# Threonine-Sensitive Aspartokinase–Homoserine Dehydrogenase Complex from *Escherichia coli*. Subunit Stoichiometry and Size of the Catalytic Unit†

D. Eugene Wampler

ABSTRACT: The subunit size of the threonine-sensitive aspartokinase–homoserine dehydrogenase complex from a derepressed mutant of *Escherichia coli* (TIR-8) has been studied using sodium dodecyl sulfate acrylamide gel disc electrophoresis, gel filtration in 6 M guanidine hydrochloride, and equilibrium sedimentation under mild dissociating conditions. Data from all three of these methods indicate a monomer molecular weight of approximately 90,000. These results indicate that the enzyme, as normally isolated, is composed of four subunits. Gel filtration and a variety of sedimentation techniques have been used to examine the extent of subunit aggregation both in the absence of biological ligands and under catalytic conditions. In the absence of ligands the enzyme exists in an association–dissociation equilibrium which tends toward dissociation at high pH and low temperature. In the presence of ligands, under catalytic conditions, the extent of association depends on the reaction conditions. Under conditions for the two forward reactions (aspartate to aspartyl phosphate and aspartic semialdehyde to homoserine) both the partition coefficient with Sephadex G-200 and the sedimentation coefficient are those of the tetramer. These coefficients do not change upon the addition of the feedback inhibitor, L-threonine. Under conditions for the homoserine dehydrogenase reaction in the reverse direction, the partition and sedimentation coefficients indicate an average molecular size smaller than the tetramer. The addition of L-threonine to the reverse reaction mixture causes a return of hydrodynamic properties toward those of the tetramer. Although the feedback inhibitor stabilizes the tetrameric form of the threonine-sensitive aspartokinase–homoserine dehydrogenase complex it is unlikely that differences in the state of aggregation play a role in the biological control mechanism.

The threonine sensitive aspartokinase–homoserine dehydrogenase complex (AK–HSDH-I)[1] from *Escherichia coli* is a polymeric enzyme carrying two catalytic functions (aspartokinase and homoserine dehydrogenase) and a regulatory function (susceptibility to the feedback inhibitor, L-threonine) (Patte *et al.*, 1966). Until recently the complex was thought to be a 360,000 oligomer composed of six subunits (Truffa-Bachi *et al.*, 1969). This subunit stoichiometry was supported by several observations (summarized by Cohen, 1969) including equilibrium sedimentation in 6 M guanidine hydrochloride and stoichiometry of NADP binding. Recently, however, Clark and Ogilvie (1972), Starnes *et al.* (1972), and I (unpublished observations) independently observed that the subunits exhibit a molecular weight of 80,000–90,000 on sodium dodecyl sulfate acrylamide gel electrophoresis. Starnes *et al.* (1972) also reinvestigated the equilibrium sedimentation behavior in 6 M guanidine hydrochloride and obtained a subunit molecular weight of 80,000 rather than 60,000. Clark and Ogilvie (1972) examined the NADP binding stoichiometry and obtained 2 moles bound per mole of enzyme rather than 3 moles as reported earlier (Janin *et al.*, 1969). Because most methods for determining subunit size or ligand stoichiometry depend on experimental quantities such as partial specific volume or molar extinction coefficient and because the recent results were in direct conflict with earlier reports, it seemed necessary to extend the investigation of subunit size to other methods. Sodium dodecyl sulfate disc electrophoresis and guanidine-HCl gel filtration are two independent measures of subunit size which appear to depend almost entirely on polypeptide chain length. This paper describes results obtained by these two methods and in addition, results of velocity sedimentation and equilibrium sedimentation in dilute salt solutions where estimates of partial specific volume are more reliable.[2]

Because AK–HSDH-I appears to dissociate to functional subunits when placed in buffers which do not contain biologically effective ligands (Wampler *et al.*, 1970) it was of interest to determine the state of subunit aggregation under catalytic conditions and to investigate the possibility that feedback inhibition might involve changes in aggregation state.

## Experimental Procedure

Bovine serum albumin, ovalbumin, β-galactosidase, and chymotrypsinogen were obtained from Mann Research Laboratories. Catalase and phosphorylase *a* were obtained from Sigma Chemical Co. Calf skin tropocollagen was the generous gift of Dr. Robert Church. Guanidine hydrochloride was Schwarz–Mann Ultra Pure and the 4% agarose gel was Bio-Gel A-15m (Bio-Rad Laboratories). AK–HSDH-I was purified from a derepressed mutant of *E. coli* K-12 (for a

† From the Department of Biochemistry, University of Connecticut Health Center, Farmington, Connecticut 06032. *Received July 3, 1972.* This work was supported by grants from the University of Connecticut Research Foundation and the National Institutes of Health (AM-13225).

[1] Abbreviations used are: AK–HSDH-I, threonine-sensitive aspartokinase–homoserine dehydrogenase; Tes, *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonate; Pipes, piperazine-*N*,*N*′-bis(2-ethanesulfonate); Caps, cyclohexylaminopropanesulfonate.

[2] After this paper was submitted for publication my attention was called to a paper, then in press (Falcoz-Kelly *et al.*, 1972), in which the subunit stoichiometry of AK–HSDH-I from *E. coli* K-12 is investigated by several independent methods. They conclude that the enzyme is composed of four identical subunits. A discussion of the relationship between the previous data which supported six subunits and the present data can be found in that paper.

description of this mutant, see Szentirmai *et al.* (1968)) which was the generous gift of Dr. H. E. Umbarger. The purification procedure (Truffa-Bachi *et al.*, 1968) yielded enzyme which was greater than 95% pure as judged by specific activity. Aspartic semialdehyde was prepared by the ozonolysis of allylglycine as described by Black and Wright (1955).

Activity of the AK–HSDH-I complex was measured in three ways. 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 in the forward or reverse directions by following the oxidation or reduction of triphosphopyridine nucleotide essentially as described by Truffa-Bachi *et al.* (1966a) and Ogilvie *et al.* (1969). The composition of the three reaction mixtures and of buffer A are compared in Table I.[3]

*Gel Filtration.* For the determination of subunit size, gel filtration experiments were conducted as described by Fish *et al.* (1969) except that $10^{-2}$ M dithiothreitol was used in the pretreatment step to reduce disulfide bonds and $10^{-3}$ M dithiothreitol was used in the eluting solvent instead of 0.1 M β-mercaptoethanol.

To measure the size of the catalytic species, enzyme was mixed with Blue Dextran and either L-tryptophan or L-[$^{14}$C]-valine and passed through a 0.9 × 27 cm column of Sephadex G-200 at a flow rate of 2.1 ml/hr. The partition coefficient ($K_d$) was calculated from the relationship

$$K_d = (V_e - V_0)/(V_p - V_0) \quad (1)$$

where $V_e$ is the elution position of enzyme activity, $V_0$ is the elution position of Blue Dextran and $V_p$ is the elution position of the amino acid. The middle of the leading edge (half-maximum height) was taken as the elution position.

*Sedimentation Analysis.* Boundary sedimentation was conducted by standard methods (Chervenka, 1969) using a photoelectric scanner. The half-height of the sedimenting boundary was taken as the measure of radial position.

Activity sedimentation was conducted as described by Cohen *et al.* (1967) and Cohen and Mire (1971), again using the scanner. In this method, sedimentation coefficients can be calculated either from: (1) the midpoint of the reaction boundary, (2) the peak of the difference between successive boundaries, (3) corrected differences between successive boundaries, or (4) the position of the sedimenting enzyme sample. When the enzyme sediments as a single band, the first three methods give essentially the same sedimentation coefficient. The fourth method, however, is less direct and can give a considerably different sedimentation coefficient under certain conditions. This last method is described in more detail in the Appendix. Except where indicated, activity sedimentation coefficients were calculated from the midpoint of the reaction boundary.

Sedimentation coefficients were corrected to standard conditions as described by Chervenka (1969). This correction was greatest in activity sedimentation since the assay mixtures contained 0.67 M salt in the forward reaction and 0.81 M salt in the reverse reaction. In these cases an approximate correction was made by treating the data as if all of the salt were KCl, using density and viscosity values from the International Critical Tables (National Research Council, 1926). Corrections for the forward reaction ranged from 1.081 at 20° to 1.070 at 25° and in the reverse reaction from 1.100 at 20° to 1.086 at 25°.

High-speed equilibrium sedimentation was conducted using the scanner and stepping motor as described previously (Wampler, 1971).

TABLE I: Composition of Buffer A and the Three Reaction Mixtures.

| Component | Concentrations (mM) | | | |
|---|---|---|---|---|
| | Buffer A | Asp → Asp~P | ASA → Hse | Hse → ASA |
| Tris ($Cl^-$) | | 127 | 51.4 | 90.3 |
| Phosphate ($K^+$) | 20 | | | |
| Aspartate ($K^+$) | | 11.4 | | |
| $Na_2ATP$ | | 3.7 | | |
| $MgOAc_2$ | 2 | 5.2 | 1.8 | |
| EDTA | 2 | | 1.8 | |
| KCl | 150 | 571 | 617 | 722 |
| Homoserine (Hse) | | | | 29 |
| Aspartic semialdehyde (ASA) | | | 1.5 | |
| NADH | | 0.26 | | |
| NADP | | | | 0.62 |
| NADPH | | | 0.2 | |
| Phosphoenolpyruvate | | 3 | | |
| L-Threonine | 0.5 | *a* | *a* | *a* |
| Dithiothreitol | 0.1 | | | |
| pH | 6.8 | 7.5 | 7.5 | 9.0 |
| Temperature (°C) | | 30 | 30 | 30 |
| Sepecific activity[b] | | 9 | 63 | 2.3 |
| Threonine sensitivity[a] | | 98 | 80 | 60 |

[a] Enzyme sensitivity to L-threonine was tested by adding L-threonine to the reaction mixture to give a final concentration of 5.6 mM. Sensitivity is expressed as per cent inhibition at this concentration. [b] Specific activity is expressed as μmoles of product formed per mg of protein per min. The protein concentration was estimated from absorbancy at 278 nm, using an extinction coefficient of 0.46 absorbance unit/(cm$^2$ mg) (Truffa-Bachi *et al.*, 1968).

## Results

*Subunit Stoichiometry.* Using collagen (100,000), phosphorylase *a* (92,500), bovine serum albumin (68,000), ovalbumin (45,000), and chymotrypsinogen (25,700) as markers, AK–HSDH-I had an electrophoretic mobility in sodium dodecyl sulfate acrylamide gels (4% acrylamide) corresponding to a molecular weight between 83,000 and 85,000 (method of Weber and Osborn, 1969). The data are not presented here since they do not differ significantly from those reported by Clark and Ogilvie (1972) or Starnes *et al.* (1972).

Using bovine serum albumin (68,000) and β-galactosidase (130,000) as markers, gel filtration in 6 M guanidine hydrochloride gave a molecular weight of 93,000 for AK–HSDH-I subunits. AK–HSDH-I which had been treated with [$^{14}$C]*N*-ethylmaleimide was also subjected to gel filtration using 0.1 M

[3] The extinction coefficient used to calculate protein concentrations in this paper is apparently incorrect. Falcoz-Kelly *et al.* (1972) report an extinction coefficient of 0.63 cm$^2$/mg at 278 nm. Using the new value results in a 27% decrease in all of the protein concentrations and a 37% increase in specific activities.

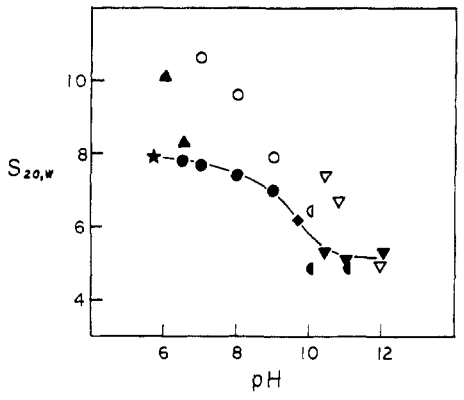


FIGURE 1: Sedimentation coefficients as a function of pH. Sedimentation was conducted in double-sector cells using scanning optics between 19 and 23° at 48,000 rpm. Experiments were conducted in 0.2 M buffer both in the presence (open symbols) and absence (filled symbols) of 0.1 M sodium chloride. The buffers used were: sodium acetate (★), Pipes (▲), Tes (●), L-glycine (◆), Caps (◖), and L-lysine (▼).

β-mercaptoethanol as the reducing agent and phosphorylase *a* (92,500) and catalase (62,500) as the markers. The peak of the radioactivity profile eluted immediately after the phosphorylase *a* peak indicating a molecular weight between 85,000 and 90,000.

It has been shown that AK–HSDH-I tends to dissociate in buffers which do not contain biologically effective ligands (substrates, potassium ion, or L-threonine) but in these solutions the enzyme is in an association–dissociation equilibrium so that the monomer molecular weight is not easily estimated (Wampler *et al.*, 1970). In an attempt to find a mild dissociating solvent which would lead to a large proportion of monomers, the sedimentation coefficient of AK–HSDH-I was measured at several pH values from 5.7 to 12 in the presence and absence of 0.1 M NaCl. At pH 5.7, 6.0, and 6.5 the mixture sedimented with two boundaries. At pH 5.7 and 6.0 the faster boundary (which accounted for 20–40% of the sample) sedimented in less than the 8-min interval between scans. From scans that were taken as the rotor approached full speed, approximate sedimentation coefficients were calculated to be in excess of 120 S at pH 5.7 and greater than 60 S in pH 6.0. At pH 6.5 the faster boundaries (which accounted for 10–20% of the sample) exhibited sedimentation coefficients of 32 and 38 S in Tes and Pipes buffers, respectively. The sedimentation coefficients of the slower boundaries are indicated in Figure 1.

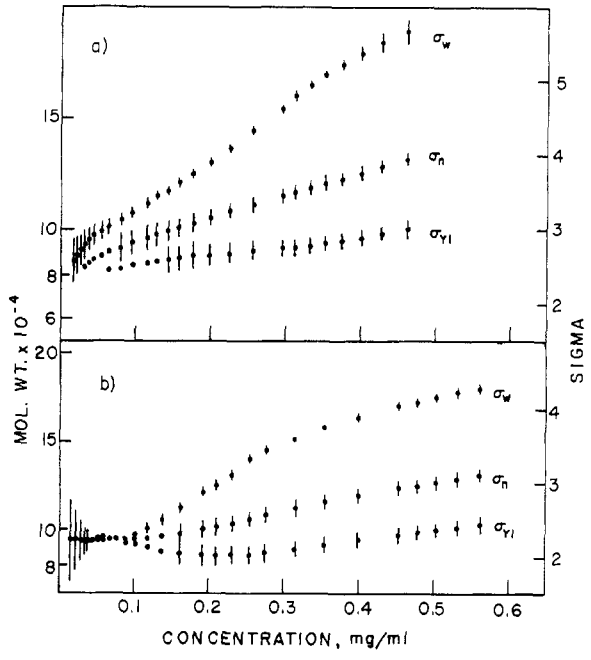


FIGURE 2: Equilibrium sedimentation at pH 11. Sedimentation was in 0.02 M lysine buffer containing 0.1 M NaCl using a 3-cm, double-sector centerpiece. The data were analyzed using the computer program developed by Roark and Yphantis (1969) which gives reduced molecular weights ($\sigma$). Molecular weights (left ordinate) were calculated from the $\sigma$ values (right ordinate) as described by Roark and Yphantis (1969). Error estimates are indicated by vertical bars except where they would confuse the data presentation. In section b the error bars at concentrations below 0.1 mg/ml are for $\sigma_w$ only. (a) 18° and 16,000 rpm, molwt = 33,332$\sigma$; (b) 7° and 14,000 rpm, mol wt = 42,254$\sigma$.

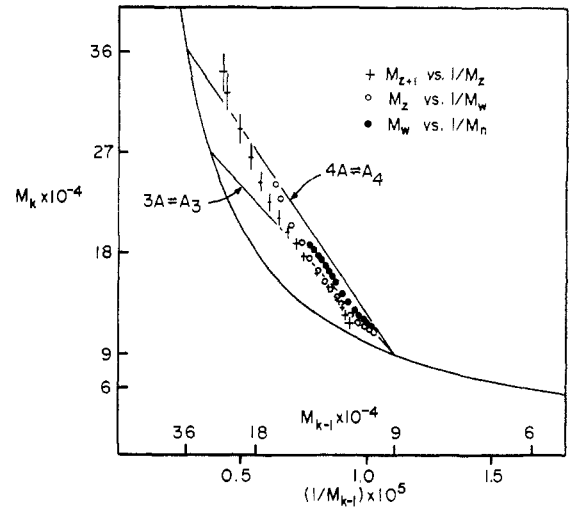


FIGURE 3: Two-species plot of data from equilibrium sedimentation at 18° and 16,000 rpm. Experimental conditions as in Figure 2a. The curved line indicates the locus of points which would be given by samples that are homogeneous with respect to molecular weight ($M_k = M_{k-1}$). Data extrapolated to this line give estimates of the monomer and oligomer molecular weights. The two straight lines in the figure are calculated lines for a 90,000 subunit involved in monomer–trimer and monomer–tetramer associations. The $M_{z+1}$ *vs.* $1/M_z$ symbols are drawn to indicate the size of the error estimate for those data. Only data with an error estimate less than ±5% of the molecular weight average at that point are included.

Because dissociation appears to be favored at high pH, equilibrium sedimentation was conducted in 0.02 M lysine buffer containing 0.1 M NaCl at pH 11. Scans were taken at 18° and 16,000 rpm (Figure 2a) and then the chamber was cooled to 7° and the rotor speed was reduced to 14,000 rpm (Figure 2b). At this elevated pH and particularly at the lower temperature, the tendency for association was greatly reduced so that a reasonable estimate of the monomer molecular weight could be made. Roark and Yphantis (1969) have presented a graphical method for analyzing association equilibria which involve only two species (monomer–dimers, monomer–trimers, etc.). This method gives an estimate of the largest and smallest members of the equilibrium as well as an estimte of the association stoichiometry. Their method is particularly useful in this case since prior knowledge of the monomer molecular weight is not required. Two-species plots (*e.g.*, Figure 3) of the data in Figure 2 indicate that the association reaction is complex (involving more than two species) and may include aggregates larger than the tetramer.

TABLE II: Partition Coefficients of AK–HSDH-I with Sephadex G-200 under Catalytic Conditions.

| Aspartokinase | | Homoserine Dehydrogenase | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | Reverse Reaction | | | | | | | |
| | | Forward Reaction | | No Thr | | 5.6 mM Thr | | Buffer A | | Tes Buffer | |
| $\mu g^a$ | $K_d$ | $\mu g$ | $K_d$ | $\mu g$ | $K_d$ | $\mu g$ | $K_d$ | $\mu g$ | $K_d$ | $\mu g$ | $K_d$ |
| | | 7.5 | 0.072 | 7.5 | 0.183 | | | | | | |
| 15 | 0.081 | | | | | 15 | 0.092 | 15 | 0.070 | | |
| | | | | 30 | 0.180 | 45 | 0.094 | | | | |
| | | 75 | 0.051 | 75 | 0.145 | | | 150 | 0.070 | 75 | 0.191 |
| 300 | 0.064 | | | | | | | 300 | 0.052 | 450 | 0.195 |

[a] $\mu g$ refers to the quantity of enzyme applied to the column (see footnote 3). The definition of $K_d$ is given in eq 1. The composition of buffer A and the reaction mixtures are given in Table I. Tes buffer is 0.02 N Na-Tes (pH 8.0) containing $10^{-4}$ M dithiothreitol.

*Size of the Catalytic Species.* In order to investigate the state of aggregation of the active enzyme, gel filtration experiments were conducted under assay conditions. The results of several experiments are summarized in Table II. Under catalytic conditions for the two forward reactions the partition coefficient for the complex ($K_d$ = 0.05–0.08) is indistinguishable from that of the tetramer in buffer A ($K_d$ = 0.05–0.07). Under conditions for the reverse reaction, however, the partition coefficient ($K_d$ = 0.15–0.18) is similar to that of the enzyme in Tes buffer at pH 8.0 ($K_d$ = 0.19). The addition of L-threonine to the reverse homoserine dehydrogenase reaction mixture caused a return to the partition coefficient of the tetramer ($K_d$ = 0.09). The amounts of enzyme given in Table II refer to the amount of enzyme in the sample (120 $\mu$l) applied to the column. The enzyme concentration in the effluent fractions can also be calculated from enzyme activity as compared to another enzyme solution diluted to a similar concentration in the column buffer when gel filtration was begun. When this is done the peaks of the enzyme concentration curves range from 1.5 $\mu$g/ml (7.5 $\mu$g applied) to 117 $\mu$g/ml (300 $\mu$g applied).

The sedimentation coefficient of the tetramer was determined by boundary sedimentation at three loading concentrations in buffer A in a four-place rotor. Scans were made at 230 nm (0.5 mg/ml), 278 nm (1.8 mg/ml), and 250 nm (4.5 mg/ml). The use of mirror optics eliminated the need to change optical settings between scans. The resulting $s_{20,w}$ values ranged between 10.9 and 11.2 S.

The sedimentation coefficient of the functioning enzyme was then investigated by activity sedimentation and the results are presented in Table III. Under catalytic conditions for the forward homoserine dehydrogenase reaction, the sedimentation coefficients ranged between 9.2 and 10.7 S and gave some indication of positive concentration dependence. For the reverse reaction sedimentation coefficients ranged between 7.0 and 7.6 S, again with the suggestion of a small concentration dependence. This lower sedimentation coefficient does not appear to be due simply to the higher pH of the reverse reaction mixture. When enzyme was sedimented through a reverse reaction mixture which had been adjusted to pH 7.8 the sedimentation coefficient was 7.9 S. The addition of 5.6 mM L-threonine to the reverse reaction mixture (at pH 9) caused an increase in these sedimentation coefficients while the addition of L-threonine had little effect on sedimentation behavior in the forward direction.

*Analysis of Activity Sedimentation Data.* As Cohen and Mire (1971) have pointed out, the technique of activity sedimentation gives artificially high sedimentation coefficients if too much enzyme is used so that the change in optical density is due primarily to the leading edge of the enzyme band. This artifact can be reduced by using a higher rotor speed so that the assay mixture is exposed to enzyme for less time and the sedimenting band has less time to diffuse. To test the possibility that the changing position of the reaction boundary at 48,000 rpm did not accurately describe the sedimentation of enzyme, 10 $\mu$l of a 60-$\mu$g/ml solution of AK–HSDH-I was subjected to activity sedimentation at 68,000 rpm in a single-sector band forming cell through the reverse homoserine dehydrogenase reaction mixture and the enzyme concentration at each radial position was calculated as described in the Appendix. The results are presented in Figure 4. A sedimentation coefficient was calculated from the changing position of the peak of the enzyme concentration curve and compared to the results from reaction boundary calculations. The data

TABLE III: Sedimentation Coefficients of AK–HSDH-I under Homoserine Dehydrogenase Reaction Conditions.

| Forward Reaction | | | | Reverse Reaction | | | |
|---|---|---|---|---|---|---|---|
| No Thr | | 5.6 mM Thr | | No Thr | | 5.6 mM Thr | |
| Concn[a] | $s_{20,w}$ (S) | Concn | $s_{20,w}$ (S) | Concn | $s_{20,w}$ (S) | Concn | $s_{20,w}$ (S) |
| 3 | 9.2 | | | | | | |
| 6 | 10.1 | 6 | 9.9 | 6 | 7.2 | | |
| 15 | 10.7 | 15 | 10.4 | 15 | 7.0 | 15 | 9.2 |
| 150 | 10.3 | | | 60 | 7.3 | 30 | 9.2 |
| | | | | 150 | 7.6 | 150 | 10.4 |

[a] Concentrations are $\mu$g of enzyme/ml in the centerpiece well. The average concentration of enzyme in the sedimenting band is approximately one-tenth that of the layered enzyme as described in the text.

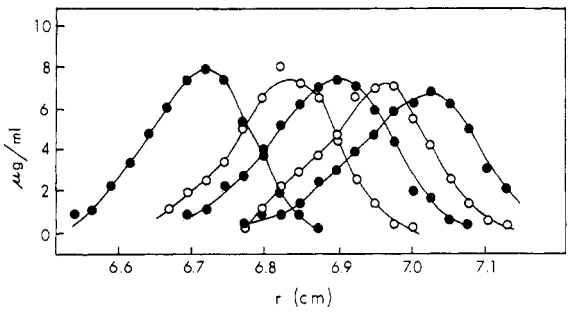


FIGURE 4: Distribution of enzyme during activity sedimentation. Enzyme concentration (expressed as μg/ml) was calulated from the change in optical density over the 4-or 6-min interval between scans as described in the text. Every other set of calculations is presented in filled symbols to facilitate reading the figure.

are presented in Figure 5. The resulting sedimentation coefficients were 7.6 S by the reaction boundary method and 7.9 S by the peak of enzyme concentration method. A similar experiment at 48,000 rpm and the same loading concentration of enzyme gave a sedimentation coefficient of 7.5 S.

Calculations of enzyme concentration were also made for a sample sedimented through the forward homoserine dehydrogenase reaction mixture at 48,000 rpm. In this case the loading concentration was 6 μg/ml and approximately 80% of the TPNH was consumed during sedimentation. The resulting sedimentation coefficients were 10.6 S by boundary half-heights and 9.9 S by enzyme concentration.

The average enzyme concentration in the sedimenting band can be estimated from the data in Figure 4. If the enzyme follows a Gaussian distribution, the average concentration will be found at 0.67 standard deviation from the radial position of the enzyme peak and the concentration will be 0.80 times the concentration at the peak. Using this approximation the average enzyme concentrations of the five curves in Figure 4 are 6.3, 5.8, 5.8, 5.6, and 5.4 μg per ml or approximately one-tenth of the concentration layered on the reaction mixture.

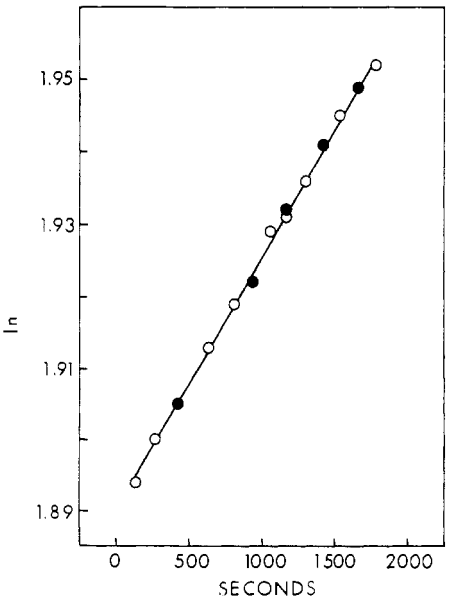


FIGURE 5: Calculation of sedimentation coefficients by two methods. The points represent the change in radial position of the reaction boundary (○) and the peak of enzyme concentration (●) illustrated in Figure 4.

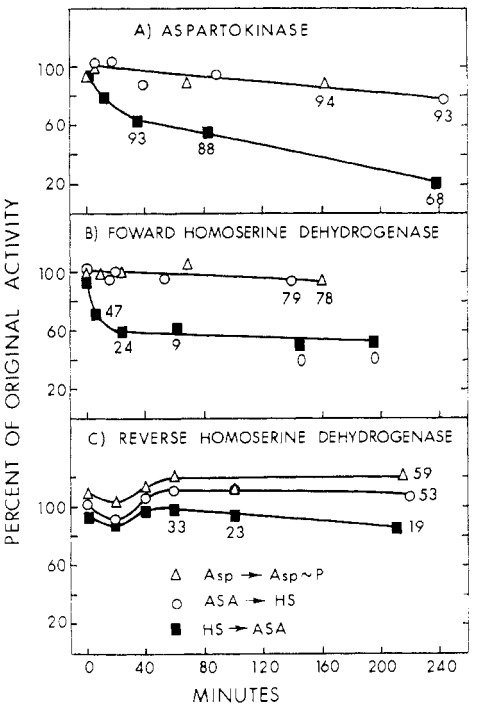


FIGURE 6: Enzyme stability in the three reaction mixtures. Enzyme was stored in either the aspartokinase assay mixture (Δ), the forward homoserine dehydrogenase assay mixture (○) or the reverse homoserine dehydrogenase assay mixture (■) and then assayed for aspartokinase (A) or homoserine dehydrogenase activity in the forward direction (B) or the reverse direction (C). The numbers refer to the per cent inhibition by 5.6 mM L-threonine.

The total amount of enzyme in the sedimenting band can also be calculated from the enzyme concentration distribution and used as an independent check on the accuracy of the calculations which produced the enzyme concentration curves. An element of the sector-shaped cell between radial positions $r_1$ and $r_2$ has an approximate volume

$$V \simeq r'\Delta r \tan \theta p \qquad (2)$$

where $r'$ is the radial position of the center of the element, $\Delta r$ is the distance from $r_1$ to $r_2$, $\theta$ is the sector angle, and $p$ is the path length of the cell. If $\Delta r$ is small, the amount of enzyme in the element ($E_{\Delta r}$) can be calculated from

$$E_{\Delta r} = V\bar{E} \simeq r'\Delta r \tan \theta p\bar{E} \qquad (3)$$

where $\bar{E}$ is the mean enzyme concentration over the interval $\Delta r$. The total enzyme ($E_t$) is related to $E_{\Delta r}$ as the total area ($A_t$) of the curve is related to the area between $r_1$ and $r_2$($A_{\Delta r}$). Thus

$$E_t = A_t E_{\Delta r}/A_{\Delta r} \qquad (4)$$

In the example illustrated in Figure 4, the total enzyme put into the centerpiece well was 1.2 μg and values of $E_t$ from the five curves were 0.77, 0.81, 0.93, 0.77, and 0.90 μg. This means that approximately 70% of the enzyme put into the well could be accounted for in the sedimenting band.

*Enzyme Stability under Catalytic Conditions.* Since both sedimentation and gel filtration experiments last for 60–90 min, it was important to investigate enzyme stability under assay conditions. To do this, enzyme was diluted into the

three reaction mixtures and at various times samples were taken and assayed for one of the three activities and for sensitivity to the feedback inhibitor. This activity was compared to that obtained from enzyme which had been kept in buffer A. (The aspartokinase, forward homoserine dehydrogenase and reverse homoserine dehydrogenase activities are 98, 80, and 60% inhibited by L-threonine when kept in buffer A.) The results are presented in Figure 6 where each panel (A, B, or C) refers to one of the three enzymatic activities while the different symbols indicate the reaction mixture in which the enzyme was stored.

Although the enzyme was relatively stable with respect to all three activities when it was stored in the reaction mixtures for the two forward reactions (Figure 6), when stored in the reaction mixture for the reverse reaction it lost 40–50% of both aspartokinase and forward homoserine dehydrogenase activities within the first hour (sections A and B). However, at least 80% of the activity for the reverse homoserine dehydrogenase reaction was retained for several hours in all three reaction mixtures (section C). The initial dip which was observed in measuring the reverse homoserine dehydrogenase activity was reproducible although in another set of experiments it occurred at 90 rather than 20 min.

In eight of the nine situations, conditions which maintained activity also maintained sensitivity to the feedback inhibitor. The one exception was that enzyme stored in the reverse reaction mixture lost 65% of its threonine sensitivity when assayed in the reverse direction, while losing only 20% of its ability to catalyze the reverse reaction.

## Discussion

It is now apparent that AK–HSDH-I from *E. coli*, as normally purified, in buffer A, is a tetramer. The molecular weight estimate of 360,000 for this "native" form is quite reliable since the sedimentation pattern in buffer A shows neither heterogeneity nor nonideality (Truffa-Bachi *et al.*, 1968; Wampler *et al.*, 1970). Subunit molecular weight estimates by sodium dodecyl sulfate acrylamide gel electrophoresis and guanidine-HCl gel filtration appear to depend only on polypeptide chain length (Tung and Knight, 1971) and thus offer a measure which depends only on the reliability of the marker molecular weights. Equilibrium sedimentation in dilute salts avoids the hazards of estimating partial specific volumes in 6 M guanidine-HCl. Results from all three of these methods give a monomer molecular weight very close to 90,000, thus establishing a tetrameric structure.

A stoichiometry of four subunits per oligomer requires a reinterpretation of the data which suggested that stable dimers were formed in Tes buffer at pH 8 (Wampler *et al.*, 1970). Since that work was published computer programs have become available which permit a statistical analysis of equilibrium data. When the data in Figure 3 of Wampler *et al.* (1970) were processed by the computer program of Roark and Yphantis (1969), it became apparent that dissociation did not stop at the dimer stage but the protein was in an association equilibrium throughout the centrifuge cell. Therefore, in the absence of allosteric ligands, the enzyme appears to be in an associating equilibrium both at pH 8 and 11.

Results of both activity sedimentation and gel filtration indicate that the predominant form of the enzyme directing catalysis in the biosynthetic direction is the tetramer. The slight decrease in sedimentation coefficient might be due either to a limited, reversible dissociation or to a change in conformation. Whatever the source of this difference, the addition of L-threonine causes no change in the sedimentation behavior. (Similar results under noncatalytic conditions were also obtained by Janin *et al.*, 1969.) Therefore, to the extent that these conditions preserve the intracellular enzyme structure, it seems unlikely that metabolic control of threonine synthesis in *E. coli* involves an association–dissociation reaction of the AK–HSDH-I complex. In fact, the only times aggregates larger than 11 S have been reported are in the absence of threonine (Cohen *et al.* (1967), Wampler *et al.* (1970), and this report) or after mercuribenzoate treatment (Truffa-Bachi *et al.*, 1966a; Barber and Bright 1968).

Under conditions for the reverse homoserine dehydrogenase reaction the enzyme quickly dissociates to a form which behaves much like enzyme in Tes buffer at pH 8. The fact that the sedimentation coefficient does not change during activity sedimentation (Figure 5) indicates that the enzyme dissociates to a stable structure (or equilibrium) within the 15–30 min required for the enzyme to sediment fully into the reaction mixture.

The observation that L-threonine shifts the structure to a more aggregated form is of dubious metabolic significance since feedback control is for metabolic flux in the opposite direction. It is more likely another illustration of the fact that L-threonine (like moderate pH and moderate temperature) tends to stabilize the enzyme against disaggregation, as has been observed in several laboratories (Patte *et al.*, 1963; Barber and Bright, 1968; Cunningham *et al.*, 1968; Wampler and Westhead, 1968).

There is little information concerning the mechanism of feedback control of aspartokinase or homoserine dehydrogenase activities in other organisms. When homoserine dehydrogenase isolated from *Rhodospirillum rubrum* was diluted into a buffer which did not contain substrates or inhibitor, the enzyme dissociated into subunits which retained activity but had lost threonine sensitivity (Mankovitz and Segal, 1969). However, these results may not be related to metabolic control. Similar dissociation and desensitization have been observed with AK–HSDH-I from *E. coli*. In the case of the *E. coli* enzyme, however, subunit formation and desensitization are observed only in the reverse reaction mixture or when biological ligands are removed (Truffa-Bachi *et al.*, 1966b; Wampler *et al.*, 1970) or when the enzyme is treated with mercurials (Truffa-Bachi *et al.*, 1966a). Threonine, the feedback inhibitor, protects against both effects. In *Pseudomonas fluorescence*, by contrast, feedback inhibition of aspartokinase by L-threonine appears to involve the formation of an inactive aggregate (Dungan and Datta, 1972).

The AK–HSDH-I complex can exist in several functional forms. The fact that substrates of one reaction inhibit the other has been interpreted by Cohen *et al.*, (1967a) as indicating that the two forward reactions are catalyzed by two different forms of the enzyme. Spectral studies (Janin and Cohen, 1969; Heck and Truffa-Bachi, 1970) and stopped-flow experiments (Barber and Bright, 1968; Janin and Iwatsubo, 1969) have investigated the transition from the two active forms to an inhibited form of the enzyme. In the reverse reaction mixture the enzyme is converted to a form which is smaller than the tetramer and although the enzyme retains activity for the homoserine dehydrogenase reaction in the reverse direction, the specific activity for both forward reactions decreases by 40–80% as does the sensitivity to feedback inhibition (Figure 6). Whether this differential change in specific activity and threonine sensitivity is simply the result of enzyme dissociation or due to some other structural changes remains to be determined.

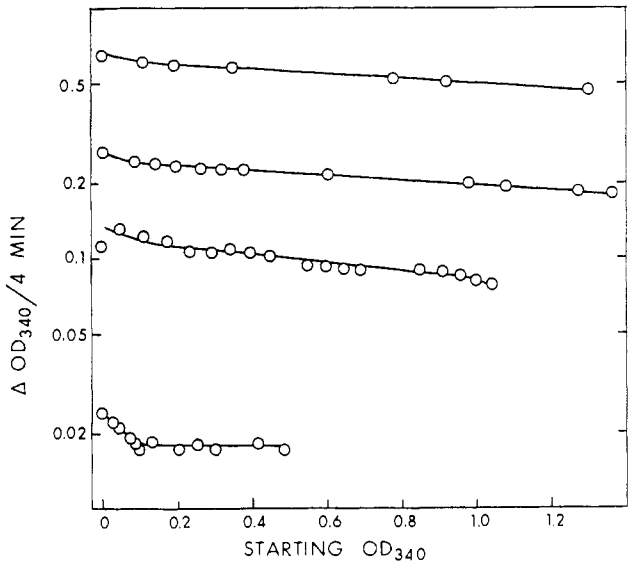


FIGURE 7: Reaction velocity as a function of the extent of reaction at four enzyme concentrations. Experimental details are given in the text.

Because the enzyme can exist in several different forms kinetic results from the reverse reaction should not be used to infer catalytic and control mechanisms for the biosynthetic reactions. Furthermore, interpretations of experiments which investigate the role of ligands in changing structure or activity must consider not only allosteric interactions but also association–dissociation reactions and the slow loss of threonine sensitivity.

## Appendix

The calculation of sedimentation coefficients from the reaction boundary in activity sedimentation is reliable only if: (1) the substrate concentrations remain saturating throughout the course of the reaction, (2) the products are not inhibitory (or do not accumulate to an appreciable extent), (3) the concentration of sedimenting enzyme is a Gaussian function of radial position, and (4) the specific activity remains constant. As Cohen and Mire (1971) have pointed out, the method may not be reliable at high enzyme concentrations where an appreciable extent of reaction occurs during sedimentation. One way to avoid this problem is to calculate the enzyme concentration at each radial position and thus measure the sedimentation coefficient of the enzyme itself. This calculation of enzyme concentration from the change in optical density requires two important corrections, one for the sedimentation and diffusion of NADPH between successive scans and another for nonlinearity of the reaction (the velocity of the reaction decreases as it approaches equilibrium).

The correction for sedimentation and diffusion uses eq 43–45 in Cohen *et al.* (1967b) which are repeated here as eq 1A–3A. In these equations $N'_0(r,T)$ is the corrected optical density at radius $r$ and time $T$, $N'(r'T - \theta)$ is the measured optical density at radius $r'$ and time $T - \theta$, $\omega$ is the angular velocity and $2\theta$ is the time interval between successive scans. The sedimentation and diffusion coefficients of the small molecule being observed (NADPH in this case) are $s'$ and $D'$, respectively. Equation 1A can be solved at each radial position by integration according to eq 4A where $i = 0$ and $i = j$

$$N'_0(r,T) = \int r'\mathrm{d}r'(E'rr')^{-1/2}(2\pi\Delta')^{-1/2}N'(r',\ T-\theta)\times \exp\{-(r-E'r')^2/2\Delta'\} \quad (1A)$$

$$E' = \exp\{2s'\omega^2\theta\} \quad (2A)$$

$$\Delta' = (D'/s'\omega^2)(E'^2 - 1) \simeq 4D'\theta \quad (3A)$$

$$N'_0(r,T) = \frac{\Delta r'}{(2\pi\Delta')^{1/2}}\sum_{i=0}^{i=j}\frac{r'_iN'}{(E'r'_ir)^{1/2}}\,e^{-(r-E'r')^2/2\Delta'} \quad (4A)$$

are the first and last radial positions in the series. Since this equation assumes that $N' = 0$ if no value is given, it is necessary to include data in the plateau region in order to generate an accurate correction through the boundary.

When the corrected optical densities are subtracted from the optical densities measured $2\theta$ sec later, the change in optical density due to the enzymatic reaction ($\Delta N'_0$) is obtained. In order to calculate the enzyme concentration from $\Delta N'_0$ it is necessary to establish the relationship between the stage of the reaction, the enzyme concentration and $\Delta N'_0$ because the amount of enzyme required to give a particular $\Delta N'_0$ changes as the substrate concentration decreases and the product concentration increases. This relationship is approximated by letting several concentrations of enzyme catalyze the reaction for an extended period of time and then plotting $\Delta N'_0$ as a function of the extent of reaction (starting optical density). Such a plot is given in Figure 7. By interpolating between the lines of Figure 7 it is possible to estimate the amount of enzyme required to give each $\Delta N'_0$ (Figure 4) and thus to calculate the sedimentation coefficient from the change in peak position.

## Acknowledgments

I am grateful to Mrs. Florence Woodiel for excellent technical assistance, to Mrs. Norma Stimler for assistance in the experiments involving $^{14}$C-labeled enzyme, to Dr. David Yphantis for several helpful discussions and to Drs. Ogilvie, G. N. Cohen, and Cox for making their results available before publication.

## References

Barber, E. D., and Bright, H. J. (1968), *Proc. Nat. Acad. Sci. U. S. 60*, 1363.

Black, S., and Wright, N. G. (1955), *J. Biol. Chem. 213*, 39.

Chervenka, C. H. (1969), A Manual of Methods for the Analytical Ultracentrifuge, Palo Alto, Calif., Beckman Instruments.

Clark, R. B., and Ogilvie, J. W. (1972), *Biochemistry 11*, 1278.

Cohen, G. N. (1969), *Curr. Top. Cell. Regul. 1*, 183.

Cohen, G. N., Patte, J.-C., Truffa-Bachi, P., and Janin, J. (1967a), *Regul. Nucl. Acid Protein Biosyn. Proc. Int. Symp. 1966*, 357.

Cohen, R., Girard, B., and Messiah, A. (1967b), *Biopolymers 5*, 203.

Cohen, R., and Mire, M. (1971), *Eur. J. Biochem. 23*, 267.

Cunningham, G. N., Maul, S. B., and Shive, W. (1968), *Biochem. Biophys. Res. Commun. 30*, 159.

Dungan, S. M., and Datta, P. (1972), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 31*, 423 Abs.

Falcoz-Kelly, F., Janin, J., Saari, J. C., Veron, M., Truffa-Bachi, P., and Cohen, G. N. (1972), *Eur. J. Biochem. 28*, 507.

Fish, W. W., Mann, K. G., and Tanford, C. (1969), *J. Biol. Chem. 244*, 4989.

Heck, H. d'A., and Truffa-Bachi, P. (1970), *Biochemistry 9*, 2776.
Janin, J., and Cohen, G. N. (1969), *Eur. J. Biochem. 11*, 520.
Janin, J., and Iwatsubo, M. (1969), *Eur. J. Biochem. 11*, 530.
Janin, J., van Rapenbusch, R., Truffa-Bachi, P., and Cohen, G. N. (1969), *Eur. J. Biochem. 8*, 128.
Mankovitz, R., and Segal, H. L. (1969), *Biochemistry 8*, 3775.
National Research Council (1926), *in* International Critical Tables Vol. 5, p 17.
Ogilvie, J. W., Sightler, J. H., and Clark, R. B. (1969), *Biochemistry 8*, 3557.
Patte, J.-C., LeBras, G., Lovings, T., and Cohen, G. N. (1963), *Biochim. Biophys. Acta 67*, 16.
Patte, J.-C., Truffa-Bachi, P., and Cohen, G. N. (1966), *Biochim. Biophys. Acta 128*, 426.
Roark, D., and Yphantis, D. (1969), *Ann. N. Y. Acad. Sci. 164*, 245.
Starnes, W. L., Munk, P., Mau, S. B., Cunningham, G. N., Cox, D. J., and Shive, W. (1972), *Biochemistry 11*, 677.
Szentirmai, A., Szentirmai, M., and Umbarger, H. E. (1968), *J. Bacteriol. 95*, 1672.
Truffa-Bachi, P., and Heck, H. d'A. (1971), *Biochemistry 10*, 2700.
Truffa-Bachi, P., Le Bras, G., and Cohen, G. N. (1966a), *Biochim. Biophys. Acta 128*, 440.
Truffa-Bachi, P., Le Bras, G., and Cohen, G. N. (1966b), *Biochim. Biophys. Acta 128*, 450.
Truffa-Bachi, P., van Rapenbusch, R., Janin, J., Gros, C., and Cohen, G. N. (1968), *Eur. J. Biochem. 5*, 73.
Truffa-Bachi, P., van Rapenbusch, R., Janin, J., Gros, C., and Cohen, G. N. (1969), *Eur. J. Biochem. 7*, 401.
Tung, J.-S., and Knight, C. A. (1971), *Biochem. Biophys. Res. Commun. 42*, 1117.
Wampler, D. E. (1971), *Anal. Biochem. 44*, 528.
Wampler, D. E., Takahashi, M., and Westhead, E. W. (1970), *Biochemistry 9*, 4210.
Wampler, D. E., and Westhead, E. W. (1968), *Biochemistry 7*, 1661.
Weber, K., and Osborn, M. (1969), *J. Biol. Chem. 244*, 4406.

# Haloacetol Phosphates. A Comparative Study of the Active Sites of Yeast and Muscle Triose Phosphate Isomerase†

I. L. Norton and F. C. Hartman*

ABSTRACT: A procedure is described for purifying triose phosphate isomerase from Bakers' yeast to homogeneity in 35–40% yields. This enzyme, like the corresponding enzyme from rabbit muscle, is inactivated by the substrate analog, 3-chloroacetol phosphate, *via* selective esterification of a single glutamyl $\gamma$-carboxylate. The amino acid sequence around this reactive residue (Ala-Tyr-Glu-Pro-Val-Trp) is identical with that found in the rabbit muscle enzyme. Sequence homologies between the segments containing the active-site glutamyl residue in these two diverse species, rabbit and yeast, suggest that the carboxyl group is functional in catalysis.

Haloacetol phosphates are active-site-specific reagents for triose phosphate isomerase. In the case of the isomerase from both rabbit and chicken muscle, inactivation results from a highly selective esterification of a single glutamyl $\gamma$-carboxylate (Hartman, 1970a; Coulson *et al.*, 1970). The unusual reactivity of this carboxylate and the observation that chloroacetol phosphate inactivates triose phosphate isomerase from diverse species led Hartman (1971) to postulate that the carboxylate is probably functional in catalysis. Comparative studies on enzymes with the same function but from different species can be pertinent to ascertaining whether a given amino acid residue is catalytically essential. The validity of this statement resides in numerous examples supporting the concept that active-site regions of enzymes have been conserved during evolution; *i.e.*, residues involved in catalysis are species invariant, and the primary sequences adjacent to these residues are highly homologous (Smith, 1970). Therefore, as a further test of the functional significance of the active-site glutamyl residue, we have characterized the site of yeast triose phosphate isomerase that is modified by 3-chloroacetol phosphate.

## Experimental Section

*Materials.* NADH, DL-glyceraldehyde 3-phosphate (as the dimethyl ketal), and glycerophosphate dehydrogenase were obtained from Boehringer Mannheim Corp. Trypsin treated with L-(tosylamido-2-phenyl)ethyl chloromethyl ketone, DFP-treated carboxypeptidase, and pepsin were products of Worthington Biochemical Corp. $^{32}POCl_3$ and $NaB^3H_4$ were purchased from Ammersham–Searle Corp. Ultra-Pure biological grades of ammonium sulfate and guanidine hydrochloride were purchased from Schwarz–Mann. Phenyl isothiocyanate and trifluoroacetic acid were obtained from Aldrich Chemical Co. Chloroacetol phosphate was synthesized as described previously (Hartman, 1970b); the $^{32}P$-labeled reagent (initial specific radioactivity of $1.28 \times 10^6$ cpm/$\mu$mole) was prepared

† From the Biology Division, Oak Ridge National Laboratory, Oak Ridge, Tennessee 37830. *Received July 3, 1972.* This is paper V in a series; paper IV (Paterson *et al.*, 1972) appeared in *Biochemistry 11*, 2070. A preliminary account of this work has appeared (Hartman and Norton, 1972). Research sponsored by the U. S. Atomic Energy Commission under contract with the Union Carbide Corp.