Variation of the Size of the Reacting Form of *Escherichia coli* K12 Threonine-Sensitive Aspartokinase–Homoserine Dehydrogenase with pH and Effectors†

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ABSTRACT: The sedimentation behavior of the threonine-sensitive aspartokinase–homoserine dehydrogenase of *Escherichia coli* K12 has been examined with the technique of R. Cohen *et al.* [(1967), *Biopolymers 5*, 203] for determining the sedimentation coefficients of the reacting forms of enzymes. Deuterium oxide (40%) was used to stabilize sedimentation boundaries (Taylor *et al.* (1972), *J. Biol. Chem. 247*, 7383). Sedimentation was observed during catalysis of both the forward and reverse homoserine dehydrogenase reactions under a variety of conditions. The sedimentation behavior of the enzyme was independent of the direction in which activity was measured. Assay conditions such as pD (or pH) and presence of effector molecules, however, had significant effects on the sedimentation behavior of the reacting enzyme. Homoserine dehydrogenase was found to exist in an active tetrameric form only around pD 8 where the sedimentation coefficient was 10.6 ± 0.14 S. When the pD was raised or lowered from 8 increasing amounts of dimeric homoserine dehydrogenase were formed. L-Threonine or L-aspartate plus potassium ion stabilized the tetrameric form at pD values other than 8.0 while L-aspartate alone and D-aspartate had no effect. Potassium ion alone appeared to shift the association–dissociation equilibrium slightly toward the tetramer. Reacting enzyme sedimentation was also conducted in the absence of D_2O. The properties of the enzyme in the H_2O system are qualitatively identical with those in the D_2O system. The pH at which the maximum sedimentation coefficient was obtained, however, shifted from about 8.0 to 7.65. In addition the dependence of the sedimentation coefficient on pH was much greater in H_2O than in D_2O. Although the reacting form of homoserine dehydrogenase exists partially in the dimeric form at most pH values and the effectors L-threonine or L-aspartate plus potassium ion influence the association state of the enzyme, it does not appear that the association–dissociation equilibrium, *per se*, is an important factor in the regulation of homoserine dehydrogenase activity.

In *Escherichia coli* K12 the threonine-sensitive aspartokinase and homoserine dehydrogenase activities are carried by a single protein molecule, aspartokinase–homoserine dehydrogenase I. Recent studies (Starnes *et al.*, 1972; Clark and Ogilvie, 1972; Falcoz-Kelley *et al.*, 1972) indicate the protein which has a molecular weight of 360,000 is composed of 4 apparently identical subunits. As control points in the biosynthesis of several aspartate family amino acids the aspartokinase and homoserine dehydrogenase activities are subject to modulation by several small molecules. L-Threonine functions as a feedback inhibitor of both enzymatic activities while L-aspartate can serve as an activator of aspartokinase as well as an inhibitor of homoserine dehydrogenase. In addition, potassium ion is an activator of both activities. Some time ago Wampler *et al.* (1970) reported that, on removal of these biological effectors, aspartokinase–homoserine dehydrogenase (AK-HSDH[1]) dissociated into smaller catalytically active units which they characterized as dimers. Since their study indicated that more than one polymeric form of the enzyme is functional in the homoserine dehydrogenase reaction it was of interest to determine the aggregation state of the molecule catalyzing the dehydrogenase reaction under a variety of conditions.

The technique of reacting enzyme sedimentation developed by R. Cohen *et al.* (1967) was chosen for this study since it permits the direct monitoring of reacting enzyme molecules without need for physical separation of protein species prior to activity determination. Extreme care, however, is necessary both in determination of experimental conditions and in interpretation of results. Wampler (1972) has recently used this technique in a study of HSDH. Our results indicate that he may have encountered some of the difficulties associated with the technique as well as difficulties resulting from the highly pH-sensitive association–dissociation equilibrium of the enzyme. The relationship of the present study to that of Wampler will be discussed later in this paper. Several reports in addition to that of Wampler indicate that biologically important ligands influence the association state of AK-HSDH. Removal of L-threonine from buffers has been found to cause dissociation of the native protein as well as loss of HSDH sensitivity to threonine (Patte *et al.*, 1963; Barber and Bright, 1968; Cunningham *et al.*, 1968; Wampler *et al.*, 1970). Finally L-aspartate in the presence of potassium ion also appears to stabilize the tetramer (Takahashi and Westhead, 1971). Consequently we have examined the effects of L-threonine, L-aspartate, and potassium ion on the sedimentation behavior of the reacting form of AK-HSDH.

The effects of pH variation on the sedimentation of reacting AK-HSDH were also determined since other reports have indicated a pH sensitivity of the HSDH activity. Truffa-Bachi *et al.* (1966a) found inactivation of AK-HSDH occurred at pH 9.0 and was partially prevented by inclusion of HSDH substrates or L-aspartate in incubation mixes. Inactivation at pH 9.0 was accompanied by loss of HSDH sen-

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[1] Abbreviation used is: AK-HSDH, threonine-sensitive aspartokinase–homoserine dehydrogenase.

sitivity to threonine, which was prevented only by threonine itself. In addition, investigators of AK-HSDH have utilized two HSDH activity assays, conducted at two different pH values.

Methods

Purification. AK-HSDH was isolated from *Escherichia coli* K12,[2] by slight modification of the procedure of Truffa-Bachi *et al.* (1968). Specific activity of HSDH obtained by this procedure was 32.

Enzyme Assays. Homoserine dehydrogenase activity in the forward reaction (aspartic semialdehyde → homoserine) was determined by measuring the decrease in optical density due to NADPH oxidation (Patte *et al.*, 1966). The components of the reaction mixture (pH 7.5) were 0.26 mM NADPH, 1 mM aspartate semialdehyde, 0.05 M Tris, 0.3 M KCl, 2 mM $MgAc_2$, and 2 mM KEDTA. Aspartate semialdehyde was prepared according to Black and Wright (1955). Activity in the reverse reaction (homoserine → aspartic semialdehyde) was determined by measuring the optical density increase due to $NADP^+$ reduction essentially as described by Ogilvie *et al.* (1969). The components of the reaction mixture (pH 8.9) were 1.3 mM $NADP^+$, 26 mM L-homoserine, 0.1 M Tris, and 0.3 M KCl. At these buffer concentrations pH changes, caused by the catalytic reaction, are insignificant and the pH remains constant during centrifugation. Unless specified reaction mixtures were made 30 or 40% in D_2O (Bio-Rad, Biological Purity Grade). pD values were obtained by adding 0.12 and 0.16 unit to pH meter readings for 30 and 40% respectively (Glasoe and Long, 1960). Both activities were measured at 20° and units of specific activity were micromoles of NADPH per minute per milligram of protein.

Protein Assays. Protein concentration was measured using the extinction coefficient 0.63 cm^2/mg at 278 mμ determined by Falcoz-Kelly *et al.* (1972).

Active Enzyme Sedimentation. Experiments were performed in a Beckman-Spinco Model E analytical ultracentrifuge. An AnD rotor was used in all experiments. Temperature was controlled and measured at approximately 20° by use of a Beckman RTIC unit. Double-sector capillary type synthetic boundary cells with quartz windows and a 12-mm optical path were used. Centrifugation was conducted at 52,000 or 60,000 rpm.

The reacting enzyme sedimentation procedure of Cohen (Cohen, 1963; Cohen and Hahn, 1965; R. Cohen *et al.*, 1967) was used. In all cases HSDH activity was followed. Deuterium oxide, 30 or 40% was used to produce positive density gradients in assay solutions as described by Taylor *et al.* (1972). Assay solutions were as described in enzyme assays with adjustments in pH and effector concentration where indicated. The main sample compartment and reference compartment were filled with 0.29 and 0.31 ml of assay solution, respectively. The sample reservoir received 6 μl of enzyme solution. Enzyme concentrations used in the forward assay at pH 7.66 were in the range 2.2–3.4 μg/ml. Concentrations used in the reverse assay at pH 9.06 ranged from 35 to 55 μg per ml. When the pH of the assay solution was changed or effectors added, enzyme concentrations were adjusted to produce the same optical density changes as in the above systems. As the band of reacting enzyme sedimented through the assay solution absorbance by NADPH was followed at 340 mμ using the photoelectric scanner at 2-min intervals.

[2] The bacterial strain used in these experiments was kindly supplied by Dr. H. E. Umbarger.

Viscosity and Density Measurements. Densities of all assay solutions were determined at 20.0 ± 0.02° in a 2.0-ml Lipkin pycnometer calibrated with twice-distilled water. The procedure of Daniels *et al.* (1962) was followed. Viscosities were determined in a 5-ml Ostwald viscosimeter with a flow time of 82.0 sec for twice-distilled water at 20°. Observed sedimentation coefficients in solvents containing deuterium oxide were corrected by a formula (Taylor *et al.*, 1972) derived from the equations of Martin *et al.* (1959) and Schachman (1959).

$$s_{20,w} = s_{obsd}\left(\frac{\eta_{t,w}}{\eta_{20,w}}\right)\left(\frac{\eta_{soln}}{\eta_{t,w}}\right)\left(\frac{(1-\bar{v}\rho)_{20,w}}{(1-\bar{v}\rho/k)_{t,soln}}\right)\frac{1}{k}$$

where k is the correction factor for deuteration of the protein (Martin *et al.*, 1959). The corrections in H_2O systems ranged from 1.07 to 1.15 while those in D_2O solutions were from 1.28 to 1.42.

Data Analysis. The technique of active enzyme sedimentation may be subject to some serious artifacts. Enzyme concentration should be low enough so that substrate levels remain well above K_m values throughout centrifugation. When excessive quantities of enzyme are used, enzyme molecules in the leading part of the sedimenting band produce more product than do molecules in the trailing part of the band. Thus that product produced in the leading part of the band is primarily measured and as the band widens due to diffusion the sedimentation coefficient becomes increasingly higher than the true value. This situation also can result when a reversible reaction, such as the homoserine dehydrogenase reaction, is studied. In this case enzyme molecules in the leading edge of the sedimenting band react in the presence of substrates alone while molecules in the trailing edge react in the presence of product. Thus, more product is again produced by molecules in the leading edge of the band (Cohen and Mire, 1971). Cohen and Mire were able to increase the apparent sedimentation coefficient of glucose-6-phosphate dehydrogenase from 6.3 to 6.9 by overloading the system. Before sedimentation studies were begun precautions were taken in an effort to avoid the artifacts discussed above. Levels of substrates in assay solutions were at least 4-fold higher than K_m values.

The optical density range in which the reverse HSDH reaction was linear was determined in a spectrophotometer. In the ultracentrifuge the range of enzyme units which gave maximum absorbance changes within this linear range was also checked and enzyme quantities within this range were utilized. The lack of exact linearity of the forward reaction made such precautions impossible in the forward system. Owing to this and to the higher enzyme concentration possible with the less sensitive reverse assay, the reverse system was utilized wherever possible.

Sedimentation coefficients were determined by the method of midpoints for all runs. The log of the distance of the sedimenting boundary from the axis of rotation at the half-height of the boundary was plotted as a function of time and the sedimentation coefficient was calculated from the slope of the line of the best fit (Schachman, 1959). As a check on the validity of the method, sedimentation coefficients were also determined by the difference curve method of Cohen and Mire (1971) for at least one run at each experimental condition. The difference in product distribution between two successive scanner traces was measured and plotted as a function of the distance from the axis of rotation. Gaussian-shaped difference curves were obtained (Figure 1B). The log of the distance from the axis of rotation at the maximum of each difference curve was plotted as a function of the mean time

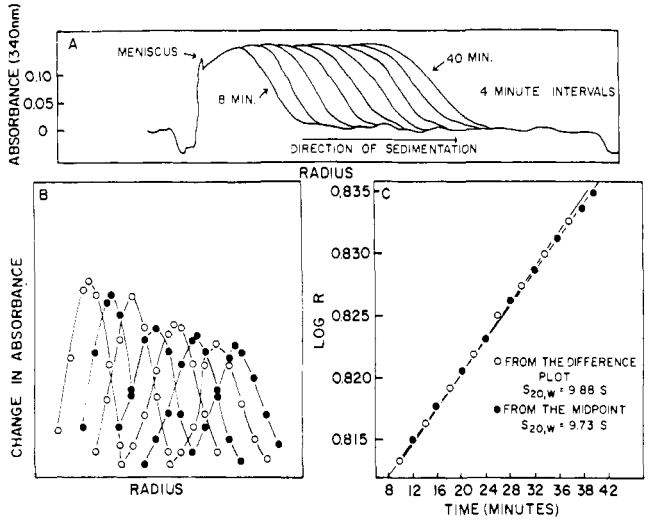

FIGURE 1: Analysis of the sedimentation of the reacting form of aspartokinase–homoserine dehydrogenase. Homoserine dehydrogenase activity was measured in the reverse direction at pH 7.65 in H_2O with no effectors. Conditions for centrifugation were: Rotor speed, 52,000 rpm; scan speed, fastest; chart speed, 25 mm/sec; noise suppression, off; wavelength, 340 nm; optical density range, 0–0.2. (A) Successive scanner traces superimposed on a single frame of reference; (B) difference curves; (C) plots of the log of the radial position as a function of time for data obtained from the difference curves (○) and from the midpoint of the boundary (●). Each $s_{20,w}$ was calculated from the slope of the line of best fit for the respective data.

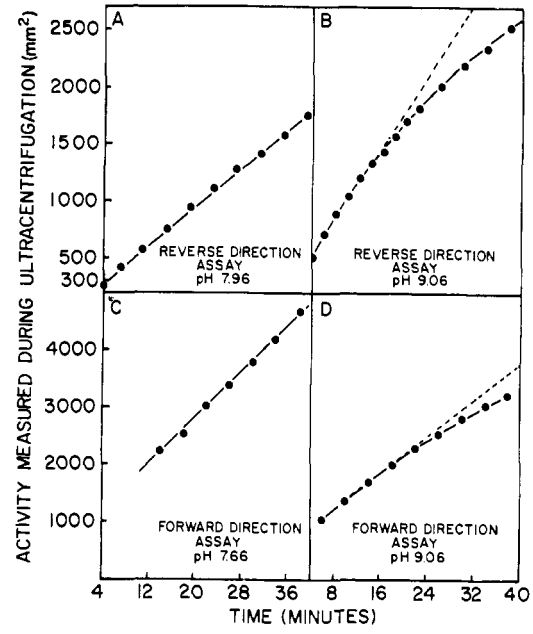

FIGURE 2: The variation of homoserine dehydrogenase activity during ultracentrifugation. Activity is expressed in terms of area under each scanner trace corrected for radial dilution. (A) Assay of homoserine dehydrogenase in the reverse reaction at pD 7.96; (B) the reverse reaction at pD 9.06; (C) the forward reaction at pD 7.66; (D) the forward reaction at pD 9.06. All examples were in D_2O in the absence of effectors. See Methods section for buffers used.

for each difference curve and the sedimentation coefficient was determined as for the midpoint method. For all experimental conditions reported, the sedimentation coefficients determined by the two methods differed by less than the scatter of the data for the particular condition (Figure 1C). As shown by Taylor (1972) the method of midpoints can provide a simple, reasonably accurate measure of the sedimentation coefficient in the technique of reacting enzyme centrifugation.

Several experiments were also analyzed for possible activity changes of the enzyme during sedimentation. Total product formed at each 2-min scanner trace was determined, corrected for radial dilution (Vinograd and Bruner, 1966), and plotted *vs.* time. Linear plots were obtained in all cases of the reverse system except for experiments at pH 9.06 with no effectors or with D-aspartate. In both of the latter conditions there was a gradual loss in activity up to 20% during the course of an experiment. This could be due to either lower activity of the dimer formed at pH 9.06 or to slight inactivation of the dimer.

In the forward system a significant activity decrease was noted only at pH 9.06. Activity dropped 12% in 38 min. Some examples are shown in Figure 2.

Sedimentation coefficients for conditions which resulted in nonlinear log R *vs.* t plots were determined in the following manner. The point at which the slope changed was determined by eye and was checked by determining the initial point which gave the highest correlation coefficient for the least-squares line of the second slope. Sedimentation coefficients reported are those resulting from this second slope. Such nonlinear log R *vs.* t plots occurred in systems in which activity was found to decrease during a run.

Results

HSDH Stability under Active Enzyme Sedimentation Conditions. Much of the following study involves use of solutions 40% in deuterium oxide. Since the presence of D_2O could result in decreased activity due to deuteration of crucial groups on the enzyme, the substrates or both, activity in D_2O systems had to be compared to that in H_2O systems. No basic activity differences were found between H_2O and D_2O systems.

The centrifuge experiments required 40–60 min and it was necessary to examine the stability of the enzyme in the sedimentation solutions for that period of time. The results of this study are presented in Figure 3. HSDH measured in the forward reaction shows maximum activity at pD 7.36. Activity falls slowly below this pD value and rapidly above it. The rapid loss of activity above pD 7.36 could be due to instability of substrates in basic solutions as well as instability of the enzyme itself. In the sedimentation studies the pD 9.06 forward system was checked carefully for possible enzyme overloading due to loss of substrates but this did not appear to cause a problem in the range of concentrations employed.

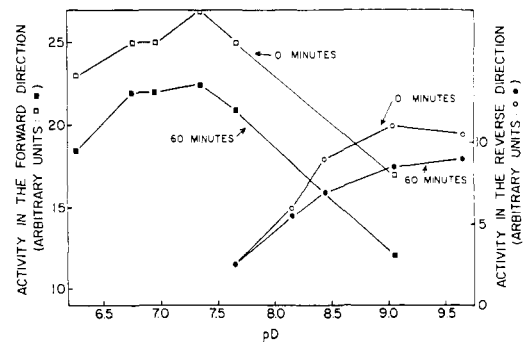

FIGURE 3: Homoserine dehydrogenase activity and stability during incubation in 40% D_2O at zero time (□) and at 60 min (■) in the forward reaction solution and at zero time (○) and at 60 min (●) in the reverse reaction solution.

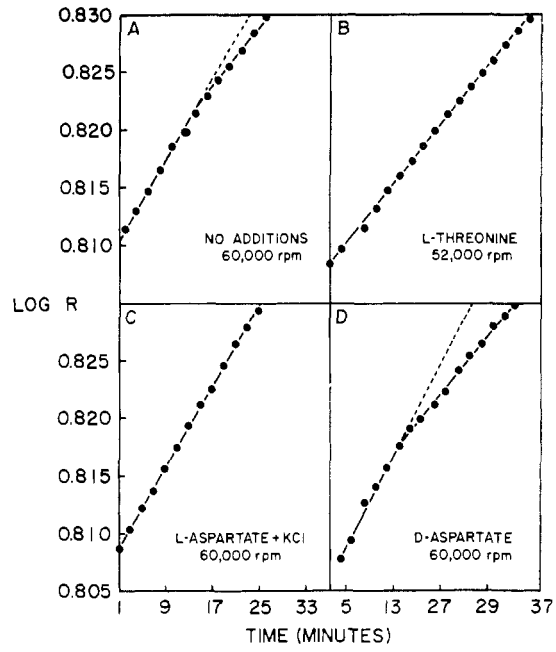

FIGURE 4: Plots of the log of the radial position (midpoint) *vs.* time for experiments at pD 9.06. Activities were measured with the homoserine dehydrogenase assay in the reverse reaction in 40% D_2O for experiments (A) with no effectors, (B) 1×10^{-2} M L-threonine, (C) 1×10^{-2} M L-aspartate plus 3×10^{-1} M KCl, and (D) 1×10^{-2} M D-aspartate plus 3×10^{-1} M KCl.

In all cases enzyme activity was lost on prolonged exposure to forward assay systems. It is thought that this activity loss did not lead to highly erroneous sedimentation coefficients for any totally inactivated enzyme would not be measured by the technique. The pH–activity profile for the reverse reaction rises from 7.66 to 9.06. This rise may reflect larger contributions from the forward reaction in the lower pH range. If artifacts due to a reversible reaction are present a concentration dependence of the apparent sedimentation coefficient should be observed. No such dependence was observed at pD 7.66 where the forward reaction should make the largest contribution. In addition, analysis of data indicated neither sedimentation artifacts nor activity decrease during sedimentation at this pH.

Forward and Reverse Homoserine Dehydrogenase Assays during Reacting Enzyme Sedimentation. HSDH activity is routinely measured in the forward reaction (hydrogenation of aspartate semialdehyde) with NADPH at pH 7.5 or in the reverse reaction (dehydrogenation of homoserine with $NADP^+$) at pH 9.0. Therefore, reacting enzyme sedimentation experiments were conducted using both assay systems. The sedimentation coefficient of AK-HSDH measured in the forward system was 10.3 ± 0.11 S while that measured in the reverse system was 7.78 ± 0.21 S. In order to determine whether the observed difference was due to the change in pH or to the direction of assay, AK-HSDH was sedimented through both forward and reverse assay systems under a variety of conditions. Results are presented in Table I. The $s_{20,w}$ obtained in the forward system did not differ significantly from that obtained in the reverse system at any pH studied. The slightly higher values consistently observed in the reverse system may be due to the 20-fold higher enzyme concentrations required in this system. It therefore appears that the initially observed sedimentation difference is due to pH variation rather than the direction of assay.

TABLE I: Comparison of Sedimentation Coefficient in Forward and Reverse Assay Systems.[a]

pH	Effector	$s_{20,w}$[b] Forward Reaction	$s_{20,w}$[b] Reverse Reaction
7.66		10.3 ± 0.1 (4)	
7.96			10.6 ± 0.14 (4)
9.06		7.63 ± 0.24 (4)	7.78 ± 0.21 (8)
9.06	1×10^{-3} M L-Thr	10.24 (1)	10.75 ± 0.11 (3)
8.00[c]		7.03 ± 0.16 (2)	7.28 ± 0.11 (2)

[a] Buffers are described in Methods. Note all buffers contain D_2O unless otherwise indicated. [b] Average ± standard deviation (number of experiments). [c] No D_2O.

Reacting Enzyme Sedimentation at pD 9.06. At pD 9.06, plots of the log of the radius *vs.* time shown in Figure 4A exhibit a breakpoint. The slope of the first portion of the plot corresponds to a sedimentation coefficient of about 10.5 S while that of the second portion gives a value of 7.78 S. The transition would appear to represent a slow dissociation from tetramer to dimer during incubation at pH 9.06. It has been reported (Truffa-Bachi *et al.*, 1966b) that HSDH undergoes a first-order inactivation when exposed to Tris buffer at pH 9.0. Substrate levels of $NADP^+$ and homoserine as well as 10^{-2} M L-aspartate were found to provide considerable protection against this inactivation. Inactivation at pH 9.0 was accompanied by desensitization to threonine inhibition which was prevented totally by addition of L-threonine itself and partially by L-aspartate plus potassium ion.[3]

In an effort to define the relationship between the pH 9.0 inactivation reported by Truffa-Bachi *et al.* (1966a) and the dissociation noted in the present study, we conducted reacting enzyme sedimentation experiments at pD 9.06 in the presence of several effectors. When L-aspartate plus potassium ion or L-threonine was present in the reaction system the log *R vs. t* plot was linear (Figure 4B,C) and sedimentation coefficients of 10.47 ± 0.22 and 10.78 ± 0.11 S were obtained with L-aspartate plus potassium ion and L-threonine, respectively. It is evident that the pD 9.06 dissociation is prevented by both L-aspartate plus potassium ion or by L-threonine. On the other hand, D-aspartate which does not protect against pH 9.0 inactivation also fails to prevent dissociation, for the log *R vs. t* plot again exhibits a break (Figure 4D) and the sedimentation coefficient is about 8.0 S. L-Aspartate in the absence of potassium ion and L-homoserine plus $NADP^+$ which protect against inactivation do not prevent dissociation at pD 9.06. In both cases the log *R vs.* time plots are characterized by two slopes and sedimentation coefficients are 8.0 ± 0.34 and 7.78 ± 0.21 S for L-aspartate and L-homoserine plus $NADP^+$, respectively.

Effect of pD Variation on Reacting Enzyme Sedimentation. The reacting enzyme sedimentation studies at pD 7.66 and 9.06 indicate a pH dependence of the association state of reacting AK-HSDH. Experiments were therefore conducted at pD values ranging from 6.26 to 9.66. The pD curve obtained in 40% D_2O is shown in Figure 5. The tetrameric form of the enzyme appears to predominate only near pD 8.0. When the pD is raised or lowered from this point AK-HSDH appears

[3] J. C. Mackall and K. E. Neet, unpublished observations.

TABLE II: Sedimentation in the Presence of Effector Molecules.[a]

pH (D)	Reaction[b]	Effector	$s_{20,w}$[c] (S)
6.8	F[d]	2×10^{-2} M L-Thr	10.7 ± 0.07 (2)
7.66	F	2×10^{-2} M L-Thr	10.62 ± 0.12 (4)
9.06	F	1×10^{-2} M L-Thr	10.24 (1)
9.06	R	1×10^{-2} M L-Thr	10.75 ± 0.11 (3)
9.66	R	2×10^{-2} M L-Thr	10.1 ± 0.09 (2)
7.96	R	2×10^{-2} M L-Asp	11.0 ± 0.2 (3)
9.06	R	1×10^{-2} M L-Asp	10.47 ± 0.22 (4)
9.66	R	2×10^{-2} M L-Asp	7.71 ± 0.25 (2)
9.06	R[e]	2×10^{-2} M L-Asp	8.00 ± 0.34 (2)

[a] Buffers are described in Methods. Note all buffers contain D_2O and 0.3 M KCl unless otherwise indicated. [b] F = assay in the forward reaction; R = assay in the reverse reaction. [c] Average ± standard deviation (number of experiments). [d] No D_2O. [e] No KCl.

to dissociate into increasing amounts of dimer. The expected concentration dependence of the sedimentation coefficient was not observed in the region of the maximum or minimum sedimentation values. This may be due to the small range of concentration available for study or to the lack of such dependence at these points. At points of intermediate sedimentation rate particularly at pD 8.16 and 8.46, however, concentration dependence was observed. This probably reflects the association–dissociation occurring in this pD region.

The study at pD 9.06 indicates that the allosteric effectors L-threonine and L-aspartate in the presence of potassium ion influence the association state of the reacting form of AK-HSDH. In order to determine whether or not these effects extended over the pH range of interest we conducted experiments at several pH(D) values in the presence of effectors. Results are presented in Table II. In the presence of L-threonine the reacting enzyme appears to exist predominantly as tetramer at all pH(D) values from 6.8 to 9.66. In contrast stabilization of tetramer by L-aspartate in the presence of potassium ion is only effective between pD 7.96 and 9.06. At pD 9.66 L-aspartate plus potassium ion do not appear to prevent dimer formation while below pD 7.96, where the forward assay is necessary, the effects of L-aspartate plus potassium ion appear more complex and are difficult to interpret due to significant boundary deterioration.

The study at pD 9.06 revealed a difference in HSDH sedimentation behavior in the presence of L-aspartate plus potassium ion or L-aspartate alone. Experiments were done to determine if the effect of L-aspartate plus potassium ion was due to the combination of L-aspartate and potassium ion or to potassium ion alone. The magnitude of the effect of potassium ion alone appears to be much less than that of L-aspartate plus potassium ion. Removal of potassium ion from buffer solutions did not lower the sedimentation coefficients of HSDH at pD 7.96 or at pH 6.8 in the presence of threonine. Raising the level of KCl from 0.3 to 0.7 M shifted the association–dissociation equilibrium slightly toward the tetrameric form. In water at pH 7.65 the sedimentation coefficient increased from 10.0 ± 0.19 S at 0.3 M KCl to 10.6 ± 0.12 S at 0.7 M KCl while at pH 8.0 the value increased from 7.28 ± 0.11 to 8.36 ± 0.3 S. The small difference which is observed may reflect a slight increase in the pK of the group(s) involved due to a conformational change caused by potassium or by a

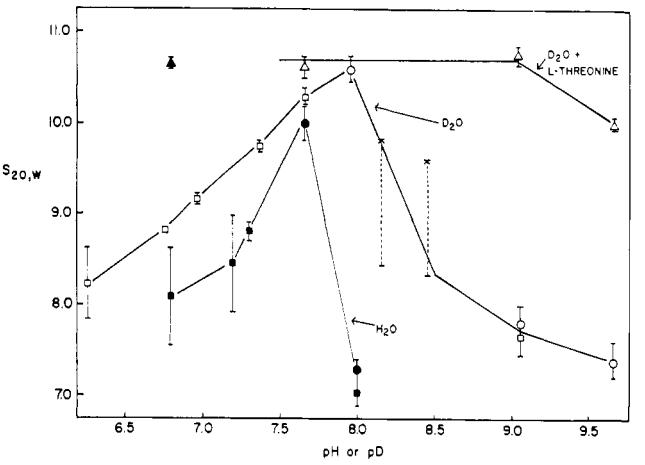

FIGURE 5: Sedimentation of aspartokinase–homoserine dehydrogenase as a function of pH or pD. Experiments in D_2O with the homoserine dehydrogenase assay in the forward reaction (□), in D_2O with the assay in the reverse reaction (○), in H_2O with the assay in the forward reaction (■), in H_2O with the assay in the reverse reaction (●), in D_2O with 2×10^{-2} M L-threonine (△), in H_2O with 2×10^{-2} M L-threonine (▲). The dotted arrows at pD 8.16 and 8.46 indicate the dependence of the sedimentation coefficient on concentration and represent the range of $s_{20,w}$ values obtained from 38 to 57 μg per ml (pD 8.16) and from 68 to 98 μg per ml (pH 8.46).

change in solution properties. The presence of L-aspartate plus potassium ion is apparently the most effective in stabilizing the tetramer.

Reacting Enzyme Sedimentation Studies in H_2O. Since the possibility existed that the results observed were a result of the D_2O system employed to stabilize the boundary, several experiments were conducted under identical conditions in the absence of D_2O. Results are presented in Figure 5. As in the D_2O system, the sedimentation coefficient of AK-HSDH exhibits a maximum. That maximum, however, has shifted from around pD 8.0 to pH 7.6. In addition the dependence of the sedimentation coefficient on pH is considerably greater than observed in D_2O. As seen in the D_2O system, L-threonine stabilizes the tetrameric form of AK-HSDH in H_2O. It therefore appears that although there are quantitative differences between the D_2O and H_2O systems, the transitions with pD observed in D_2O are not peculiar to that system.

Discussion

The technique of R. Cohen *et al.* (1967) for determining the sedimentation coefficients of reacting enzymes was exceptionally useful in our study. In addition, the use of deuterium oxide to create a positive density gradient in assay solutions as suggested by Taylor *et al.* (1972) was unexpectedly advantageous. Not only did D_2O provide sufficient density and eliminate the need of high salt concentrations which could effect the enzyme, it also spread out and thus magnified the pH (pD) dependence of the sedimentation coefficient. The pH dependence of the sedimentation coefficient is exceedingly strong in water (see Figure 5) and effects due to small pH changes could easily be attributed to other factors.

In both D_2O and H_2O systems the sedimentation coefficients measured in the forward and reverse HSDH assay systems are identical when measured under the same conditions. The size of the reacting form of AK-HSDH is totally independent of the direction of catalysis. This finding is at variance with the conclusion of Wampler (1972) who reported a sedimenta-

tion coefficient of 9.2–10.7 S for the forward HSDH reaction at pH 7.5 and 7.9 S for the reverse reaction at pH 7.8. Owing to the large variation over the small pH range he concluded that the difference was not due to pH alone. Using Wampler's assay conditions we found a sedimentation coefficient of 10.6 S for the reverse reaction at pH 7.65 and 8.36 S for the reverse reaction at pH 8.0. Thus, the large difference between the two values observed by Wampler probably resulted from the fact that he was working in the region where the value of the sedimentation coefficient is highly pH dependent.

Cohen and Mire (1971) demonstrated that when a reacting enzyme system is overloaded the apparent sedimentation coefficient rises as enzyme concentration is increased. Below the concentration at which the system is overloaded the value of the sedimentation coefficient remains constant with increasing concentration unless there exists a true concentration dependence of the sedimentation coefficient. Our experiments were in a narrow concentration range in which substrate utilization was below 20%. Concentration dependence was not observed except at pH 8.16–8.46 where association–dissociation probably occurs (see Figure 5). It is suggested that the concentration dependence reported by Wampler (1972) over a 60-fold concentration range may be due to inadequate concentrations of substrates at higher levels of enzyme. The 80% NADPH utilization he reports in one experiment is well above the substrate utilization of a few per cent suggested by Cohen and Mire (1971). Such a rise in sedimentation coefficient with enzyme concentration should warn the investigator of possible overloading. A conclusion of a concentration dependence of the sedimentation coefficient should be made only with extreme caution.

Our sedimentation studies indicate that L-threonine and L-aspartate plus potassium ion, which protect against loss of HSDH activity and sensitivity to threonine at pH 9.0, also prevent dissociation of AK-HSDH. L-Aspartate and $NADP^+$ plus homoserine which also prevent HSDH activity loss but not loss of HSDH sensitivity to threonine, do not prevent dissociation at pD 9.06. It is apparent that protection by L-threonine and L-aspartate plus potassium ion differs from that by L-aspartate and HSDH substrates. L-Threonine and L-aspartate plus potassium ion may afford protection by preventing dissociation of the enzyme to a dimer which is more susceptible to inactivation than is the tetramer. L-Aspartate and substrates may prevent inactivation of the dimer once it is formed. The threonine sensitivity of the tetramer appears to be considerably more stable than that of the dimer for only those ligands which prevent dissociation protect against loss of sensitivity to threonine.

Several investigators (Patte *et al.*, 1963; G. N. Cohen *et al.*, 1967; Wampler *et al.*, 1970) have found that when AK-HSDH is subjected to treatments causing dissociation, the enzyme loses varying amounts of HSDH activity and becomes totally insensitive to threonine. While L-homoserine, $NADP^+$, and L-aspartate were found to protect against activity losses, only L-threonine or L-aspartate plus potassium ion prevented loss of sensitivity to threonine. It appears that the initial step in various inactivation processes may be dissociation of AK-HSDH to a less active dimer which is but transiently sensitive to threonine and is more subject to inactivation than is the tetramer. It appears that the tetramer is the only species capable of retaining HSDH sensitivity to threonine. This might explain why only threonine or L-aspartate plus potassium prevent loss of HSDH threonine inhibition while several ligands protect against activity loss. It should be emphasized that while we propose stable HSDH threonine sensitivity for the tetramer alone, we do not intend to imply that threonine exerts its control through stabilization of the tetramer. Although those same ligands which modify the allosteric equilibrium of HSDH influence the association–dissociation behavior of the molecule, there is no obvious relationship between the two processes. Indeed no role is seen for the dimeric form in regulation of HSDH activity. In fact the existence of active dimers with altered responsiveness to effector molecules poses a significant problem for experimental workers. Accurate interpretations of the results of studies of AK-HSDH are possible only when the experimentor is aware of the effects of various experimental conditions on the association state of the molecule and of the properties of that association state.

The shape of the pH(D) curve obtained in this study (see Figure 5) indicates that there are probably two dissociation processes occurring, one process very sensitive to pH changes above pH 8.0 and another less sensitive to pH below pH 8.0. The two dissociation processes could be due to titration of different groups involved in subunit interactions in the two pH regions. The pH range involved indicates that dissociation above pH 8.0 may involve titration of a sulfhydryl group(s). The observation that AK-HSDH dissociates when treated with sulfhydryl reagents (G. N. Cohen *et al.*, 1967) lends support to this possibility. Dissociation below pH 8.0 may be due to titration of a histidine residues(s). The pH dependence of the sedimentation coefficient is more severe in H_2O than in D_2O and the pH at which the maximum sedimentation coefficient is observed is higher in D_2O. Both differences between the H_2O and D_2O systems may be attributed to differences in solution properties between 0 and 40% D_2O. In 40% D_2O the higher pH maximum and the slower transition above pH 8.0 are probably due to the increased stability of the base–D bond over the base–H bond. Reduction of acid strength in D_2O has long been recognized (Bunton and Shiner, 1961).

The sedimentation coefficient for the AK-HSDH tetramer found here at pH 6.8 in presence of threonine, 10.7 ± 0.07 S, is lower than the $s^0_{20,w}$ of 11.4 S reported for noncatalytic conditions by Starnes *et al.* (1972), Truffa-Bacha *et al.* (1968) and confirmed in our laboratory. There are several possible explanations for the discrepancy. First, it could result from solution differences such as the presence of substrates in the active enzyme sedimentation studies. Preliminary sedimentation velocity studies with forward enzyme assay mix in which aspartate semialdehyde has been replaced by homoserine indicate that this is not the case.[3] A second possible explanation arises from the fact that sedimentation and diffusion of products during centrifugation were not included in calculation of the results. R. Cohen *et al.* (1967) have estimated this could cause an error of 3–5%. Finally the discrepancy could represent a conformational change when catalysis is occurring.

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Lanthanide Ions Activate α-Amylase†

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ABSTRACT: *Bacillus subtilis* α-amylase, which has had its calcium removed, can be reactivated under carefully controlled conditions by lutetium(III) as well as by a variety of other lanthanides. The reactivation process is extremely sensitive to concentrations of the lanthanide ions and buffer system. Thus at pH 6.9 in Hepes (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) buffer, concentrations of Lu^{3+} above 10^{-4} M inhibit the enzyme, whereas in maleate buffer at pH 6.9, apoamylase is activated by concentrations up to 10^{-3} M Lu^{3+}.

The lanthanide ions have recently been suggested as calcium ion substitutes for calcium ion binding sites in proteins and enzymes. (Darnall and Birnbaum, 1970; Birnbaum *et al.*, 1970; Williams, 1970). In contrast to the calcium ion, however, the varied magnetic and spectral properties of the rare earth metal ions should make excellent spectroscopic probes of the metal ion binding sites in proteins. It has been shown recently that the lanthanide ions isomorphously replace the calcium ion in α-amylase (Smolka *et al.*, 1971), in thermolysin (Colman *et al.*, 1972), and in the activation of trypsinogen to trypsin (Darnall and Birnbaum, 1970). In addition it has been shown that lanthanide ions replace Mg^{2+} ion in leucine tRNA synthetase (Kayne and Cohn, 1972) and pyruvate kinase (Valentine and Cottam, 1973). Lanthanide ions also replace the calcium ion and the transition metal ion in concanavalin A (Sherry and Cottam, 1973).

α-Amylase from *Bacillus subtilis* contains two identical subunits each of which appears to bind two calcium ions (Stein *et al.*, 1964; Imanishi, 1966). Removal of calcium from the enzyme results in loss of activity whereas addition of calcium back to the apoenzyme results in the recovery of full activity. Smolka *et al.* (1971) showed that the rare earth metal ions are capable of substituting for the calcium ion in *B. subtilis* α-amylase and produce active enzymes. Levitzki and Reuben (1973) utilizing somewhat different experimental conditions were unable to obtain activation of α-amylase with lanthanide ions and as a result have challenged our assertion that lanthanide ions can activate α-amylase. They reported that each subunit of α-amylase binds two gadolinium ions which do not activate the enzyme and hence lanthanides form only "abortive complexes" with α-amylase.

We have repeated and extended our original experiments and herein resolve the apparent discrepancies between the two earlier reports.

Materials and Methods

Crystalline *B. subtilis* α-amylase (lot 108B-0590, Sigma) was used without further purification. The enzyme sedimented as a single symmetrical boundary in the ultracentrifuge and gave a single band upon polyacrylamide gel electrophoresis.

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