Homoserine Dehydrogenase–Aspartokinase of *Escherichia coli*. Comparison of Threonine Saturation and Enzyme Conformation*

M. Takahashi† and E. W. Westhead

ABSTRACT: The homoserine dehydrogenase–aspartokinase complex of *Escherichia coli* K_{12} exhibits cooperative binding of the feedback inhibitor threonine. The effects of threonine binding upon enzyme conformation have been studied using ultraviolet difference spectra, sulfhydryl group reactivity, enzyme inhibition, and hydrogen exchange. Changes in enzyme conformation detected by these methods, which reflect quite different aspects of protein structure, are all coincident when studied as a function of threonine concentration. On the other hand, the saturation of threonine binding sites studied by ultrafiltration was found to occur at higher threonine concentrations than are required to effect the changes in protein conformation or state. This result is compatible with the Monod–Wyman–Changeux concerted model for allosteric transitions but not with the diagonal Koshland sequential model. The hydrogen-exchange measurements provide a technique for studying conformational changes which does not perturb enzyme structure, gives an idea of the size of the perturbation caused by ligand binding, and, additionally, provides a measure of the "tightness" or "looseness" of the enzyme. Upon threonine binding to homoserine dehydrogenase–aspartokinase, approximately 40% additional hydrogens are sequestered in the slowly exchanging core.

The aspartokinase I–homoserine dehydrogenase I–enzyme complex found in *Escherichia coli* K_{12} catalyzes both the phosphorylation of aspartate by ATP and the NADPH-linked reduction of aspartic semialdehyde to homoserine. Work of Patte *et al.* (1963) and Wampler and Westhead (1968) has shown that both enzyme activities show cooperative inhibition by the feedback inhibitor threonine. The native enzyme is a hexamer of mol wt 360,000, with a total of six threonine binding sites. Comparison of threonine saturation curves for the enzyme to changes in ultraviolet difference spectra at 289 mμ induced by threonine binding (Janin *et al.*, 1969) showed a close parallelism between conformational changes in the protein and threonine binding when studied at high potassium ion concentrations. Conformational changes measured by changes in fluorescence of the protein, on the other hand, did not correlate with the threonine saturation curve at lower potassium ion concentrations. Since the relationship of the state function to the saturation function (Monod *et al.*, 1965) gives fundamental information about the nature of the cooperative transition, we decided to examine other measures of the state function based upon parameters which do not reflect changes in the tyrosine–tryptophan environment. In addition to this correlation, examination of a variety of parameters could be expected to give some idea of the extent of the conformational change involved in the allosteric regulation of the enzyme. We have studied, as a function of threonine concentration, the hydrogen-exchange behavior of the enzyme, sulfhydryl group reactivity, and inhibition of homoserine dehydrogenase activity. In addition, ultraviolet difference changes at 289 mμ were studied as a function of threonine concentration in order to be able to compare results to those of Janin *et al.* (1969).

Materials

Buffers used were buffer G (0.02 M potassium phosphate, pH 7.2, 2 mM magnesium acetate, 2 mM K-EDTA, 0.5 mM L-threonine, and 0.1 mM DTT[1]) and buffer D (0.02 M potassium phosphate, pH 7.5, 0.08 M KCl, 0.1 mM DTT, 2 mM potassium aspartate, and 2 mM EDTA.

Aspartic semialdehyde was prepared by ozonalysis of D,L-allylglycine (Cyclo Chemicals) according to the method of Black and Wright (1955). PMB was obtained from Sigma Chemicals. Tritiated water (specific radioactivity 1 Ci/g) and [^{14}C]threonine (specific radioactivity 164 mCi/mmole) were obtained from New England Nuclear Corp. All other chemicals were obtained from commercial sources and were the purest grades available.

Methods

Enzyme Purification. *E. coli* K_{12} cultures were a gift of Dr. Martin Freundlich. *E. coli* cells grown in minimal glucose media (Davis and Mingioli, 1960) and harvested at late log phase were obtained as a frozen paste from the New England Enzyme Center. The purification of the enzyme was essentially that of Truffa-Bachi *et al.* (1968), with minor modifications. The purification consists of sonification, streptomycin sulfate treatment, ammonium sulfate fractionation, DEAE-Sephadex column chromatography, followed by chromatography on hydroxylapatite and Sephadex G-200. Enzyme obtained with this procedure had a specific activity of greater than 65 units/mg (homoserine dehydrogenase assay) and was stable when stored at a concentration greater than 5 mg/ml in buffer G at 4°. Protein concentrations were calculated using a molar extinction coefficient of 165,000 at 278 mμ (Truffa-Bachi *et al.*, 1968). This preparation appears to be greater than 98% pure as judged by disc gel electrophoresis.

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[1] Abbreviations used are: DTT, dithiothreitol; PMB, *p*-mercuribenzoate.

Standard Enzyme Assays. Homoserine dehydrogenase activity was followed by monitoring NADPH disappearance at 340 mμ upon initiation of the reaction by addition of enzyme to the assay mixture at 30°, as described previously (Wampler and Westhead, 1968). Aspartokinase activity was followed at 340 mμ with a coupled enzyme assay utilizing pyruvate kinase and lactic dehydrogenase, under the conditions reported previously (Wampler and Westhead, 1968).

The retention of properties characteristic of the "native" enzyme were verified whenever possible, at the beginning and end of experiments. Native enzyme was taken to be enzyme showing homoserine dehydrogenase specific activity of greater than 55, coupled with greater than 80% inhibition by saturating levels of threonine (10 mM).

Studies of Threonine Inhibition of Homoserine Dehydrogenase. Homoserine dehydrogenase inhibition in the presence of aspartate was studied in buffer D to which was added 2 mM magnesium acetate, 2 mM NADPH, and 0.175 mM aspartic semialdehyde. Enzyme assays were done at 30°, the reaction being initiated by addition of aspartic semialdehyde.

Hydrogen-Exchange Studies. The rapid dialysis technique of Englander and Crowe (1965) was used to study the hydrogen-exchange behavior of the enzyme. Exchange-in was conducted at room temperature for greater than 24 hr in a buffer containing 0.02 M potassium phosphate (pH 7.2), 20 mM potassium aspartate, 0.1 mM DTT, 0.1 M KCl, 2 mM EDTA, and tritiated water of specific radioactivity of 6.25 mCi/ml. Threonine was added to give a final concentration of 1 mM at the end of the incubation, and the sample was stored at 4° thereafter. Control experiments showed that longer periods of exchange-in at room temperature did not substantially change the results obtained. Exchange-out was measured using 0.5 ml of enzyme solution (protein concentration 1.6 mg/ml) inside prestretched pencil size dialysis tubing which was secured to the Englander rapid dialysis apparatus. The apparatus was immersed in a graduated cylinder containing 500 ml of buffer D containing the desired concentration of threonine. Exchange-out experiments were conducted at 27 ± 2°. Samples (25 μl) were removed from the dialysis tubing with micropipets and diluted 1:10 for measurement of protein concentration. The diluted sample (0.1 ml) was subsequently counted in 10 ml of scintillation fluid (Bray, 1960). Samples were counted in a Packard Model 3310 Tri-Carb scintillation spectrometer. The size of the "core" of slowly exchanging protons is the most reproducible parameter of hydrogen exchange, and the most convenient to measure. Preliminary experiments showed that a linear extrapolation to zero time could be made consistently from that portion of the exchange-out curve obtained after about 10 hr. Hourly samples were taken in the 10–25-hr exchange-out period. Numbers of residual hydrogens were calculated as done by Englander and Crowe (1965), and the results expressed as moles of unexchanged hydrogens per mole of enzyme (360,000).

Sulfhydryl Group Reactivity. Boyer's (1954) method of studying rates of sulfhydryl group reaction with PMB was used. Changes in absorbance at 250 mμ were measured either in a Gilford Model 240 spectrophotometer equipped with a Honeywell Elektronik 194 recorder or in a Durram-Gibson stopped-flow apparatus (Durram Instruments, Palo Alto, Calif.). An extinction coefficient of 7600 (Boyer, 1954) for the mercaptide formed was used to calculate the number of sulfhydryl groups reacted. Reactions were carried out in buffer D lacking EDTA and DTT, using 0.5 μM enzyme and a greater than tenfold excess of PMB (108 μM) to ensure pseudo-first-order kinetics. Measurements were made at 27°, and rate constants were calculated from first-order semilog plots. The rate constants reported correspond to the reactivity characteristic of the first 30% of the reactive sulfhydryl groups (18–20 total, Truffa-Bachi *et al.*, 1968). The reaction studied on the Gilford was initiated by addition of concentrated enzyme which had been previously dialyzed against a buffer containing 0.02 M potassium phosphate (pH 7.5), 0.1 mM threonine, 20 mM potassium aspartate, and 80 mM KCl.

Measurement of the rate of PMB reaction required use of the stopped-flow technique at the lower threonine concentrations. Protein and PMB concentrations after mixing were the same as those used in the Gilford studies. Temperature was maintained at 27°. Measurements were done at the smallest slits (0.5 mm) compatible with good signal to noise ratios.

Threonine Binding Studies. Threonine binding to the enzyme was measured at 27° using the ultrafiltration cell developed by Paulus (1969) which was obtained from Metaloglas, Inc., Boston, Mass. Threonine binding studies were done in buffer D with [^{14}C]threonine (specific activity ~1.1 mCi/mole) at protein concentrations of 0.9 mg/ml. Fractions (0.2 ml) of solutions containing enzyme and threonine were passed through the Amicon UM-10 filter disks. After washing the bottom sides of the filter disks with 3 ml each of ethylene glycol, the filters were then transferred to 1 ml of distilled water. Scintillation fluid (10 ml) (Bray, 1960) was added and the samples were counted on a Packard Model 3310 Tri-Carb scintillation spectrometer. Appropriate blanks (lacking protein) to compensate for the 2.5–3 μl of solution trapped in the UM-10 filters were run through the ultrafilter and these values were subtracted from the enzyme sample values.

Ultraviolet Difference Measurements at 289 mμ. Janin *et al.* (1969) have shown that significant ultraviolet difference spectra are obtained at 289 mμ upon threonine binding to the enzyme. Ultraviolet difference measurements were made at 289 mμ under buffer conditions suitable for our studies. The enzyme concentration was 11 μM, and the buffer consisted of 0.02 M potassium phosphate (pH 7.5), containing 0.08 M KCl, 0.1 mM dithiothreitol, and 2 mM potassium aspartate. Ultraviolet difference measurements were made on a Perkin-Elmer Model 356 spectrophotometer by addition of concentrated threonine solution to the sample cell together with suitable buffer additions to the reference cell. Measurements were made at 27° in 1-cm cells.

Results

Hydrogen-Exchange Studies. The results of three hydrogen-exchange experiments conducted over a range of threonine concentrations are presented in Figure 1. It can be seen that reliable back-extrapolations to obtain values for the number of slowly exchanging core hydrogens were possible at times after 10–11 hr. In the absence of threonine during the exchange-out process, there are approximately 220 core hydrogens, while in the presence of threonine at saturating concentrations the number of core hydrogens is increased to 310. The binding of threonine evidently tightens the enzyme structure and increases the core size about 40%. The homoserine dehydrogenase–aspartokinase complex in the inhibited form has substantially reduced solvent accessibility to the slowly exchanging core. At lower concentrations of threonine in the exchange-out medium, the number of residual slowly

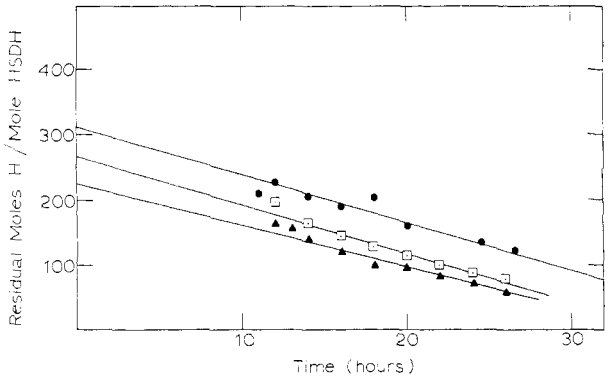

FIGURE 1: Exchange-out of residual hydrogens from tritiated homoserine dehydrogenase–aspartokinase. Experiments were conducted in buffer D at 27° at protein concentrations of 4.5 μM. Threonine concentrations are zero threonine (▲), 0.05 mM threonine (□), and 1 mM threonine (●).

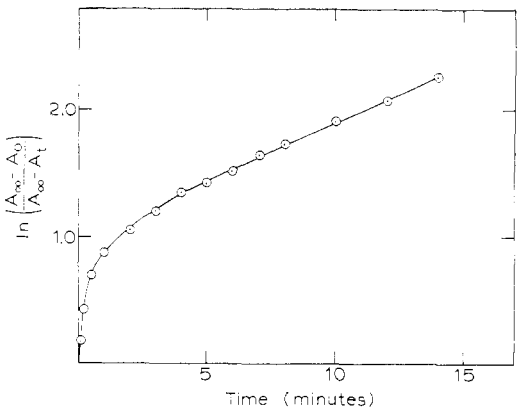

FIGURE 2: First-order rate plot for reaction of PMB (108 μM) with homoserine dehydrogenase (0.5 μM) in 0.02 M potassium phosphate buffer (pH 7.5) containing 2 mM potassium aspartate, 0.08 M KCl, and 1.09 mM threonine. Measurements were made at 250 mμ at 27°.

exchanging hydrogens is correspondingly reduced as is tabulated in Table I.

Studies of the Reactivity of Sulfhydryl Groups. Truffa-Bachi *et al.* (1968) have shown that the rate of DTNB reaction with the sulfhydryl groups of the enzyme is strongly reduced by the binding of threonine. Our studies of sulfhydryl group reactivity with PMB were made using Boyer's method of following changes in optical density at 250 mμ. The experiments were conducted using protein concentrations of 0.5 μM and a tenfold excess of PMB to insure pseudo-first-order reaction kinetics. The apparent first-order rate constants observed were verified to be independent of protein concentration over a tenfold range of protein concentration (from 0.11 to 0.76 μM). Figure 2 shows a typical reaction profile observed. It can be seen, that, in contrast to the "all-or-none" reactivity of the sulfhydryl groups observed for aspartate transcarbamylase (Gerhart and Schachman, 1968), the sulfhydryl groups of the homoserine dehydrogenase–aspartokinase complex show at least two classes of reactive sulfhydryl groups. The first 6–8 sulfhydryl groups react more readily than do the remaining 10–12 which react. The rate of reaction of the faster reacting sulfhydryl groups proved to be sensitive to the threonine concentration of the medium, while the slower reacting sulfhydryl groups proved to be insensitive to threonine concentration. The rate of reaction of the more reactive sulfhydryl groups was studied as a function of threonine concentration. The lowering of the rate of reaction of these sulfhydryl groups with increasing threonine concentration is shown in Table II. It can be seen that a substantial difference in sulfhydryl group reactivity exists between fully inhibited and fully active states of the enzyme. Under the conditions chosen for this study, the enzyme resulting from these measurements is presumed to be desensitized, with a progressive loss of homoserine dehydrogenase activity resulting as more than 8–10 sulfhydryl groups reacted with PMB. Experiments conducted at lower PMB concentrations (threefold excess) and at saturating threonine concentrations, where the rate of reaction is slowest, permitted sampling of the reaction mixture for homoserine dehydrogenase assay when only small numbers of sulfhydryl groups had reacted. Under these conditions, after approximately 8–10 sulfhydryl groups had reacted, the homoserine dehydrogenase activity was desensitized. Further reaction of sulfhydryl groups resulted in almost complete loss of homoserine dehydrogenase activity.

Inhibition of Homoserine Dehydrogenase by Threonine. Figure 3 shows the inhibition of homoserine dehydrogenase activity in the assay buffer containing aspartate. The Hill coefficient of this inhibition curve is 3.4; the Hill coefficient is derived from the slope of a plot of ln $[(v - v_{uninhibd})/(V_0 - v_{uninhibd})]$ *vs.* ln $(1 + ([\text{threonine}]/k_T)$ (Janin and Cohen, 1969), where $v_{uninhibd}$ represents the 6.5% of homoserine dehydrogenase activity that remains uninhibited even at greater than saturating threonine concentrations, and k_T is the intrinsic dissociation constant for the threonine–enzyme complex. It should be noted that this 94% inhibition found under these assay conditions is substantially higher than the normal 80–85% inhibition found for saturating levels of threonine in the standard assay. This enhanced inhibition

TABLE I: Hydrogen Core Size *vs.* Threonine Concentration.

Thr Concn (mM)	Core Size
0	224
0.025	241
0.05	266
0.125	288
0.2	297
0.3	305
1.0	310

TABLE II: Pseudo-First-Order Rate Constant for PMB Reaction with Homoserine Dehydrogenase–Aspartokinase.

Thr Concn (mM)	k (sec^{-1})
2.2	0.0553
1.09	0.0673
0.218	0.077
0.109	0.12
0.0546	0.14
0.0364	0.188
0.0273	0.233
0	0.30

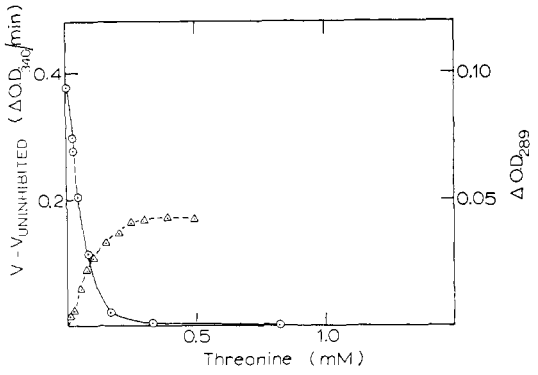

FIGURE 3: Homoserine dehydrogenase inhibition and ultraviolet difference spectral changes at 289 mμ. Homoserine dehydrogenase inhibition (left ordinate scale, ⊙) was assayed at 30° in the special assay buffer (*cf.* Methods). Ultraviolet difference measurements (right ordinate scale, △) were conducted in buffer D lacking EDTA, at a protein concentration of 11 μM.

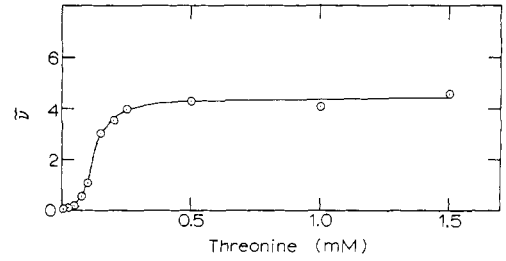

FIGURE 4: Threonine saturation of the enzyme. The number of moles of threonine bound per mole of enzyme, $\bar{\nu}$, was determined as a function of threonine concentration in the Paulus ultrafiltration cell. Experiments were conducted in buffer D, at protein concentrations of 0.9 mg/ml. (Temperature was 27°.)

probably results from a cumulative inhibition caused by both threonine and aspartate, since aspartate alone is a weak inhibitor of homoserine dehydrogenase activity. Since aspartate is known to be a noncompetitive inhibitor with respect to aspartic semialdehyde for both the native (Patte *et al.*, 1963) and dimeric (Wampler *et al.*, 1970) forms of the enzyme, the added inhibition is not due to aspartate competition with aspartic semialdehyde. It should also be noted that the point of 50% inhibition ($I_{0.5}$) is 50 μM in contrast to the usual value of 250 μM found at 0.6 M KCl; this behavior results from the fact that potassium ion and threonine are antagonists in shifting the allosteric equilibrium between the active and inhibited forms.

Ultraviolet Difference Changes at 289 mμ. Figure 3 also displays changes in ultraviolet difference spectra at 289 mμ resulting from titration with concentrated threonine solutions. It can be seen that the point of 50% change corresponds well to the $I_{0.5}$ for the homoserine dehydrogenase inhibition which is displayed on the same figure. The total change in absorbance at 289 mμ of 0.042 *A* unit/4-mg per ml concentration of enzyme is smaller than the value of 0.060 reported by Janin *et al.* (1969). This discrepancy may be due to the lower KCl concentration used (0.08 M *vs.* 0.15 M) or due to the presence of aspartate.

Threonine Binding Studies. Studies of threonine saturation of the enzyme were undertaken since there is no binding data available under the conditions of our state function determinations. Since both potassium ion and aspartate are threonine antagonists in shifting the allosteric equilibrium between active and inactive forms, direct binding data determinations are necessary to establish the exact saturation curve. Results obtained using the Paulus ultrafiltration device are presented in Figure 4. The Hill coefficient for threonine binding is 3.1 with a total of 4.8 threonine molecules bound per mole of enzyme. The intrinsic dissociation constant for the threonine-inhibited enzyme complex was determined to be 35 μM from Scatchard plots of the data.

Discussion

During the course of the work described above, considerable effort was devoted to making sure that the homoserine dehydrogenase–aspartokinase complex was in the native state at both the beginning and end of the longer term experiments. The studies of hydrogen exchange and sulfhydryl reactivity of the enzyme required leaving the enzyme for extended periods in buffers lacking threonine. In early experiments it was soon discovered that slow desensitization of the enzyme occurred despite the presence of KCl and dithiothreitol in phosphate buffer. For example, in earlier studies of PMB reaction with the enzyme in buffer D lacking aspartate, all 18 of the reactive sulfhydryl groups reacted with the same apparent rate constant when there was no threonine in the solution. Upon further investigation, it was discovered that this loss of differentiation between fast and slow sulfhydryl groups was due to desensitization of the enzyme, probably resulting from dissociation of the enzyme into dimers (Wampler *et al.*, 1970). To prevent occurrence of such adventitious effects, 2 mM potassium aspartate was added to the buffer. Aspartate in the presence of potassium ion appears to be a highly efficient protector of the native enzyme, perhaps fully as effective as threonine.

Figure 5 summarizes all the data obtained in this study and compares the fractional change in the various state functions with threonine binding data obtained with the ultrafiltration cell. It can be seen that there is good correlation for the various parameters used to monitor the conformation of the enzyme; in particular, the results from ultraviolet difference spectra agree quite well with the hydrogen-exchange and PMB data. Thus, the ultraviolet difference spectral changes appear to reflect changes representative of the whole

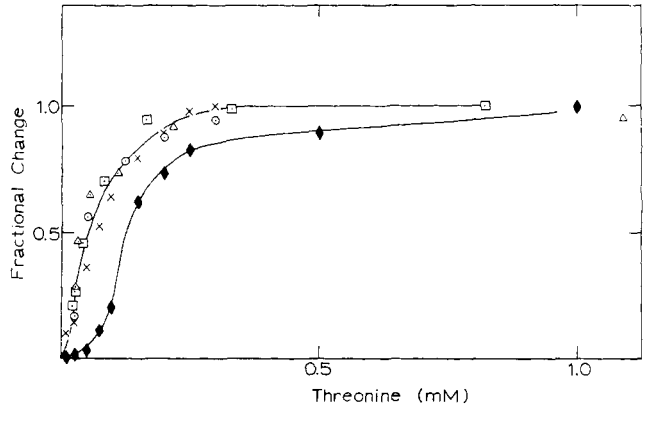

FIGURE 5: Percentage changes in saturation and state functions as a function of threonine concentration. Data of Figures 1–3 and Tables I–II have been converted to a fractional change basis. Legend: hydrogen exchange (⊙), homoserine dehydrogenase inhibition (⊡), PMB reactivity (△), ultraviolet difference changes at 289 mμ (×), and threonine binding studies (◆).

molecule. The direct binding data show that the changes in protein conformation do precede the saturation of the threonine binding sites. The latest results of Janin and Cohen (1969), using fluorescence as a measure of the state of the enzyme, are in accord with the variety of parameters we have measured here. Their previous data which showed the state and saturation functions superimposing were obtained at higher KCl concentrations. It appears that the lower potassium ion concentrations permit the separation of the two processes; this separation of the two processes occurs despite the presence of aspartate in the medium. It is a little surprising that such clear separation of state and saturation functions is seen under our conditions, since in 0.15 M KCl (less than a twofold increase in potassium ion concentration) the state function measured by ultraviolet difference spectra and the saturation function coincide (Janin *et al.*, 1969). *A priori*, it was expected that the presence of both 0.08 M KCl and 2 mM aspartate (both antagonistic effectors towards threonine) should, similarly, result in superposition of state and saturation functions. Our choice of KCl and aspartate concentrations was fortuitous in that the point of half-maximal change in the state function occurred at a threonine concentration nearly identical with the value of k_T, the intrinsic dissociation constant for the threonine-inhibited enzyme complex. Under our conditions, the apparent cooperativity of threonine binding is low enough to observe appreciable concentrations of inhibited forms of the enzyme which are not yet fully saturated with threonine. This result points out the importance of the choice of concentrations of antagonistic effectors (where possible) when attempting to discriminate between state and saturation functions. In our case, this means working at KCl and/or aspartate concentrations where the point of half-maximal change in the state functions is not $\gg k_T$. This choice of conditions may not in fact be necessarily identical with those for which one observes maximum cooperativity, *i.e.*, when the observed Hill coefficient observed is maximized (Rubin and Changeux, 1966).

Our data thus are compatible with the concerted Monod–Wyman–Changeux model (Monod *et al.*, 1965) for threonine-induced conformational changes in the homoserine dehydrogenase–aspartokinase complex but are not in accord with the simplest diagonal Koshland sequential model (Koshland *et al.*, 1966). Our conclusions are therefore in agreement with those of Janin and Cohen (1969). Plots of $[T/(1 - T)]^{1/n}$ *vs.* threonine concentration yield straight lines when $n = 3$ (state function data are derived from Figure 5, plotted according to Buc (1967), where T = fraction of enzyme in the inhibited form). Extrapolation to the abscissa gives an intrinsic threonine binding constant $k_T = 35 \pm 10$ μM. This value agrees with the binding constant $k_T = 35 \pm 15$ μM obtained from a Scatchard plot of the saturation data of Figure 5 (Changeux and Rubin, 1968) and the value of $k_T = 40$ μM obtained by Janin and Cohen (1969). Thus the data of Figure 5 are quantitatively in agreement with the concerted model since the Buc treatment is based upon the Monod–Wyman–Changeux model. However, both our results and those of Janin and Cohen do not rule out the possibility that the transition can be fitted by the more generalized Koshland models (Koshland *et al.*, 1966). Since the probes of enzyme conformation used in this study are grossly different from those used by Janin, we feel that the cumulative evidence is quite convincing that the changes seen reflect representative changes in the conformation of the enzyme and cannot be explained by localized masking effects due to threonine binding. The hydrogen-exchange core size should certainly reflect changes characteristic of the interior of the molecule and seems to be an especially suitable tool to investigate allosteric conformational changes. Since hydrogen core size is a measure of the relative motility of the molecule, one also obtains an idea of the relative "tautness" or looseness of the molecule. In addition, one obtains a rough quantitative estimate of the magnitude of the conformation change. Our estimate is that saturation of the enzyme by threonine results in a 40% increase in the number of tightly sequestered hydrogens. This number of core hydrogens, is, of course, very small relative to the total number of exchangeable hydrogens in the molecule and is low compared with the core size of many other proteins (Hvidt and Nielsen, 1966). This small "core" may reflect an unusually labile conformation, may be due to pH dependence of core size peculiar to this protein, or may result from the exchange-in procedure used. In order to stabilize the enzyme during exchange-in, we found it necessary to incubate in the presence of aspartate. The stabilizing effect of aspartate may also be manifest as a limitation in accessibility of core protons. One cannot therefore make definitive statements about the absolute size of the perturbation of the molecule that occurs.

Janin and Cohen (1969) have found that the inhibited form of homoserine dehydrogenase–aspartokinase is the state of higher enthalpy and entropy. Their results can be considered to be compatible with our hydrogen-exchange data which show that the threonine binding form (inhibited form) is the less motile state if one considers that the thermodynamic data include energetic contributions from the solvent. In particular, loss of structured water around hydrophobic groups buried as a result of threonine binding could be expected to decrease the overall entropy of the system. Consistent with this interpretation, our results show an increase in core size upon binding of threonine, indicating a tighter structure with the hydrophobic interior of the enzyme becoming less accessible to the solvent. This result agrees with the nature of the ultraviolet difference spectral changes which correspond to the transfer of tyrosine or tryptophan groups to a less polar environment when threonine is bound (Janin *et al.*, 1969), and also with the decreasing accessibility of the sulfhydryl groups.

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Conformational Changes in Rabbit Muscle Aldolase. Ultraviolet Spectroscopic Studies*

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ABSTRACT: The binding of D-arabinitol 1,5-diphosphate (Ara-P_2) to rabbit muscle aldolase (EC 4.1.2.13) produces a spectral change which indicates that both tryptophyl and tyrosyl residues are affected. The titration curves are not hyperbolic indicating the possibility of cooperativity in the binding process or differences between sites. Tryptophan appears to be present in, or close to, the binding site. The temperature-induced difference spectrum of aldolase does not vary in intensity linearly with temperature over the range 0–50° supporting the view that the enzyme can undergo a temperature-induced conformational change. The temperature-induced difference spectra of a number of model compounds have been examined and shown to vary in intensity linearly with temperature. Ara-P_2 binding does not stabilize the enzyme against heat denaturation. Ara-P_2 binding does affect the difference spectrum produced by lowering the temperature indicating that binding may produce a similar conformation at all temperatures.

The effect of temperature on K_m and V_{max} for the cleavage of fructose 1,6-diphosphate (Fru-P_2[1]) by rabbit muscle aldolase and on K_I for the binding of D-arabinitol 1,5-diphosphate (Ara-P_2) by the enzyme has been described previously (Lehrer and Barker, 1970). Van't Hoff and Arrhenius plots of the binding and velocity data all showed curvature and were interpreted as indicating the existence of at least two active forms of the enzyme which would be present in equal amounts at approximately 28°, the transition temperature for their interconversion. It was not possible to determine the enthalpy of interconversion of the two forms of the enzyme, and appreciable quantities of both forms of the enzyme may be present over the temperature range of interest (4–45°).

The aldolase reaction can be thought of as consisting of three steps, binding, catalysis, and unbinding. The thermodynamics of the binding and catalysis steps were obtained, and it was found that the binding step was most strongly influenced by temperature. Below the transition temperature the thermodynamic parameters for binding were $\Delta G° = -7.2$ kcal mole^{-1}, $\Delta H° = 11.5$ kcal mole^{-1}, $\Delta S° = +63.8$ eu. Above the transition temperature these values were $\Delta G° = -7.4$ kcal mole^{-1}, $\Delta H° = -13.8$ kcal mole^{-1}, and $\Delta S° = -20.9$ eu.[2] Activation parameters were not so dramatically affected. These findings led to the proposal that the enzyme undergoes a conformational change as the temperature is varied, but that when the substrate is bound the same catalytically active form is induced (Scheme I). If a conformational change is involved it might be sufficiently large to result in the perturbation of tyrosine or tryptophan chromophores.

Alternatively, the observed changes in ΔH with temperatvre can be explained on the basis of a large and constant difference in heat capacity (ΔC_p) between the enzyme and the enzyme–substrate (or inhibitor) complex. In this case, ΔH will be zero at some temperature and will be of different sign above and below that temperature (Glasstone, 1946). This explanation does not require a conformational change in the protein at the temperature where ΔH is zero and the temperature perturbation of the enzyme would be linear (Bello, 1969).

In an attempt to distinguish between these possibilities, the temperature difference spectra of aldolase and of some model compounds were obtained, and the effect of binding Ara-P_2 on the temperature-induced difference spectrum of aldolase was determined. It is important to ascertain whether transitions in ΔH and ΔS for enzymatic processes are due to conformational changes or to heat capacity differences, since, in some cases, these changes are important to the function of the organism (Somero and Hotchachka, 1968)

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[1] Abbreviations used are: Fru-P_2 = D-fructose 1,6-diphosphate; Ara-P_2 = D-arabinitol 1,5-diphosphate.

[2] In the original publication errors were made in the signs for ΔS which are corrected here.