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 define the overall kinetic mechanism of adenosine 3',5'-monophosphate dependent protein kinase catalytic subunit and also to elaborate the kinetic mechanism in the direction of peptide phosphorylation, we have determined its kinetic mechanism in the direction of MgADP phosphorylation. Studies of initial velocity as a function of uncomplexed Mg^{2+} (Mg_f) in the absence and presence of dead-end inhibitors were used to define the kinetic mechanism. Data are consistent with the overall kinetic mechanism in the direction of MgADP phosphorylation being random with both the pathways allowed, i.e., the pathway in which MgADP binds to the enzyme prior to phosphorylated peptide and the pathway in which phosphorylated peptide binds to enzyme prior to MgADP. In addition, depending on the concentration of Mg_f, one or the other pathway predominantes. At low (0.5 mM) Mg_f, the mechanism is steady-state ordered with the pathway in which phosphorylated peptide binds first being preferred; at high (10 mM) Mg_f, the kinetic mechanism is equilibrium ordered, and the pathway in which MgADP binds first is preferred. This change in mechanism to equilibrium ordered at higher concentration of Mg_f is due to an increase in affinity of the enzyme for MgADP and a decrease in affinity for the phosphorylated peptide. The Haldane relationship gives a K_{eq} of $2 \pm 1 \times 10^3$ at pH 7.2, in agreement with the values obtained from ^{31}P NMR ($1.6 \pm 0.8 \times 10^3$) and direct determination of reactant concentrations at equilibrium ($3.5 \pm 0.6 \times 10^3$).

Adenosine 3',5'-monophosphate dependent protein kinase (ATP:protein phosphotransferase, EC 2.7.1.37) is a heterotetramer consisting of a regulatory dimer coordinated to two catalytic monomers (Taylor et al., 1990). In the tetrameric form, the C-subunit[1] is inactive. The two R-subunits bind four molecules of cAMP, releasing the two molecules of C-subunit, which are then active. The C-subunit phosphorylates a variety of different proteins in vivo, modulating their physiological activity (Bramson et al., 1984).

A steady-state random kinetic mechanism has been suggested for the C-subunit in the direction of SP phosphorylation, with ordered release of products, PSP being released prior to MgADP (Cook et al., 1982). In contrast to the random mechanism, Whitehouse et al. (1983) concluded that the mechanism was steady-state ordered, with MgATP adding prior to peptide and with ordered release of PSP prior to MgADP. Based on isotope partitioning studies and initial velocity studies at high concentrations of SP, Kong and Cook (1988) concluded that the mechanism in the direction of SP phosphorylation is indeed steady-state random, with both binary complexes, E·MgATP and E·SP, allowed but with the pathway with MgATP binding first preferred. In experiments designed to determine whether an E·MgADP complex could be trapped, no such trapping was observed even at high (5 mM) concentrations of PSP in the chase. The latter observation suggests that, in the direction of MgADP phosphorylation, either the kinetic mechanism is rapid-equilibrium or PSP must bind prior to MgADP.

In this paper, we report initial velocity and dead-end inhibition studies of the C-subunit in the direction of MgADP phosphorylation. The data are consistent with a steady-state ordered kinetic mechanism, with PSP binding first, at low (0.5 mM) Mg_f. The data also indicate a change in kinetic mechanism at high (10 mM) Mg_f to equilibrium ordered with MgADP binding first.

MATERIALS AND METHODS

Chemicals. α-D-Glucose, L-serine, DTT, AMPCP, G6PDH, and HK were obtained from Sigma. The reagent TNBS was from Fluka, DE52 resin was from Whatman, and Bradford reagent was from Bio-Rad.

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. The gradients were linear from 0 to 30% acetonitrile and were 0.1% in TFA. The column effluent was monitored at 215 nm, and the major peaks were collected and dried on a rotary evaporator. The residual solids

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[1] Abbreviations: cAMP, adenosine 3',5'-monophosphate; AMPCP, 5'-phosphoadenosine α,β-methylene diphosphonate; PMSF, phenylmethanesulfonyl fluoride; Mops, 3-(*N*-morpholino)propanesulfonic acid; Mes, 2-(*N*-morpholino)ethanesulfonic acid; TNBS, 2,4,6-trinitrobenzenesulfonic acid; TFA, trifluoroacetic acid; $NADP^+$, β-nicotinamide adenine dinucleotide phosphate; G6PDH, yeast glucose-6-phosphate dehydrogenase; HK, yeast hexokinase; 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+}; EDTA, ethylenediaminetetraacetic acid; DTT, DL-dithiothreitol; βME, 2-mercaptoethanol; HPLC, high-performance liquid chromatography; C18RP, C18 reverse phase; DP, Asp-peptide (Leu-Arg-Arg-Ala-Asp-Leu-Gly); E, free (unliganded) C-subunit; E_t, total enzyme.

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were dissolved in 50 mM Mops, pH 7, and the pH of the resulting solution was adjusted to 7 with KOH. Amino acid compositions and sequences were determined and confirmed the identity of the synthetic peptides. Peptide concentrations were determined by the method of Plapp et al. (1971), which makes use of amino group modification with TNBS; L-serine was used as a standard. The modification reaction was monitored at 367 nm using a Gilford 260 spectrophotometer attached to a strip chart recorder and was initiated by addition of peptide and allowed to proceed until the rate slowed to the background of TNBS hydrolysis observed prior to initiation of the reaction.

C-Subunit Preparation. The C-subunit was purified by an adaptation of the methods of Sugden et al. (1976) and of Olsen and Uhler (1989). Four bovine hearts were homogenized in 10 mM potassium phosphate, pH 6.9, 1 mM PMSF, 1 mM EDTA, 15 mM βME, and 1 mM leupeptin. The homogenate was centrifuged at 15 000*g* for 30 min. The supernatant was filtered through glass wool and cheesecloth. The filtrate was then batch-loaded for 4–5 h onto 1.2 L of DE52 resin preequilibrated in 55 mM potassium phosphate, pH 6.9, 1 mM EDTA, and 15 mM βME (buffer A). The resin was then poured into a column and washed overnight with 15–20 column volumes of buffer A. The C-subunit was then eluted from the column with 45 mM potassium phosphate, pH 6.9, which contained 100 μM cAMP and 15 mM βME. The cAK activity in the eluate was determined by the method of Cook et al. (1982). The fractions containing activity were pooled and then loaded onto an affinity column (Olsen & Uhler, 1989) in which the affinity ligand consists of a nonapeptide portion of the heat-stable inhibitor protein (Gly-Arg-Thr-Gly-Arg-Arg-Asn-Ala-Ile-NH_2) coupled to Bio-Rad Affi-Gel 10. The column was washed according to the method of Olsen and Uhler (1989). The C-subunit was eluted with a 55–1000 mM potassium phosphate gradient at pH 6.9 containing 10% glycerol and 1 mM DTT. Fractions of 4 mL each were collected, and those containing cAK activity were pooled and concentrated by Amicon ultrafiltration. Protein concentration was determined by the method of Bradford (1976).

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. A unit of C-subunit is defined as the amount of enzyme required to catalyze the formation of 1 μmol of PSP per min at pH 7 and 25 °C. The reaction mixture was incubated overnight at room temperature and then titrated with perchloric acid to pH 2. This acidification results in enzyme denaturation and precipitation of some of the K^+ as $KClO_4$. The solution was centrifuged to remove all solid material, and the supernatant was loaded onto a preparative scale C18RP column attached to a Beckman System Gold HPLC. The column was developed with a gradient of 0–30% acetonitrile that was 0.1% in TFA; the effluent was monitored at 215 nm. The fluorescamine-positive peak was collected, rotary-evaporated, and suspended in 50 mM Mops, pH 7. The pH of the solution was adjusted to 7 with KOH. The resulting solution was assayed for ATP, ADP, and SP and was found to be free of these contaminants. The amount of the peptide in solution was then determined by the method of Plapp et al. (1971) as described above.

Enzyme Assay. The activity of the C-subunit was determined using a coupled enzyme assay system, where the production of MgATP is coupled to the appearance of NADPH at 340 nm via the reactions catalyzed by HK and G6PDH. A typical assay at pH 7.2 contained the following components in a final volume of 0.4 mL: 100 mM Mops, pH 7.2; 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. Under all conditions, plots of velocity versus the C-subunit concentration were linear. All data were collected by using a Gilford 260 spectrophotometer connected to a strip chart recorder. 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; and KADP, 210 mM (Cook et al., 1982; Dawson et al., 1969; Martell & Smith, 1979). The dissociation constant for MgPSP was taken to be 20 mM (G. D. McClure, Jr., unpublished observation). The MgAMPCP dissociation constant was assumed to be equal to that of MgADP.

Initial Velocity Studies. Initial velocity patterns in the absence of inhibitors were obtained by varying the concentrations of PSP at different fixed levels of MgADP using the assay described above. Data were obtained in the presence of 100 mM KCl at several fixed levels of Mg_f, including 0.5, 1, 2, 5, and 10 mM, and in the absence of KCl at 0.5 and 10 mM Mg_f. Dead-end inhibition patterns were obtained by varying one reactant with the second fixed at K_m and at several different levels of the inhibitor. In order to be certain the inhibitors did not affect the coupling enzyme activities, velocity was measured as a function of enzyme concentration at the lowest concentrations of reactants and the highest concentrations of inhibitors. In all cases these plots were linear.

Equilibrium Constant. The equilibrium constant for the C-subunit was determined at pH 6.0 (100 mM Mes) as follows. Reaction mixtures were prepared at different ratios of [PSP]/[SP] and a constant [MgADP]/[MgATP] ratio. Enzyme was added and, after a 2-h incubation, the C-subunit was denatured by vortexing the mixture with a few drops of perchloric acid. The final SP concentration was determined enzymatically as described by Cook et al. (1982).

^{31}P NMR Spectra. ^{31}P NMR spectra were recorded at 36.3 MHz on a JOEL FX-90Q NMR spectrometer with HOD as the locking signal. Spectra were obtained by using a 90° pulse of 25 μs duration, a pulse delay of 3 s, and a pulse acquisition time of 3.4 s. Typically, 120 scans were accumulated, and the sweep width was 1200 Hz. Chemical shifts were referenced to 85% H_3PO_4 as an external standard. All measurements were made using 1-cm tubes at a probe temperature of 28 °C.

Data Processing. Reciprocal initial velocities were plotted versus reciprocal substrate concentrations; all plots and their replots were linear. Data were fitted by using the appropriate rate equation and the FORTRAN programs developed by Cleland (1979). Initial velocity patterns obtained at 0.5 and 1 mM Mg_f, varying PSP at several fixed levels of MgADP, were fitted using eq 1, while those obtained at 2, 5, and 10 mM Mg_f were fitted using eq 2. Data for competititve, noncompetititve, and uncompetitive inhibition were fitted using eqs 3–5, respectively.

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

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

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

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

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

In eqs 1–5, v is the initial velocity, V is the maximum velocity, K_a and K_b are K_m values for reactants A and B, I is inhibitor concentration, while K_{ia}, K_{is}, and K_{ii} are inhibition constants for A, slope, and intercept, respectively.

RESULTS

Armstrong et al. (1979) reported the presence of a metal binding site on the C-subunit which binds Mg^{2+} or Mn^{2+} (this is in addition to the Mg^{2+} bound to ATP); this site on the C-subunit will be referred to as the second metal binding site. Occupancy of this second site by metal results in a decrease in the maximum velocity and an increase in V/K for the metal nucleotide complex as a result of an increased affinity of the enzyme for nucleotide (Kong & Cook, 1988). Therefore the steady-state kinetic analysis reported here was performed at different fixed levels of Mg_f. At the extreme concentrations of Mg_f, data were also collected in the presence or absence of 100 mM KCl. The KCl concentrations are the same as those used in the studies of Cook et al. (1982) in the direction of SP phosphorylation and, thus, allow a direct comparison of the data in both reaction directions.

Initial Velocity Studies in the Absence of Added Inhibitors. An initial velocity pattern was obtained at 0.5 mM Mg_f in the absence of products and dead-end inhibitors by varying MgADP at several fixed levels of PSP; the pattern intersects to the left of the ordinate (Figure 1A). At 10 mM Mg_f, however, the pattern intersects on the ordinate when $1/v$ is plotted vs PSP (Figure 1B). The data obtained at 0.5 mM Mg_f suggest a sequential kinetic mechanism, while those obtained at 10 mM Mg_f suggest a specific sequential mechanism, i.e., an equilibrium-ordered mechanism with MgADP binding prior to PSP. In addition to the data discussed above, initial velocity patterns were collected at Mg_f concentrations of 1, 2, and 5 mM Mg_f. At 1 mM Mg_f, the pattern still intersects to the left of the ordinate, while at Mg_f concentrations of 2 mM and higher, the initial velocity patterns intersect on the ordinate (data not shown).

To determine the effect of KCl, initial velocity patterns were obtained at 0 and 100 mM KCl with Mg_f at 0.5 and 10 mM. At 10 mM Mg_f, no changes in the kinetic parameters are observed in the presence or absence of 100 mM KCl. However, at 0.5 mM Mg_f, significant differences in the kinetic parameters are observed. Kinetic parameters are summarized in Table I.

Inhibition Studies. Dead-end inhibition studies were carried out in order to define further the kinetic mechanism at 0.5 and 10 mM Mg_f. With Mg_f at 0.5 mM, MgAMPCP, a dead-end analog of MgADP, is competitive vs MgADP when PSP is fixed near its K_m and is uncompetitive vs PSP. A dead-end analog of PSP, DP, is competitive vs PSP when MgADP is fixed near its K_m and is noncompetititve vs MgADP. At 10 mM Mg_f, MgAMPCP is competitive vs PSP. Kinetic parameters are summarized in Table II. α-Aminobutyryl peptide, a dead-end analog of PSP, has an α-aminobutyryl group in place of phosphoserine; this peptide has an estimated K_i of 1.3 mM. Glu-peptide, another dead-end analog of PSP which has a glutamate residue in place of phosphoserine, has an estimated K_i of 2 mM.

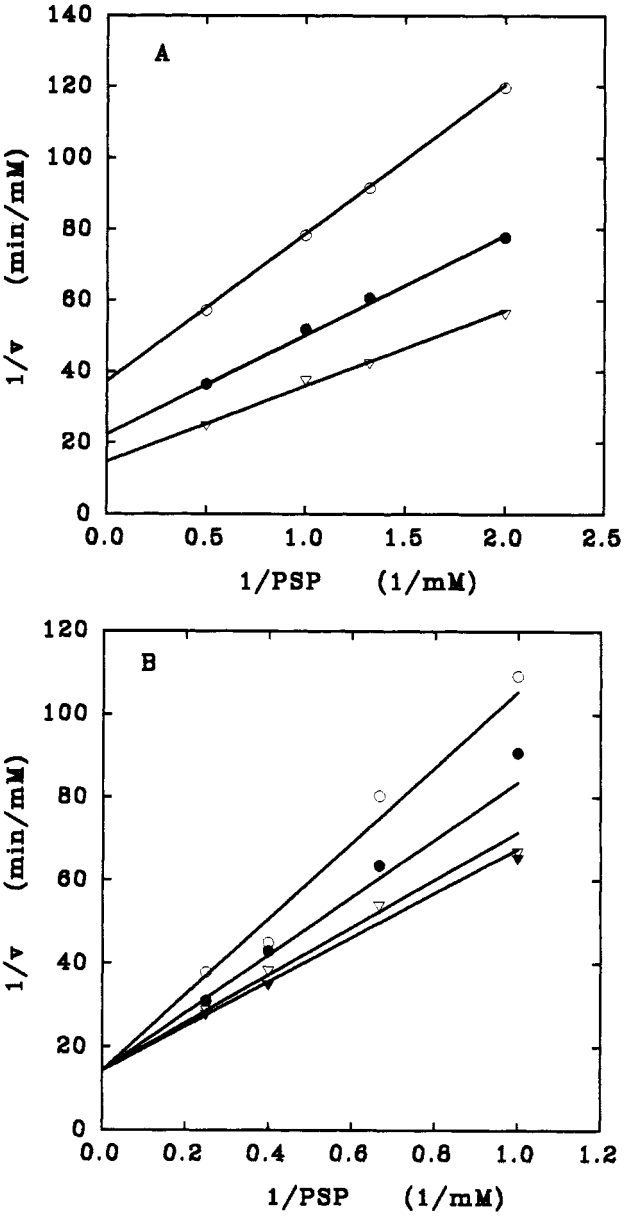


FIGURE 1: Figure 1 (Panel A): Initial velocity pattern in the direction of MgADP phosphorylation at pH 7.2. All assays were carried out at 0.5 mM Mg_f and 100 mM KCl. PSP was varied at several fixed levels of MgADP including 20.5 (○), 41 (●), and 82 μM (▽). All other conditions are as described under Materials and Methods. (Panel B): Initial velocity pattern in the direction of MgADP phosphorylation at pH 7.2. All assays were carried out at 10 mM Mg_f and 100 mM KCl. PSP was varied at several fixed levels of MgADP including 2.2 (○), 4.4 (●), 9.8 (▽), and 16.5 μM (▼). All other conditions are as described under Materials and Methods.

Equilibrium Constant Determination. Addition of C-subunit to reaction mixtures containing different concentrations of SP and PSP but constant MgADP and MgATP produces a change in SP as equilibrium is approached (Figure 2). SP concentrations were measured according to the method of Cook et al. (1982). The change in the SP concentration is plotted against the initial [PSP]/[SP] ratio, with K_{eq} being taken as the point where the resulting curve crosses the ΔSP axis. The equilibrium constant ([PSP][MgADP]/[SP][MgATP]) measured using this method at pH 6.0 and 25 °C is $2.2 \pm 0.4 \times 10^2$. The lower pH was used in these studies to facilitate measurement. The K_{eq} for cAK is pH dependent. A pH of 6 was used so that sufficient SP remained at equilibrium to measure.

Table I: Kinetic Parameters of cAMP-Dependent Protein Kinase as a Function of Mg_f at pH 7.2[a]

	Mg_f						
	0.5 mM	1 mM	2 mM	5 mM	10 mM	10 mM[b]	0.5 mM[b]
K_{MgADP} (mM)	0.085 (0.011)	0.013 (0.008)					0.030 (0.009)
K_{PSP} (mM)	2.0 (0.3)	2.3 (0.8)	0.6 (0.3)	1.1 (0.2)	3.4 (0.5)	4.1 (0.7)	2.0 (0.6)
K_{iMgADP} (mM)	0.038 (0.003)	0.07 (0.02)	0.05 (0.02)	0.011 (0.002)	0.0020 (0.0003)	0.0019 (0.0003)	0.06 (0.02)
K_{iPSP} (mM)	0.9 (0.1)	12 (6)					3.7 (1.1)
V/E_t (s^{-1})	4.2 (0.4)	2.3 (0.4)	1.2 (0.1)	1.22 (0.05)	1.9 (0.1)	1.9 (0.2)	1.7 (0.2)
$V/K_{MgADP}E_t$ (mM^{-1} s^{-1})	44 (2)	170 (80)					57 (10)
$V/K_{PSP}E_t$ (mM^{-1} s^{-1})	2.1 (0.1)	1.0 (0.2)	1.9 (0.8)	1.1 (0.1)	0.57 (0.04)	0.47 (0.04)	0.9 (0.2)

[a] All parameters were determined at 100 mM KCl unless indicated otherwise. All other conditions are as noted in the text. Numbers in parentheses are standard errors. [b] Determined in the absence of KCl.

Table II: Dead-End Inhibition as a Function of Mg_f at pH 7.2[a]

variable substrate	fixed substrate	inhibitor	pattern	K_{is} (mM)	K_{ii} (mM)
		0.5 mM Mg_f			
PSP	MgADP	DP	C[b]	2.9 ± 0.4	
MgADP	PSP	DP	NC[c]	4 ± 1	15 ± 5
MgADP	PSP	MgAMPCP	C	1.2 ± 0.1	
PSP	MgADP	MgAMPCP	UC[d]		3.1 ± 0.3
		10 mM Mg_f			
PSP	MgADP	MgAMPCP	C	0.13 ± 0.02	

[a] All data were obtained at 100 mM KCl. All other conditions are as described in the text. [b] C, competitive. [c] NC, noncompetitive. [d] UC, uncompetitive.

The ^{31}P NMR spectrum of the reaction mixture was also used to determine the equilibrium constant (Figure 3). A reaction mixture was prepared away from the equilibrium position, and the spectrum was recorded prior to the addition of enzyme and as a function of time after the addition of enzyme to ensure that equilibrium had been attained. The percent increase and decrease in given phosphate resonances were used to adjust the reactant concentrations. The concentrations of MgADP and MgATP were recalculated, and the amount of MgPSP was assumed to be insignificant. The equilibrium constant calculated in this manner is $1 \pm 0.5 \times 10^2$ at pH 6.0.

The maximum velocities in the forward and reverse directions were determined using a single cAK stock solution while keeping constant the ratio of the reactant concentrations. In the direction of SP phosphorylation, the ratio SP/MgATP was kept constant and V was determined from the $1/v$ vs 1/SP plot by extrapolation to infinite SP. In the direction of MgADP phosphorylation, PSP/MgADP was kept constant and V was determined from the $1/v$ vs 1/PSP plot.

DISCUSSION

Kinetic Mechanism in the Direction of MgADP Phosphorylation. Occupancy of the second metal binding site on the C-subunit by Mg^{2+} or Mn^{2+} results in an increase in 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 affinity is obtained when the Mg^{2+} bound at the second site coordinates the α- and γ- (and perhaps also the β-) phosphates of MgATP as well as residues on the enzyme. Therefore we performed steady-state kinetic analyses at different fixed levels of Mg_f, from 0.5 to 10 mM.

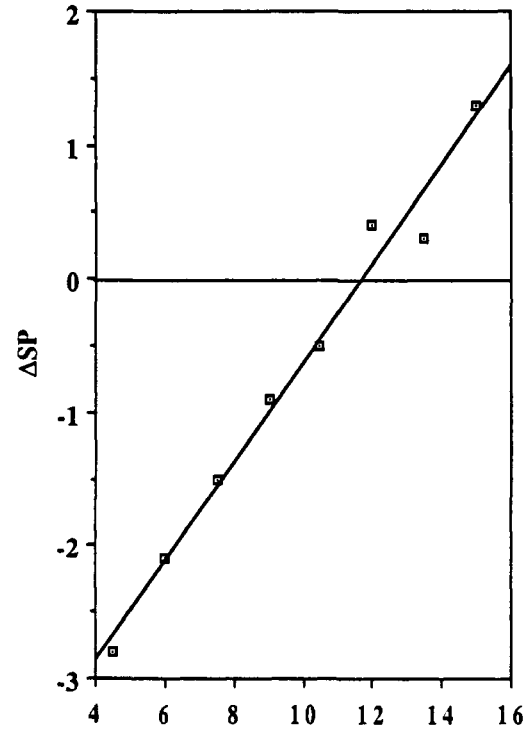


FIGURE 2: Equilibrium constant for C-subunit. The PSP/SP ratio was varied at a constant MgADP/MgATP ratio at pH 6. Correction was made for the concentrations of chelate complexes as described under Materials and Methods.

At 0.5 and 1 mM Mg_f, initial velocity patterns intersect to the left of the ordinate, indicative of a sequential kinetic mechanism, in agreement with the kinetic mechanism proposed in the direction of SP phosphorylation (Cook et al., 1982; Whitehouse et al., 1983; Whitehouse & Walsh, 1983). However, at 2, 5, and 10 mM Mg_f, the patterns intersect on the ordinate when $1/v$ is plotted vs 1/PSP. The latter is diagnostic for an equilibrium-ordered mechanism where MgADP must bind to the enzyme prior to PSP. The kinetic mechanism observed at low Mg_f could be either random or ordered. The dead-end inhibition patterns allow a distinction to be made between these two possibilities. The uncompetitive inhibition pattern obtained with MgAMPCP vs PSP is diagnostic for an ordered mechanism in which PSP adds prior

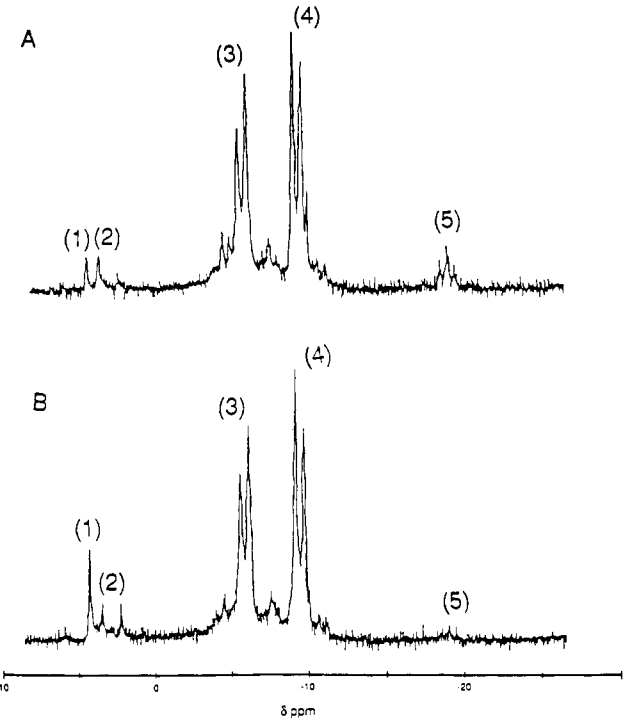
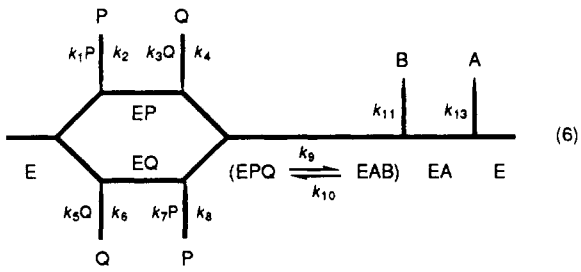


FIGURE 3: Fourier transform ^{31}P NMR of the C-subunit equilibrium mixture. (Panel A): Reaction mixture minus enzyme. The initial total concentration of reactants is as follows: Mg^{2+}, 30 mM; PSP, 10 mM; ATP, 7.7 mM; ADP, 281.2 mM; Mes (pH 6), 100 mM; and 30% D_2O. (Panel B): Reaction mixture 2 h after the addition of 3 units of C-subunit. Probe temperature was 28 °C. Enzyme activity was redetermined after the spectrum was taken with no change in activity. Both spectra are the result of 100 scans with proton decoupling. Chemical shifts were referenced to 85% H_3PO_4 as external standard. The resonances indicated are as follows: (1) PSP; (2) inorganic phosphate; (3) γ-phosphate of ATP and β-phosphate of ADP; (4) α-phosphate of ATP and ADP; and (5) β-phosphate of ATP.

to MgADP. This mechanism is supported by the noncompetitive pattern obtained with DP vs MgADP (Cleland, 1977). These data suggest that the overall kinetic mechanism is random with both pathways allowed, i.e., the pathway in which MgADP binds to enzyme prior to PSP and the pathway in which PSP binds to enzyme prior to MgADP. In addition, it appears that, depending on the concentration of Mg_f, one or the other pathway predominates, also in agreement with previous studies (Kong & Cook, 1988).

Consider the following mechanism:



(6)

where A, B, P, and Q represent MgATP, SP, PSP, and MgADP, respectively. At low Mg_f, the top pathway in mechanism 6 predominates, while at high Mg_f the bottom pathway predominates. Based on the data shown in Table I, it is apparent that increasing the concentration of Mg_f increases the enzyme's affinity for MgADP. In order for the kinetic mechanism to change from steady-state ordered with PSP binding first to equilibrium ordered with MgADP adding first, the binding of MgADP to free enzyme must come to equilibrium with the enzyme at high Mg_f, i.e., the off-rate for MgADP must be much greater than the overall net rate for conversion of E·MgADP to products. Thus, an apparent dilemma must be explained, i.e., the affinity for MgADP increases as Mg_f concentration increases, yet the off-rate for MgADP must exceed the net rate of product formation. In order to reconcile these results, Mg_f must have other effects.

On the basis of mechanism 6, the following expressions represent the kinetic parameters V and V/K for reactants for the two pathways, i.e., PSP adding first or MgADP adding first.

PSP adds prior to MgADP:

$$V/K_{MgADP}E_t = k_9/[K_{IMgADP}(1 + k_9/k_4 + k_{10}/k_{11})] \quad (7)$$

$$V/K_{PSP}E_t = k_1 \quad (8)$$

$$V/E_t = k_9/[1 + k_9(1/k_{11} + 1/k_{13}) + k_{10}/k_{11}] \quad (9)$$

MgADP adds first:

$$V/K_{PSP}E_t = k_9/[K_{IPSP}(1 + k_9/k_8 + k_{10}/k_{11})] \quad (10)$$

$$V/K_{MgADP}E_t = (k_5k_7k_9P/k_6k_8)/(1 + k_9/k_8[1 + k_7P/k_6] + k_{10}/k_{11}) \quad (11)$$

The expression for V is independent of the pathway used to attain EPQ and, thus, is identical for both pathways. In the above expressions, K_{IMgADP} and K_{IPSP} are dissociation constants from the ternary E·MgADP·PSP complex. In order for the mechanism to become equilibrium ordered at high concentrations of Mg_f, the off-rate for MgADP from the E·MgADP complex (k_6) must become much greater than $V/K_{PSP}E_t$. Since k_6 is decreasing as Mg_f increases (see changes in K_{iMgADP} in Table I), another term in the denominator of the expression for V/K_{PSP} must be increasing in order to decrease this macroscopic rate constant or k_9 must decrease at high Mg_f. The latter is unlikely since it has been shown that all of the effects of Mg_f can be explained in terms of an increase in the affinity for MgATP and MgADP (Kong & Cook, 1988). In addition, the term k_{10}/k_{11} is present in all of the above expressions and affects all those parameters equally, making an increase in this term unlikely. Finally, the affinity of the enzyme for PSP has decreased considerably compared to SP, making it unlikely that the former is sticky, i.e., k_9/k_8 is likely near zero. It is expected that the most probable effect of increasing Mg_f is an increase in K_{IPSP}, that is, a decrease in the affinity for PSP in the E·MgADP·PSP·Mg complex. In support of this, the affinity for PSP in the E·PSP·Mg complex decreases drastically as the Mg_f concentration increases only slightly (see K_{iPSP} in Table I). Thus, even though k_6, the off-rate for MgADP, is decreasing as the concentration of Mg_f increases, the affinity for PSP binding to enzyme decreases even more, resulting in a change from steady-state-ordered addition of PSP prior to MgADP to equilibrium-ordered addition of MgADP prior to PSP. These data corroborate the suggested steady-state random mechanism in the direction of SP phosphorylation (Cook et al., 1982; Kong & Cook, 1988), but with increasing concentration of Mg_f resulting in a preference for the pathway in which MgATP adds first and MgADP is released last from the enzyme.

In the direction of MgADP phosphorylation, an increase in the affinity of enzyme for MgADP is reflected in the observed decrease in K_{iMgADP} from 38 to 2 μM as Mg_f is increased from 0.5 to 10 mM Mg_f. The decrease is even more pronounced when the values obtained for the same enzyme form are compared. From 2 to 10 mM Mg_f (E·MgADP

complex), a pronounced trend is observed, i.e, K_{iMgADP} decreases from 50 to 2 μM. This decrease in K_{iMgADP} is similar to the decrease in K_{MgATP} observed by Cook et al. (1982) in the direction of SP phosphorylation. The decrease in K_{iMgADP} is probably due to a decrease in the off-rate for MgADP since the decrease in K_{MgATP} was shown by Kong and Cook (1988) to be due to a decrease in the off-rate for MgATP. These authors showed that the off-rate for MgATP from E·MgATP decreased from 14 s^{-1} at 0.5 mM Mg_f to 0.3 s^{-1} at 10 mM Mg_f. Likewise, the decrease reported here in V/E_t from 4.2 s^{-1} at 0.5 mM Mg_f to 1.9 s^{-1} at 10 mM Mg_f is probably due to a decrease in the off-rate for MgATP (Kong & Cook, 1988), that is, at the higher Mg_f concentration, the release of MgATP becomes the rate-limiting step whereas, at the lower Mg_f, some step other than the release of MgATP is rate-limiting. If this is true, one expects V/E_t to become equal to the off-rate of 0.3 s^{-1} for MgATP observed by Kong and Cook (1988) at 10 mM Mg_f. If the values determined for V/E_t at 0.5, 1, 2, 5, and 10 mM Mg_f reported above are fitted to a hyperbolic function, the value obtained for V/E_t at an infinite concentration of Mg_f is 1 ± 0.7 s^{-1}, which is within the confidence limits of the value reported by Kong and Cook (1988).

Cook et al. (1982) reported that in the presence of 100 mM KCl, the binding constants for MgATP and MgADP increased 2–4-fold at low Mg_f concentrations. This increase was not considered to be due to competitive inhibition by Cl^-, but was interpreted as indicative of some rearrangement of tertiary structure in the vicinity of the nucleotide binding site due to the increase in ionic strength (Cook et al., 1982). A steady-state kinetic analysis was performed in the studies reported here in the absence or presence of 100 mM KCl at 0.5 and 10 mM Mg_f. At 10 mM Mg_f, there is no change in the kinetic mechanism or parameters as a function of KCl concentration, i.e., Mg^{2+} appears to override the KCl effect. This lack of any change due to KCl at 10 mM Mg_f is consistent with the observation of Cook et al. (1982). However, at 0.5 mM Mg_f, K_{MgADP} increases from 30 μM in the absence of KCl to 85 μM in the presence of KCl. This increase of K_{MgADP} in the presence of KCl is consistent with the 2–4-fold increase in the binding constants for MgATP and MgADP observed by Cook et al. (1982) and can be accounted for by an increase in the off-rate for MgADP in the presence of KCl. At 0.5 mM Mg_f, a change is also observed in V/E_t, which increases from 1.7 s^{-1} in the absence of KCl to 4.2 s^{-1} in the presence of KCl. This increase in V/E_t may be due to an increase in the off-rate for MgATP in the presence of KCl, which is consistent with the increase in K_{MgATP} observed by Cook et al. (1982) in the presence of KCl. Thus, the off-rate for MgATP becomes the rate-limiting step as discussed previously for the Mg_f effect. At 0.5 mM Mg_f, K_{iPSP} increases from 1 mM in the presence of 100 mM KCl to about 4 mM in the absence of KCl. The increase in affinity for PSP again likely reflects an effect of KCl decreasing the affinity for MgADP, resulting in an increase in the affinity for PSP as discussed above for change in the kinetic mechanism with Mg_f concentration.

Dead-End Inhibition Studies. To confirm the kinetic mechanism of the C-subunit, dead-end inhibition studies were performed. In order to show that the inhibiton data are quantitatively internally consistent and thus support the qualitative interpretation suggested above, the observed K_i values were corrected for the concentration of the fixed reactant to give an estimate of the true K_i values. The apparent K_i value, obtained from the uncompetitive inhibition of MgAMPCP vs PSP, can be corrected using the equation app $K_{ii} = K_i(1 + MgADP/K_{MgADP})$, giving a true $K_{iMgAMPCP}$ of 1.4 mM. This can be compared to a value of 0.9 mM obtained from the competitive inhibition pattern vs MgADP, in which app K_{is} has been corrected using the expression $K_i(1 + K_{iPSP}/PSP)$. Similarly, the K_i values estimated from the noncompetitive inhibition of DP vs MgADP, using app $K_{is} = K_i(1 + PSP/K_{iPSP})$ and app $K_{ii} = K_i(1 + PSP/K_{PSP})$, give corrected K_i values of 1.9 and 9.8 mM, respectively. The first of these is in agreement with the K_{iDP} of 2.9 mM obtained from the DP vs PSP inhibition. The latter is not as close but has a large standard error.

Equilibrium Constant. The equilibrium constant for the reaction catalyzed by the C-subunit using SP as a substrate has been determined using two different methods: (1) varying the SP/PSP ratio at a fixed MgATP/MgADP ratio and measuring the change in SP concentration after equilibrium is reached and (2) measuring intensity changes via ^{31}P NMR starting with different initial reactant concentrations. The K_{eq} values obtained from these methods at pH 6 are 220 and 100, giving values of 3500 and 1600 at pH 7.2. When kinetic data are correctly determined, kinetic parameters will adhere to the Haldane relationship. A kinetic Haldane relationship for a bi–bi mechanism (Cleland, 1982) is as follows:

$$K_{eq} = (V_f)(K_{MgADP})(K_{iPSP})/(V_r)(K_{SP})(K_{iMgATP}) \quad (12)$$

Substitution of the different values in eq 12 gives an estimate for K_{eq} of 2 ± 1 × 10^3 at pH 7.2, in good agreement with the directly determined K_{eq} values given above.

Conclusions. The data presented above collectively suggest that MgADP will in general bind to the enzyme before the addition of PSP can take place. However, at lower concentrations of Mg_f, when the second metal site is empty, the affinity of the enzyme for MgADP is significantly lower than when the site is occupied. At low Mg_f, when PSP attempts to bind the enzyme in the presence of MgADP, either (1) there is a significant amount of charge repulsion because PSP bears two additional negative charges to the active site or (2) the conformation of the site does not accommodate PSP well. Thus, in the absence of MgADP at the active site, PSP binds with higher affinity. At high Mg_f, the affinity of the enzyme for MgADP increases about 25-fold. This tighter binding of MgADP is due to the second Mg^{2+} site now being filled, allowing coordination of the α- and β-phosphate groups of MgADP by the second metal. The Mg^{2+} occupying the second site thus either neutralizes any charge repulsion from MgADP and/or enzyme that PSP would have to encounter or molds the site to accommodate PSP more easily or both.

In their crystallographic study of the C-subunit, Knighton et al. (1991) found that the γ-phosphate of MgATP was close to Asp^{184} and concluded that this invariant residue is probably involved in chelation of Mg^{2+} in the complex of MgATP with the enzyme. Perhaps it is the case that Asp^{184} chelates the second Mg^{2+}; that is, when Asp^{184} is not coordinated to Mg^{2+}, there may be electrostatic repulsion between the Asp^{184} carboxylate and the β-phosphoryl group of MgADP. Once Asp^{184} binds Mg^{2+}, its negative charge will be sequestered from the catalytic site, and the repulsive force will no longer be present, and this will allow MgADP to bind with higher affinity. Knighton et al. (1991) also postulate that, once Asp^{184} is sequestered from the catalytic loop, other invariant residues such as Lys^{72} and Glu^{91} rearrange in order to maximize the nucleophilicity of the SP serine hydroxyl moiety that is poised to attack the γ-phosphoryl of MgATP. Based on the present studies, we now further suggest that Glu^{91} may add to the repulsion of the PSP phosphate and hence the low affinity for PSP. The effect of KCl in increasing K_{MgADP} and V/E_t can

be explained by KCl decreasing the affinity for the nucleotides as discussed previously (Cook et al., 1982). The decrease in affinity of the enzyme for MgADP results in an increase in affinity for PSP as discussed above. The weak effect of KCl is effectively eliminated as Mg_f stabilizes the enzyme form that has higher affinity for the nucleotide reactants.

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REFERENCES

Armstrong, R. N., Kondo, H., Granot, J., Kaiser, E. T., & Mildvan, A. S. (1979) *Biochemistry 18*, 1230.

Bradford, M. M. (1976) *Anal. Biochem. 72*, 248.

Bramson, H. N., Mildvan, A. S., & Kaiser, E. T. (1984) *CRC Crit. Rev. Biochem. 15*, 93.

Cleland, W. W. (1977) *Adv. Enzymol. 45*, 273.

Cleland, W. W. (1979) *Methods Enzymol. 63*, 103.

Cleland, W. W. (1982) *Methods Enzymol. 87*, 366.

Cook, P. F., Neville, M. E., Jr., Vrana, K. E., Hartl, F. T., & Roskoski, R., Jr. (1982) *Biochemistry 21*, 5794.

Dawson, R. M. C., Elliot, D. C., Elliott, W. H., & Jones, K. M. (1969) *Data for Biochemical Research*, 2nd ed., Oxford University Press, London.

Gutte, B., & Merrifield, R. B. (1969) *J. Am. Chem. Soc. 91*, 501.

Knighton, D. R., Zheng, J., Ten Eyck, L. F., Ashford, V. A., Xuong, N.-h., Taylor, S. S., & Sowadski, J. M. (1991) *Science 253*, 407.

Kong, C.-T., & Cook, P. F. (1988) *Biochemistry 27*, 4795.

Martell, A. E., & Smith, R. M. (1979) *Critical Stability Constants*, Vol. 3, Plenum Press, New York.

Olsen, S. R., & Uhler, M. D. (1989) *J. Biol. Chem. 264*, 18662.

Plapp, B. V., Moore, S., & Stein, W. H. (1971) *J. Biol. Chem. 246*, 939.

Sugden, P. H., Holladay, L. A., Reimann, E. M., & Corbin, J. D. (1976) *Biochem. J. 159*, 409.

Taylor, S. S., Buechler, J. A., & Yonemoto, W. (1990) *Annu. Rev. Biochem. 59*, 971.

Whitehouse, S., & Walsh, D. A. (1983) *J. Biol. Chem. 258*, 3682.

Whitehouse, S., Feramisco, J. R., Casnellie, J. E., Krebs, E. G., & Walsh, D. A. (1983) *J. Biol. Chem. 258*, 3693.