Threonine Inhibition of the Aspartokinase–Homoserine Dehydrogenase I of *Escherichia coli*. Stopped-Flow Kinetics and the Cooperativity of Inhibition of the Homoserine Dehydrogenase Activity†

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ABSTRACT: Threonine interactions with the threonine-sensitive aspartokinase–homoserine dehydrogenase I (AK–HSD) from *Escherichia coli* K12 have been investigated by studying the relaxation times from the uninhibited to the inhibited form of the enzyme and the kinetic inhibition of the steady state activity. Comparison of changes in the relaxation time, changes in the cooperativity of the inhibition, and changes in binding cooperativity have been made under a range of comparable conditions. The progress curve lag in threonine inhibition of the HSD activity was studied by stopped-flow rapid kinetic techniques as a function of pH, temperature, ionic strength, and ATP concentration. The protein concentration independence and the activation energy of the apparent first-order rate constant for the lag, k_1, demonstrated its identity to the R to T isomerization previously studied. Observed relaxation times ranged from 10 ms to 1.5 s. Limiting values of the relaxation time, τ_{min}, and the threonine concentration at half the maximal rate of transition, K_k, were obtained from analysis of double-reciprocal plots of k_1 vs. threonine concentration. τ_{min} was substantially decreased with increasing temperature. Other changes in experimental conditions resulted in smaller decreases in τ_{min}. Kinetic Hill coefficients obtained from standard spectrophotometric procedures were unaffected by changes in temperature, remaining constant at about 2.2. A small increase of the Hill coefficient with increasing pH and a small decrease on addition of 0.8 M choline chloride were observed. Lack of correlation of changes in τ_{min} and n_H ruled out a kinetic contribution of the lag of inhibition to the cooperativity observed for threonine inhibition of the HSD activity. A comparison of K_I, the threonine inhibition constant, and K_k, the threonine concentration at half the maximal rate of transition, revealed that no correlation existed between these two parameters, and that the value of K_I is an order of magnitude lower than that of K_k. Values of the Hill coefficient and dissociation constant for binding to the high affinity site under various conditions were equivalent to the corresponding values for inhibition of the HSD steady-state kinetics. The high affinity sites are proposed to be responsible for HSD inhibition and the kinetic cooperativity for this activity is solely a reflection of the binding cooperativity. We conclude that the cooperative inhibition of HSD activity by threonine is best described by rapid equilibrium binding of threonine to the high affinity site with subsequent site–site interactions.

The presence of two distinct, separate sites of threonine binding to aspartokinase–homoserine dehydrogenase I (AK–HSD)[1] of *E. coli* (Bearer & Neet, 1978) has raised the question of the role of each in the inhibition of homoserine dehydrogenase activity, and the mechanism by which inhibition occurs. Thus, a study of the initial and steady-state kinetics of the HSD inhibition was undertaken. The characteristics of the steady-state inhibition of HSD by threonine vary according to direction and conditions of assay. Under forward assay conditions (aspartic β-semialdehyde to homoserine) threonine inhibition is noncompetitive vs. aspartic β-semialdehyde (Patte et al., 1963). When studied at high pH (8.5) and low potassium (5 mM), no cooperativity is observed (Truffa-Bachi et al., 1974). At lower pH and in the presence of 0.15 M or higher potassium concentration or 2 mM aspartate, kinetic homotropic cooperativity is obtained with threonine. Hill coefficients vary from 2 to 3.4 depending on conditions of assay (Patte et al., 1966; Barber & Bright, 1968; Takahashi & Westhead, 1971).

Progress curve transients are characteristic of the time course of inhibition. These transients were first observed by Barber & Bright (1968) while viewing early reaction times of HSD on a stopped-flow apparatus. Upon rapid mixing of threonine with AK–HSDI, initial lags in onset of threonine inhibition occurred. The observed rate constants for the lag times were independent of protein concentration of threonine. The authors concluded that the most likely explanation for this effect was a reversible isomerization of active to inactive enzyme with distant site–site interactions. By examining spectrofluorometric changes upon rapid addition of threonine, Janin & Iwatsubo (1969) were able to find a relaxation of the protein on the same order of time as the stopped-flow kinetics. Two states of the enzyme were defined by this data—an R state with low affinity for threonine and a T state with high affinity for threonine. Therefore, the transients seen by both kinetic analysis and spectroscopy were postulated to be due to a redistribution of enzyme between the R and T states (Janin & Iwatsubo, 1969).

This paper describes experiments to examine the relationship between these two effects of threonine on HSD, the cooperative steady-state inhibition and the progress curve transients, and to correlate them with the two classes of threonine binding sites. The values of the kinetic Hill coefficient, n_H, and τ_{min},

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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.

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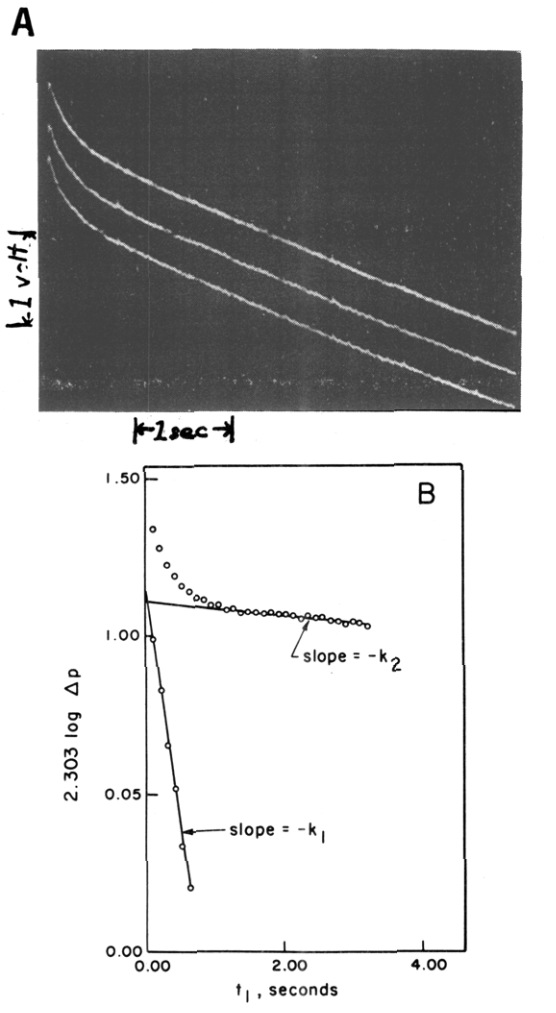

FIGURE 1: (A) Typical photograph of stopped-flow oscilloscope with three separate progress curves. Assay conditions were 10 mM THR, pH 7.2, 20 °C in buffer B. Photograph taken at 0.5 V/vertical division and 0.5 s/horizontal division. (B) Guggenheim treatment of progress curve as described in Methods. The slopes correspond to a k_1 of 3.7 s^{-1} and k_2 of 0.06 s^{-1} for 4.0 mM [THR], pH 7.2, 20 °C in buffer B.

the limiting value of the reciprocal of the observed rate of transition, were determined under a variety of conditions. If the same molecular mechanism is contributing to both n_H and τ_{min}, corresponding changes in these parameters would be expected as conditions change. The Hill coefficients of threonine binding, n_b, and the dissociation constant, K_D, as measured under the same conditions (Bearer & Neet, 1978), are used for comparison with the kinetically derived constants.

Experimental Section

Methods

Enzyme Purification. Enzyme was purified as previously described (Ogilvie et al., 1969; Bearer & Neet, 1978). Enzyme purified through the Sephadex A-50 step was used for most of this work, had a specific activity of 20 units/mg (aspartic β-semialdehyde to homoserine) at 27 °C, and a ratio of 3:1 for activity in the absence and presence of 10 mM threonine measured in the reverse (homoserine to aspartic β-semialdehyde) direction. Enzyme retained full activity and threonine sensitivity when incubated in buffer B at room temperature for at least 7 h (the duration of experiments). Several points were checked for the transient and cooperativity with enzyme further purified on Sepharose 4B to specific activity of 47 (Bearer & Neet, 1978). Identical results were obtained. Buffers and assays are as previously described (Bearer & Neet, 1978).

Stopped-Flow Procedure. AK–HSDI was desalted by passage through a Sephadex G-25 column equilibrated with buffer B. Enough enzyme was added to buffer B such that the enzyme solution contained 2.25 units/mL (measured in the reverse direction). NADPH was then added to 0.3 mM. The solutions containing threonine were composed of buffer B plus 8.0 mM ASA. The threonine concentration range was 1.00 mM to 20.0 mM threonine. The final concentrations after mixing in the stopped-flow were: enzyme, 1.125 units/mL; NADPH, 0.15 mM; ASA, 4.0 mM; and THR, 0.5–10.0 mM.

A Durrum Gibson apparatus was used for kinetic measurements of light absorbance at 340 nm with a deuterium lamp. Photomultiplier noise was reduced by using a variable filter with a time constant less than 5% of the time per division setting. Mixing was complete after 5 ms. Three separate curves on the oscilloscope were photographed with a Polaroid Land Camera for subsequent analysis.

Stopped-Flow Data Analysis. Progress curves were digitized by hand using a transparent grid. The resulting raw data were fit to a double exponential Guggenheim equation (see Appendix in supplementary material). The Guggenheim equation (Shoemaker & Garland, 1967) was used because the absorbance at infinite time cannot be measured on the oscilloscope.

A program for a Hewlett-Packard 9100 calculator was used to plot data as ln ΔP vs. t_1 and calculate values of k_1, k_2, ψ_1, ψ_2, and ψ_i where:

k_1 = first-order rate constant for decay of the initial velocity to the steady-state velocity (eq A14);

k_2 = rate constant for conversion of substrate to product in the steady state (eq A11);

ψ_1 = contribution to the observed initial velocity of the rapidly decaying species (eq A13);

ψ_2 = initial velocity for the slow process extrapolated to zero time (eq A9);

ψ_i = observed initial velocity (eq A23);

ΔP = the concentration of product produced in the constant time interval Δt (eq A20, A24);

$\Delta t = t_2 - t_1$.

Since the same enzyme solution is used for one complete threonine curve, the ψ_i values should be the same. Consistency in ψ_i values is indicative of proper choice of Δt and oscilloscope settings.

Plots of k_1^{-1} vs. THR^{-1} were linear. A Hewlett-Packard 9100 calculator was used to plot data, draw a least-squares line through the points, and calculate values for τ_{min}, where τ_{min} is k_1^{-1} at infinite threonine, and K_k (the threonine concentration at which $k_1 = 0.5\ (\tau_{min})^{-1}$).

Steady-State Inhibition by Threonine. Steady-state velocities were measured on a Gilford spectrophotometer at 340 nm using a tungsten lamp and a Perkin-Elmer strip chart recorder. Assay mix containing 3×10^{-3} M ASA, and 1.5×10^{-4} M NADP in buffer B, was allowed to temperature equilibrate. AK–HSDI was desalted before use by passage through a Sephadex G-25 column.

Kinetic Hill coefficients were derived from Hill plots of the steady-state inhibition. Log $[(V_m - v)/(v - v_s)]$ was plotted vs. log [threonine] where V_m is the velocity in the absence of threonine, V_s is the velocity in the presence of 10 mM threonine, and v is the velocity at any specified threonine concentration. The range of threonine concentrations giving maximal slope was estimated, and a least-squares line was drawn

Liposomes from Ionic, Single-Chain Amphiphiles†

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ABSTRACT: In studies of the minimum physicochemical requirements for lipid membrane formation, we have made liposomes from dilute, aqueous dispersions of C(8)–C(18) single-chain amphiphiles. In general, membrane formation from ionic soaps and detergents requires the presence of uncharged amphiphiles. Vesicles were characterized by phase-contrast microscopy, by trapping of ionic dyes, as well as by negative-stain and freeze-fracture electron microscopy. They were typically heterogeneous in size, but the average diameter could be experimentally varied in some cases over the range of 1 to 100 μm. Uni-, oligo-, and multilamellar vesicles were observed. Membrane permeability to various solutes was determined in part by a new technique which utilized phase-contract microscopy; when impermeable vesicles exclude added solutes such as sucrose, refractive index differences are created between vesicle contents and surrounding medium, so that the vesicles appear bright in the phase microscope. Permeant solutes do not produce this effect. Spectrophotometric permeability determinations confirmed the results of this technique and provided quantitative measures of permeability. Monoalkyl liposomes have potential uses as models of biomembranes and in drug delivery. They are also relevant to the prebiotic origin of biomembranes.

Lipid bilayer membranes form spontaneously when membrane lipids isolated from most living organisms are dispersed in water. These bilayer-forming compounds almost universally consist of various glycerolipids or sphingolipids containing two long hydrocarbon chains. The few exceptions include flagellar membrane lipids, found in a protozoan. These are composed of single-chain hydrocarbons containing sulfate and chlorine, together with sterols and free fatty acids (Haines, 1973; Chen et al., 1976). While some single-chain lipids (fatty acids and lysophospholipids) are normally found in biomembranes in low concentrations, these compounds have been considered incapable of forming stable lipid bilayers in water at low lipid concentrations (Tanford, 1973). However, Gebicke and Hicks have published accounts of lipid vesicles forming spontaneously from *cis*-Δ^9-octadecenoic acid (oleic acid) in dilute aqueous dispersions (Gebicke and Hicks, 1973, 1976). We have been interested in the minimum physicochemical requirements for lipid membrane formation and became aware that single-chain lipids can form stable liposomes during experiments employing dodecanoate, a 12-carbon saturated fatty acid. Subsequently, we have determined that a wide variety of saturated single-chain amphiphiles varying in length from 8 to 18 carbons are capable of forming lipid vesicles.

The purpose of this paper is to define the conditions under which liposomes can be prepared from monoalkyl[1] compounds and to describe some characteristics of these vesicles.

Before proceeding, we must define our use of some terms which might otherwise be ambiguous. We have used the word "liposome" to describe certain spherical or tubular structures formed by amphiphiles dispersed in an aqueous medium. These structures contain lipid bilayers and enclose aqueous compartments and are thereby differentiated from micelles. We have operationally defined liposomes by their microscopic appearance and by their ability to entrap small, polar molecules. We have coined the term "oligolamellar" to describe liposomes which, by phase-contrast and electron microscopy, appear to contain an aqueous compartment surrounded by one or several concentric lipid bilayers. We feel that the term "unilamellar" should be used only when chemical or ultrastructural evidence is presented which can differentiate between these two vesicle types. Using our operational criteria, "multilamellar" vesicles are those which appear dark by phase-contrast microscopy and which fail to become bright on dilution of vesicle dispersions with an impermeant nonelectrolyte such as sucrose (see Materials and Methods for details of this technique).

Materials and Methods

Sources and Purity of Materials. C(8:0)–C(16:0) and C(18:1$^{\Delta 9}$) (oleic) acids, decanol, and NaDodSO$_4$[2] were purchased from Sigma Chemical Co., St. Louis, Mo.; dodecanol was obtained from Aldrich, Milwaukee, Wis. All were used as received. Thin-layer chromatography of these compounds was performed on silica gel f-254 plates (EM Laboratories, Elmsford, N.Y.) in hexane–ethyl ether–acetic acid (80:20:1) and in chloroform–methanol–water (65:25:4). The separated components were visualized with a Rhodamine 6G spray and illuminated at 366 nm. Each compound revealed only a single spot when 1 and 10 μg of lipid were analyzed. Gas–liquid chromatography of saturated fatty acids was performed using the boron trifluoride–methanol reagent (Metcalfe and Schmitz, 1961) and a 10% DEGS column. Oleic acid was received sealed under nitrogen, then diluted with chloroform, purged with argon, and stored at −20 °C. Except for C(8) fatty acid [>97.4% pure, containing small amounts of C(10) and C(12) fatty acids], all saturated fatty acids were greater than 99.7% pure. The dye 6-carboxyfluorescein (6CF) was pur-

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[1] "Monoalkyl" and "single-chain" will be used interchangeably to describe amphiphiles with a single hydrocarbon chain derived from either fatty acids or fatty alcohols.

[2] Abbreviations used: CMC, critical micelle concentration; EDTA, ethylenediaminetetraacetic acid, disodium salt; HTMAB, hexadecyltrimethylammonium bromide; PC, phosphatidylcholine; PS, phosphatidylserine; NaDodSO$_4$, sodium dodecyl sulfate; TBS, 10 mM triethylaminoethanesulfonic acid (TES), 130 mM NaCl, 10 mM KCl; 6CF, 6-carboxyfluorescein; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol.

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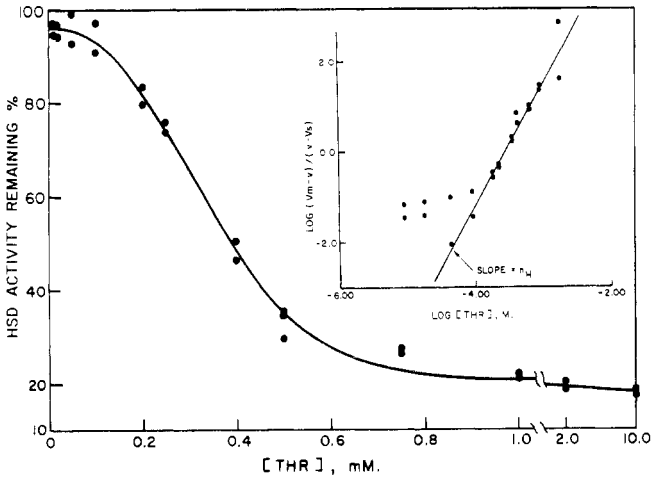

FIGURE 3: Threonine inhibition of HSD steady-state activity in the forward direction. HSD assay conditions were pH 7.2, 20 °C in buffer B. One hundred percent activity is measured in the absence of threonine. Inset: Hill plot of data. Solid line is least-squares fit to data points at maximum slope of the plot. The kinetic Hill coefficient, n_H, is the slope of this fit.

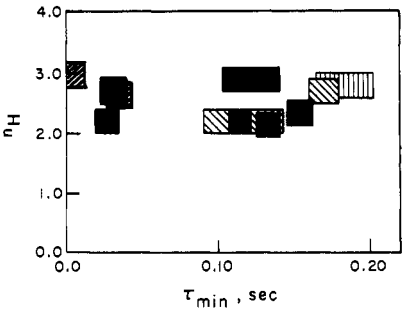

FIGURE 4: Comparison of n_H with τ_{min} for HSD. Kinetic Hill coefficients compared with minimum relaxation times measured under the same conditions show no correlation when conditions of assay varied. Boxes denote standard errors of parameters. Data from Table I.

0.17 to 0.13 s (Table I). No significant effect on K_k, however, was measured. It appears that increases in ionic strength slightly increase the rate of isomerization in both directions while threonine concentration dependence remains the same.

Variation of τ_{min} with MgATP. The addition of 0.25 mM and 0.50 mM MgATP has a complex effect on τ_{min} and K_k (Table I). τ_{min} decreases from 0.17 to 0.11–0.13 s in the presence of this ligand. The values, for K_k, however, increase over the range of ligand concentration studied. Thus, it appears that the rate constant for the isomerization becomes larger in the presence of MgATP whereas the threonine effect is reduced, either by a decrease in affinity for threonine (stabilization of R form), or by decreasing the effect of bound threonine such that the T form is modified.

Variations of n_H. The steady-state velocities obtained from the stopped-flow experiments could not be used because of an order of magnitude difference in K_I (steady-state inhibition constant of threonine) and K_k. Velocities obtained from the stopped-flow experiment did overlap those obtained by spectrophotometry at the higher threonine concentrations. A typical inhibition curve is shown in Figure 3. Data points from these curves were replotted as described in Methods to obtain a kinetic Hill coefficient, n_H (Figure 3, inset).

The inhibition curves were repeated several times. For a set of conditions, the same range of threonine concentrations was chosen for calculation of the Hill coefficient. Constants obtained in this way agreed within ±0.2 unit for a set of experiments. Deviations of the slope of the Hill plot at low and high threonine concentrations are expected (Cornish-Bowden & Koshland, 1975) with a value of one at each extreme. At low concentration, however, an activation of HSD was consistently observed which could be due to a stabilization of the tetramer of AK-HSD by low concentrations of threonine (Cunningham et al., 1968).

Increasing temperature from 20 to 37 °C at pH 6.9 or from 20 to 27 °C at pH 7.2 or 7.6 had no effect on the kinetic Hill coefficient (Table I). In buffer A at pH 7.4 the changes between 20 and 37 °C also appear to be insignificant. Trends in K_I values are harder to assess in that the error in determination is large. No significant variation in K_I can be observed from the values obtained.

Kinetic Hill coefficients also appeared to be pH independent. Variations of pH from 6.5 to 7.6 did show a slight increase from 2.3 to 2.8. This increase, however, may not be significant because of the amount of error in determination of the Hill coefficients. The increase in K_I values was also not large enough to establish any significance.

The addition of choline chloride or MgATP appeared to have borderline effects on either the Hill coefficient or the values of K_I. The addition of 0.8 M choline chloride to assay mix caused a decrease in the Hill coefficient from 2.7 to 2.15 with a concomitant decrease in the K_I from 2.7 mM to 2.1 mM threonine. Both these decreases, however, are of questionable validity because of the size of error in both measurements. Likewise, the decrease in K_I and n_H with addition of 0.25 mM MgATP is probably not significant because its magnitude is less than two standard errors.

Discussion

Variations of K_I and K_k. Examination of Table I shows no significant correlation of changes in K_I and K_k values with varying conditions. It is noteworthy, however, that the K_I values are an order of magnitude less than the K_k values. We have shown elsewhere (Bearer, 1977) that K_I from steady-state inhibition and K_k from transient saturation are nonidentical functions of the other rate constants for either a concerted equilibrium model or a slow transition kinetic model. Thus it is quite possible for these constants to differ by an order of magnitude.

Variations of τ_{min} and n_H. From the results in Table I, several comparisons can be made. With increasing temperature, τ_{min} decreased substantially from 0.15 to 0.03 s in buffer B. Under the various conditions employed, this change in τ_{min} was the largest measured. Arrhenius plots of the rate of the conformational transition with respect to temperature have demonstrated an activation energy of 22 kcal/mol, for the isomerization (R → T) in the presence of 0.5 mM threonine (Janin & Iwatsubo, 1969). The finding of an activation energy of 18.9 kcal/mol for the kinetic process measured here is good evidence that the two processes are the same. As measured under the same conditions, however, the Hill coefficient for threonine inhibition was independent of temperature, remaining essentially constant at 2.2. This temperature independence of the Hill coefficient was also found at pH 7.2 and pH 7.6 over a temperature range of 20–27 °C and in buffer A between 20 and 37 °C. From these data it would appear that changes in τ_{min} are not paralleled by changes in kinetic cooperativity. The lack of correlation is diagrammatically demonstrated in Figure 4.

It has been shown that pH has a substantial effect on the HSD activity and cooperativity in the forward direction over a range of 6.5–9.0 (Patte et al., 1966; Barber & Bright, 1968; Mackall & Neet, 1973). Therefore, we studied the effect of

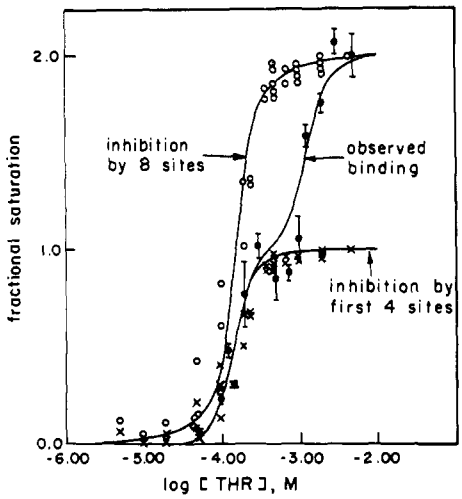

FIGURE 5: Comparison of binding of threonine (Bearer & Neet, 1978) to HSD inhibition by threonine in buffer B, pH 6.9 and 20 °C. (●) Moles of threonine bound per mol of AK-HSDI; (X) fractional inhibition assuming 4 mol bound per mol of AK-HSDI correlates with maximum inhibition; (○) fractional inhibition assuming 8 mol bound per mol correlates with maximum inhibition. Solid lines are single Monod fits for kinetic data and a sum of two Monod equations for binding data. For binding data: (first fit) $K_R = 0.02$, $K_T = 1.5 \times 10^{-4}$, $L = 10^{-4}$, and $n = 4.0$; (second fit) $K_R = 0.02$, $K_T = 1.25 \times 10^{-4}$, $L = 10^{-4}$, and $n = 4.0$. For HSD inhibition, assuming 4 mol of threonine per mol of enzyme: $K_R = 0.02$, $K_T = 1.5 \times 10^{-5}$, $L = 10^{-4}$, and $n = 4.0$. Assuming 8 mol of threonine per mol of enzyme: $K_R = 10^{-3}$, $K_T = 4 \times 10^{-5}$, $L = 10^{-5}$, and $n = 8.0$.

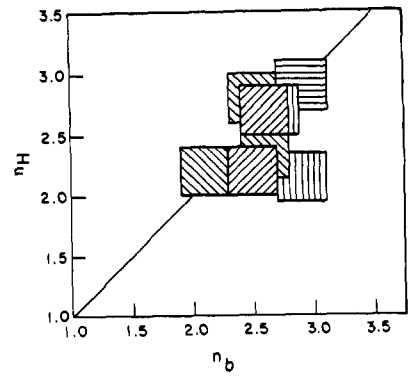

FIGURE 6: Comparison of Hill coefficients obtained from binding, n_b, with Hill coefficients of HSD inhibition, n_H, under comparable conditions. n_H values are from Table I; n_b values are from Bearer & Neet (1978). The line is drawn for the situation in which $n_H = n_b$.

variation of pH on τ_{min}. The pH range studied, 6.9 to 7.6, was chosen to minimize association-dissociation effects (Mackall & Neet, 1973). Increasing pH caused a small increase in the τ_{min} value, from 0.15 to 0.18 s and an insignificant increase in Hill coefficient from 2.3 at pH 6.5 to 2.8 at pH 7.6 (Table I).

Lack of a correlated change in n_H and τ_{min} was also observed for addition of salt. Evidence has been presented that increases in ionic strength of a neutral salt (choline chloride) have a conformational effect on AK-HSDI (Ogilvie et al., 1975). As the salt concentration was increased to 0.8 M, τ_{min} decreased from 0.17 to 0.13 s. The Hill coefficient decreased from 2.7 to 2.15 over the same range of salt concentrations, but this decrease is not significant because of the error associated with values of n_H.

Similar conclusions are reached with studies of MgATP. Because of the multiple effect of MgATP on the HSD activity (Patte et al., 1966; Cunningham et al., 1968; Broussard et al., 1972; Ogilvie et al., 1975), the effect of its addition to stopped-flow solutions was examined. The decrease in τ_{min} upon addition of 0.25 MgATP was small but significant, but no significant change was observed for the Hill coefficients (Figure 4). Since both 0.25 and 0.50 mM concentrations of MgATP had maximum effect on HSD_{rev} initial velocity, it is not surprising the τ_{min} for both concentrations is the same. This lack of correlation between kinetic Hill coefficients and transient rate constants is inconsistent with kinetic mechanisms such as the slow transition model (Ainslie et al., 1972) in which there is a mechanistic relationship between the two parameters.

Correlation of High Affinity Binding Site and HSD Inhibition. Comparison of the K_D data from binding studies (Bearer & Neet, 1978) with the K_I data (Table I) indicates that the K_I values for HSD inhibition are very similar to the K_D values of the higher affinity threonine binding site. In fact, in buffer B for nine different experimental conditions between pH 6.9 and 7.6, between 20 and 37 °C, and, in the presence of choline Cl or ATP, the average value of K_I (0.22 ± 0.06 mM) is not significantly different from the average K_D (0.16 ± 0.03 mM) for the high affinity site. Thus, it appears that threonine binding to the higher affinity sites is responsible for the HSD inhibition. The higher values of K_I and K_D in buffer A compared with buffer B are due to an increase in the concentration of K^+ (Janin et al., 1969).

Further evidence that higher affinity binding and HSD inhibition are caused by the same threonine binding site is presented in Figure 5. Binding and inhibition data have been plotted on the same scale assuming either that the first 4 mol of threonine bound are responsible for all of the HSD inhibition (symbol X), or that all 8 mol of threonine must be bound for maximal HSD inhibition (open circles). Better agreement of binding and kinetic data occurs when it is assumed that the first 4 mol bound per mol of enzyme cause maximal HSD inhibition. The inconsistency of the kinetic data with the binding data when it is assumed that 8 mol per mol must be bound for maximum inhibition is especially obvious at higher concentrations of threonine. In this range, the two curves are clearly distinct. The assumption that only the second 4 threonines bound are responsible for HSD inhibition gives equally poor agreement (not shown). Thus, it was concluded that the higher affinity threonine binding site is solely responsible for the threonine inhibition of the HSD activity. This is in agreement with the assignment of inhibitory sites according to work from Takahashi's laboratory (Wright & Takahashi, 1977a,b; Wright et al., 1976).

The Mechanism of Kinetic Cooperativity of Threonine Inhibition of HSD. The inhibition of the HSD activity by threonine shows a kinetic cooperativity which exactly parallels the cooperativity of binding to the high affinity sites (Figure 5). The n_H (kinetic) is equal to the n_b (equilibrium) under all experimental conditions tested (Figure 6). Thus, for the HSD activity, the cooperativity of inhibition is solely a result of equilibrium binding of threonine with no kinetic component in the mechanism. This conclusion is in agreement with that reached by comparison of the transient in the inhibition by threonine to the cooperativity of the inhibition (Figure 4).

In summary, we have shown that: (1) the high affinity threonine binding sites inhibit the HSD activity; and (2) the kinetic cooperativity of threonine inhibition of HSD activity is best described as due to a rapid equilibrium binding of threonine with site-site interactions which is independent of the rate of the isomerization.

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Supplementary Material Available

A proof (Appendix) establishing that the Guggenheim method is applicable for analysis of double exponential decays (6 pages). Ordering information is given on any current masthead page.

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