Participation of ADP Dissociation in the Rate-Determining Step in cAMP-Dependent Protein Kinase†

Jie Zhou and Joseph A. Adams*

Department of Chemistry, San Diego State University, San Diego, California 92182-1030

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ABSTRACT: Pre-steady-state kinetic analyses of the catalytic subunit of cAMP-dependent protein kinase showed that the rate constant for phosphoryl transfer is fast and either the release of one or both of the products or a conformational change controls turnover [Grant, B., & Adams, J. A. (1996) *Biochemistry 35*, 2022−2029]. To determine which step or steps control turnover in the wild-type enzyme, we used a catalytic trapping technique to measure directly the dissociation rate constant for ADP. The phosphorylation of two peptide substrates, LRRASLG and GRTGRRNSI, was monitored using a rapid quench flow technique under conditions where saturating concentrations of ADP were preequilibrated with the enzyme before excess ATP and one of the substrates were added to trap the free enzyme and to start the phosphorylation reaction. Under ADP preequilibration conditions, no 'burst' phase was observed, and although the rate of linear, steady-state turnover was unaffected, the net production of phosphopeptide lagged behind the non-preequilibrated control. This phenomenon occurs due to the slow release of the product, and kinetic modeling suggests that this effect can be explained if the dissociation rate constant for ADP is 24 s^{-1} and solely limits turnover (k_{cat} = 23 s^{-1}) for the phosphorylation of LRRASLG. Using GRTGRRNSI, the dissociation rate constant for ADP is 35 s^{-1} and limits turnover (k_{cat} = 29 s^{-1}) if the reaction is initiated by the addition of enzyme. Under preequilibration conditions with either ATP or GRTGRRNSI, turnover is approximately 50% lower, suggesting that ADP release may partially control this parameter. This preequilibration effect can be explained by slowly interconverting enzyme forms with specific peptide-induced turnover properties. These studies indicate that ADP release is an essential rate-limiting component for turnover but also suggests that other factors contribute subtly when the structure of the substrate is altered.

The role of protein phosphorylation in normal cell function is an area of continued interest in biological chemistry. The protein kinases are now recognized as key enzymes that facilitate this posttranslational modification, and their catalytic activity has been shown to moderate many processes such as sugar and lipid metabolism, DNA replication and transcription, secretory pathways, and cell cycle progression. The prevalence of these enzymes in signal transduction pathways has prompted intensive investigations into the structure of these enzymes. X-ray crystal structures for several protein kinases in differing stages of activation are now available and set the stage for thorough structure−function studies. The demonstration that cyclin A binding and phosphorylation alter the structure of the cyclin-dependent kinase, Cd*k*2, producing a fully active enzyme that controls cell cycle progression, illustrates the advances made in recent years (DeBont et al., 1993; Jeffrey et al., 1995; Russo et al., 1996). Despite the considerable gains extended by crystallographic analyses, less is known about the kinetic mechanisms of these catalysts. The apparent lag between structural and kinetic studies arises from several factors including low protein expression in host cells, difficulty in obtaining fully active forms, and difficulty in physically separating regulatory and catalytic portions within larger multidomain proteins.

The best understood protein kinase from a kinetic perspective is the catalytic subunit of cAMP-dependent protein kinase (PKA).[1] PKA phosphorylates short peptide substrates with the general consensus sequences R-R-X-S/T-hyd and R-X-X-R-X-X-S/T-hyd, where X is variable and hyd is any hydrophobic amino acid (Kemp et al., 1976, 1977; Zetterqvist et al., 1990). PKA binds randomly ATP and the seven residue peptide substrate Kemptide (LRRASLG), although the initial binding of ATP is preferred in the absence of catalysis at high Mg^{2+} concentrations (Cook et al., 1982b; Kong & Cook, 1988). Pre-steady-state kinetic analyses demonstrate that the rate of Kemptide phosphorylation (500 s^{-1}) in the active site is 25-fold larger than turnover, explaining why the K_m is more than 10-fold lower than the K_d for Kemptide (Grant & Adams, 1996). While a viscosity dependence on turnover suggests that the release of one or both of the products, ADP or phosphokemptide, is rate-determining for k_{cat}, the participation of a slow, viscosity-dependent conformational change cannot be ruled out (Adams & Taylor, 1992). In fact, stopped-flow kinetic studies have demonstrated that maximum turnover for an acrylodan-labeled mutant of PKA is partially controlled by

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* Author to whom correspondence should be addressed. Telephone: (619) 594-6196. Fax: (619) 594-1879. E-mail: jadams@sundown.sdsu.edu.

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[1] Abbreviations: Kemptide, peptide sequence LRRASLG; MTCN, 50 mM Mes, 25 mM Tris, 25 mM Caps, 50 mM NaCl; phosphokemptide, phosphorylated Kemptide [LRRAS(P)LG]; PKA, catalytic subunit of cAMP-dependent protein kinase; PKI, protein kinase inhibitor; PKS-(14−22), peptide sequence GRTGRRNSI.

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a conformational change (Lew et al., 1997). Although the steady-state kinetic parameters for this mutant are only modestly perturbed, these results do not conclusively show that a conformational change is rate-determining for the wild-type enzyme. Finally, steady-state kinetic analyses of the reverse reaction (i.e., ADP phosphorylation) demonstrate that product binding is ordered (Qamar et al., 1992), supporting a mechanism in which phosphopeptide is released prior to ADP in the forward direction. While this is useful information that helps to establish a complete kinetic mechanism for PKA, it does not define the rate-determining step for turnover.

We developed a rapid quench flow method for measuring directly the rate constant for ADP dissociation from the wild-type catalytic subunit of PKA. In the absence of ADP, PKA rapidly binds and phosphorylates Kemptide in the first 10 ms of the reaction, producing a large, enzyme stoichiometric 'burst' in phosphokemptide formation. If ADP release is the rate-determining step for k_{cat}, it is expected that pre-equilibration of the enzyme with this product prior to rapid mixing with excess ATP and substrate will displace the 'burst' phase since catalysis would be delayed by the dissociation of this ligand. Likewise, if ADP release is very fast, then little or no effect of preequilibration on the 'burst' phase would be observed. Intermediate cases where ADP release is partially rate-limiting can be discerned by modeling the 'burst' reaction. We have employed this methodology to determine the rate at which ADP dissociates from the active site of wild-type PKA. The method shows definitively that while conformational changes may be essential for catalysis, the sole, rate-determining step in turnover is ADP dissociation for the phosphorylation of Kemptide. In contrast, turnover for a tight binding substrate, GRTGRRNSI, is partially limited by ADP dissociation when the enzyme is preequilibrated with ATP. The data provide an active role for the substrate in controlling turnover.

MATERIALS AND METHODS

Materials. Adenosine triphosphate (ATP), 3-(cyclohexylamino)-1-propanesulfonic acid (Caps), 2-(*N*-morpholino)-ethanesulfonic acid (Mes), pyruvate kinase (type II from rabbit muscle), lactate dehydrogenase (type II from bovine heart), nicotinamide adenine dinucleotide, reduced form (NADH), 3-(*N*-morpholino)propanesulfonic acid (Mops), sodium chloride, phosphoenolpyruvate, and tris(hydroxymethyl)aminomethane (Tris) were purchased from Sigma Chemicals. 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. Kemptide and PKS(14−22) were synthesized and purified by the USC Microchemical Core Facility. The concentrations of Kemptide and PKS(14−22) were determined by complete turnover with PKA under conditions of limiting peptide in the spectrophotometric assay. Recombinant catalytic 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), and its activity was measured using Kemptide as a substrate.

Activity Assay. The activity of the catalytic subunit was determined in a coupled enzyme assay as described previously (Cook et al., 1982a). 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. All reactions were measured in a Beckman DU640 spectrophotometer equipped with a microcuvette holder. Typical steady-state kinetic assays were performed in 50 mM Mops (pH 7) or MTCN buffer (pH 7) in a final volume of 60 μL at 24 °C. PKA (30−60 nM) was typically incubated with 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 for several minutes before initiating the reaction with peptide. The free concentration of divalent metal ion was approximately 10 mM in all assays.

Rapid Quench Flow Measurements. Pre-steady-state kinetic measurements were made using a KinTek Corp. Quench Flow Apparatus Model RGF-3. Quench flow experiments were typically executed by preequilibrating enzyme and magnesium chloride in one sample loop and peptide, magnesium chloride, and [γ-^{32}P]ATP in the other. Final concentrations of the reactants upon mixing were 1.5−2.3 μM PKA, 10 mM free magnesium chloride, 0.2−1.9 mM [γ-^{32}P]ATP (1000−2000 cpm/pmol), and 100 μM peptide substrate in 50 mM Mops (pH 7) or pH 7 MTCN buffer. All reported concentrations are those in the mixing chamber of the instrument prior to reaction start. The reactions were quenched with 30% acetic acid, and the phosphorylated peptide 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, washed 4 times with 0.5% phosphoric acid and once 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 or PKS(14−22) in the presence of quench] and phosphopeptide retention on washed filter disks according to previously published procedures (Grant & Adams, 1996). Enzyme, buffer, and magnesium chloride in one syringe were mixed with a solution containing 30% acetic acid, ATP, and peptide substrate in the another syringe. This percentage of acetic acid effectively quenched the phosphorylation reaction since background phosphorylation represented only 5% of the total phosphorylation over 100 ms when buffer replaced the acetic acid in one of the sample loops. The fraction of phosphopeptide retained on washed phosphocellulose disks was determined by spotting known amounts of ^{32}P-labeled phosphopeptide and calculating the percent retention after washing. The labeled phosphopeptide was synthesized by complete turnover of limiting amounts of Kemptide or PKS(14−22) with PKA. Between 5 and 500 pmol of labeled phosphopeptide was spotted with 4500−5000 pmol of Kemptide or PKS(14−22). These amounts represent the predicted quantity range of phosphopeptide produced and spotted on the filter disks in the rapid quench flow experiments based on steady-state kinetic parameters. In control experiments, 60 ± 4% of the phosphokemptide and 72 ± 5% of the PKS(14−22) phosphopeptide were retained after washing. The time-dependent concentration of phosphopeptide 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 phosphopeptide retention on washed filter disks.

Catalytic Trapping Experiments. A catalytic trapping experiment was performed to measure the effects of ligand preequilibration on the phosphorylation reaction. In this experiment, PKA (2−2.3 μM), $MgCl_2$ (10 mM), and varying amounts of ADP (100−600 μM, final concentration) were preequilibrated in the sample loop before being mixed with Kemptide or PKS(14−22) (100 μM), $MgCl_2$ (10 mM), and varying amounts of ATP (400−1500 μM) from the other sample loop. Control experiments were performed under identical ATP and substrate levels without ADP preequilibration for comparison. The isolation and quantification of the phosphopeptide were conducted as already described in the previous section.

Data Analysis. The steady-state kinetic parameters, V_{max} and K_m, were obtained by fitting the initial reaction velocity to the Michaelis−Menten equation. Data in each quench flow time course were fitted to an empirical function containing a single exponential and a linear component in the absence of ADP:

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

where y is the concentration of phosphopeptide, α is the observed 'burst' amplitude, k_b is the observed exponential 'burst' rate constant, and L is the observed linear rate. The data were fitted using the Macintosh computer graphics program Kaleidagraph (Synergy Software), which utilizes an iterative least-squares algorithm. The pre-steady-state kinetic traces in the absence and presence of ADP preequilibration were modeled using HopKINSIM, a Macintosh version of the numerical integration program KINSIM (Barshop et al., 1983).

RESULTS

Pre-Steady-State Kinetic Studies in the Absence of ADP Preequilibration. The phosphorylation of Kemptide on short time scales was monitored using a rapid quench flow instrument. Figure 2 shows the formation of phosphokemptide in the first 100 ms under conditions of 1.0 mM ATP, 100 μM Kemptide, 1.9 μM PKA, and 10 mM free Mg^{2+} in 50 mM Mops (pH 7). The production of product is biphasic with a rapid exponential 'burst' phase followed by a linear phase. The data were fit to eq 1, and the values for α, k_b, and k_L (L/[E]) are reported in Table 1. Similar experiments were performed on the phosphorylation of PKS(14−22). Figure 3 shows the production of phosphopeptide as a function of time under conditions of 1.0 mM ATP, 100 μM PKS(14−22), 2.2 μM PKA, and 10 mM free Mg^{2+} in pH 7 MTCN buffer. This transient also observes 'burst' kinetics as in the case of Kemptide. The data were fitted to eq 1, and the parameter fits are displayed in Table 1. These results for the phosphorylation of both peptide substrates are consistent with previously published values under similar reaction and buffer conditions (Grant & Adams, 1996; Zhou & Adams, 1997).

Effects of ADP on the 'Burst' Kinetics. Rapid quench flow studies were performed for both peptides when PKA was preequilibrated with ADP. Figure 2 shows the formation of phosphokemptide in the first 100 ms when PKA was preequilibrated with 400 μM ADP prior to the addition of ATP and Kemptide in 50 mM Mops (pH 7). The amounts

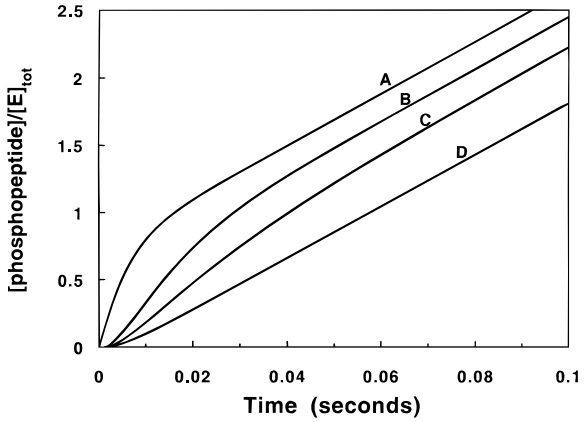


FIGURE 1: Predicted time-dependent formation of phosphokemptide either in the absence (A) or in the presence of ADP preequilibration (B−D) when the dissociation rate constant for ADP, k_{off}, has the following values: $k_{off} = 4 \times k_4$ (B); $k_{off} = 2 \times k_4$ (C); $k_{off} = k_4$ (D). All curves were generated using the kinetic simulation program KINSIM (Barshop et al., 1983) and the kinetic mechanism outlined in Scheme 1. The following kinetic rate constants were used to obtain all curves: $k_2 = 10\ \mu M^{-1}\ s^{-1}$; $k_{-2} = 2000\ s^{-1}$; $k_3 = 500\ s^{-1}$; $k_4 = 23\ s^{-1}$. The binding of ATP is assumed to be rapid and favorable so that ideal trapping conditions are obtained (i.e., ADP does not reassociate with the enzyme).

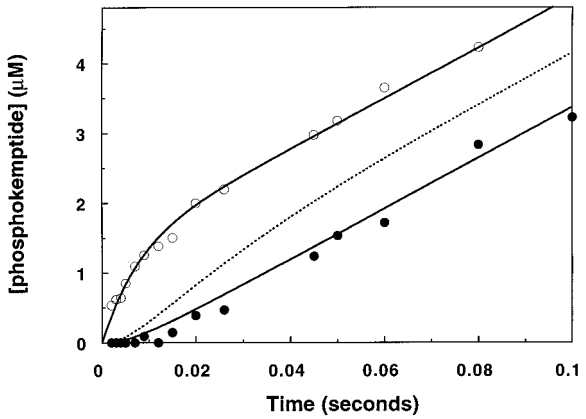


FIGURE 2: Production of phosphokemptide in the absence (○) and presence (●) of ADP at 10 mM free Mg^{2+}. The concentrations of PKA, ATP, and Kemptide are 1.9 μM, 1.0 mM, and 100 μM, respectively, in 50 mM Mops (pH 7.0). PKA was preequilibrated with 400 μM ADP (concentration in the mixing chamber) prior to the addition of ATP and Kemptide for one of the two data sets. The lines drawn through the data were obtained from simulated data using Scheme 1 and the following rate constants: $k_2 = 20\ \mu M^{-1}\ s^{-1}$; $k_{-2} = 4000\ s^{-1}$; $k_3 = 400\ s^{-1}$; $k_4 = 24\ s^{-1}$; $k_{off} = 24\ s^{-1}$. The dotted line was generated using KINSIM (Barshop et al., 1983) and the above rate constants for Scheme 1 except $k_{off} = 46\ s^{-1}$ under conditions of ADP preequilibration.

of PKA, ATP, and Kemptide are equivalent to those used in the control experiment (i.e., no ADP preequilibration) in Figure 2. To ensure that the concentration of ADP was sufficient to bind all the free enzyme prior to reaction and that the concentration of ATP was sufficient to trap all the free enzyme after ADP dissociation, several concentrations of ATP and ADP were used. Increasing the concentrations of ADP and ATP from 400 and 1000 μM to 600 and 1500 μM, respectively, had no effect on the net production of phosphopeptide (data not shown). Furthermore, the linear rates of phosphopeptide formation were similar to the control (i.e., no ADP). Table 2 shows the linear fits (k_L) for data points between 20 and 100 ms. Only when equivalent amounts of ADP and ATP were used did the linear rate

Table 1: Parameter Fits to Pre-Steady-State Kinetic Transients for the Phosphorylation of Kemptide and PKS(14−22) in the Absence and Presence of ADP[a]

	substrates	
parameter	Kemptide	PKS(14−22)
k_b (s^{-1})[b]	130 ± 20	180 ± 30
$\alpha/[E]_o$[b]	0.70 ± 0.05	0.82 ± 0.04
$L/[E]_o$ (s^{-1})[b]	21 ± 1.0	29 ± 1.0
K_d (μM)[c]	200	0.10
k_3 (s^{-1})[d]	400	170
k_4 (s^{-1})[d]	24	35
k_{off} (s^{-1})[e]	24	33

[a] Data fitting of kinetic transients in Figures 2 and 3. [b] k_b, $\alpha/[E]_o$, and $L/[E]_o$ are obtained from data fitting to eq 1 in the absence of ADP. [c] The K_d for Kemptide was previously reported (Grant & Adams, 1996), and that for PKS(14−22) was set at 0.1 μM. The values of k_2 and k_{-2} were set at 20 μM^{-1} s^{-1} and 4000 s^{-1} for Kemptide and 250 μM^{-1} s^{-1} and 25 s^{-1} for PKS(14−22). [d] The values of k_3 and k_4 were determined by modeling the data in the absence of ADP using Scheme 1. [e] The values of k_{off} were determined by modeling the data in the presence of ADP using k_3, k_4, and K_d (k_2 and k_{-2}).

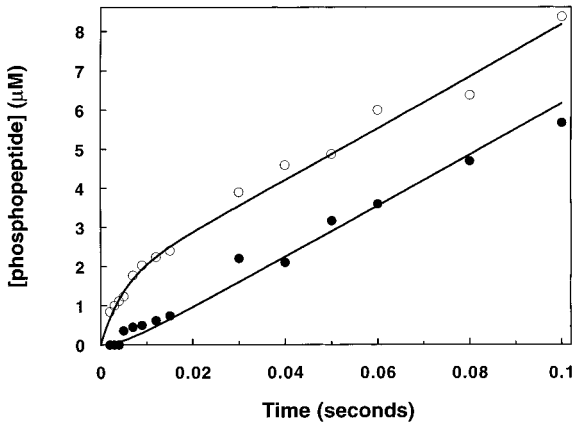


FIGURE 3: Production of phosphopeptide in the absence (○) and presence (●) of ADP at 10 mM free Mg^{2+} using the substrate PKS(14−22). The concentrations of PKA, ATP, and PKS(14−22) are 2.0 μM, 600 μM, and 100 μM, respectively, in pH 7.0 MTCN buffer. PKA was preequilibrated with 400 μM ADP (concentration in the mixing chamber) prior to the addition of ATP and PKS(14−22) for one of the two data sets. The lines drawn through the data were obtained from simulated data using Scheme 1 and the following rate constants: $k_2 = 250$ μM^{-1} s^{-1}; $k_{-2} = 25$ s^{-1}; $k_3 = 170$ s^{-1}; $k_4 = 35$ s^{-1}; $k_{off} = 33$ s^{-1}.

decrease below the control ($v^o/v = 1.6$ at 0.4 mM ADP and ATP).

Similar experiments were performed using the substrate PKS(14−22). Figure 3 shows the formation of phosphopeptide when ADP (400 μM) was preequilibrated with PKA prior to reaction with ATP (1.0 mM) and PKS(14−22) (100 μM) in pH 7 MTCN buffer. The amounts of ATP and PKS(14−22) were identical to those used in the non-preequilibrated kinetic trace in Figure 3. Likewise, ATP and ADP levels were adjusted to ensure that all the enzyme was bound with ADP prior to reaction and all the free PKA was trapped by ATP. For example, increasing the concentrations of ADP and ATP from 200 and 1000 μM to 300 μM and 1.9 mM or 100 μM and 1.0 mM, respectively, had no effect on the net production of phosphopeptide (data not shown). Likewise, linear fitting of these data sets between 20 and 100 ms gave rates similar to the control experiment (Table 2).

Preequilibration Effects on the Phosphorylation of Peptides. The pre-steady-state kinetic experiments shown in Figures 2 and 3 were measured when ATP and peptide substrates were not preequilibrated with PKA prior to mixing. To determine whether ligand preequilibration affects turnover, the formation of phosphopeptide was monitored by rapid quench flow mixing and the coupled enzyme assay for both substrates. For Kemptide, kinetic profiles identical to that shown in Figure 2 were obtained when ATP was preequilibrated with PKA (data not shown). Pre-steady-state experiments were not performed under conditions where PKA was preequilibrated with Kemptide prior to the addition of ATP. Preequilibration with ATP did not affect the observed 'burst' rate constant or the linear turnover rate using identical concentrations of enzyme, ATP, and Kemptide. The lack of an effect on turnover rate was confirmed using the coupled enzyme assay. Again, no effect was measured when identical concentrations of PKA, ATP, and Kemptide were added in different order (data not shown). Using the coupled enzyme assay, extrapolation of the initial velocity data to infinite Kemptide concentration gave a turnover number of 23 s^{-1}. The slightly slower linear rate of 21 s^{-1} (Table 1), derived from the rapid quench flow studies (Figure 2), is consistent with the reported K_m of 9 μM (Grant & Adams, 1996).

Table 2: Effects of ATP and ADP Concentrations on the Production of Phosphopeptide Using Rapid Quench Flow Analysis[a]

substrate	[ADP] (mM)[b]	[ATP] (mM)	k_L (v/E_o)[e]	v^o/v^e
Kemptide[c]	0	1.0	22 ± 1.0	1.0
	0.4	0.4	14 ± 0.8	1.6
	0.4	1.0	21 ± 0.40	1.0
	0.6	1.5	22 ± 0.41	1.0
PKS(14−22)[d]	0	1.0	28 ± 2.0	1.0
	0.10	1.0	24 ± 3.0	1.2
	0.20	1.0	28 ± 2.0	1.0
	0.30	1.9	27 ± 2.4	1.0

[a] In all experiments, PKA is mixed with varying amounts of ATP and fixed peptide either in the absence or in the presence of varying amounts of preequilibrated ADP. [b] These values are the concentrations of ADP in the mixing chamber. [c] All experiments with this substrate were performed with 1.5 μM PKA and 100 μM Kemptide in 50 mM Mops buffer (pH 7.0). [d] All experiments with this substrate were performed with 2.2 μM PKA and 100 μM PKS(14−22) in pH 7 MTCN buffer. [e] The values of k_L were measured using a linear function on all data between 20 and 100 ms. v^o/v is the ratio of the linear rate in the absence and presence of preequilibrated ADP.

For PKS(14−22), the turnover rate was substantially different when PKA was first preequilibrated with either ATP or substrate prior to the start of the reaction. Figure 4 shows comparative traces for the phosphorylation of PKS(14−22) (100 μM) either when PKA (2.0 μM) is preequilibrated with ATP prior to the addition of peptide or when the reaction is started by the addition of PKA to ATP and peptide. Identical concentrations of all three components are used for both experiments to ensure that the observed differences are not due to differences in enzyme or ligand concentrations. The fitted values for k_b, α, and L either in the absence or in the presence of ATP preequilibration are 160 ± 35 s^{-1}, 1.4 ± 0.23 μM, and 59 ± 4 μM/s, respectively, or 170 ± 27 s^{-1}, 1.7 ± 0.20 μM, and 32 ± 2 μM/s, respectively. The fits to the linear portions of both curves provide turnover numbers of 30 and 16 s^{-1} in the absence and presence of ATP preequilibration. These observations were confirmed using the coupled enzyme assay. When PKA (49−61 nM) is preequilibrated with either ATP (200 μM) or PKS(14−22) (40−80 μM), maximum turnover is 17 s^{-1}. If PKA is not

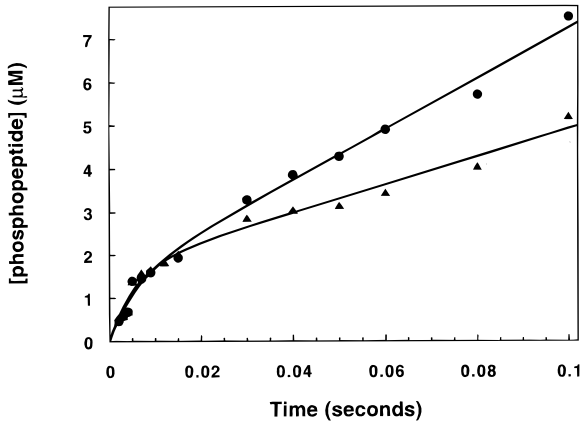


FIGURE 4: Production of phosphopeptide either when PKA is preequilibrated with ATP prior to the addition of PKS(14−22) (▲) or when the reaction is started with the addition of PKA (●). In both experiments, the concentrations of PKA, ATP, and PKS(14−22) are 2.0, 200, and 100 μM, respectively. The data are fit to eq 1.

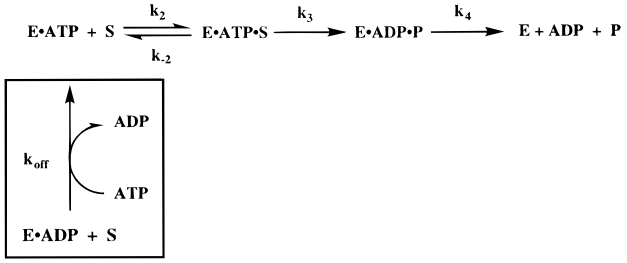
preequilibrated with either ATP or PKS(14−22), maximum turnover is 29 s^{-1}. These values compare well with the turnover rates measured in the rapid quench flow experiment in Figure 4.

DISCUSSION

A thorough understanding of the mechanism of protein phosphorylation catalyzed by protein kinases requires the unambiguous identification of individual kinetic steps comprising the catalytic pathway. Rapid quench flow studies showed that the phosphorylation of short peptide substrates by PKA follows 'burst' kinetics consistent with a kinetic mechanism in which phosphoryl transfer occurs at a rate constant (500 s^{-1}) that exceeds turnover by 25-fold (Grant & Adams, 1996). With the first observation of the phosphoryl transfer step, attention now focuses on the isolation and characterization of the substrate and product binding steps. Steady-state kinetic approaches have shown previously that this enzyme phosphorylates short peptide substrates with a random, steady-state kinetic mechanism (Kong & Cook, 1988). In contrast, the release of products, ADP and phosphokemptide, is ordered based on inhibition studies. At high concentrations of free Mg^{2+} (10 mM), phosphokemptide release occurs prior to ADP dissociation. Isotope partitioning (Kong & Cook, 1988) and viscosometric (Adams & Taylor, 1992) studies support rate-determining ADP release, but these assertions rely ultimately on a thermodynamic consideration without detailed assessment of kinetic pathways. More recently, stopped-flow fluorescence studies of an acrylodan-labeled mutant of PKA showed that ADP release is partially rate-determining with a slow, viscosity-sensitive conformational change contributing to k_{cat} (Lew et al., 1997). Since this mutant displays modestly altered steady-state kinetic parameters, this conformational change may be relevant for the wild-type enzyme. Given the ambiguous results from steady-state and transient kinetic approaches, a direct method for the measurement of the dissociation rate constant for ADP from wild-type PKA is needed.

Predicting the Effects of ADP Preequilibration on the 'Burst' Phase. The phosphorylation of peptides by PKA is characterized by a rapid, exponential 'burst' in phosphopeptide followed by a slower, linear phase that observes Michaelis−Menten kinetics (Grant & Adams, 1996; Zhou & Adams, 1997). The possibility that attainment of the active ternary complex, E·ATP·S, can be impeded by product release offers an effective approach for the direct measurement of the ADP dissociation rate constant and a role for this step in rate-limiting turnover. The effects of substrate concentration and metal ion character were used previously to define a kinetic mechanism for Kemptide phosphorylation (Grant & Adams, 1996). This mechanism shown in Scheme 1 incorporates rapid equilibrium binding of substrate followed by fast phosphoryl transfer and slow product dissociation.

Scheme 1



The individual rate constants in this kinetic mechanism ($k_3 = 500\ s^{-1}$, $k_4 = 21\ s^{-1}$, $k_{-2}/k_2 = 200\ \mu$M, and $k_{-2} \gg k_3$), determined from an analytical solution (Grant & Adams, 1996), can be used to predict the pre-steady-state kinetic transients at all levels of substrate when ATP concentrations are saturating. The numerical integration program KINSIM (Barshop et al., 1983) was used to simulate the 'burst' kinetics at 100 μM Kemptide (Figure 1). If ADP is preequilibrated with PKA prior to mixing with excess ATP and Kemptide, the time-dependent formation of the active ternary complex, E·ATP·S, will be obstructed by ADP dissociation and substrate binding. Since the binding of substrate was previously shown to be rapid ($k_2[S] \gg k_3 \ll k_{-2}$), the formation of this complex will be affected only by the dissociation rate constant for ADP (k_{off}) when ATP levels are high. In this mechanistic variation (Scheme 1), PKA is preequilibrated with ADP so that no free enzyme exists prior to the addition of ATP and Kemptide. In the presence of ADP (Figure 1), the 'burst' phase disappears and is replaced by a small lag followed by linear turnover. Since ideal trapping conditions are a condition of this kinetic model (i.e., fast binding of ATP), linear turnover is unaffected although the net production of phosphopeptide lags behind the control. Likewise, if the dissociation rate constant for ADP is fast relative to the 'burst' rate constant, no significant perturbation in the 'burst' phase is observed (simulation not shown in Figure 1). If the dissociation rate constant for ADP is 2- and 4-fold larger than k_{cat}, the kinetic transients are sigmoidal and distinguishable. Finally, this mechanism assumes a stepwise release of ADP and association of Kemptide prior to the establishment of steady-state conditions, but dissociation of ADP from a dead-end ternary complex, E·ADP·S, is possible under some conditions. However, the high K_d for Kemptide ($\geq 200\ \mu$M) measured in several ternary complexes (Cook et al., 1982a; Grant & Adams, 1996) suggests that little of the dead-end complex forms under steady-state turnover or trapping conditions so that the mechanism in Scheme 1 approximates the dissociation pathway for ADP (Cook et al., 1982a).

Role of ADP Dissociation in Peptide Phosphorylation. The kinetic transient in the absence of ADP in Figure 2 was modeled using the simulation program KINSIM (Barshop et al., 1983) and kinetic parameters previously measured from an analytical solution to the data (Grant & Adams, 1996). The simulated data closely match the experimental data points when the rate constants, k_3 and k_4, shown in Table 1 are used. Since association and dissociation rate constants for Kemptide are unknown, reasonable estimates were selected that are consistent with the measured thermodynamic dissociation constant (Grant & Adams, 1996). Acceptable simulations of the data were obtained when k_3 was set at 400 s^{-1} rather than 500 s^{-1}, the previously reported phosphoryl transfer rate constant obtained from a substrate extrapolation (Grant & Adams, 1996). In the presence of ADP, the initial 'burst' phase disappears although linear turnover after 20 ms is unaffected. Since an analytical solution does not exist for the complete kinetic mechanism in Scheme 1, we used KINSIM to model the experimental data assuming that all the enzyme is initially in the binary form E·ADP and that sufficient amounts of ATP are added to trap all the freely dissociated enzyme. The attainment of these ideal trapping conditions is confirmed by demonstrating that higher concentrations of either ADP and ATP do not affect the net production of phosphokemptide (Table 2). With these conditions adequately met, the kinetic transient in Figure 2 was modeled using only one variable, the dissociation rate constant for ADP (k_{off}). The best visual modeling of the experimental data occurs when this parameter is 24 s^{-1}. Given the fast rate of phosphoryl transfer (400 s^{-1}) and the turnover rate for Kemptide (23 s^{-1}), the data indicate that the rate-determining step must be the release of ADP.

These experiments were repeated for the phosphorylation of the nine residue peptide, PKS(14−22). A low K_m (0.1 μM) and high k_{cat} (2-fold larger than that for Kemptide) have been reported (Mitchell et al., 1995), endowing it with the highest second-order phosphorylation rate constant of all known PKA substrates (∼200 μM^{-1} s^{-1}). The kinetic transient for the phosphorylation of this peptide in the absence of ADP (Figure 3) is best modeled when k_3 and k_4 are 170 and 35 s^{-1} (Table 1), values that are consistent with the measured k_{cat} of 29 s^{-1}. Also, the observed 'burst' rate constant of 180 s^{-1} (Table 1) is close in value to the expected sum of k_3 and k_4 ($k_b = k_3 + k_4$). The kinetic transient in the presence of ADP is best modeled when the dissociation rate constant for ADP is set at 33 s^{-1}. Given the turnover number for PKS(14−22), the data show that the rate-determining step for PKS(14−22) is primarily ADP dissociation. While ADP dissociates primarily from the binary complex, E·ADP, for the phosphorylation of Kemptide, it is presumed that a dead-end complex forms for the phosphorylation of PKS(14−22) owing to the high affinity of this substrate (Mitchell et al., 1995) and ADP dissociates from the ternary complex, E·ADP·PKS(14−22). Kinetic simulations were performed using a variation of the mechanism in Scheme 1 in which ADP is released from the dead-end complex and replaced by ATP, k_3 is the same, and k_4 is now expanded to include a substrate binding step followed by ADP release (phosphopeptide release is presumed fast and the peptide binding step to E·ATP, k_2, is removed). The kinetic simulation which results from this mechanism is indistinguishable from that in Figure 3. Finally, it is possible that the observed differences in trapping rates of 24 and 34 s^{-1} for ADP using both substrates result from dissociation from the binary complex, E·ADP, for Kemptide versus the dead-end complex, E·ADP·PKS(14−22), for PKS(14−22).

Effects of Substrate and ATP Preequilibration. The k_{cat} value of 29 s^{-1} for PKS(14−22) is 1.8-fold higher than the previously reported value at the same pH and buffer conditions (Zhou & Adams, 1997). The results presented herein demonstrate that this phenomenon is due to ligand preequilibration. Although no lag phases are observed at either low or high enzyme concentrations in the steady-state or pre-steady-state kinetic experiments using the radiochemical assay, the linear turnover rate is slower when the enzyme is preequilibrated with either ATP or peptide compared to kinetic studies where the enzyme is added to buffer containing both ATP and peptide (Figure 4). This phenomenon cannot be accounted for by synergistic effects between PKS(14−22) and ADP (i.e., substrate reduces the dissociation rate constant for ADP) since the steady-state measures multiple turnovers and preequilibration should not affect this when the concentrations of enzyme, ATP, and substrate are identical. To explain this unusual result, we presume that PKS(14−22) can either induce or select two slowly interconverting enzyme forms. While both forms permit efficient phosphoryl transfer, one form allows a slightly higher turnover number than the other. While the structural source of this altered turnover is unknown, the similarities of the measured dissociation rate constant for ADP (35 s^{-1}) and the turnover numbers (29 and 16 s^{-1}) suggest that ADP release is a contributing element for rate limitation for the phosphorylation of PKS(14−22) but other factors may be involved.

REFERENCES

Adams, J. A., & Taylor, S. S. (1992) *Biochemistry 31*, 8516−8522.

Barshop, B. A., Wrenn, R. F., & Frieden, C. (1983) *Anal. Biochem. 130*, 134−145.

Cook, P. F., Neville, M. E., Vrana, K. E., Hartl, F. T., & Roskoski, J. R. (1982a) *Biochemistry 21*, 5794−5799.

Cook, P. F., Neville, M. E., Vrana, K. E., Hartl, F. T., & Roskoski, J. R. (1982b) *Biochemistry 21*, 5794−5799.

DeBont, H. L., Rosenblatt, J., Jancarik, J., Jones, H. D., Morgan, D. O., & Kim, S. H. (1993) *Nature 363*, 595−602.

Grant, B., & Adams, J. A. (1996) *Biochemistry 35*, 2022−2029.

Jeffrey, P. D., Russo, A. A., Polyak, K., Gibbs, E., Hurwitz, J., Massague, J., & Pavletich, N. P. (1995) *Nature 376*, 313−320.

Kemp, B. E., Benjamini, E., & Krebs, E. G. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73(4)*, 1038−1042.

Kemp, B. E., Graves, D. J., Benjamini, E., & Krebs, E. G. (1977) *J. Biol. Chem. 252*, 4888−4894.

Kong, C.-T., & Cook, P. F. (1988) *Biochemistry 27*, 4795−4799.

Lew, J., Taylor, S. S., & Adams, J. A. (1997) *Biochemistry 36*, 6717−6724.

Mitchell, R. D., Glass, D. B., Wong, C., Angelos, K. L., & Walsh, D. A. (1995) *Biochemistry 34*, 528−534.

Qamar, R., Yoon, M.-Y., & Cook, P. F. (1992) *Biochemistry 31*, 9986−9992.

Russo, A. A., Jeffrey, P. D., & Pavletich, N. P. (1996) *Nat. Struct. Biol. 3*, 696−700.

Yonemoto, W., McGlone, M. L., Slice, L. W., & Taylor, S. S. (1991) in *Protein Phosphorylation (Part A)* (Hunter, T., Sefton, B. M., Eds.) pp 581−596, Academic Press, Inc., San Diego.

Zetterqvist, Ö. Z., Ragnarsson, U., & Engstrom, L. (1990) in *Peptides and Protein Phosphorylation* (Kemp, B. E., Ed.) pp 171−187, CRC Press, Inc., Boca Raton.

Zhou, J., & Adams, J. A. (1997) *Biochemistry 36*, 2977−2984.

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