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Magnetic Resonance Studies of Substrate and Inhibitor Binding to Porcine Muscle Adenylate Kinase†

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ABSTRACT: Nuclear magnetic relaxation and electron paramagnetic reasonance (epr) techniques have been used to examine binding of substrates and inhibitors to porcine muscle adenylate kinase. The results show that there is one binding site for MnATP or ATP per mole of enzyme, with dissociation constants of 45 and 35 μM, respectively. The binding parameters for dATP are essentially identical with those of ATP, and the water proton relaxation enhancement for the ternary complexes with ATP and dATP are also similar ($\sim$15 at 24.3 MHz). The dissociation constants for Mn(II) complexes of GTP and tripolyphosphate are an order of magnitude higher than that of MnATP. Ternary complexes of GTP and tripolyphosphate also gave much lower water proton relaxation enhancements than did ATP and dATP. Diadenosine pentaphosphate (Ap_5A) forms a tight complex with the enzyme with a dissociation constant of 1.5 μM; the dissociation constant of the $MnAp_5A$ complex from the enzyme is even smaller (<0.5 μM). Ap_5A is a potent inhibitor of adenylate kinase: 50% inhibition occurs at an Ap_5A concentration of 0.2 μM in the presence of $MnCl_2$. The epr spectrum of the ternary complex, enzyme–MnATP, resembles that of MnATP. Addition of AMP to give the equilibrium mixture results in a considerable broadening of the epr spectrum. On the other hand, the epr spectrum of $MnAp_5A$ changes markedly upon addition of the enzyme, giving rise to a spectrum which resembles that of the equilibrium mixture.

Adenylate kinase is a widely occurring enzyme which catalyzes the following reaction

$$2ADP \overset{M^{2+}}{\rightleftharpoons} ATP + AMP$$

The enzyme is important in maintaining equilibrium among the various species in the adenine nucleotide pool (Noda, 1962). The adenylate kinase reaction is presumed to be a major pathway for phosphorylation of AMP to the level of ADP in the cell.

The interaction of nucleotide substrates with adenylate kinase from rabbit muscle has been examined previously by ultracentrifugation (Kuby *et al.*, 1968) and magnetic resonance techniques (O'Sullivan and Noda, 1968). However, because of the multiplicity of equilibria involved in this system, uncertainties remain in both the dissociation constants and binding stoichiometries. In particular, earlier magnetic resonance studies (O'Sullivan and Noda, 1968) covered only a limited portion of the total ligand saturation curve. A subsequent numerical analysis of these data (Reed *et al.*, 1970) showed that one could not establish the number of binding sites for MnATP from the limited data. We have therefore carried out PRR[1] measurements with the porcine muscle enzyme over a much wider range of nucleotide concentration to enable an unequivocal analysis of the dissociation constants and binding stoichiometries for substrates. We have also investigated the enzyme's interaction with the potent in-

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[1] Abbreviations used are: PRR, proton relaxation rate; Ap_5A, P^1,P^5-di(adenosine-5′) pentaphosphate; PSTD, per cent relative standard deviation; PPP, tripolyphosphate; Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; Ap_4A, P^1,P^4-di(adenosine-5′) tetraphosphate.

hibitor Ap_5A (Lienhard and Secemski, 1973) and measured the electron paramagnetic resonance (epr) spectrum of Mn(II) bound to the enzyme in the presence of various nucleotides and tripolyphosphate.

Materials and Methods

Porcine muscle adenylate kinase was a generous gift from Drs. L. Noda and G. McDonald. The twice-recrystallized enzyme has a specific activity of 1930 units/mg assayed by the coupled, hexokinase pH-Stat method (Kress *et al.*, 1966). The kinetic inhibition experiments reported here were carried out using the coupled, spectrophotometric procedure involving lactate dehydrogenase and pyruvate kinase. The following conditions were used: in a total volume of 1 ml; 200 μM AMP; 15 μM ATP; 1 mM P-enolpyruvate; 10 mM $MgCl_2$ or $MnCl_2$; 100 μM NADH; 25 μg of lactate dehydrogenase; 25 μg of pyruvate kinase. The reaction was initiated by addition of approximately 0.3 μg of adenylate kinase, and oxidation of NADH was monitored at 340 nm. For all of the experiments described in this paper the ammonium sulfate suspension of enzyme was dialyzed against 50 mM K^+–Hepes at pH 8.0 containing 0.2 M tetramethylammonium chloride and 1 mM dithioerythritol. The enzyme did not lose activity when stored in this medium for a period of several days. All of the experiments reported here were carried out in solutions with this composition.

Enzyme concentrations were determined spectrophotometrically at 280 nm using the published values of the extinction coefficient and molecular weight (Noda, 1962). ATP, GTP, and GDP were obtained as the sodium salts from P-L Biochemicals; dATP (sodium salt) from Sigma Chemical Co. The sodium salt of Ap_5A was a generous gift of Dr. J. G. Moffatt (Institute of Molecular Biology, Syntex Research). The Ap_5A migrated as a single spot in thin-layer chromatography on silica gel in the two solvent systems: saturated ammonium sulfate–1 M sodium acetate–isopropyl alcohol (80:18:2, v/v) and 1-butanol–acetone–acetic acid–5% ammonium hydroxide–water (4.5:1.5:1:1:2, v/v), with R_F values of 0.6 and 0.17, respectively. Other reagents were of the highest grade commercially available.

The PRR of water was measured at 24.3 MHz using a pulsed nuclear magnetic resonance (nmr) spectrometer as previously described (Cohn and Leigh, 1962). Electron paramagnetic resonance (epr) spectra were recorded at 9.1 GHz on a Varian E-3 spectrometer with samples contained in high-purity quartz capillary tubing. PRR data was analyzed using the methodology outlined previously for creatine kinase and rabbit muscle adenylate kinase (Reed *et al.*, 1970). The equilibria characterizing the various interactions in the system are summarized below.[2] A considerable economy in computer time was realized by making a preliminary visual fit to the data, using a Tektronix Graphic Terminal to display the data and theoretical curves. The values of K_2, K_s, and ϵ_t were obtained by fitting the titration curves (E_T and M_T constant, S_T variable) simultaneously for at least two values of E_T. The procedure gives reasonably unique values for these parameters (O'Sullivan *et al.*, 1972).

TABLE I: Dissociation Constants and Enhancement Parameters for Substrates and an Inhibitor in the Adenylate Kinase Reaction.[a]

Nucleotide	K_2 (μM)	K_s (μM)	ϵ_t	PSTD[b]
ATP[c]	44 (100)	35 (35)	14.8 (15)	10 (20)
dATP[c]	44 (100)	35 (32)	16.2 (16.6)	12 (21)
GTP	380	1,200	5.0	10
PPP	300	240	4.5	4
GDP	$\geq$2000	$\geq$10,000	5	
Ap_5A	$\leq$0.5	$\leq$1.5	5.0	

[a] All measurements were carried out in 50 mM Hepes–K^+ at pH 8.0 with 0.2 M $(CH_3)_4N^+Cl^-$ and 1 mM dithioerythritol. The frequency was 24.3 MHz, and the temperature was 24 $\pm$ 1°. The following values for dissociation constants and ϵ were used for the calculations in the table: K_D = 9 mM, ϵ_b = 4; for the nucleoside triphosphate K_1 = 10 μM, ϵ_a = 1.6; for GDP, Ap_5A and PPP, K_1 = 30, 8, 6 μM, respectively; ϵ_a = 1.6, 1.6, and 1.1, respectively. [b] Per cent standard deviation of ϵ_t, calculated for each data point, from the mean. [c] Numbers in parentheses refer to the best fit for two binding sites per mole of enzyme.

Results

Binary Mn(II) Complexes. In agreement with previous findings (O'Sullivan and Noda, 1968) there was only a weak interaction between the enzyme and Mn(II) in the absence of substrates. Using a combination of PRR and epr methods the K_D was determined to be approximately 9 mM with ϵ_b = 4.[3] Under the conditions described in the Methods section, the values of K_1 for Mn(II) with ATP, dATP, GTP, GDP, Ap_5A, and PPP were 10, 10, 10, 30, 8, and 6 μM, respectively, as determined by epr titrations (Cohn and Townsend, 1954). ϵ_a for all of the binary Mn(II) nucleotide complexes was determined to be 1.6, and that of the MnPPP complex 1.1.

Ternary Complexes. Figure 1 shows a plot of PRR enhancement as a function of ATP concentration at three different values of E_T. The solid curves are computed with the values of the constants listed in Table I. Figure 1a shows the best fit to the data for a stoichiometry of one site for MnATP or ATP per 21,000 molecular weight enzyme. Figure 1b shows the best fit assuming that there are two sites per mole of enzyme. The one-site model clearly gives a superior fit to the data as is reflected in the relative standard deviations for best fit in the two models (*cf.* Table I). In a previous analysis of PRR titration data for rabbit muscle adenylate kinase the fit was not changed appreciably when a binding stoichiometry of one of two sites was assumed (Reed *et al.*, 1970). Although the data in the lower saturation range (ATP < 100 μM) could be fitted satisfactorily by either a one-site or a two-site model, only the one-site model provides a reasonable fit to the data over the entire ligand concentration range. It is noteworthy that the titration curves in Figure 1 also allow a determination of K_s.

[2] K_1 = (M)(S)/(MS); K_2 = (E)(MS)/(EMS), K_s = (E)(S)/(ES); K_D = (E)(M)/(EM). (E_T), (M_T), (S_T) are the total concentrations of enzyme, metal ion, and substrate, respectively. ϵ_a, ϵ_b, ϵ_t are the characteristic PRR enhancement parameters of the MS, EM, and EMS complexes respectively. ϵ^* is the observed PRR enhancement under any given set of conditions.

[3] The earlier values of K_D = 2 mM and ϵ_b = 1.3 for the rabbit muscle enzyme were estimated from data taken at lower enzyme concentrations where the extrapolation to infinite enzyme concentration is questionable (O'Sullivan and Noda, 1968). Since the K_D is generally much higher than constants for other equilibria involving the metal ion, the EM species constitutes a very minor component of the solution under almost all conditions. Thus, the precise values of K_D and ϵ_b do not significantly affect the data analysis.

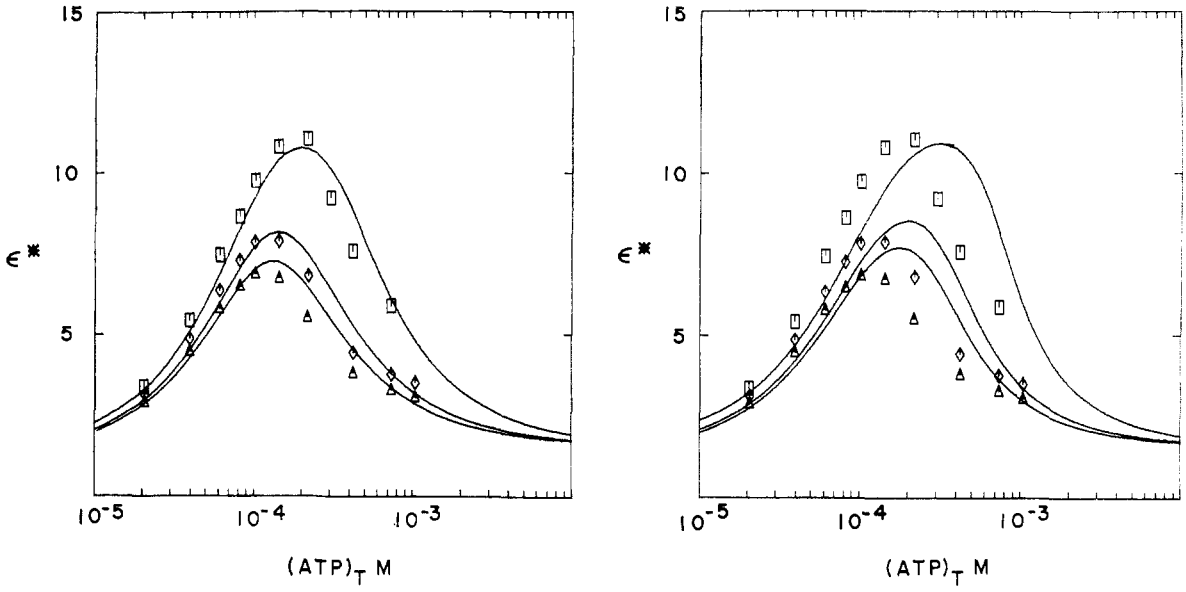

FIGURE 1: PRR enhancement at 24.3 MHz, 24° for solutions of Mn(II)–adenylate kinase as a function of ATP concentration. Solid curves were drawn with the constants given in Table I. The solutions contained 50 mM Hepes-K^+ (pH 8.0); 0.2 M $(CH_3)_4N^+Cl^-$; 1 mM dithioerythritol; M_T = 100 μM. (□) E_T = 313 μM; (◇) E_T = 156 μM; (△) E_T = 125 μM. (a, left) Solid curves represent best fit for one binding site per mole of enzyme; (b, right) solid curves represent the best fit for two binding sites per mole of enzyme.

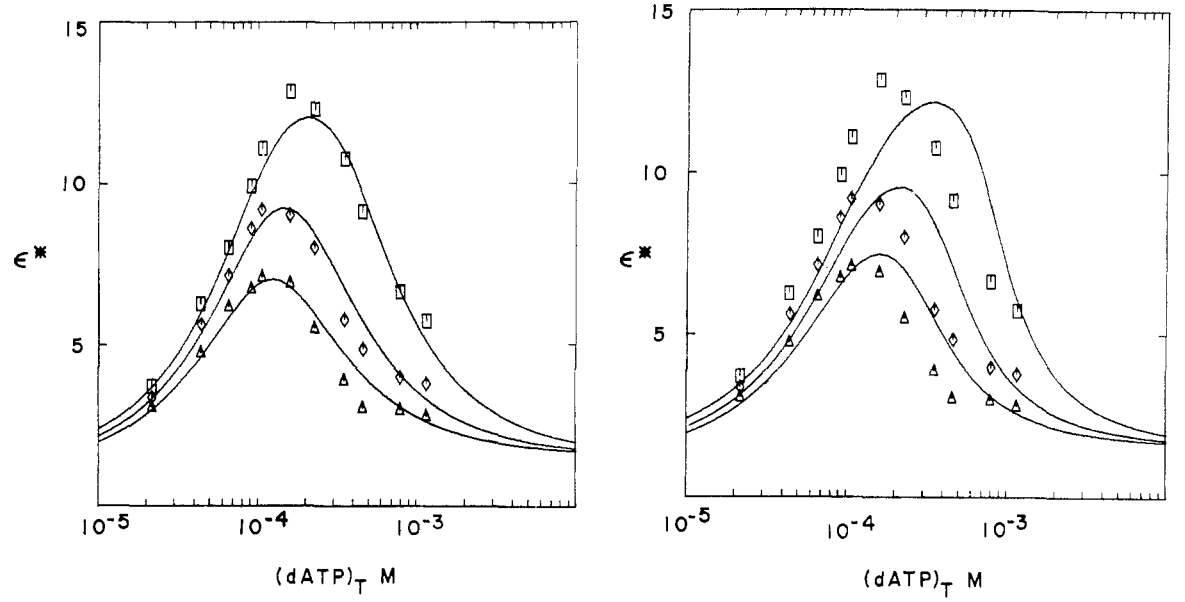

FIGURE 2: PRR enhancement for solutions of Mn(II)–adenylate kinase as a function of dATP concentration. Solid curves were drawn with the constants given in Table I. (□) E_T = 341 μM; (◇) E_T = 170 μM; (△) E_T = 102 μM. (a, left) Best fit for one binding site per mole of enzyme; (b, right) best fit for two binding sites per mole of enzyme. All other conditions are identical with those given for Figure 1.

The value of K_s is slightly lower than that of K_2, which accounts for the very effective competition of ATP for the MnATP binding site. This competition results in a sharp decrease in ϵ^* when ATP_T is appreciably larger than Mn_T.

Results of PRR titrations for dATP are shown in Figure 2. Again the one-site model gives the most reasonable fit to the experimental data (*cf.* Figure 2a,b). ϵ_t for dATP (16.2) is slightly greater than that for ATP (14.8). However, since the error limit is ~10% on each of these values, the apparent difference in ϵ_t is probably not significant.

PRR results for titrations with GTP and PPP are shown in Figure 3. The measured enhancements are much lower for these triphosphate substrates. Consequently, the larger experimental errors present greater problems in the data analysis. Nevertheless, it seems clear that K_2 is approximately an order of magnitude larger for these substrates than for ATP, and ϵ_t is about one-third that for ATP.

PRR titration curves for GDP[4] at two enzyme concentrations are shown in Figure 4. As with GTP and PPP, the measured enhancements are quite low. However, it appears that K_2 for GDP is significantly higher than K_2 for GTP. It is also apparent that K_s is also very much higher than K_2 because even at 1 mM GDP_T the free nucleotide is not providing effective competition for metal–nucleotide binding (*i.e.*, ϵ^* has not

[4] GDP is presumed to bind only at the subsite in which it can accept a phosphoryl group (the triphosphate subsite) since GMP is neither a substrate nor an inhibitor of the enzyme.

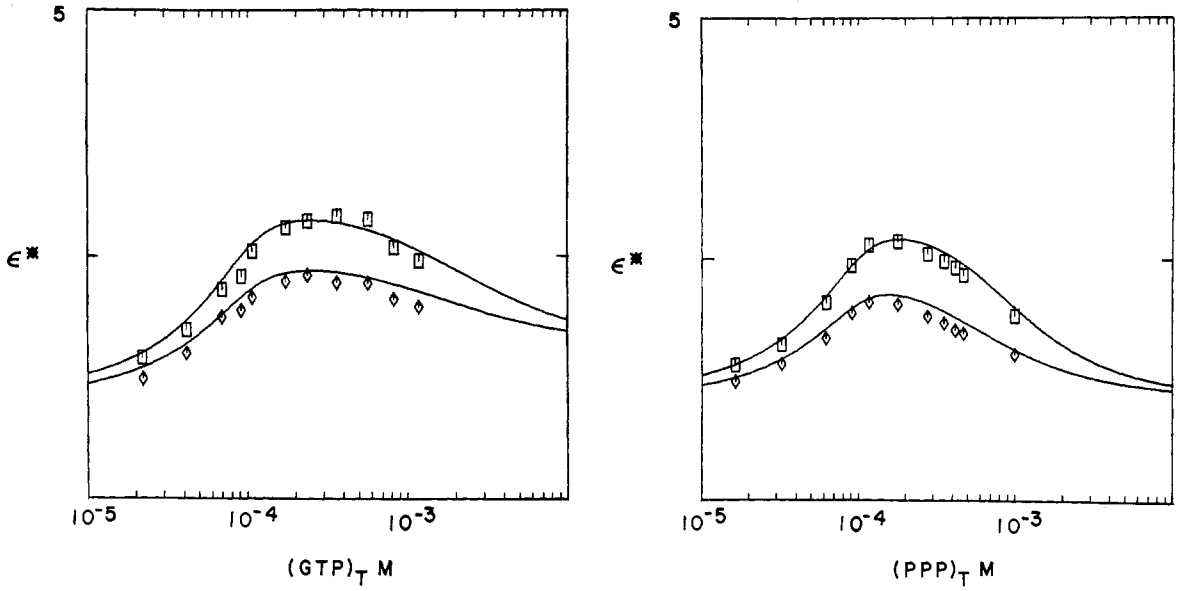

FIGURE 3: PRR enhancement for solutions of Mn(II)–adenylate kinase as a function of GTP (a) or PPP (b) concentration. Solid curves were drawn with the constants given in Table I. (a, left) (□) E_T = 317 μM; (◇) E_T = 158 μM; (b, right) E_T = 423 μM; (◇) E_T = 212 μM. All other conditions are identical with those given for Figure 1.

reached a maximum). The sizeable experimental errors involved in the determination of these low enhancements and the weak binding obviated a more rigorous treatment of the data.

Figure 5 shows the results of PRR titrations for Ap_5A. The observed enhancements coincide (Figure 5a) at the two different enzyme concentrations when $[Ap_5A]_T < [Mn]_T$, which shows that binding of $MnAp_5A$ to the enzyme is exceedingly tight. Under these conditions it is impossible to determine K_2 and K_s exactly, but upper limits of 0.5 and 1.5 μM, respectively, can be assigned to these constants (the constants can be somewhat lower without significantly altering the shape of the curve). Taking advantage of the very tight binding of $MnAp_5A$ to the enzyme, it is possible to confirm the stoichiometry of the complex by titrating the enzyme with Ap_5A under conditions where $Mn_T > E_T$. Such an experiment is shown in Figure 5b (where the abscissa is linear in Ap_5A concentration). With 102 μM enzyme and 300 μM Mn(II), the breakpoint occurs at 95 μM Ap_5A, confirming the 1:1 stoichiometry of the complex. This finding is strong evidence for the existence of only one active site (comprising the AMP and ATP subsites) per mole of enzyme, as indicated in the ATP titrations described above. These experiments also indicate that only one Mn(II) is involved at the active site. Since the "breakpoint" in Figure 5a occurs at ~100 μM Ap_5A (and Mn_T = 100 μM), this indicates that the stoichiometry is 1:1:1 Ap_5A:Mn(II):enzyme. Previous kinetic studies by Noda (1958) had indicated a maximal activity with $[Mg^{2+}]$ = 0.5 [ADP] in the forward direction and with $[Mg^{2+}]$ = [ATP] in the reverse direction. Thus, Noda (1958) had suggested a stoichiometry of one metal ion per active site.

Inhibition by Ap_5A. It has recently been reported that Ap_5A is a very potent inhibitor of rabbit muscle adenylate kinase (Lienhard and Secemski, 1973). The very small K_i (~10^{-8} M with Mg(II) as activating cation) suggests that Ap_5A bridges the two subsites on the enzyme. With the porcine muscle enzyme and Mn(II) as the activating cation, 50% inhibition of the enzyme occurs at 2×10^{-7} M Ap_5A. On the other hand, 50% inhibition occurs at 5×10^{-8} M Ap_5A with Mg(II) ion present. The value of K_2 determined from PRR measurements is in the range of the kinetic inhibition constant.

Epr Spectra. Previous epr studies of the Mn–nucleotide complexes with creatine kinase have provided some insight into the nature of the coordination sphere of the bound divalent cation (Reed and Cohn, 1972). The epr spectrum of the ternary complex of MnATP with adenylate kinase (Figure 6a), like that of MnATP with creatine kinase, is not markedly different from that of the MnATP binary complex.[5] Comparison of the spectrum for enzyme–MnATP with that of the equilibrium mixture (Figure 6a,b) shows that the environment of the Mn(II), as reflected by the epr spectrum, is sensitive to the binding of AMP or ADP or both at the active site. Unfortunately, the epr lines are too broad to allow a more detailed interpretation of the spectral parameters.

The epr spectrum of the binary $MnAp_5A$ complex (Figure 6c) is virtually identical to that of MnATP (Reed and Cohn, 1972). However, there is a striking change in the spectrum of $MnAp_5A$ when the complex is bound to adenylate kinase (Figure 6d). This is in marked contrast to the situation with MnATP where little change is observed. It should be pointed out that although a detailed interpretation of the spectrum of the enzyme–$MnAp_5A$ complex is not feasible, this spectrum is quite similar to that of the equilibrium mixture (compare Figures 6b,d). The broadened epr spectrum of enzyme Mn-Ap_5A indicates a shortened electron spin relaxation time relative to that of enzyme–MnATP. Since the PRR enhancement for enzyme–$MnAp_5A$ is also lower than that for enzyme–MnATP, this probably indicates that the electron spin relaxation makes a major contribution to the correlation time for water proton relaxation (*cf.* Reuben and Cohn, 1970; Reed *et al.*, 1972).

Epr spectra were also recorded for solutions containing ternary complexes of enzyme, Mn(II) and GTP, PPP, and

[5] A similar experiment was reported by O'Sullivan and Noda (1968). Under their conditions, approximately 50% of the total Mn(II) was present as the enzyme–MnATP complex. In our experiment 85% of the total Mn(II) is present as the ternary complex.

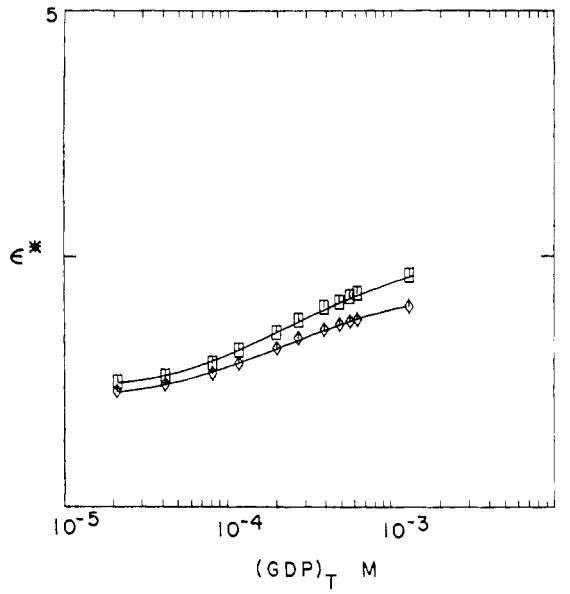

FIGURE 4: PRR enhancement for solutions of Mn(II)–adenylate kinase as a function of GDP concentration. Solid curves are sketched through the data points. (□) E_T = 423 μM; (◇) E_T = 212 μM. All other conditions are identical with those given for Figure 1.

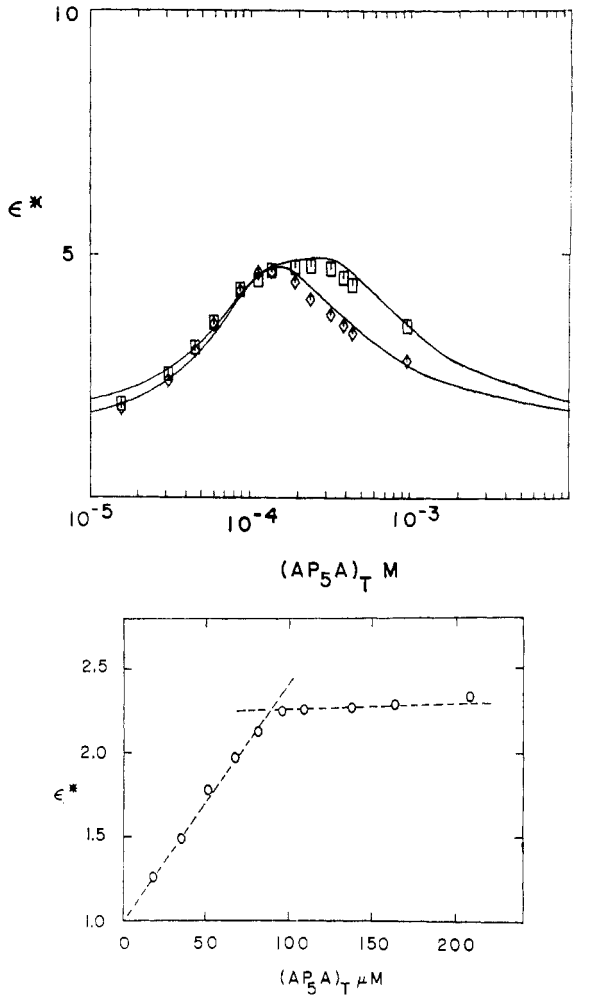

FIGURE 5: PRR enhancement for solutions of Mn(II)-adenylate kinase as a function of Ap_5A concentration. (a, top) (□) E_T = 340 μM; (◇) E_T = 170 μM; M_T = 100 μM. Solid curves drawn with constants given in Table I. (b, bottom) E_T = 102 μM; M_T = 300 μM. All other conditions are identical with those given in Figure 1.

GDP. In these cases, the spectra narrowed considerably at higher temperatures indicating that there were considerable spectral contributions from the binary Mn–substrate complexes (Reed and Ray, 1971) (*cf.* the higher K_2 values for these substrates in Table I). It was therefore impossible to reach any conclusions about the spectra of the enzyme-bound species.

Similar studies with Mn(II) complexes in the creatine kinase system (Reed and Cohn, 1972) have shown that addition of anions such as nitrate or formate to enzyme–MnADP and creatine leads to formation of very distinctive complexes whose epr and PRR parameters differ significantly from those of the simple enzyme–Mn–ADP–creatine complex. It has been suggested by Milner-White and Watts (1971) that the anions occupy the site of the missing phosphoryl group in this complex and thereby produce a complex resembling the transition state. Attempts to produce a potential transition state analog with adenylate kinase by addition of nitrate to solutions containing enzyme, Mn(II), GDP, and AMP were not successful. There were no changes in the epr spectrum following addition of nitrate. Although one would expect the binding of MnGDP to the enzyme to be enhanced if such a transition state analog complex were formed, it is possible that binding would still be too weak to facilitate epr detection of the complex.

Discussion

The data summarized in Table I and Figures 1–6 give some information on the relationship between the divalent metal ion and nucleotides in the complexes with adenylate kinase. Since the values of K_s and K_2 for ATP (and for dATP) are very similar, it is probable that the Mn(II) ion is not directly coordinated to the protein in these ternary complexes. This conclusion is supported by the similarity of the epr spectra of the enzyme–MnATP and MnATP complexes.

Substitution of guanine for the adenine moiety of ATP weakens K_2 by approximately an order of magnitude. In fact it appears that the guanosine moiety contributes little to the total binding energy of MnGTP to the enzyme, since MnPPP appears to bind equally well. In the absence of Mn(II), PPP binds more tightly to the enzyme than does GTP, but still more weakly than do ATP and dATP.

Kuby *et al.* (1968) have investigated the binding of ATP to rabbit muscle adenylate kinase in the absence and presence of Mg(II) using a sedimentation gradient procedure. Results from their studies showed that K_s was approximately equal to K_2, in agreement with the present findings for the porcine muscle enzyme in the presence of Mn(II). However, the indication of approximately two equivalent binding sites for ATP or MgATP from the sedimentation measurements on the rabbit muscle enzyme does not agree with the finding of one site from our PRR data for the porcine enzyme. Where the concentrations of enzyme and substrate overlap, the PRR data for the porcine muscle and rabbit muscle enzymes are indistinguishable. There is little basis to suspect a species difference because the properties of the porcine muscle and rabbit muscle enzymes appear to be very similar in other respects (*cf.* Noda and Kuby, 1957; Schirmer *et al.*, 1970). There is additional evidence that the two subsites of the active site of adenylate kinase are not equivalent. Thus, the triphosphate subsite shows only limited substrate specificity since GTP, dATP, ITP, and PPP can all substitute for ATP to some ex-

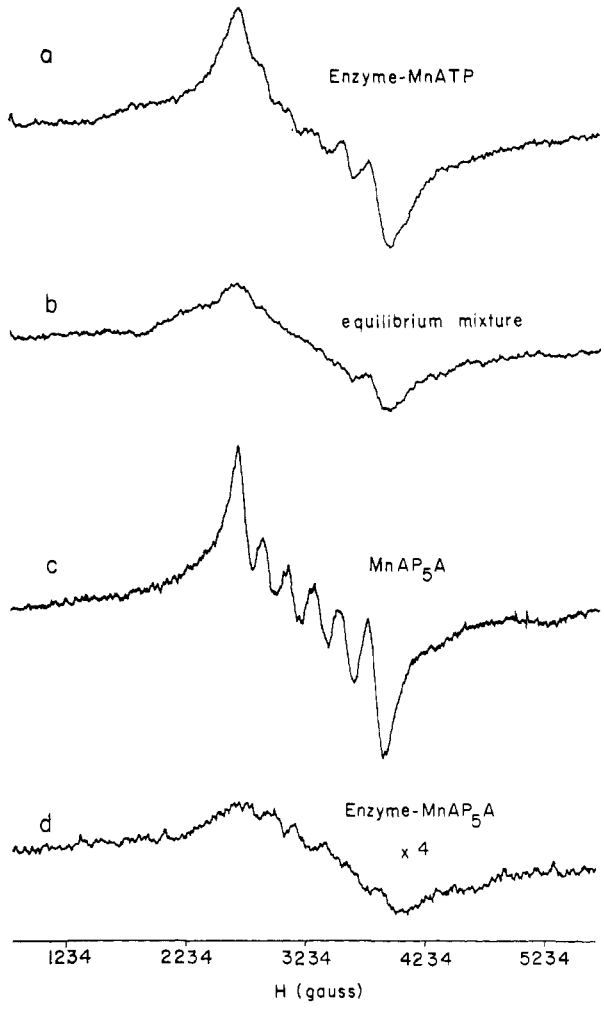

FIGURE 6: Comparison of epr spectra for Mn(II) complexes of adenylate kinase. All spectra were recorded at 1°. (a) E_T = 1.9 mM; Mn_T = 0.4 mM; ATP_T = 1.8 mM; (b) equilibrium mixture of substrates, total nucleotide concentration 2.5 mM; E_T = 1.9 mM; Mn_T = 0.4 mM; (c) binary complex $MnAp_5A$; Mn_T = 0.4 mM; Ap_5A_T = 1.3 mM; (d) ternary complex, enzyme-$MnAp_5A$; E_T = 1.9 mM; Mn_T = 0.4 mM; Ap_5A_T = 1.3 mM. Buffer solution composition is given in legend for Figure 1.

tent (O'Sullivan and Noda, 1968). By contrast, the monophosphate subsite appears to be highly specific for AMP, since no activity is observed when 1,N^6-ethenoAMP (Secrist *et al.*, 1972), IMP, GMP, or 3'AMP (M. Cohn unpublished observations) is substituted for AMP. That there is only one catalytic site per mole of adenylate kinase may be inferred from the stoichiometry of inactivation by 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole (Price, 1972) and from the stoichiometry of binding of Ap_5A.

For Ap_5A the value of K_2 is significantly less than that of K_s. There is also a striking difference between the epr spectra of the enzyme-$MnAp_5A$ complex and of $MnAp_5A$. These observations would suggest that either the Mn(II) ion is directly coordinated to the enzyme in the ternary complex or that the metal ion is involved in producing a favorable orientation of the pentaphosphate chain. In the latter case, the broadened epr spectrum would result from geometric distortions associated with protein–polyphosphate interactions. It is remarkable that Ap_5A is a much more powerful inhibitor than Ap_4A (Purich and Fromm, 1972) particularly since the latter compound contains the same number of phosphate groups as the active substrate combination. Since the estimated dissociation constant for the enzyme–Ap_4A complex (24 μM) (Purich and Fromm, 1972) is only slightly lower than our value of K_s for ATP, it is very unlikely that Ap_4A is a true transition state analog. It would seem that Ap_4A, in contrast to Ap_5A, binds only to one of the two subsites of the catalytic site. A plausible hypothesis is that binding of the substrates AMP and/or ATP–plus divalent metal ion leads to a disposition of the substrate subsites which optimally facilitates phosphoryl transfer. Evidence for substrate-induced conformational changes has been obtained from studies of the effects of substrates on the reactivity of the SH groups of the enzyme, on the fluorescence intensity of a suitably labeled enzyme derivative (Price, 1972) and on the epr spectrum of a spin-labeled enzyme derivative (N. C. Price and M. Cohn unpublished results).

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