied by a sharp drop in the cooperativity of L-threonine inhibition and a progressive loss of threonine sensitivity until the homoserine dehydrogenase activity is less than 5% inhibited and the aspartokinase activity is 50–60% inhibited by saturating L-threonine concentration. Except for a reduction in specific activity, subunit formation does not appear to alter substantially the kinetic properties of the aspartokinase and homoserine dehydrogenase activities, but does change the kinetics of threonine inhibition and the apparent K_m for potassium ion.

The work reported here makes it clear that specific buffer effects make a great difference in the production and stability of these subunits. Spontaneous subunit formation in phosphate buffers was slow and incomplete, with secondary loss of both activities (Wampler and Westhead, 1968). At low concentrations in buffers HEPES and TES, the process is complete and the dimer is stable. At high protein concentrations the solution becomes heterogeneous with the appearance of high molecular weight aggregates. In the presence of ligands such as aspartate and potassium the enzyme exhibits a sedimentation coefficient intermediate between that characteristic of native enzyme and dimers. These sedimentation coefficients probably reflect an equilibrium between dimers and hexamers. The idea that potassium stabilizes the hexameric form in such an equilibrium is supported by the observation that the hexameric form shows a lower K_m for potassium than the dimers (Table I). Potassium ion has also been shown to stabilize a tetrameric form of homoserine dehydrogenase from *Rhodospirillum rubrum* (Datta and Gest, 1965). When desensitized enzyme from *E. coli* is exposed to high concentrations of potassium ions large aggregates form. We (unpublished data), as well as Barber and Bright (1968), have observed species with sedimentation coefficients of 30–40 S. These large aggregates have no aspartokinase activity and the homoserine dehydrogenase activity is not inhibited by threonine.

Kinetic studies of the aspartokinase–homoserine dehydrogenase complex have been interpreted as indicating that the native enzyme exists in two conformations, one active and the other inhibited. This idea has recently received support with the use of absorption and fluorescence spectroscopy (Janin and Cohen, 1969). Because the changes in fluorescence intensity fit a third-order dependence on aspartate and threonine concentrations they proposed that the cooperative unit in the aspartokinase–homoserine dehydrogenase complex is a trimer. Our results show that functional dimers are readily formed from the native enzyme under appropriate conditions; further, threonine saturation curves for these aspartokinase active dimers still exhibit cooperative interactions (Hill coefficients of 1.5–2.0). However, dissociation of the native hexamer into functional dimers under certain conditions does not

exclude the possibility that the molecule functions as two trimers under other conditions. Janin *et al.* (1969) have reported that there are only three binding sites for NADPH, and this presumably means that there are only three active sites for the homoserine dehydrogenase activity; three active sites are, of course, most easily accommodated among three dimers.

Homoserine dehydrogenase isolated from *R. rubrum* has some striking similarities and differences as compared to the enzyme isolated from *E. coli*. In *R. rubrum* aspartokinase and homoserine dehydrogenase activities are on separate molecules, yet it is a striking fact that the substrates of aspartokinase inhibit the homoserine dehydrogenase activity (Mankowitz and Segal, 1969). The enzyme from *R. rubrum* is normally a tetramer (rather than a hexamer) but on removal of threonine it forms dimers which are insensitive to threonine, as does the *E. coli* enzyme. In both cases these threonine insensitive dimers retain sensitivity to aspartate and ATP inhibition.

One fundamental question concerning the *E. coli* enzyme is whether the six polypeptide chains are identical. We have not been able to separate aspartokinase and homoserine dehydrogenase activities on DEAE-Sephadex when the enzyme is in dimeric form. We hope that stable dimers will serve as starting material for production of active monomers which could be used to test for the separateness of catalytic subunits. Further studies of the structure and activity of the aspartokinase–homoserine dehydrogenase complex will require more detailed kinetic and sedimentation analysis. Such work is currently under way.

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