Biochemistry

Volume 11, Number 24 November 21, 1972

Threonine-Sensitive Aspartokinase–Homoserine Dehydrogenase of *Escherichia coli* K 12. Evidence for a Cooperative Tetramer†

Henry d'A. Heck

ABSTRACT: Equilibrium dialysis, protein fluorescence, and the inactivation of the aspartokinase function by the adenosine triphosphate (ATP) analog, 6-mercapto-9-β-D-ribofuranosylpurine 5′-triphosphate (SH-TP), were used to investigate threonine binding and the conformation of aspartokinase I–homoserine dehydrogenase I in Tris buffer, and the results were compared with inhibition of the aspartokinase activity under closely similar conditions. The binding process was interpreted quantitatively in terms of the simple allosteric model of Monod, Wyman, and Changeux, in which four threonine sites form a single cooperative unit. The Hill coefficient is 2.3 and the total interaction energy is 2.1 ± 0.9 kcal. The "kinetic Hill coefficient" of 4 reported by others was confirmed, and can be simply explained by assuming that the inhibition of the aspartokinase function directly reflects the conformational state of the cooperative unit. The concerted tetramer is one model which appears to be capable of interpreting both the equilibrium and steady-state properties of aspartokinase I–homoserine dehydrogenase I.

Aspartokinase I–homoserine dehydrogenase I (aspartokinase or ATP:L-aspartate 4-phosphotransferase, EC 2.7.2.4; homoserine dehydrogenase or L-homoserine:NADP oxidoreductase, EC 1.1.1.3) is a bifunctional enzyme catalyzing two nonsequential steps on the pathway of threonine biosynthesis from aspartic acid in *Escherichia coli* K 12. Both enzymatic activities can be inhibited by L-threonine, which has led to the proposal that the enzyme has a regulatory function (Cohen, 1969). Investigations on the aspartokinase activity at catalytic concentrations of the complex have been reported by several authors (Patte *et al.*, 1966; Wampler and Westhead, 1968; Wampler *et al.*, 1970). In Tris buffer, the aspartokinase function is inhibited by the end product, L-threonine, in a strongly cooperative manner, characterized by a "kinetic Hill coefficient" (Changeux, 1963)

$$n' = \frac{\mathrm{d} \log \left(\frac{v_0 - v}{v - v_s} \right)}{\mathrm{d} \log [\text{L-threonine}]} \cong 4 \qquad (1)$$

where v_0 is the catalytic rate under specified conditions in the absence of threonine, and v and v_s are the corresponding rates at intermediate and saturating concentrations of the inhibitor, respectively. These observations provide a framework within which any proposed regulatory mechanism ought to fit.

Concurrently with these investigations, a variety of experiments on the binding of threonine to the enzyme and on the conformational effects of ligand binding was performed (Janin *et al.*, 1969; Janin and Cohen, 1969; Heck and Truffa-Bachi, 1970; Takahashi and Westhead, 1971). At equilibrium in phosphate buffer, threonine binding appears to conform well to the simple two-states allosteric model of Monod, Wyman, and Changeux (1965) (Janin and Cohen, 1969; Takahashi and Westhead, 1971; Janin, 1972). The mechanism proposed by Janin and Cohen envisages two independent cooperative units per protein molecule, each unit composed of three identical and intrinsically independent threonine binding sites.

Although this mechanism seems to account satisfactorily for threonine binding to the enzyme at equilibrium, it does not explain the high value of n' observed under steady-state conditions in Tris buffer. If the independent, cooperative trimer model is correct, it is necessary to propose a more complex regulatory mechanism, manifesting itself under the aspartokinase assay conditions, in order to account for the high value of the observed kinetic Hill coefficient.

At least three possible alternative mechanisms can be envisaged. First, it might be proposed that the two proposed trimer units are not completely independent under the kinetic

† From the Department of Chemistry, University of California, Berkeley, California 94720. *Received May 8, 1972.* The results of this investigation were presented in part at the 56th Annual Meeting of the Federation of American Societies for Experimental Biology, Atlantic City, N. J., April 1972. This research was supported by the U. S. Public Health Service Grant No. AM 13164-03.

conditions, but interact more or less weakly with one another so as to raise the Hill coefficient to 4. A similar mechanism was suggested for hemoglobin (Wyman, 1967). Second, it is conceivable that one of the many nonequilibrium models which have been proposed to account for sigmoidal ligand saturation curves might be applicable to this system (Whitehead, 1970). Aspartokinase I–homoserine dehydrogenase I has already been cited as a primary example of a "hysteretic" enzyme (Frieden, 1970), *i.e.*, an enzyme which responds slowly to rapid changes in ligand concentration. It is known that, in principle, such enzymes can exhibit sigmoidal saturation curves even in the absence of cooperative interactions (Frieden, 1970). Third, the proposed size of the basic cooperative unit, a trimer, could be incorrect.

In order to understand the significance of the kinetic observations, it is necessary that the binding equilibria and associated conformational changes be examined under conditions which closely resemble the kinetic measurements. This paper describes the results of such experiments. While these studies were in progress, Clark and Ogilvie (1972), as well as Falcoz-Kelly *et al.* (1972), reinvestigated the structure of the enzyme, and concluded that aspartokinase I–homoserine dehydrogenase I is a tetramer, rather than a hexamer. These recent observations on the protein structure corroborate the binding studies reported here, and suggest a plausible mechanistic interpretation for the results.

Materials and Methods

Materials. E. coli K 12, strain Tir 8 (Szentirmai *et al.*, 1968), was grown on a minimal medium containing 1% glucose as the carbon source. The cells were harvested in the late-log phase and stored at −15°. The homogeneous protein was isolated from bacterial extracts and purified as described by Truffa-Bachi *et al.* (1968). The enzyme preparation, suspended in 50% ammonium sulfate containing 5 mM L-threonine, was maintained at 5°. Stored under these conditions, the enzyme was stable both with respect to catalytic activities and threonine sensitivity for a period of several months.

L-[^{14}C]Threonine was purchased from International Chemical and Nuclear Corp. The compound had a specific activity of 140 Ci/mole. SH-TP[1] was kindly supplied by Dr. Joseph A. Duke of the University of California, San Francisco. All other reagents and enzymes were obtained from commercial sources.

Buffer A contained 0.12 M Tris, 0.6 M KCl, 10 mM potassium L-aspartate, and 5 mM $MgCl_2$ (pH 7.40). Buffer B is the same as buffer A including 1 mM dithioerythritol.

Apparatus. Spectrophotometric measurements were made on a Heath Model 701 spectrophotometer containing a thermostatted cell holder. Fluorometric titrations were performed using thermostatted cuvettes in a Perkin-Elmer MPF-2A spectrofluorometer. Ligand samples were added to enzyme solutions using an Agla microsyringe assembly (Burroughs-Wellcome). Measurements of pH were made using a Radiometer Model 22 pH meter.

Equilibrium dialysis experiments were carried out in Lucite microdialysis cells constructed according to a design of O. Uhlenbeck of the University of Illinois, Urbana (personal communication). Visking dialysis membranes were treated according to Myer and Schellman (1962) and stored under ethanol. Before use, the membranes were rehydrated with buffer B, then blotted dry. Radioactive samples were counted in a Beckman LS-250 scintillation counter.

Equilibrium Dialysis. A sample of the enzyme preparation, resuspended from ammonium sulfate, was equilibrated with buffer B by dialyzing for 12 hr at 5°. The absorbancy of the stock solution was measured at 278 nm, and 50-μl volumes of the protein solution and of ligand solution in the same buffer were placed on opposite sides of the membranes. The filling pores were then sealed, and the cell block was placed in a circulating water bath, kept at a constant temperature of 20° for 12 hr. Preliminary tests showed that equilibrium was reached in less than 9 hr.

The stability of the enzyme was checked in a separate experiment by measuring the aspartokinase activity and sensitivity toward threonine of a similar protein solution in buffer B over a period of several days. At 20°, the aspartokinase activity decreased to approximately one-half its original value after about 6 days, without undergoing detectable desensitization. Microbial growth in the solution eventually precluded an exact measurement of the inactivation rate constant; however, there was no detectable loss in enzymatic activity during the first 24 hr. The enzyme appeared, therefore, to be sufficiently stable in buffer B so that the experiments could be performed. The high stability of the enzyme under these conditions was foreseen in view of the reports of other investigators of the stabilizing effects on the enzyme of aspartate and potassium ion (Patte *et al.*, 1963; Wampler *et al.*, 1970).

Samples for radioactive analysis were taken from each side of the dialysis cell with 3-μl micropipets without dismantling the cell, and were transferred to vials containing 0.1 ml of water, which was used to rinse the pipets. A 10-ml aliquot of scintillation fluid was then placed in each vial. The fluid contained 5% (v/v) BioSolv III (Beckman) and 4.2% (v/v) Spectrafluor (Amersham–Searle) in toluene solvent. The samples so obtained had activities of approximately 12,000 dpm.

The concentration of threonine was calculated from the known (weighed) amount of nonradioactive compound added and the assumed specific activity of the radioactive isotope. The concentration of threonine binding sites was determined from the absorbancy of the protein solution at 278 nm. The measured absorbancy was related to the concentration of threonine binding sites in two ways: first, a direct estimate of the number of threonine sites was made from threonine binding experiments at relatively high ligand concentrations. This approach involves considerable uncertainty owing to the fact that threonine is not strongly bound under the experimental conditions. Second, an indirect estimate of the number of threonine sites was made by measuring the concentration of NADPH sites: a sample of protein of known absorbancy in buffer B was mixed with an equal volume of an NADPH solution in the same buffer, and subjected to gel filtration at 5° on a column of Bio-Gel P-10. The column had been previously equilibrated with an NADPH solution of the same total concentration as in the final protein mixture. Standard methods were used to calculate the moles of bound NADPH in the sample (Fairclough and Fruton, 1966; Bush *et al.*, 1971). The stoichiometry of threonine binding in phosphate buffer suggests that the number of threonine binding sites is twice as large as the number of NADPH sites (Janin *et al.*, 1969; Falcoz-Kelly *et al.*, 1972). The values for the concentration of threonine sites obtained by the two methods were consistent with one another.

Fluorometric Titrations. In order to ensure that all of the homoserine dehydrogenase sites were occupied in the gel

[1] Abbreviation used is: SH-TP, 6-mercapto-9-β-D-ribofuranosylpurine 5′-triphosphate.

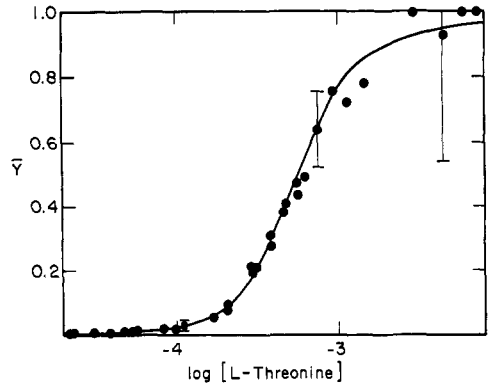

FIGURE 1: Binding of [^{14}C]threonine to the protein in buffer B at 20° by equilibrium dialysis. The concentration of enzyme was about 26 μM.

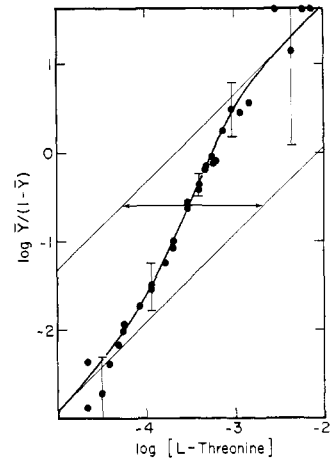

FIGURE 2: Hill plot of L-threonine binding. The total interaction energy, per site, is 2.1 ± 0.9 kcal.

filtration experiment described above, the dissociation constant for NADPH from protein sites in buffer B was determined by fluorometric titration. The apparent dissociation constant at 25° is about 2 μM, a value significantly larger than that reported for the same reaction in phosphate buffer in the absence of aspartate (Janin *et al.*, 1969). The concentration of NADPH in the gel filtration experiment exceeded this value by approximately a factor of 50.

Threonine binding to enzyme samples in buffer B was followed by fluorescence methods using the excitation and emission wavelengths recommended by Janin and Cohen (1969). In one experiment, the buffer was identical with B, except for the presence of 1 mM aspartate. In all cases, the measured fluorescence intensities were corrected for dilution of the protein samples.

Aspartokinase Inactivation with SH-TP. The reaction of the enzyme with SH-TP in buffer A was followed by observing the disappearance of the aspartokinase activity, as described previously (Truffa-Bachi and Heck, 1971).

Aspartokinase Assay. The aspartokinase activity was measured in buffer B to which was added 0.3 mM NADH, 6 mM adenosine triphosphate (ATP), 0.3 μM pyruvate kinase, and 0.9 μM lactate dehydrogenase. The pH of the assay system was adjusted to that of buffer B.

Results

Binding of Threonine. The affinity of the enzyme for threonine in buffer B is lower than that which has been found under other conditions (Janin *et al.*, 1969; Takahashi and Westhead, 1971). As indicated in Figure 1, the point of half-saturation occurs at the relatively high L-threonine concentration of 0.57 mM. A low affinity of the enzyme for threonine was predicted from the fact that buffer B contains both a high concentration of potassium ion and a moderate concentration of aspartate. These conditions were chosen for their similarity to those of the normal aspartokinase assay.

At the highest concentrations of threonine, less than 5% of the total threonine molecules in solution are bound to the enzyme. At these high levels of threonine, the error in the fractional occupation of sites, Y, in repeated measurements was estimated to be approximately 50%.

The low affinity of the enzyme for threonine in buffer B introduces uncertainty in the shape of the ligand equilibrium curve at high levels of threonine. The smooth curve of Figure 1 fits the data reasonably well, with particular attention being given to the points below 1 mM threonine, which are known with greater precision. The curve was calculated using the constants of the simple allosteric model of Monod *et al.* (1965) to be described later.

If the binding data are recast in the form of a Hill plot (Figure 2), the cooperative nature of the binding process can be visualized more clearly. The solid curve, which is theoretical, has a maximum slope approximately equal to 2.3. This result agrees well with the value 2.5 estimated by Janin *et al.* (1969) for threonine binding in a different buffer system. The total "interaction free energy" (Wyman, 1964), which corresponds to the decrease in the free energy of ligand binding, per site, as the macromolecule becomes saturated, is given by

$$\Delta G^\circ_I = 2.303RT(\Delta \log x) \quad (2)$$

where x is the free ligand activity. The quantity in parentheses in eq 2 is the difference between the asymptotes of the Hill plot at any fixed value of $\bar{Y}$, signified in Figure 2 by the double-headed arrow. For the present system, the interaction free energy is 2.1 ± 0.9 kcal.

It should, perhaps, be stressed that the interaction free energy was not calculated empirically using eq 2, since a simple statistical analysis shows that the error in the ordinate of a Hill plot approaches infinity at the extremes of the titration curve (Watts-Tobin, 1967). Rather, the interaction free energy was calculated directly from the constants of the allosteric model, using

$$\Delta G^\circ_I = RT \ln \frac{K_R}{K_T} \frac{(1 + L')}{\left(1 + L' \frac{K_R}{K_T}\right)} \quad (3)$$

where K_R and K_T are the intrinsic dissociation constants for threonine from sites in the R and T conformations, respectively, and $L' = T_0/R_0$ is the conformational equilibrium constant in the absence of threonine under the experimental conditions. Equation 3 is derived by defining the T conformation to be the state of greater ligand affinity. The values of the constants in eq 3, determined directly from the binding measurements, and supported by other experiments to be described, are given in Table I.

Conformational Transitions. Janin and Cohen (1969) suggested that when the aromatic amino acids of aspartokinase

TABLE I: Equilibrium Constants of the Allosteric Model.[a]

	T Form	*R* Form
	Dissociation Constants (mM)	
Threonine	0.22 ± 0.10	20 ± 10
Aspartate		28 ± 20
	Allosteric Constants	
L′	0.015 ± 0.018	
L	0.06 ± 0.15	

[a] At 20° in buffer B.

I-homoserine dehydrogenase I are excited with ultraviolet light of wavelengths shorter than 280 nm, the protein fluorescence changes reflect primarily conformational changes of the protein. The rate of the rapid reaction of cysteine residues of the protein with *p*-chloromercuribenzoate has also been reported to be a conformational probe rather than an index of ligand binding (Takahashi and Westhead, 1971). I investigated the effect of threonine binding on the rate of inactivation of the aspartokinase function by SH-TP, since, of the two sulfhydryl reagents, SH-TP reacts much more specifically and with apparently simpler kinetic characteristics (Truffa-Bachi and Heck, 1971).

For the interpretation of the changes in protein fluorescence and in the kinetics of the aspartokinase inactivation by SH-TP upon threonine binding, a two-states model was assumed. This incorporates a bias against more complicated mechanisms, and such mechanisms are, therefore, not excluded by this study, but this hypothesis is the simplest consistent with the measurements. No assumptions were made at the outset concerning the validity of concerted (Monod *et al.*, 1965) or sequential (Koshland *et al.*, 1966) models.

Thus, the fractional change in protein fluorescence was expressed by

$$\bar{T} = \frac{F_R - F_{obsd}}{F_R - F_T} \tag{4}$$

where F_R may be regarded as the total protein fluorescence

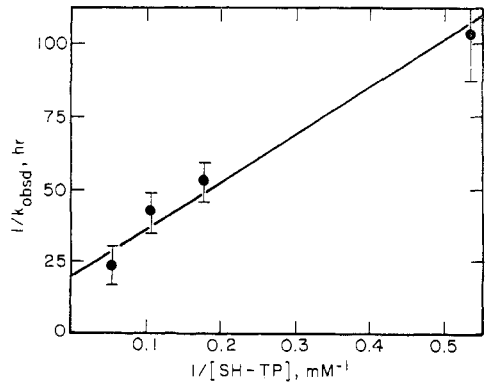

FIGURE 3: Plot of the inverse of the observed rate constant for aspartokinase inactivation by SH-TP at 25° *vs.* the inverse of the concentration of SH-TP. The solutions contained 0.7 M potassium phosphate, 0.12 M KCl, 10 mM L-threonine, 1.2 μM aspartokinase I-homoserine dehydrogenase I (pH 7.61), and various concentrations of SH-TP as shown.

when all subunits are in the *R* conformation, and F_{obsd} and F_T are the corresponding values of the protein fluorescence at intermediate and saturating concentrations of threonine, which we define to bind preferentially to the *T* state. According to the concerted model, $\bar{T}$ is identically the fraction of protein molecules in the *T* conformation, whereas, in the sequential model, $\bar{T}$ represents simply the fraction of subunits in that form. The decision as to which of these models is applicable rests upon comparing the saturation function with the state function (Ogawa and McConnell, 1967).

The changes in the rate of inactivation of the aspartokinase function by SH-TP in buffer A were treated in a similar way. It was observed that the inactivation rate decreased sharply upon addition of threonine, although high concentrations of threonine (10 mM) do not entirely prevent the inactivation from occurring. The results of the inactivation experiments could be readily analyzed by assuming that, in the presence of threonine, two different reactivities toward SH-TP are available to a given reaction site

$$\begin{array}{ccc} AK_R + \text{SH-TP} \overset{K_S}{\rightleftharpoons} AK_R - \text{SH-TP} & \xrightarrow{k_R} & AK_R\text{-SS-TP} \\ \uparrow\downarrow & & \uparrow\downarrow \\ AK_T + \text{SH-TP} \overset{K_S}{\rightleftharpoons} AK_T - \text{SH-TP} & \xrightarrow{k_T} & AK_T\text{-SS-TP} \end{array} \tag{5}$$

where AK_R and AK_T represent free enzyme in the two different reactivity states, AK_R-SH-TP and AK_T-SH-TP are enzyme–SH-TP complexes, and AK_R-SS-TP and AK_T-SS-TP are the products of covalent reaction occurring within the complexes. For this mechanism, the apparent pseudo-first-order inactivation rate constant is given by

$$k_{obsd} = \left(\frac{k_R[AK_R] + k_T[AK_T]}{[AK_R] + [AK_T]}\right)\left(\frac{1}{1 + K_S/[\text{SH-TP}]}\right) \tag{6}$$

The constancy of K_S in the above mechanism implies that the binding of SH-TP does not affect the amounts of enzyme in the two different reactivity states. This is supported by the observation that a plot of the inverse of k_{obsd} is proportional to the inverse of the SH-TP concentration (eq 6 and Figure 3) for the inactivation reaction in the presence of 10 mM L-threonine, where $[AK_R] \cong 0$. Similar results were observed in the absence of threonine, where $[AK_T] \cong 0$ (Truffa-Bachi and Heck, 1971). In both instances, the dissociation constant for SH-TP is 9 mM. Although K_S is unchanged by threonine binding, k_R and k_T differ by approximately two orders of magnitude, as can be discerned by comparing the results of Figure 3 to those obtained earlier for the same conditions in the absence of threonine (Truffa-Bachi and Heck, 1971).

At a given essentially constant SH-TP concentration, the second factor on the right of eq 6 is constant. In this case, the fraction of sites in the reactivity state *T* can be studied as a function of threonine concentration, using

$$\bar{T} = \frac{k_R - k_{obsd}}{k_R - k_T} \tag{7}$$

which follows directly from eq 6. The same general interpretation may be given to eq 7 as was applied to eq 4.

When the changes in protein fluorescence and in the rate constant for reaction of sulfhydryl groups toward SH-TP are investigated at different concentrations of threonine, the

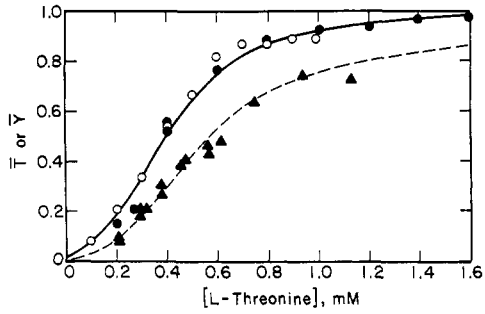

FIGURE 4: Fractional changes, $\bar{T}$, in protein fluorescence in buffer B (○), and in the rate constant for aspartokinase inactivation by SH-TP in buffer A (●) at 20° *vs.* the concentration of L-threonine. The concentration of protein was 15 μM in the fluorometric titrations, and 9.5 μM in the SH-TP inactivations. Representative data from Figure 1 for the fractional occupation of sites by L-threonine, $\bar{Y}$, are included for comparison (▲).

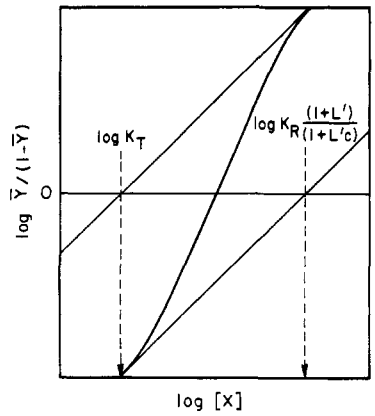

FIGURE 5: Evaluation of the constants of the model of Monod *et al.* from a Hill plot of ligand binding data; x is the free ligand activity. The T state is assumed to be the state of greater affinity.

two $\bar{T}$ functions (eq 4 and 7) appear to overlap closely. Typical data from one set of measurements are shown in Figure 4. This result implies that the different properties of the protein which are detected by these probes change in a similar way with variations in threonine concentration.

On the other hand, representative binding data included in Figure 4 show that the alterations in the above two variables do not correspond identically with changes in the occupation of enzyme sites by threonine. Assuming that the threonine binding sites are equivalent, these results support the hypotheses of Janin and Cohen (1969) and of Takahashi and Westhead (1971) that changes in the protein fluorescence and sulfhydryl group reactivities directly measure alterations in the protein conformation rather than in the amount of ligand bound, and provide further evidence for the interpretation of the results in terms of an all-or-none, concerted cooperative model. Both curves of Figure 4 are calculated, using the constants given in Table I and the model to be discussed.

According to the simple allosteric mechanism of Monod *et al.* (1965), the ratio of protein molecules in T and R conformations is given by

$$\frac{\bar{T}}{\bar{R}} = L'\left(\frac{1 + [\text{L-threonine}]/K_T}{1 + [\text{L-threonine}]/K_R}\right)^n \qquad (8)$$

where n is the number of threonine binding sites in the cooperative unit. Assuming that the intrinsic dissociation constant for threonine from the R conformation is much larger than from the T state, a linear relationship should exist between log $(\bar{T}/\bar{R})$ and log $(1 + [\text{L-threonine}]/K_T)$, the slope of which provides an estimate of n, and the intercept a measure of L'.

For the plot described, the evaluation of K_T could be made by displaying the ligand binding data in the form of the Scatchard equation (Changeux and Rubin, 1968). The Hill plot can also be used to estimate this constant. As shown in Figure 5, the asymptote (with unit slope) corresponding to saturation of the protein with ligand has a value on the ordinate equal to zero when log $[x]$ = log K_T. The other asymptote, which also has a slope of unity, may be used to estimate K_R. This particular interpretation only applies, of course, when the T conformation is defined to be the state of greater affinity.

Since the error in estimating K_T by any method is large, the above technique was used initially to obtain only an approximate value of K_T, and subsequent refinements were made visually, fitting the entire binding curve, until the best values of K_T, K_R, and L' were obtained. These values are those given in Table I. The given maximum error limits include possible values for the constants which could yield a curve fitting the data within the precision of the experimental points.

With K_T = 0.22 mM, the changes in fluorescence and in the aspartokinase inactivation rate constant with SH-TP were plotted according to eq 8 (Figure 6). The solid line of the figure corresponds to experimental conditions which are the same as those used for the threonine binding measurements. The unweighted least-squares slope of the data is equal to 3.8, and the intercept gives $L' = 0.02 \pm 0.03$, consistent with the estimate of L' using the equilibrium dialysis technique (Table I).

The above experiments were done in buffers A or B, both of which contain 10 mM L-aspartate. In order to obtain an estimate of the effect of aspartate on the apparent allosteric equilibrium constant, as well as an approximate value of the intrinsic aspartate dissociation constant from the enzyme, additional fluorescence titrations with threonine were carried out in the presence of 1 mM aspartate. Some representative data are shown in Figure 6. It was tacitly assumed for the interpretation of these data that aspartic acid binds to four sites. This assumption is presently open to serious doubt, since the number of aspartokinase sites in the enzyme has not been determined. Janin and Cohen (1969) suggested that three aspartate sites per protein molecule interact cooperatively, but this proposal seems unlikely in the light of recent redeterminations of the physical properties of the enzyme.

Assuming, however, that four aspartate sites interact cooperatively, that the aspartate and threonine sites are intrinsically independent, and that aspartate binds almost exclusively to the R conformation (Janin and Cohen, 1969), the apparent allosteric equilibrium constant, L', may be written as

$$L' = \frac{L}{\left(1 + \frac{[\text{L-aspartate}]}{K'_R}\right)^4} \qquad (9)$$

where L is the allosteric constant in the absence of aspartate, and K'_R is the intrinsic dissociation constant for aspartate from sites in the R state. The constants, L and K'_R, can be evaluated from the two intercepts of Figure 6 using eq 9, and are given in Table I. The least-squares slope of the dashed

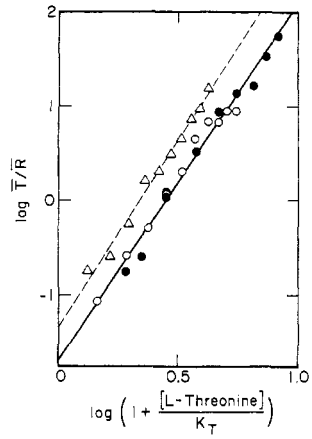

FIGURE 6: Plot of the logarithm of the $\bar{T}/\bar{R}$ ratio *vs.* the logarithm of $(1 + [\text{L-threonine}]/K_T)$, where $K_T = 0.22$ mM; data from protein fluorescence in buffer B (○), and from the rate constant for aspartokinase inactivation in buffer A (●), both of which contain 10 mM aspartate. In a solution containing 1 mM aspartate, but otherwise identical to buffer B, the fluorescence changes indicate an increase in the $\bar{T}/\bar{R}$ ratio (△).

line in Figure 6 is identically 4.0, suggesting that also at low aspartate, the cooperative unit for threonine binding is composed of four sites.

Inhibition by Threonine of the Aspartokinase Activity. A "kinetic Hill plot" of the aspartokinase activity as a function of threonine concentration, under conditions which closely resemble the threonine binding measurements described above, is shown in Figure 7. On the same graph are depicted the changes in protein fluorescence and in the rate constant for sulfhydryl group reaction with SH-TP expressed as the $\bar{T}/\bar{R}$ ratio. The solid curve through these points is a theoretical function based on eq 8.

The error in estimating $(v_0 - v)/(v - v_S)$ is proportionally greatest at the extremes of the kinetic Hill plot. If the point corresponding to the lowest threonine concentration is disregarded for reasons of possible error, a least-squares plot through the kinetic data can be shown to yield a slope of 4, confirming the reports of other investigators (Patte *et al.*, 1966; Wampler and Westhead, 1968; Wampler *et al.*, 1970). There is, of course, no general physical requirement that a

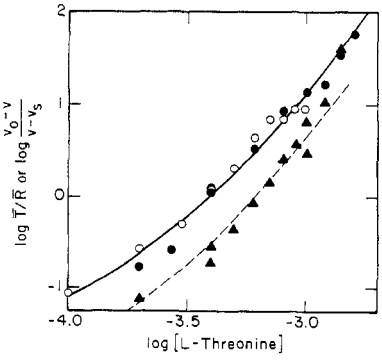

FIGURE 7: Kinetic Hill plot of aspartokinase activity [log $(v_0 - v)/(v - v_S)$] *vs.* the logarithm of the threonine concentration, under the aspartokinase assay conditions (about 5 nM aspartokinase I–homoserine dehydrogenase I) (▲). Data from protein fluorescence in buffer B (○) and the rate constant for aspartokinase inactivation in buffer A (●) are included for comparison.

kinetic Hill plot should be linear. Indeed, Figure 7 shows that a smooth curve can be drawn through the kinetic data which has, within experimental error, the same shape as the theoretical $\bar{T}/\bar{R}$ function. This suggests that the kinetic Hill coefficient of 4 can be explained simply by assuming that the $(v_0 - v)/(v - v_S)$ function directly mirrors the $\bar{T}/\bar{R}$ ratio of a cooperative tetramer, in which the R conformation is enzymatically active. In accordance with the allosteric mechanism, alterations in the $\bar{T}/\bar{R}$ ratio do not directly parallel changes in the amount of threonine bound, where, as noted earlier, the maximal Hill coefficient is only 2.3.

A possible explanation for the horizontal displacement of the two curves in Figure 7 from one another is that the experimental conditions are not precisely identical in the two kinds of experiments. In addition to employing different concentrations of aspartokinase I–homoserine dehydrogenase I, the kinetic measurements include ATP as well as auxiliary enzymes and their substrates; one or more of these factors might conceivably effect the $\bar{T}/\bar{R}$ ratio. For example, it is known that aspartate binding is facilitated in the presence of adenosine diphosphate (J. Janin, personal communication). If ATP has a similar influence on aspartate binding, a higher threonine concentration should be required in the kinetic than in the equilibrium measurements to achieve the same value of $\bar{T}/\bar{R}$, since aspartate binds preferentially to the R form of the enzyme (Janin and Cohen, 1969). This explanation agrees with the results of Figure 7.

Discussion

A major goal of investigations of enzymatic regulation is the deduction of regulatory mechanisms for enzymes under nonequilibrium conditions. The least ambiguous experimental findings are, however, often made at, or close to, equilibrium. It may, therefore, be asked whether inferences made about systems at equilibrium are also applicable to systems far from equilibrium, since it is known that, under such conditions, sigmoidal saturation curves can be generated even in the absence of cooperative behavior (Rabin, 1967; Sweeny and Fisher, 1968; Frieden, 1970).

The results of the present study show that at equilibrium, in Tris buffer, aspartokinase I–homoserine dehydrogenase I seems to bind threonine according to the simple allosteric model of Monod *et al.* (1965). This result was not unexpected in view of the earlier findings of Janin and Cohen (1969) and Takahashi and Westhead (1971). The essential difference between the earlier measurements and those of the present paper is the proposed number of threonine binding sites in the cooperative unit. The postulate of four sites clearly accounts for the measurements reported here and is more consistent with revised estimates of the protein subunit structure (Clark and Ogilvie, 1972; Falcoz-Kelly *et al.*, 1972).

More important, however, is the fact that this correction provides a reasonable explanation for the inhibition of the enzyme by threonine under nonequilibrium, steady-state conditions. If the conformational change between R and T forms of the enzyme is concerted, the kinetic Hill coefficient of 4 reported in several laboratories can be interpreted in a formally straightforward manner in terms of a tetrameric model. On the other hand, a trimeric model cannot explain, in a simple manner, the observed kinetic Hill coefficient.

The alternative cooperative mechanism most widely cited for regulatory proteins, the sequential model of Koshland *et al.* (1966), seems to be less likely. First, the changes in fluorescence and in the rate constant for the inactivation of

the aspartokinase function by SH-TP are at variance with the simplest form of this model, since the observed changes do not parallel corresponding changes in the saturation of the enzyme by threonine. Second, the simplest sequential model does not provide a clear explanation for the strong cooperativity of inhibition by threonine observed under steady-state conditions. The simplest sequential model implies that the inhibition of the enzyme should correspond directly to the binding of the inhibitor, which is not the case here.

Although the simplest sequential mechanism seems to be ruled out, it must be emphasized that more complicated forms of this model are not excluded. Characteristic of all simple models is the assumption of only two states for each subunit. In a more general case, the number of states should be allowed to vary depending upon whether or not neighboring sites are occupied with ligand. Such a mechanism greatly increases the number of adjustable parameters, and might be used to fit state functions and saturation functions of any desired complexity. A general discussion of this mechanism has already been given by Haber and Koshland (1967). Although no attempt was made to fit the data in terms of this more complicated scheme, it is clear that it represents a possible alternative interpretation.

Acknowledgments

I thank Dr. Joseph A. Duke for the gift of SH-TP used in this work, Dr. James C. Wang for the use of his scintillation counter, and Dr. Melvin Calvin for the use of the spectrofluorometer.

References

Bush, K., Mahler, H. R., and Shiner, V. J., Jr. (1971), *Science 172*, 478.

Changeux, J.-P. (1963), *Cold Spring Harbor Symp. Quant. Biol. 28*, 497.

Changeux, J.-P., and Rubin, M. M. (1968), *Biochemistry 7*, 553.

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

Cohen, G. N. (1969), *Curr. Top. Cell. Regul. 1*, 184.

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

Fairclough, G. F., and Fruton, J. S. (1966), *Biochemistry 5*, 673.

Frieden, C. (1970), *J. Biol. Chem. 245*, 5788.

Haber, J. E., and Koshland, D. E. (1967), *Proc. Nat. Acad. Sci. U. S. 58*, 2087.

Heck, H. d'A., and Truffa-Bachi, P. (1970), *Biochemistry 9*, 2776.

Janin, J. (1972), *Cold Spring Harbor Symp. Quant. Biol. 36*, 193.

Janin, J., and Cohen, G. N. (1969), *Eur. J. Biochem. 11*, 520.

Janin, J., van Rapenbusch, R., Truffa-Bachi, P., and Cohen, G. N. (1969), *Eur. J. Biochem. 8*, 128.

Koshland, D. E., Nemethy, G., and Filmer, D. (1966), *Biochemistry 5*, 365.

Monod, J., Wyman, J., and Changeux, J.-P. (1965), *J. Mol. Biol. 12*, 88.

Myer, Y. P., and Schellman, J. A. (1962), *Biochim. Biophys. Acta 55*, 361.

Ogawa, S., and McConnell, H. M. (1967), *Proc. Nat. Acad. Sci. U. S. 58*, 19.

Patte, J.-C., LeBras, G., Loviny, 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.

Rabin, B. R. (1967), *Biochem. J. 102*, 22c.

Sweeny, J. R., and Fisher, J. R. (1968), *Biochemistry 7*, 561.

Szentirmai, A., Szentirmai, M., and Umbarger, H. E. (1968), *J. Bacteriol. 95*, 1672.

Takahashi, M., and Westhead, E. W. (1971), *Biochemistry 10*, 1700.

Truffa-Bachi, P., and Heck, H. d'A. (1971), *Biochemistry 10*, 2700.

Truffa-Bachi, P., van Rapenbusch, R., Janin, J., Gros, C., and Cohen, G. N. (1968), *Eur. J. Biochem. 5*, 73.

Wampler, D. E., Takahashi, M., and Westhead, E. W. (1970), *Biochemistry 9*, 4210.

Wampler, D. E., and Westhead, E. W. (1968), *Biochemistry 7*, 1661.

Watts-Tobin, R. J. (1967), *J. Mol. Biol. 23*, 305.

Whitehead, E. (1970), *Progr. Biophys. Mol. Biol. 21*, 321.

Wyman, J. (1964), *Advan. Protein Chem. 19*, 223.

Wyman, J. (1967), *J. Amer. Chem. Soc. 89*, 2202.