# Isotope Partitioning in the Adenosine 3′,5′-Monophosphate Dependent Protein Kinase Reaction Indicates a Steady-State Random Kinetic Mechanism†

Cheng-Te Kong and Paul F. Cook*

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

*Received September 21, 1987; Revised Manuscript Received January 22, 1988*

ABSTRACT: Isotope partitioning beginning with the binary E·MgATP and E·*N*-acetyl-Leu-Arg-Arg-Ala-Ser-Leu-Gly (Ser-peptide) complexes indicates that the kinetic mechanism for the adenosine 3′,5′-monophosphate dependent protein kinase is steady-state random. A total of 100% of the initial radioactive E·MgATP complex is trapped as phospho-Ser-peptide at infinite Ser-peptide concentration at both low and high concentration of uncomplexed $Mg^{2+}$, suggesting that the off-rate of MgATP from the E·MgATP·Ser-peptide complex is slow relative to the catalytic steps. $K_m$ for Ser-peptide in the trapping reaction decreases from 17 μM at low $Mg^{2+}$ to 2 μM at high $Mg^{2+}$, indicating that $Mg^{2+}$ decreases the off-rate for MgATP from the E·MgATP complex. A total of 100% of the radioactive E·Ser-peptide complex is trapped as phospho-Ser-peptide at low $Mg^{2+}$, but only 40% is trapped at high $Mg^{2+}$ in the presence of an infinite concentration of MgATP, suggesting that the off-rate for Ser-peptide from the central complex is much less than catalysis at low but not at high $Mg^{2+}$. In support of this finding, the $K_i$ for Leu-Arg-Arg-Ala-Ala-Leu-Gly (Ala-peptide) increases from 0.27 mM at low $Mg^{2+}$ to 2.4 mM at high $Mg^{2+}$. No trapping was observed at either high or low $Mg^{2+}$ for the E·MgADP complex up to a phospho-Ser-peptide concentration of 5 mM. Thus, it is likely that in the slow-reaction direction the kinetic mechanism is rapid equilibrium. Finally, no substrate inhibition was observed by Ser-peptide at any concentration of $Mg^{2+}$ or MgATP when the coupled spectrophotometric assay with pyruvate kinase and lactate dehydrogenase was used.

The catalytic subunit of adenosine 3′,5′-monophosphate (cAMP) dependent protein kinase catalyzes the phosphorylation of a variety of proteins according to eq 1, with phosphorylation usually resulting in a change in the physiological activity of the protein.

$$\text{MgATP} + \text{protein} \rightarrow \text{phosphoprotein} + \text{MgADP} \quad (1)$$

The specific amino acid sequence representing the phosphorylation site of the protein substrate has been extensively studied (Zetterquist et al., 1976; Kemp et al., 1977; Feramisco et al., 1979, 1980; Kemp, 1980), and a number of peptides have been shown to serve as phosphoryl acceptor. Generally, one or two positively charged amino acids are required N-terminal to the serine (or threonine) to be phosphorylated, followed by a small nonpolar amino acid residue with a second small nonpolar amino acid C-terminal to the serine. The best peptide substrate found for the kinase is Leu-Arg-Arg-Ala-Ser-Leu-Gly (Hjelmquist et al., 1974).

The kinetic mechanism has been suggested by Cook et al. (1982) to be steady-state random in the direction of Ser-peptide phosphorylation with a dead-end E·MgADP·Ser-peptide product complex and with ordered release of phosphopeptide and MgADP. An alternative mechanism (Whitehouse & Walsh, 1983; Whitehouse et al., 1983) in which an ordered addition of MgATP and peptide followed by an ordered release of phosphopeptide and MgADP has been proposed. In the latter mechanism, the authors suggest the presence of a dead-end E·Ser-peptide complex.

In this study, isotope partitioning and substrate inhibition were used to investigate and distinguish between the possible kinetic mechanisms proposed for the cAMP-dependent protein kinase catalytic subunit from bovine heart. Data are consistent with a steady-state random mechanism at both low and high $Mg^{2+}$ concentrations with the pathway in which MgATP adds to the enzyme first preferred.

## Materials and Methods

*Chemicals and Enzymes.* Phosphoenolpyruvate, NADH, ATP, ADP, Mops,[1] and SP-Sephadex were from Sigma. [$\gamma$-$^{32}$P]ATP was a generous gift of Dr. R. A. Masaracchia of the Department of Biochemistry, Texas College of Osteopathic Medicine. [$^3$H]Acetic anhydride was obtained from Du Pont and [2-$^3$H]ADP was obtained from Amersham. Aqua Mix was obtained from ICN Radiochemicals. The liquid scintillation system was from Searle Analytic, Model 6892.

The peptides were prepared with a Biosearch SAM II peptide synthesizer. The t-Boc amino acids and resin with t-Boc-glycine attached (0.28–0.32 mmol/g of resin) were obtained from Biosearch. Methylene chloride (HPLC grade) and acetonitrile (HPLC grade) were from Mallinckrodt. Diisopropylethylamine, diisopropylcarbodiimide, anisole, and acetylimidazole were from Aldrich. Dimethylformamide was obtained from J. T. Baker. Trifluoroacetic acid was either from Pierce or from Advanced ChemTech. Synthesis made use of a 6.7 molar excess of t-Boc amino acid over resin. After

† This work was supported by NIH Grant GM 37057, Grant B-1031 from the Robert A. Welch Foundation, and Grant BRSG S07 RR 07195 awarded by the Biomedical Research Grant Program, Division of Research Resources, National Institutes of Health. P.F.C. is the recipient of NIH Research Career Development Award AM 01155 and a research fellowship from the Alexander von Humboldt Stiftung, Bonn, West Germany (1987).

[1] Abbreviations: Mops, 3-(*N*-morpholino)propanesulfonic acid; t-Boc, *tert*-butyloxycarbonyl; Taps, 3-[[tris(hydroxymethyl)methyl]amino]-propanesulfonic acid; Ser-peptide, Leu-Arg-Arg-Ala-Ser-Leu-Gly; Ala-peptide, Leu-Arg-Arg-Ala-Ala-Leu-Gly; PEP, phosphoenolpyruvate; cAPK, adenosine 3′,5′-monophosphate dependent protein kinase catalytic subunit; $Mg_f$, uncomplexed form of $Mg^{2+}$; NADH, reduced nicotinamide adenine dinucleotide; HPLC, high-performance liquid chromatography; TFA, trifluoroacetic acid; NOE, nuclear Overhauser effect.

0006-2960/88/0427-4795$01.50/0 

synthesis, the peptide was cleaved from the resin and deblocked by a Peninsula Laboratories HF cleavage apparatus with the hydrogen fluoride gas from Alphagaz. The peptide was then purified with a Bio-Rad HPLC system and a Hi-Pore 318 Prep (250 × 21.5 mm) reverse-phase column, developed with a linear gradient of 0–50% acetonitrile in 0.1% TFA. The amino acid composition was consistent with the peptide sequence. The Altex Ultrasil-AX HPLC column was from Rainin.

*Enzymes.* Pig heart lactate dehydrogenase and rabbit muscle pyruvate kinase were from Boehringer Mannheim Biochemicals. The catalytic subunit of cAMP-dependent protein kinase (cAPK) was purified according to the method of Sugden et al. (1976). The enzyme had a final specific activity of 25–30 units/mg of protein obtained with 0.5 mM uncomplexed $Mg^{2+}$. The protein concentration was determined by the method of Bradford (1976) with bovine serum albumin as a standard.

*Enzyme Activity Determination.* The cAPK activity in the direction of peptide phosphorylation was determined by coupling the production of MgADP to 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, 0.2 mM NADH, 2 mM ATP, 2.75 mM $MgCl_2$, 0.2 mM Ser-peptide, 18 units of lactate dehydrogenase, 17 units of pyruvate kinase, and variable amounts of cAPK. The reaction was initiated by the addition of cAPK after sufficient data were collected to determine the background rate in the absence of cAPK. The background rate was subtracted from the plus enzyme rate to obtain the initial velocity. Under all conditions, the rate was a linear function of enzyme concentration. Data were collected on a Gilford 260 spectrophotometer equipped with strip chart recorder. The temperature was maintained at 25 °C by a circulating water bath with the capacity to heat and cool the thermospacers of the cell compartment. All substrate concentrations were corrected for the concentration of the metal chelate complexes with $Mg^{2+}$ as discussed previously (Cook et al., 1982) by using the following values for dissociation constants: MgPEP, 5 mM; MgNADH, 19.5 mM; MgATP, 0.0143 mM; Mg-Ser-peptide, 316 mM (Dawson et al., 1979; Martell & Smith, 1982). The stability constant for Mg-Ala-peptide was assumed equal to that of Mg-Ser-peptide.

*Initial Velocity Studies.* Assays to determine whether substrate inhibition was observed were carried out as discussed above with uncomplexed $Mg^{2+}$ at 0.5 or 10 mM concentration. At 10 mM uncomplexed $Mg^{2+}$, MgATP was maintained either at 20 times its $K_m$ (0.4 mM) or at 2 times its $K_m$ (0.04 mM), and Ser-peptide was varied from 0.02 to 5 mM with 0.008 unit of cAPK per assay. At 0.5 mM uncomplexed $Mg^{2+}$, the MgATP was maintained either at 20 times its $K_m$ (4 mM) or at its $K_m$ (0.2 mM), and Ser-peptide was varied from 0.02 to 3 mM with 0.014 unit of cAPK per assay.

For Ala-peptide inhibition, assays were carried out at 10 mM uncomplexed $Mg^{2+}$ as previously described. The concentration of MgATP was maintained saturating (1 mM), and Ser-peptide was varied around its $K_m$ at Ala-peptide concentrations of 0, 2, 4, and 8 mM.

*Isotope Partitioning Studies.* Isotope partitioning studies were performed according to Rose et al. (1974). Three enzyme–substrate complexes (E·MgATP, E·Ser-peptide, and E·MgADP) were tested for isotope trapping.

E·MgATP trapping experiments were carried out at both 0.5 and 10 mM $Mg_f$. At low $Mg^{2+}$ concentration, the 0.05-mL pulse solution contained 210 μg of cAPK (0.1 mM), 0.2 mM [$\gamma$-$^{32}$P]ATP (specific activity 195 cpm/pmol), 0.7 mM $MgCl_2$, and 20 mM Mops, pH 7. This solution was added to a vigorously stirred 5-mL chase solution containing 100 mM Mops, pH 7, 3.2 mM ATP, and 3.7 mM $MgCl_2$, with a Ser-peptide concentration of 10 μM. The experiment was then repeated at Ser-peptide concentrations of 50 and 200 μM. The above experiment was then carried out with 10 mM $Mg_f$. In this case the pulse solution contained 105 μg of cAPK (0.05 mM), 0.1 mM [$\gamma$-$^{32}$P]ATP (specific activity 146 cpm/pmol), 10.1 mM $MgCl_2$, and 20 mM Mops, pH 7, while the chase solution contained 100 mM Mops, pH 7, 2 mM ATP, and 12 mM $MgCl_2$, with Ser-peptide concentrations of 1.19, 8.3, and 11.86 μM. In each case, the reaction was terminated by adding acetic acid to a final concentration of 30%, 2 s after the pulse solution was mixed with the chase solution. The resulting [$^{32}$P]phosphopeptide was detected by scintillation counting after being spotted on phosphocellulose paper and washed according to de la Houssaye and Masaracchia (1983).

The E·Ser-peptide trapping experiments were carried out at 0.5 and 10 mM $Mg_f$. At low uncomplexed $Mg^{2+}$ concentration, the 0.05-mL pulse solution contained 210 μg of cAPK (0.1 mM), 0.5 mM [$^3$H]acetyl-Ser-peptide (specific activity 21.33 cpm/pmol), 0.5 mM $MgCl_2$, and 20 mM Mops, pH 7. [$^3$H]Acetyl-Ser-peptide was prepared according to Riordan and Vallee (1967). The chase solution contained 100 mM Mops, pH 7, 1.5 mM Ser-peptide, and a MgATP concentration of 0.1 mM. The experiment was then repeated at MgATP concentrations of 0.4, 0.7, and 1 mM. After termination of the reaction as above, the reaction mixture was lyophilized and dissolved in 5 mL of 20 mM Taps, pH 9. A 0.5-mL aliquot of this solution was applied to a SP-Sephadex column (Bio-Rad Econo-Column), preequilibrated with 20 mM Taps, pH 9, and eluted with a 50-mL linear gradient of 0–0.5 M NaCl, collecting fractions of 0.9 mL. An aliquot of 0.5 mL of each fraction was counted for radioactivity. The above experiment was then repeated at 10 mM $Mg_f$. In this case the pulse solution contained 210 μg of cAPK (0.1 mM), 0.5 mM [$^3$H]acetyl-Ser-peptide (specific activity 21.33 cpm/pmol), 10 mM $MgCl_2$, and 20 mM Mops, pH 7, while the chase solution contained 100 mM Mops, pH 7, and 1.5 mM Ser-peptide with MgATP concentrations of 0.1, 0.2, 0.4, and 1 mM at a $Mg_f$ concentration of 10 mM.

E·MgADP trapping experiments were carried out at 0.5 and 10 mM $Mg_f$. At low $Mg^{2+}$, the 0.05-mL pulse contained 210 μg of cAPK (0.1 mM), 0.2 mM [2-$^3$H]ADP (specific activity 30 cpm/pmol), 0.7 mM $MgCl_2$, and 20 mM Mops, pH 7. The chase solution contained 100 mM Mops, pH 7, 1 mM ADP, and 1.5 mM $MgCl_2$, and phospho-Ser-peptide was varied from 1 to 5 mM. The phospho-Ser-peptide was prepared from Ser-peptide enzymatically by cAPK reaction and purified as above for E·Ser-peptide trapping. After termination of the reaction as above, the reaction mixture was lyophilized and dissolved in 5 mL of 10 mM phosphate buffer, pH 4.5. An aliquot of 0.2 mL was applied to an Altex Ultrasil-AX HPLC column (Rainin) and eluted with a linear gradient of 0.3–1 M KCl. Fractions of 1.5 mL were collected, and a 0.5-mL aliquot of each fraction was counted for radioactivity. The experiment was then repeated at 10 mM $Mg_f$. In this case the pulse solution contained 210 μg of cAPK (0.1 mM), 0.2 mM [2-$^3$H]ADP (specific activity 30 cpm/pmol), 10.2 mM $MgCl_2$, and 20 mM Mops, pH 7, while the chase solution contained 100 mM Mops, pH 7, 1 mM ADP, and 11 mM $MgCl_2$, and phospho-Ser-peptide was varied from 1 to 5 mM.

In each of the above cases a control was run in which the same amount of radiolabeled substrate in the pulse solution

was added to the chase solution and the reaction started by adding the same amount of enzyme in a 0.05-mL pulse. All the reactions were performed at 25 °C.

*Data Processing.* Reciprocal initial velocities (or amount of radioactive product formed) were plotted against reciprocal substrate concentration, and all plots were linear. Data were fitted according to the appropriate rate equation and FORTRAN programs developed by Cleland (1979). Data for isotope partitioning were fitted by using eq 2. Data for competitive inhibition studies with Ala-peptide were fitted by using eq 3.

$$P^* = P^*_{max}A/(K'_a + A) \quad (2)$$

$$v = VA/[K_a(1 + I/K_i) + A] \quad (3)$$

In eq 2 and 3, $P^*_{max}$ is the maximum amount of trapping, $P^*$ is the amount of trapping at a giving concentration of $A$, $K'_a$ is the concentration of substrate in the chase solution that gives half the maximum amount of trapping ($K_m$ for trapping), $K_a$ is the $K_m$ for $A$, $V$ is the maximum velocity, $A$ and $I$ are the concentrations of substrate and inhibitor, respectively, and $K_i$ is the inhibition constant for $I$.

## Results

*Initial Velocity Studies.* Substrate inhibition of the cAPK reaction by Ser-peptide has been reported by Cook et al. (1982) and Whitehouse et al. (1983). In both of these studies, a filter-binding fixed-time assay was used. Plots of initial velocity obtained by using the coupled spectrophotometric assay vs Ser-peptide concentration at saturating MgATP and either 0.5 or 10 mM $Mg_f$ give no indication of substrate inhibition. In addition, at 0.5 mM $Mg_f$ with MgATP equal to its $K_m$ or at 10 mM $Mg_f$ with MgATP equal to 2 times its $K_m$, no substrate inhibition is observed.

Inhibition by Ala-peptide vs Ser-peptide at 0.5 mM $Mg_f$ and saturating MgATP is competitive with a $K_i$ of 270 $\mu$M (Yoon & Cook, 1987). The inhibition by Ala-peptide vs Ser-peptide at 10 mM $Mg_f$ is also competitive with a $K_i$ for Ala-peptide of 2.4 ± 0.4 mM at pH 7.0.

*Isotope Partitioning Studies.* Three binary enzyme–substrate complexes (E·MgATP, E·Ser-peptide, and E·MgADP) were used for isotope partitioning studies, and each of these is discussed below.

*E·MgATP Partitioning.* On the basis of a $K_d$ of 160 $\mu$M (Cook et al., 1982) for E·MgATP, a pulse solution containing 2430 pmol of E·[$\gamma$-$^{32}$P]MgATP was prepared for isotope partitioning studies at 0.5 mM $Mg_f$ (also contained in the pulse solution). At 10 mM $Mg_f$ concentration, on the basis of a $K_d$ of 0.01 mM (Armstrong et al., 1979a,b), the pulse solution contained 2130 pmol of E·[$\gamma$-$^{32}$P]MgATP. Each of the above was subjected to isotope partitioning at variable levels of Ser-peptide contained in the chase solution. In both cases, radiolabeled phosphopeptide was produced, but note that at the higher $Mg_f$ concentration the range of Ser-peptide concentrations used to trap E·[$\gamma$-$^{32}$P]MgATP is less by a factor of 10.

For both $Mg_f$ concentrations, ca. 100% of the initial radiolabeled binary complex is trapped at infinite concentration of Ser-peptide in the chase solution. Kinetic parameters for the trapping reaction are shown in Table I.

*E·Ser-peptide Partitioning.* On the SP-Sephadex column, phospho-Ser-peptide was eluted approximately between fractions 15 and 20, whereas Ser-peptide was eluted between fractions 25 and 30. The amount of E·[$^3$H]acetyl-Ser-peptide in the pulse solution was 3200 pmol at both 0.5 and 10 mM $Mg_f$, calculated with a $K_d$ of 0.25 mM (Feramisco & Krebs, 1978). The pulse solutions were used in isotope partitioning experiments with varied levels of MgATP in the chase solution. In both cases, radiolabeled phosphopeptide was produced. With 0.5 mM $Mg_f$ apparently 100% of the initial radiolabeled binary complex was trapped at infinite concentration of MgATP, while with 10 mM $Mg_f$ only 40% was trapped. Kinetic parameters are shown in Table I.

Table I: Summary of Kinetic Constants from Isotope Partitioning

| parameter[a] | $Mg_f$ 0.5 mM | $Mg_f$ 10 mM |
|---|---|---|
| E·[$\gamma$-$^{32}$P]MgATP Trapping | | |
| EA* (pmol) | 2430 | 2130 |
| $P^*_{max}$ (pmol) | 2427 ± 53 | 2115 ± 100 |
| $K_{Ser\text{-}peptide}$ ($\mu$M) | 17 ± 2 | 2.0 ± 0.8 |
| $P^*_{max}$/EA* (%) | 99 ± 2 | 99 ± 4 |
| $k_2$ ($s^{-1}$) | 14 ± 2 | 0.34 ± 0.14 |
| $k_1$ ($M^{-1}$ $s^{-1}$) | (8.75 ± 1.25) × $10^4$ | (2.8 ± 1.3) × $10^4$ |
| E·[$^3$H]Acetyl-Ser-peptide Trapping | | |
| EA* (pmol) | 3200 | 3200 |
| $P^*_{max}$ (pmol) | 3136 ± 67 | 1173 ± 20 |
| $K_{MgATP}$ (mM) | 0.58 ± 0.05 | 0.18 ± 0.01 |
| $P^*_{max}$/EA* (%) | 98 ± 2 | 36.7 ± 0.6 |
| $k_8$ ($s^{-1}$) | 64 ± 30 | |
| $k_7$ ($M^{-1}$ $s^{-1}$) | (2.6 ± 1.2) × $10^5$ | |

[a] EA* is the initial amount of the radiolabeled binary complex present in the pulse solution for each experiment.

*E·MgADP Partitioning.* The amount of E·[2-$^3$H]ADP in the pulse solution was 3600 pmol at 0.5 mM $Mg_f$, on the basis of a $K_d$ of 0.05 mM, and 4722 pmol at 10 mM $Mg_f$, calculated with a $K_d$ of 0.005 mM (Cook et al., 1982). These were subjected to isotope partitioning experiments at variable levels of phospho-Ser-peptide. No trapping of the E·[2-$^3$H]MgADP binary complex was observed at either 10 or 0.5 mM $Mg_f$ up to a phospho-Ser-peptide concentration of 5 mM.

## Discussion

*Substrate Inhibition Studies by Ser-peptide.* Existing studies agree that the kinetic mechanism of cAPK is a sequential mechanism. Cook et al. (1982) proposed that the kinetic mechanism was steady-state random with substrate inhibition by Ser-peptide at a concentration of 0.5 mM as a result of the formation of a E·MgADP·Ser-peptide dead-end complex. Whitehouse et al. (1983) suggested an ordered mechanism in which the nucleotide binds first and also observed substrate inhibition by Ser-peptide at a concentration of 0.1 mM, suggesting formation of an E·Ser-peptide nonproductive binary complex. In both cases in which substrate inhibition was observed, the radioassay with [$\gamma$-$^{32}$P]ATP was used, which makes use of [$^{32}$P]phospho-Ser-peptide binding to phosphocellulose paper. However, both the substrate, Ser-peptide, and the product, [$^{32}$P]phospho-Ser-peptide, will bind to the paper. Thus, at higher concentrations of Ser-peptide, it is likely that the paper becomes saturated, resulting in an underestimate of product produced. This could be the reason why substrate inhibition was observed with the radioassay.

To test whether Ser-peptide exhibits substrate inhibition, the coupled spectrophotometric assay was used. At high $Mg_f$ concentration, no substrate inhibition by Ser-peptide was observed at either saturating or nonsaturating MgATP over the range 20 $\mu$M to 5 mM Ser-peptide. Similar results were obtained at 0.5 mM $Mg_f$ concentration.

Circular dichroic studies (Reed et al., 1985) have shown that binding of the peptide substrate involves a series of conformational changes in the absence of nucleotide. Equilibrium dialysis studies (Whitehouse et al., 1983) have also detected the E·Ser-peptide binary complex. That no substrate inhibition

is observed by Ser-peptide suggests that the E·Ser-peptide complex is a productive binary complex.

*Isotope Partitioning Studies of E·MgATP.* At low $Mg_f$ concentration, a value of 100% trapping is obtained for MgATP, indicating that either MgATP has a high commitment to catalysis once bound or that the mechanism is ordered with MgATP binding before Ser-peptide. Under conditions where 100% trapping occurs, the off-rate of MgATP from the E·MgATP binary complex, $k_2$, can be calculated from the product of $K'_b$ (the $K_m$ for Ser-peptide for trapping) and $V/K_bE_t$ from initial velocity studies (Rose et al., 1974). The $K'_{Ser\text{-}peptide}$ for the trapping reaction is 17 ± 2 μM, whereas the steady-state kinetic parameter $V/E_tK_{Ser\text{-}peptide}$ is (8.5 ± 0.3) × $10^5$ $M^{-1}$ $s^{-1}$ (Yoon & Cook, 1987). Thus $k_2$ is equal to 14 ± 2 $s^{-1}$. The on-rate for MgATP, $k_1$, is obtained from $K_d = k_2/k_1$ in which $K_d$ is 0.16 mM (Cook et al., 1982). A value of (8.75 ± 1.25) × $10^4$ $M^{-1}$ $s^{-1}$ is obtained, which is about 4 orders of magnitude lower than the diffusion limit. These data suggest that the binding of MgATP to the enzyme requires at least two steps. The conformational change accompanying MgATP binding may be reflected in the tight binding of adenosine with a strained torsional glycosylic angle of 78° and puckering of the ribose ring as shown by the NOE studies of Rosevear et al. (1983). In addition, the circular dichroic studies of Reed and Kinzel (1984) using blue dextran also suggest that ATP binding to the enzyme causes a conformational change.

An increase in the concentration of uncomplexed $Mg^{2+}$ is known to increase the affinity for MgATP (Armstrong et al., 1979a; Cook et al., 1982). As a result, the trapping experiments were repeated with 10 mM $Mg_f$ in both pulse and chase solutions. The maximum trapping remains at 100%, indicating that MgATP is still released slowly from the central E·MgATP·Ser-peptide complex compared to product formation, but $K'_{Ser\text{-}peptide}$ for trapping decreases to 2.0 ± 0.8 μM. The value for $k_2$ can again be calculated as above with $V/E_tK_{Ser\text{-}peptide}$ equal to (1.70 ± 0.06) × $10^5$ $M^{-1}$ $s^{-1}$ (Cook et al., 1982), giving an apparent off-rate for MgATP of 0.34 ± 0.14 $s^{-1}$, about 40-fold lower than that obtained at low $Mg^{2+}$ concentration. $K_d$ for MgATP under these conditions has been measured by Armstrong et al. (1979b), using equilibrium dialysis, as 12 μM, allowing calculation of the apparent on-rate as (2.8 ± 1.3) × $10^4$ $M^{-1}$ $s^{-1}$. These data suggest that the increase in affinity in the presence of high concentrations of $Mg^{2+}$ is a result of a decrease in the effective off-rate with little apparent effect on the on-rate. However, even at high $Mg^{2+}$ concentrations, the on-rate is about 4 orders of magnitude lower than the diffusion limit, suggesting a minimum two-step process for MgATP binding independent of the $Mg^{2+}$ concentration.

*Isotope Partitioning Studies of E·Ser-peptide.* At low $Mg^{2+}$, 100% trapping is obtained for the E·Ser-peptide complex indicating tht (1) E·Ser-peptide does form and is a productive binary complex and (2) Ser-peptide is tightly bound in the E·MgATP·Ser-peptide central complex as is also true for MgATP. Thus, the off-rate of Ser-peptide from E·Ser-peptide, $k_8$, can be calculated from the product of $K'_a$ (the $K_m$ for MgATP for the trapping reaction) and $V/E_tK_a$ from initial velocity studies (Rose et al., 1974). $K'_{MgATP}$ for trapping is 0.58 ± 0.05 mM, whereas the kinetic parameter $V/E_tK_{MgATP}$ is (1.1 ± 0.5) × $10^5$ $M^{-1}$ $s^{-1}$ (Yoon & Cook, 1987). Thus, $k_8$ is equal to 64 ± 30 $s^{-1}$. The on-rate for Ser-peptide is thus calculated by using a $K_d$ of 0.25 mM (Feramisco et al., 1979) as (2.6 ± 1.2) × $10^5$ $M^{-1}$ $s^{-1}$. This value is about 3 orders of magnitude lower than the diffusion limit and as for MgATP suggests a minimum two-step process for Ser-peptide binding. Recent experiments (Reed & Kinzel, 1984; Reed et al., 1985) using the circular dichroism of blue dextran bound to the C-subunit suggest that peptide binding occurs in three steps, with the first involving interaction of the Arg-Arg subsite with enzyme resulting in a closed conformation. This closing of the site is followed by a second change that results in the peptide assuming a specific coil conformation, and this is followed by a third change that results in some movement of the serine hydroxyl group.

These trapping experiments were repeated with 10 mM $Mg_f$ in both pulse and chase solutions. The maximum trapping for Ser-peptide decreases to 40%, suggesting that the $Mg_f$ concentration effects the binding of Ser-peptide to the central complex, increasing its off-rate. To test this possibility, $K_i$ for Ala-peptide was measured at saturating MgATP and 10 mM $Mg_f$. $K_i$ for Ala-peptide under these conditions is 2.4 ± 0.4 mM, whereas $K_i$ at 0.5 mM $Mg_f$ is 0.27 ± 0.07 mM (Yoon & Cook, 1987). If one assumes that the on-rate for Ala-peptide remains the same, the off-rate for Ala-peptide and thus presumably also the off-rate for Ser-peptide must increase at high $Mg_f$. Since a $K_d$ of 0.25 mM was used for E·Ser-peptide at 10 mM $Mg_f$, identical with that used for E·Ser-peptide at low $Mg_f$, and $K_d$ is likely higher than this value, the maximum amount of trapping cannot be ascertained since the initial concentration of E·Ser-peptide is not known. $K_d$ for Ser-peptide has been reported as 0.25 mM in the absence and presence of MgAMP-PCP, a nonhydrolyzable analogue of MgATP (Feramisco & Krebs, 1978) but is likely higher than this in the presence of high $Mg_f$ as suggested above. Thus, one has the interesting case in which $K_m$ for Ser-peptide is identical at low and high $Mg_f$ but $K_d$ increases as the $Mg_f$ concentration increases. Consider the minimal mechanism for the kinase at saturating MgATP:

$$EA \underset{k_4}{\overset{k_3B}{\rightleftharpoons}} EAB \underset{k_{14}}{\overset{k_{13}}{\rightleftharpoons}} EPQ \overset{k_9}{\rightarrow} EQ \overset{k_{11}}{\rightarrow} \qquad (4)$$

where A, B, P, and Q are MgATP, Ser-peptide, phospho-Ser-peptide, and MgADP, and $K_b$ is given by eq 5 where $K_{ib}$

$$K_b = \frac{K_{ib}k_{11}(k_{14} + k_9 + k_{13}k_9/k_4)}{k_{11}(k_{14} + k_9) + k_{13}(k_9 + k_{11})} \qquad (5)$$

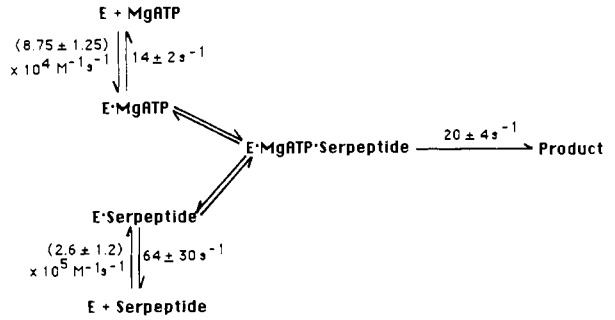
is $k_4/k_3$. The off-rate for phospho-Ser-peptide is much greater than catalysis as indicated by the $K_d$ value >30 mM (Granot et al., 1981), and some step after catalysis must limit the rate at least partially for $K_m$ (20 μM) to be less than $K_d$ (≥250 μM). With these qualifiers the above simplifies to eq 6 (Cook

$$K_b = K_{ib}(k_{11}/k_4 + k_{11}/k_{13}) \qquad (6)$$

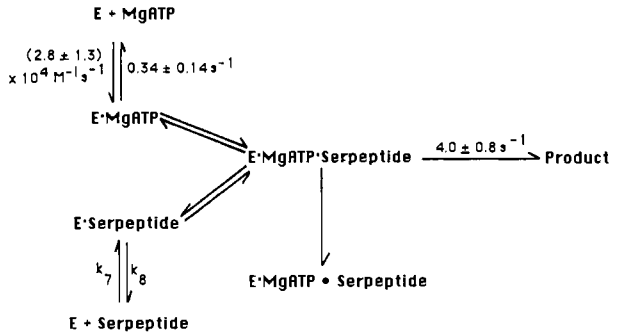
et al., 1982). Since the off-rate for Ser-peptide ($k_4$) increases, $k_{11}$ must decrease by the same amount in order that $K_b$ remain the same as $K_{ib}$ increases. In addition, $k_{11}/k_{13}$ must not be a very important term; that is, the catalytic step(s) is (are) not rate limiting. Thus, as suggested previously, the release of MgADP from E·MgADP must at least partially limit at low $Mg_f$ and become more rate limiting at high $Mg_f$, likely accounting for the 5-fold decrease in $V_{max}$ observed as the concentration of $Mg_f$ increases (Cook et al., 1982; Yoon & Cook, 1987). This is not surprising since $Mg^{2+}$ increases the affinity for MgATP by bridging enzyme and the polyphosphate chain of the nucleotide and will likely do the same with MgADP.

*Isotope Partitioning Studies of E·MgADP.* There are several possibilities for no trapping being obtained with MgADP at either high or low $Mg_f$ concentration. (1) The

Scheme I




Scheme II




E·MgADP binary complex does not form. (2) The off-rate of MgADP from E·MgADP is much faster than $k_3$[phospho-Ser-peptide] even at the highest [phospho-Ser-peptide]. (3) MgADP is released from E·MgADP·phospho-Ser-peptide much faster than it turns over.

Kinetic studies have shown that $K_d$ for E·MgADP is 0.05 mM (Cook et al., 1982), and thus E·MgADP does form and MgADP binds reasonably tightly. As discussed above, the MgADP release step is probably rate limiting in the phosphorylation direction. For the cAPK reaction, the ratio of $V_{max}$ in the direction of Ser-peptide phosphorylation to that in the direction of MgADP phosphorylation is 250 (unpublished results of M.-Y. Yoon in this laboratory), indicating that MgADP must be released from the central product complex at least 250 times faster than the slowest step in the direction of MgADP phosphorylation; in other words, the mechanism is likely rapid equilibrium in this direction, suggesting that the third possibility is most likely.

*Summary of Isotope Partitioning Studies.* The data obtained from isotope partitioning studies are summarized in Scheme I for low $Mg_f$ and in Scheme II for high $Mg_f$. At low $Mg_f$, both MgATP and Ser-peptide are released from the central complex slowly compared to catalysis. In addition, on the basis of the magnitude of the on-rate, the binding processes require more than one step in both cases. When $Mg_f$ concentration increases, the affinity of MgATP for the enzyme is increased as a result of a decrease in the off-rate for MgATP (as seen, for example, in the E·MgATP binary complex), but it results in an increase in the off-rate of Ser-peptide at least from the E·Ser-peptide·MgATP ternary complex.

The isotope partitioning studies of cAPK suggest a steady-state random kinetic mechanism in the direction of protein phosphorylation since trapping was obtained from both E·[$\gamma$-$^{32}$P]MgATP and E·[$^3$H]acetyl-Ser-peptide. At high $Mg_f$ conditions, there is a preference for MgATP binding to the enzyme first.

ACKNOWLEDGMENTS

We thank Theresa Rhodes for typing the manuscript.

REFERENCES

Armstrong, R. N., Kondo, H., Granot, J., Kaiser, E. T., & Mildvan, A. S. (1979a) *Biochemistry 18*, 1230.
Armstrong, R. N., Kondo, H., & Kaiser, E. T. (1979b) *Proc. Natl. Acad. Sci. U.S.A. 76*, 722.
Bolen, D. W., Stingelin, J., Bramson, H. N., & Kaiser, E. T. (1980) *Biochemistry 19*, 1176.
Bradford, M. M. (1976) *Anal. Biochem. 72*, 248.
Cleland, W. W. (1975) *Biochemistry 14*, 3220.
Cleland, W. W. (1979) *Methods Enzymol. 63*, 103.
Cook, P. F., Neville, M. E., Vrana, K. E., Hartl, F. T., & Roskoski, R., Jr. (1982) *Biochemistry 21*, 5794.
Dawson, R. M. C., Elliott, N. H., & Jones, K. M. (1979) *Data for Biochemical Research*, 2nd ed., p 423, Oxford University Press, London.
de la Houssaye, B. A., & Masaracchia, R. A. (1983) *Anal. Biochem. 128*, 54.
Feramisco, J. R., & Krebs, E. G. (1978) *J. Biol. Chem. 253*, 8968.
Feramisco, J. R., Kemp, B. E., & Krebs, E. G. (1979) *J. Biol. Chem. 254*, 6987.
Feramisco, J. R., Glass, D. B., & Krebs, E. G. (1980) *J. Biol. Chem. 255*, 4220.
Granot, J., Mildvan, A. S., Bramson, H. N., & Kaiser, E. T. (1981) *Biochemistry 20*, 602.
Hjelmquist, G., Anderson, J., Edlund, B., & Engstrom, L. (1974) *Biochem. Biophys. Res. Commun. 61*, 559.
Kemp, B. E. (1980) *J. Biol. Chem. 225*, 2914.
Kemp, B. E., Graves, D. J., Benjamine, E., & Krebs, E. G. (1977) *J. Biol. Chem. 252*, 4888.
Martell, A. E., & Smith, R. M. (1982) *Critical Stability Constants*, Vol. 5, Plenum, New York.
Promerantz, A. H., Allfrey, G., Merrifield, R. B., & Johnson, E. M. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 4261.
Reed, J., & Kinzel, V. (1984) *Biochemistry 23*, 968.
Reed, J., Kinzel, V., Kemp, B. E., Cheng, H.-C., & Walsh, D. A. (1985) *Biochemistry 24*, 2967.
Riordan, J. R., & Vallee, B. L. (1967) *Methods Enzymol. 2*, 565.
Rose, I. A., O'Connell, E. L., Litwin, S., & Bar-Tana, J. (1974) *J. Biol. Chem. 249*, 5163.
Rosevear, P. R., Bramson, H. N., O'Brian, C., Kaiser, E. T., & Mildvan, A. S. (1983) *Biochemistry 22*, 3161.
Sugden, P. H., Holladay, L. A., Reiman, E. M., & Corbin, J. D. (1976) *Biochem. J. 159*, 409.
Walsh, D. A., & Krebs, E. G. (1973) *Enzymes (3rd Ed.) 8*, 555.
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.
Yoon, M. Y., & Cook, P. F. (1987) *Biochemistry 26*, 4118.
Zetterquist, O., Ragnarsson, U., Humble, E., Berglund, L., & Engstrom, L. (1976) *Biochem. Biophys. Res. Commun. 70*, 696.