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Threonine-Sensitive Aspartokinase from *Escherichia coli*. Magnetic Resonance and Binding Studies†

Robert S. Ehrlich and Mark Takahashi*

ABSTRACT: The interaction of manganese and manganese–ATP with the threonine-sensitive aspartokinase–homoserine dehydrogenase complex of *E. coli* K 12, Tir-8 has been investigated by nuclear magnetic resonance and direct binding techniques. An enhancement in the proton relaxation rate of 14.1 was found for the binary metal–enzyme complex. This enhancement was reduced to 10.1 in the presence of threonine with no significant change in the affinity of the enzyme for manganese. The ternary complex of aspartokinase with metal–ATP had an enhancement of 10.2. Threonine did not change the enhancement but increased the dissociation constant for Mn–ATP from 1 to 2.8 mM. We have found from kinetic studies apparent competitive inhibition between threonine and Mn–ATP. The binding studies indicate that there are four sites for manganese and ATP and are consistent with four sites for Mn–ATP. Manganese–ATP appears to bind the aspartokinase as a metal-bridge complex. A change at the Mn binding site occurs upon addition of threonine which is reflected by the relaxation rate of water at the active site but the cause of this change in enhancement has not been determined.

Threonine-sensitive aspartokinase of *Escherichia coli* is part of an enzyme complex consisting of aspartokinase I–homoserine dehydrogenase I (ATP:L-aspartate 4-phosphotransferase, EC 2.7.2.4; L-homoserine:NADP oxidoreductase, EC 1.1.1.3) which catalyzes the first and third steps in the sequence of reactions leading to the biosynthesis of threonine from aspartate. It has recently been shown that the catalytically active form of aspartokinase–homoserine dehydrogenase consists of four identical subunits (Falcoz-Kelly *et al.*, 1972; Wampler, 1972) and that the two activities reside on opposite ends of each polypeptide chain (Véron *et al.*, 1972). Binding studies have indicated four sites for NADPH (Janin *et al.*, 1969; Falcoz-Kelly *et al.*, 1972) and six to eight sites for threonine (Janin and Cohen, 1960; Takahashi and Westhead, 1971). It has been assumed (Heck, 1972) that the enzyme has four binding sites for aspartate and ATP. In the present report we will show data indicating four binding sites for manganese and ATP and also data which is consistent with four manganese–ATP sites.

Both enzyme activities are subject to inhibition by threonine. For homoserine dehydrogenase the inhibition by threonine is noncompetitive with respect to NADPH and aspartic semialdehyde (Patte *et al.*, 1963). For aspartokinase, Stadtman *et al.* (1961) have found competitive inhibition between threonine and aspartate, but the competitive nature of the inhibition has been questioned by Wampler and Westhead (1968). Wampler and Westhead (1968) have also found that the curve of per cent inhibition *vs.* threonine concentration is shifted toward higher threonine concentrations as ATP is increased, indicating antagonism between ATP and threonine. Our kinetic studies show threonine is competitive with ATP at saturating aspartate.

It has been postulated that control of both enzyme activities is produced by a threonine-induced change from a relaxed configuration of the enzyme to a tight configuration

† From the Department of Biochemistry, College of Medicine and Dentistry of New Jersey, Rutgers Medical School, University Heights, Piscataway, New Jersey 08854. *Received May 14, 1973.* Supported by U. S. Public Health Service Grant GM-18628.

(Cohen, 1969). Such a configurational change has been monitored by uv difference spectroscopy, protein fluorescence (Janin and Cohen, 1969), and sulfhydryl group reactivity (Heck and Truffa-Bachi, 1970; Heck, 1972). While these techniques measure the overall enzyme conformation, nuclear magnetic resonance (nmr) is sensitive to the specific conformation at the active site. The water proton relaxation rate[1] (prr) method has been used extensively to study the interaction of metal and ligands at enzyme active sites (Mildvan and Cohn, 1970). We have used prr with a manganese(II) probe to detect changes in the metal-enzyme complex upon addition of threonine. Prr measurements also allowed calculations of enzyme–ligand dissociation constants and made possible the distinction between ATP–metal bridge and metal–ATP bridge complexes with aspartokinase.

Experimental Section

Materials. Aspartokinase was isolated from *E. coli* K 12, Tir8 by a modification of the method of Truffa-Bachi *et al.* (1968). Cells were grown in 500-l. batches at the Waksman Institute of Microbiology, Rutgers University. About 1 kg of wet paste was suspended in 20 mM potassium phosphate buffer (pH 7.2) containing 0.15 M KCl, 2 mM magnesium acetate, 2 mM EDTA, 1 mM L-threonine, and 0.1 mM dithiothreitol and passed twice through a Manton-Gaulin homogenizer at 2°. The rest of the purification followed the published method. The enzyme was stored at 4° in 20 mM potassium phosphate buffer (pH 7.2) containing 0.1 mM L-threonine, 0.1 mM EDTA, 1 mM magnesium acetate, and 0.1 mM dithiothreitol. Protein concentration was determined by utilizing a value for the molar extinction coefficient at 278 nm of 209,000 (M. Takahashi, unpublished result). The molecular weight of the enzyme was assumed to be 360,000 daltons (Truffa-Bachi *et al.*, 1968). The enzyme had a specific activity of 100 μmol min^{-1} mg^{-1} protein assayed with the homoserine dehydrogenase assay (Wampler and Westhead, 1968) and showed less than 20% loss of activity when kept at 4° over a period of up to 1 year. ATP, L-threonine, and Hepes were obtained from Sigma. Manganese solutions were prepared from dried reagent grade $MnCl_2$ and the concentrations were checked by atomic absorption (Varian Techtron Model AA4). All other materials were reagent grade. Water used was triply deionized. ^{54}Mn was obtained from New England Nuclear Corporation. ATP-γ-^{32}P was obtained from Dr. Charles Brostrom (Department of Pharmacology, Rutgers Medical School). Before use the enzyme was dialyzed twice against greater than 100-fold volumes of the appropriate buffer at room temperature for over 5 hr.

The buffer used for most of these studies (KN.5) contained 10 mM Hepes (pH 7.5), 1 mM dithiothreitol, and 0.5 M KNO_3. Other buffers used in the nmr studies contained different amounts of potassium ion.

Electron Spin Resonance (esr) Studies. Spectra were run on a Varian E-12 spectrometer at 9.4 GHz. Enzyme concentrations of 50–60 μM were used. The samples were contained in 1-mm i.d. quartz capillaries. The sum of amplitudes of the six hyperfine peaks was used as a measure of the free manganese concentration.

Binding Studies. Ligand binding to enzyme was measured with a membrane ultrafiltration device (Paulus, 1969) supplied by Medical Research Apparatus Corp. Diaflo PM-10 membranes (Amicon) were used. These membranes did not bind Mn or ATP but retained 2.7 μl of solution, or about twice as much as the UM-10 membranes. For the manganese binding studies the ^{54}Mn solution in 0.1 M HCl was neutralized with 0.05 M Hepes and then diluted with $MnCl_2$ solution to give a specific activity of 5 Ci/mol. Samples containing varying amounts of labeled manganese and approximately 10^{-5} M aspartokinase in a volume of 0.2 ml of KN.5 buffer were forced through the membranes under 40 psi nitrogen. The bottoms of the membranes were washed with ethylene glycol and the membranes were placed in Aquasol (New England Nuclear Corp.) and counted using a Packard 3310 TriCarb liquid scintillation counter. ATP-γ-^{32}P was diluted to a specific activity of about 0.5 Ci/mol. All points are an average of three or more determinations.

Nuclear Magnetic Resonance Studies. Measurements of the longitudinal relaxation time (T_1) of water were made on a Magnion pulsed spectrometer at 30 MHz with probe modified to allow temperature control. Samples of 75–125 μl were contained in small glass tubes. The variation in amplitude after a 180–90° pulse sequence or the null method (Carr and Purcell, 1954) were used to determine T_1. When the null method was used the null time of a standard manganese solution was also measured to check the effects of errors in pulse-length duration. Errors in relaxation time determinations are estimated to be about ±5%. Titrations of manganese with aspartokinase were performed by successive dilutions of solutions containing 0.1 mM Mn(II) and a high enzyme concentration with buffer containing 0.1 mM manganese. Titrations with the ligands ATP and threonine were performed by adding small quantities of concentrated solution containing 0.1 mM Mn(II) and the ligand to manganese–aspartokinase solutions; alternatively, aliquots of a manganese–aspartokinase solution containing ligand were added to an identical solution lacking ligand. In the first case small corrections were made for the effect of dilution.

Results are expressed in terms of observed enhancements (Eisinger *et al.*, 1962), $\epsilon = (1/T_{1_p})_E/(1/T_{1_p})$, where $(1/T_{1_p})$ and $(1/T_{1_p})_E$ are the paramagnetic contributions to the relaxation rate in the absence and presence of enzyme. Results for binary complexes were fit by choosing values of K_D = [E][Mn]/[EMn] and calculating from this the free [Mn] and bound [EMn] concentrations of manganese. These were used to determine the intrinsic enhancement of the binary complex, ϵ_b, from

$$\epsilon = [Mn]/[Mn]_t + [EMn]\epsilon_b/[Mn]_t \quad (1)$$

where $[Mn]_t$ is the total manganese concentration. The value of K_D giving the smallest standard deviation in ϵ_b was chosen.

For the ternary complexes, an analogous equation was used to calculate the enhancement of the ternary EMn–ATP complex, ϵ_t.

$$[Mn]_t\epsilon = [Mn] + [Mn\text{–}ATP]\epsilon_a + [EMn]\epsilon_b + [EMn\text{–}ATP]\epsilon_t \quad (2)$$

The enhancement, ϵ_a, of the Mn–ATP complex and dissociation constant K_1 = [Mn][ATP]/[Mn–ATP] have been determined by titration of the prr to be $\epsilon_a = 1.7$ and $K_1 = 27 \times 10^{-6}$ M. The ATP–aspartokinase dissociation constant, K_S = [aspartokinase][ATP]/[aspartokinase–ATP], was determined using ultrafiltration. Values for K_1, K_S, and K_D were fixed, and [Mn], [Mn–ATP], [E–Mn], and [E-Mn–ATP] were calculated

[1] Abbreviations used are: prr, proton relaxation rate; Hepes, *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid.

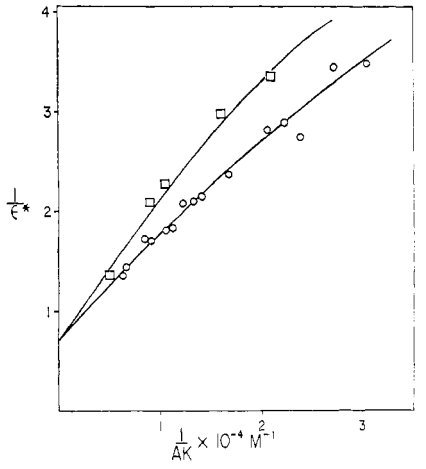

FIGURE 1: Titration of manganese with aspartokinase. The reciprocal of the enhancement (ϵ^*) in proton relaxation rate is plotted against reciprocal enzyme concentration. The solution contains 0.1 mM (○) or 0.5 mM (□) Mn in KN.5 buffer. The lines are computer solutions of eq 1 and the equilibrium equation for K_D = 670 μM and ϵ_b = 14.1.

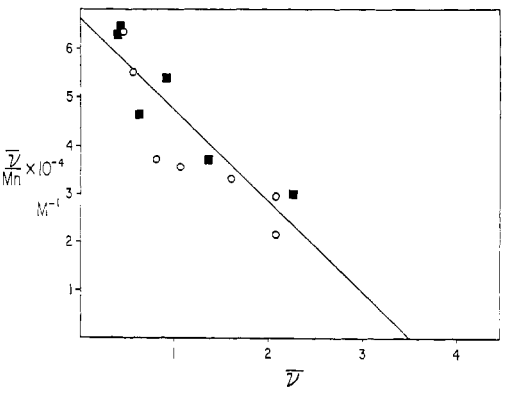

FIGURE 2: Scatchard plot for the binding of manganese to aspartokinase in KN.5 buffer. The measurements using esr signal amplitudes (■) use samples containing 50–60 μM aspartokinase, and the ultrafiltration samples (○) contain about 10 μM aspartokinase, $\bar{\nu}$ = [EMn]/[E]$_t$, where [EMn] is the concentration of bound manganese and [E]$_t$ the total enzyme concentration. [Mn] is the concentration of free manganese. The straight line is a least-squares fit to the combined data.

for various values of K_2 = [Mn–ATP][E]/[EMn–ATP] using a computer routine obtained from Reed (Reed *et al.*, 1970). Several different enzyme concentrations were used and the resulting values averaged. The value of K_2 was chosen to give minimum standard deviation in ϵ_t. Systematic errors larger than the quoted error are possible because the weak binding allows only a small part of the saturation curve to be covered.

Kinetic Studies. Aspartokinase activity was measured by the ferric chloride assay (Wampler and Westhead, 1968) and by an enzymatic assay. The latter mixture contained: 120 mM Tris (pH 7.5), 0.6 M KCl, 16 mM aspartate, varying amounts of Mn(II)–ATP, and up to 0.04 unit of aspartokinase. The reaction was allowed to proceed 2 min after initiation by addition of enzyme and stopped with threonine (10 mM). Phosphoenolpyruvate (3.5 mM), Mg (1.5 mM), and NADH (0.31 mM) were added and the optical density at 340 nm was read. Pyruvate kinase (15 units) and lactic dehydrogenase (25 units) were added and the optical density at 340 nm was read when equilibrium was reached. Activities quoted are the average of duplicate assays.

Results

Manganese–Enzyme Binary Complex. Enhancements of the proton relaxation rate of water produced by manganese in aspartokinase solutions were measured as a function of enzyme concentration. Reciprocals of the enhancements are shown in Figure 1 as a function of reciprocal enzyme concentrations for total manganese concentrations of 0.1 and 0.5 mM. Solutions containing 0.5 M potassium were used because early experiments showed a large number of manganese binding sites (>5) at high manganese concentrations when 0.1 M potassium was used. A fitting procedure was used in which the dissociation constant was chosen and ϵ_b calculated for each point by means of eq 1. A minimum in the standard deviation for all such values of ϵ_b from their mean was sought assuming four binding sites. Since several minima were found for the titrations at 0.1 mM manganese, the degeneracy was removed by using data taken at 0.5 mM manganese. The values that best fit the data are K_D = 670 ± 70 μM and ϵ_b = 14.1 ± 0.9.

To confirm that this fit was satisfactory and that the assumption of four binding sites was correct, direct binding experiments were done. A Scatchard (Scatchard, 1949) plot of data on the binding of manganese to aspartokinase in buffer KN.5 is shown in Figure 2. The number of binding sites and dissociation constants obtained from the ultrafiltration data is n = 3.3 ± 0.9 and K_2 = 510 ± 150 μM while the values from esr data are n = 3.7 ± 0.9 and K_2 = 550 ± 130 μM. An average of these sets of results gives 3.5 ± 0.9 sites and K_D = 530 ± 140 μM.

An attempt to observe an esr signal from bound manganese was made but no spectrum could be obtained. This probably means that manganese bound to aspartokinase is in a region of large zero field splitting (Reed and Cohn, 1970).

Effect of L-*Threonine on Binary Complex.* Since threonine is known to produce a conformational change in aspartokinase we investigated its effect on the prr of the Mn–aspartokinase solution. The enhancement of the proton relaxation rate as a function of reciprocal L-threonine concentration is shown in Figure 3A. The enhancements are extrapolated to infinite threonine concentration for each enzyme concentration. The results of these extrapolations are plotted against enzyme concentration in Figure 3B. These data were fit in the same way as the data in the absence of threonine, with the assumption that binding of manganese to threonine could be ignored.

The dissociation constant for manganese from aspartokinase in the presence of threonine is found to be 670 ± 80 μM, and the enhancement of bound manganese 10.1 ± 0.7. In an ultrafiltration experiment 2.5 mM threonine was found to change the ratio of bound manganese to total enzyme by less than 10% confirming the result derived from prr data which showed that threonine does not change the affinity of the enzyme for free manganese. Proton relaxation rates in a sample containing threonine were compared with prr in a sample without threonine over the temperature range 2–40°. No difference in the temperature dependence of the prr was found. The temperature coefficient in both cases is negative, indicating that chemical exchange is not the rate-limiting process.

The values of threonine concentration, $K_{1/2}$, at which the enhancement was changed by 50% was determined from Figure 3A for each enzyme concentration, the average value is 0.45 mM. In another experiment in which the potassium concentration was lowered to 0.02 M but ionic strength was kept at 0.5 with $NaNO_3$, the $K_{1/2}$ was found to be 0.14 mM.

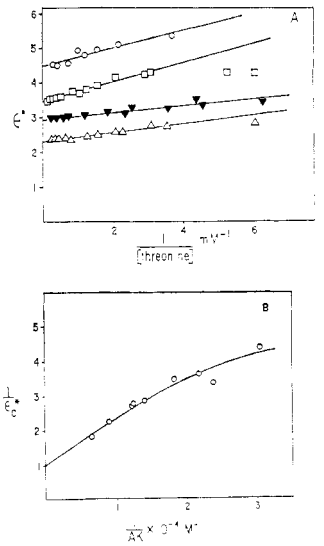

FIGURE 3: (A) Titration of manganese–aspartokinase with L-threonine. The observed enhancements are plotted as a function of reciprocal threonine concentration for four concentrations of aspartokinase: (○) 112, (□) 71, (▼) 42, (△) 33 μM. All solutions contain 0.1 mM manganese in KN.5 buffer. The lines show extrapolation to infinite threonine concentration to obtain ϵ_c.* (B) Plot of enhancements extrapolated to infinite threonine concentration (ϵ_c*). A reciprocal plot as in Figure 1 is used. Additional data not in Figure 3A are included here. The line is the calculated curve for $\epsilon_b = 10.1$ and $K_D = 670$ μM.

The values for half-saturation by threonine ($K_{1/2}$) are consistent with values from direct binding experiments; Heck (1972) finds half-saturation at 0.57 mM (0.6 M KCl–10 mM aspartate) while Cohen (1969) finds half-saturation at 0.040 mM at 0.02 M KCl. The assumption that binding of manganese to threonine could be ignored was tested by measuring the Mn–L-threonine dissociation constant using esr. A dissociation constant of 90 ± 10 mM was found. An additional measure of the dissociation constant was obtained by adding D-threonine (which does not bind to the enzyme but binds Mn as well as L-threonine does) to a Mn–enzyme solution. A manganese–threonine dissociation constant of 100 mM was found. Since the concentration of L-threonine in the experiments shown in Figure 3 is less than 6 mM, binding of manganese by threonine could be ignored.

Ternary Complex with Mn–ATP. Since the binding of ATP and Mn–ATP to aspartokinase is fairly weak, errors involved

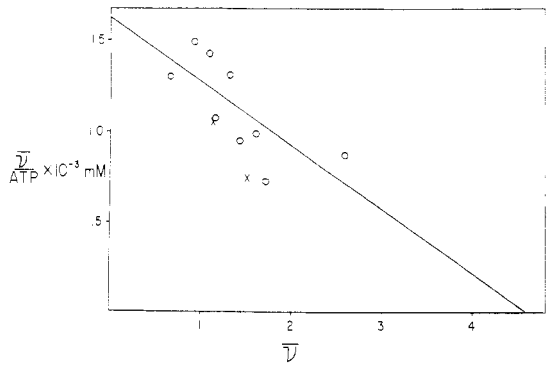

FIGURE 4: Scatchard plot of ATP-γ-^{32}P binding to aspartokinase. $\bar{\nu}$ = concentration of bound ATP/total enzyme concentration; [ATP] = concentration of free ATP. The line is a least-squares fit giving 4.6 ± 1.6 sites and K_S = [ATP][E]/[EATP] = 2.8 ± 1.0 mM: (○) experiments done in the absence of threonine; (×) experiments in the presence of 2.5 mM L-threonine.

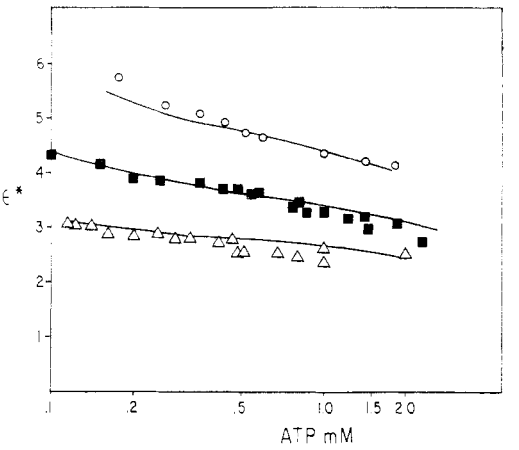

FIGURE 5: A plot of observed enhancement as a function of ATP concentration for three enzyme concentrations: (○) 151, (■) 83, (△) 43 μM. The lines are computed using K_S = 2.8 mM, K_1 = 27 μM, K_D = 600 μM, K_2 = 1 mM, ϵ_a = 1.7, ϵ_b = 14.1, and ϵ_t = 10.2. All solutions contain 0.1 mM Mn in KN.5 buffer.

in direct determination of binding constants using ultrafiltration are large. Figure 4 shows a Scatchard plot of the data for ATP-γ-^{32}P binding to aspartokinase in the absence of metal. The best computer fit gives 4.6 ± 1.6 sites and K_S = [E][ATP]/[EATP] = 2.8 ± 1.0 mM. These values were used in the treatment of data on the ternary complex. The prr enhancement is decreased when ATP is added to manganese–aspartokinase solutions (Figure 5). Part of the decrease in enhancement is due to the decreased binding of Mn–ATP compared to Mn in these samples. A fit by the method described under the Experimental Section with K_2 = 1000 ± 350 μM was obtained giving ϵ_t = 10.2 ± 0.9. This dissociation constant for Mn–ATP was checked by ultrafiltration using labeled Mn and labeled ATP. In analyzing these data the assumption of four sites is made and the multiequilibria are solved for the total amount of labeled species bound. The values of K_2 determined from each experimental point were averaged. The result is 1030 ± 200 μM for the case where labeled ATP-γ-^{32}P was used to determine bound Mn(II)–ATP (Figure 6A). The data using ^{54}Mn are given in Table I and a dissociation constant for Mn(II)–ATP from these data is K_2 = 960 ± 160 μM. The consistency of the results using two labels lends confidence in the reliability of the values determined for the dissociation constant for Mn–ATP.

When aspartokinase is added to a Mn–ATP solution the electron spin resonance spectrum is observed to decrease in amplitude. This and the reduction in enhancement in going from binary to ternary complex are indicative of metal-bridge complexes (Mildvan and Cohn, 1970).

Effects of Threonine on the Ternary Complex. Since threonine has an effect on the binding and the prr enhancement of manganese in the binary complex, similar experiments were done on the ternary complex which is expected to more closely resemble the active complex. Addition of 2.5 mM threonine to aspartokinase–Mn–ATP solutions results in a reduction in the amount of bound Mn–ATP (Figure 6). At 0.75 mM Mn–ATP the decrease in bound metal–nucleotide (Figure 6B) reaches a plateau at threonine concentrations over 1 mM. Analysis of the data (Figure 6A) for Mn–ATP binding in the presence of threonine yields a dissociation constant K_2 for Mn–ATP in the presence of threonine of 2.6 ± 0.5 mM.

The prr was examined to see if threonine decreases the enhancement in the same manner as for the binary complex or if its effect is only on the dissociation constant, K_2. Threonine

TABLE I: Manganese Binding in the Presence of ATP.[a]

Manganese (mM)	ATP (mM)	Enzyme (μM)	Mn(II) Bound Total Enzyme
0.25	2.75	10.7	0.56
0.5	2.75	10.7	0.76[b]
0.75	2.75	10.7	0.96
1.0	2.75	10.7	1.70
1.5	2.75	10.7	2.48
0.4	2.14	11.3	0.97[c]
0.4	5.7	11.3	0.76
0.25	1.78	12.1	0.56
0.5	1.78	12.1	0.96
0.75	1.78	12.1	1.57
1.00	1.78	12.1	1.68
0.3	2.50	10.3	0.52
0.5	2.5	10.3	0.77
0.7	2.5	10.3	1.08
1.0	2.5	10.3	1.42

[a] Binding was measured using ^{54}Mn as described in the Experimental Section. [b] Addition of 2.5 mM threonine reduced this ratio to 0.30. [c] Addition of 2.5 mM threonine reduced this ratio to 0.28.

causes a decrease in the observed enhancement. The observed ternary complex enhancements were extrapolated to infinite threonine concentration in the same manner as was done for binary complexes. The resulting enhancements are shown in Figure 7 plotted as a function of ATP concentration. The best fit to our results gives $K_2 = 3.0 \pm 0.7$ mM, with $\epsilon_t = 9.9 \pm 1.3$. Thus, threonine does not change the enhancement of bound Mn–ATP significantly but does change the affinity of aspartokinase for manganese–ATP.

Kinetic Studies. The finding of competition between threonine and Mn–ATP binding using prr and ultrafiltration prompted us to look at the effect of threonine on the kinetics of Mn–ATP saturation of aspartokinase. Figure 8 shows a double

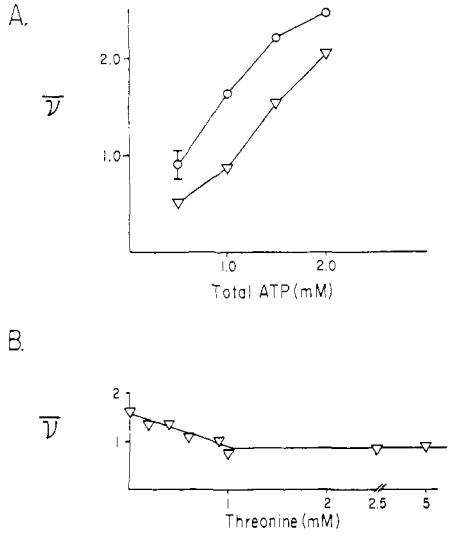

FIGURE 6: (A) Binding of Mn–ATP-γ-^{32}P to aspartokinase without (○) and with (▽) 2.5 mM threonine. The Mn concentration is $^3/_4$ the ATP concentration. $\bar{\nu}$ = total ATP bound/total enzyme concentration. (B) Binding of ATP-γ-^{32}P in a solution of 1 mM ATP–0.75 mM $MnCl_2$ in KN.5 buffer as a function of added threonine. The lines have no theoretical significance.

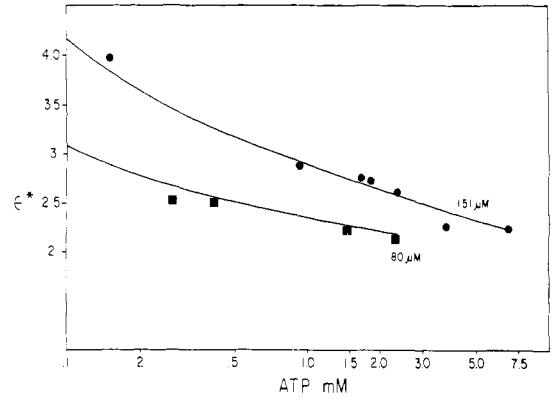

FIGURE 7: Extrapolated enhancements of solutions containing Mn, ATP, and aspartokinase. The extrapolations to infinite threonine concentrations were done as in Figure 3A. The observed enhancements are plotted as a function of ATP concentration for two enzyme concentrations: (●) 151, (■) 80 μM. Curves are computed with parameters of Figure 5 except $K_2 = 3.0$ mM; $\epsilon_t = 9.9$.

reciprocal plot of aspartokinase activity done as a function of Mn–ATP at saturating aspartate concentration. ATP was kept at a concentration of 1.5 times that of Mn(II) so that at all concentrations little inhibition by free manganese or ATP is expected. The result of the absence of threonine is an apparent K_m of 0.19 mM for Mn(II)–ATP. Threonine at three different concentrations is seen to produce competitive inhibition.

It should be noted that the data shown in Figure 8 indicate a nonhyperbolic dependence on threonine concentration. Measurement of the threonine binding and inhibition curves has been made by many authors (Janin *et al.*, 1969; Heck, 1972). The binding and inhibition are cooperative. A detailed study of threonine binding is currently being pursued.

At high ATP (3–11 mM) an apparent K_m for Mn(II) of 0.22 ± 0.05 mM was obtained using both enzymatic and ferric chloride assays. Inhibition is observed when Mn concentration exceeds ATP concentration and gives an approximate inhibition constant of 0.7 ± 0.3 mM for free Mn.

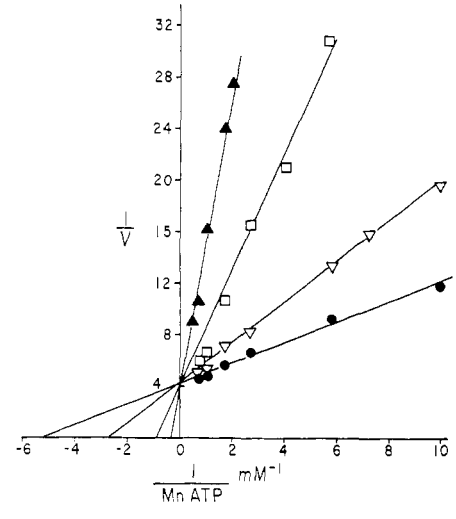

FIGURE 8: Plot of reciprocal of aspartokinase activity *vs.* reciprocal of the concentration of Mn–ATP at the various concentrations. The solutions contained ATP and manganese in the ratio 3:2 and the velocities were measured by the pyruvate kinase assay as described under the Experimental Section. The solutions contain: (●) no threonine, (▽) 0.8 mM threonine, (□) 1.2 mM threonine, (▲) 1.6 mM threonine. Velocities are measured as change in absorbance at 340 nm/min.

TABLE II: Summary of Results.

Ligand	Prr Enhancement	Dissociation Constant[a] (mM)
Mn	14.1 ± 0.9	0.6 ± 0.15
Mn + threonine	10.1 ± 0.7	0.67 ± 0.08
ATP		2.8 ± 1.0
Mn–ATP	10.2 ± 0.9	1.0 ± 0.3
Mn–ATP–threonine	9.9 ± 1.3	2.8 ± 0.6

[a] Dissociation constants were averages of results from direct binding and nmr.

Discussion

The data presented show that there are four ATP and four Mn(II) sites per 360,000 molecular weight of enzyme and are consistent with four Mn–ATP sites. These results support the presence of both kinase and dehydrogenase activities on identical subunits since previous findings have shown four homoserine dehydrogenase sites per 360,000 molecular weight. The dissociation constants obtained directly by esr and ultrafiltration agree with those derived from nmr titrations. Table II summarizes these dissociation constants and the resulting enhancements. The binding constant for manganese is consistent with the inhibition constant for excess manganese. It is thus likely that the Mn binding is at the active site. Free ATP has been found to bind weakly to the enzyme and inhibition at high ATP concentrations has been noted previously (D. E. Wampler, private communication). The dissociation constant found for Mn–ATP is six times the apparent K_m (0.19 mM) found there. This value for K_m is in agreement with that (0.19 mM) found by Wampler and Westhead (1968) for ATP in the presence of saturating aspartate and 5.7 mM Mg^{2+} using a pyruvate kinase coupled assay. It is possible that ATP binding is altered by the presence of aspartate. Since ADP is known to enhance aspartate binding (Truffa-Bachi and Heck, 1971; Heck, 1972) and aspartate influences ADP binding, an interaction between ATP binding and aspartate binding is also possible.

The large enhancement in the binary Mn–aspartokinase system and the reduction upon adding ATP to form a ternary complex are indicative of a metal-bridge complex (Mildvan and Cohn, 1970). Supportive evidence for this conclusion is the observation that there is inhibition by Ca^{2+}. It has been found (Mildvan, 1970) that enzyme systems where metal–nucleotide–enzyme complexes form are generally activated by Ca^{2+} while those where nucleotide–metal-enzyme complexes form are inhibited by Ca^{2+}. Further confirmatory evidence for a metal-bridge complex was obtained by examining the esr spectrum of Mn–ATP complex. Upon addition of aspartokinase the amplitude of this spectrum is reduced, presumably because of broadening caused by binding of the complex to the enzyme. Binding through a nucleotide bridge would not be expected to alter the esr spectrum since the ligand field at the manganese atom is unaltered.

The relaxation rate for the enzyme–metal system could be determined by T_{1M}, the relaxation time for protons in water coordinated to bound manganese, or by τ_m, the residence time of H_2O in the manganese coordination sphere. The latter is ruled out if one finds a negative temperature coefficient for $1/T_{1P}$ (Mildvan and Cohn, 1970). Such temperature dependence has been found for Mn–aspartokinase (unpublished data). T_{1M} itself could be determined by τ_r, the rotational correlation time, τ_s, the electron spin relaxation time, or τ_m. A contribution from τ_r is unlikely because of the large molecular weight of aspartokinase. A preliminary finding that the relaxation time increases in going from 30 to 10 MHz (unpublished data) shows that τ_s plays an important role in the determination of T_{1M}. Such a frequency dependence has also been found for pyruvate kinase (Reuben and Cohn, 1970) which also forms metal-bridge complexes.

The reduction in enhancement from 14.1 to 10.1 upon addition of threonine could be caused by a decrease in the electron spin relaxation time, a decrease in the exchange rate $1/\tau_m$ of H_2O molecules into the Mn inner sphere, or could be due simply to a reduction in the number of H_2O molecules in the inner coordination sphere of manganese. The first two effects could arise from a change in conformation at the active site paralleling the change in enzyme conformation observed by Janin and Cohen (1969). A reduction in the number of H_2O molecules in the coordination sphere by displacement by threonine or a threonine-induced change in the number of manganese–protein ligands has not been ruled out.

The enhancement of the ternary ATP–Mn–aspartokinase complex is not significantly changed by threonine although the binding of Mn–ATP is reduced. The reduction in binding is not nearly complete even at threonine concentrations that produce close to 100% inhibition in the kinetic assay. This is in contrast to the competitive inhibition by threonine observed in the kinetic assay done with varying ATP. The kinetic observation of competitive inhibition is consistent with binding of threonine at the active site. The possibility that ATP stabilizes the active form also exists and stabilization of the active form by aspartate is the probable explanation for the apparent competition observed (Stadtman *et al.*, 1961) between threonine and aspartate. Wampler and Westhead (1968) have shown a shift in the threonine inhibition curve in going from 0.52 to 3.3 mM ATP. The threonine concentration required for 50% inhibition increases in going from low to high ATP. An increase in the half-saturation value for threonine in the presence of Mn–ATP has been observed in direct binding experiments (unpublished data). Further experiments are in progress to determine whether threonine binds directly at the aspartokinase active site, a possibility which would be the simplest explanation for our results.

Acknowledgments

Preliminary nmr experiments were performed by Dr. Barry Pass. We wish to thank Professor H. Y. Carr, Physics Department, Rutgers University, for use of the pulsed nmr spectrometer, Mrs. E. Anderson, Dr. T. Niki, and Professor E. Wasserman, Chemistry Department, Rutgers University, for esr measurements, Mrs. N. Smith for maintenance and preparation of *E. coli* Tir8, and J. Hospodka and E. Nagy of Carter-Wallace for use of the Manton-Gaulin homogenizer.

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Interaction of Streptokinase and Rabbit Plasminogen†

Lloyd A. Schick and Francis J. Castellino*

ABSTRACT: The ability of rabbit plasminogen and plasmin to form complexes with streptokinase has been studied and compared to the human system. At 1:1 or 10:1 ratios of streptokinase to rabbit plasminogen, no complex was observed by sucrose density centrifugation; however, almost all the rabbit plasminogen was converted to plasmin. Under identical conditions with human plasminogen, a complex was formed which consisted of altered streptokinase and human plasmin. When the acylating agent *p*-nitrophenyl *p'*-guanidinobenzoate was added to either human or rabbit plasminogen prior to addition of a molar equivalent of streptokinase, reactive complexes were formed in each case which consisted of human or rabbit plasminogen and native streptokinase. Upon treatment of human plasmin with a molar equivalent of streptokinase, a complex is formed which consists of altered streptokinase and human plasmin. This complex possesses the ability to activate bovine plasminogen. Human plasmin, inactivated with diisopropyl fluorophosphate, retains the capability of complexing with streptokinase but this complex does not activate bovine plasminogen. Upon treatment of rabbit plasmin or diisopropyl fluorophosphate inactivated rabbit plasmin with streptokinase no complexes or bovine plasminogen activator activity results. These results coupled with kinetic observations suggest that only rabbit plasminogen can serve as a plasminogen proactivator with streptokinase, whereas either human plasminogen or human plasmin can serve as a plasminogen proactivator with streptokinase. It is further observed that the reason for the increased streptokinase sensitivity of human plasminogen compared to rabbit plasminogen can be explained by the relative stability of streptokinase in the human plasminogen activator complex compared to the rabbit plasminogen activator complex.

Plasminogen is a single-chain protein of mol wt 81,000–88,000, depending on the species (Barlow *et al.*, 1969; Sodetz *et al.*, 1972), and plasmin is a two-chain molecule of approximately the same molecular weight, stabilized by disulfide bond(s) (Barlow *et al.*, 1969; Sodetz *et al.*, 1972). Many agents mediate the conversion of plasminogen to plasmin, among which is the bacterial endotoxin streptokinase. The mechanism of activation of human plasminogen by streptokinase has been a widely studied subject. The problem resolves itself into the manner in which the nonproteolytic protein, streptokinase, catalyzes the proteolytic cleavage required for conversion of plasminogen into plasmin. Further, the fact that not all species of plasminogen are capable of activation by streptokinase raises other interesting questions.

It has been demonstrated that human plasmin and streptokinase can form a 1:1 complex (Zylber *et al.*, 1959; Kline and Fishman, 1961; Markus and Werkheiser, 1964; Ling *et al.*, 1965; Hummel *et al.*, 1965). This complex possesses the ability to convert any species of plasminogen into plasmin (Blatt *et al.*, 1964; Wulf and Mertz, 1969) and is called "plasminogen activator." It was also found that "activator" can form whether human plasminogen or plasmin is used as the starting material (McClintock and Bell, 1971; Reddy and Markus, 1972). A possible mechanism of activation of human plasminogen by streptokinase, employing all the above considerations, has been nicely formulated by Reddy and Markus (1972).

Recently, some details of the mechanism of the streptokinase-mediated activation of human plasminogen proposed by Reddy and Markus (1972) have been challenged by Taylor and Beisswenger (1973). The latter authors propose that streptokinase can only react with the human plasmin, which usually contaminates human plasminogen preparations. This interaction leads to formation of a modified streptokinase capable of directly activating human plasminogen. However,

† From the Department of Chemistry, Program in Biochemistry and Biophysics, the University of Notre Dame, Notre Dame, Indiana 46556. *Received May 16, 1973.* Supported in part by Grant HL-13423 from the National Heart and Lung Institute, National Institutes of Health, a grant-in-aid from the Indiana Heart Association, a cooperative grant-in-aid from the American and Indiana Heart Associations, and a grant-in-aid from Miles Laboratories.