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Stereochemical and Kinetic Studies on the Action of the Catalytic Subunit of Bovine Cardiac Muscle Adenosine 3′,5′-Monophosphate Dependent Protein Kinase Using Metal Ion Complexes of ATPβS†

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ABSTRACT: The phosphotransferase activity of bovine cardiac muscle protein kinase catalytic subunit has been investigated by the use of metal ion complexes of ATPβS diastereomers ("A" and "B") as donor substrates in peptide phosphorylation. It was found that MgATPβS "A" was used by the enzyme 500-fold faster than MgATPβS "B" but that Cd^{II}ATPβS "B" was preferred by about 20-fold over Cd^{II}ATPβS "A" isomer. It is argued that the β,γ-bidentate complexes of MgATPβS "A" and of Cd^{II}ATPβS "B", as well as the Δ isomer of $Co^{III}(NH_3)_4$ATP, have identical stereochemistry about the β-phosphorus position and that this structure is preferentially recognized by the enzyme. A comparison of double-reciprocal kinetic plots suggested that the reaction with MgATPβS "A" proceeds by a ping-pong mechanism while that with MgATP follows an ordered sequence. Further analysis including product inhibition and γ-^{32}P exchange studies showed that a ping-pong mechanism is not plausible for MgATPβS "A" and that, like MgATP, phosphorylation employing the "A" isomer probably proceeds by an ordered sequence. The enzyme uses MgATPβS "A" and MgATP as substrates in very much the same manner since the same k_{cat} (~640 min^{-1}) and K_m (10.7 μM) values are obtained with either substrate. Kinetic differences between the reactions of the two nucleotide triphosphate substrates arise from different K_m values for peptide and, presumably, from different dissociation constants for the enzyme–Mg–nucleotide triphosphate complexes.

The stereochemistry of the metal ion–nucleotide triphosphate complexes accepted as substrates by pyruvate kinase and yeast hexokinase has recently been examined (Jaffe & Cohn, 1978) through the use of the "A" and "B" diastereomers of ATPβS (Eckstein & Goody, 1976).[1] Cornelius & Cleland (1978) showed that hexokinase reacts with the Λ diastereomer of the stable metal ion β,γ-bidentate complex $Co^{III}(NH_3)_4$ATP, and the absolute configuration of the Co(III)–triphosphate moiety has been established (Merritt et al., 1978). The center of asymmetry of $Co^{III}(NH_3)_4$ATP and MgATPβS in the triphosphate chain is the β-phosphate position, and for each β,γ-bidentate complex of $Co^{III}(NH_3)_4$ATP there exists a MgATPβS diastereomer of the same geometric configuration. On the basis of the stereoselectivity of hexokinase for the MgATPβS "B" diastereomer, Jaffe & Cohn (1978) assigned this isomer as having the same stereochemistry as the Λ isomer of $Co^{III}(NH_3)_4$ATP.

To elucidate the stereochemistry of the metal ion–nucleotide triphosphate complex which reacts with the catalytic subunit of bovine cardiac muscle cAMP-dependent protein kinase (EC 2.7.1.37; ATP:protein phosphotransferase), we have investigated the action of the catalytic subunit on metal ion complexes of ATPβS. Granot et al. (1979) have shown that the Δ isomer of β,γ-bidentate $Co^{III}(NH_3)_4$ATP reacts with the protein kinase catalytic subunit, albeit very slowly, while the Λ isomer does not appear to serve as a substrate. Though there is a definite preference exhibited by a number of enzymes for reaction with either the Λ or Δ isomer of $Co^{III}(NH_3)_4$ATP, the rate of utilization of the preferred Co(III) complex is invariably quite slow (Danenberg & Cleland, 1975; Cornelius & Cleland, 1978; Li et al., 1978; Dunaway-Mariano et al., 1979). Because of the low rate of reaction, we found it desirable to employ additional stereochemical probes involving

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[1] Abbreviations used: we have used the same designation ("A" and "B") for the diastereomers of ATPβS as employed by Eckstein & Goody (1976); PEI, poly(ethylenimine); DTT, dithiothreitol.

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substrates which react at rates more comparable to MgATP.

In this paper we have used [γ-^{32}P]ATPβS "A" and "B" diastereomers to explore not only the stereochemistry but also the kinetic behavior of the metal ion–nucleotide triphosphate complexes which react with protein kinase catalytic subunit. Our studies, taken in conjunction with those of Granot et al. (1979), provide an important confirmation of the relationship between the stereochemistry of divalent metal ion–ATPβS complexes and the diastereomers of $Co^{III}(NH_3)_4ATP$, the relationship first proposed by Jaffe & Cohn (1978).

Experimental Section

[γ-^{32}P]ATP ($\sim$540 μCi/μmol) was purchased from Amersham, and [^{32}P]phosphate was supplied by New England Nuclear. [γ-^{32}P]ATPβS "B" diastereomer ($\sim$6 μCi/μmol) was prepared by enzymic synthesis using acetate kinase, acetyl [^{32}P]phosphate, and ADPβS, as described by Stingelin et al. (1980). [γ-^{32}P]ATPβS "A" ($\sim$220 μCi/μmol) was prepared by way of a coupled enzyme system involving phosphoglycerate phosphokinase, malate dehydrogenase, and glyceraldehyde-3-phosphate dehydrogenase in the presence of [^{32}P]phosphate, glyceraldehyde 3-phosphate, oxaloacetate, NAD^+, and ADPβS (Stingelin et al., 1980). The synthetic heptapeptide substrate Leu-Arg-Arg-Ala-Ser-Leu-Gly was prepared and characterized as described by Armstrong et al. (1979a). A minimum amount of reagent grade HNO_3 was used to dissolve ultrapure CdO (Roc/Ric, Sun Valley, CA), and the cadmium solution formed was dried by using a stream of nitrogen. The sources of magnesium and cadmium ions in the kinetic experiments were $MgCl_2$ and $Cd(NO_3)_2$. All other reagents were of the highest quality commercially available.

Protein kinase catalytic subunit from bovine heart was purified to homogeneity as previously described (Armstrong et al., 1979a), and reaction velocities were measured by determining ^{32}P incorporation into the heptapeptide by the following modification of the Witt & Roskoski (1975) procedure. A mixture (75-μL volume) at pH 7.5 containing 50 mM Tris, 0.15 M KCl, 13.33 mM $MgCl_2$, 0.209% BSA, [γ-^{32}P]ATP or [γ-^{32}P]ATPβS "A" or "B", and heptapeptide was incubated for 5 min at 30 °C. An aliquot (25 μL) of protein kinase catalytic subunit ($\sim$8 nM) also in 50 mM Tris, 0.15 M KCl, and 1.0 mM DTT at pH 7.5 was added to initiate the peptide phosphorylation reaction maintained at 30 °C. In experiments containing Cd(II), no DTT was present and 13.33 mM $Cd(NO_3)_2$ was used in place of 13.33 mM $MgCl_2$. Aliquots (25 μL each) were removed at time intervals of 1, 2, and 3 or 5 min and quickly applied to numbered (1 × 2 cm) phosphocellulose paper strips which were then immersed in 2 L of 10% glacial acetic acid solution. Duplicate or triplicate measurements were made for each initial velocity measurement. After at least 30 min in 10% glacial acetic acid at room temperature, the phosphocellulose strips were rinsed with six 2-L volumes of deionized water followed by successive drying with 3 × 200 mL of acetone and 3 × 200 mL of pentane. After being air-dried, the strips were placed into scintillation vials containing 6 mL of toluene–Omnifluor and assayed for radioactivity. Appropriate controls were run to determine background radioactivity. Inhibition studies involving ADPβS were performed in the above manner using saturating conditions of heptapeptide (2.66 mM). Initial velocity data represent less than 3, 5, and 10% consumption of the substrate present in the least amount for studies involving ATP, ATPβS, and ADPβS, respectively.

Peptide phosphorylation is not the only activity of protein kinase catalytic subunit since the enzyme is known to exhibit ATPase activity under certain conditions (Armstrong et al., 1979b). The question arose as to whether nucleotide triphosphate hydrolase activity could be a competing reaction in the presence of ATPβS "A" and heptapeptide. We examined this point by performing PEI thin-layer chromatography of aliquots from a reaction mixture containing MgATPβS "A", catalytic subunit, and heptapeptide. The chromatogram was further subjected to autoradiography to locate radioactive spots, and it was observed that [$^{32}P_i$]phosphate did not increase with time and that [^{32}P]phosphopeptide accounted for essentially all of the activity of the enzyme under the conditions of our kinetic experiments.

Table I: Initial Rates of Reaction of Nucleotide Triphosphate Substrates with Protein Kinase in the Presence of Divalent Metal Ions[a]

nucleotide triphosphate	Mg(II)	Cd(II)
[γ-^{32}P]ATP	636.6	0.217
[γ-^{32}P]ATPβS "A"	535.0	0.051
[γ-^{32}P]ATPβS "B"	1.1	0.893

[a] Initial rates are expressed in micromoles of ^{32}P incorporation into peptide per minute per micromole of enzyme at 30 °C, pH 7.5. Final concentrations were 200 μM nucleotide triphosphate, 525 μM heptapeptide, and 10 mM metal ion as indicated. Enzyme concentration was 2 and 250 mM for Mg(II) and Cd(II)-containing solutions, respectively, and all other conditions were as described under Experimental Section.

The radioisotope exchange experiment was performed by preparing a mixture (pH 7.5) containing 0.15 M KCl, 50 mM Tris, 200 μM ATPβS "A", 10 mM $MgCl_2$, 200 μM [^{14}C]ADP (31 μCi/μmol), and either 236 or 1.95 nM protein kinase catalytic subunit. The mixture was incubated at 25 °C, and 5-μL aliquots were removed at time intervals and applied to PEI sheets. Thin-layer chromatography of the PEI sheets was performed by elution with 0.75 M potassium phosphate buffer at pH 3.4 followed by autoradiography using a previously described procedure (Stingelin et al., 1980). This method is able to distinguish between all radioactively labeled nucleotide di- and triphosphates.

Kinetic parameters listed in Table II were evaluated from primary (parts a and b of Figure 1) and secondary (parts a and b of Figures 3 and 4) plots by use of a BASIC computer program (ENZKIN) written by Burstein et al. (1976) and patterned after Cleland (1963). The program was kindly provided by Professor John Westley and gives an iterative least-squares best fit to a hyperbolic function by assuming equal variances for the experimental velocities.

Results

In the presence of Mg(II), ATPβS "A" was found to be a much better substrate than the "B" diastereomer for the phosphorylation of peptide by protein kinase catalytic subunit. The data in Table I demonstrate that the MgATPβS "A" complex reacts with the enzyme approximately 500-fold faster than does MgATPβS "B". This substrate selectivity was found to be reversed when Cd(II) was replaced by Mg(II). In this case, Cd^{II}ATPβS "B" reacts approximately 20-fold faster than does Cd^{II}ATPβS "A". It should be pointed out that these selectivity ratios are only approximate, since they result from initial velocity measurements under conditions in which the concentration of the metal ion–nucleotide triphosphate and/or peptide substrates may or may not be saturating. In lieu of K_m and k_{cat} data for the Cd^{II}ATPβS "A" and "B" and Mg^{II}ATPβS "B" complexes, we have taken the relative velocities as a reflection of the stereochemical preference of the enzyme for the various metal ion–nucleotide triphosphate substrates.

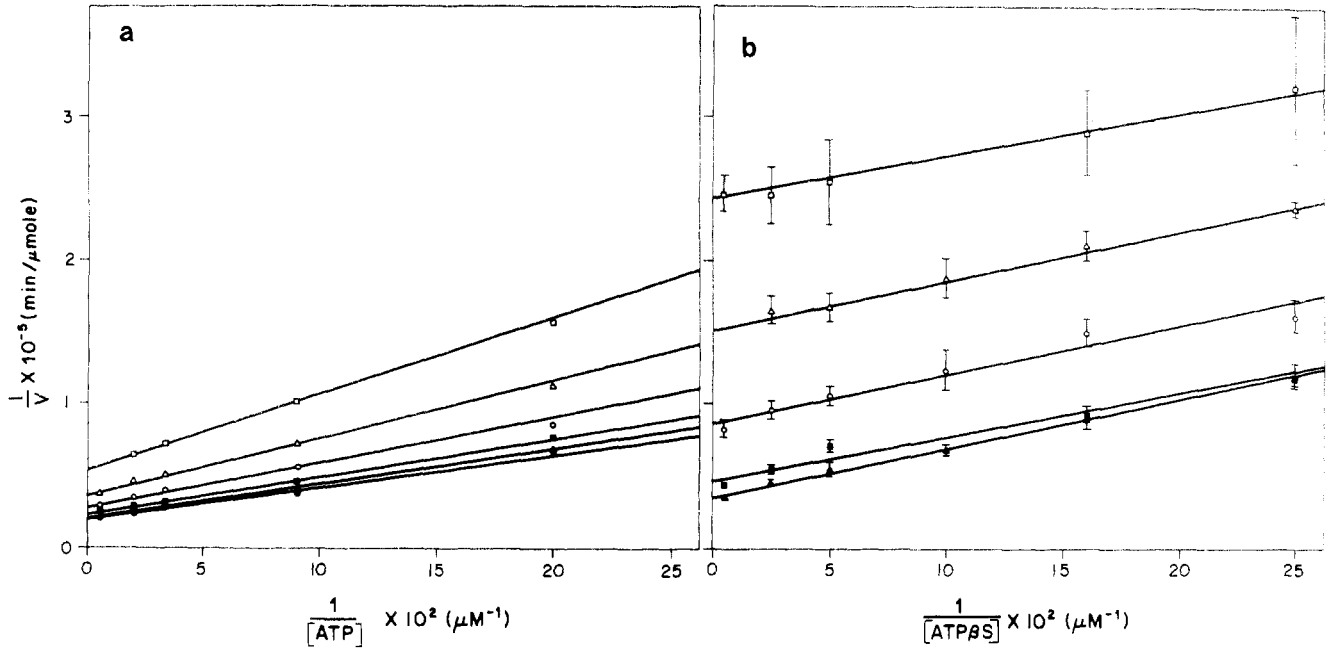

FIGURE 1: (a) Lineweaver–Burk plot for phosphorylation of heptapeptide using catalytic subunit, [γ-^{32}P]ATP, and a final heptapeptide concentration of 9.8 (□), 19.17 (△), 34.08 (○), 63.9 (■), 127.8 (▲), and 426.1 (●) μM. Plots of $1/V$ vs. $1/$[peptide] at several fixed concentrations of ATP give a pattern very similar to that of Figure 1a (not shown). Data points represent the average of duplicate initial velocity measurements. (b) Lineweaver–Burk plot for phosphorylation of heptapeptide using catalytic subunit, [γ-^{32}P]ATPβS "A", and heptapeptide concentrations of 42.6 (□), 79.9 (△), 159.8 (○), 532.5 (■), and 2663 (▲) μM. Plots of $1/V$ vs. $1/$[peptide] also give parallel lines at several fixed ATPβS "A" concentrations (not shown). Data points represent the average of two sets of triplicate measurements. The experimental results using 532.5 μM heptapeptide were obtained 1 day after the results using the other peptide concentrations.

The stereochemical conclusions presented here and elsewhere (Jaffe & Cohn, 1978) rest on the assumption that the stereochemistry of the most reactive Mg–thio-ATP diastereomer corresponds to the stereochemistry of the MgATP complex preferred by the enzyme. This further implies that the pathway for the enzyme-catalyzed reaction of the most favored Mg–thio-ATP diastereomer substrate should be like that for MgATP. To investigate this point, we have compared the kinetic behavior of MgATPβS "A" in the phosphorylation of the synthetic peptide substrate with that using MgATP under identical conditions. Our results generally support the basic premise that protein kinase acts on MgATP and MgATPβS "A" in a similar manner, but some surprising differences in details of the kinetics of the reactions of the two metal–nucleotide complexes have been found.

When MgATP and peptide were substrates, the initial velocity pattern given in Figure 1a was obtained. These results are reminiscent of the initial velocity pattern for MgATP and peptide found by Pomerantz et al. (1977) using the catalytic subunit from calf thymus protein kinase. The data appear to fit a sequential mechanism, and Pomerantz et al. (1977) have interpreted their data in that fashion (i.e., both substrates must add before either product is released).

The corresponding data obtained using MgATPβS "A" and peptide as substrates appear to fit a remarkably different pattern, as seen in Figure 1b. Here, the initial velocity pattern is what one would expect for a classical ping-pong sequence in which the first product must dissociate from the enzyme before the second substrate can bind. Similar results have been obtained by Moll & Kaiser (1976) for bovine brain protein kinase holoenzyme, using MgATP and histone as substrates. A variety of tests exist to establish whether the parallel lines of the double-reciprocal plots are truly parallel, giving added credence to the interpretation of a ping-pong reaction sequence. One such criterion is based on the prediction that a plot of $K_{m_{app}}$ vs. $V_{m_{app}}$ for either substrate will result in a straight line with

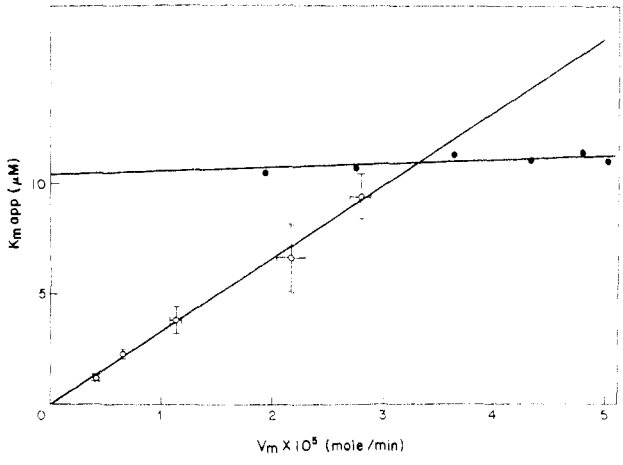

FIGURE 2: Plot of apparent K_m and V_m data evaluated by using heptapeptide and ATP (●) or ATPβS "A" (○) as substrates.

zero intercept if a ping-pong mechanism is operating or a positive intercept if another mechanism is applicable (Koster & Veeger, 1968; Slater, 1955). Within error, the intercept of Figure 2 with ATPβS "A" as the substrate passes through the origin, giving supporting evidence for a ping-pong sequence involving ATPβS "A".

We have evaluated the kinetic parameters for MgATP and peptide by use of secondary plots (see parts a and b of Figure 3) of the data in Figure 1a. For this analysis we assumed that the catalytic subunit mediates peptide phosphorylation by an ordered sequence mechanism with MgATP as the first substrate to add to the enzyme.[2] For comparison we have also

[2] Product inhibition studies have demonstrated that MgADP is a competitive inhibitor of MgATP (H. Kondo and E. T. Kaiser, unpublished experiments). This supports the assignment of MgATP as the first substrate to add to the enzyme.

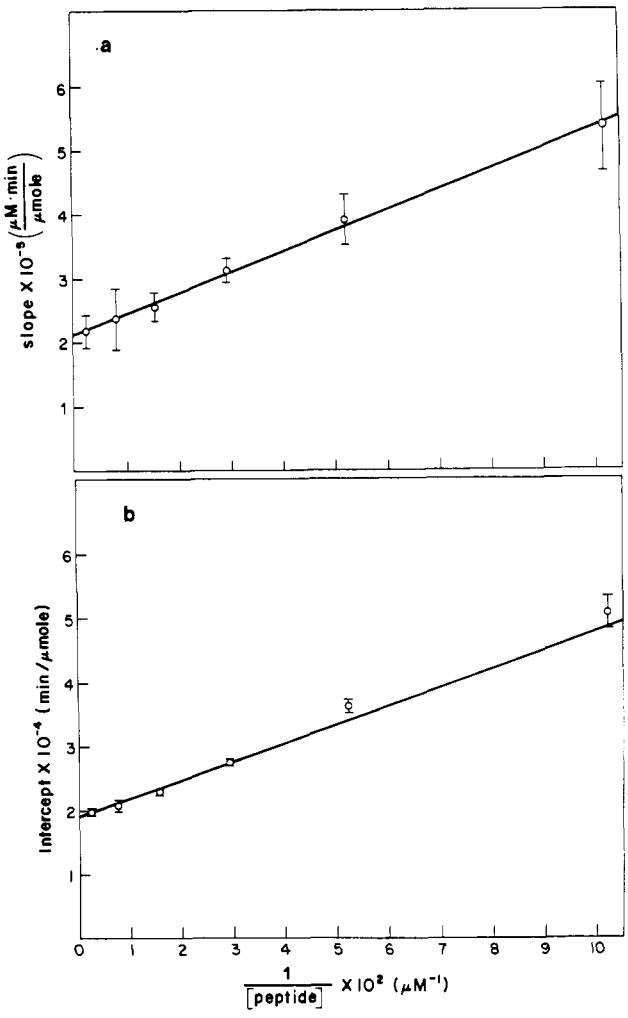

FIGURE 3: (a) Plot of slopes of Figure 1a vs. $[peptide]^{-1}$. (b) Plot of intercepts of Figure 1a as a function of $[peptide]^{-1}$.

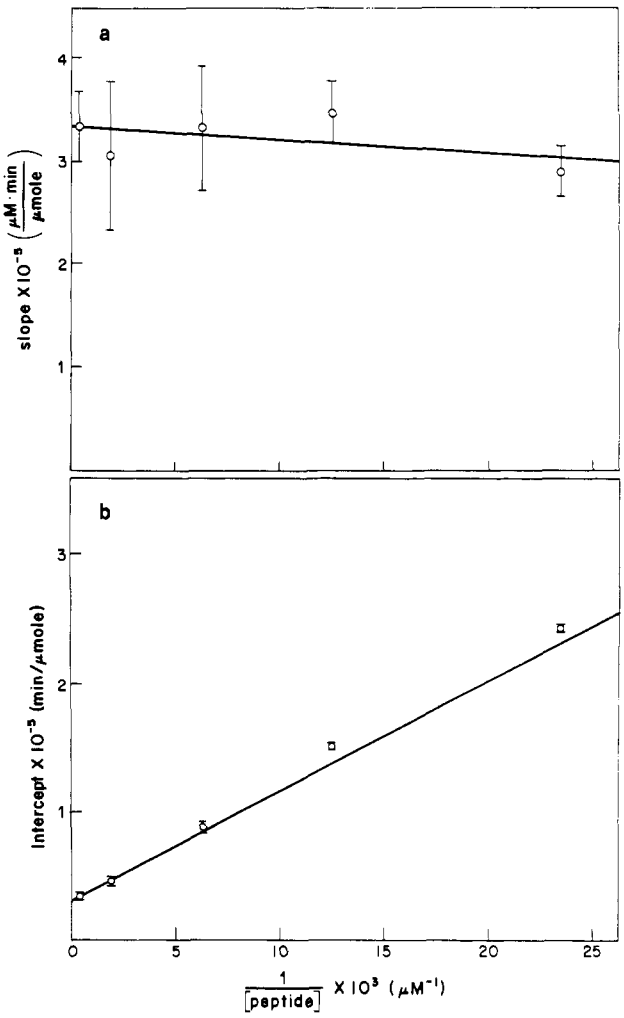

FIGURE 4: (a) Slopes of lines in Figure 1b as a function of $[peptide]^{-1}$. (b) Intercepts of lines in Figure 1b as a function of $[peptide]^{-1}$.

Table II: Kinetic Constants for Phosphorylation of Heptapeptide in the Presence of [γ-^{32}P]ATP or [γ-^{32}P]ATPβS "A"

	substrate	
	[γ-^{32}P]ATP	[γ-^{32}P]ATPβS
K_{m_B} (μM)	15.38 ± 1.02	274.5 ± 13.4
K_{m_A} (μM)	10.7 ± 0.26	11.0 ± 0.59
k_{cat} (min^{-1})	613.2 ± 8.9	657.1 ± 5.1
K_{D_A} (μM)	12.75 ± 1.35	

evaluated the kinetic constants for the peptide plus MgATPβS "A" system by the use of secondary plots (see parts a and b of Figure 4) of the data in Figure 1b. The kinetic constants for both substrate systems are listed in Table II.

Not all of the data which we have are supportive of a ping-pong sequence for ATPβS "A". Since the sequence requires the transient formation of a phosphoenzyme, we attempted to detect the existence of such an intermediate by measuring the exchange of the donor substrate with nucleotide diphosphate product. We have assumed in this experiment that ADP would interact with enzyme in a manner identical with ADPβS. An enzyme solution containing MgATPβS "A" was incubated with [^{14}C]MgADP at 25 °C, and aliquots of the mixture removed at regular intervals were chromatographed on PEI sheets. After elution of the PEI sheets with 0.75 M K_2HPO_4 buffer at pH 3.4, autoradiography was performed on the chromatogram to detect radioactive spots. Even with this very sensitive technique, there was never any indication of ^{14}C-labeled nucleotide triphosphate formation. Even if the equilibrium lies strongly in favor of phosphenzyme, detection of at least minute amounts of ^{14}C-labeled nucleotide triphosphate would be expected. For this reason the likelihood of MgATPβS utilization by means of a true ping-pong mechanism is seriously questioned.

Inhibition studies using MgADPβS gave further evidence against a ping-pong pathway for MgATPβS "A". The first product (MgADPβS) released by the enzyme should exhibit no inhibitory effects under conditions in which the second substrate (heptapeptide) is saturating. Figure 5 demonstrates that MgADPβS is not only inhibitory but also that the inhibition pattern is strictly competitive. This type of inhibition has also been observed with MgADP by using MgATP and heptapeptide as substrates and is consistent with the inhibition pattern expected for an ordered sequence mechanism[2] (Fisher & Hoagland, 1968).

Despite the question as to the appropriate kinetic mechanism for MgATPβS "A", the kinetic parameters for MgATP and MgATPβS "A" are remarkably similar. MgATPβS "A" is approximately as good a substrate as MgATP in terms of their k_{cat} values. Furthermore, the K_m values for MgATP and MgATPβS "A" are essentially identical. Only the K_m for peptide appears to be markedly different for the two systems with the peptide K_m in the presence of MgATP being almost 20-fold lower than it is in the presence of MgATPβS "A". It is also seen from Table II that the dissociation constant for MgATP appears to be equal to the K_m for this substrate. The

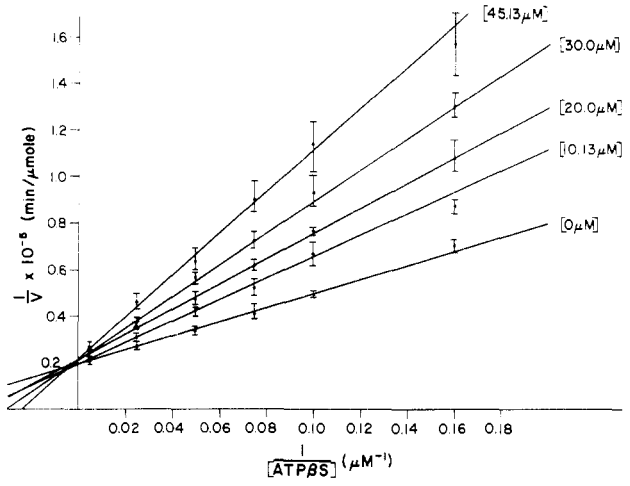

FIGURE 5: Product inhibition of the phosphorylation of heptapeptide with ATPβS "A" using ADPβS. Final concentrations were 2.66 mM heptapeptide, 10 mM $MgCl_2$, 2.46 nM catalytic subunit, and ADPβS as indicated. All other solution conditions were as described under Experimental Section. Within error, all intercepts are identical and entirely consistent with the strictly competitive inhibition by ADPβS. A K_i value of 25.1 ± 2.2 μM for ADPβS was determined from these data.

equivalence of these values is probably fortuitous.

Discussion

It has recently been demonstrated that stable complexes of ATP with Co(III) may serve as substrates for a number of enzymes, and the particular isomer preferred by an enzyme provides a means for determining the chelate structure as well as the absolute stereochemistry of the productive metal ion–ATP complex (Cornelius & Cleland, 1978; Merritt et al., 1978; Danenberg & Cleland, 1975; Li et al., 1978). The β,γ-bidentate complexes of $Co^{III}(NH_3)_4ATP$ exist in two geometric forms (Δ and Λ), and the absolute configurations of these structures are known (Merritt et al., 1978). Cornelius & Cleland (1978) have shown that only the Λ isomer of β,γ-bidentate $Co^{III}(NH_3)_4ATP$ is a substrate for hexokinase and have concluded that the preferred geometry of the β,γ-bidentate MgATP complex with the enzyme must be identical with that of the Λ isomer of β,γ-bidentate $Co^{III}(NH_3)_4ATP$.

Recently, Granot et al. (1979) demonstrated that bovine cardiac muscle protein kinase catalytic subunit was able to phosphorylate the heptapeptide Leu-Arg-Arg-Ala-Ser-Leu-Gly by using only the Δ isomer of β,γ-bidentate $Co^{III}(NH_3)_4ATP$ as substrate. Thus, the β,γ-bidentate complex of MgATP having the same geometry as the Δ isomer of $Co^{III}(NH_3)_4ATP$ is expected to be the preferred substrate for this enzyme. However, because of the very low rate of the catalytic action of the protein kinase on the Δ isomer of β,γ-bidentate $Co^{III}(NH_3)_4ATP$, the application of an alternative stereochemical test involving the use of a more reactive substrate seemed desirable. For this reason the possibility that ATPβS might serve as a reactive substrate for the protein kinase was explored. Indeed, as shown in Table I, the "A" isomer of β,γ-bidentate MgATPβS was found to be utilized very efficiently by the catalytic subunit of protein kinase.

The center of asymmetry in the diastereomers of β,γ-bidentate $Co^{III}(NH_3)_4ATP$ complexes which can be a focal point in studies of enzyme stereospecificity is the phosphorus of the β-phosphate group, as is the case for metal ion complexes of ATPβS. Accordingly, the configurations of the β,γ-bidentate metal ion complexes of the two diastereomers of ATPβS correspond to the two respective stable stereoisomers of bidentate $Co^{III}(NH_3)_4ATP$. It is logical to equate the structure of the metal ion–ATPβS diastereomer which is preferred by the enzyme with that of the preferred isomer of $Co^{III}(NH_3)_4ATP$. On the basis of this assumption and the results of Table I, one can conclude that the β,γ-bidentate MgATPβS "A" isomer should have the same geometric configuration as the Δ isomer of β,γ-bidentate $Co^{III}(NH_3)_4ATP$. In order to verify that it is the chirality at the β-phosphorus of the β,γ-bidentate metal ion complex that is the principal determinant for selectivity by the enzyme, the Cd(II) complexes of ATPβS were used as substrates. Jaffe & Cohn (1978) have shown that while Mg(II) binds predominantly to the oxygens in complexing with ATPβS, Cd(II) coordinates primarily to the sulfur rather than the oxygen atom at the β position. Because of these metal ion preferences, the β,γ-bidentate MgATPβS "A" isomer is expected to be geometrically equivalent to the β,γ-bidentate Cd^{II}ATPβS "B" isomer. In such a case the β,γ-bidentate Cd^{II}ATPβS "B" complex should be preferred by the enzyme over the corresponding "A" diastereomer Cd^{II}ATPβS complex. This prediction is borne out as seen in Table I, and from these results one can conclude that protein kinase catalytic subunit accepts as a preferred substrate the β,γ-bidentate MgATP "A" complex having the absolute geometric configuration corresponding to the Δ isomer of β,γ-bidentate $Co^{III}(NH_3)_4ATP$.

Our assignments of the equivalence of the geometries of the β,γ-bidentate complexes of $Co^{III}(NH_3)_4ATP$ (Δ isomer), MgATPβS "A" isomer, and Cd^{II}ATPβS "B" isomer are entirely consistent with the assignments of Jaffe & Cohn (1978) in their studies on the stereochemistry of the metal ion–nucleotide triphosphate substrates preferred by hexokinase. The configuration at the β-phosphorus of the $Co^{III}(NH_3)_4ATP$ substrates preferred by hexokinase is opposite to that of the β,γ-bidentate $Co^{III}(NH_3)_4ATP$ complex preferred by protein kinase catalytic subunit. Since these two enzymes function with substrates having opposite configurations at the β-phosphorus and the catalytic preferences of the enzymes for Mg(II) and Cd(II) complexes of ATPβS diastereomers are also exactly opposite, the internal agreement of metal ion–nucleotide triphosphate structural assignments in both studies is an important confirmation of the stereochemical concepts originally described by Jaffe & Cohn (1978). Recently, Richard et al. (1978) have independently determined the absolute configurations of the diastereomers of ATPβS with results in agreement with those of Jaffe & Cohn (1978).

Though β,γ-bidentate MgATP exists in the same geometric forms as MgATPβS "A" and MgATPβS "B", the transformation from one form of MgATP to another occurs very rapidly in solution. The active site of an enzyme may well prefer one of these metal ion–nucleotide triphosphate configurations, and this preference should be reflected in the relative reaction rates of the enzyme for MgATPβS "A" or MgATPβS "B". It follows that the preferred MgATPβS diastereomer would be an excellent model for the productive β,γ-bidentate MgATP complex. Accordingly, if the sulfur atom does not significantly alter the chemistry of the enzyme-mediated γ-phosphoryl transfer reaction, then the preferred MgATPβS diastereomer should have kinetic characteristics very similar to those of MgATP. We have investigated this point in some detail with protein kinase catalytic subunit by obtaining initial velocity data for peptide phosphorylation using MgATP and also MgATPβS "A".

A most striking difference in the kinetic behavior of MgATPβS "A" and MgATP is evident from the double-reciprocal plots for these substrates (parts a and b of Figure 1). The initial velocity pattern exhibited by MgATP is charac-

teristic of a sequential mechanism while that observed for MgATPβS "A" is generally interpreted to indicate a ping-pong reaction sequence. Further analysis of the kinetics of MgATPβS "A" demonstrated that the data conform very well to ping-pong kinetics (see Figure 2) and that the slopes of Figure 1b are independent of the MgATPβS "A" concentration (Figure 4a). Nevertheless, ping-pong and sequential pathways are so different mechanistically that it is quite unlikely that the same enzyme would be capable of performing such very different tasks on geometrically identical substrates.

The most direct evidence for the existence of a ping-pong mechanism in this system would be to observe the exchange of radiolabel between donor substrate (ATPβS) and nucleotide diphosphate product in the absence of peptide acceptor. The lack of available radiolabeled ADPβS prevented us from performing this particular experiment, so [^{14}C]ADP was used instead along with the assumption that binding of ADP would mimic ADPβS binding to the putative phosphoenzyme. In the experiment, catalytic subunit was incubated with MgATPβS "A" and Mg[^{14}C]ADP in the absence of peptide and autoradiography of PEI chromatograms was performed on samples taken from the reaction mixture at various time intervals. This very sensitive technique failed to give any evidence whatsoever of [^{14}C]ATP appearance even after extended incubation times. The failure to observe exchange indicates either that [^{14}C]ADP is unable to interact with enzyme in a manner conducive for exchange or that the reaction of MgATPβS "A" does not proceed by a ping-pong sequence despite the implications of Figure 1b.

Additional evidence against a ping-pong mechanism for ATPβS comes from product inhibition studies involving ADPβS, with ATPβS and heptapeptide as substrates. For this mechanism the phosphate donor (ATPβS) must be the first substrate to combine with enzyme, and the corresponding nucleotide diphosphate (ADPβS) is the first product released. If the concentration of the second substrate (heptapeptide) is saturating, then peptide phosphorylation should not be inhibited by the presence of ADPβS (Fisher & Hoagland, 1968). By contrast, if ATPβS functions by an ordered sequence mechanism as ATP does, then ADPβS should be a competitive inhibitor of ATPβS with heptapeptide saturating. The results of Figure 5 demonstrate that ADPβS is inhibitory and the inhibition is competitive. This gives very strong evidence that phosphate transfer from ATPβS is catalyzed by the same mechanism as in the case of ATP and is consistent with an ordered reaction sequence.

The interpretation of steady-state kinetic data can be ambiguous, and there are circumstances under which apparent ping-pong kinetics can be observed for an enzyme system actually operating by way of a sequential mechanism (Segel, 1975). This follows from consideration of the rate equation

$$\frac{1}{V} = \frac{1}{V_m} + \frac{K_{m_A}}{V_m}\frac{1}{[A]} + \frac{K_{m_B}}{V_m}\frac{1}{[B]}\left(1 + \frac{K_D}{[A]}\right) \quad (1)$$

for an ordered sequence reaction with [A] representing the Mg–nucleotide triphosphate substrate concentration, [B] representing the peptide substrate concentration, K_{m_A} and K_{m_B} the corresponding Michaelis constants for A and B, and K_D the thermodynamic dissociation constant for A. The numerical value of $1 + (K_D/[A])$ is exceedingly important in determining the magnitude of the slope change in a $1/V$ vs. $1/[B]$ plot at several fixed concentrations of A. If $K_D/[A]$ remains very small in comparison to unity over the full range of A concentration, then the $1/V$ vs. $1/[B]$ plots will exhibit no slope changes, resulting in data like those of Figure 1b. If, however $K_D/[A]$ is significant in comparison to unity over the concentration range of A, data as indicated in Figure 1a would be expected.

If this interpretation is indeed correct for the data obtained for peptide phosphorylation by MgATP and MgATPβS "A" catalyzed by the protein kinase, it should be possible to make an estimate of the limiting value of K_D for MgATPβS "A". On the basis of the accuracy of the slopes measured from Figure 1b, a 20% increase in slope in going from the highest concentration of MgATPβS "A" to the lowest should be detectable. By use of this estimate along with the lowest concentration of MgATPβS "A" (4 μM) employed in our experiments, the observation of apparently parallel lines (Figure 1b) implies that the K_D for this substrate has a limiting value of ≤0.8 μM. This is to be compared with an evaluated K_D for MgATP of 13 μM (Table II).

While this explanation is quite plausible, it is very difficult to evaluate the dissociation constant for the binding of ATPβS "A" to the catalytic subunit. Since the catalytic subunit exhibits significant phosphohydrolase activity in the micromolar range of enzyme concentration, it has not been feasible to use time-requiring techniques for measuring K_D, like equilibrium dialysis. The use of filter binding assays to measure K_D for the MgATPβS "A"–protein kinase complex also poses problems because the dissociation constant is not small enough for the method to be accurate.

Behavior similar to that seen in parts a and b of Figure 1 has been observed for substrates of brain hexokinase (Fromm & Ning, 1968). A kinetic pattern characteristic of ping-pong kinetics was observed with glucose and ATP as substrates, while a pattern corresponding to ordered sequence kinetics was found by using fructose and ATP. Since glucose and fructose appear to react at the same site on the enzyme, Fromm & Ning claimed that the enzyme functions by means of a sequential mechanism and that ping-pong kinetics are observed only because the Dalziel coefficient (ϕ_{12}) in the rate equation is much smaller with glucose as substrate than with fructose. The consequence of their explanation is essentially the same as we have suggested in the case of the protein kinase catalytic subunit.

As seen from the data in Table II, MgATP and MgATPβS "A" have identical K_m values and are equally good substrates in terms of their k_{cat} values. This is expected if both substrates are utilized by the protein kinase in a very similar fashion. The major differences between the two substrates lie in the peptide K_m values and, presumably, the K_D values for nucleotide triphosphates. With MgATPβS "A" as substrate, the K_m for peptide is ~20 times larger than the K_m for peptide determined with MgATP as cosubstrate. The K_D for MgATP is 13 μM, and if the MgATPβS "A" substrate follows the same sequential mechanism, its K_D must be ≤0.8 μM, suggesting that the "A" isomer is bound an order of magnitude more strongly than MgATP. The close correspondence of the kinetic constants for MgATP and MgATPβS "A" provides general support for the conclusion that MgATPβS "A" is not only an excellent model for the geometrically preferred form of MgATP but also that the kinetic differences represent rather small modulations of the catalytic action.

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Proximity of Antibody Binding Sites Studied by Fluorescence Energy Transfer†

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ABSTRACT: Fluorescence energy transfer experiments by steady-state and nanosecond monophoton techniques were carried out with a covalently linked hybrid rabbit IgG antibody containing one antilactose site and one anti-Dns [5-(dimethylamino)-1-naphthalenesulfonyl] site. The hybrid antibody was prepared from antilactose and anti-Dns antibody by mild reduction, dissociation into half-molecules in acid, and random reassociation with re-formation, to the extent of 80%, of the single disulfide bond between the heavy chains. Fractionation with an antilactose-specific immunoadsorbent yielded a population in which each IgG molecule contained no more than one anti-Dns site per antibody. The acceptor molecules used for intramolecular energy transfer were derivatives of *p*-aminophenyl β-lactoside (PAPL): (dimethylamino)benzeneazo-PAPL and *N*-fluorescyl-PAPL. The fluorescence lifetime (24 ns) and quantum yield (0.57) of the bound Dns group were unaffected by the presence of the acceptor in the adjacent site. Three models were used to calculate the minimum distance between the adjacent sites of the IgG antibody based on the overlap in the emission and absorption spectra of the donor–acceptor pairs and the segmental flexibility of the immunoglobulin molecule. The calculations yielded values in the range of 5.5–7.0 nm for the minimum distance of separation between the antibody sites in solution and demonstrated a substantial energy barrier to the closer approach of the sites.

The property of segmental flexibility inherent and distinctive to the immunoglobulin molecule has been established by sedimentation velocity (Noelken et al., 1965), by electron microscopy (Valentine & Green, 1967; Schumaker et al., 1979), and by time-dependent fluorescence polarization using monophoton technology (Yguerabide et al., 1970; Chan & Cathou, 1977; Lovejoy et al., 1977). Such molecular flexibility undoubtedly plays an important role in the ability of antibody to establish multivalent attachment when identical reiterated antigenic determinants are presented, as in the cases of viral, bacterial, and neoplastic surface antigens. These multiple interactions result in enhancement of intrinsic affinities, thereby increasing the efficiency of immunological responses. In addition, it may be that segmental flexibility plays a role in the regulation of complement activation by immunoglobulin in which diminution of flexibility due to antigen binding allows for more efficient recognition of the Clq binding site of the $C_\gamma 2$ domain.

In an ingenious attempt to define the limits of molecular flexibility and to delineate the overall shape of IgG in solution, Werner et al. (1972) attempted to determine the average distance between the two antigen combining sites of IgG by measuring the efficiency of fluorescent energy transfer between a Dns[1] donor and a fluorescein acceptor positioned in the

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