Chemical Mechanism of the Adenosine Cyclic 3′,5′-Monophosphate Dependent Protein Kinase from pH Studies†

Moon-Young Yoon and Paul F. Cook*

Department of Biochemistry, North Texas State University/Texas College of Osteopathic Medicine, Denton, Texas 76203

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ABSTRACT: The pH dependence of kinetic parameters and inhibitor dissociation constants for the adenosine cyclic 3′,5′-monophosphate dependent protein kinase reaction has been determined. Data are consistent with a mechanism in which reactants selectively bind to enzyme with the catalytic base unprotonated and an enzyme group required protonated for peptide (Leu-Arg-Arg-Ala-Ser-Leu-Gly) binding. Binding of the peptide apparently locks both of the above enzyme residues in their correct protonation state. MgATP preferentially binds fully ionized and requires an enzyme residue (probably lysine) to be protonated. The maximum velocity and V/K_{MgATP} are pH independent. The V/K for Ser-peptide is bell-shaped with pK values of 6.2 and 8.5 estimated. The pH dependence of $1/K_i$ for Leu-Arg-Arg-Ala-Ala-Leu-Gly is also bell-shaped, giving pK values identical with those obtained for $V/K_{Ser\text{-}peptide}$, while the K_i for MgAMP-PCP increases from a constant value of 650 μM above pH 8 to a constant value of 4 mM below pH 5.5. The K_i for uncomplexed Mg^{2+} obtained from the Mg^{2+} dependence of V and V/K_{MgATP} is apparently pH independent.

The catalytic subunit of protein kinase is responsible for the phosphorylation of specific protein substrates by MgATP[1] resulting in a change in the activity of the substrate protein (Bramson et al., 1983). The sequence specificity of the kinase for its protein substrate has been extensively studied (Feramisco & Krebs, 1978; Feramisco et al., 1979, 1980; Kemp, 1980) with the clear requirement for a positively charged subsite (usually comprised of two arginines) N-terminal to the serine to be phosphorylated. In addition, the specificity for the phosphoryl donor has been studied with MgATP the most efficient donor but MgITP and MgGTP able to substitute (Walsh & Krebs, 1973; Cook et al., 1982). A random sequential kinetic mechanism has been proposed from initial velocity studies (Cook et al., 1982; Whitehouse & Walsh, 1983; Whitehouse et al., 1983).

The enzyme apparently prefers the Δ isomer of β,γ-bidentate $Co(NH_3)_4ATP$ (Granot et al., 1979) and binds the adenosine portion of the nucleotide tightly. On the basis of NMR studies, Rosevear et al. (1983) have shown that the anticonformation is bound with a high torsional glycosylic angle (78°) and puckering of the ribose ring. Also from NMR studies, Granot et al. (1981) suggest the peptide substrate Leu-Arg-Arg-Ala-Ser-Leu-Gly is bound as a coil. Distance measurements from NMR and EPR studies with substrate analogues (Granot et al., 1980) have been used to suggest either a metaphosphate-like intermediate or a bimolecular nucleophilic substitution mechanism in which a conformation change brings the reactants closer together.

A number of chemical modification studies have been carried out on the catalytic subunit. A lysine (lysine-72) in the vicinity of the β- or γ-phosphate of MgATP has been identified by using the affinity label 5′-[*p*-(fluorosulfonyl)-benzoyl]adenosine (Zoller et al., 1981; Zoller & Taylor, 1979). The use of an affinity label analogue of the peptide substrate mentioned above most recently showed the presence of cysteine 199 at the active site (Bramson et al., 1982), although this had also been suggested by others previously [see Bramson et al. (1983) and references cited therein]. Using the bifunctional reagent *o*-phthalaldehyde, Puri et al. (1985) have shown the above two residues to be quite close to one another. Additionally, a tyrosine or histidine and glutamate residue has also been identified at the active site [see Bramson et al. (1983) and references cited therein].

Although an abundance of information is available on many aspects of the mechanism of the cAMP-dependent protein kinase reaction, very little is known of the chemical mechanism, particularly the acid–base chemistry. The kinase is an excellent candidate for such studies as a result of the extensive study it has received, particularly with respect to active-site modification. In this paper we report the pH dependence of the kinetic parameters of the cAMP-dependent protein kinase reaction. Data suggest a mechanism involving a single general base and a binding group that helps orient the peptide for phosphoryl transfer.

Materials and Methods

Chemicals. Phosphoenolpyruvate, ATP, NADH, Mes, Hepes, Mops, Taps, BTP, and AMP-PCP were from Sigma. Gold label (100 atom % D) D_2O was from Aldrich. All peptides were prepared by using the solid-phase synthesis of

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[1] Abbreviations: BTP, 1-[bis(hydroxymethyl)amino]-3-tris(hydroxymethyl)propane; Mes, 2-(*N*-morpholino)ethanesulfonic acid; Mops, 4-morpholinepropanesulfonic acid; Taps, 3-[[tris(hydroxymethyl)methyl]-amino]propanesulfonic acid; PEP, phosphoenolpyruvate; FSBA, 5′-[*p*-(fluorosulfonyl)benzoyl]adenosine; AMP-PCP, 5′-adenylyl methylenediphosphate; cA protein kinase, adenosine cyclic 3′,5′-monophosphate dependent protein kinase; Mg_f^{2+}, the uncomplexed form of Mg^{2+}, that is, corrected for chelate complex formation; Ser-peptide, Leu-Arg-Arg-Ala-Ser-Leu-Gly; Ala-peptide, Leu-Arg-Arg-Ala-Ala-Leu-Gly; HPLC, high-performance liquid chromatography; ATP, adenosine 5′-triphosphate; GTP, guanosine 5′-triphosphate; ITP, inosine 5′-triphosphate; ADP, adenosine 5′-diphosphate; NADH, reduced nicotinamide adenine dinucleotide.

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Gutte and Merrifield (1969) via a Biosearch SAM II peptide synthesizer. The *t*-Boc amino acids were from Sigma or Biosearch with the guanidinium group of arginine nitro-protected. Resin was obtained from Sigma with *t*-Boc-glycine attached at a substitution of 0.4 mmol/g of resin. Methylene chloride and acetonitrile were from Fisher. Dimethylformamide, diisopropylcarbodiimide, diisopropylethylamine, anisole, and acetylimidazole were from Aldrich. Trifluoroacetic acid was from Pierce. After synthesis, the peptide was cleaved from the resin and deblocked by using a Penninsula Laboratories HF-cleavage apparatus with HF gas purchased from Alphagaz. The peptide was then purified by HPLC equipped with a gradient maker using reverse phase on a C_{18} column with a linear gradient of 0–50% acetonitrile in 0.1% trifluoroacetic acid. Amino acid composition was used to confirm the product.

Enzymes. Pig heart lactate dehydrogenase and rabbit muscle pyruvate kinase were from Sigma. Adenosine cyclic 3′,5′-monophosphate dependent protein kinase (cA protein kinase) was purified according to the method of Sugden et al. (1976).

Enzyme Assay. The cA protein kinase was assayed by coupling the production of MgADP to the pyruvate kinase and lactate dehydrogenase reactions and continuously monitoring the disappearance of NADH at 340 nm (Cook et al., 1982). The assay contained the following in a 0.4-mL volume: 100 mM Mops, pH 7; 100 mM KCl; 1.1 mM PEP; 0.3 mM NADH; 3.56 mM ATP; 4.14 mM $MgCl_2$; 0.19 mM Ser-peptide; 18 units of lactate dehydrogenase; 17 units of pyruvate kinase; and cA protein kinase. The ATPase activity of the kinase was assayed under identical conditions except that the Ser-peptide was not present and the cA protein kinase concentration was 750 times greater than for the normal assay. Reaction was initiated by the addition of cA protein kinase after sufficient data were collected to determine a background rate in the absence of the kinase. The background rate was subtracted from the plus enzyme rate to obtain the initial velocity. Data were collected by using a Gilford 260 spectrophotometer with strip chart recorder. Temperature was maintained at 25 °C by a circulating water bath with the capacity to heat and cool the thermospacers of the cell compartment. The coupled enzyme assay was optimized according to Cleland (1979a) so that the lag would occur during mixing. When inhibitors were used, the assay was tested by obtaining velocity vs. enzyme patterns at the highest inhibitor concentration used at the lowest concentration of variable substrate. In all cases, the rate was a linear function of cA protein kinase concentration. The concentrations of reactants were corrected for the concentration of the metal chelate complexes according to Park et al. (1984) except for MgATP and MgAMP-PCP by using the following values for dissociation constants: MgPEP, 5 mM; MgNADH, 19.5 mM; MgAMP, 3.2 mM; MgATP, 0.0143 mM; Mg-Ser-peptide, 316 mM (Dawson et al., 1979; Martell & Smith, 1977). The stability constants for MgAMP-PCP and Mg-Ala-peptide were assumed equal to those for MgATP and Mg-Ser-peptide.

pH Studies. The pH stability of the cA protein kinase was determined by incubating enzyme at the desired pH and assaying aliquots as a function of time up to 10 min at pH 7 as described above. Enzyme is stable with no activity lost from pH 6 to 9. At pH 5.5, some denaturation occurs with a $t_{1/2}$ of 14.7 min, while at pH 10 a $t_{1/2}$ of 5 min is obtained. However, since initial velocities are measured, since substrates are present in the assay, which is likely to afford some protection against denaturation, and since enzyme is added from a stock solution at pH 7 to the assay mixture at the desired pH, the small activity loss will not affect the measurement of initial velocities.

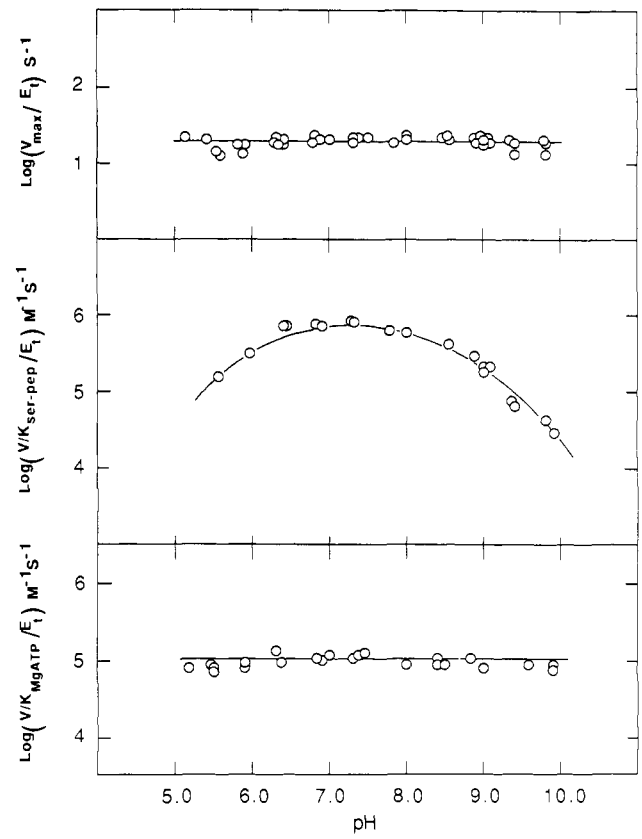


FIGURE 1: pH dependence of the kinetic parameters for phosphorylation of Ser-peptide in the cA protein kinase reaction. The V/K value for Ser-peptide was obtained at saturating concentrations of MgATP (4 mM) with the concentration of Ser-peptide varied around its K_m (50–500 μM, pH 6 and below; 10–100 μM, pH 6.4–8.55; 50–500 μM, pH 9; 0.1–1 mM, pH 9.4 and above). The V/K value for MgATP was obtained at saturating concentrations of Ser-peptide (4 mM, pH 5.93 and below; 0.6 mM, pH 6.4–8.55; 2.3 mM, pH 8.9–9.4; 8 mM, pH 9.9) with the concentration of MgATP varied around its K_m (0.1–1 mM). All reactants except ATP were corrected for the concentration of metal chelate complexes as described in Material and Methods. The concentration of uncomplexed Mg^{2+} was maintained at 0.5 mM. The points shown are experimental values. The curve in the case of the V/K for Ser-peptide is from a fit to the data using eq 5, while the lines are the calculated average value in the case of V and V/K_{MgATP}.

The velocity vs. enzyme profile was measured at pH 5 and 10. In both cases, the velocity is a linear function of cA protein kinase activity. Initial velocity data were obtained as a function of pH under conditions in which either MgATP was saturating or Ser-peptide was saturating. The pH was maintained by using the following buffers at 100 mM concentrations: Mes, 5–6.5; Mops, 6.5–7.5; Taps, 7.5–8.5; BTP, 8.5–10. The pH was recorded before and after initial velocity data were recorded.

Inhibition data were obtained for inhibitors competitive with the variable substrate at a saturating concentration of the fixed substrate. Full inhibition patterns were obtained at the pH extremes (5 and 10) with the substrate varied at several different fixed levels of the inhibitor, including zero. Once the competitive nature of the inhibition was established as pH independent, Dixon experiments were carried out in which the variable substrate was fixed at a concentration equal to its K_m and the inhibitor concentration varied over a range (including zero) that gave inhibition. The measured K_i was then divided by 2 to obtain the true K_i.

Solvent Perturbation of the pK Values. There was some question as to whether the V profile in H_2O decreased at low pH. To test this possibility, the V profile was repeated in D_2O. Enzyme stability, linearity of the assay, and the pD dependence of the kinetic mechanism were redetermined at the pD extremes with results qualitatively similar to those obtained in H_2O. All reagents except enzyme and peptide were made up in D_2O with buffers titrated to the desired pD with KOD. The final concentration of D_2O was approximately 95%. The pH of the solution was measured and the pD calculated by adding 0.4 to the pH reading as a result of the isotope effect on the electrode (Schowen, 1977).

5'-[p-(Fluorosulfonyl)benzoyl]adenosine Inactivation. Inactivation of the catalytic subunit of cAMP-dependent protein kinase has been reported previously (Zoller & Taylor, 1979). At pH 7 and 8, 0.12 unit of catalytic subunit was incubated in separate inactivation mixtures with 100 mM buffer (as indicated under pH studies above) and several different concentrations of FSBA: pH 7 (0–0.4 mM); pH 8 (0–0.1 mM). At time intervals of 10 min, aliquots were withdrawn and assayed for activity with pH 7 200 mM Mops using saturating concentrations of MgATP and Ser-peptide. The apparent first-order rate constant for inactivation at each FSBA concentration was obtained from a plot of the natural logarithm of the velocity vs. time.

Data Processing. Reciprocal initial velocities were plotted against reciprocal substrate concentrations, and all plots and replots were linear except for the data collected by varying the MgATP concentration at different fixed levels of uncomplexes Mg^{2+}. Data were fitted by using the appropriate rate equation and FORTRAN programs developed by Cleland (1979b). Data for substrate saturation curves (not shown) used to obtain the pH dependence of the kinetic parameters were fitted by using eq 1, while data for sequential initial

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

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

$$v = \frac{VA}{K_a(1 + I/K_{is}) + A} \quad (3)$$

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

$$\log y = \log C/(1 + H/K_1 + K_2/H) \quad (5)$$

$$\log y = \log C/(1 + H/K_1) \quad (6)$$

$$\log y = \log \frac{Y_L + Y_H(K_1/H)}{1 + K_1/H} \quad (7)$$

$$y = a(1 + I/K_{IN})/(1 + I/K_{ID}) \quad (8)$$

velocity patterns and competitive and noncompetitive inhibition were fitted by using eq 2–4. Data for bell-shaped pH profiles in which the limiting slopes were +1 and −1 were fitted by using eq 5, while ATPase data, which decrease with a limiting slope of +1 at low pH, were fitted by using eq 6. Data for the pH dependence of $1/K_{i,MgAMP\text{-}PCP}$, which decreased from a constant value at high pH to a lower constant value at low pH, were fitted by using eq 7. Data for the uncomplexed Mg^{2+} dependence of V and V/K_{MgATP} were fitted by using eq 1 for each saturation curve with the hyperbolic replots fitted by using eq 8. In eq 1–4 v is the initial velocity, V is the maximum velocity, and K_a and K_b are the K_m values for A and B, respectively. A, B, and I are substrate and inhibitor concentrations, K_{ia}, K_{is}, and K_{ii} are observed inhibition constants for A, slope, and intercept, respectively. In eq 5–7, y is the observed value of the parameter of interest, e.g., $V/K_{Ser\text{-}peptide}$, at a given pH, C is the pH-independent value of y, H is the hydrogen ion concentration, K_1 and K_2 are acid dissociation constants for enzyme, substrate, or inhibitor functional groups, and Y_L and Y_H are the pH-independent values of $1/K_{i,MgAMP\text{-}PCP}$ at low and high pH, respectively. In eq 8, y is the value of V or V/K_{MgATP} at a given Mg_f^{2+} concentration, I is the concentration of Mg_f^{2+}, a is the value of y at $I = 0$, $a(K_{ID}/K_{IN})$ is the value of y at $I = \infty$, K_{ID} is the dissociation constant for Mg_f^{2+} from the E:Ser-peptide:Mg_f^{2+} complex when V/K_{MgATP} is plotted and from the E:Ser-peptide:MgATP:Mg_f^{2+} complex when V is plotted, and K_{IN} is a ratio of rate constants that causes the hyperbola to level off at a finite value.

Results

In order to obtain information on the acid–base chemistry catalyzed by the catalytic subunit of cAMP-dependent protein kinase, the pH dependence of kinetic parameters was determined. However, prior to a determination of the pH dependence of the kinetic parameters to facilitate interpretation of the results in terms of the predominant enzyme present under any given set of conditions, pH stability and the pH dependence of the kinetic mechanism must first be obtained. As pointed out in Materials and Methods, the enzyme is sufficiently stable over the pH range 5–10 to collect initial velocity data, particularly since the pH is instantaneously jumped from 7 to the desired pH. The kinetic mechanism is random with a preferred addition of MgATP prior to the peptide substrate (Cook et al., 1982; Whitehouse & Walsh, 1983; Whitehouse et al., 1983). The diagnostic pattern for this mechanism is the noncompetitive inhibition obtained by using a dead-end analogue of Ser-peptide at varying MgATP concentrations. A repeat of the initial velocity pattern in the absence of inhibitors at pH 5 and 10 gives a double-reciprocal plot that intersects to the left of the ordinate, suggesting a sequential mechanism. In addition, the dead-end inhibition pattern obtained by using Leu-Arg-Arg-Ala-Ala-Leu-Gly, an analogue of the substrate hepapeptide in which the serine to be phosphorylated is replaced by alanine, is noncompetitive at both pH values. As a result, the kinetic mechanism appears to be pH independent and random over the pH range 5–10.

pH Dependence of Kinetic Parameters. The kinetic parameters in the direction of Ser-peptide phosphorylation are shown in Figure 1. The maximum velocity is essentially pH independent. The V/K_{MgATP} is pH independent over the entire pH range but decreases slightly at both low and high pH. The V/K for Ser-peptide is bell-shaped, decreasing at both low and high pH with slopes of 1 and −1, respectively. Values of 6.2 ± 0.2 and 8.5 ± 0.1 are obtained for the pKs. The pH-independent values of V/E_t, $V/(K_{Ser\text{-}peptide})E_t$ and $V/(K_{MgATP})E_t$ are 20 ± 4 s^{-1}, (8.5 ± 0.3) × 10^5 M^{-1} s^{-1}, and (1.1 ± .5) × 10^5 M^{-1} s^{-1}.

The enzyme also catalyzes an ATPase reaction at a rate $^1/_{1300}$ the rate of Ser-peptide phosphorylation (Armstrong et al., 1979b). Since transfer to water is very slow, the phosphoryl transfer rate is almost certain to be rate determining. The pH dependence of the maximum velocity and the V/K for MgATP are shown in Figure 2. Both are pH dependent, giving a pK value of 6.0 ± 0.1. The pH-independent values of V/E_t and $V/(K_{MgATP})E_t$ are 0.016 ± 0.002 s^{-1} and 55 ± 4 M^{-1} s^{-1}.

pH Dependence of the K_i for Inhibitory Analogues. Dead-end inhibitors competitive with the variable substrate bind to the same enzyme form as the variable substrate. Since the variable substrate is limiting under conditions in which inhibition is observed, true dissociation constants are obtained

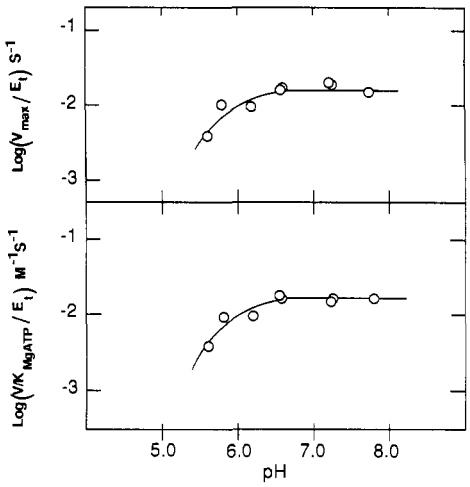


FIGURE 2: pH dependence of the kinetic parameters from the ATPase reaction of cA protein kinase. The concentration of MgATP was varied around its K_m with the uncomplexed Mg^{2+} concentration maintained at 0.5 mM. The points are experimental values. The curves for V and V/K_{MgATP} are theoretical from a fit of eq 6 to the data.

and the pH dependence of the dissociation constants will yield intrinsic pK values. The pK_i profile[2] for Ala-peptide is shown in Figure 3A. The value of $1/K_i$ decreases at low and high pH, giving pK values of 6.4 ± 0.3 and 8.5 ± 0.2, identical with those observed in the $V/K_{Ser\text{-}peptide}$ pH profile. The pH-independent value of K_i is 0.27 ± 0.07 mM.

The pK_i profile for MgAMP-PCP is depicted in Figure 3B. The $1/K_i$ value is pH independent above pH 8 with a value of 0.65 ± 0.03 mM and decreases to become constant below pH 5.5 with a value of 4.0 ± 0.4 mM. A pK value of 6.8 ± 0.1 is obtained for the decrease in $1/K_i$, and this value is perturbed to 6.0 ± 0.1, which is measured where the $1/K_i$ value levels off at low pH.[3]

Adenosine 5′-monophosphate is also a competitive inhibitor vs. MgATP. The pK_i profile for AMP is shown in Figure 3C. The affinity for AMP increases as the pH decreases with $1/K_i$, increasing by a factor of 10 per pH unit below pH 7. A value of about 6 mM is obtained for K_i at pH 9, which a value of 90 μM is observed at pH 5.4.

Solvent D_2O Perturbation of pK Values. Some of the data obtained for V_{max} (not shown in Figure 1) indicated a decrease in the parameter at low pH. The decrease was not reproducible so that it becomes important to determine whether there is a real decrease. It is known (Schowen, 1977) that the pK values of nitrogen and oxygen bases increases by 0.4–0.6 pH units in D_2O. A redetermination of the V and $V/K_{Ser\text{-}peptide}$ pH dependence in D_2O should then give the break in the V profile at about pD 6.0, while pK values around 6.6 and 8.9 are expected for the $V/K_{Ser\text{-}peptide}$ profile. The latter act as an internal control as to whether the method works. The V profile in D_2O is pH independent from pH 5.4 to 9.5, while the $V/K_{Ser\text{-}peptide}$ decreases at low and high pH giving pK values of 6.6 ± 0.1 and 8.8 ± 0.1 (Figure 4). Thus, the V profile does not decrease at low pH. The pH-independent values of

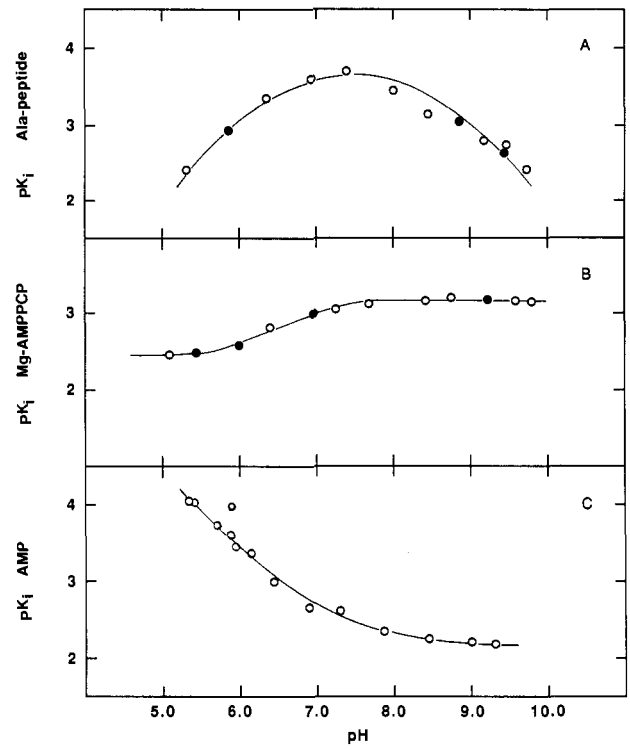


FIGURE 3: pH dependence of inhibitor dissociation conatnts for cA protein kinase. (A) pH dependence of the Ala-peptide dissociation constant. The filled circles represent data obtained from full inhibition patterns with MgATP maintained at a saturating concentration and Ser-peptide varied around its K_m (as in legend to Figure 1) at several different fixed Ala-peptide concentrations (2–8 mM, pH 5.84–8.85 and 9.4). The open circles represent data from Dixon plots varying Ala-peptide (0–8 mM, pH 5.3 and 9.7; 0–1.2 mM, pH 6.45–8.5) at a saturating concentration of MgATP and Ser-peptide equal to $K_{Ser\text{-}peptide}$. Under these conditions the true K_i is half the experimentally determined value. The poits are experimental, while the curve is from a fit of eq 5 to the data. (B) pH dependence of the MgAMP-PCP dissociation constant. The filled circles represent data from full inhibition patterns with Ser-peptide maintained at a saturating concentration and MgATP varied around its K_m (levels listed in legend to Figure 1) at several different fixed MgAMP-PCP concentrations (2–8 mM, pH 5.45–6; 0.5–2 mM, pH 6.26; 3–12 mM, pH 9.28). The open circles represent data from Dixon plots varying MgAMP-PCP (0–8 mM, pH ≤6; 0–3.6 mM, pH >6) at a saturating concentration of Ser-peptide and MgATP equal to its K_m. The points are experimental, while the curve is theoretical based on a fit of eq 7 to the data. (C) pH dependence of the AMP dissociation constant. All data were obtained from Dixon plots varying the concentration of AMP (0–0.6 mM, pH 5.43–5.9; 0–24 mM, pH 6.47–7.3; 0–40 mM, pH ≥7.85) at a saturating concentration of Ser-peptide and MgATP equal to its K_m. The points are experimental, and the curve is drawn by eye.

V/E_t and $V/(K_{Ser\text{-}peptide})E_t$ at 18 ± 3 s^{-1} and (7 ± 4) × 10^5 M^{-1} s^{-1}. The ratio of the pH-independent values in H_2O and D_2O gives no significant solvent isotope effects. A direct comparison at pH(D) 7.5, however, indicates there is a significant isotope effect on V of 1.6 ± 0.4.

pH Dependence of the K_i for Mg_f^{2+}. A metal in addition to the one required to form the MgATP chelate complex has been implicated in the mechanism of the catalytic subunit (Armstrong et al., 1979a; Bolen et al., 1980; Granot et al., 1979, 1980; Cook et al., 1982). The dissociation constant for the second metal can be determined kinetically (Cook, 1982; Cook et al., 1982) by obtaining the saturation curve for MgATP (the $V/K_{Ser\text{-}peptide}$ is not affected) as a function of Mg_f^{2+} and then plotting V and V/K_{MgATP} vs. Mg_f^{2+}. The concentration of Mg_f^{2+} that gives half the change in the parameter represents the dissociation constant for E:Ser-peptide:Mg and E:Ser-peptide:MgATP:Mg in the case of

[2] Plotted as log $1/K_i$ so that a decrease represents a decrease in affinity.

[3] Note that this is a log–log plot and thus the pK is obtained at the pH where the pH-independent value has decreased by a factor of 2 (0.3 log unit).

[4] Data obtained from isotope partitioning experiments by C.-T. Kong in this laboratory (unpublished) also indicate that catalysis is not slow compared to the off-rate for MgATP and MgADP. About 100% of the E:[γ-^{32}P]MgATP is trapped as [^{32}P]phosphopeptide with a K_m for the trapping reaction for Ser-peptide equal to the K_m for the phosphotransferase reaction.

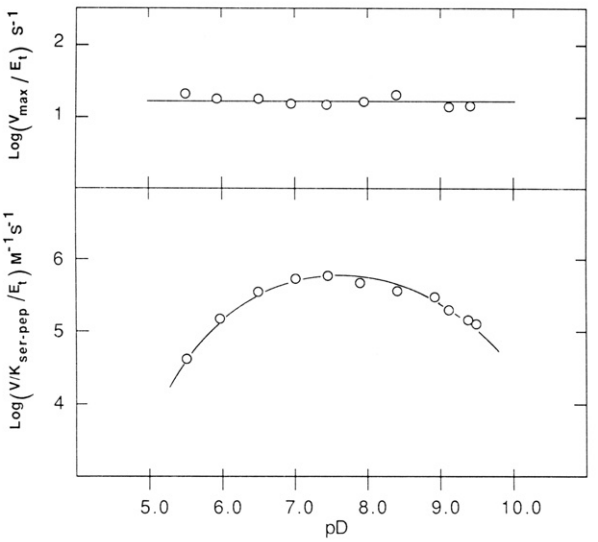


FIGURE 4: pD dependence of V and $V/K_{Ser\text{-}peptide}$. Conditions are as listed in the legend to Figure 1 except that all reagents were prepared in D_2O. Reported pD values are the recorded pH values plus 0.4. The points are experimental. The curve for $V/K_{Ser\text{-}peptide}$ is theoretical from a fit of eq 5 to the data. The line for V is the calculated average value.

V/K_{MgATP} and V, respectively. An example is shown in Figure 5 determined at pH 8.8.

The above experiment was repeated as a function of pH, and the pH dependence of V and V/K_{MgATP} was obtained at zero and infinite concentrations of Mg_f^{2+}. Values obtained at zero Mg_f^{2+} were identical with those reported in Figure 1. Data obtained at infinite Mg_f^{2+} are also pH dependent, with V about 5-fold lower and V/K_{MgATP} about 8-fold higher than those obtained at 280 Mg_f^{2+}. The K_i values obtained for Mg_f^{2+} have little, if any, pH dependence within standard error. It is possible that there is some pH dependence for the K_i for Mg_f^{2+} from E:Ser-peptide:MgATP:Mg but it is difficult to tell because of the large standard error. Thus, if Mg_f^{2+} has titrable ligands, they are likely outside the pH range 5–9.

Inactivation by 5′-[p-(Fluorosulfonyl)benzoyl]adenosine. The reagent 5′-[*p*-(fluorosulfonyl)benzoyl]adenosine covalently modifies the catalytic subunit of cAMP-dependent protein kinase, resulting in inactivation with a stoichiometry of 1 per subunit (Zoller & Taylor, 1979; Hixson & Krebs, 1979). A complex is formed between enzyme and FSBA as shown by a double-reciprocal plot of $1/k_{inact}$ vs. 1/FSBA that intersects on the ordinate. The substrate, MgATP, affords complete protection against inactivation by FSBA. A peptide containing a modified lysine has been isolated and sequenced (Zoller & Taylor, 1979).

There is no evidence for a lysine in the V/K_{MgATP} profile that covers the pH range 5–10. It was of interest to determine the value of the pK for the lysine modified by FSBA. Unfortunately, the solubility and stability of FSBA only allowed determinations to be made at pH 7 and 8. Plots of ln v vs. t are linear at all FSBA concentrations used, and a double-reciprocal plot of the data is also linear (data not shown). Values of 0.037 and 0.06 min^{-1} are obtained at pH 7 and 8, respectively, with a value of 1.31×10^3 M^{-1} min^{-1} obtained for k_{max}/K_{FSBA} at pH 8. Data collected by Hixson and Krebs (1979) at pH 7.5 normalized to the same enzyme concentration give a value 0.054 min^{-1} for k_{max} and 3.6×10^2 M^{-1} min^{-1} for k_{max}/K_{FSBA}.

DISCUSSION

Interpretation of the pH Dependence of Kinetic Parameters. The maximum velocity is independent of pH over the range

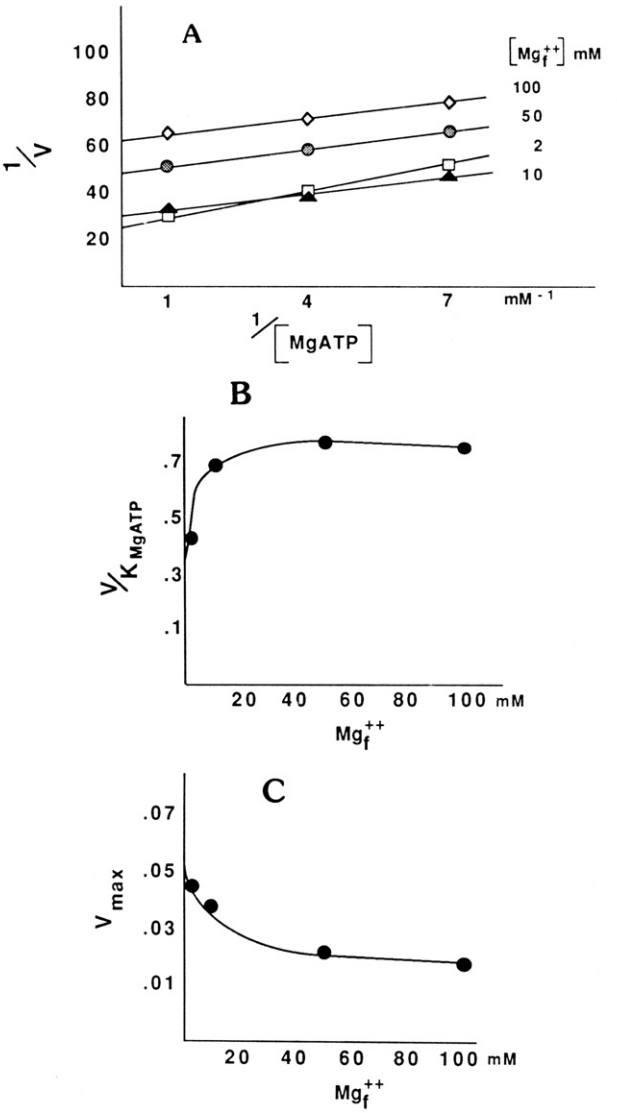


FIGURE 5: Dependence of V and V/K_{MgATP} on concentration of Mg_f^{2+} at pH 8.8. (A) Initial velocity pattern obtained by varying MgATP (concentrations indicated) at the fixed concentration of Mg_f^{2+} indicated. Ser-peptide was maintained saturating. (B) Replot of V/K_{MgATP} against Mg_f^{2+}. (C) Replot of V against Mg_f^{2+}. Points in A are experimental, while curves are theoretical as a result of a fit of eq 1 to data obtained at each Mg_f^{2+} concentration. Points are calculated V and V/K values for B and C, while curves are theoretical from a fit of eq 8 to the data.

5.5–10. Below pH 5.5 it is possible, on the basis of data presented in Figure 1, that the rate decreases below this pH. In order to test this possibility, the solvent deuterium isotope effect was obtained (Schowen, 1977). If V is dependent on pH, it should decrease at a higher pD than pH by 0.4–0.6 as a result of the equilibrium isotope effect on the pK value. A determination of the maximum rate as a function of pD indicates it is pD independent from 5.4 to 9.5 and does not decrease below a pD of 6.0–6.1 as expected if V were pH dependent. Since V is pH independent, a mechanism in which reactants bind selectively to the correctly protonated form of the enzyme is suggested (Cleland, 1977). For a mechanism of this type intrinsic pK values are observed for enzyme and substrate functional groups in the pH–rate profiles. The solvent isotope effect of 1.6 on V indicates a proton involved in a slow step after release of the first product and prior to addition at substrates. This aspect is being pursued presently.

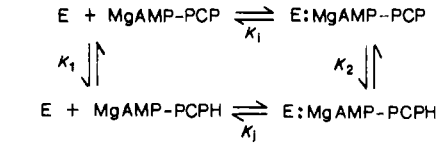
The V/K for MgATP is pH independent from pH 5.5 to 9.5 but decreases slightly below and above these pH values.

One would expect to see the pK of MgATP unless both the protonated and unprotonated forms bind equally well and are both catalytically competent, and this is unlikely. The pK obtained for protonation of the γ-phosphate of MgATP at an ionic strength of 0.1 is 4.6 (Martell & Smith, 1982), outside the range of the present studies. An enzyme lysine residue is known to be in the vicinity of the β- or γ-phosphate of MgATP (Zoller & Taylor, 1979; Hixson & Krebs, 1979). The protonation state of this group is almost certain to affect the binding of the nucleotide. A pK value greater than 9 is estimated for this lysine from the pH dependence of the inactivation rate by FSBA since the data obtained at pH values from 7 to 8 suggest that k_{max}/K_{FSBA} is increasing by a factor of 10 per pH unit. As a result, the pK of the lysine modified by FSBA must be at least 9 and is probably higher and, as a result, if it does affect catalysis and/or nucleotide binding, its pK is again outside the range of these studies.

The V/K for Ser-peptide decreases at low and high pH, giving pK values of 6.2 and 8.5 representing titrable groups of Ser-peptide or E:MgATP. The peptide has no pK values in this pH range so that the decrease in rate with changes in pH must reflect the titration of enzyme residues. General base catlaysis is expected in the case of the kinase reaction with a base accepting the proton from the serine hydroxyl concomitant with attack on the γ-phosphate. The group with a pK of 6.2 is most likely the general base. In addition to this base, however, there is clearly the requirement for a second enzyme residue with a pK of 8.5 that must be protonated for activity. The simplest explanation is that this enzyme residue is a binding group for the peptide (or protein substrate) that may serve to aid in proper orientation of the substrate for subsequent chemistry. If so, there are only a few reasonable possibilities in terms of the functional groups on peptide that could be bound by this enzyme residue. Of the amino acid residues in the peptide substrate (Leu-Arg-Arg-Ala-Ser-Leu-Gly) the N-terminal and C-terminal residues can be removed with only a 2–4-fold decrease in V/K for the peptide (Kemp et al., 1977), and thus these are not likely candidates for orienting groups. The two argine residues are required for tight binding and represent the initial interaction of the peptide substrate with enzyme (Reed & Kinzel, 1984; Reed et al., 1985). This presumably occurs via combined electrostatic and hydrogen-bonding interaction with active-site glutamate residues.[5] As a result, these residues are already well anchored in the active site. Three possible residues of the peptide remain, Ala-Ser-Leu, any or all of which could be bound to the group with a pK of 8.5. However, since the serine is phosphorylated, it is most likely the serine that is bound. Since the enzyme group must be protonated for activity, the interaction is most likely a hydrogen bond with the carbonyl oxygen of the peptide backbone. Further experimental evidence will be required to test this hypothesis.

An ATPase reaction that is intrinsic to the protein kinase affords an excellent opportunity to look at a rate-determining transfer of the γ-phosphate of MgATP (the ATPase is $^1/_{1300}$ the rate of the kinase reaction). The maximum rate and the V/K for MgATP decrease at low pH, reflecting the protonation of the catalytic base. The pK is within experimental error identical with that obtained from the $V/K_{Ser\text{-}peptide}$ profile, in agreement with the fact that true pK values are expected in the latter. In addition, since the pK of the catalytic base is essentially the same when determined from the ATPase reaction representing free enzyme (6.0 ± 0.1) and from the $V/K_{Ser\text{-}peptide}$ for the kinase reaction representing E:MgATP (6.2 ± 0.2), the presence of MgATP in the active site does not affect the pK of the base. Water is a very poor acceptor for the phosphoryl compared to peptide as measured not only by the difference in turnover numbers for the two (20 and 0.016 s^{-1}, respectively) but also by the V/K_{MgATP} values (10^5 and 55 M^{-1} s^{-1}). Both parameters reflect the decreased rate of chemical transfer since the ratio is similar (1300–2000) in both cases.

Scheme I



Interpretation of the pK_i Profiles. The mechanism proposed above suggests that intrinsic pK values should be observed in the $V/K_{Ser\text{-}peptide}$ profile. This aspect can be tested through the use of competitive inhibitors. These inhibitors mimic the variable substrate and bind to the same enzyme forms and thus should be affected by the protonation state of the same groups that affect the binding of substrates. Since the K_i for a competitive inhibitor is its thermodynamics dissociation constant, true pK values will be observed. The inhibitor closest in structure to the substrate Ser-peptide is Ala-Peptide in which the hydroxyl to be phosphorylated is replaced by a proton. The pK_i profile for Ala-peptide gives pK values essentially identical with those obtained from the $V/K_{Ser\text{-}peptide}$ profile, corroborating the above proposed mechanism. The peptide substrate only binds to enzyme in which the group with a pK of 6.2 is unprotonated and the group with a pK of 8.5 is protonated. The pH-independent value of the K_i is 270 μM, identical with the K_i of 250 μM for *N*-acetylleucyl-Ser-peptide obtained by direct binding studies (Feramisco & Krebs, 1978) in the presence or absence of MgAMP-PCP. The data for acetyl-Ser-peptide binding and the fact that the K_m for the acetylated peptide is more than 10-fold lower than its K_d were interpreted by Cook et al. (1982) as indicating rate-limiting release of MgADP. Since the K_i for Ala-peptide is identical with that for Ser-peptide, it can further be stated that the serine hydroxyl does not contribute significantly to the overall binding affinity. Of course, the assumption must be made that E:MgAMP-PCP is identical with the E:MgATP complex so that the lack of change of the Ser-peptide K_i in the presence of MgAMP-PCP is also true with MgATP bound. This does not appear to be a bad assumption according to the NMR studies of Armstrong et al. (1979a) even though the diphosphonate analogue binds about 4-fold weaker as shown by K_i values of 650 μM for MgAMP-PCP (these studies) and 160 μM for MgATP obtained at 0.5 mM Mg_f^{2+} (Cook et al. 1982).

Very little pH dependence is observed in the V/K_{MgATP} profile, suggesting that no binding groups for the nucleotide titrate over the pH range 5.5–9.5. To test this, the pK_i profile for MgAMP-PCP was obtained. The K_i is pH independent with a value of 650 μM above pH 8 but increases to a new constant value of 4 mM at low pH. Behavior of this kind can be described by a model in which either an enzyme residue or MgAMP-PCP loses affinity upon protonation but can still bind with reduced affinity once fully protonated. The pK for AMP-PCP is 8.4 (Yount et al., 1971), and the dissociation constant for MgAMP-PCP is 0.038 mM, only 3-fold higher than that for MgATP. The pK for ATP decreases 2 pH units

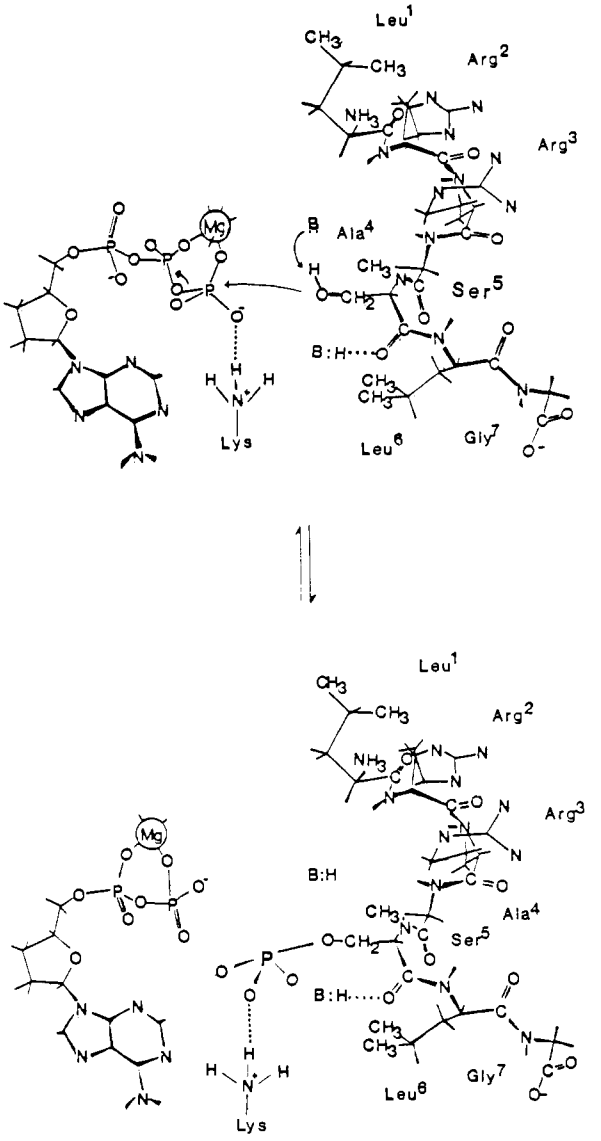
[5] The glutamate residues have been shown to be present by Matsuo et al. (1980) using glycine ethyl ester as a chemical modifying agent. The only reactant that protects against inactivation of the glycine ethyl ester is the peptide. The residues were identified by isolating the modified amino acids.

with Mg bound, and a decrease of about 1.5 pH units is expected in the case of AMP-PCP, giving a pK of about 6.9 for MgAMP-PCP, similar to the observed value of 6.8. As a result, the pH dependence of MgAMP-PCP binding can be described by Scheme I. The values for K_i and K_j are 650 μM and 4 mM, respectively. The value for pK_1 will be obtained at the pH where K_i has increased 2-fold to a value of 1.3 mM, while the value of pK_2 will be obtained at the pH where K_j has decreased 2-fold to a value of 2 mM. Thus, when bound, the pK for MgAMP-PCP is only decreased by 0.8 pH unit from 6.8 to 6.0.[6] Of interest, the binding of MgAMP-PCP is not eliminated when the γ-phosphate is protonated. These data suggest that this may also be true for MgATP, but data cannot be collected to test this since the pK for MgATP is too low.

The pK_i profile for AMP is also quite interesting. Cook et al. (1982) suggested on the basis of K_d values for adenosine (40 μM), AMP (8 mM), MgADP (40 μM), and MgATP (160 μM) that there existed in the vinicity of the α-phosphate a hydrophobic region or a negatively charged residue. Work by Bhatnagar et al. (1983), using a number of nucleotide analogues having positive, negative, and hydrophobic moieties at the 2′, 3′, and 5′ positions of the ribose, indicates that the region in the vicinity of the α-phosphate is hydrophobic. As a result, the K_i for AMP is expected to decrease with decreasing pH. The pK values for AMP are 6.4 and <1.0 (Dawson et al., 1969). The K_i decreases below pH 7, and below pH 6 it is decreasing by a factor of 10 per pH unit. The phosphate pK of 6.4 is expected in this profile as discussed above. However, the increase in affinity for AMP as a result of protonating the phosphate is probably buried in the data since there is only a slight inflection in the data around pH 6–7. The continued increase in affinity as the pH decreases must be a result of protonating some enzyme residue with a pK less than 5 that either neutralizes a negative charge or puts a proton on a group that is within hydrogen-bonding distance of the phosphate. A possibility for this residue is one of the glutamate residues thought to hydrogen bond the arginines of the peptide substrate. If a value of 4.7 is used as an estimate of this putative glutamate pK (similar to acetic acid since the α-amine and α-carboxyl of glutamate are in peptide linkage), a pH-independent value (with monoprotonated AMP and the enzyme residue protonated) of 20 μM is obtained for the K_i of AMP. The latter is within error equal to that obtained for adenosine (40 μM) and MgADP (40 μM). Whether the group titrated is a glutamate will have to await further study.

Chemical Mechanism. The data suggest a mechanism in which a minimum of three active-site residues participate in catalysis and binding, with the reactants Ser-peptide and MgATP selectively binding to enzyme only when these residues are in their correct protonation state (Scheme II). This is suggested by the pH independence of the V profiles for both the kinase and ATPase reactions and the pK_i profile for Ala-peptide. In addition, MgATP binds with highest affinity when ionized but may still bind when protonated as suggested by the MgAMP-PCP pK_i profile. When Ser-peptide is bound, all groups are apparently locked in their correct protonation state since the pH dependence of the catalytic base is not observed in the V/K_{MgATP} profile.

Scheme II



The nucleotide is bound via the adenosine moiety most tightly (Rosevear et al., 1983) but the tripolyphosphate portion of the molecule also contributes since guanosine alone at a concentration of 1 mM or Mg-tripolyphosphate alone at a concentration of 10 mM does not inhibit when MgGTP is a substrate with a K_m of 1 mM (Cook et al., 1982). The contribution of the tripolyphosphate moiety is likely a result of interaction of the γ-phosphate with a protonated ϵ-amine of lysine that has been shown to be in the vicinity of the β- or γ-phosphates by using the affinity analogue FSBA (Zoller & Taylor, 1979; Zoller et al., 1981). The lysine could thus also serve as an electrophile neutralizing the formal negative charge on the γ-phosphate in preparation for nucleophilic attack.

The peptide binds in three stages as suggested by CD evidence (Reed & Kinzel, 1984; Reed et al., 1985). It first interacts via the Arg-Arg subsite with glutamate residues present, then assumes a specific coil-type orientation that probably orients the serine hydroxyl proton toward the catalytic base and facilitates in-line attack of the serine oxygen on the γ-phosphate of MgATP. The group with a pK of 8.5 could certainly play a role in the bound peptide conformation change by hydrogen bonding the serine carbonyl oxygen.

Once proper orientation has occurred, the catalytic base accepts a proton from the serine hydroxyl concomitant with nucleophilic attack on the γ-phosphate. Whether an additional

[6] Since the γ-phosphate of MgATP is suggested to be hydrogen-bonded to an active-site lysine and this is expected to be a reasonably strong interaction, the small perturbation of the MgAMP-PCP pK probably indicates the γ-phosphate of MgAMP-PCP, unlike that of MgATP, may not be proper position for a strong interaction with the lysine.

conformational change accompanies this process to bring the hydroxyl and the γ-phosphate closer together or the reaction coordinate is elongated [5.3 ± 0.7 Å (Granot et al., 1980)] is not known at present.

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