pH Dependence of the Kinetic Mechanism of the Adenosine 3′,5′-Monophosphate Dependent Protein Kinase Catalytic Subunit in the Direction of Magnesium Adenosine 5′-Diphosphate Phosphorylation†

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ABSTRACT: In order to determine the acid–base chemical mechanism of the adenosine 3′,5′-monophosphate dependent protein kinase catalytic subunit, the pH dependence of the kinetic mechanism in the direction of MgADP phosphorylation has been determined using initial velocity studies in the presence and absence of dead-end inhibitors. The kinetic mechanism in the direction of MgADP phosphorylation is random, with changes in the preference of substrate binding as a function of pH. At pH 7.2 and below, the kinetic mechanism is ordered with phosphorylated peptide binding prior to MgADP. At pH 7.6, the opposite pathway with MgADP binding prior to phosphorylated peptide is preferred. The pH independence of V/E_t is consistent with a mechanism in which reactants only bind to the correctly protonated form of the enzyme. The V/K_{MgADP} value decreases, as the pH increases, giving a pK of about 7 which is likely that of a general acid, the same group that serves as a general base in the opposite reaction direction [Yoon, M.-Y., & Cook, P. F. (1987) *Biochemistry 26*, 4118]. The pK_{iPSP} decreases at low pH giving a pK of about 6.5, probably reflecting the phosphate group of the peptide.

A sequential kinetic mechanism has been suggested for the catalytic subunit of cAK[1] in the direction of SP phosphorylation (Cook et al., 1982; Kong & Cook, 1988; Whitehouse et al., 1983; Whitehouse & Walsh, 1983). On the basis of initial velocity (Cook et al., 1982) and isotope partitioning (Kong & Cook, 1988) studies, the kinetic mechanism in the direction of SP phosphorylation has been shown to be steady-state-random with ordered release of PSP prior to MgADP. The mechanism of the C-subunit in the direction of MgADP phosphorylation has recently been reported by Qamar et al. (1992) as being dependent upon the concentration of Mg_f. At low Mg_f, the mechanism is steady-state-ordered with PSP binding prior to MgADP, while at high Mg_f the mechanism is equilibrium-ordered with MgADP binding prior to PSP. The change in mechanism to equilibrium-ordered at the higher concentration of Mg_f has been attributed to an increase in the affinity of the enzyme for MgADP and a decrease in affinity for PSP.

In the direction of SP phosphorylation, the C-subunit has a pH-independent kinetic mechanism (Yoon & Cook, 1987). On the basis of the pH dependence of the kinetic parameters and dead-end inhibitor dissociation constants of the C-subunit, these authors suggested a chemical mechanism requiring general-base catalysis of the phosphoryl-transfer reaction and a binding group that must be protonated for activity, which is implicated in orienting the peptide for phosphoryl transfer. On the basis of the crystal structure, Knighton et al. (1991) suggest that Asp[166], one of only four invariant carboxyl groups in the protein kinase family, is the most likely candidate to be the catalytic general base, since it is the only one of the four that is oriented toward the Ala side chain at the pseudophosphorylation site of the bound inhibitor peptide in the crystal structure.

In this article, we report the pH dependence of the kinetic mechanism of the C-subunit and, from these data, the pH dependence of the kinetic parameters in the direction of MgADP phosphorylation. The kinetic mechanism changes with pH such that at low pH PSP binds first, while at high pH the kinetic mechanism is steady-state-ordered with MgADP binding first. Data are consistent with the requirements for a general acid which has to be protonated for activity and for the phosphoryl group of the peptide to be unprotonated for optimum binding.

MATERIALS AND METHODS

Chemicals. α-D-Glucose, AMPCP, G6PDH, and HK were obtained from Sigma.

Peptide Synthesis, Purification, and Quantification. All peptides were synthesized by solid-phase methods (Gutte & Merrifield, 1969). After synthesis, the peptides were purified on a preparative-scale C18RP column from Dynamax attached to a Beckman System Gold HPLC, as previously described (Qamar et al., 1992). Amino acid compositions and sequences were determined, confirming the identity of the synthetic peptides. Peptide concentrations were determined by the method of Plapp et al. (1971), with a slight modification as previously described (Qamar et al., 1992).

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[1] Abbreviations: cAMP, adenosine 3′,5′-monophosphate; cAK, cAMP-dependent protein kinase from bovine heart; C-subunit, catalytic subunit of cAK; R-subunit, regulatory subunit of cAK; SP, serpeptide Leu-Arg-Arg-Ala-Ser-Leu-Gly; PSP, phosphorylated SP; Mg_f, uncomplexed Mg^{2+}; DP, Asp peptide Leu-Arg-Arg-Ala-Asp-Leu-Gly, AMPCP, 5′-phosphoadenosine α,β-methylene diphosphonate; $NADP^+$, β-nicotinamide adenine dinucleotide 2′-phosphate; G6PDH, yeast glucose-6-phosphate dehydrogenase; HK, yeast hexokinase; Mops, 3-(*N*-morpholino)propanesulfonic acid; Mes, 2-(*N*-morpholino)ethanesulfonic acid; Taps, 3-[*N*-[tris(hydroxymethyl)methyl]amino]propanesulfonic acid; C18RP, C18 reverse phase; HPLC, high-performance liquid chromatography.

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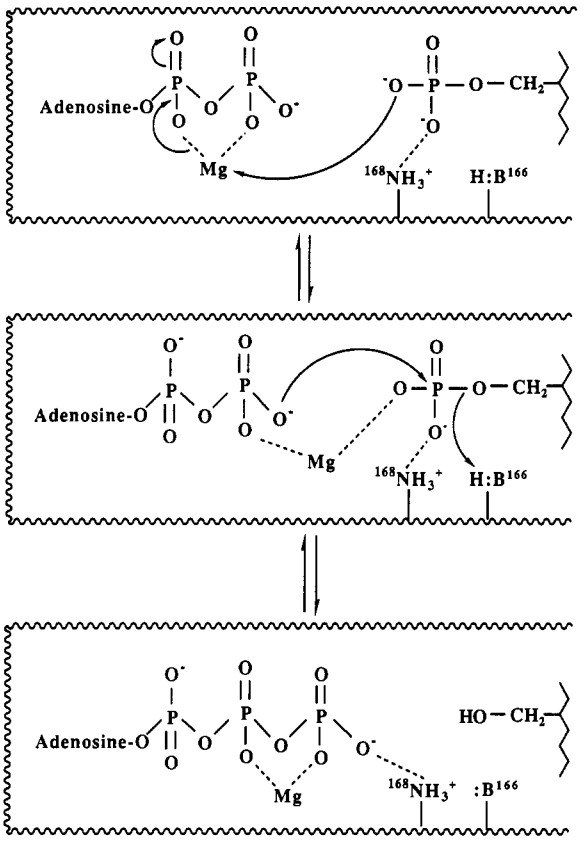
Scheme I: Proposed Chemical Mechanism in the Direction of MgADP Phosphorylation



C-Subunit Preparation. The C-subunit was purified by an adaptation of the methods of Sugden et al. (1976) and Olsen and Uhler (1989) and has been described in detail previously (Qamar et al., 1992).

Preparation of PSP. PSP was prepared by enzymatic phosphorylation of SP by the C-subunit. The reaction mixture had the following components in a 2-mL volume: 200 mM Mops, pH 7; 80 mM ATP; 84 mM $MgCl_2$; 20 mM SP; and 5 units of C-subunit. One unit of C-subunit is defined as the amount of enzyme required to catalyze the formation of 1 μmol of PSP per minute at pH 7 and 25 °C. After incubation of the reaction mixture overnight at room temperature, it was titrated with perchloric acid to pH 2, purified, and quantitated as described previously (Qamar et al., 1992).

Enzyme Assay. The activity of the C-subunit was determined by coupling the production of MgATP to the HK and G6PDH reactions and continuously monitoring the appearance of NADPH at 340 nm. A typical assay contained the following components in a final volume of 0.4 mL: 100 mM buffer; 100 mM KCl; 3 mM α-D-glucose; 1 mM $NADP^+$; 0.2 mM ADP; 0.7 mM $MgCl_2$; 10 mM PSP; 15 units of G6PDH; 78 units of HK; and 1 unit of C-subunit. In order to obtain the initial velocity, the background rate was obtained in the absence of C-subunit and was subtracted from the rate obtained upon the addition of C-subunit. All data were collected using a Gilford 260 spectrophotometer connected to a strip chart recorder. Under all conditions, plots of velocity versus the C-subunit concentration were linear. The substrate concentrations were corrected for the formation of Mg^{2+} chelate complexes, as discussed previously (Cook et al., 1982), by using the following values for dissociation constants: MgADP, 0.25 mM; MgNADP, 19.5 mM; KADP, 210 mM; and MgPSP, 20 mM (Cook et al., 1982; Dawson et al., 1969; Martell & Smith, 1979; Qamar et al., 1992). The MgAMPCP dissociation constant was assumed to be equal to that of MgADP.

pH Studies. cAK was shown by Yoon and Cook (1987) to be quite stable with no loss in activity from pH 6 to 9. At pH 5.5, some denaturation occurs with a $t_{1/2}$ of 14.7 min. However, the initial velocities measured will not be affected because enzyme is added to initiate the reaction from a stock solution maintained at pH 7 and the presence of substrates will afford some protection.

Plots of velocity versus C-subunit concentration were measured at pH 5.5, 7, and 8. In all cases, the plots are linear. Initial velocity patterns were obtained as a function of pH in the absence of inhibitors by varying the concentration of PSP at several fixed levels of MgADP using the assay described above. Dead-end inhibition patterns were obtained as a function of pH by measuring the initial velocities at varying concentrations of one reactant, while maintaining the second at K_m, and repeating this experiment at several levels of an inhibitor. The pH was maintained by using one of the following buffers over the indicated pH range at 100 mM final concentration: Mes, 5.5–6.5; Mops, 6.5–7.5; and Taps, 7.5–8. The pH was recorded before and after the initial velocity data were obtained, with no change observed.

Data Processing. Reciprocal initial velocities were plotted versus reciprocal substrate concentrations; all plots and their replots were linear. Data were fit by using the appropriate rate equation and the FORTRAN programs developed by Cleland (1979). Initial velocity patterns obtained at pH 6.2 and higher were fit using eq 1, while those obtained at pH 6 and lower were fit using eq 2. Data for competitive, noncompetitive, and uncompetitive inhibition were fit using eqs 3–5, respectively. Data for the V/K_{MgADP} pH profile, which decreases with a limiting slope of −1 at high pH, were fit using eq 6, while the pK_{iPSP} profile, which has a limiting slope of +1 at low pH, was fit using eq 7. In eqs 1–5, v is the

$$v = VAB/(K_{ia}K_b + K_aB + K_bA + AB) \quad (1)$$

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

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

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

$$v = VA/[K_a + A(1 + I/K_{ii})] \quad (5)$$

$$\log y = \log C/(1 + K_2/H) \quad (6)$$

$$\log y = \log C/(1 + H/K_1) \quad (7)$$

initial velocity, V is the maximum velocity, K_a and K_b are K_m values for A and B, I is inhibitor concentration, and K_{ia}, K_{is}, and K_{ii} are inhibition constants for A, slope, and intercept, respectively. In eqs 6 and 7, y is the observed value of the parameter of interest (i.e., V/K or $1/K_i$), C is the pH-independent value of y, H is the hydrogen ion concentration, and K_1 and K_2 are acid dissociation constants for enzyme, substrate, or inhibitor functional groups.

RESULTS

The pH dependence of kinetic parameters was determined to allow elucidation of the acid–base chemical mechanism of the C-subunit. The pH data can only be rationally interpreted

Table I: pH Dependence of Kinetic Parameters in the Direction of MgADP Phosphorylation

	pH 5.8[a]	pH 6.0[a]	pH 6.2[b]	pH 6.6[b]	pH 7.2[b]	pH 7.6[b]
K_{MgADP} (mM ± SE)	0.036 ± 0.023	0.035 ± 0.005	0.018 ± 0.005	0.022 ± 0.004	0.085 ± 0.011	0.085 ± 0.025
K_{PSP} (mM ± SE)			0.26 ± 0.14	0.59 ± 0.15	2.01 ± 0.25	0.88 ± 0.20
K_{iMgADP} (mM ± SE)			0.19 ± 0.09	0.05 ± 0.01	0.038 ± 0.003	0.38 ± 0.07
K_{iPSP} (mM ± SE)	4.9 ± 2.0	2.1 ± 0.4	2.7 ± 0.5	1.4 ± 0.2	0.9 ± 0.1	4.0 ± 1.0
V/E_t (s^{-1} ± SE)	3.6 ± 1.2	3.7 ± 0.2	2.4 ± 0.3	2.6 ± 0.3	4.2 ± 0.3	2.5 ± 0.3
$V/K_{MgADP}E_t$ (mM^{-1} s^{-1} ± SE)	101.7 ± 36.2	105.7 ± 11.6	134.7 ± 21.0	122.7 ± 12.0	48.5 ± 2.1	28.9 ± 5.7
$V/K_{PSP}E_t$ (mM^{-1} s^{-1} ± SE)			9.3 ± 4.2	4.5 ± 0.6	2.1 ± 0.1	2.8 ± 0.4

[a] Data were fit using eq 2. [b] Data were fit using eq 1.

if the kinetic parameters reflect the same predominant enzyme form. Thus, it is important to determine the pH dependence of the kinetic mechanism. All assays were carried out at low (0.5 mM) Mg_f concentrations, unless otherwise indicated.

Initial Velocity Studies in the Absence of Added Inhibitors. Initial velocity patterns were obtained at neutral, acidic, and basic pH. At neutral and basic pH, initial velocity patterns intersect to the left of the ordinate, indicating a sequential kinetic mechanism. At acidic pH, the patterns intersect on the ordinate when plotted as $1/v$ versus 1/MgADP, indicative of an equilibrium-ordered mechanism with PSP binding prior to MgADP. Since a pH dependence of the kinetic mechanism was observed, initial velocity patterns were obtained as a function of pH in order to obtain kinetic parameters; these are summarized in Table I. When Mes was used as the buffer, a decrease in $V/K_{MgADP}E_t$ was observed compared to the value obtained in Mops. In order to verify the existence of the buffer effect, a Mes versus MgADP inhibition pattern was obtained and gave competitive inhibition, with a K_{iMes} value of 108 mM (data not shown). The K_{iMes} was used to correct the observed K_{MgADP} by dividing the observed value by (1 + Mes/K_{iMes}).

pH Dependence of Kinetic Parameters. The pH dependence of kinetic parameters in the direction of MgADP phosphorylation is shown graphically in Figure 1. The maximum velocity is pH-independent, while $V/K_{MgADP}E_t$ decreases to a limiting slope of −1 at basic pH, giving a pK of 7.1 ± 0.1. The pK_{iPSP} decreases as the pH is decreased, giving a pK of 6.5 ± 0.2. The V/K_{PSP} is not defined at a sufficient number of pH values to give a reliable pK estimate.

Inhibition Studies. The kinetic mechanism was further defined at different pH values by performing dead-end inhibition studies. At pH 6, MgAMPCP, a dead-end analog of MgADP, is uncompetitive versus PSP when MgADP is fixed near its K_m, while DP, a dead-end analog of PSP, is competitive versus MgADP when PSP is fixed near its K_i. At pH 6.6 and 7.2, MgAMPCP is competitive versus MgADP and uncompetitive versus PSP when MgADP is fixed near its K_m, while DP is competitive versus PSP and noncompetitive versus MgADP when PSP is fixed near its K_m. At pH 7.6, MgAMPCP is competitive versus MgADP and noncompetitive versus PSP when MgADP is fixed near its K_m, while DP is competitive versus PSP and uncompetitive versus MgADP when PSP is fixed near its K_m. Data are summarized in Table II.

pH Dependence of K_i for Dead-End Inhibitors. As can be seen from the corrected K_i values of the two different inhibitors (in Table II), the parameters K_{iDP} and $K_{iMgAMPCP}$ are both pH-independent, within experimental error, over the pH range examined.

DISCUSSION

Armstrong et al. (1979) reported that, in addition to Mg^{2+} binding to ATP, the C-subunit also has a metal binding site,

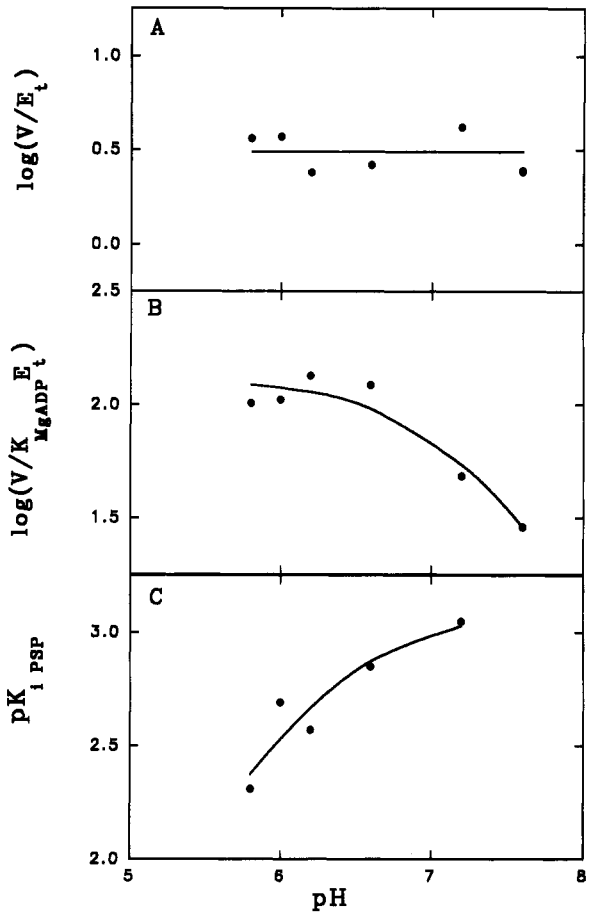


FIGURE 1: pH dependence of kinetic parameters in the direction of MgADP phosphorylation: (A) V/E_t; (B) $V/K_{MgADP}E_t$; (C) $1/K_{iPSP}$. Data are from Table I. Points are experimental, while the curves are from a fit of eqs 6 ($V/K_{MgADP}E_t$) and 7(pK_{iPSP}) to the data.

which will be referred to as the second metal binding site since the other metal is coordinated only to ATP. It has been shown that, when the metal occupies this second site, there is a decrease in the maximum velocity and an increase in V/K for the metal–nucleotide complex as a result of an increase in the affinity of the enzyme for MgATP and MgADP (Armstrong et al., 1979; Cook et al., 1982; Kong & Cook, 1988). These authors concluded that this increase in the affinity of the enzyme for the metal–nucleotide complex when Mg^{2+} is bound at the second metal site is due to Mg^{2+} coordinating the α- and γ-phosphates (and perhaps also the β) of MgATP or MgADP as well as residues on the enzyme. We have recently reported that, at pH 7.2, the binding of Mg^{2+} at the second site results in an increase in the affinity of the enzyme for MgADP and a decrease in affinity for PSP. These changes in affinity for MgADP and PSP result in changes in the order of substrate binding. When the second site does not contain bound Mg^{2+}, the enzyme has an ordered mechanism and binds PSP first. Occupation of the second site with Mg^{2+} reverses

Table II: pH Dependence of the Dissociation Constants for Dead-End Inhibitors

variable substrate	fixed substrate	inhibitor	pattern	K_{is} (mM ± SE)	K_{ii} (mM ± SE)
pH 6.0					
MgADP	PSP	DP	C[a]	3.1 ± 0.3 (2.1 ± 0.2)[b]	
PSP	MgADP	MgAMPCP	UC		1.8 ± 0.1
pH 6.6					
PSP	MgADP	DP	C	3.1 ± 0.4	
MgADP	PSP	DP	NC	15.5 ± 5.2 (8.8 ± 3.0)	4.8 ± 0.5 (1.7 ± 0.2)
MgADP	PSP	MgAMPCP	C	1.0 ± 0.1 (0.42 ± 0.04)	
PSP	MgADP	MgAMPCP	UC		0.7 ± 0.1 (0.37 ± 0.05)
pH 7.2[c]					
PSP	MgADP	DP	C	2.9 ± 0.4	
MgADP	PSP	DP	NC	4.2 ± 1.0 (1.9 ± 0.5)	15.2 ± 5.2 (9.8 ± 3.4)
MgADP	PSP	MgAMPCP	C	1.2 ± 0.1 (0.9 ± 0.1)	
PSP	MgADP	MgAMPCP	UC		3.1 ± 0.3 (1.4 ± 0.1)
pH 7.6					
PSP	MgADP	DP	C	4.2 ± 0.5 (1.3 ± 0.2)	
MgADP	PSP	DP	UC		4.1 ± 0.3 (2.0 ± 0.2)
MgADP	PSP	MgAMPCP	C	1.0 ± 0.2	
PSP	MgADP	MgAMPCP	NC	4.1 ± 0.3 (2.7 ± 0.2)	0.8 ± 0.1 (0.24 ± 0.03)

[a] Values in parentheses are corrected for the fixed reactant concentration. [b] C, NC, and UC represent competitive, noncompetitive, and uncompetitive, respectively. [c] From Qamar et al. (1992).

the order of addition, with MgADP binding before PSP, and in addition the metal–nucleotide complex binding comes to equilibrium (Qamar et al., 1992).

pH Dependence of Kinetic Mechanism. The data suggest a kinetic mechanism for the C-subunit at pH 7.2 and 0.5 mM Mg_f that is ordered, with PSP binding prior to MgADP. Diagnostic patterns for this mechanism are uncompetitive inhibition by MgAMPCP versus PSP and noncompetitive inhibition by DP versus MgADP. As can be seen from the data of Table II, this mechanism is also indicated at pH 6.6 and presumably also at pH 6.2, since an intersecting initial velocity pattern similar to that at pH 6.6 is oberved (Table I). At pH 6.0 and below, however, the initial velocity pattern intersects on the ordinate when the reciprocal initial velocity is plotted against the reciprocal of the MgADP concentration. These data are indicative of an equilibrium-ordered addition of PSP prior to MgADP. The latter mechanism is confirmed by the uncompetitive inhibition by MgAMPCP versus PSP suggesting the mechanism is still ordered, and the competitive inhibition by DP versus MgADP, indicating that all of the enzyme can be converted to the central complex by increasing MgADP to infinity (Cleland, 1977). The mechanism again changes as the pH is increased from 7.2 to 7.6. At pH 7.6, DP is uncompetitive versus MgADP and MgAMPCP is noncompetitive versus PSP, indicating the mechanism is now ordered with MgADP binding prior to PSP. Thus, the mechanism changes with pH as it does with Mg_f concentration. This is consistent with an overall kinetic mechanism that is random, but with the pathway that is allowed to be dependent on pH. These results are in agreement with Qamar et al. (1992) and the previous studies of Kong and Cook (1988).

In order for the kinetic mechanism to change from steady-state-ordered with PSP binding first to equilibrium-ordered as the pH is lowered, the binding of PSP must come to equilibrium at the lower pH values, i.e., the off-rate for PSP from the E:PSP complex must become much greater than the overall net rate of conversion of E:PSP to products, as explained previously for the change in mechanism as a function of Mg_f (Qamar et al., 1992). As can be seen from the data in Table I, K_{iPSP} increases as the pH is decreased to below 7.2. The increase in K_{iPSP} could result from either an increase in the off-rate for PSP or a decrease in the on-rate, but is likely a result of the former. The off-rate for PSP from the E:PSP complex must become much greater than the overall net rate of conversion of E:PSP to products, i.e., the binding of PSP comes to equilibrium. This results in the change in the mechanism from steady-state-ordered to equilibrium-ordered.

The change in the preference of substrate binding as the pH is increased from 7.2 to 7.6 is probably due to a change in the affinity of the free enzyme for MgADP and PSP. The change in affinity can perhaps be explained by the amount of negative charge brought to the active site. At pH values below 7, the general acid (Asp^{166}; *vide supra*) will be protonated and neutral and one of the two phosphate oxygens of PSP will be largely protonated, giving a net charge of −1 with the latter possibly neutralized by an enzyme residue, e.g., Lys^{168}; MgADP will have a charge of −1. As the pH is increased, additional negative charge results from deprotonation of the phosphate of PSP and the catalytic general acid, leading to charge repulsion. Under these conditions, MgADP has a net charge of only −1 compared to the −2 of PSP, and thus there will be a preference for binding MgADP prior to PSP at high pH.

On the basis of crystallographic studies of the catalytic subunit, Knighton et al. (1991) have proposed that the invariant residue Asp^{184} may be the residue that is involved in the chelation of Mg^{2+} in the complex of MgATP with the enzyme; this is also likely to be the case for MgADP chelation. Therefore, an alternative explanation of the change in the preference of substrate binding as the pH is increased from 7.2 to 7.6 is that, at pH 7.2, Asp^{184} is protonated and therefore does not chelate the Mg^{2+} in the complex of MgADP. However, at pH 7.6 Asp^{184} is largely unprotonated and thus able to chelate the Mg^{2+} in the MgADP complex; this results in an increase in the affinity of the enzyme for MgADP, and therefore, the change in the preference of substrate binding occurs from PSP binding first at the lower pH to MgADP binding first at the higher pH.

pH Dependence of Kinetic Parameters. Information on the pH dependence of kinetic parameters, and thus the acid–base chemistry, is obtained from the data in Table I. However, only the kinetic parameters that reflect the same enzyme form can be compared. Thus, V/K_{MgADP} from pH 5.8 to 7.2 reflects predominantly E:PSP and free substrate, while at pH 7.6 it reflects free enzyme and free substrate and this value cannot be used. Likewise, K_{iPSP} reflects E:PSP over the same range. There is insufficient data available to analyze V/K_{PSP}.

The maximum velocity is pH-independent over the range examined, suggesting that only the correctly protonated reactants and enzyme form a productive complex. The V/K_{MgADP} is pH-dependent and decreases at high pH, giving a pK of 7.1. The pK of the β-phosphate of ADP is in the range 6.1–6.7 (Dawson et al., 1969) but will be lower for MgADP, and thus the observed pK reflects an enzyme residue, probably that of the catalytic general acid. The general base must be unprotonated in the direction of SP phosphorylation, and it has a pK of 6.5 (Yoon & Cook, 1987). In the direction of MgADP phosphorylation, the general base is a general acid and must be protonated for activity, in order to donate a proton to the serine being formed. The pK is slightly higher, probably

as a result of the pK reflecting E:MgATP in the forward direction but E:PSP in the reverse direction.

The pK of 6.5 obtained from the pK_{iPSP} profile is likely that of the phosphoryl group of PSP, which must be unprotonated for optimum binding. Since the K_{PSP} is defined at only three pH values, the V/K_{PSP} profile is not very reliable. The latter profile reflects predominantly free enzyme and free PSP. A fit of V/K_{PSP} versus pH to eq 6 gives a pK of 6.3 ± 0.3, which is in reasonably good agreement with the pK of 6.5 obtained for the PSP phosphate from the pK_{iPSP} profile. These data are consistent with the acid–base mechanism proposed by Yoon and Cook (1988).

Dead-End Inhibition Studies. To confirm the kinetic mechanism of the C-subunit at various pH values, dead-end inhibition studies were performed. In order to demonstrate that the dead-end data are internally quantitatively consistent and thus support the qualitative data, the observed K_i values were corrected for the concentration of the fixed reactant to give an estimate of the true K_i values of the inhibitors. At pH 6, the apparent K_i value, obtained from the competitive inhibition of DP versus MgADP, can be corrected using the equation $K_i(app) = K_i(1 + PSP/K_{iPSP})$, giving a true K_{iDP} of 2.1 mM.

At pH 6.6 the apparent K_i value obtained from the uncompetitive inhibition of MgAMPCP versus PSP can be corrected by using the equation $K_{ii}(app) = K_i(1 + MgADP/K_{MgADP})$, which gives a true $K_{iMgAMPCP}$ of 0.37 mM. This can be compared with the value of 0.42 mM obtained from the competitive inhibition of MgAMPCP versus MgADP, where $K_{is}(app)$ has been corrected using the equation $K_i(app) = K_i(1 + K_{iPSP}/PSP)$. The K_i values estimated from the noncompetitive inhibition of DP versus MgADP, using $K_{is}(app) = K_i(1 + PSP/K_{iPSP})$ and $K_{ii}(app) = K_i(1 + PSP/K_{PSP})$, give K_i values of 8.8 and 1.7 mM, respectively. The first of these values is not very close to the value for K_{iDP} of 3.1 mM obtained from the DP versus PSP inhbition, but it has a large standard error. The latter value is in much better agreement with the value obtained from the DP versus PSP inhibition.

Corrected values for K_i of dead-end inhibitors at pH 7.2 have been reported (Qamar et al., 1992). At pH 7.6, the apparent K_i value obtained from the uncompetitive inhibition of DP versus MgADP can be corrected using the equation $K_{ii}(app) = K_i(1 + PSP/K_{PSP})$, which gives a true K_{iDP} of 2 mM. This can be compared to a value of 1.3 mM, obtained from the competitive inhibition of DP versus PSP, in which $K_{is}(app)$ has been corrected using the expression $K_i(1 + K_{iMgADP}/MgADP)$. The K_i values obtained from the noncompetitive inhibition of MgAMPCP versus PSP can be corrected using the equations $K_{is}(app) = K_i(1 + MgADP/K_{iMgADP})$ and $K_{ii}(app) = K_i(1 + MgADP/K_{MgADP})$. These give K_i values of 2.7 and 0.24 mM, respectively, which are in reasonable agreement with the value of 1 mM obtained for $K_{iMgAMPCP}$ from the MgAMPCP versus MgADP inhibition. The K_i values for both DP and MgAMPCP are pH-independent, within error.

Conclusions. On the basis of the data reported here and those recently reported (Qamar et al., 1992), it has been clearly established that the kinetic mechanism of the C-subunit in the direction of MgADP phosphorylation is random, but with only one of the two pathways is allowed, dependent on the pH and the Mg_f concentration.

The acid–base chemistry suggested by the pH dependence of kinetic parameters in the direction of MgADP phosphorylation is fully consistent with the mechanism of Yoon and Cook (1988), shown in Scheme I. The requirement for a general acid is observed and reflects the same group observed in the opposite direction. The reaction may initially involve a ligand displacement mechanism, in which the oxygen of the α-phosphate (or water) is displaced by an oxygen of the PSP phosphate group, followed by a nucleophilic attack of a MgADP β-phosphate oxygen on the phosphate group of PSP. The group with a pK of about 8 required to be protonated for activity and thought to orient the peptide for phosphorylation is not observed in these studies since it is difficult to study the reaction at pH values higher than 7.5. Finally, there is a requirement for the phosphate of PSP to be unprotonated for optimum binding.

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