Threonine Inhibition of the Aspartokinase–Homoserine Dehydrogenase I of *Escherichia coli*. A Slow Transient and Cooperativity of Inhibition of the Aspartokinase Activity†

Cynthia F. Bearer‡ and Kenneth E. Neet*

ABSTRACT: The threonine inhibition of the steady-state kinetics of the aspartokinase (AK) activity of the threonine-sensitive aspartokinase–homoserine dehydrogenase I of *Escherichia coli* has been examined under a variety of experimental conditions. Kinetic studies of AK activity revealed the presence of a lag of threonine inhibition. This lag was distinct from the homoserine dehydrogenase (HSD) transient because: (1) the half-time of the lag was on the order of minutes for AK as compared with seconds for HSD, and (2) the initial velocity of AK activity (at about 10 s) was inhibited whereas the initial velocity of HSD activity (at about 100 ms) was not. The K_I for inhibition of the AK initial velocity (after 10 s) was similar to that of the HSD steady-state inhibition. These data suggest that the same threonine site was responsible for inhibition of the initial AK velocity and the steady-state HSD velocity. The kinetic Hill coefficient for inhibition of the AK initial velocity was relatively constant with assay conditions at about 4.0, but was significantly higher than that previously observed for the HSD steady-state inhibition or for threonine binding, about 2.2 (Bearer, C. F., & Neet, K. E. (1978) *Biochemistry 17* (preceding paper in this issue)). Threonine inhibition of the initial AK velocity (after 10 s) was saturated at about 85–95% inhibition, whereas the further inhibition in the steady state, after the slow transient, continued to 100% inhibition. The inhibition of the steady state was saturated at threonine concentrations of 3–5 mM. A lag in the serine inhibition was also observed with a similar half-time for the transient but with a lower degree of cooperativity in the initial velocity (n_H = 2.7). We have interpreted these data to indicate two conformational transitions of AK-HSD induced by threonine: (1) A relatively slow transition (ms) resulting in 80–95% inhibition of both AK and HSD activities, but with different degrees of cooperativity. (2) A slower transition (min) resulting in no further inhibition of HSD but with complete inhibition of the AK activity. A model for the cooperative binding and inhibition of AK–HSD has been proposed and tested by examination of the steady-state velocities of the AK activity as a function of threonine concentration. The model consists of a tetrameric enzyme with biglobular protomers which undergoes a series of conformational transitions in the presence of threonine. An equation has been derived which relates the AK activity in the steady state to the distribution among four enzyme states: the latter distribution is determined by Hill coefficients and dissociation constants which have been obtained from independent kinetic or equilibrium experiments. The inhibition curve obtained from this equation gave a satisfactory fit to the experimental data under four different sets of conditions, using values for three of the binding parameters obtained from threonine binding experiments. A Hill coefficient of four for the high affinity sites adequately fit the data, indicating that the kinetic cooperativity in the first set of sites was consistent with the model. A physiological rationale for the complex behavior of AK–HSD is proposed. A single set of high affinity threonine binding sites allows both activities of the bifunctional enzyme to be inhibited (to 80–90%) but an additional, subtle control is exerted by the difference in cooperativity of the two activities generated by the hysteretic cooperativity of the AK inhibition. The low affinity set of threonine sites allows for complete shutdown of the AK activity but with a "damping" effect through the second hysteretic transition.

Aspartokinase–homoserine dehydrogenase (AK–HSD)[1] I of *E. coli* possesses two catalytic activities, each of which is feedback inhibited by threonine. Extensive studies of this inhibition have been made (see Truffa-Bachi et al., 1974), generally under different conditions so that comparisons between the two activities are difficult. We have undertaken investigations on the threonine inhibition of aspartokinase activity of AK–HSD in order to elucidate the relationship between the inhibition of the HSD activity and of the AK activity. Two lines of evidence have made this study pertinent: (1) description of two sets of threonine sites by direct binding studies and by labeling; and (2) kinetic Hill coefficients derived from AK inhibition curves and binding Hill coefficients for threonine which are not equal.

By a technique involving the formation of covalent Co (III)–AK adducts, Ryzewski & Takahashi (1975) have been able to locate 4 of the 8 threonine binding sites on the aspartokinase domain, adjacent to the aspartokinase active site. As shown in the first paper of this series, we have delineated high and low affinity threonine binding sites (Bearer & Neet, 1978a). One might expect different kinetic effects from each. To determine if the same set of threonine sites are responsible for both the HSD and AK inhibitions, we have studied the AK activity under the same conditions of assay as HSD activity and threonine binding (Bearer & Neet, 1978a,b).

A discrepancy in the Hill coefficients of a ligand determined

† From the Department of Biochemistry, Case Western Reserve University, Cleveland, Ohio, 44106. *Received January 5, 1978; revised manuscript received May 24, 1978.* Supported by a grant from the National Institutes of Health, U.S. Public Health Service, AM12881.

‡ Predoctoral Trainee of the U.S. Public Health Service, Training Grant 5-T01-GM00035 from the National Institutes of Health. This work was submitted to Case Western Reserve University in partial fulfillment of the requirements for a Doctor of Philosophy degree (1977). Present address: Department of Cell Biology, Baylor College of Medicine, Houston, Texas 77030.

[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; PK/LDH, pyruvate kinase/lactate dehydrogenase.

0006-2960/78/0417-3523$01.00/0

by kinetics and by binding is diagnostic for a kinetic contribution to the mechanism of cooperativity. Several laboratories have reported kinetic Hill coefficients of 4 for threonine inhibition of aspartokinase (Patte et al., 1966; Truffa-Bachi et al., 1969; Wampler & Westhead, 1968; Jacques & Truffa-Bachi, 1976; Wampler et al., 1970), whereas binding Hill coefficients greater than 3.1 have never been reported (Janin et al., 1969; Heck, 1972; Takahashi & Westhead, 1971). Binding Hill coefficients from our own results for both sites were no greater than 2.9. During the initial investigation of the threonine inhibition of AK, we observed a lag in the threonine inhibition of AK. In a kinetic mechanism of cooperativity, the progress curve transient and steady-state Hill coefficients are different combinations of the same rate constants (Ainslie et al., 1972; Bearer, unpublished). A change in one necessitates a change in the other. We have compared these two parameters of the AK inhibition in order to determine their relationship and to determine if the AK inhibition by threonine involves a kinetic component in the mechanism of cooperativity.

These studies have allowed us to correlate the structural and mechanistic data on the enzyme and arrive at a model for the enzyme which accommodates most of the experimental findings and suggests a novel regulatory mechanism based upon the interplay of threonine binding and hysteretic regulation of the enzyme. In addition we have been able to predict the behavior of the steady state velocity of the AK activity from the model and compare the experimental data to it.

Experimental Section

Materials. Pyruvate kinase/lactate dehydrogenase (PK/LDH) was obtained from Boehringer Mannheim. All other reagents were obtained in the highest possible grade from standard sources.

Methods. Enzyme was purified as described (Bearer & Neet, 1978a). Before use, AK-HSDI was desalted by passage through a G-25 column. Buffers were prepared as previously described (Bearer & Neet, 1978a).

Aspartokinase Assay. The PK/LDH coupled assay of Wampler & Westhead (1968) was employed with slight modification. To either buffer B or buffer A the following constituents were added such that the final concentrations were: 10.0 mM aspartate, 5.0 mM $MgCl_2$, 0.3 mM NADH, 3.3 mM ATP, 2.8 mM phosphoenolpyruvate, 0.3 μM PK, and 0.9 μM LDH. Appropriate aliquots of threonine were added by microliter pipet to 2 mL of assay mix. Assays were initiated by addition of enzyme, unless otherwise stated, with a mixing time on the order of 10 s. The production of ADP by aspartokinase was coupled to the oxidation of NADH by PK/LDH and observed at 340 nm on a Gilford spectrophotometer and Perkin-Elmer strip chart recorder. One unit of enzyme is defined as the amount of enzyme which produces 1 μmol of ADP per min. The concentration of protein was determined as described (Falcoz-Kelly et al., 1972; Bearer & Neet, 1978a). Since the activity of AK is about 20 times higher in buffer A than in buffer B, the former was used for most experiments.

Data Analysis. Both initial and steady-state velocities, V_i and V_S, were obtained from the optical density decrease at 340 nm. The progress curve transient was shown to be a first order process by digitization of representative assays. Semilog plots of the data by the Guggenheim method (Shoemaker & Garland, 1967; Bearer & Neet, 1978b) yielded straight lines. The apparent first-order constant for the transient, k, for all other assays was calculated from the x intercept, x, of the progress curve utilizing the following formula (Frieden, 1970):

$$k = \frac{V_S - V_i}{xV_S}$$

A typical progress curve is shown in Figure 1. Values for the Hill coefficient, K_I, K_K, and τ_{min} were obtained as described previously (Bearer & Neet, 1978b). K_I is the inhibition constant of the steady state velocity. K_K is the threonine concentration at which $k = 0.5(\tau_{min})^{-1}$. τ_{min} is k^{-1} at infinite threonine concentration.

Theoretical Section

The relationship between conformations, threonine binding, and enzymatic rate are analyzed here in order to relate the steady-state AK velocity to the threonine concentration.

For the analysis of AK-HSDI we define: a state R, which has full activity for both activities; a state T, which is mostly (80-90%) inhibited for both activities; a state U, which has the same HSD activity as the T state but zero AK activity; and a state R/U, which has the full HSD activity but zero AK activity. (For a more detailed presentation of these states, see Discussion.) Since the conversions between R and T and U are slow, velocity analysis is done in terms of the species of enzyme to which none, one, or two threonines are bound per subunit: E is enzyme with no threonine bound, Et1 is enzyme with threonine bound to the site t1, Et2 is enzyme with threonine bound to t2, Et1t2 is enzyme with both sites t1 and t2 occupied, and E_0 is total enzyme.

The further assumption is made that threonine binds randomly and independently between the t1 and t2 sites, but that interactions occur among the four t1 (or four t2) sites in the tetramer. Because of the velocities and time dependencies of each of the conformations has been defined, the rate expressions of each activity can be expressed as a function of the proportion of each conformation.[2] For HSD, the initial velocity, V_{Hi}, is uninhibited and equal to the velocity in the absence of threonine, V_{HO}. In terms of species:

$$\frac{V_{Hi}}{V_{HO}} = 1 = \frac{[E]}{[E]_0} + \frac{[Et1]}{[E_0]} + \frac{[Et2]}{[E_0]} + \frac{[Et1t2]}{[E_0]} \quad (1)$$

The steady-state velocity, V_{HS}, after the faster transient (the slow transient has no effect on HSD activity) is:

$$\frac{V_{HS}}{V_{HO}} = \frac{[E]}{[E_0]} + \frac{[Et2]}{[E_0]} + \frac{V_{HSS}}{V_{HO}}\left(\frac{[Et1]}{[E_0]} + \frac{[Et1t2]}{[E_0]}\right)$$
$$= 1 + \left(\frac{V_{HSS}}{V_{HO}} - 1\right)\left(\frac{[Et1] + [Et1t2]}{[E_0]}\right) \quad (2)$$

where V_{HSS} is the HSD steady state activity at saturating threonine. From inspection of eq 2, the steady-state velocity of HSD is a direct function of threonine binding to site t1. Such a relationship was found to be the case (see Figure 5 of Bearer & Neet, 1978b).

For the AK activity, the initial velocity, V_{Ai}, was measured after the R to T transient, as indicated by complete HSD inhibition, but before the T to U transient. Therefore:

$$\frac{V_{Ai}}{V_{AO}} = \frac{[E]}{[E_0]} + \frac{[Et2]}{[E_0]} + \frac{V_{AiS}}{V_{AO}}\left(\frac{[Et1]}{[E_0]} + \frac{[Et1t2]}{[E_0]}\right)$$
$$= 1 + \left(\frac{V_{AiS}}{V_{AO}} - 1\right)\left(\frac{[Et1] + [Et1t2]}{[E_0]}\right) \quad (3)$$

where V_{AiS} is the initial activity at saturating threonine. From eq 3, it is evident that V_{Ai} is also a direct function of threonine saturation at t1. For the AK steady-state velocity V_{AS}, all enzyme forms with threonine bound to t2 are totally inhibited. Therefore

$$\frac{V_{AS}}{V_{AO}} = \frac{[E]}{[E_0]} + \frac{V_{AiS}}{V_{AO}}\left(\frac{[Et1]}{[E_0]}\right) \quad (4)$$

[2] Symbols used previously (Bearer & Neet, 1978b) are redefined here for consistency. The relationships are $\psi_1 = V_{Hi}$; $\psi_2 = V_{HS}$.

From eq 4, it can be seen that V_{AS} is not a direct function of any measurable quantity and is thus difficult to analyze directly.

If it is assumed that the binding of threonine, T, to t1 is in equilibrium and unaffected by threonine bound to t2, then the dissociation constant, K_1, and the Hill coefficient, n_1, for binding to t1 can be expressed as:

$$\frac{[E][T]^{n_1}}{[Et1]} = \frac{[Et2][T]^{n_1}}{[Et1t2]} = K_1^{n_1} \quad (5)$$

Rearranging and dividing by total enzyme concentration:

$$\frac{[E]}{[E_0]} = \frac{[Et1]}{[E_0]}\frac{K_1^{n_1}}{[T]^{n_1}} \text{ and } \frac{[Et2]}{[E_0]} = \frac{[Et1t2]}{[E_0]}\frac{K_1^{n_1}}{[T]^{n_1}} \quad (6)$$

and solving for [E] + [Et1]:

$$\frac{[E] + [Et1]}{[E_0]} = \left(1 + \frac{K_1^{n_1}}{[T]^{n_1}}\right) = \left(\frac{[Et1]}{[E_0]}\right) \quad (7)$$

Substitution of eq 6 into eq 4 gives:

$$\frac{V_{AS}}{V_{AO}} = \left(\frac{[Et1]}{[E_0]}\right)\left(\frac{K_1^{n_1}}{[T]^{n_1}} + \frac{V_{AiS}}{V_{AO}}\right) \quad (8)$$

Substitution of eq 7 into eq 8 leads to:

$$\frac{V_{AS}}{V_{AO}}\left(\frac{K_1^{n_1}}{[T]^{n_1}} + \frac{V_{AiS}}{V_{AO}}\right)\left(1 + \frac{K_1^{n_1}}{[T]^{n_1}}\right)^{-1}\left(\frac{[E] + [Et1]}{[E_0]}\right) \quad (9)$$

The last term in eq 9 can be related to the second set of threonine binding sites which have a dissociation constant, K_2, and a Hill coefficient, n_2:

$$K_2^{n_2} = \frac{[Et1][T]^{n_2}}{[Et1t2]} = \frac{[E][T]^{n_2}}{[Et2]} \quad (10)$$

Rearrangement of eq 10 leads to the normal binding expression for a cooperative system:

$$\frac{[Et1t2] + [Et2]}{[E_0]} = \frac{[T]^{n_2}}{K_2^{n_2} + [T]^{n_2}} \quad (11)$$

And since

$$\frac{[E] + [Et1]}{[E_0]} = 1 - \frac{[Et1t2] + [Et2]}{[E_0]} = \left(1 + \frac{[T]^{n_2}}{K_2^{n_2}}\right)^{-1} \quad (12)$$

Equation 9 becomes:

$$\frac{V_{AS}}{V_{AO}} = \left(1 + \frac{[T]^{n_1}}{K_1^{n_1}}\frac{V_{AiS}}{V_{AO}}\right)\left(1 + \frac{[T]^{n_1}}{K_1^{n_1}}\right)^{-1}\left(1 + \frac{[T]^{n_2}}{K_2^{n_2}}\right)^{-1} \quad (13)$$

Equation 13 expresses the relative AK steady-state velocity (V_{AS}/V_{AO}) entirely in terms of parameters derived from independent experiments. V_{AiS} and V_{AO} are essentially normalization factors to the experimental data. Values for n_1 and K_1 can be obtained from either kinetic or binding experiments related to the first set of threonine sites. Values for n_2 and K_2 can be obtained from binding experiments related to the second set of threonine sites.

Results

Observation and Characterization of a Transient. While studying threonine inhibition of AK activity, a progress curve transient was observed, qualitatively similar to that described for HSD (Bearer & Neet, 1978b); however, the AK transient was much slower (Figure 1). The transient was unaffected by buffer changes since it was observed both in buffers A and B. In order to characterize the transient, preincubation studies were carried out. In the usual assay in which a transient occurred, initiation was accomplished by addition of enzyme to the assay mix which included threonine. If enzyme was included in the assay mix and aspartate was added to initiate the reaction, no transient appeared. For both conditions of initiation, the steady-state velocity, V_{AS}, was the same. If more threonine was added during the course of the assay, an additional lag of inhibition occurred. This was especially obvious when the assay was initiated in the absence of threonine and then threonine was added. In the latter case, enzyme was diluted prior to threonine addition, indicating that the transient cannot be explained by a simple dissociation of AK-HSDI. These studies showed that the transient was a property of threonine inhibition of AK-HSDI and not an artifact of the coupled assay procedure; furthermore, increasing the amount of coupling enzyme had no effect. A transient was also observed with serine inhibition of AK at concentrations 100× greater than threonine. Similar observations were made with enzyme additionally purified through Sepharose 4B to a specific activity of 50 units/mg (Bearer & Neet, 1978a), indicating that the lag of inhibition is a property of homogeneous AK-HSDI.

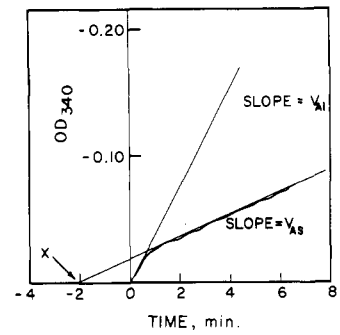

FIGURE 1: Progress curve for threonine inhibition of AK activity. Assay conditions are pH 6.9, 24 °C, and 1.25 mM L-threonine, buffer A. Initiation is with addition of enzyme.

The magnitude of the half-time for the transient of AK was 100-fold greater than that of HSD (Bearer & Neet, 1978b). Half-times of this magnitude exceed the longest time measurable by stopped-flow techniques so that traces were obtained from a Gilford spectrophotometer. The error in the determination of k for AK was much larger than for HSD because more noise was observed for AK assays on the Gilford than for HSD assays on the stopped-flow and better reproducibility was attained with stopped-flow measurements.

The effect of protein concentration on k was determined over a 10-fold range at 1.0 mM threonine. The value of k in buffer A varied from 2.5 ± 0.14 to 2.7 ± 1.0 min over a protein concentration of 0.034 to 0.0034 mg/mL. The value of τ_{min} in buffer B was the same (0.14 ± 0.07 and 0.3 ± 0.1 min) over a similar 10-fold range of enzyme concentration. This result ruled out an association reaction as an explanation for the transient.

τ_{min} Analysis. Due to the large error involved in obtaining the value of k, there was a large uncertainty in the calculation of τ_{min}, the extrapolated value of k^{-1} at infinite threonine concentration. Negative values of τ_{min} have no realistic, physical meaning. Positive values of τ_{min} appear to range from 6 to 18 s or roughly 100 times the τ_{min} values for HSD. Variations of conditions, as shown in Table I, have no large effect on τ_{min} within the limits of error with one exception: no transient was observed when the temperature was raised to 37 °C. The lack of a transient could be due either to a large decrease or increase in k with temperature. Plots of k^{-1} vs. THR^{-1} at 20 and 24 °C (Figure 2) seemed to indicate the latter. The activity at 37 °C was almost completely inhibited by high threonine concentration also suggesting that the observed ve-

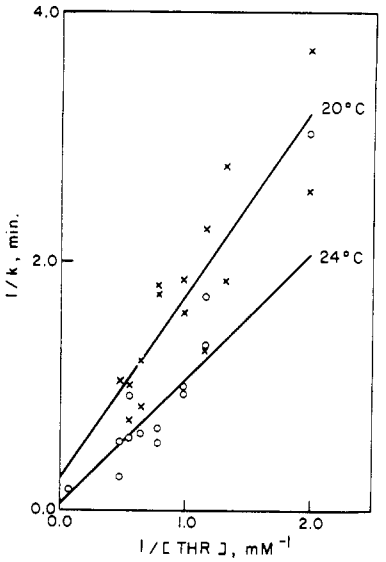

FIGURE 2: Plot of k^{-1} vs. $[THR]^{-1}$ at two different temperatures. AK assay conditions were pH 6.9 in buffer A. Solid lines are least-squares approximations to the experimental data.

TABLE I: Inhibition of AK Initial Velocity.

pH	temp (°C)	additions[a]	n_H (± 0.3)[b]	K_I (mM) (± 0.05)[b]	τ_{min} (±SE) (min)
			Threonine		
6.9	24		3.2	0.62	0 (± 0.2)
6.9	20		3.8	0.57	0.2 (± 0.3)
7.2	20		3.7	0.85	0.3 (± 0.1)
7.2	27		4.0	1.10	
7.6	20		4.2	0.96	−0.3 (± 0.3)
7.2	20	0.1 M choline	4.3	0.86	0.1 (± 0.3)
7.2	20	0.8 M choline	4.4	0.68	−0.8 (± 0.5)
7.2	20	0.15 M KCl[c]	3.5	0.37	0.06 (± 0.05)
7.2	20	K_m of ATP[d]	3.9	0.55	
7.2	20	B[e]	4.0	0.46	0.14 (± 0.07)
			Serine		
7.2	20		2.7	58.4	−0.8 (± 0.5)

[a] Buffer A: 0.12 M Tris, 0.6 M KCl, 10 mM Asp, 3.3 mM ATP, 5 mM $MgCl_2$, and 1 mM DTT (unless otherwise noted). [b] Largest standard deviation. Values obtained from Hill plots. [c] [KCl] lowered to 0.15 M, buffer A. [d] [ATP] lowered to 0.18 mM. [e] Parameters obtained in buffer B: 0.06 M potassium phosphate, 3 mM EDTA, 0.5 mM DTT, 0.15 M KCl.

locity is the steady-state velocity (see later). The transient induced by serine had a τ_{min} consistent with τ_{min} values obtained with threonine (Table I).

The uncertainty in determinations of τ_{min} resulted in larger uncertainties in the calculation of K_k (the threonine concentration required for k to reach half its maximal value). However, the magnitude of K_k (data not shown) was found consistently to be on the order of 10 mM, which is 10-fold greater than K_I.

Initial Velocity, V_{Ai}, Analysis. Unlike the initial velocity, V_{Hi}, for the HSD activity (Bearer & Neet, 1978b), the initial velocity, V_i, for AK activity was already inhibited by threonine at the earliest observable time, 10 s. The inhibition of V_{Ai} is shown in Figure 3. Two characteristics of this plot stand out: (1) the sigmoidicity of the curve, and (2) the decrease of the initial velocity to a limiting value at saturating concentrations of threonine. Hill plots of this data, as described (Bearer & Neet, 1978b), gave the Hill coefficients and K_I values in Table I. With increases in temperature from 20 to 24 °C at pH 6.9 or 20 to 27 °C at pH 7.2, no significant effect on the value of n_H was apparent. With the larger temperature range, a small but significant increase in K_I occurred. Increases in pH from 6.9 to 7.6 also had no effect on n_H, but a small consistent increase in K_I was observed which was significant over the whole range of pH. Addition of 0.8 M choline chloride to the assay mix had minimal effect on the cooperativity of inhibition and caused only a slight decrease in K_I. Decreasing the ATP concentration to its K_m value, 0.18 mM (Wampler & Westhead, 1968; Truffa-Bachi et al., 1968), decreased the K_I from 0.85 to 0.55 mM, but did not affect the cooperativity. Lowering the potassium chloride concentration from 0.6 M to 0.15 M had no effect on the Hill coefficient, but dropped the K_I value from 0.85 mM to 0.37 mM. These values corresponded to those found in buffer B, suggesting that the difference in kinetic properties found in the two buffers was a result of the difference in KCl concentration. Serine inhibition of the AK activity demonstrated a marked difference in cooperativity when compared with threonine. The Hill coefficient for serine was 2.7, whereas for threonine under the same conditions it was 3.7 (Table I).

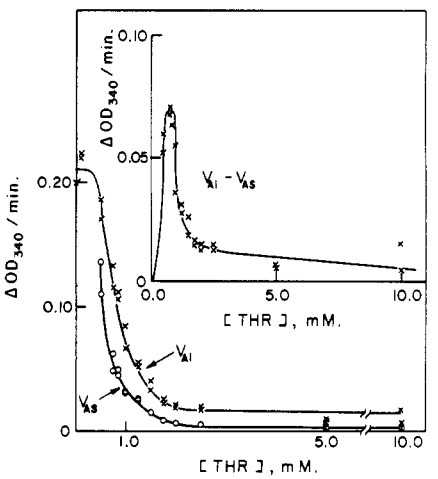

FIGURE 3: Threonine inhibition of AK activity. Initial (V_{Ai}) and steady-state (V_{AS}) velocities plotted as function of threonine concentrations. Assay conditions were pH 7.2, 20 °C, buffer A. Inset: data plotted as $V_{Ai} - V_{AS}$ vs. concentration of threonine.

Steady-State Velocity, V_{AS}, Analysis. The steady-state velocity, V_{AS} (Figure 3), proved difficult to analyze but two characteristics were apparent. At saturating threonine $V_{AS} = 0$, in contrast to the V_{Ai} which always approached a plateau at about 5–15% of uninhibited velocity. Inhibition of initial velocity occurred at lower concentrations of threonine than those required for visualization of the transient. However, the apparent K_I for V_{Ai} and V_{AS} were very similar, although the K_I for V_{AS} appeared to be slightly greater than the K_I for V_{Ai}. Because of these relationships, plots of $V_{Ai} - V_{AS}$ (Figure 3, inset) go through a maximum. Since this inhibition was superimposed on the inhibition of the initial velocity, there was no simple method of analysis to obtain good values for K_I. One parameter which can be estimated was the concentration of threonine which totally inhibited the steady-state velocity. These concentrations were all found to be between 3 and 5 mM under all conditions studied (see Figure 3).

Discussion

Correspondence of V_{Ai} to V_{HS} of HSD. There is good evidence that the V_{Ai} of AK activity (this study) and the V_{HS} of

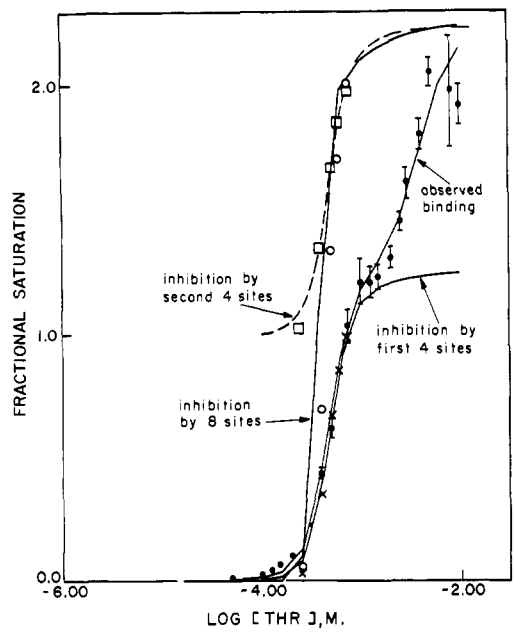

FIGURE 4: Comparison of binding of threonine to AK initial activity inhibition (Bearer & Neet, 1978b) by threonine in buffer A, pH 7.2, and 20 °C. AK activity was assayed in the presence of 0.18 mM ATP. (●) Moles of threonine bound per mole of AK-HSDI; (X) fractional inhibition assuming the first 4 mol of threonine bound per mol of AK-HSDI correlates with maximum inhibition; (○) fractional inhibition assuming 8 mol bound per mol correlates with maximum inhibition; (□) fractional inhibition assuming the second 4 mol of threonine per mol correlates with maximum inhibition. Solid lines are single Monod fits to kinetic data and a sum of two Monod equations for binding data. For binding data: (first fit) $K_R = 0.02$, $K_T = 1.65 \times 10^{-4}$, $L = 10^{-3}$, and $n = 5.5$; (second fit) $K_R = 0.2$, $K_T = 6 \times 10^{-4}$, $L = 10^{-3}$, and $n = 3.5$. For AK inhibition, assuming 4 mol of threonine per mol of enzyme: $K_R = 0.2$, $K_T = 8.5 \times 10^{-5}$, $L = 10^{-4}$, and $n = 5$. Assuming 8 mol of threonine per mol of enzyme: $K_R = 0.2$, $K_T = 7.5 \times 10^{-5}$, $L = 10^{-7}$, and $n = 9$. (Allowing the n value to go to 5 or 9 gave slightly better fits to the Monod equation.)

the HSD activity (Bearer & Neet, 1978b) reflect the same conformation of AK-HSDI induced by threonine. First both V_{Ai} and V_{HS} are inhibited to a limiting, finite value at saturating threonine. Secondly, both inhibitions occur within 10 s. Thirdly, the K_I values for threonine are the same order of magnitude under the same conditions of assay. At pH 7.2, 20 °C in buffer B, the K_I for V_{Ai} is 0.46 mM and for V_{HS} is 0.27$_5$ mM. At pH 7.2, 20 °C in buffer A, the K_I for V_{Ai} is 0.85 mM and for V_{HS} is 0.19 mM. Finally, parallel changes in the values of the K_I occur with variation of assay conditions. Increases in temperature or pH cause small increases in K_I for V_{Ai} or V_{HS}. Addition of choline chloride to the assay mix decreased the value of the K_I for both activities, although for HSD the change is of questionable significance. Small discrepancies between the K_I for the two activities are not contradictory to the interpretation that the same threonine site is responsible for both inhibitions because AK substrates appear to increase the value of K_I for AK (see Table I).

Correlation of High Affinity Site and V_{Ai} Inhibition. As a corollary to the above hypothesis, the high affinity sites which are responsible for the HSD inhibition (Bearer & Neet, 1978b) must also cause the initial velocity inhibition of AK. Better correlation exists between binding and kinetic data (Figure 4) when it is assumed that the first 4 mol of threonine bound per mol of enzyme saturate the inhibition of the initial velocity (symbol X). Neither the assumption that all 8 sites are responsible for inhibition (open circles) nor the assumption that the second set of 4 sites is responsible (open squares) fits the binding curve well (Figure 4).

TABLE II: Comparison of AK Threonine Inhibition to High Affinity Threonine Binding Sites.

pH	buffer[a]	n_H[b]	n_b[c]	K_I[b] (mM)	K_D[c] (mM)
7.2	B		2.7		0.12
7.2	B (+ ATP)		2.5		0.16
7.2	B (Asp, ATP)	4.0		0.46	
7.4	A (Asp)		2.5		0.45
7.2	A (Asp, ATP)	3.7		0.85	
7.2	A (Asp, ATP at K_m)	3.9		0.55	
7.6	A (Asp, ATP)	4.2		0.96	

[a] Buffers A and B as in Table I. Substrates in parentheses are indicated to emphasize differences between different conditions. 10 mM aspartate, 3.3 mM ATP, or 0.18 mM ATP for the experiment at K_m. [b] Hill coefficient and inhibition constant from AK initial velocity, Table I. [c] Hill coefficient and dissociation constant from threonine binding studies (Table I; Bearer & Neet, 1978a).

Direct comparison of K_I and K_D under identical conditions of assay are only possible in two cases because different buffers were used for the two different assays. An observed substrate effect on K_I may be responsible for differences in K_I vs. K_D. When ATP is decreased to its K_m value of 0.18 mM, the K_I is decreased from 0.85 mM to 0.55 mM. This agrees well with the K_D of 0.45 mM (Table II).

Kinetic Mechanism for Cooperativity of V_{Ai} Inhibition by Threonine. From the results of other papers in this series (Bearer & Neet, 1978a,b) we have postulated the HSD cooperativity reflects binding cooperativity because Hill coefficients from both kinetics and binding are 2.2 to 2.9, depending on conditions of assay. However, Hill coefficients for the inhibition by threonine of the V_{Ai} of AK are all approximately 4, independent of substrate concentration. It seems from this result that a kinetic mechanism is involved in the threonine cooperativity of inhibition of AK. AK inhibition is, therefore, the sum of both binding and kinetic cooperativity. The slow transition mechanism (Ainslie et al., 1972) is capable of introducing additional kinetic terms such that the kinetic cooperativity is 4 whereas the binding cooperativity is 2.5. Serine inhibition which has been reported as noncooperative for HSD (Truffa-Bachi et al., 1974; Costrejean & Truffa-Bachi, 1977) has a Hill coefficient of 2.7 for AK. For serine it may be that there is no binding cooperativity but that kinetic cooperativity is still expressed.

AK Transient. The AK transient observed in these studies was different in many respects from the HSD transient. τ_{min} obtained from AK data appeared to have a limiting value on the order of 6 to 18 s when extrapolated to infinite threonine concentrations. τ_{min} of HSD, on the other hand, had a limiting value on the order of tenths of seconds. Over the same range of threonine concentrations, the transients had different time dependencies. For example, at pH 7.2, 20 °C in buffer B, 1.0 mM threonine corresponded to a k_1 value of 1.36 s^{-1} for HSD. Under the same conditions, k for the AK transient was 0.041 s^{-1}. Moreover, the initial velocities for the HSD activity were equal to the velocity in the absence of threonine, whereas the AK initial velocities (after a 10-s mixing time) were inhibited to an extent which depended upon the threonine concentration. From these results it appears that the two transients reflect separate molecular events.

There is evidence that the low affinity site induces the slower transition in the enzyme and produces the inhibition of the steady-state velocity of AK. Because of the difficulty in interpreting the steady-state inhibition, no parameter has been

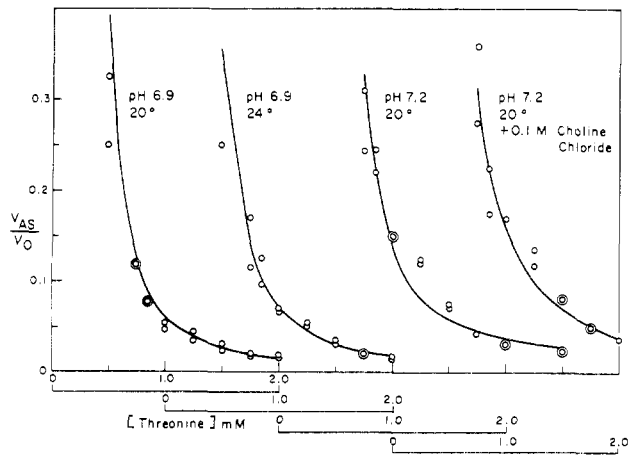

FIGURE 5: Comparison of theoretical and experimental values of the steady-state velocity of aspartokinase activity, V_{AS}. Points are experimental data obtained in buffer A under the conditions indicated. Continuous lines are calculated from eq 13 with the following values of the constants:

	n_1	K_1	n_2	K_2	V_{AiS}	V_0
pH 6.9, 20 °C	4	0.45	2.4	1.2	0.01	0.151
pH 6.9, 24 °C	4	0.45	2.4	1.2	0.014	0.167
pH 7.2, 20 °C	4	0.6	2.6	2.6	0.01	0.21
pH 7.2, 20 °C + choline Chl (0.1 M)	4	0.6	2.4	1.2	0.02	0.118

The threonine concentration scale is shifted to the right for each curve as indicated.

calculated to compare with the K_D for the second binding site. However, we have shown that concentrations of threonine on the order of 3–5 mM are required to saturate the process that is responsible for the steady-state inhibition (Figure 4). The saturation of the low affinity site corresponds to this range of threonine concentrations (Bearer & Neet, 1978a). The high affinity site appears to be completely saturated at 1 mM threonine. It seems reasonable to assume, then, that the low affinity site for threonine is responsible for the steady state inhibition of the AK activity.

Correlation of Model with Experimental Results. We have compared data obtained from the steady-state velocities of AK activity (after the 1–2-min transient) with the predictions of the analysis in the theoretical section. Four conditions were examined (Figure 5) in which the data were sufficient (solid circles) to make an adequate comparison. The solid lines of Figure 5 were calculated from eq 13 with the parameters listed in the legend to the figure. The values of n_1, K_1, n_2, and K_2 were chosen within the limitation of the values obtained in independent kinetic or binding experiments (Table I and Bearer & Neet, 1978a,b) with a slight adjustment to give the best visual fit to the data. V_{AiS} and V_0 were obtained from the initial velocity at saturating threonine and the uninhibited velocity, respectively, for the same conditions. No statistical fitting method to obtain the parameters was used so that the process used represents a test of the model developed above and the parameters measured separately.

For all the calculated curves, a value of 4 for n_1 was used since this is the extent of cooperativity measured for the initial velocity of AK and differs from the n_b value from binding data. The shape of the curves is not very sensitive to the value of n_1 and equally good fits were obtained with a value of $n_1 = 2.5$, with slight adjustments to the other parameters. Thus, analysis of the steady-state velocities of this model does not yield information on the cooperativity of the initial velocity. It is clear from analysis of the initial velocity (Table I), however, that the *kinetic* value for n_1, as defined in eq 6 is about 4.

For the pH 7.2, 20 °C curve the values used for n_2 and K_2 (Figure 5) were taken directly from the binding data under the same conditions. The value of K_1 was adjusted upward slightly (0.45 to 0.66 mM) which presumably reflects the kinetic effect of ATP on the interaction, although we have shown (Bearer & Neet, 1978a) that ATP has no effect on the binding of threonine in buffer B. For the pH 7.2 plus choline curve (Figure 5) the same values of the parameters provided a good fit, except for a lowering (2.6 to 1.2 mM) of the value for K_2. This twofold effect of choline on K_2 was expected from the observed effect on threonine binding (Bearer & Neet, 1978a).

For the pH 6.9 curves the value of K_1 was adjusted down slightly (0.6 to 0.45 mM) relative to the pH 7.2 curve because of the effect of the lower pH on K_1 from initial velocity measurements. The values for n_2 and K_2 presented (Figure 5) were those obtained from binding studies in buffer B. The expected value of K_2 nearer to the 2.6 mM observed for pH 7.2 in buffer A did not fit nearly as well. Although less kinetic data were available in buffer B, a reasonable fit was also obtained using the same process described above for buffer A.

Obviously, the satisfying agreement between experiment and data presented here does not prove that the model is correct, but only that the data are consistent with it. None of the data suggests that a different degree of cooperativity (n_2 = 2.4–2.6) is required for the kinetics than for the binding at the second set of threonine sites. Thus the second, slow (1–2 min) transition in the AK activity does not appear to contribute to the cooperativity of the kinetics.

Model for Conformational Changes of AK-HSD. We propose herein a model for the monomer of AK-HSDI (Figure 6) which takes into account various aspects of conformational changes and inhibited states upon interaction with threonine. The important features of this model are presented below.

(1) The AK-HSDI monomer is a biglobular protein with the AK and HSD activities residing in the separate halves, based upon limited proteolysis (Veron et al., 1972, 1973; Mackall & Neet, 1974) and mutant enzyme (Veron et al., 1972) studies.

(2) Two separate, distinct sites for threonine binding per monomer of AK-HSDI were identified by binding and kinetic studies (Bearer & Neet, 1978a). One site of threonine binding is proximal to the AK active site for two reasons: (a) the inhibition by threonine of AK activity is competitive vs. aspartate, and (b) recent studies by Tilak et al. (1976) have shown a distance of 4.4 Å between Mn(III)-ATP and threonine. The exact location of the other threonine site, the allosteric site, has not been located yet, although the proteolysis experiments suggest that it is also on the AK half of the molecule (Veron et al., 1972, 1973; Mackall & Neet, 1974).

(3) The high affinity site, t1, is responsible for the inhibition of the steady-state velocity of HSD, V_{HS} (Bearer & Neet, 1978b; Wright & Takahashi, 1977a), and the initial velocity of AK, V_{Ai} (this paper).

(4) The mechanism of cooperativity of inhibition of the two activities is different. Kinetic cooperativity for HSD appears to reflect binding cooperativity, excluding any purely kinetic terms (Bearer & Neet, 1978b). In contrast, the kinetic Hill coefficients for the AK initial velocity are significantly larger than the Hill coefficient obtained from binding (Table II). This result suggests the possibility of a kinetic mechanism for cooperativity of AK inhibition by threonine and could be adequately explained by a slow transition (hysteretic) model (Ainslie et al., 1972; Frieden, 1970).

(5) The low affinity site, t2, correlates with the inhibition

of steady-state velocity of AK activity. The steady-state inhibition and the low affinity threonine binding curve become saturated in the same range of threonine concentrations (Figures 3 and 4).

(6) At least three different conformations (R, T, U) of AK-HSDI are needed to explain the results of this study. A fourth (R/U) (Figure 6) is implicated by kinetic analysis. The recent kinetic and binding data, plus other data from the literature, enable a physical and kinetic description to be made of each conformation.

(a) The R conformation (Figure 6) has no threonine bound to either site t1 or t2. The velocities for both activities are defined as V_{AO} and V_{HO} for AK and HSD, respectively.[2] The distance between the active sites in this conformation is about 29 Å (Wright & Takahashi, 1977b). Subunit interactions between monomers in the R conformation are relatively weak compared with other conformations (Cunningham et al., 1968; Mackall & Neet, 1973). The R conformation is also characterized by 6 to 8 sulfhydryls reactive to PMB (Takahaski & Westhead, 1971) and 24 to 26 to DTNB (Truffa-Bachi et al., 1968).

(b) The binding of 1 mol of threonine per mol of monomer to t1 produces the conformational state T. The velocities of the two activities in the T state are defined as V_{HS}, the steady-state velocity at saturating threonine for HSD (Bearer & Neet, 1978b) and V_{AiS}, the initial velocity at saturating threonine for AK. Both domains of the monomer undergo a conformational change (Figure 6) because both enzymatic velocities are cooperatively inhibited. The T conformation is less susceptible to proteases (Veron et al., 1972, 1973; Mackall & Neet, 1974), possibly as a result of greater subunit interactions. Tyrosine and tryptophan residues are buried in the T conformation as compared with R (Janin & Cohen, 1969), sulfhydryls are protected (Takahashi & Westhead, 1971; Truffa-Bachi et al., 1968), and fewer hydrogens are available for solvent exchange (Takahashi & Westhead, 1971).

(c) The U conformation is invoked as a distinct conformation because of the cooperativity of binding at the second threonine site (Bearer & Neet, 1978a) and because of the AK progress curve transients (Figure 1). Since no further spectroscopic change or inhibition of HSD activity is observed, only the AK domain is shown undergoing a conformational change. The velocity for HSD remains as V_{HS} while the velocity for AK, V_{AS}, is zero. At 5 mM threonine, where U would be the predominant conformation, the distance between the two active sites is about 35 Å, 7 Å greater than the distance in the R conformation (Wright & Takahashi, 1977b). The binding site, t2, is about 4.4 Å from the ATP binding site in this conformation (Tilak et al., 1976).

(d) R/U, the conformation shown in brackets in Figure 6 has been shown to exist because of data from AK-Co(III)-Thr adducts (Wright & Takahashi, 1977a) and kinetic studies (this paper). The HSD domain is unaffected because addition of free threonine results in threonine binding comparable to native enzyme (Wright & Takahashi, 1977a). The AK domain is shown to be in a conformation similar to that of U because AK activity is totally inhibited. The velocities for the R/U conformation are V_{HO} for HSD and zero for AK. The R/U form is significant upon inhibition of native enzyme by threonine. If threonine binding to t1 was prerequisite to binding at t2, i.e., no R/U conformation, then ($V_{Ai} - V_{AS}$) would coincide with the low affinity binding curve (Figure 3). Adequate fitting of the experimental data was obtained with the assumption that binding to the t1 and t2 sites was random and independent (Figure 5). It may be possible that the S conformation observed by Janin & Iwatsubo (1969) is the same as the R/U conformation.

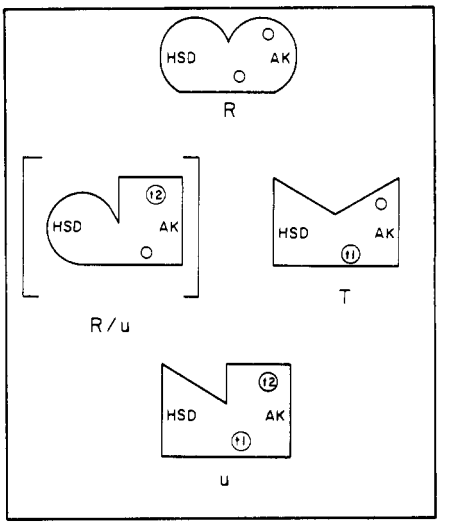

FIGURE 6: A model for the conformations and threonine binding of the monomeric unit of AK-HSDI. R, T, U, and R/U represent the four conformational states of the enzyme. Shapes of each domain (globule) represent specific conformations of the AK or HSD domain which may change semiindependently. Threonine binding sites are represented by t1 and t2. Brackets around the R/U conformation indicate that the evidence for this form is indirect. See text for discussion.

(7) Ligand binding to each of the conformations is presumed to be rapid but transitions between forms are not rapid. The R to T isomerization is slow ($\tau \sim 30$ to 200 m, Bearer & Neet, 1978b) but the T to U isomerization is even slower ($\tau \sim 0.1$ min, Table I).

(8) The enzyme is predominately in a tetrameric state of association, under the conditions of the experiments. The various conformations of Figure 6 are represented simply as a single monomer for reasons of clarity but should more properly be represented as occurring within the tetramer. Interaction of subunits to form the tetramer have been shown to occur through the AK domains (Veron et al., 1973; Mackall & Neet, 1974). The cooperativity of binding (Bearer & Neet, 1978a) is clearly due to the differing subunit interactions in the differing states, R_4, T_4, U_4.

Physiological Regulation of AK-HSD. Physiological advantage of the mechanism of inhibition of AK-HSD by threonine. The interpretation of the mechanism of inhibition of AK-HSD presented in this series of papers has two interesting features which bear upon the physiological role and regulation of this enzyme. The first of these is the 80 to 90% inhibition of both activities by a single set of binding sites for the metabolic regulator, threonine. The second feature is the involvement of a hysteretic mechanism in one of these activities (AK) but not the other (HSD). These two aspects fit together into an understanding of the functioning of the enzyme to control metabolic flux through the pathway for amino acid biosynthesis. The results presented in this series of papers makes it clear that the two activities are regulated simultaneously, at least to 80-90%, by a single set of threonine binding sites. The advantage to the organism of having the majority of the inhibition coordinated to a changing threonine concentration is clear. However, the observation that there is a difference in cooperativity between the two inhibitions, V_{HS} and V_{Ai}, due to the kinetic contribution in the AK activity, allows for an additional, subtle control. At low levels of threonine the HSD activity ($n = 2.3$) would commence to be inhibited first but, as the levels continued to rise, the AK activity would be inhibited more steeply ($n = 4$) and reach its maximum inhibition at a slightly

TABLE III: Comparison of the Cooperativity of Inhibition of HSD and AK by Threonine.[a]

	Thr concn required for % inhibition				ratio Thr required for 81 and 9% inhibition
	9%	45%	81%	99%	
HSD	0.08	0.2	0.52		6.75
AK	0.12	0.2	0.34	4.82	3
Michaelis—Menten enzyme	0.022	0.2	1.8		81

[a] Percentages of inhibition are based on a residual 10% activity for HSD and AK (first set of sites) at "saturating threonine". No further inhibition of HSD occurs but AK activity is completely inhibited at sufficient threonine to saturate the second set of sites. Values are calculated for $K_1 = 0.2$; n_1(HSD) = 2.3; n_1 (AK) = 4.0; n_2 (AK) = 2.5; K_2 (AK) = 2.0. Values calculated for the "Michaelis-Menten enzyme" are for $K_1 = 0.2$; $n_1 = 1.0$; 10% residual activity.

lower threonine concentration. These conditions are calculated for several threonine concentrations in Table III. Utilization of aspartic semialdehyde for synthesis of lysine might be possible at intermediate concentrations of threonine but not above about 1 mM. The important point to notice is that this fine tuning of inhibition by threonine occurs with a single threonine binding site per polypeptide chain by utilizing both cooperativity through subunit interactions and cooperativity through the slow transition mechanism. Such a process as described here gives an explanation for, and an evolutionary advantage of, having cooperativity through a ligand induced slow transition in an oligomeric enzyme where one might not expect it. The importance of looking for hysteresis and accompanying cooperativity in all enzymes is emphasized.

With AK-HSD there is an additional inhibition of the AK activity as threonine concentrations become even higher, due to binding at the second set of sites. This inhibition accounts for complete inactivation of the AK activity and might be important in assuring that no leakage through the pathway to threonine occurs at exceedingly high threonine concentrations. The occurrence of a second transient (minutes) in the AK inhibition might serve the function of damping the response time of the system to high levels of threonine. The study of Szczesiul & Wampler (1976) of systems containing the six enzymes involved in the biosynthetic pathway of threonine is consistent with the observation of a transient in the progress curve of aspartokinase. They found that inhibition of aspartokinase by threonine was the most important step in regulation. Additional data showed that threonine was produced rapidly for the first few minutes of reaction followed by a slower rate of production. During the slower rate of production of threonine, the concentrations of intermediates decreased. It appeared that the inhibition of aspartokinase was responsible for the decrease in threonine production and that this inhibition occurred with a time lag of several minutes. It is interesting to speculate that this lag is a reflection of the lag of inhibition of purified AK reported in this study.

Acknowledgments

We would like to thank Dr. H. E. Umbarger and his laboratory for supplying a culture of *E. coli* K12 TIR 8. We would also like to thank Dr. J. W. Ogilvie's group for their helpful outline of AK-HSDI purification.

References

Ainslie, G. R., Shill, J. P., & Neet, K. E. (1972) *J. Biol. Chem. 247,* 7088.
Bearer, C. F., & Neet, K. E. (1978a) *Biochemistry 17* (first of three papers in a series in this issue).
Bearer, C. F., & Neet, K. E. (1978b) *Biochemistry 17* (second of three papers in a series in this issue).
Costrejean, J.-M., & Truffa-Bachi, P. (1977) *J. Biol. Chem. 252,* 5332.
Cunningham, G., Maul, S., & Shive, W. (1968) *Biochem. Biophys. Res. Commun. 30,* 159.
Falcoz-Kelly, F., Janin, J., Saari, J. C., Veron, M., Truffa-Bachi, P., & Cohen,, G. N. (1972) *Eur. J. Biochem. 28,* 507.
Frieden, C. (1970) *J. Biol. Chem. 245,* 5788.
Heck, H. d'A. (1972) *Biochemistry 11,* 4428.
Jacques, Y., & Truffa-Bachi, P. (1976) *Eur. J. Biochem. 62,* 485.
Janin, J., & Cohen, G. N. (1969) *Eur. J. Biochem. 11,* 520.
Janin, J., & Iwatsubo, M. (1969) *Eur. J. Biochem. 11,* 530.
Janin, J., van Rapenbusch, R., Truffa-Bachi, P., & Cohen, G. N. (1969) *Eur. J. Biochem. 8,* 128.
Mackall, J. C., & Neet, K. E. (1973) *Biochemistry 12,* 3483.
Mackall, J. C., & Neet, K. E. (1974) *Eur. J. Biochem. 49,* 195.
Patte, J.-C., Truffa-Bachi, P., & Cohen, G. N. (1966) *Biochim. Biophys. Acta 128,* 426.
Ryzewski, C., & Takahashi, M. T. (1975) *Biochemistry 14,* 4482.
Shoemaker, D. P., & Garland, C. W. (1967) *Experiments in Physical Chemistry,* p 222, McGraw-Hill, New York, N.Y.
Szczesiul, M., & Wampler, D. E. (1976) *Biochemistry 15,* 2236.
Takahashi, M., & Westhead, E. W. (1971) *Biochemistry 10,* 1700.
Tilak, A., Wright, K., Damle, S., & Takahashi, M. (1976) *Eur. J. Biochem. 69,* 249.
Truffa-Bachi, P., Van Rapenbusch, R., Janin, J., Gros, C., & Cohen, G. N. (1968) *Eur. J. Biochem. 5,* 73.
Truffa-Bachi, P., van Rapenbusch, R., Janin, J., Gros, C., & Cohen, G. N. (1969) *Eur. J. Biochem. 7,* 401.
Truffa-Bachi, P., Veron, M., & Cohen, G. N. (1974) *Crit. Rev. Biochem. 2,* 379.
Veron, M., Falcoz-Kelly, F., & Cohen, G. N. (1972) *Eur. J. Biochem. 28,* 520.
Veron, M., Saari, J. C., Villar-Palasi, C., & Cohen, G. N. (1973) *Eur. J. Biochem. 38,* 325.
Wampler, D. E., & Westhead, E. W. (1968) *Biochemistry 7,* 1661.
Wampler, D. E., Takahashi, M., & Westhead, E. W. (1970) *Biochemistry 9,* 4210.
Wright, J. K., & Takahashi, M. (1977a) *Biochemistry 16,* 1541.
Wright, J. K., & Takahashi, M. (1977b) *Biochemistry 16,* 1548.