Pre-Steady-State Kinetic Analysis of cAMP-Dependent Protein Kinase Using Rapid Quench Flow Techniques†

Bruce D. Grant‡ and Joseph A. Adams*,§

Department of Chemistry, San Diego State University, San Diego, California 92182-1030, and Department of Chemistry and Biochemistry, University of California, San Diego, La Jolla, California 92093-0654

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ABSTRACT: The phosphorylation of a peptide substrate by the catalytic subunit of cAMP-dependent protein kinase was monitored over short time periods (2−1000 ms) using a rapid quench flow mixing device and a radioactive assay. The production of phosphokemptide [LRRAS(P)LG] as a function of time is characterized by a rapid "burst" phase (250 s^{-1}) followed by a slower, linear phase ($L/[E]_t = 21\ s^{-1}$) at 100 μM Kemptide. The amplitude of this "burst" phase varies linearly with the enzyme concentration and represents approximately 100% of the total enzyme concentration, indicating that the "burst" phase is not due to product inhibition. The observed rate constants for the "burst" and linear phases and the "burst" amplitude vary hyperbolically with the substrate concentration. From these dependencies, a maximum "burst" rate constant of 500 ± 60 s^{-1} and a K_m and K_d for Kemptide of 4.9 ± 1.4 and 200 ± 60 μM were determined. The k_{cat} and K_m data extracted from the linear portion of the rapid quench flow transients are indistinguishable from those obtained by standard steady-state kinetic analyses using low catalytic subunit concentrations and a spectrophotometric, coupled enzyme assay. Both rate constants for the "burst" and linear phases decreased in the presence of Mn^{2+}. The data imply that the phosphorylation of Kemptide by the catalytic subunit occurs by a mechanism in which the substrate is loosely bound, is rapidly phosphorylated at the active site, and is released at a steady-state rate that is likely controlled by the dissociation rate constant for ADP. The combined pre-steady-state kinetic data establish a comprehensive, kinetic mechanism that predicts all the steady-state kinetic and viscosometric data. This study represents the first chemical observation and characterization of phosphoryl transfer at the active site of a protein kinase and will be useful for further structure−function studies on this and other protein kinases.

Protein phosphorylation controls many key biological processes within the cell ranging from short-term effects such as carbohydrate metabolism to long-range events including DNA transcription and cell division. The enzymes that catalyze phosphoryl transfer from ATP to serine, threonine, and tyrosine residues are known as protein kinases. Over the last decade, the scientific community has witnessed an explosion in the number of discovered protein kinases. Over 200 protein kinases have been identified, to date, but it is possible that as many as 2000 may be encoded in vertebrate genomes (Hunter, 1994). Their essential role in cell function is underpinned by the observation that aberrant forms of protein kinases have been associated with a host of diseases. In most cases, these abnormalities can be linked to defects in the mechanism of regulation of these enzymes. These findings indicate that catalytic efficiency is controlled stringently in the normal cell so that the protein kinase is only transiently active upon signalling. Although a significant amount of energy has been expended on understanding the nature and significance of these signals, little is known about the kinetic processes that occur at the active site of protein kinases. In particular, it is still unclear how fast phosphoryl transfer from ATP to substrates occurs in the active site cavity. A thorough understanding of these processes is germane to an informative study of the structure and dynamics of this enzyme class.

The overexpression of large amounts of protein kinases in various host cells has allowed the first steps to be made toward understanding how structure and function relate in this important class of catalysts. The X-ray crystal structures of several protein kinases from both the serine-specific (DeBont et al., 1993; Hu et al., 1994; Knighton et al., 1991; Xu et al., 1995; Zhang et al., 1994) and tyrosine-specific class (Hubbard et al., 1994) have now been determined. Although these structures have some distinguishing features, they share many common structural attributes. For example, they are composed of two subdomains—a small ATP binding subdomain and a larger substrate binding subdomain. The active site lies in a cleft created by the two lobes and is composed of a number of conserved residues that appear to be important for catalysis. In the active site of the catalytic subunit (C-subunit) of cAMP-dependent protein kinase (cAPK),[1] several residues converge to stabilize the triphosphates of ATP (Lys-72, Lys 168, Gly-50, Gly-52), to bind the primary (Asp-184) and secondary (Asn-171) metals, and to serve as a potential catalytic base (Asp-166) for the

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* Address correspondence to this author at the Department of Chemistry, San Diego State University. Telephone: (619) 594-6196. Fax: (619) 594-1879. E-mail: jadams@sundown.sdsu.edu.

‡ University of California, San Diego.

§ San Diego State University.

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[1] Abbreviations: cAPK, cAMP-dependent protein kinase; C-subunit, catalytic subunit of cAPK; Kemptide, peptide sequence L-R-R-A-S-L-G; Mops, 3-(*N*-morpholino)propanesulfonic acid; phosphokemptide, phosphorylated Kemptide [L-R-R-A-S(P)-L-G].

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hydroxyl of serine in the substrate. Since these amino acid side chains originate from both domains, the orientation of both lobes is critical for catalysis. The crystal structures of the inactive protein kinases, cdk2 (DeBont et al., 1993) and MAP kinase (Zhang et al., 1994), for example, show disruptions in the position of these two lobes compared to the active C-subunit structure (Knighton et al., 1991).

Despite the large number of expressed protein kinases for which three-dimensional information is available, elucidation of the catalytic mechanisms of these enzymes have been delayed with one noted exception. A variety of kinetic studies on the C-subunit of cAPK has been applied to understand the mechanism of catalysis of this enzyme. It has been shown through viscosometric studies that maximum turnover (i.e., k_{cat}) is controlled by a diffusion-controlled step (Adams & Taylor, 1992). This presumption implies that the rate constant for phosphoryl transfer is, at least, 10-fold higher than k_{cat}. The tighter binding of ADP compared to phosphokemptide (Cook et al., 1982a; Qamar et al., 1992; Whitehouse et al., 1983; Whitehouse & Walsh, 1983) and the dependence of metal ions on k_{cat} (Adams & Taylor, 1993a; Armstrong et al., 1979) suggest that the release of ADP limits maximum turnover. Other studies have shown that the K_m for Kemptide is more than 1 order of magnitude lower than the K_d (Cook et al., 1982b; Whitehouse & Walsh, 1983). The rapid rate of chemistry at the active site and slow release of ADP explain this phenomenon. However, no kinetic study has isolated the phosphoryl transfer step at the active site of cAPK or any protein kinase, to date, so that only a lower limit can be placed on this step.

The use of cAPK as a model system to study the pre-steady-state kinetics of protein phosphorylation has several advantages. First, the recombinant C-subunit of cAPK can be obtained in high yields and in a highly purified state from *Escherichia coli* (Slice & Taylor, 1989; Yonemoto et al., 1991). Second, the C-subunit is fully phosphorylated and active and contains no regulatory sequences. Third, detailed steady-state kinetic information is available to help interpret the pre-steady-state kinetics. The enzyme phosphorylates good substrates with a random, kinetic mechanism (Kong & Cook, 1988). In the ternary complex, E·ATP·S, the γ phosphate of ATP is transferred to the hydroxyl of serine by direct, nucleophilic displacement based on stereochemical arguments (Ho et al., 1988). The binding of a wide variety of nucleotide analogs (Bhatnagar et al., 1983; Hoppe et al., 1978) and peptide inhibitors (Glass et al., 1988, 1989; Kemp et al., 1988) has been studied. Extensive peptide studies have shown that the enzyme will efficiently phosphorylate substrates with the following consensus sequence: R-R-X-S/T-Hyd, where X is variable and Hyd is any hydrophobic amino acid (Kemp et al., 1977; Zetterqvist et al., 1990). The effects of pH on the phosphorylation of Kemptide have been measured, which suggest the possible participation of a general-base catalyst (Yoon & Cook, 1987). The internal equilibrium constant for the reaction has been measured using ^{31}P NMR and found to be highly favorable in the direction of ADP production (Qamar et al., 1992).

Rapid quench flow analyses have been used in this paper to isolate a phosphorylated peptide substrate at the active site of the C-subunit of cAPK in the millisecond time range. Pre-steady-state kinetic investigations show that this peptide is in rapid exchange with the enzyme and is rapidly phosphorylated at the active site before slower, steady-state kinetic turnover is established. The dependence of substrate concentration on the "burst" rate and amplitude provides detailed mechanistic information that can be used to predict all the steady-state kinetic data. This mechanism not only confirms the rate estimations of Adams and Taylor (1992) based on indirect viscosometric measurements but also specifies a rate value to the phosphoryl transfer step. This sets the stage for detailed kinetic analyses of mutant protein kinases and the unambiguous assignment of the individual steps in the catalytic mechanism.

MATERIALS AND METHODS

Materials. Adenosine 5′-triphosphate (ATP), 3-(*N*-morpholino)propanesulfonic acid (Mops), manganese chloride, lactate dehydrogenase, pyruvate kinase, nicotinamide adenine dinucleotide, reduced (NADH), *p*-nitrophenylacetate, and phosphoenolpyruvate were purchased from Sigma Chemicals. Magnesium chloride, phosphoric acid, and liquid scintillant were obtained from Fisher Scientific. Phosphocellulose filter disks were purchased from Whatman, and [γ-^{32}P] ATP was obtained from NEN Products.

Peptide and Enzyme. The heptameric peptides, LRRASLG (Kemptide) and LRRAALG, were synthesized at the Peptide and Oligonucleotide Facility at the University of California, San Diego. Kemptide was purified by high-performance liquid chromatography after synthesis. Kemptide concentration was determined by turnover with the C-subunit under conditions of limiting peptide in the spectrophotometric assay. Recombinant C-subunit was expressed in *E. coli* and purified according to previously published procedures (Yonemoto *et al.*, 1991). The concentration of the enzyme was measured by its absorbance at 280 nm ($A_{0.1\%} = 1.2$).

Coupled Enzyme Assay. The enzymatic activity of the C-subunit was determined as described previously (Cook *et al.*, 1982). The oxidation of NADH, monitored spectrophotometrically as an absorbance decrease at 340 nm, is coupled to the production of ADP by lactate dehydrogenase and pyruvate kinase. C-Subunit (10−50 nM) was typically incubated in a 1 mL containing 50 mM Mops (pH 7.0), 0.2 mM ATP, 10 mM magnesium chloride, 1 mM phosphoenolpyruvate, 0.3 mM NADH, 12 units of lactate dehydrogenase, and 4 units of pyruvate kinase at 23 ˚C in a Hewlett Packard spectrophotometer. For inhibition studies, the peptide, LRRAALG, was preequilibrated with the enzyme for 1 min in a total volume of 60 μL using a quartz microcell (50 μL minimum volume) in a DU 640 Beckman spectrophotometer. All reactions were initiated by adding varying amounts of Kemptide.

Solution Viscosity Measurements. The relative viscosity (η^{rel}) of buffers containing glycerol or sucrose was measured relative to a 50 mM Mops buffer at pH 7.0, 23.0 °C, using an Ostwald viscometer (Shoemaker & Garland, 1962). Relative solvent viscosities of 1.0 and 2.7 were obtained for buffers containing 0% and 30% (w/v) sucrose. The measurements were made in triplicate and did not deviate by more than 2%.

Rapid Quench Flow Measurements. Pre-steady-state kinetic measurements were made using a KinTek Corp. Model RGF-3 quench flow apparatus. The apparatus consists of three syringes driven by a stepping motor. Each reactant is pushed from a sample line into a reaction line by buffer

driven from two of the syringes. Varying reaction lines and stepping motor speeds were used to determine the reaction time (2−1000 ms). The third syringe delivers a solution that quenches the aged reaction as it exits the reaction line, and the quenched reaction is collected in a 1.5 mL plastic vial. Delivery volumes and the volumes of various lines in the apparatus were calibrated with an average error of 1% by following the absorbance of the yellow dye, p-nitrophenylacetate, under alkaline conditions (0.1 N NaOH) at 400 nm.

Quench flow experiments were typically executed by loading enzyme, buffer, magnesium chloride, and ATP into one sample loop and Kemptide into the other. Final concentrations of the reactants upon mixing were 1−4 μM C-subunit, 50 mM Mops (pH 7.0), 10 mM magnesium chloride, 0.2 mM [γ-^{32}P]ATP (600−2000 cpm pmol^{-1}), and 5−500 μM Kemptide. The reactions were quenched using 30% acetic acid, and final concentrations in the quenched reaction ranged from 21 to 25% acetic acid depending on which reaction line was utilized. Phosphorylated Kemptide was separated from unreacted ATP by a filter binding assay (Kemp et al., 1977). A portion of each quenched reaction (55 μL) was spotted onto a phosphocellulose filter disk and was washed 4 times with 0.5% phosphoric acid. The filter disks were rinsed with acetone, dried, and counted on the ^{32}P channel in liquid scintillant.

Control experiments were performed to determine the background phosphorylation (i.e., phosphorylation of Kemptide in the presence of quench) and phosphokemptide retention on washed filter disks. Enzyme, buffer, magnesium chloride, and ATP in one syringe were mixed with a solution containing 30% acetic acid and Kemptide in the another syringe such that the concentration of acetic acid in the mixed reaction loops was 15%. This percentage of acetic acid effectively quenched the phosphorylation reaction since background phosphorylation represented only 5% of the total phosphorylation over 500 ms when buffer replaced the acetic acid in one of the sample loops. The fraction of phosphokemptide retained on washed phosphocellulose disks was determined by spotting known amounts of ^{32}P-labeled phosphokemptide and calculating the percent retention after washing. The labeled product was synthesized by complete turnover of limiting amounts of Kemptide with the C-subunit. Between 5 and 500 pmol of phosphokemptide was spotted with 4500 pmols of Kemptide. These amounts represent the predicted quantity range of phosphokemptide produced and spotted on the filter disks in the rapid quench flow experiments based on steady-state kinetic parameters. In all control experiments, 62 ± 4% phosphokemptide was retained after washing. The time-dependent concentration of phosphokemptide was then determined by considering the total counts per minute (cpm) on each disk, the specific activity of the [γ-^{32}P]ATP label, the total collected volume, the background phosphorylation, and the phosphokemptide retention on washed filter disks.

Data Analysis. Data in each quench flow time course were fitted to an empirical function containing a single exponential and a linear component:

$$y = \alpha[E]_t[1 - \exp(-k_b t)] + Lt \quad (1)$$

where y is the concentration of phosphokemptide, α is the observed "burst" amplitude, k_b is the observed single-exponential "burst" rate constant, and L is the observed linear rate. Plots of the observed values for α , L, and k_b as a function of substrate concentration were fit empirically to eqs 2−4, respectively:

$$\alpha = \left(\frac{\alpha_{max}[S]}{[S] + K_\alpha}\right)^2 \quad (2)$$

$$L = \frac{L_{max}[S]}{[S] + K_L} \quad (3)$$

$$k_b = \frac{k_{max}[S]}{[S] + K_b} + k_o \quad (4)$$

where α_{max}, L_{max}, and k_{max} are the maximum values for α, L, and k_b at infinite substrate concentrations, respectively, and K_α, K_L, and K_b are observed half-maximal values for these substrate dependencies. Extrapolation of eq 4 to zero substrate concentration yields the rate constant k_o. The data were fitted using the Macintosh computer graphics program Kaleidagraph (Synergy Software), which utilizes an iterative least-squares algorithm.

Table 1: Steady-State Kinetic, Viscosometric, and Inhibition Parameters for the Phosphorylation of Kemptide by the C-Subunit of cAPK[a]

parameter	experimental	calculated[b]
k_{cat} (s^{-1})	22 ± 2	21
K_m (μM)	6.9 ± 1.7	8.1
k_{cat}/K_m (μM^{-1} s^{-1})	3.2 ± 0.84	2.5
K_I (μM)	190 ± 20	
$(k_{cat})^{\eta}$ [c]	1.0	0.96
$(k_{cat}/K_m)^{\eta}$ [c]	∼0	

[a] The data were collected using low C-subunit concentrations in the spectrophotometric enzyme-coupled assay. The variable substrate is Kemptide (LRRASLG) so that K_m reflects the apparent affinity constant for this peptide. [b] The calculated data were determined using the kinetic mechanism described in Scheme 2 and eqs 6 and 7 and the relationship $(k_{cat})^{\eta} = k_3/(k_3 + k_4)$ (Adams & Taylor, 1992). [c] These parameters were determined from two relative viscosity measurements at 1 and 2.7: $\{(k_{cat})^0/(k_{cat}) - 1\}/(\eta^{rel} - 1) = (k_{cat})^{\eta}$ and $\{(k_{cat}/K_m)^0/(k_{cat}/K_m) - 1\}/(\eta^{rel} - 1) = (k_{cat}/K_m)^{\eta}$.

RESULTS

Steady-State Kinetic, Viscosometric, and Inhibitor Studies. The k_{cat} and K_m values for Kemptide, shown in Table 1, were determined from plots of initial velocity *vs* Kemptide concentration. The data were generated under saturating concentrations of ATP (200 μM; K_m=10 μM) and 10 mM free Mg^{2+} by the enzyme coupled spectrophotometric assay (Yoon & Cook, 1987). The effect of solvent viscosity on k_{cat} was determined by measuring this parameter in the absence and presence of 30% sucrose ($\eta^{rel} = 2.7$) according to Adams and Taylor (1992). The ratio of k_{cat} in the absence and presence of this viscosogen (k_{cat}^0/k_{cat}) is 2.7 ± 0.20. This value represents the expected decrease for a steady-state kinetic parameter that is diffusion-controlled [$\{(k_{cat}^0/k_{cat}) - 1\}/(\eta^{rel} - 1) = 1$] (Brouwer & Kirsch, 1982). This effect on k_{cat} has been observed previously for the C-subunit under a variety of buffer conditions (Adams & Taylor, 1992, 1993a,b) . In these cases, the data were interpreted based on a reaction scheme that incorporated fast phosphoryl transfer and rate-determining, diffusion-controlled release of

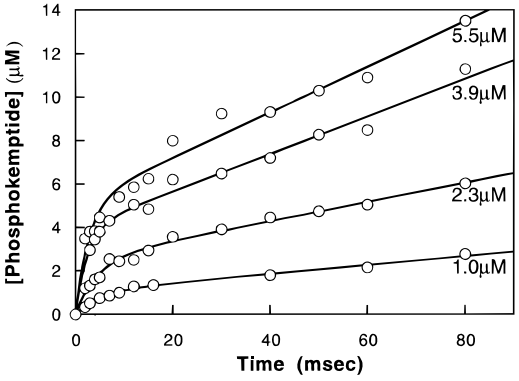

FIGURE 1: Effects of C-subunit concentration on the pre-steady-state kinetic transients using 200 μM [γ-^{32}P] ATP, 10 mM free Mg^{2+}, 100 μM Kemptide, and the indicated enzyme concentrations, 1.0, 2.3, 3.0, and 5.5 μM in 50 mM Mops (pH 7.0). The data sets were fit to eq 1, and the values for the "burst" amplitudes and the "burst" and linear rate constants are listed under Results.

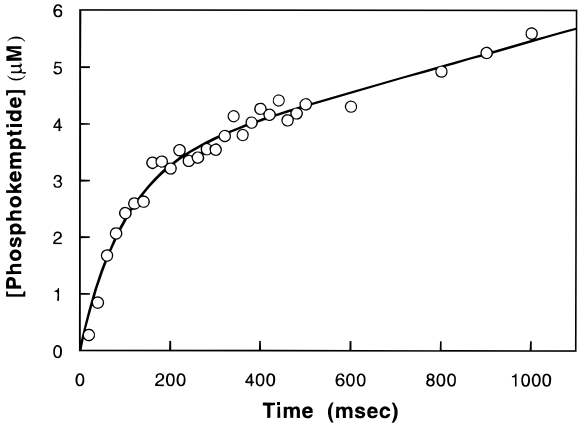

FIGURE 2: Effects of Kemptide concentration on the pre-steady-state kinetic transient using 200 μM [γ-^{32}P]ATP, 10 mM free Mn^{2+}, 3.5 μM C-subunit, and 300 μM Kemptide in 50 mM Mops (pH 7.0). Data were fit to eq 1, and values for k_b and k_L (L/[E]$_t$) of 10.4 $\pm$ 1.0 s^{-1} and 0.70 $\pm$ 0.08 s^{-1}, respectively, were obtained.

the product, ADP. The similarity of these data with those presented here for k_{cat} implies that there is no change in the rate-determining step under these new buffer conditions. Since the K_m for Kemptide is low (Table 1), it was difficult to measure accurately the effects of solvent viscosity on k_{cat}/K_m. To circumvent this problem, solvent viscosity measurements were made in the presence of 2 mM LRRAALG, a competitive inhibitor, in the absence and presence of 30% sucrose ($\eta^{rel} = 2.7$). The competitive inhibitor raised the apparent K_m for the substrate without affecting k_{cat}. No effect of solvent viscosity was detected on the apparent k_{cat}/K_m [$\{(k_{cat}/K_m)^0/(k_{cat}/K_m) - 1\}/(\eta^{rel} - 1) = 0$] (data not shown). These data are consistent with other viscosity data for the C-subunit and imply that Kemptide is in rapid exchange with the enzyme under these buffer conditions (Adams & Taylor, 1992, 1993a). The K_I for LRRAALG was measured at 0 and 30% sucrose and found to be 190 $\pm$ 20 μM (Table 1) under both conditions, indicating that viscous buffers do not influence inhibitor binding. This provides a control for the determination of the apparent k_{cat}/K_m in the presence of a competitive inhibitor.

Effects of Enzyme Concentration and Divalent Metal Ions on the "Burst" Parameters. Plots of phosphokemptide concentration *vs* time were generated using four different C-subunit concentrations (1.0, 2.3, 3.9, and 5.5 μM), 200 μM [γ-^{32}P]ATP, 10 mM free Mg^{2+}, and 100 μM Kemptide. The kinetic transients at each enzyme concentration are displayed in Figure 1. The data were collected from 2 to 80 ms and fitted to eq 1. The observed "burst" amplitudes (α) for each curve fit represented approximately 100% of the total enzyme concentration ([E]$_t$, α[E]$_t$: 1.0, 1.03 $\pm$ 0.09; 2.3, 2.49 $\pm$ 0.16; 3.9, 3.91 $\pm$ 0.23; and 5.5, 5.11 $\pm$ 0.38 μM). The observed exponential "burst" rate constant (k_b) measured from these curves was 250 $\pm$ 40 s^{-1}, and the linear rate constant (L/[E]$_t$) was 21 $\pm$ 1.3 s^{-1}. This linear rate constant matched the C-subunit's maximum turnover rate of 22 $\pm$ 2 s^{-1} measured in the enzyme-coupled spectrophotometric assay (Table 1).

The phosphorylation of Kemptide was monitored in the presence of the divalent metal ion Mn^{2+}. Figure 2 shows the production of phosphokemptide *vs* time when 3.5 μM enzyme, 200 μM [γ-^{32}P]ATP, and 10 mM free Mn^{2+} were mixed with 300 μM Kemptide. Metal substitution had pronounced effects on both k_b and k_L (L/[E]$_t$). The decrease in the magnitude of the observed "burst" rate permitted a more detailed analysis of this phase. In Figure 1, approximately 60% of the "burst" amplitude is lost in the instrument deadtime (2 ms). The fitting of the "burst" phase in Figure 2 to a single, slower exponential indicates that the rapid phosphorylation of Kemptide occurs in a single, observable step.

Substrate Concentration Effect on the "Burst" Phase. Plots of phosphokemptide concentration vs time were generated using 1−3 μM enzyme, 200−600 μM [γ-^{32}P]ATP, and 10 mM free Mg^{2+} under conditions of variable concentrations of Kemptide (5−400 μM). The plots of the normalized "burst" amplitude (α) and observed linear rate constants (k_L) *vs* Kemptide concentration are displayed in Figure 3A. The data points for α and k_L were fit to eqs 2 and 3, respectively. The maximal value for the "burst" amplitude, α_{max}, and the half maximal value, K_α, are listed in Table 2. The maximal linear rate constant, k_L^{max} (L_{max}/[E]$_t$), and the half-maximal value, K_L, are also listed in Table 2**.** A plot of the observed "burst" rate constant *vs* Kemptide concentration is depicted in Figure 3B. The data were fit to eq 4 to obtain the maximum value for the "burst" rate constant, k_{max}, the half-maximal value, K_b, and the *y*-intercept, k_o. These parameter fits are summarized in Table 2.

As shown in Figure 3B, Kemptide concentrations of 400 μM (data not shown) were the highest values used in the rapid quench flow apparatus. Above this value, decreases in the linear rate were detected (data not shown). This substrate inhibition has been observed previously and has been interpreted according to a kinetic mechanism involving the population of an unproductive enzyme−substrate complex (Adams & Taylor, 1992). In this mechanism, a slow, viscosity-independent conformational change precedes phosphoryl group transfer and lowers the apparent k_{cat}. Increasing the concentration of ATP above 600 μM restores the expected k_{cat} value of 21 s^{-1} (Table 2) but makes an accurate measurement of the "burst" phase difficult owing to a low specific activity for ATP (data not shown).

DISCUSSION

The widespread involvement of protein kinases in signal transduction pathways has sparked an intensive investigation of the structure and catalytic properties of these enzymes.

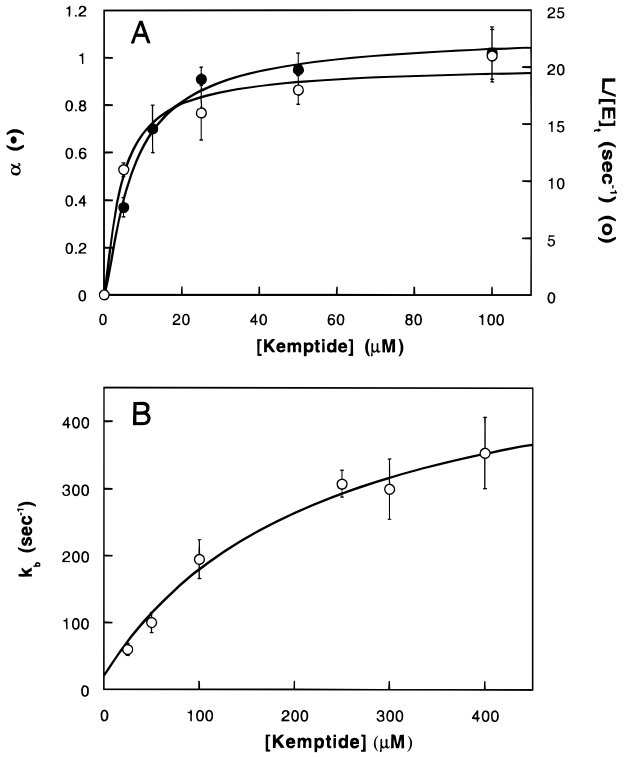

FIGURE 3: Effects of substrate concentration on the "burst" and linear rate constants and "burst" amplitude. Each plot was generated from a series of pre-steady-state kinetic transients at different Kemptide concentrations (5−400 μM) using 200−600 μM [γ-^{32}P]-ATP, 10 mM free Mg^{2+}, and 1−3 μM C-subunit in 50 mM Mops (pH 7.0). The individual rate and amplitude parameters were determined from eq 1. (A) The observed "burst" amplitudes, α (filled circles), and the linear rate constants, $k_L = L/[E]_t$ (open circles), are plotted as a function of Kemptide concentration using eqs 2 and 3, respectively. (B) The "burst" rate constant (k_b) is plotted as a function of Kemptide concentration using eq 4.

Table 2: Parameter Fits to Rapid Quench Flow Data for the Phosphorylation of Kemptide by the C-Subunit of cAPK[a]

parameters	Scheme 1[b]	experimental	calculated[c]
α_{max}	$[k_3/(k_3 + k_4)]^2$	1.0 ± 0.01	0.92
$L_{max}/[E]_t$ (s^{-1})	k_4	21 ± 1	
k_{max} (s^{-1})	k_3	500 ± 60	
k_0 (s^{-1})	k_4	18 ± 6	
K_α (μM)	$K_d[k_4/(k_3 + k_4)]$	3.4 ± 0.32	8.1
K_L (μM)	$K_d[k_4/(k_3 + k_4)]$	4.9 ± 1.4	8.1
K_b (μM)	K_d	200 ± 60	

[a] These experimental data were determined by fitting the rapid quench flow data to eqs 2−4) [b] These rate and thermodynamic constants are extracted from Scheme 1 and eqs 5−7. [c] The calculated data were obtained from the evaluation of eq 5 using the empirical fits to eq 4.

The field has been advanced by the recent crystal structure solutions for several protein kinases including the first structure, the C-subunit of cAPK (Knighton et al., 1991). The atomic resolution of individual amino acids within the structure has prompted speculation on the catalytic mechanism for protein phosphorylation. The available structures suggest that ATP and peptide substrates are positioned in the active site for general-base-catalyzed phosphoryl group transfer. Asp-166 in the C-subunit is strictly conserved in all protein kinases and makes a well-defined hydrogen bond with the hydroxyl group of the substrate (Madhusudan et al., 1994). This finding underpins speculation that Asp-166 acts as a general-base catalyst in phosphoryl transfer. Other residues in the active site appear to be important for nucleotide and substrate recognition. Proof for the specific involvement of these and other residues in certain aspects of phosphoryl group transfer will come, in part, from the kinetic study of mutant forms of the enzymes.

A necessary prerequisite for the assignment of certain active-site residues in protein phosphorylation is a detailed kinetic mechanism for the wild-type enzyme. The use of viscosometric studies on the C-subunit of cAPK showed that maximum substrate turnover is diffusion-controlled, placing a lower limit on the rate of phosphoryl transfer at the enzyme's active site of 200 s^{-1} (Adams & Taylor, 1992). Although the rate of this transfer does not influence maximum turnover, its value directly impacts the specificity constant of the enzyme, k_{cat}/K_m. In order to understand the catalytic efficiency of protein kinases and to establish a framework for the comparison of mutant enzyme forms, it is essential to measure the rate of phosphoryl transfer at the active site. We have chosen the C-subunit of cAPK as a model system for the first pre-steady-state kinetic analysis of a protein kinase. We selected this enzyme based on its ease of expression in bacterial hosts (Slice & Taylor, 1989; Yonemoto et al., 1991), the availability of steady-state kinetic studies (Adams & Taylor, 1992; Kong & Cook, 1988; Whitehouse & Walsh, 1983; Yoon & Cook, 1987), and the X-ray solution of several forms of the enzyme including binary and ternary complexes of nucleotide and peptide substrate, product, and inhibitor molecules (Knighton et al., 1991; Madhusudan et al., 1994; Zheng et al., 1993). In this paper, we use rapid quench flow experiments to monitor the production of phosphorylated peptide in the ms time range. The results of this study place a discreet value on the rate constant for phosphoryl group transfer at the active site of the enzyme and provide a complete kinetic mechanism describing all steps of the kinetic mechanism.

Viscosometric and Inhibitor Studies. The phosphorylation of Kemptide was monitored at low C-subunit concentrations using a coupled spectrophotometric assay. The steady-state kinetic parameters are measured under conditions of 10 mM free Mg^{2+} and 200 μM ATP in 50 mM Mops (pH 7.0) and are reported in Table 1. Solvent viscosity was found to influence profoundly these parameters. A maximum effect of viscosity on k_{cat} was measured while no effect was measured on the second-order rate constant, k_{cat}/K_m (Table 1). These results are consistent with previous published data on the phosphorylation of Kemptide under different buffer conditions of 100 mM Tris, pH 8.0 (Adams & Taylor, 1992), and imply that the substrate is in rapid exchange with the enzyme (i.e., the dissociation of the substrate is fast relative to phosphoryl transfer) and is phosphorylated by a rate constant that is, at least, 10-fold larger than the net release rate of the products. Although this provides valuable information on the rate-determining step in the enzyme reaction, it places only a lower limit on the rate constant for phosphoryl transfer. Furthermore, only a lower limit on the dissociation rate constant for Kemptide can be measured so that no exact K_d can be determined.

The value of K_m for Kemptide under these buffer conditions (Table 1) is lower than those measured under other buffer conditions. For example, the K_m values for Kemptide are 60 and 30 μM in 100 mM Tris, pH 8 (Adams & Taylor, 1992), and 100 mM Mops, pH 7 (Yoon & Cook, 1987), respectively. Since the K_m value is a complex collection of

terms that include the binding of the peptide, phosphoryl transfer, and product release, the K_I for a competitive inhibitor was measured to determine whether the lower K_m under our conditions may be due, in part, to a tighter affinity of the peptide. The K_I for the competitive inhibitor, LRRAALG, was measured using a Dixon plot and was found to be 190 ± 20 μM (Table 1) . This compares with the value of 2400 μM in 100 mM Mops, pH 7 (Kong & Cook, 1988), and 800 μM in a multicomponent buffer system (Adams & Taylor, 1993b). The binding of the inhibitor is clearly sensitive to buffer conditions which implies that the binding of Kemptide may also show some differences depending upon the selection of the buffer. Furthermore, these differences are not limited to the nature and concentration of the buffer components but are also dependent upon the concentration of free Mg^{2+}. At 0.5 mM free Mg^{2+}, a K_I value of 270 μM has been reported for LRRAALG (Kong & Cook, 1988) in 100 mM Mops, an order of magnitude lower than the value at 10 mM free Mg^{2+}. Owing to these apparent differences in the binding of peptides to the C-subunit, the comparison of data under identical conditions of buffer, pH, and divalent metal ion concentration is important. This is particularly relevant for this study since the data from steady-state and pre-steady-state kinetic analyses are compared.

Pre-Steady-State Kinetic Measurements. Although steady-state kinetic measurements are important first steps toward characterizing an enzyme, they are limited in their ability to provide detailed mechanistic information regarding how a substrate is converted to product in the active site (Jencks, 1969). However, the steady-state kinetic approach can be coupled to more powerful techniques such as pH, isotope, or viscosometric studies to provide added mechanistic information. The use of pre-steady-state kinetic analyses is perhaps the most useful tool for the elucidation of enzyme reaction mechanisms. Rapid quench flow techniques have been applied in this paper to elucidate fully the kinetic mechanism of the C-subunit of cAPK. The viscosometric data presented in Table 1 predict that a "burst" of phosphorylated peptide [LRRAS(P)LG] will be measured in the first enzyme turnover and that subsequent turnover will be limited by a diffusive step, namely the release of the product ADP. Furthermore, these data indicate that the substrate is in rapid exchange with the enzyme and that the low K_m for Kemptide is due to the slow desorption of the product (Adams & Taylor, 1992).

The time-dependent measurement of phosphokemptide shows a rapid exponential formation of product ("burst") within the first 10 ms of the reaction (Figure 1). This "burst" phase ($k_b = 250\ s^{-1}$) then quickly curtails into a linear phase that is consistent with maximum steady-state turnover ($L/[E]_t = k_L = 21\ s^{-1}$). The amplitude of the "burst" phase is enzyme-dependent and represents on average 100% of the total enzyme concentration. These observations indicate decisively that the "burst" phase is not due to rapid product inhibition and that all the enzyme active sites are available for catalysis (Hartley & Kilby, 1954). Since the first available time point in these studies is taken at 2 ms (instrument deadtime) and the observed "burst" rate at 100 μM Kemptide is 250 s^{-1}, approximately 60% of the single-exponential phase is unobservable. To address the concern that the actual "burst" phase may be more complicated than a simple, single exponential, rapid quench studies were performed using 10 mM Mn^{2+} (Figure 2). Previous viscosometric studies demonstrated that the rate of phosphoryl transfer using this divalent metal ion is 14 s^{-1}, a value that is more than 15-fold lower than the value in the presence of Mg^{2+} (Adams & Taylor, 1993a). The production of phosphokemptide in the presence of Mn^{2+} is similar in form to that in the presence of Mg^{2+} (Figure 1) although the "burst" rate constant and linear rate are lower. The 50- and 30-fold lower values for k_b and k_L measured in the presence of Mn^{2+} are thoroughly consistent with estimations based on viscosometric data (Adams & Taylor, 1993a). The ability to measure a single-exponential "burst" phase over 200 ms indicates that the rapid production of phosphokemptide occurs in a single, observable step in the presence of Mn^{2+}. We presume that this is also true for the "burst" phase in the presence of Mg^{2+}.

The effects of Kemptide concentration on the pre-steady-state kinetic transients were measured to gain further insights into the kinetics of phosphoryl group transfer. The three critical parameters that define the "burst", α, k_b, and L, vary hyperbolically with the concentration of Kemptide (Figure 3A,B). In order to describe mathematically these phenomena, a kinetic mechanism was written for the phosphorylation of Kemptide by the C-subunit. Scheme 1 describes a three-step mechanism for Kemptide turnover at saturating ATP concentration.

Scheme 1

$$E\cdot ATP + S \overset{K_d}{\rightleftharpoons} E\cdot ATP\cdot S \xrightarrow{k_3} E\cdot ADP\cdot P \xrightarrow{k_4} E + ADP + P$$

In this mechanism, K_d is the thermodynamic dissociation constant for the substrate in the ternary E·ATP·S complex, k_3 is the absolute rate constant for phosphoryl transfer from ATP to the substrate at the active site, and k_4 is the net dissociation constant for the products. The latter step is presumed to be limited by the release of ADP owing to the relative K_d values for both products (Cook et al., 1982a; Whitehouse et al., 1983; Whitehouse & Walsh, 1983). The viscosity data in Table 1 show that Kemptide is released from the ternary complex at a rate that is much higher than the rate of phosphoryl transfer so that substrate binding in the first step can be treated as a rapid equilibrium (K_d). The integrated rate law for Scheme 1 that describes the appearance of product and enzyme-bound product as a function of time has been previously determined for a general enzyme reaction (Gutfreund, 1971, 1975) and is presented in eq 5 for the kinetic mechanism in Scheme 1

$$\frac{[E\cdot ADP\cdot P] + [P]}{[E]_t} = \alpha[1 - \exp(-k_b t)] + k_L t \qquad (5)$$

where

$$\alpha = \left\{\frac{k_3}{k_3 + k_4}\left(\frac{[S]}{[S] + K_m}\right)\right\}^2$$

$$k_b = \frac{k_3[S]}{K_d + [S]} + k_4$$

$$k_L = \frac{k_{cat}[S]}{K_m + [S]}$$

Equation 5 has the same form as the empirical description

of eq 1 and predicts that the appearance of phosphokemptide will occur in two distinct phases—an exponential and a linear phase, and that the three defining parameters, α, k_b, and k_L, will vary hyperbolically with the substrate concentration. The kinetic transients in Figures 1, 2, and 3 are consistent with the predictions of eq 5. By fitting the data in Figure 3A,B to eqs 2−4, all the kinetic and thermodynamic parameters in Scheme 1 can be calculated (Table 2). Fitting of the observed amplitude data (α) to eq 2 provides α_{max} and K_α, which correspond to $[k_3/(k_3 + k_4)]^2$ and K_m, respectively. Fitting of the observed linear rate (L) to eq 3 provides L_{max} and K_L, which correspond to k_{cat} and K_m, respectively. Also, fitting the observed "burst" rate (k_b) data to eq 4 provides k_{max}, K_b, and k_o, which correspond to k_3, K_d, and k_4, respectively, in Scheme 1. Fitting of the pre-steady-state kinetic data in Figure 3B to eq 4 establishes a kinetic solution for Scheme 1. This new mechanism is depicted in Scheme 2.

Scheme 2

$$E\cdot ATP + S \underset{}{\overset{200\ \mu M}{\rightleftharpoons}} E\cdot ATP\cdot S \xrightarrow{500\ s^{-1}} E\cdot ADP\cdot P \xrightarrow{21\ s^{-1}} E + ADP + P$$

The kinetic mechanism in Scheme 2 offers two important parameters not available from the viscosometric data. First, the rate constant for phosphoryl group transfer, k_3, is now evaluated directly. Adams and Taylor (1992) predicted that this rate constant is, at least, 200 s^{-1}. Analysis of the pre-steady-state kinetic transients shows that the true rate constant is 2.5-fold larger than this lower limit. Second, the pre-steady-state kinetic data provide a direct measurement of the K_d for Kemptide to the active ternary complex, E·ATP·S. Prior to this study, the binding of Kemptide to the C-subunit could only be estimated from mock ternary complexes with ATP analogs (Whitehouse et al., 1983). The larger values of K_I and K_d for the inhibitor peptide, LRRAALG, compared to the K_m for Kemptide have led some to conclude that the γ-phosphate of ATP improves the binding of the substrate (Whitehouse et al., 1983). The pre-steady-state kinetic data prove categorically that this assertion is false. The dependence of the observed burst rate constant on Kemptide concentration (Figure 3B), when fitted to eq 5, provides directly the dissociation constant for substrate in the ternary E·ATP·S complex. The equivalence between the true K_d for Kemptide to the active ternary complex and the K_I for LRRAALG (Table 2) indicates that any potential H-bond between Asp-166 and the serine hydroxyl group of Kemptide does not stabilize the substrate at the active site and that this competitive inhibitor can be used to measure the true affinity of the substrate. We conclude from these data that the hydrogen bond, determined from the X-ray solution of a substrate complex of the C-subunit (Madhusudan et al., 1994), does not lower the ground state energy of the Michaelis complex (i.e., lower K_d) but rather isolates a productive rotamer for in-line phosphoryl transfer.

Correlation between Steady- and Pre-Steady-State Kinetic Data. The kinetic mechanism in Scheme 2, derived from pre-steady-state kinetic data, is consistent with all the steady-state kinetic data determined at low C-subunit concentrations in identical buffer conditions. The steady-state kinetic parameters for Scheme 1, k_{cat} and K_m, are shown in eqs 6 and 7:

$$k_{cat} = \frac{k_3 k_4}{k_3 + k_4} \tag{6}$$

$$K_m = k_d\left(\frac{k_4}{k_3 + k_4}\right) \tag{7}$$

By substituting the values derived from pre-steady-state data for K_d, k_3, and k_4 (Scheme 2) into eqs 6 and 7, the steady-state kinetic parameters can be predicted. The calculated values for k_{cat}, K_m, and k_{cat}/K_m are presented along with their experimental values in Table 1. Furthermore, the maximal amplitude, α_{max}, and the half-maximal values for this parameter and that for the linear rate, L, can also be calculated from the kinetic mechanism in Scheme 2 using eq 5. These calculated values are listed along with their corresponding experimental values in Table 2. The agreement between these data sets verifies that the kinetic mechanism in Scheme 2 is an appropriate solution.

The studies in this paper detail the first observation of phosphoryl group transfer at the active site of a protein kinase and, as such, contribute significantly to our understanding of this group of catalysts. This new kinetic mechanism describes an enzyme that transfers the γ-phosphate of ATP to a peptide substrate at a remarkable rate constant of 500 s^{-1}. In fact, this rate constant is only 50% lower than the rate constant for hydride transfer in the active site of the NADPH-dependent dihydrofolate reductase (Fierke et al., 1987). This rate constant is, by no means, an upper limit for phosphoryl exchange at the active site of an enzyme. For example, the rate constant for nucleotide incorporation into DNA, catalyzed by T7 polymerase, is estimated to be 9000 s^{-1} or more (Patel et al., 1991). What is the advantage of phosphorylating proteins with rate constants that vastly exceed the rate of release of the product, ADP? The answer to this query lies in the steady-state kinetic parameter, k_{cat}/K_m. For cAPK, k_{cat}/K_m is equivalent to the ratio of the phosphoryl transfer rate constant and the dissociation constant for the substrate (i.e., $k_{cat}/K_m = k_3/K_d$). The C-subunit maintains a high value for this parameter by optimizing the rate constant for the chemical step. Any changes in k_3 would be directly reflected in k_{cat}/K_m. This compares with enzymes that bind their substrates tightly, show diffusion-limited kinetics for k_{cat}/K_m, and do not benefit from any increases in the chemical step. The kinetic mechanism in Scheme 2 permits the evaluation of the catalytic efficiency of cAPK and serves as a foundation for the study of mutant forms of the enzyme so that the value of specific residues at the active site can be assessed.

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