# Threonine Inhibition of the Aspartokinase–Homoserine Dehydrogenase I of *Escherichia coli*. Threonine Binding Studies†

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ABSTRACT: Both activities of the aspartokinase–homoserine I (AK-HSD) of *Escherichia coli* are inhibited by threonine. Careful threonine binding studies have now been done which have allowed us to distinguish the various effects of threonine on the enzyme. The ultrafiltration technique of H. Paulus ((1969) *Anal. Biochem. 32,* 101) for measuring ligand binding was shown to be comparable with equilibrium dialysis techniques. Reduction in error by utilization of this procedure enabled us to obtain evidence for two different sets of threonine sites by direct binding studies. The binding data were mathematically consistent with two independent classes of threonine sites, each of which contained four sites per tetramer and had a Hill coefficient of about 2.3–2.5. $K_D$ for the second set of sites was five- to tenfold greater than the high affinity sites, depending upon conditions. The data now suggest that the sequential model for site–site interactions adequately describes the cooperativity of threonine binding to the high affinity set of sites.

Aspartokinase–homoserine dehydrogenase I from *Escherichia coli* catalyzes the first and third steps in the biosynthetic pathway from aspartate to the amino acids threonine, isoleucine, and methionine (Truffa-Bachi et al., 1974b). The aspartokinase I and homoserine dehydrogenase I activities are known to occur on the same polypeptide chain (Patte et al., 1966; Veron et al., 1972). L-Threonine is a feedback inhibitor of both activities of aspartokinase–homoserine dehydrogenase I (AK–HSD)[1] of *E. coli* (Truffa-Bachi et al., 1974a). This ligand has several measurable effects on the enzyme: threonine (a) produces a steady-state inhibition of both activities with homotropic kinetic cooperativity under some assay conditions (Patte et al., 1963; Cohen & Truffa-Bachi, 1965), (b) changes several state functions of the enzyme (Janin & Iwatsubo, 1969; Janin & Cohen, 1969; Truffa-Bachi et al., 1968), (c) protects against inactivation of both activities by several agents (Veron et al., 1972, 1973; Mackall & Neet, 1974; Takahashi & Westhead, 1971; Truffa-Bachi et al., 1968), (d) stabilizes the tetrameric form of the enzyme (Cunningham et al., 1968; Mackall & Neet, 1973), and (e) exhibits a time dependency for onset of homoserine dehydrogenase inhibition (Barber & Bright, 1968). The relationship between threonine binding and these effects is not yet clear.

In buffers containing high potassium ion concentrations, threonine inhibition displays kinetic homotropic positive cooperativity (Patte et al., 1966) with a Hill coefficient of about 4 for the aspartokinase activity (Patte et al., 1966; Heck, 1972; Wampler & Westhead, 1968) and about 2 to 3 for the homoserine dehydrogenase activity (Patte et al., 1966; Barber & Bright, 1968; Takahashi & Westhead, 1971). Direct binding studies have shown maximal binding of 8 mol of threonine/tetramer, or two per subunit (Janin et al., 1969; Veron et al., 1973; Janin & Cohen, 1969). The binding of threonine under conditions of high potassium ion concentration is also cooperative with a Hill coefficient of 2.3 (Janin et al., 1969; Heck, 1972). These experiments had suggested that the threonine binding sites were all identical, but recent evidence has suggested that there are two classes of binding sites for threonine (Ehrlich & Takahashi, 1973; Tilak et al., 1976; Wright & Takahashi, 1977a,b; Ryzewski & Takahashi, 1975). We have investigated the binding of threonine to the native enzyme in order to establish the mechanism of inhibition by bound threonine, and to determine the extent to which binding cooperativity contributes to kinetic cooperativity.

## Experimental Section

### *Materials*

Ammonium sulfate UltraPure was purchased from Schwarz/Mann. Choline chloride was the product of Matheson, Coleman and Bell. EDTA, dipotassium salt was obtained from Eastman Kodak. Sephadex A-50 was the product of Pharmacia. Ozone was generated by a Weisbach Ozonator.

Purity of [$^{14}$C]threonine (230 mCi/mmol) was checked by periodate oxidation followed by paper chromatography in an l-butanol:acetic acid:water (2:1:1) system. The purity of [$^{14}$C]threonine obtained from Amersham/Searle was better than that from other commercial sources and was used for all ultrafiltration studies. Aqueous counting scintillant was also obtained from Amersham/Searle. Visking membranes were obtained from Union Carbide. All other reagents were obtained from standard sources at the highest purity available.

### *Methods*

*Enzyme Purification.* AK–HSD I was purified from *E. coli* K12 TIR8, a thiaisoleucine-resist mutant derepressed for the enzyme. The procedure was a slightly modified version of the preparation by Ogilvie et al. (1969). Enzyme with HSD (forward) specific activity of 50 at 27 °C was used for equilibrium dialysis studies and preliminary ultrafiltration experiments. All other work was carried out using enzyme with a specific

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[1] Abbreviations used: AK, aspartokinase; AK–HSD, aspartokinase–homoserine dehydrogenase; ASA, aspartic β-semialdehyde; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; HS, homoserine; HSD, homoserine dehydrogenase; PMB, *p*-mercuribenzoate.

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activity of 47 at 27 °C. Enzyme activity and threonine sensitivity were found to be stable at room temperature for 24 h.

*Buffers.* Buffer F: 0.06 M potassium phosphate, 3.0 mM potassium EDTA, 0.5 mM DTT, and 0.01 M L-threonine, pH 7.6. Buffer B: 0.06 M potassium phosphate, 3.0 mM potassium EDTA, 0.5 mM DTT, 0.15 M KCl, pH 7.6. Buffer A: 0.12 M Tris, 0.6 M KCl, 10 mM potassium L-aspartate, 3 mM $MgCl_2$, and 1 mM DTT, pH 7.4.

*Protein Assay.* Protein concentration was measured at 278 nm using the extinction coefficient of 0.63 $cm^2/mg$ found by Falcoz-Kelly et al. (1972).

*Enzyme Assays.* One unit of enzyme is the amount of enzyme which uses 1 μmol of substrate per min or which produces 1 μmol of product per min. Three different activities can be measured for AK-HSD I: HSD in the reverse direction, HSD in the forward direction, and aspartokinase (AK) activity.

Activities are expressed in units of the forward HSD activity unless otherwise noted. The reverse HSD assay contained: 0.1 M Tris-HCl (pH 8.9), 0.025 M homoserine, 0.33 mM $NADP^+$, and 0.4 M KCl. Activity was measured as the absorbance increase at 340 nm due to $NADP^+$ reduction. Threonine sensitivity was measured by addition of 0.01 M threonine to the assay mixture. The reaction in the forward direction was measured by the absorbance decrease at 340 nm as NADPH becomes oxidized. The assay mix contained 4.0 mM aspartic β-semialdehyde (ASA) and 0.3 mM NADPH in buffer B.

ASA was synthesized using the method of Black & Wright (1955). Ozone was bubbled through a mixture of allylglycine and 1 N HCl and ASA purified on a column of Dowex 50W, $H^+$ form. The concentration of ASA was measured by the total $A_{340}$ change of the forward reaction mix when the amount of ASA added was limiting.

*Polyacrylamide Electrophoresis.* Purity of AK-HSD I was checked by polyacrylamide electrophoresis on 7.5% acrylamide, pH 8.3 gels (Davis, 1964), and stained with 35% perchloric acid–0.04% Coomassie Blue (G250) (Reisner et al., 1975). L-Threonine was included in both gels and running buffer to stabilize the tetramer under conditions of electrophoresis (Truffa-Bachi et al., 1968). Gels overloaded with 100 μg of AK-HSD I (sp act. 50) only showed minor impurities and the enzyme was considered to be adequately pure (>98%) for binding studies.

*Equilibrium Dialysis.* Measurements of threonine binding to AK-HSD I by equilibrium dialysis were done in 60-μL Lucite cells with boiled dialysis tubing. Enzyme was centrifuged, resuspended in buffer B, and dialyzed against buffer B to remove threonine. Twenty-five microliter of protein at about 5 mg/mL was placed in one side of Lucite dialysis cells. Twenty-five-microliter aliquots of the appropriate dilution of [$^{14}$C]threonine in buffer B containing 0.02 μCi were added to the other side of the cells; equilibrium was reached after 16 h. Two 10-μL samples were removed from each side of the cell for counting along with two 10-μL samples from the corresponding threonine solution. Concentration of threonine bound was calculated from the difference between dpm in experimental and blank cells and from the specific radioactivity of the corresponding threonine solution.

*Ultrafiltration.* Ultrafiltration binding studies by the method of Paulus (1969) were performed using a 22-channel Lucite apparatus made by John Hoegler of Case Western Reserve University. Visking membranes were prepared by boiling in 1 mM EDTA and were stored in 0.1 mM EDTA at 4 °C. Enzyme was centrifuged, dissolved in buffer B, and run through a small G-25 fine column to remove ammonium sulfate and threonine. Aliquots of enzyme were added to [$^{14}$C]-threonine solutions of known concentration to a protein concentration of approximately 2 mg/mL. Two 5-μL samples were taken from each solution to determine the specific activity of the threonine. For each threonine concentration of the ultrafiltration apparatus, one or two blanks and four experimental channels were filled with 100 μL of the appropriate solution. Nitrogen gas was applied at 30 psi for 2.5 h until filtration was complete. The bottoms of the membranes were rinsed twice each with 5 mL of ethylene glycol and removed for scintillation counting. The following formula was used to convert DPM to moles of threonine bound per mole of enzyme, $\bar{\nu}$:

$$\bar{\nu} = (M_w)10^4\left(\frac{0.63}{A_{278}}\right)\left(\frac{DPM_{exp}}{sa_{exp}} - \frac{DPM_{blank}}{sa_{blank}}\right)$$

where $M_w$ is the molecular weight of AK-HSDI, $A_{278}$ is the absorbance at 278 nm, 0.63 is the extinction coefficient, $DPM_{exp}$ and $DPM_{blank}$ are the means of the calculated DPM for enzyme and blank solutions respectively, and $sa_{exp}$ and $sa_{blank}$ are the calculated specific radioactivities.

*Scintillation Counting.* Samples from equilibrium dialysis experiments were placed in scintillation vials and 4 mL of aqueous counting scintillant (ACS) was added. Membranes and 5-μL samples from ultrafiltration studies were placed in scintillation vials containing 1.0 mL of distilled water. Membranes were allowed to soak in water overnight. Subsequently, 8 mL of ACS was added to each vial. All counting was done on a Nuclear Chicago Mark I scintillation counter using an external standard ratio quench curve to calculate the DPM of each sample. Counting efficiencies were always greater than 70%.

*Data Analysis.* Because of the complexities of the binding curves, the data were analyzed in several ways for cooperativity. (1) Hill plots were drawn assuming 8 mol of threonine are bound per mol of AK-HSDI. (2) With the assumption of two independent sites for threonine, Hill coefficients were calculated for $n = 4$ (where $n$ is the maximum number of moles of threonine bound to one site per mole of tetramer). All binding data where less than 4 mol of threonine was bound per mol of enzyme were used for calculation of $n_b$, the binding Hill coefficient, for the higher affinity sites. Binding data greater than 4 mol of threonine per mol of enzyme were used to calculate $n_b$ for the lower affinity sites from $\log (4 - (\bar{\nu} - 4))/(\bar{\nu} - 4)$, where $\bar{\nu}$ is moles bound per mole. (3) Hill coefficients were calculated by curve fitting with a sum of two Monod-symmetry model equations (Monod et al., 1965) using the 9100A Hewlett-Packard calculator. The initial portion of the binding curves were fit to the following equation:

$$\bar{Y}_f = \frac{LC\alpha(1 + C\alpha)^{n-1} + \alpha(1 + \alpha)^{n-1}}{L(1 + C\alpha)^n + (1 + \alpha)^n}$$

where $L$ is the ratio of the concentrations of the T state of the enzyme to the R state in the absence of threonine; $C$ is the ratio of the dissociation constant of the R state, $K_R$, to the T state, $K_T$; $\alpha$ is the concentration of threonine divided by $K_T$; $n$ is the number of homologous sites; and $\bar{Y}_f$ is the fractional saturation. Values of $K_R$, $K_T$, $L$, and $n$ were varied to obtain a reasonable fit. The curve described by these parameters was then subtracted from the rest of the data. These subtracted values were fit to a curve using the same equation as described above. The sum of the two saturation functions was then plotted to determine the fit to the whole binding curve. Each equation was then plotted as $\log (1 - \bar{Y}_f)/\bar{Y}_f$ vs. log threonine to determine the separate Hill coefficients.

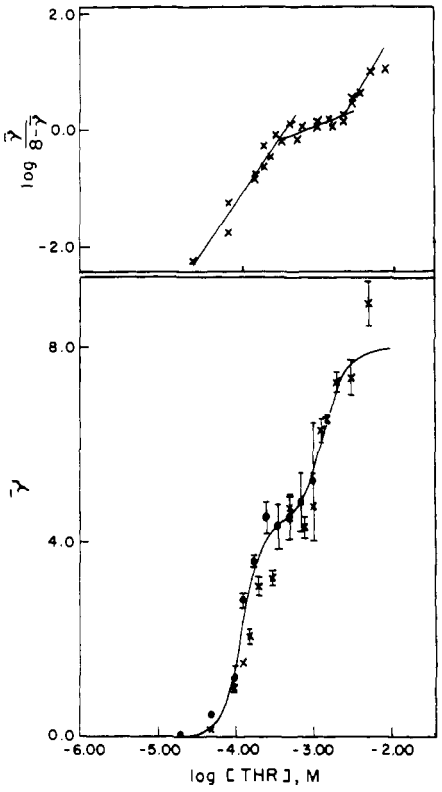


FIGURE 1: (A, Bottom) Binding of L-[$^{14}$C]threonine to AK-HSDI in buffer B, pH 7.2 and 20 °C. (X) Ultrafiltration; (●) equilibrium dialysis. Vertical bars indicate the standard deviation of three to five replicate points. The solid line is the sum of two Monod models fitted to the data. First fit: $K_R = 0.02$, $K_T = 3.5 \times 10^{-5}$, $L = 10^{-3}$, and $n = 4.75$. Second fit: $K_R = 2.0$, $K_T = 4.5 \times 10^{-5}$, $L = 10^{-5}$, and $n = 3.25$. (B, Top) Hill plot of data in A. Solid lines are tangents to the data at points of maximum or minimum slope.

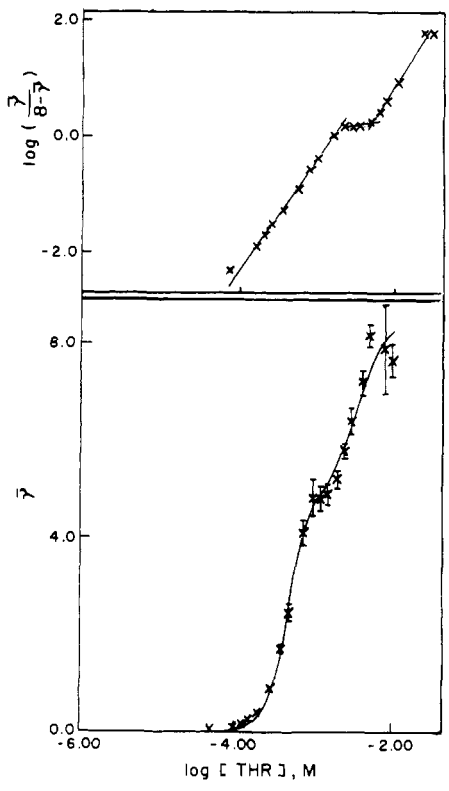


FIGURE 2: (A, Bottom) Binding of L-[$^{14}$C]threonine to AK-HSDI in buffer A, pH 7.4 and 20 °C. Vertical bars indicate the standard deviation of 3 to 5 replicate points. Solid line is the sum of two Monod equations fitted to the data. First fit: $K_R = 0.2$, $K_T = 1.5 \times 10^{-4}$, $L = 10^{-3}$, and $n = 5.0$. Second fit: $K_R = 2.0$, $K_T = 1 \times 10^{-4}$, $L = 10^{-4}$, and $n = 3.25$. (B, Top) Hill plot of data in A. Solid lines are tangents to the data at points of maximum or minimum slope.

## Results

*Equilibrium Dialysis.* The results of equilibrium dialysis in buffer B, pH 7.2, 20 °C, are shown in solid circles in Figure 1A. Protein concentrations were 4–7 mg/mL. Values obtained at low concentrations of threonine have low standard deviations. At concentrations of threonine greater than $5 \times 10^{-4}$ M, much larger errors were observed.

*Ultrafiltration.* Results of ultrafiltration binding experiments with L-[$^{14}$C]threonine in buffer B, pH 7.2, and 20 °C are also shown in Figure 1A. Saturation of the binding sites appeared to be reached in the 3 mM range where about 8 mol of threonine are bound per mol of AK-HSDI. The data at lower concentrations (Figure 1A) show excellent agreement with that obtained using equilibrium dialysis. The error in equilibrium dialysis measurements increases with increasing threonine concentration, while the error remains essentially constant for ultrafiltration determinations. Data obtained by ultrafiltration also show a plateau region at about $2.5 \times 10^{-4}$ M threonine, confirming the observation made by equilibrium dialysis. A Hill plot of these data (Figure 1B) shows positive, negative, and positive cooperativity with Hill coefficients and $K_D$ values of 2.2 and 0.22 mM, 0.44 and 0.32 mM, and 2.53 and 0.80 mM. The plateau region was observed under all conditions for binding, including buffer A (Figure 2). Hill values and $K_D$ in this Tris buffer are 2.2 and 0.74 mM, 0.50 and 0.58 mM, and 1.5 and 1.27 mM (Figure 2).

*Analysis of Binding Data.* Because of the complex shape of the binding curves (Figures 1 and 2) and the correspondingly poor fit to a single binding function, the binding data were analyzed as the sum of binding to two separate, distinct sets of sites (see also Discussion). Thus, Hill values were computed for values of $\bar{\nu}$ less than 4 and for values of $\bar{\nu}$ greater than 4 with moles bound per mole, $\bar{\nu}$, equal to $\bar{\nu} - 4$. One hundred percent saturation was assumed at 8 threonine mol bound per mol of enzyme as established elsewhere (Janin et al., 1969; Veron et al., 1973; Janin & Cohen, 1969). Although this value cannot be derived directly from the data presented here, our data are consistent with $n = 8$. Maximal slopes were derived from plots of $\log((4 - \bar{\nu})/\bar{\nu})$ vs. log[threonine]. $K_D$ values were calculated as the $x$ intercept of the tangent to the maximum slope. Results are summarized in Table I for several experimental conditions.

For $\bar{\nu} < 4$, the binding Hill coefficients, $n_b$, appear to be relatively independent of experimental conditions. For temperature changes from 20 to 37 °C, $n_b$ decreases from 2.6 to 2.1, while the $K_D$ increases from 0.15 mM to 0.21 mM. Due to the errors in determination, it is doubtful whether these changes are indicative of a trend. These changes, however, are the largest observed under the conditions studied. For changes in pH and addition of neutral salt or ATP the Hill coefficient does not change significantly from 2.7 ± 0.2. The $K_D$ values in buffer B are also all within experimental error (±0.05) of 0.16 mM. It is interesting to note that, in buffer A, which is a Tris buffer containing 0.6 M KCl, the Hill coefficient is the same as measured in buffer B, 2.5, but the $K_D$ has increased to 0.45 mM. This value of $K_D$ is significantly increased over the values of $K_D$, 0.15 mM, observed in buffer B.

For $\bar{\nu} > 4$ the values of $K_D$ are 4 to 11 times larger than the corresponding values of $K_D$ for the high affinity site. The experimental conditions appear to have more of an effect on the Hill coefficient for $\bar{\nu} > 4$, although the experimental uncertainty is somewhat greater. Addition of 0.1 M choline chloride

TABLE I: Binding Parameters for High and Low Affinity Threonine Binding Sites.[a]

| pH | temp (°C) | additions[b] | $\nu < 4$ $n_b$ (± 0.2)[c] | $\nu < 4$ $K_D$ (mM) (± 0.05)[f] | $\nu > 4$ $n_b$ (± 0.2)[c] | $\nu > 4$ $K_D$ (mM) (± 0.05)[c] |
|---|---|---|---|---|---|---|
| 6.9 | 20 | | 2.6 | 0.15 | 2.4[d] | 1.2[e] |
| 6.9 | 24 | | 2.6 | 0.12 | | |
| 6.9 | 37 | | 2.1 | 0.21 | | |
| 7.2 | 20 | | 2.7 | 0.12 | 2.7 | 1.3 |
| 7.6 | 20 | | 2.5 | 0.17 | | |
| 7.2 | 20 | 0.1 M choline Cl | 2.6 | 0.16 | 2.2 | 0.65 |
| 7.2 | 20 | 0.8 M choline Cl | 2.9 | 0.17 | | |
| 7.2 | 20 | 0.25 mM ATP[f] | 2.5 | 0.16 | 1.2[d] | 0.8 |
| 7.2 | 20 | 0.50 mM ATP[f] | 2.9 | 0.15 | | |
| 7.4 | 20 | [g] | 2.5 | 0.45 | 2.6 | 2.6 |

[a] Data were analyzed by constructing Hill plots for the first four binding sites ($\nu < 4$) separately from the second four sites ($4 < \nu < 8$); see Methods. [b] Buffer B: 0.06 M potassium phosphate, 3 mM EDTA, 0.5 mM DTT, 0.15 M KCl (unless otherwise noted). [c] Largest standard deviation, unless noted in footnotes *d* and *e*. [d] ± 0.8. [e] ± 0.2. [f] Equimolar addition of $MgCl_2$ and $Na_2ATP$. [g] Measured in buffer A: 0.12 M Tris, 1 mM DTT, 0.6 M KCl, 3 mM $MgCl_2$, 10 mM aspartate.

decreases $n_b$ from 2.7 to 2.2, with a decrease in $K_D$ from 1.30 mM to 0.65 mM. Addition of 0.25 mM ATP also reduces the Hill number with a concomitant decrease in the $K_D$ value from 1.30 to 0.78 mM. Changes in $n_b$ and $K_D$ with respect to pH are difficult to assess due to the large error in determination of these parameters at pH 6.9 and 20 °C. As is consistent with the results of the higher affinity site, the cooperativity of threonine binding remains within the same range when measured in buffer A. However, the $K_D$ is increased to 2.6 mM.

*Curve Fitting.* The binding curves were fit to a sum of two Monod models as described under Methods. Examples of curve fitting are shown as the solid line in Figures 1 and 2. Reasonable fits were obtained with several sets of values for $K_R$, $K_T$, $L$, and $n$. Ranges for the Hill coefficients and $K_D$ values assuming two independent sites were determined (see paragraph concerning supplementary material at the end of this paper). These values agreed well with those determined from Hill plots in Table I.

## Discussion

*Comparison of Equilibrium Dialysis vs. Ultrafiltration.* The ultrafiltration technique of Paulus (1969) proved very useful in the study of threonine binding to AK-HSDI because of its speed and accuracy. Equilibrium dialysis experiments used solutions of high protein concentrations, 4-7 mg/mL. Because desalting by G-25 chromatography causes some dilution, ammonium sulfate and threonine from the storage buffer had to be removed by dialysis. Preliminary experiments showed that 6 h was required for complete removal of threonine. After the Lucite cells were filled, it took an additional 16 h for the two compartments to equilibrate and therefore at least 22 h was required to obtain binding data. Another major drawback of equilibrium dialysis was the low reproducibility of $\bar{\nu}$ at high threonine concentrations. Even at enzyme concentration of 7 mg/mL, the difference in threonine concentration across the dialysis membrane was only 3% at $5 \times 10^{-3}$ M.

Ultrafiltration had already been shown to be a viable technique for measuring threonine binding to AK-HSDI under some experimental conditions (Takahashi & Westhead, 1971). For ultrafiltration, protein concentrations were on the order of 2 mg/mL. Therefore, ammonium sulfate and threonine could be rapidly removed by G-25 chromatography and the protein concentration was still high enough for use. The accuracy at high threonine concentrations for the ultrafilter is much higher than equilibrium dialysis. Blank values showed 0.2% retention of sample by membrane alone. If enzyme is saturated at a threonine concentration of $5 \times 10^{-3}$ M, 1% of the threonine will be bound at an enzyme concentration of 2 mg/mL. This will give a fivefold difference between blank and experimental DPM on membrane. By maintaining a high specific activity of threonine, highly significant values were obtained at high threonine concentrations. The good agreement in values obtained by equilibrium dialysis and ultrafiltration for AK-HSDI (Figure 1) shows that any surface phenomena occurring during ultrafiltration were not significantly affecting the binding of threonine. Therefore, ultrafiltration became the method of choice for threonine binding measurements.

Because of these improvements in technique we have been able to obtain binding data up to high threonine concentrations with much smaller standard errors than previous reports (Janin et al., 1969; Heck, 1972; Veron et al., 1973; Takahashi & Westhead, 1971; Janin & Cohen, 1969). These more precise binding curves have allowed definition of a "bump" in the curves and the separation of two types of threonine binding sites.

*Two Threonine Binding Sites.* The ultrafiltration binding data were interpreted as the sum of two threonine binding sites for the following reasons: (a) the plateau region (bump) was consistently observed under all experimental conditions; (b) Hill plots demonstrated two breaks in the curve; (c) the $K_D$ of the higher affinity site was comparable to the $K_I$ of the HSD activity (Bearer & Neet, 1978a), (d) the saturation of the lower affinity site paralleled the total inhibition of the AK steady-state activity (Bearer & Neet, 1978b), and (e) recent findings in the literature (Wright & Takahasi, 1977; Wright et al., 1976a,b) also distinguish two separate classes of threonine binding sites.

*The Mechanism of Cooperativity of Threonine Binding.* The sum of two Monod models fit separately to the two sites agreed well with all binding data. Examples are shown in Figures 1 and 2. The Monod concerted model was used to fit the data because of its extensive use in the literature with this enzyme (Heck, 1972; Takahashi & Westhead, 1971; Janin & Iwatsubo, 1969; Janin & Cohen, 1969; Janin, 1972). Although this model agrees well with the data, it does not constitute proof that AK-HSDI follows a concerted model. It does provide further support to the existence of two separate, distinct threonine binding sites per monomer. Reinterpretation of data in terms of these two sets of threonine sites removes the primary argument against a sequential model (Koshland et al., 1966) for cooperativity of threonine binding to HSD, namely, that

the R to T transition appears to be complete before the enzyme is saturated with threonine (Heck, 1972; Takahashi & Westhead, 1971; Janin & Cohen, 1969). The R to T transition has been measured by several methods: HSD inhibition, hydrogen exchange, mercuribenzoate reactivity, ultraviolet spectra difference changes, and protein fluorescence below 280 nm. For several of these measurements, our new interpretation would be that only the saturation of the first four threonine binding sites should be compared with the R to T transition for the following reasons: (a) Wright & Takahashi (1977a) discovered that the protein fluorescence of their AK-Co(III)-Thr adduct was very similar to native AK-HSDI. On addition of free threonine, the protein fluorescence changed as expected for the R to T transition along with inhibition of the HSD activity. Thus, the change in protein fluorescence emission appears to be associated only with the four threonine sites regulating the HSD activity. Further evidence for this result was found on the addition of 3-hydroxypipecolic acid (3-OH-PIP) to native AK-HSDI (Tilak et al., 1977). (b) That inhibition of the AK activity cannot be followed by changes in fluorescence emission may be due to the centralized location of the AK domain in the quaternary structure of AK-HSDI (Veron et al., 1973; Mackall & Neet, 1974). This location may also prevent hydrogen exchange of the AK domain. It is possible then that hydrogen exchange also measures only conformational changes in the HSD domain and is thus a measure of only the first 4 molecules of threonine binding. (c) PMB has been shown to have no effect on the AK threonine sensitivity (Truffa-Bachi et al., 1966). PMB reactivity, then, may also only reflect changes in the HSD domain due to the higher affinity threonine sites. (d) Evidence has been presented that only the first 4 molecules of threonine bound to enzyme inhibit the HSD activity (Bearer & Neet, 1978a).

We have reviewed the data in the literature which compare these conformational parameters with threonine binding where 100% maximum effect is correlated to 8 mol of threonine bound per mol of AK-HSDI. The recalculation of these data to correlate 100% effect with 4 mol of threonine binding per mol of AK-HSDI reveals the interesting fact that threonine binding correlates well with the R to T transition; i.e., the state function closely follows the binding function. If this interpretation is correct, then the sequential model for the cooperativity of the binding of threonine to AK-HSDI is more compatible with the experimental results.

## Acknowledgments

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## Supplementary Material Available

A table (Table II) provides the fitted parameters of $n$ and $K_D$ from the best Monod fit to the high and low affinity threonine binding sites under ten experimental conditions (1 page). Ordering information is given on any current masthead page.

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