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Adenosine Cyclic 3′,5′-Monophosphate Dependent Protein Kinase: Kinetic Mechanism for the Bovine Skeletal Muscle Catalytic Subunit†

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ABSTRACT: The kinetic mechanism for adenosine cyclic 3′,5′-monophosphate dependent protein kinase was determined from initial velocity studies in the absence and presence of the product MgADP and dead-end inhibitors. Data are consistent with random addition of MgATP and Ser-peptide and ordered release of phospho-Ser-peptide and MgADP with a dead-end E–MgADP–Ser-peptide complex. In addition to the metal required for the nucleotide, we also characterized the binding of Mg^{2+} to a second site. Increasing the Mg^{2+} results in a 5–6-fold decrease in V_{max} in the presence or absence of 0.1 M KCl. There is a 5-fold increase in V/K_{MgATP} in the absence of KCl and a 13-fold increase in V/K_{MgATP} at 0.1 M KCl. The effect of increasing free Mg^{2+} on V_{max} and V/K was also obtained with MgITP (20% the V_{max} with MgATP) and MgGTP (10% the V_{max} with MgATP) as substrates. The dissociation constant for Mg^{2+} from E–Ser-peptide–Mg^{2+} and central complexes is 2–3 mM. At low concentrations of free Mg^{2+} and no added KCl, competitive inhibitors of MgATP (K_i = 160 μM) decrease in the order adenosine = MgADP ($K_i \simeq 40$ μM) > AMP ($K_i \simeq 8$ mM). At saturating free Mg^{2+}, K_i values are 10 μM (MgATP), 10 μM (MgADP), 40 μM (adenosine), and 850 μM (AMP). In addition, guanosine (1 mM) and $MgPPP_i$ (10 mM) failed to inhibit at low or high free Mg^{2+}. A mechanism for nucleotide interaction is proposed on the basis of a hydrophobic site or negatively charged group in the region of the α-phosphate in the nucleotide binding site and the presence or absence of a negative charge on the α-phosphate. We also made use of a spectrophotometric assay for protein kinase that couples the production of MgADP to the pyruvate kinase and lactate dehydrogenase reactions. On the basis of determinations of the amount of phosphorylatable Ser-peptide by spectrophotometric or radioisotopic determinations, we find that commercial samples contain 25–50% nonpeptide material by weight.

Adenosine cyclic 3′,5′-monophosphate dependent protein kinase catalyzes the phorphorylation of a variety of proteins according to

$$MgATP + protein \rightarrow phosphoprotein + MgADP$$

Activation by cAMP[1] occurs via dissociation of the inactive holoenzyme (R_2C_2) to a regulatory dimer (R_2·$cAMP_4$) and two catalytic monomers (C). A common feature of the amino acid sequence of many cAMP-dependent protein kinase substrates is the presence of one or two arginyl residues on the amino-terminal side of the phosphorylated serine or threonine (Zetterquist et al., 1976; Kemp et al., 1977; Feramisco et al., 1979, 1980; Kemp, 1980). Guanethidine sulfate, a guanidinium containing analogue, is a competitive inhibitor of protein kinase with respect to the protein substrate (Witt & Roskoski, 1980). The nucleotide specificity of the enzyme has been reported by Walsh & Krebs (1973).

A combination of magnetic resonance (Granot et al., 1979, 1980) and kinetic studies (Armstrong et al., 1979; Bolen et

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[1] Abbreviations: cAMP, adenosine cyclic 3′,5′-monophosphate; AMPPCP, 5′-adenylyl methylenediphosphate; Ser-peptide, Leu-Arg-Arg-Ala-Ser-Leu-Gly; R, regulatory subunit; C, catalytic subunit; E_t, total enzyme concentration; Mops, 4-morpholinepropanesulfonic acid; DPNH, reduced nicotinamide adenine dinucleotide.

0006-2960/82/0421-5794$01.25/0

al., 1980) led to the proposal of an inhibitory divalent metal site. At high concentrations, both Mn^{2+} and Mg^{2+} occupy a site on the enzyme (in addition to the metal nucleotide) that coordinates the enzyme and the α and γ or α, β, and γ positions of metal ATP. Occupation of this inhibitory site decreases the maximum velocity and increases the V/K for metal nucleotide as a result of an increased nucleotide affinity and decreased K_m. Granot et al. (1980) reported that saturating Mn^{2+} decreases the dissociation constant of MnAMPPCP 17-fold. It also decreases the maximal velocity about 70-fold (MnATP as substrate). Interactions of Mg^{2+} with this inhibitory site were less well characterized. Armstrong et al. (1979), however, showed that saturating Mg^{2+} decreases the V_{max} about 5-fold; the dissociation constant for Mg^{2+} was about 2.3 mM.

Studies carried out by Feramisco & Krebs (1978) have shown that the K_d for acetyl-Ser-peptide is 250 μM when obtained in the presence or absence of Mg^{2+} and 5′-adenylyl imidodiphosphate, yet the K_m for this same peptide is 3 μM. This effectively rules out a completely rapid equilibrium random mechanism. A sequential kinetic mechanism has been suggested by Pomerantz et al. (1977) and a compulsory ordered addition of MgATP and Ser-peptide has been suggested by Bolen et al. (1980). We have determined the kinetic mechanism of the bovine skeletal muscle catalytic subunit with Ser-peptide and MgATP as substrates under several conditions. These include low (0.5 mM free) and high Mg^{2+} (10 mM free) with 0.1 M KCl. Data were also obtained that provide mechanistic information concerning Mg^{2+} interactions at its inhibitory site and the interaction of the substrates MgITP and MgGTP at the nucleotide site.

Materials and Methods

Chemicals and Enzymes. Ismelin sulfate (guanethidine) was obtained from CIBA-Geigy. Bovine skeletal muscle cAMP-dependent protein kinase catalytic subunit (type II) was prepared by a modification of the method of Sugden et al. (1976). The final specific activity was 19 units/mg (at 10 mM Mg^{2+} with Ser-peptide as substrate). Other enzymes and chemicals were obtained from Sigma Chemical Co.

Protein Kinase Activity Measurements. The protein kinase C subunit activity was determined by two methods. The first was the phosphocellulose radioisotopic procedure (Witt & Roskoski, 1975) modified for use with Ser-peptide as substrate. For resolution of labeled ATP from the phospho-Ser-peptide, portions (25 μL) of the reaction mixture were applied onto 1 × 2 cm phosphocellulose strips and immersed in 75 mM phosphoric acid (10 mL/strip; 5 mL of 85% H_3PO_4/L of H_2O) to terminate the reaction. The strips were swirled gently for 2 min, the phosphoric acid was decanted, and the phosphocellulose strips were washed twice more (2 min each) in phosphoric acid with gentle agitation. Radioactivity was measured by liquid scintillation spectrometry. After the application of 250 000 cpm, blanks were less than 250 cpm. With a fourth wash, blanks were less than 100 cpm. We also made use of a spectrophotometric assay for protein kinase that couples the production of MgADP to the pyruvate kinase and lactic dehydrogenase reactions. A typical assay contained the following components in a 1-mL total volume unless otherwise noted: 100 mM Mops, pH 7.1, 15 units of pig heart lactic dehydrogenase, 7 units of rabbit muscle pyruvate kinase, 100 mM KCl, 1 mM phosphoenolpyruvate, 200 μM DPNH, 1.5 mM $MgCl_2$, 1 mM ATP, and 0.25 mM Ser-peptide. The reaction was initiated by the addition of 0.017 unit of C subunit (based on 19 units/mg obtained with 10 mM Mg^{2+}) after allowing sufficient time to record a background rate due to the ATPase activity of pyruvate kinase. This rate was substracted from the rate obtained after the addition of protein kinase. Rates were obtained on a Gilford 220 spectrophotometer and strip-chart recorder with the wavelength selector at 340 nm and signal output at 0.05–0.1 OD full scale. The cell compartment was maintained at 25 °C with a heating–cooling water bath and thermospacers. Optimum concentrations of lactic dehydrogenase and pyruvate kinase were determined according to Cleland (1979a) and checked by varying the protein kinase concentrations. A plot of velocity against concentration of protein kinase was linear up to at least 0.08 unit. No lag was observed in any of the time courses. Data obtained with the spectrophotometric assay correlated exactly with data obtained with the radioassay.

For calculation of the concentration of uncomplexed Mg^{2+} and Mg–nucleotides, correction was made for competitive chelation by K^+ and other nucleotides that were present in the reaction mixture. The stability constants [written as K = (metal)(ligand)/(metal–ligand)] used are as follows: MgATP, 0.0143 mM; MgADP, 0.25 mM; MgAMP, 3.2 mM; K-ATP, 100 mM; K-ADP, 210 mM; MgPEP, 5 mM; Mg–Ser-peptide, taken as 316 mM, which is the value for magnesium acetate; K-AMP, K-PEP, and K–Ser-peptide are not significant for conditions used in this study. Stability constants for MgATP and MgADP are from Morrison (1979) while all others are from Dawson et al. (1979). The stability constants used do not vary enough with ionic strength to significantly effect levels of *free* and complexed species present in the reaction mixtures for the metal–ligand combinations used.

Determination of Ser-peptide Concentration and Purity. The concentration of phosphorylatable Ser-peptide can readily by obtained by the coupled spectrophotometric assay. Because the couple depends on the MgADP produced (converting it to MgATP), the concentration of nucleotide substrate remains constant, the reaction goes to completion (that is, until Ser-peptide is depleted), and the concentration of Ser-peptide is directly proportional to the ΔOD_{340} that results from the decrease in the concentration of DPNH. A typical concentration assay contained in a 1-mL total volume the following: 100 mM Mops, pH 7.1, 15 units of pig heart lactic dehydrogenase, 21 units of rabbit muscle pyruvate kinase, 0.085 unit of protein kinase C subunit, 100 mM KCl, 1 mM phosphoenolpyruvate, 1 mM ATP, 1.5 mM $MgCl_2$, and 200 μM DPNH. The above reaction mixture is incubated at 25 °C for 5 min (during this time any ADP present is converted to ATP), and a small background ATPase rate is recorded. The initial absorbance is recorded just before the addition of Ser-peptide, and a small amount of Ser-peptide (calculated to give 0.6–1.0 absorbance change at 340 nm, based on weight) is added. The reaction quickly goes to completion, and the rate returns to the base-line ATPase value. The absorbance change is corrected for the minor background rate, and the concentration of Ser-peptide is calculated from an extinction coefficient of 6220 M^{-1} cm^{-1} for DPNH at 340 nm.

Ser-peptide was purchased from three commercial sources: Sigma Chemical Co., Boehringer-Manheim, and Peninsula Laboratories. Materials obtained from the above three sources (several lot numbers in the case of Sigma and Peninsula) were 55 ± 2%, 75 ± 3%, and 76 ± 2% Ser-peptide, respectively, by either the spectrophotometric or radioenzymatic procedure.

High-performance liquid chromatograms of these peptides were obtained by means of a Beckman high-performance liquid chromatograph with a C_{18} column by using an Altec pump running reverse phase with 50 mM phosphate/50% methanol. The effluent was monitored for absorbance at 210 nm.

Table I: Product Inhibition by MgADP as a Function of Mg_{free} and KCl Concentration[a]

variable substrate	$[Mg_{free}]$ = 0.5 mM				$[Mg_{free}]$ = 10 mM			
	pattern	K_m[b] (μM)	K_{is} (μM)	K_{ii} (μM)	pattern	K_m[b] (μM)	K_{is} (μM)	K_{ii} (μM)
				[KCl] = 0				
MgATP[c]	C	92 ± 6	52 ± 4		C	12.9 ± 0.8	14.3 ± 0.9	
				[KCl] = 0.1 M				
MgATP[c]	C	177 ± 13	227 ± 26		C	14.4 ± 0.9	9.4 ± 0.5	
Ser-peptide[d]	NC	9.6 ± 0.8	107 ± 24	221 ± 32	NC	8.1 ± 0.7	19 ± 4	18.1 ± 1

[a] All data were obtained by the radioassay. [b] K_m for the variable substrate. [c] Data obtained at saturating and nonsaturating concentrations of Ser-peptide are identical. [d] Patterns were obtained with [MgATP] = K_{MgATP}.

Chromatograms indicate that the Sigma product is about 90% Ser-peptide whereas the other two are about 96%. Since the contaminants are mainly invisible at 210 nm, either they are salt and water or else they elute with the same retention time as Ser-peptide (the latter possibility is unlikely). Amino acid analyses were also obtained on a Technicon amino acid analyzer. Molar ratios based on serine obtained for the above peptides are 1.7:2.5:1.4:1.0:0.93, 2.0:2.3:1.2:1.0:1.0, and 2.2:2.3:1.0:1.0:1.2, respectively, for Leu:Arg:Ala:Ser:Gly (standard error is 10%). Significant deviations were consistently obtained for arginine in all three products, alanine in the case of both Sigma and Boehringer-Manheim, and glycine for Peninsula. All three Ser-peptide preparations gave K_m and V_{max} values that were essentially identical when Ser-peptide was varied.

Data Processing. Reciprocal initial velocities were plotted against reciprocal substrate concentrations, and all plots were linear. Data were fitted to the appropriate rate equation by using the FORTRAN programs of Cleland (1979b). Data for MgGTP and MgITP obtained at saturating Ser-peptide and several free Mg^{2+} concentrations were fitted line by line to eq 1. Initial velocity patterns obtained at different free Mg^{2+}

$$v = VA/(K + A) \quad (1)$$

concentrations varying MgATP at several Ser-peptide concentrations were fitted to eq 2. Data for the dependence of

$$v = VAB/(K_{ia}K_b + K_aB + K_bA + AB) \quad (2)$$

kinetic parameters on free Mg^{2+} concentrations for MgATP, MgGTP, and MgITP were fitted to eq 3. Data for compe-

$$y = a(1 + P/K_{I,num})/(1 + P/K_{I,denom}) \quad (3)$$

titive and noncompetitive inhibition were fitted to eq 4 and

$$v = \frac{VA}{K(1 + I/K_{is}) + A} \quad (4)$$

5, respectively. In eq 1, 4, and 5, V is the maximum velocity,

$$v = \frac{VA}{K(1 + I/K_{is}) + A(1 + I/K_{ii})} \quad (5)$$

K is the Michaelis constant, and K_{is} and K_{ii} are slope and intercept inhibition constants. In eq 2, K_{ia} is the dissociation constant for A and K_a and K_b are Michaelis constants for A and B. In eq 3, y is the observed value of V or V/K, a is the value of y at $P = 0$ ($aK_{I,denom}/K_{i,num}$ is the value of y at $P = \infty$), P is free Mg^{2+} concentration, $K_{I,denom}$ is the dissocation constant for free Mg^{2+}, and $K_{I,num}$ is a ratio of rate constants that causes the kinetic parameter of interest to level off at a finite value as free Mg^{2+} goes to infinity.

Results

Initial Velocity Studies. Armstrong et al. (1979) reported that Mn^{2+} or Mg^{2+} binds to protein kinase at an inhibitory site, as well as to the β- and γ-phosphates of ATP. Therefore, steady-state kinetic analyses were carried out with free Mg^{2+} at 0.5 or 10 mM in combination with either 100 mM or no KCl. Under all of these conditions, MgADP is a competitive inhibitor with respect to MgATP (Table I). With both low and high KCl, moreover, increasing the Mg^{2+} concentration from 0.5 to 10 mM resulted in a decrease in the K_m for MgATP and the K_i for MgADP. The salt significantly affected the values obtained for the kinetic parameters only at low Mg^{2+}. As KCl is increased from 0 to 100 mM, both the K_m for MgATP and the K_i for MgADP are increased (Table I). Salt has no effect on these parameters at 10 mM free Mg^{2+}.

Experiments were also performed in the presence of other nucleotide analogues and guanethidine (a peptide substrate analogue). When the pattern was obtained with an analogue of the variable substrate, the fixed substrate concentration was saturating (more than 10-fold the K_m). When the pattern was obtained with an analogue of the fixed substrate, nonsaturating concentrations of the latter were used. Analogues of MgATP (adenosine and AMP) were competitive vs. MgATP at both low and high free Mg^{2+}. Increasing the Mg^{2+} concentration has no effect on the K_i for adenosine but decreases the K_i for AMP. This finding is consistent with the data of Granot et al. (1979), which indicate that inhibitory Mg^{2+} coordinates the α- and γ-phosphates of MgATP (and may also coordinate the β-phosphate) with the enzyme. Guanethidine, an analogue of Ser-peptide, is competitive with respect to Ser-peptide at both low and high free Mg^{2+} with a constant K_i. When guanethidine was used as an inhibitor with MgATP as the variable substrate, however, noncompetitive inhibition was observed with no change in the K_{is} as free Mg^{2+} was increased. Adenosine is a noncompetitive inhibitor with respect to Ser-peptide. At high free Mg^{2+}, the K_{ii} is unchanged, indicating that adenosine binding to the E–Ser-peptide complex is not altered. On the other hand, the K_{is} is increased nearly 3-fold, indicating that adenosine binding to the free enzyme is reduced at high Mg^{2+}. In addition to the inhibitors listed, guanosine (1 mM) and magnesium tripolyphosphate (10 mM) fail to inhibit at low or high Mg^{2+}.

Determination of Mg^{2+} Dissociation Constants. Experiments were performed as discussed by Cook (1982) and Viola & Cleland (1981) in which the initial velocities were determined at varying Ser-peptide and each of several fixed concentrations of MgATP and a single fixed concentration of free Mg^{2+}. This experiment was repeated at three different concentrations of free Mg^{2+}. The dissociation constants for Mg^{2+} were determined from a plot of V_{max} and V/K_{MgATP} against free Mg^{2+} (Figure 1A,B) and were 1.7 ± 0.8 mM and 3.2 ± 0.6 mM, respectively. The K_d for free Mg^{2+} obtained from the V_{max} plot approximates that obtained from the V/K_{MgATP} plot. The K_m for Ser-peptide failed to change significantly: 20 ± 1 μM at 0.5 mM free Mg^{2+} and 16 ± 1.6 μM at 10 mM

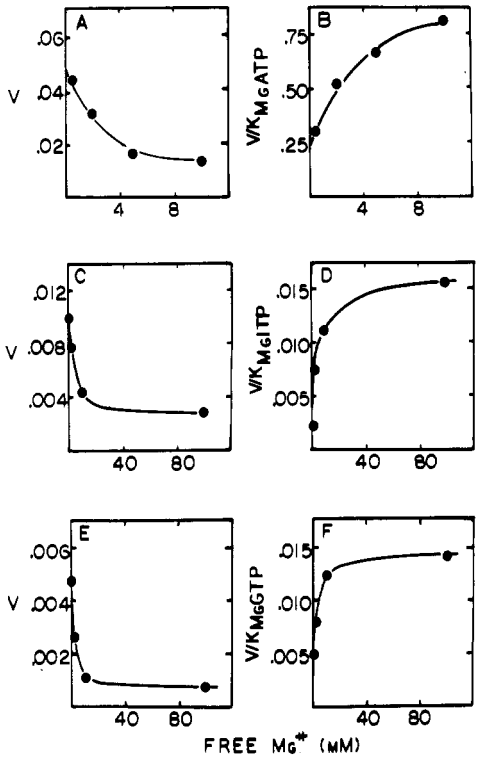


FIGURE 1: Dependence of V_{max} and V/K on concentration of free Mg^{2+}. (A and B) MgATP was the nucleotide substrate; curves are theoretical fits to eq 3. (C and D) MgITP was the nucleotide substrate; curves are drawn by eye. (E and F) MgGTP was the nucleotide substrate; curves are drawn by eye. All data were obtained with the spectrophotometric assay at pH 7 and 25 °C. Ser-peptide was saturating at 250 μM for panels C–F. Data in (A) and (B) are obtained from initial velocity patterns at each free Mg^{2+} concentration so that Ser-peptide is extrapolated to saturating concentration. Each datum point in (A) and (B) comes from a fit of the data for the initial velocity pattern to eq 2. At zero free Mg^{2+} concentrations, the following values for kinetic parameters corrected for enzyme concentration were obtained: $V_{MgATP}/E_t = 3100\ min^{-1}$; $V/(K_{MgATP}E_t) = 1.4 \times 10^7\ M^{-1}\ min^{-1}$; $V_{MgITP}/E_t = 640\ min^{-1}$; $V/(K_{MgITP}E_t) = 1.3 \times 10^5\ M^{-1}\ min^{-1}$; $V_{MgGTP}/E_t = 310\ min^{-1}$; $V/(K_{MgGTP}E_t) = 2.8 \times 10^5\ M^{-1}\ min^{-1}$.

free Mg^{2+}. The decrease in $V/K_{Ser\text{-}peptide}$ is mainly due, therefore, to a decrease in V_{max} at the high Mg^{2+} concentration. This supports the notion of an inhibitory Mg^{2+} site on the enzyme. The K_i for MgATP decreased from 160 ± 20 μM at 0.5 mM free Mg^{2+} to 7.0 ± 2.7 μM at 10 mM free Mg^{2+}. To determine whether this holds for alternate substrate nucleotides (Walsh & Krebs, 1973), we performed experiments with MgITP and MgGTP at saturating Ser-peptide (250 μM). Dissociation constants of Mg^{2+} estimated from titrations of both V and V/K were about 3 mM (in agreement with the data for MgATP). The V_{max} values for MgGTP and MgITP were about 10% and 20%, respectively, that of MgATP (Figure 1). Michaelis constants were 1.1 ± 0.1 mM and 4.38 ± 0.6 mM for MgGTP and MgITP, respectively, at 0.5 mM free Mg^{2+}, which decreased to 0.054 ± 0.016 mM and 0.145 ± 0.035 mM, respectively, at 100 mM free Mg^{2+}. Thus, the V/K values for MgGTP and MgITP were 40- and 100-fold lower, respectively, than that of MgATP. The V/K values for the two alternate substrates, however, were similar. Because these nucleotides are unlikely to be sticky (i.e., dissociate slower than they react to give products), this similarity in V/K values may indicate that the same step is limiting for both nucleotides (assuming no perturbations in the transition-state structure). Free Mg^{2+} decreases the MgATP, MgITP, and MgGTP V_{max} by 5.5-, 4.5-, and 6-fold and the K_m by 25-, 30-, and 20-fold, respectively.

Discussion

On the basis of a sequential initial velocity pattern and a competitive product inhibition pattern by MgADP against MgATP at saturating Ser-peptide concentrations, the kinetic mechanism was suggested to be compulsory ordered (Bolen et al., 1980). The pattern used by Bolen et al. (1980) to suggest order, however, is consistent with several other possibilities including a Theorell–Chance mechanism in which Ser-peptide adds before MgATP with a very rapid release of MgADP after addition of MgATP. Another Theorell–Chance possibility is the addition of MgATP before Ser-peptide with the very fast release of phospho-Ser-peptide after the addition of Ser-peptide. Still another possibility is a rapid equilibrium random mechanism with a dead-end E–ser-peptide–MgADP complex [a completely rapid equilibrium mechanism has been ruled out by the data of Feramisco & Krebs (1978) as stated above]. The product inhibition patterns obtained for MgADP vs. both Ser-peptide and MgATP are consistent with any of the above mechanisms as well as the ordered mechanism proposed previously (Bolen et al., 1980).

Dead-end inhibition patterns, however, are able to differentiate the above mechanisms. An ordered mechanism or Theorell–Chance mechanism in which MgATP binds before Ser-peptide requires that the dead-end inhibitor guanethidine (a Ser-peptide analogue) give an uncompetitive pattern when MgATP is varied. Since a noncompetitive pattern was obtained, these mechanisms would require a dead-end E–guanethidine complex. This would, however, produce an intercept effect when Ser-peptide is varied in the presence of guanethidine, and the patterns were clearly competitive. The Theorell–Chance mechanism with Ser-peptide adding first is still possible because addition of guanethidine before MgATP will give a noncompetitive pattern with the slope effect arising from partial reversal of the inhibition as a result of increasing MgATP. This mechanism, however, is eliminated by the noncompetitive pattern for adenosine against Ser-peptide. Thus, the ordered mechanisms fail to correspond with the patterns observed. However, all patterns are certainly consistent with random addition of MgATP and Ser-peptide. Two combinations of guanethidine are allowed: one with free enzyme and the other with E–MgATP. The first would be competitive and the second uncompetitive to produce the net noncompetitive inhibition that is observed. Similarly, two combinations of adenosine are allowed (one with free enzyme and one with E–Ser-peptide) to produce the observed noncompetitive inhibition with respect to varying Ser-peptide. Release of phospho-Ser-peptide and MgADP adheres to either ordered release of phospho-Ser-peptide and MgADP or rapid release of both products. In either case, a dead-end E–Ser-peptide–MgADP complex must be formed to account for competitive inhibition by MgADP vs. MgATP at saturating Ser-peptide (Table II; Bolen et al., 1980). Granot et al. (1981) estimated the K_d for phospho-Ser-peptide as ≥ 30 mM, which suggests that this product binds very weakly and is probably released very fast with respect to catalysis. Thus, even though an E–phospho-Ser-peptide complex may form, it does not accumulate in the steady state in the direction of peptide phosphorylation. The K_d for Ser-peptide does not change in the presence and absence of a nonhydrolyzable Mg–nucleotide analogue (Feramisco & Krebs, 1978), but the K_m is smaller than K_d. Even though the mechanism is random, it can be depicted as follows since the K_m for Ser-peptide is obtained at saturating MgATP:

$$EA \underset{k_4}{\overset{k_3,\,B}{\rightleftharpoons}} EAB \underset{k_6}{\overset{k_5}{\rightleftharpoons}} EPQ \xrightarrow{k_7} EQ \xrightarrow{k_9} E \qquad (6)$$

Table II: Dead-End Inhibition as a Function of Mg_{free} Concentration[a]

variable substrate	inhibitor	pattern	K_{is} (mM)	K_{ii} (mM)
		$[Mg_{free}]$ = 0.5 mM		
MgATP[b]	guanethidine	NC	2.4 ± 0.4	4.3 ± 0.4
	adenosine	C	0.047 ± 0.006	
	AMP[c]	C	8.7 ± 0.5	
Ser-peptide[b]	guanethidine	C	5.88 ± 0.75	
	adenosine	NC	0.104 ± 0.03	0.079 ± 0.01
		$[Mg_{free}]$ = 10 mM		
MgATP[b]	guanethidine	NC	2.2 ± 0.7	14.5 ± 2.1
	adenosine	C	0.035 ± 0.003	
	AMP[c]	C	0.64 ± 0.06	
Ser-peptide[b]	guanethidine	C	6.1 ± 0.8	
	adenosine	NC	0.27 ± 0.08	0.087 ± 0.004

[a] All assays were run with the spectrophotometric assay except those with AMP as a dead-end inhibitor for which identical results were obtained with both assays. All assays contained 0.1 KCl. [b] Ser-peptide was maintained at $K_{Ser\text{-}peptide}$ for the guanethidine patterns and saturating for all others when MgATP was varied. MgATP was maintained at K_{MgATP} for the adenosine patterns and saturating for all others when Ser-peptide was varied. [c] With KCl = 0, patterns were competitive vs. MgATP with K_i values of 4.7 ± 0.6 mM and 0.77 ± 0.07 mM at 0.5 and 10 mM Mg_{free} concentrations, respectively.

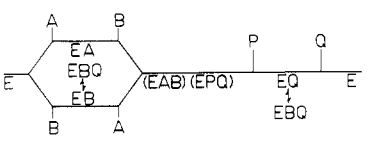
where A, B, P, and Q are MgATP, Ser-peptide, phospho-Ser-peptide, and MgADP. For the above mechanism K_b is given by eq 7 ($K_{ib} = k_4/k_3$). As stated above, the off-rate

$$K_b = \frac{K_{ib}k_9(k_6 + k_7 + k_5k_7/k_4)}{k_9(k_6 + k_7) + k_5(k_7 + k_9)} \quad (7)$$

for phospho-Ser-peptide is most likely very fast with respect to catalysis (that is, $k_7 \gg k_5$ and k_6). In addition, substrate inhibition by Ser-peptide is observed at concentrations above 0.5 mM as a result of formation of the E–Ser-peptide–MgADP complex (data not shown), which indicates the off-rate for MgADP is slow with respect to catalysis and some E–MgATP does accumulate during the steady state. Thus, $k_5 > k_9$; $k_7 \gg k_9$. Simplifying gives eq 8. In order that K_b be less than

$$K_b = K_{ib}(k_9/k_4 + k_9/k_5) \quad (8)$$

K_{ib}, it is necessary that k_9 be less than k_5, but it may also be less than k_4. Thus, the mechanism may involve either rapid equilibrium addition of both substrates with slow release of MgADP ($k_9 < k_5 < k_4$) or sticky Ser-peptide ($k_4 < k_5$), indicative of steady-state random addition of substrates or a combination of both. Isotope exchange at equilibrium can be used to distinguish between the above possibilities. The general form of the mechanism in the short-hand notation of Cleland (1963) is



where A, B, P, and Q have the same definition as before.

A 5–6-fold inhibition of protein kinase activity occurs as free Mg^{2+} is increased from zero to infinity when MgATP is saturating. Under assay conditions commonly employed for protein kinase activity determinations (10 mM $MgCl_2$, 100 μM ATP), the enzyme is therefore inhibited about 4-fold. This inhibition is manifested as a decrease in both V_{max} (representing E–Ser-peptide–MgATP and all complexes up to regeneration of free enzyme) and the V/K for Ser-peptide (representing E–MgATP). Dissociation constants for Mg^{2+} of 1.7 mM and 3 mM were obtained from central complexes and the E–Ser-peptide complex, respectively. The 5-fold inhibition of V_{max} agrees with the data of Armstrong et al. (1979). In addition, however, activation is obtained at low concentrations of MgATP. This activation is manifested by a 5-fold increase in V/K_{MgATP} and gives a K_d for free Mg^{2+} of 3.2 mM (for binding to E–Ser-peptide). This decrease in V_{max} and increase in V/K correspond to a 25–30-fold decrease in K_{MgATP} and are comparable to the 17-fold decrease in K_d for MnAMPPCP and CrAMPPCP with Mn^{2+} as inhibitory metal (Bolen et al., 1980). It appears that Mg^{2+} binds with equal affinity to binary and central complexes. This phenomenon is not restricted to adenine nucleotides since comparable results were obtained with both MgGTP and MgITP.

On the basis of magnetic resonance studies, Granot et al. (1979) found substantial changes in the torsional angle of the glycosidic linkage, suggesting that the enzyme interacts very strongly with the adenosine portion of the nucleotide. This is substantiated by the low K_i (40 μM) that we find for adenosine as well as the observation that other purine and pyrimidine nucleotides are poor substrates (Walsh & Krebs, 1973). Guanosine fails to bind to the enzyme since its K_i is >10 mM. The C-6 amino group of adenine is a hydrogen-bond donor, and the most prevalent tautomeric forms of guanosine and inosine are hydrogen-bond acceptors, which may represent the difference in binding characteristics. This contention is supported by the work of Hoppe et al. (1979), who reported that ITP and GTP bind poorly to the enzyme (K_i > 2mM). When the Mg^{2+} inhibitory site is empty, guanosine and inosine triphosphates have K_m values in the millimolar range. Since guanosine does not inhibit at this concentration, there must be interaction with the tripolyphosphate side chain. Tripolyphosphate, however, does not bind at 10 mM concentrations so that a combination of the nucleotide and phosphate side chain is required.

At low salt, the following K_i values were observed for binding of the nucleoside–nucleotide series to E–Ser-peptide at low free Mg^{2+}: adenosine, 0.04 mM; AMP, 5 mM; ADP, 0.05 mM; ATP, 0.10 mM. The affinity of adenosine and MgADP is essentially the same. This suggests that binding strength for these molecules may involve only interaction of the nucleoside portion of the molecule for MgADP since adenosine lacks an α-phosphate while the α-phosphate is not charged in MgADP. When the position of coordination is changed to β and γ as in MgATP, the α-phosphate bears a formal charge, and the dissociation constant increases by 2-fold. When AMP is used, two formal charges reside on the α-phosphate, and the dissociation constant increases 125-fold. These data suggest that either a negative charge or a hydrophobic site is present in the area of the α-phosphate in the nucleotide binding site. When the α-phosphate is charged, it is repulsed by either this negative charge or the hydrophobicity of the site. MgGTP and MgITP bind at 1–2 mM even though guanosine (1 mM) and magnesium tripolyphosphate (10 mM) do not bind. The combined effect of the binding of both nucleotide and tripolyphosphate portions must contribute to the enhanced binding strength of nucleotide over nucleoside or tripolyphosphate.

As free Mg^{2+} is increased to high concentrations so that the inhibitory site is filled, additional binding strength is obtained as a result of coordination to at least α- and γ-phosphates (which neutralizes charge on the α-phosphate). Thus, at low salt, charges on these positions produce a decrease in K_i of

about 10-fold (except for MgADP, which is already tightly bound).

At high concentrations of KCl, the binding constants for MgATP, MgADP, and AMP are increased at low free Mg^{2+} concentrations by 2–4-fold. This increase is not due to competitive inhibition by Cl^- because the change is a partial one with a maximum occurring at about 100 mM (data not shown). In addition, Na_2SO_4 also produces the same phenomenon, which indicates it is not peculiar to either K^+ or Cl^-. This effect may be indicative of some rearrangement of tertiary structure in the vicinity of the nucleotide binding site as the ionic strength is increased.

There are a number of advantages and disadvantages of the coupled spectrophotometric assay compared with the radioisotopic assay procedure. The choice of procedure depends upon the purpose of the experiment. The spectrophotometric assay is valuable in enzyme kinetic studies because it is continuous. To ensure that a linear time course is obtained with the discontinuous radioassay, for example, requires that samples be taken and processed at several time points. Any nucleotide that participates in the pyruvate kinase couple can be used, and synthesis of radiolabeled nucleoside triphosphates are not required. The reagents required for the spectrophotometric assay, moreover, are 50-fold less expensive. The spectral assay, however, requires about 20-fold more C subunit than the radioassay (200 vs. 10 ng, respectively). With impure enzyme fractions, one must distinguish between Ser-peptide-dependent ADP generation from the C subunit and other ATPase or ADP-generating activities. The results for both procedures with purified enzyme, however, were experimentally indistinguishable. In the development of the spectrophotometric assay, we found that the quantity of commercially supplied Ser-peptide was 50–75% that stated by the supplier. Analysis by high-performance liquid chromatography showed that this 25–50% impurity far exceeded the peptide contaminants, and we concluded, therefore, that the unaccounted for mass was probably salts and water. Ser-peptide can also be quantitated with the radioassay by using 20-fold excess [^{32}P]ATP compared with acceptor peptide.

Acknowledgments

We thank Dr. Jack Herbert for the amino acid analyses and Dr. Syed Alam for the use of his high-performance liquid chromatograph. We also thank Wanda Santa Marina and Karen L. Vrana for typing the manuscript.

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