Phosphorylation of Peptide Substrates for the Catalytic Subunit of cAMP-Dependent Protein Kinase*

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The steady-state kinetic parameters for the phosphorylation of four peptides by the catalytic subunit of cAMP-dependent protein kinase were measured as a function of pH. For peptides containing the minimum consensus sequence, R-R-X-S-hyd (where hyd is a hydrophobic residue), the \( k_{cat}/K_{peptide} \) profile is bell-shaped with \( pK \) values of 6.4 and 9.4. Inhibition studies with the peptide LR3RNAI indicate that the lower \( pK \) corresponds to an intrinsic \( pK \) on the enzyme, whereas the higher \( pK \) is perturbed upward by 1 pH unit. Viscosity studies verify that substrate stickiness accounts for the kinetic perturbation of the higher \( pK \) in \( k_{cat}/K_{peptide} \). Substitution of the P-3 arginine with alanine (where serine is the P-site) yields a \( k_{cat}/K_{peptide} \) versus \( pH \) profile that is also bell-shaped, although both \( pK \) values are intrinsic acid dissociation constants of the enzyme. Replacement of the P-2 arginine with alanine removes the lower \( pK \) in the \( pH \)-rate profile without altering the higher \( pK \). These results indicate that recognition of the P-2 arginine residue is a hallmark of enzyme residues. This result implies that if the catalytic subunit mechanism involves general base catalysis, the identification of this base is not manifested in the \( pH \)-rate profiles.

\[ 4 \text{cAMP} + R_2C = \rightarrow 4 \text{cAMP} - R_2 + 2C \]  
(Eq. 1)

This process frees the C-subunit from the inactive holoenzyme (R_2C, where R_2 is a regulatory dimer) in the cytoplasm. Active C-subunit (40,900 Da) then regulates the function of a number of proteins by phosphoryl transfer from ATP to serine and threonine residues. Although the first protein kinase, phosphorylase kinase, was purified in the 1950s (Kreb's et al., 1959), a regulatory function for this class of enzyme was delineated 2 decades later. Today, protein kinases clearly control a vast number of cellular processes including metabolism, growth, and gene expression (Hanks et al., 1985; Cooper, 1990). Despite the discovery of a large number of protein kinases, to date, cAMP-dependent protein kinase remains unique. Its ability to be easily dissociated from its regulatory components provides a simple model for the entire protein kinase family. Detailed physical studies of this enzymatic mechanism have been greatly enhanced by two recent breakthroughs: 1) the overexpression and purification of recombinant mammalian catalytic subunit from Escherichia coli (Slice and Taylor, 1989; Yonemoto et al., 1991) and 2) the x-ray crystallographic structure solution of the catalytic subunit with an inhibitor peptide bound at the active site (Knighton et al., 1991a, 1991b).

Peptide studies have shown that the C-subunit will preferentially phosphorylate serine and threonine residues in the minimum consensus sequence R-R-X-S/T-hyd, where X is variable and hyd is a hydrophobic residue (Kemp et al., 1977; Zetterqvist et al., 1990). Replacement of one of the arginines with alanine or lysine lowers the specificity constant, \( V/K \), by approximately 2-3 orders of magnitude (for review, see Djar and Ragnarsson (1991)). Both steady-state kinase (Cook et al., 1982) and isotope partitioning studies (Kong and Cook, 1988) indicate that the C-subunit binds ATP and substrate randomly, although initial binding of the nucleotide is preferred. The 2 orders of magnitude difference in \( K_d \) compared to \( K_d (K_d > K_m) \) for the heptapeptide Kemptide, LRASLG, suggests that a rapid step occurs after ternary complex (E-ATP-Kemptide) formation. Viscosity studies support a kinetic mechanism involving fast phosphoryl transfer from the \( \gamma PO_4 \) of ATP to the hydroxyl group acceptor of the peptide followed by rate-limiting release of the product, ADP (Adams and Taylor, 1992). Phosphorylation of the peptide considerably weakens its affinity for the C-subunit so that it has no energetic consequence on the transition state for \( k_{cat} \) (Whitehouse et al., 1988). Stereochemical studies indicate a direct, in-line attack of the hydroxyl group on the \( \gamma PO_4 \) of ATP (Ho et al., 1988).

The kinetic processing of substrate depends greatly on pH. Yoon and Cook (1988) showed that the binary E-ATP complex can populate three protonation states, of which only one binds peptide and transfers phosphate. This conclusion derives from the bell-shaped \( pH \) dependence of \( V/K \) and the \( pH \) insensitivity of \( V \). It is not clear which residue on the enzyme needs to be protonated for full activity. However, a compelling role for the group with the lower \( pK \) is general base catalysis. The C-subunit may efficiently deliver phosphate by providing an ionizable group close to the hydroxyl proton of the serine-containing peptide. Removal of this proton would increase the nucleophilicity of the attacking group. We studied the \( pH \) dependence values of peptide analogs on the steady-state kinetic parameters to ask whether these ionizable residues are important for positioning the peptide for attack or for general base catalysis. Kinetic mechanisms were established...
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for four peptides that differ in size and overall charge. For two substrates, a neutral residue, alanine, systematically replaced each arginine in the consensus sequence. It is postulated that alterations in the peptide distal from the site of phosphorylation should not affect the acid-base chemistry of the pH-rate profile if a true, general base mechanism is operative and is manifested in the pH-rate profile.

EXPERIMENTAL PROCEDURES

Materials—Adenosine 5'-triphosphate (ATP), phosphoenolpyruvate, magnesium chloride, nicotinamide adenine dinucleotide, reduced NADH, Mes, Tris, Caps, pyruvate kinase (rabbit muscle), and lactate dehydrogenase (bovine heart) were purchased from Sigma. Sucrose was purchased from Mallinckrodt. Sodium chloride and glycerol were purchased from Fisher.

Peptides and Protein—All peptides were synthesized at the Peptide and Oligonucleotide Facility at the University of California, San Diego. Peptides were purified by reverse phase preparative high performance liquid chromatography. The concentration of some peptides was determined by turnover with the catalytic subunit under conditions of limiting peptide. The recombinant C-subunit was expressed and purified from E. coli according to previously published procedures (Konemoto et al., 1991). The concentration of enzyme was measured by A280 (A280 = 1.2).

Kinetic Assay—The enzymatic activity of the C-subunit was measured spectrophotometrically (Cook et al., 1982). This assay couples the production of ADP with the oxidation of NADH by pyruvate kinase and lactate dehydrogenase. Typically, 2–5 mM ATP was pre-equilibrated with catalytic subunit in a buffer containing 1 mM phosphoenolpyruvate, 0.3 mM NADH, 12 units of lactate dehydrogenase, and 4 units of pyruvate kinase in a final volume of 1 ml. Reactions were initiated by adding varying amounts of peptide. Inhibition studies were done by pre-equilibrating 2 mM ATP, C-subunit, and inhibitor before adding substrate. All reactions were done in a buffer containing 50 mM Mes, 25 mM Tris, 25 mM Caps, and 50 mM NaCl (MTCN buffer) either in the presence or absence of glycerol or sucrose. The pH of the buffers was adjusted by adding small volumes of concentrated HCl or NaOH. All kinetic measurements were performed at 23.0 °C, pH 6–10.8, and 10 mM free Mg+.

The C-subunit was sufficiently stable at both low and high pH extremes within the time frame of ligand pre-equilibration and assay. The C-subunit could be pre-equilibrated in pH 6 MTCN buffer for 10 min with no loss of activity. At pH 10.8, the C-subunit was inactivated in about 30 min in MTCN buffer.

For peptides III and IV (Table I), steady-state kinetic parameters were also determined by a discrete time point assay in addition to the continuous, coupled enzyme assay. In this technique, 0.35–0.55 µM C-subunit, 5 mM ATP, 15 mM MgCl2, and varying amounts of peptide (6–47 mM for III and 22–90 mM for IV) were mixed in a total volume of 100 µl of MTCN buffer (pH 7.5, or 8). After 60–240 s of reaction time, the samples were diluted to 1.0 ml in an assay mix containing 1 mM phosphoenolpyruvate, 0.3 mM NADH, 12 units of lactate dehydrogenase, and 4 units of pyruvate kinase in pH 10 MTCN buffer. The initial amount of the C-subunit in the 100-µl reaction sample and the pH of the assay mix were chosen so that, upon dilution, little or no peptide was further phosphorylated. The amount of ADP produced during the reaction period time (before dilution) was measured by the total absorbance change at 340 nm (ΔA340). The absorbance change due to dilution of the assay mix (ΔA340) was added to the reaction sample was subtracted from ΔA340 by recording the absorbance change after adding 100 µl of water to 990 µl of assay mix. The corrected absorbance change (ΔA340 = ΔA340 – ΔA340) due solely to ADP production was used to measure reaction velocities as a function of substrate concentration. This method was used for measuring kcat values for weakly bound substrates.

Solution Viscosity Measurements—The relative viscosity (ηr) of buffers containing arginine or sucrose was measured relative to MTCN buffer at pH 8.0, 23.0 °C, using an Ostwald viscometer (Shoemaker and Garland, 1962). 20% and 39% glycerol buffers (w/w %) were used to obtain relative viscosity values of 1.5 and 1.8, respectively. Solutions of 26, 32, and 39% sucrose (w/w %) were used to obtain relative viscosities of 2.0, 2.4, and 2.9, respectively. All measurements of viscosity were performed in triplicate.

Data Analysis—The values of kcat and Kpeptide, determined from plots of initial velocity versus substrate concentration according to Equation 2.

\[
v = \frac{V_{\text{max}} [S]}{K_{\text{peptide}} + [S]}
\]

(Eq. 2)

v is the initial velocity, [S] is the concentration of the varied substrate, Vmax is the maximal velocity, and Kpeptide is the Michaelis constant. The maximal velocity was then converted to kcat by dividing Vmax by the total enzyme concentration. For several peptides, kcat/Kpeptide values were obtained by plotting kcat versus peptide concentration and fitting the data by linear regression. Plots of kcat/Kpeptide ascertained either by Equation 2 or by linear regression were fit to Equations 3, 4, or 5.

\[
y = C + C^* 10^{pK_a - pK_a}
\]

(Eq. 3)

\[
y = C 10^{-(pK_a - pK_a)}
\]

(Eq. 4)

\[
y = C 10^{-(pK_a - pK_a)}
\]

(Eq. 5)

y is the observed kcat/Kpeptide at a given pH, C and C* are the maximum and minimum values of kcat/Kpeptide, respectively, and pK_a and pK_b are the lower and higher acid dissociation constants, respectively. Competitive inhibition data were fit by a Dixon plot (Segel, 1975). Plots of the apparent inhibition constant, Kip versus pH were fit to Equation 6.

\[
1/K_{\text{ip}} = \frac{1/K_{\text{cat}}}{1} + \frac{1}{10^{pK_a - pK_a}}
\]

(Eq. 6)

K_{\text{ip}} is the pH-independent dissociation constant and pK_a and pK_b are the lower and higher acid dissociation constants, respectively.

RESULTS

dpH-dependent Steady-state Kinetic Parameters—The steady-state kinetic parameters, kcat and kcat/Kpeptide, for peptides I and II (Table I) were measured as a function of pH under conditions of saturating ATP (2 mM) and 10 mM free Mg++. Using the continuous coupled enzyme assay. As illustrated in Fig. 1A, kcat/Kpeptide is bell-shaped and kcat is constant over the pH range 6–10 for peptides I and II (kcat data is not shown). The kcat/Kpeptide data were fit to equation 3 to yield the pH-independent kcat/Kpeptide and the two pK values. Table I lists the results of these fits. For peptides III and IV (Table I), kcat/Kpeptide values were determined from linear plots of initial reaction velocity versus substrate concentration. Fig. 1B illustrates the pH dependence values of kcat/Kpeptide for these peptides. Like the di-arginine class of peptides (I and II), peptide III has a bell-shaped pH-rate profile. The pH dependence of peptide IV gives the ionization of a single residue. Both peptides III and IV show a plateau in kcat/Kpeptide at high pH (pH > 9). This is in contrast to the di-arginine peptides, I and II, that apparently approach zero at high pH. Equations 4 and 5 satisfy mathematically the shapes of the pH dependence values of III and IV, respectively. Table I compiles the best fits of kcat/Kpeptide, kcat/Kpeptide, pK_a, and pK_b. The pK_a values for peptides I, II, and III are statistically identical. Likewise, the pK_b values for I and II are the same. However, the pK_b values for peptides III and IV are statistically lower than those of peptides I and II.

The kcat values for peptides III and IV could not be determined directly from the continuous coupled enzyme assay owing to their high Kpeptide values for the C-subunit (Table I). Instead, a discrete time point assay was used to take advantage of smaller reaction volumes and to lower the total quantity of peptide needed to saturate the C-subunit in a 1-ml assay. In this modified assay, the C-subunit is combined with ATP and peptide in a small reaction volume (100 µl) for a desired time period before stopping the reaction by a 10-fold dilution into...
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TABLE I
pH-dependent steady-state kinetic parameters in MTNCN buffer

| Peptide   | $k_{cat}$ | $k_{cat}/K_{m,peptide}^*$ | $k_{cat}/K_{peptide}^*$ | $pK_a$ | $pK_b$ |
|-----------|-----------|--------------------------|-------------------------|--------|--------|
| I (LRRASLG) | 22 ± 2 | 880 ± 50 | 6.5 ± 0.10 | 9.3 ± 0.10 |
| II (LRRNSI) | 22 ± 3 | 1800 ± 130 | 6.3 ± 0.12 | 8.4 ± 0.13 |
| III (LARNSI) | 6.6 ± 0.03 | 0.21 ± 0.010 | 0.061 ± 0.010 | 6.3 ± 0.10 | 8.5 ± 0.16 |
| IV (LRANSI) | 9.0 ± 0.26 | 0.58 ± 0.012 | 0.099 ± 0.003 | 8.3 ± 0.08 |
| V (LRRNALG) | 70 ± 10 | | | | |

$^*$ These values were determined by discrete time point assays (see "Materials and Methods") using 5 mM ATP and 10 mM free Mg$^{2+}$ in pH 6 MTNCN buffer for peptide III and pH 7.5 MTNCN buffer for peptide IV at 23 °C. At pH 8, $k_{cat} = 8.7 ± 0.90$ s$^{-1}$ for peptide III and, at pH 6, $k_{cat} = 8.8 ± 0.60$ s$^{-1}$ for peptide IV.

![Fig. 1. Plots of $k_{cat}/K_{peptide}$ for peptides I–IV as a function of pH in MTNCN buffer, 10 mM free Mg$^{2+}$, 2 mM ATP at 23 °C.](image)

A. $k_{cat}/K_{peptide}$ for peptides I (C) and II (O). Equation 3 was used to fit these data (see text and Table I). B. $k_{cat}/K_{peptide}$ for peptides III (C) and IV (O). These data were determined from the slopes of the initial reaction velocity versus peptide concentration and were fit to Equations 4 and 5, respectively (see text and Table I).

A high pH buffer (pH 10 MTNCN buffer) containing the coupling reagents. The total amount of ADP produced was calculated from the corrected total absorbance change at 340 nm. The total production of ADP did not exceed 10% of the initial peptide or ATP concentrations and was linearly dependent on the C-subunit concentration (data not shown).

The values for $k_{cat}$ were determined at two pH values for peptides III and IV. Table I compiles these results. For both peptides, $k_{cat}$ did not change between pH 6 and 8. The reduced values of $k_{cat}/K_{peptide}$ for peptides III and IV are due to high $K_{peptide}$ values. For peptide III, $K_{peptide} = 31 ± 2.1$ mM at pH 6; for peptide IV, $K_{peptide} = 16 ± 0.55$ mM at pH 7.5. These latter parameters are obtained directly from the ratio of $k_{cat}$ and $k_{cat}/K_{peptide}$ in Table I.

Effect of Viscosity on the Kinetics of Peptide I and IV—$k_{cat}$ and $k_{cat}/K_{peptide}$ for peptides I and IV were measured at varying solvent viscosity in MTNCN buffer under conditions of saturating ATP (2–5 mM), 10 mM free Mg$^{2+}$, and varying peptide concentrations. Fig. 2 shows the plots of the ratio of $k_{cat}$ and $k_{cat}/K_{peptide}$ in the absence and presence of viscosogen as a function of relative solvent viscosity, $\eta_m$.

Both steady-state kinetic parameters are linearly dependent on solvent viscosity for peptide I and IV. The data for $k_{cat}/K_{peptide}$ for peptide I were removed for clarity. The slopes of these lines for peptide I were 1.0 ± 0.08 and 0.97 ± 0.10 for $k_{cat}$ and $k_{cat}/K_{peptide}$, respectively. For peptide IV the slope for $k_{cat}$ is 0.30 ± 0.06. However, $k_{cat}/K_{peptide}$ for peptide IV is insensitive to solvent viscosity. All $k_{cat}/K_{peptide}$ values for peptide IV were determined from the slopes of initial velocity versus peptide concentration in the absence and presence of viscosogen. All $k_{cat}$ values for peptide IV were determined from the time point assay method. For all values of $\eta_m$, the observed enzymatic
velocities were dependent linearly on the C-subunit concentration, indicating that added viscosity did not limit the rate at which the coupling enzymes converted ADP (data not shown).

pH-dependent Inhibition Kinetics—Peptide V is a competitive inhibitor of the C-subunit with respect to peptide I (Salerno et al., 1990). The apparent dissociation constant (Kpp) for this inhibitor was measured as a function of pH in MTCN buffer. Varying amounts of inhibitor, saturating ATP (2 mM), 12 mM MgCl₂, and fixed amounts of C-subunit were pre-equilibrated before adding a fixed amount of peptide I (80–125 μM). Plots of 1/v versus inhibitor concentration (Dixon plot) were used to extrapolate to the value of Kpp (Segel, 1975). Fig. 3 illustrates the pH dependence of 1/Kpp. The data gave a bell-shaped curve that was fit to Equation 6. Table I compiles the pH-independent dissociation constant, Ki, pKa, and pKb for all peptides. Conversely, pKb is identical to pK2 for peptides III and IV but not peptides I and II.

DISCUSSION

Kinetic Mechanism for Peptides I and II—Yoon and Cook (1987) showed previously that the apparent second order rate constant for ATP, kcat/KATP, and the maximal rate constant, kcat, are pH-independent in the range of 6–10 when using peptide I as a phosphoryl acceptor. Alternatively, the pH dependence of kcat/Kpeptide is bell-shaped with two defining pK values of 6.2 and 8.5. These data imply that the free and ATP-bound C-subunit exist in three ionization states and that ATP does not discriminate between these forms. In contrast, peptide I binds a single ionization state that supports phosphate transfer. Scheme I illustrates this mechanism at high ATP concentrations. Fully protonated (H₂·E·ATP·S) and fully ionized (E·ATP·S) ternary complexes are not populated. Under these conditions the pH dependence in kcat/Kpeptide gives intrinsic pK values for both free and ATP-bound C-subunit. pH-dependent studies of the competitive inhibitor, LRRAALG, confirmed that these pK values are, indeed, intrinsic (Yoon and Cook, 1987).

Our data for peptides I and II are consistent with this steady-state kinetic mechanism. kcat is independent of pH, and kcat/Kpeptide is bell-shaped between pH 6 and 10 (Fig. 1A). Furthermore, the magnitudes of the steady-state kinetic parameters are equivalent except that pKb for both peptides is approximately 1 unit higher (9.4 versus 8.5; Table I). We performed pH-dependent inhibition studies to see if this elevated pK value reflects the intrinsic pK of the enzyme (Fig. 3). Although pKb for the inhibition kinetics is the same as pKb for the steady-state phosphorylation kinetics (Table I), pKb is approximately 1 unit higher than pKb. Thus, the basic region of the pH-rate profile for peptides I and II does not reflect the intrinsic pK of an ionizing group on the enzyme. It is not clear why our data indicate a perturbed pK. However, the consistency of pKb and pKb compared to that of Yoon and Cook (1987) argues that the buffer components have no different effect on the ionization of the ATP-bound enzyme.²

Scheme I was modified to satisfy the inhibition and steady-state kinetic data. In MTCN buffer, peptides I and II must bind to the fully ionized enzyme-ATP complex so that the measured pKb reflects a perturbed pK on the enzyme. Scheme II shows this new mechanism. This scheme allows for the perturbation of pKb by substrate stickiness (i.e. ks > k-). We presume that the fully protonated form (H₂·E·ATP) does not bind peptide since pKb in the inhibition kinetics and pKb II in the steady-state kinetic studies are identical. Consideration of the steady-state kinetics of the other peptides will confirm this assertion (see “Kinetic Mechanism of Peptides III and IV”). Since the steady-state kinetic parameters for both peptides I and II are similar, both arginines are important recognition elements for the pH-dependent binding of peptides of 6–7 residues in length.

Interpretation of Viscosity Data—Solvent viscosity affects the phosphorylation of peptide I by the C-subunit (Adams and Taylor, 1992). The individual effects on kcat and kcat/Kpeptide are consistent with the Stokes-Einstein equation and are interpreted with the microscopic rate constants for substrate/product binding and phosphoryl transfer. Large effects of viscosity on kcat imply that product dissociation limits this parameter, whereas smaller effects on kcat/Kpeptide indicate that substrate is in near rapid equilibrium with the enzyme in 100 mM Tris buffer (pH 8). Since the predominant kinetic pathway in Scheme II at pH 8 involves the binding and phosphorylation of peptide through the mono-protonated species, the pathways involving the fully protonated and fully ionized species can be ignored. Since the diffusion rate constants,

7 Yoon and Cook (1988) used 100 mM concentrations of Mes for pH 5–6.5, Mops for pH 6.5–7.5, Taps for pH 7.5–8.5, and BPT for pH 8.5–10.
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$k_t$, $k_2$, and $k_4$, are indirectly proportional to the intrinsic solvent viscosity of the buffer and $k_2$ is insensitive to viscosity, $k_{cat}$ and $k_{cat}/K_{peptide}$ are related to the relative viscosity ($\eta^*$). In this mechanism, phosphorylated peptide dissociates rapidly owing to its large $k_2$ relative to that of ADP (Whitehouse et al., 1983). Thus, $k_4$ is the dissociation rate constant for ADP alone. Equations 7 and 8 relate the slopes of the plots of $k_{cat}$ and $k_{cat}/K_{peptide}$ in the absence and presence of viscosogen as a function of $\eta^*$.

$$\frac{(k_{cat})^{*}}{(k_{cat})} = \frac{k_4}{k_3 + k_4} \quad (\text{Eq. 7})$$

$$\frac{(k_{cat}/K_{peptide})^{*}}{(k_{cat}/K_{peptide})} = \frac{k_4}{k_3 + k_4} \quad (\text{Eq. 8})$$

$(k_{cat})^*$ and $(k_{cat}/K_{peptide})^*$ are the slopes of the plots of $k_{cat}^*/k_{cat}$ and $(k_{cat}/K_{peptide})^*/k_{cat}/K_{peptide}$, respectively. The superscript postscript "**" refers to the steady-state kinetic parameter in the absence of viscosogen. These slope values lie between the theoretical limits of 0 and 1 and give information on the contribution of product dissociation to the rate-determining step in $k_{cat}$ and the stickiness of the substrate. Since $(k_{cat})^* \approx 1$, the rate constant for phosphotransfer must greatly exceed that for product dissociation (i.e. $k_4 \gg k_3$) so that the rate-determining step in $k_{cat}$ is product release ($k_4 \approx k_{cat}$). Due to the error measurement in $(k_{cat})^*$, $k_4$ must equal or exceed 250 s$^{-1}$. Furthermore, since $(k_{cat}/K_{peptide})^* \approx 1$, the rate of phosphotransfer must greatly exceed the rate of substrate dissociation from the ternary complex (i.e. $k_3 \gg k_2$). In other words, peptide I is a sticky substrate in MTNE buffer and $k_t = k_{cat}/K_{peptide} = 0.88 \mu M^{-1} s^{-1}$ (Table I). We have found that the level of substrate stickiness is greatly dependent on buffer conditions. In 100 mM Tris, pH 8, peptide I is no longer a sticky substrate (Adams and Taylor, 1992). Varying buffer conditions, though, have no measurable effects on the sensitivity of $k_{cat}$ to viscosogen. Since both values for $(k_{cat})^*$ and $(k_{cat}/K_{peptide})^*$ are near the diffusion-controlled limit, all proton transfers needed to achieve the active ternary complex, H.E-ATP-S, are fast and thermodynamically favorable at pH 8.

For the mechanism outlined in Scheme II, Equation 9 relates the displacement of the pK for $k_{cat}/K_{peptide}$ ($pK_2$) to the intrinsic pK ($pK_3$) and the microscopic rate constants (Cleland, 1977).

$$pK_2 = pK_3 + \log(1 + k_3/k_{-3}) \quad (\text{Eq. 9})$$

Since steady-state kinetic and competitive inhibition studies (Table I) measure $pK_3$ and $pK_2$, the stickiness term, $k_3/k_{-3}$, is approximately 10. This value is consistent with the viscosity data and Equation 8. Substitution of the stickiness term into Equation 8 gives $(k_{cat}/K_{peptide})^* \approx 1$. This provides an internal check on both experimental methods. A lower limit for $k_2$ can be set ($k_2 \geq 25$ s$^{-1}$) from the stickiness term and the lower limit for $k_2$. Although changes in buffer components have effects on the stickiness of peptide I, it is not clear whether these effects are manifested separately on $k_3$ and $k_2$ or both.

**Kinetic Mechanism for Peptides III and IV**—The discrete time point assay method was used to measure $k_{cat}$ values for peptides III and IV. Both parameters were unchanged by pH but were lower in value than those of peptides I and II. Since the rate-determining step in $k_{cat}$ is product release for the di-arginine class of peptides, we wondered whether the same is true for these peptides. The effect of solvent viscosity on $k_{cat}$ for the phosphorylation of peptide IV is lower compared to the effect on peptide I (Fig. 2). We have shown previously that other di-arginine-containing peptides behave similarly (Adams and Taylor, 1992). The slope of $k_{cat}/k_{cat}$ versus $\eta^*$ for peptide IV is intermediate between a diffusion and a non-diffusion-controlled reaction ($\eta^* = 0.30 \pm 0.06$ for peptide IV). Equation 7, $k_{cat}$ and $(k_{cat})^*$ were used to calculate the rate constants for phosphoryl transfer ($k_3$) and product release ($k_4$) ($k_3 = 13 \pm 1.2 s^{-1}$ and $k_4 = 30 \pm 6.1 s^{-2}$). For this peptide the chemical rate is similar in value to the product release rate. Additionally, the value of $30 s^{-1}$ for ADP release is close to the value of $22 s^{-1}$ for peptide I. However, the replacement of 1 of the consensus arginines with alanine lowers the rate of phosphoryl transfer more than 19-fold (compare $k_4 \geq 250 s^{-1}$ for peptide I). We presume that replacement of the other arginine with alanine in peptide III has a similar reduction in catalysis. Certainly, the lower value for $k_{cat}$ for this peptide compared to those of peptides I and II supports this point. The diminution in the rate of phosphotransfer for alanine-containing peptides underpins the tight relationship between substrate binding and catalysis in this enzyme.

Using the continuous coupled enzyme assay, only $k_{cat}/K_{peptide}$ values could be derived owing to the weak affinities of peptides III and IV with the C-subunit. Nevertheless, the $k_{cat}/K_{peptide}$ values listed in Table I, which were determined from the slopes of initial velocity versus substrate concentration, are consistent with values reported in the literature (Kemp et al., 1977). The shapes of the $k_{cat}/K_{peptide}$ versus pH profiles show three distinct differences from those of peptides I and II: 1) the higher pH values ($pK_4$) are approximately 1 unit lower than those of the other peptide; 2) peptide IV has no lower $pK_3$; 3) both peptides have plateaus in $k_{cat}/K_{peptide}$ at high pH.

Since $pK_4$ and $pK_2$ for peptides III and IV are equal (Table I), the enzyme residue needed for optimum activity of the di-arginine peptides (I and II; Table I) is the same for the other peptides. It is not clear, however, if this enzyme residue makes a direct or indirect contact with the substrate. The plateau observed in $k_{cat}/K_{peptide}$ at high pH implies that the fully ionized ternary complex in Scheme II can form and support phosphotransfer for peptides III and IV. It is also possible that peptides I and II may support catalysis from this complex. However, the perturbation of $pK_4$ places the plateau in an unobservable pH region (pH > 10). It is likely that the similarity of $k_{cat}/K_{peptide}$ and $k_{cat}/K_{peptide}$ (Table I) for peptides III and IV implies that $pK_4$ and $pK_2$ are close in value since $k_{cat}$ is constant and $K_{peptide}$ closely represents the true thermodynamic dissociation constants when the substrate is not sticky and $k_4$ does not greatly exceed $k_3$. However, since no measurable plateau was found at high pH in the competitive inhibition studies (Fig. 3), the presence of both arginines in the ternary complex must raise $pK_4$ sufficiently above $pK_4$ in Scheme II. In other words, positively charged residues in the peptide reduce the acidity of the basic enzyme group.

The lack of a lower pK for the phosphorylation of peptide IV argues that a functional group on the enzyme ionizes to interact directly with the P-2 arginine.3 This residue does not, however, contact the P-3 arginine since the lower pK is observed in peptide III. It is not likely that the residue with the lower pK is still operative in the mechanism for peptide IV but is perturbed to even lower pK values since this peptide binds weakly and offers no means of kinetic perturbation through substrate stickiness. Since solvent viscosity does not affect $k_{cat}/K_{peptide}$ for peptide IV (Fig. 2), this peptide is in rapid equilibrium with the E-ATP complex (i.e. $k_{cat} \gg k_4$ in

3 For all peptides studied in this manuscript, the site of phosphorylation is the P-site. All positions N-terminal to the serine are designated P-1, P-2, P-3, etc. For example, asparagine in peptide II is the P-1 asparagine.
Scheme II). Thus, pKᵢ is not shifted out of the studied pH range of 6–10. Since pKᵢ and pKᵤ for peptides I, II, and III are identical and kₑᵣ is pH-independent, these peptides do not bind the fully protonated species.

**General Base Catalysis**—Yoon and Cook (1987) have postulated that the acidic pKᵦ observed in kₑᵣ/Kₚ is due to the ionization of a general base catalyst near the site of chemical transformation. This residue would greatly facilitate phosphoryl transfer by removal of the hydroxyl group proton of serine-containing peptides. However, the data presented here are not consistent with this hypothesis since substitution of the substrate’s P-2 arginine removes pKᵦ. We cannot argue that the replacement of the arginine in peptide IV has local effects on the positioning of the serine relative to a putative general base. Since replacement of the P-3 arginine with alanine in peptide III does not remove the acidic pKᵦ, the putative base should still be functional for this peptide. However, there is no significant difference in kₑᵣ/Kₚ or kₑᵣ (Table I) for peptides III and IV. One would expect large rate reductions for substrates that did not exploit the general base catalysis. Since this is not the case, we conclude that the replacement of the P-2 arginine with alanine in peptide IV implies that positioning of the substrate is critical for efficient catalysis.

The C-subunit positions several amino acid side chains near the P-2 and P-3 arginines of the peptide based on a crystal structure resolved to 2.0 Å (Knighton et al., 1993). The carboxyl group of Glu-127 and the phenolic oxygen of Tyr-330 interact with the P-3 arginine. In contrast, the carboxyl groups of Glu-170 and Glu-230 interact with the P-2 arginine. The ionization of either Glu-127 or Tyr-330 is unlikely to influence the pH-rate profile since peptide III has no effect on pKᵢ (Table I). Therefore, these residues are not responsible for the pH dependence in kₑᵣ/Kₚ. Chemical modification studies with hydrophobic and water soluble carbodiimides (Buechler and Taylor, 1988, 1990) suggest that Glu-170 and Glu-328 are exposed to solvent and Glu-230 is partially buried. Since pKᵦ is 2 units higher than the solvent exposed pK of the glutamate side chain, Glu-230 is the likely candidate for pKᵦ in Fig. 1. We are currently testing this hypothesis using site-directed mutagenesis.

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