Is There a Catalytic Base in the Active Site of cAMP-Dependent Protein Kinase? †

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ABSTRACT: The carboxyl group of an aspartic acid in the active site of the serine-specific protein kinase, cAMP-dependent protein kinase, is poised near the hydroxyl proton of a peptide substrate in the X-ray crystallographic structure (Madhusudan et al., 1994), suggesting that this residue may act as a general-base catalyst in the phosphoryl transfer reaction. Indeed, several proposals have been made in this regard. We measured the pre-steady-state kinetics in this enzyme using a rapid quench flow technique to understand the role of this putative base. The phosphorylation of the peptide substrate, GRTGRRNSI, by cAMP-dependent protein kinase exhibited "burst" kinetics consistent with a mechanism in which the peptide is phosphorylated rapidly (154 s^{-1}) and the product(s) is (are) released slowly (16 s^{-1}). The replacement of Mg^{2+} with Mn^{2+} leads to a 13-fold reduction in this observed "burst" rate constant, suggesting that this transient is limited either by the phosphoryl transfer step or by a metal ion-dependent conformational change step. The influence of deuterium oxide on the pre-steady-state kinetics was monitored in the presence of both divalent metal ions, and no solvent isotope effect was measured on either "burst" phase. A large solvent isotope effect is observed on k_{cat} in the presence of either metal ion, and a proton inventory analysis in the presence of Mg^{2+} indicates that two or more protons are transferred in the product release step. Finally, no pH dependence is observed on the "burst" rate constant using either Mg^{2+} or Mn^{2+} over the pH range of 6−9. The combined data do not support a mechanism involving a general-base catalyst whose pK_a is greater than 5 or less than 10 if the "burst" phase is cleanly limited by the phosphoryl transfer step. If the "burst" phase is limited by a metal ion-dependent conformational change step, the measurement of the phosphoryl transfer step is obscured, and the participation of a base catalyst is indeterminate.

The role of protein phosphorylation in normal cell function is an area of continued interest in the scientific community. The regulation of phosphoryl transfer reactions, catalyzed by protein kinases, is of particular interest. These enzymes transfer the γ-phosphate of ATP to serine, threonine, or tyrosine side chains in target proteins and, subsequently, affect a variety of physiological responses including metabolism, DNA transcription, secretion, differentiation, and cell cycle control. The protein kinases are grouped into two large subclasses based on their specificity for serine/threonine and tyrosine. The activity of these catalysts is tightly regulated by a number of mechanisms which include phosphorylation and intrasteric regulation. The recent X-ray structure solutions of several protein kinases now allow a detailed molecular approach toward understanding the catalytic and regulatory properties of these important enzymes (Bossemeyer et al., 1993; DeBont et al., 1993; Hu et al., 1994; Hubbard et al., 1994; Jeffrey et al., 1995; Knighton et al., 1991; Xu et al., 1995; Zhang et al., 1994). The first protein kinase structure solved, the catalytic subunit of cAMP-dependent protein kinase (PKA),[1] is as a paradigm for this enzyme class. The structures of active PKA have now been solved in several cocrystals containing various nucleotides, divalent metal ions, and inhibitor, substrate, and product peptides (Knighton et al., 1991; Madhusudan et al., 1994; Zheng et al., 1993a,c) providing useful insights into the mechanism of phosphoryl transfer.

The catalytic subunit of PKA phosphorylates enzymes and proteins that possess the minimum consensus sequence R-R-X-S/T-hyd, where X is variable and hyd is a hydrophobic residue (Kemp et al., 1976, 1977; Zetterqvist et al., 1990). Steady-state kinetic analyses demonstrate that the catalytic subunit phosphorylates the substrate, LRRASLG, by a random kinetic mechanism but the initial binding of ATP is preferred (Cook et al., 1982; Kong & Cook, 1988). Although the transfer efficiency is best in the presence of Mg^{2+}, the enzyme will accept a number of other divalent metals (Bhatnagar et al., 1983). The k_{cat} value for PKA in the presence of Mn^{2+} is 20-fold lower than in the presence of Mg^{2+} (Adams & Taylor, 1993a). The effects of solvent viscosity on the steady-state kinetic parameters suggest that maximum turnover is limited by product release, namely, ADP dissociation, in the presence of either metal ion (Adams & Taylor, 1992, 1993a). A "burst" of phosphopeptide is observed in pre-steady-state kinetic transients, indicating that phosphoryl transfer occurs at a rate constant of 500 s^{-1}, a value 25-fold higher than k_{cat} (Grant & Adams, 1996). These results explain why the K_m for Kemptide is more than 1 order

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[1] Abbreviations: Caps, 3-(cyclohexylamino)-1-propanesulfonic acid; Kemptide, peptide sequence LRRASLG; Mes, 2-(*N*-morpholino)-ethanesulfonic acid; MTCN, 50 mM Mes, 25 mM Tris, 25 mM Caps, 50 mM NaCl; PKA, cAMP-dependent protein kinase; PKI, protein kinase inhibitor; PKS(14−22), peptide sequence GRTGRRNSI; PTPase, protein tyrosine phosphatase; SIE, solvent isotope effect; Tris, tris-(hydroxymethyl)aminomethane.

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of magnitude lower than its K_d (Whitehouse et al., 1983; Whitehouse & Walsh, 1983) and, for the first time, provide a detailed kinetic scheme for the application of structure−function studies.

The X-ray structures of PKA suggest that a number of key interactions in the active site may facilitate a rapid rate of phosphoryl transfer. In particular, an attractive hypothesis for the stabilization of the transition state in PKA invokes the participation of Asp-166 as a general-base catalyst. Evidence that supports this mechanism comes from a variety of kinetic and structural sources. First, the pH dependence of the second-order rate constant, V/K, for Kemptide phosphorylation is bell-shaped with pK_a values of approximately 6.2 and 8.5 (Yoon & Cook, 1987). Second, the X-ray crystal structure of the catalytic subunit complexed with a peptide substrate demonstrates a hydrogen bond interaction between the hydroxyl and the strictly conserved active-site aspartate (Madhusudan et al., 1994). Third, removal of this aspartate in the yeast PKA by "charge-to-alanine" scanning produces a mutant enzyme with a k_{cat} value that is approximately 300-fold lower than that for the wild-type enzyme (Gibbs & Zoller, 1991). Fourth, stereochemical analyses indicate that the phosphoryl transfer reaction proceeds with inversion of configuration, consistent with direct, in-line attack of the hydroxyl on the γ-phosphate of ATP and the formation of a pentavalent, phosphorane species (Ho et al., 1988). Although these studies may be supportive, no direct kinetic studies have been performed on the isolated phosphoryl transfer step that would either affirm or controvert this mechanism.

The inability to define the mechanism of catalysis for PKA originates from a poor understanding of the individual steps in the kinetic mechanism. If the transfer of the γ-phosphoryl group of ATP to a peptide substrate were subject to general-base catalysis, the forward rate constant for this step should increase with increasing pH and may be sensitive to varying amounts of deuterium oxide. Unfortunately, the actual phosphoryl transfer step is not expressed directly in either steady-state kinetic parameter, k_{cat} or k_{cat}/K_m (Adams & Taylor, 1992; Grant & Adams, 1996). To circumvent this problem, we used a combination of pre-steady-state kinetics and solvent isotope, pH dependence, and metal ion studies to gain insight into the phosphoryl transfer step catalyzed by PKA. We monitored the "burst" phase in PKA using rapid quench flow techniques and showed that the rate constant for this phase is pH independent between 6 and 9 and is not affected by solvent deuterium when the reaction is followed in the presence of Mg^{2+} or Mn^{2+}. These data are not consistent with the participation of a general-base catalyst whose pK_a is between 5 and 10 if the "burst" phase is limited by the phosphoryl transfer step. If this phase is limited by a slow, metal ion-dependent conformational change preceding phosphoryl transfer, the participation of a base catalyst is inconclusive.

MATERIALS AND METHODS

Materials. Adenosine 5′-triphosphate (ATP), 3-(cyclohexylamino)-1-propanesulfonic acid (Caps), 2-(*N*-morpholino)ethanesulfonic acid (Mes), manganese chloride, sucrose, pyruvate kinase (type II from rabbit muscle), lactate dehydrogenase (type II from bovine heart), nicotinamide adenine dinucleotide, reduced form (NADH), 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. The peptide GRTGRRNSI [PKS(14−22)] was a gift from Dr. Susan S. Taylor at the University of California, San Diego. The seven residue peptide Chocktide, LRRWSLG, was synthesized and purified by the USC Microchemical Core Facility. The concentrations of Chocktide and PKS(14−22) were determined by complete turnover with the catalytic subunit 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 Chocktide.

Coupled Enzyme Assay. The activity of the catalytic subunit was determined in a coupled enzyme assay 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. All reactions were measured in a Beckman DU640 spectrophotometer equipped with a microcuvette holder. Typical steady-state kinetic reactions were performed in a buffer containing MTCN (varying pH) in a final volume of 60 μL either in the presence or absence of sucrose or in the presence or absence of D_2O at 27 °C. Catalytic subunit (30−50 nM) was typically incubated with 0.2 mM ATP, 10 mM magnesium chloride or manganese 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 varying amounts of peptide (10−200 μM). The free concentration of divalent metal ion was approximately 10 mM.

Solution Viscosity Measurements. The relative viscosity (η^{rel}) of buffers containing sucrose was measured relative to MTCN buffer at pH 7.0, 27 °C, using an Ostwald viscometer (Shoemaker & Garland, 1962). Each viscosity measurement was carried out using 5.0 mL of buffer containing varying amounts of viscosogen. Relative solvent viscosities of 1, 1.8, and 2.7 were obtained for buffers containing 0, 20, and 31% (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. Quench Flow Apparatus Model RGF-3 equipped with two sample loops and three drive syringes as previously reported (Grant & Adams, 1996). Quench flow experiments were typically executed by loading enzyme, buffer, magnesium chloride or manganese chloride, and ATP into one sample loop and PKS(14−22) into the other. Final concentrations of the reactants upon mixing were approximately 2 μM catalytic subunit, 10 mM magnesium chloride or manganese chloride, 0.2 mM [γ-^{32}P]ATP (1000−2000 cpm pmol^{-1}), and 10−200 μM PKS(14−22). The reactions were quenched with 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 PKS(14−22) 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 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, magnesium chloride, and ATP in one syringe were mixed with a solution containing 30% acetic acid and PKS(14−22) 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 1000 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 phosphopeptide and calculating the percent retention after washing. The labeled phosphopeptide was synthesized by complete turnover of limiting amounts of PKS(14−22) with the catalytic subunit. Between 5 and 500 pmol of labeled phosphopeptide was spotted with 4500−5000 pmol of 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 all control experiments, 72 ± 5% phosphopeptide was 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.

Data Analysis. The steady-state kinetic parameters, V_{max} and K_m, were obtained by plotting the initial reaction velocity versus the total substrate concentration according to eq 1:

$$v = \frac{V_{max}[S]}{K_m + [S]} \quad (1)$$

where v is the initial reaction velocity, [S] is the total peptide concentration, V_{max} is the maximal reaction velocity, and K_m is the Michaelis constant. The maximal reaction velocity was converted to k_{cat} by dividing V_{max} by the total enzyme concentration. The pH dependency in k_{cat} in the presence of Mn^{2+} was fit to eq 2:

$$y = \frac{(C)10^{-pH} + (C^*)10^{-pK_a}}{10^{-pH} + 10^{-pK_a}} \quad (2)$$

where y is the observed k_{cat} at a given pH, C and C^* are the minimum and maximum values of k_{cat}, respectively, and pK_a is the acid dissociation constant. Data in each quench flow time course were fitted to an empirical function containing a single exponential and a linear component:

$$y = \alpha[1 - \exp(-k_b \cdot t)] + L \cdot t \quad (3)$$

where y is the concentration of phosphopeptide, α is the observed "burst" amplitude, k_b is the observed single 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.

Table 1: Steady-State Kinetic Parameters for the Phosphorylation of PKS(14−22) in Mg^{2+} and Mn^{2+} [a]

parameter	Mg^{2+}	Mn^{2+}
k_{cat} (s^{-1})	16 ± 2	0.50 ± 0.10[b]
pK_a (V)		7.8 ± 0.10
$(k_{cat})^{\eta}$	0.90 ± 0.10	

[a] All reactions were performed in MTCN buffer with 10 mM free divalent metal ion, 200 μM ATP, and variable PKS(14−22) concentrations, 27 °C. [b] These parameters are reported from data collected in pH 7 MTCN.

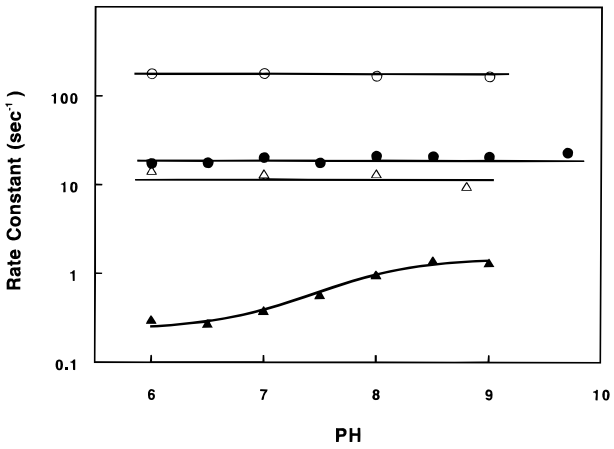

FIGURE 1: pH dependence of k_{cat} in the presence of Mg^{2+} (●) and Mn^{2+} (▲) and of k_b in the presence of Mg^{2+} (○) and Mn^{2+} (△) for the phosphorylation of PKS(14−22) using 200 μM ATP and 10 mM free divalent metal ion.

RESULTS

Steady-State Kinetics. The steady-state kinetic parameters for the PKA-catalyzed phosphorylation of PKS(14−22) were determined from plots of initial velocity vs PKS(14−22) concentration (Table 1). These data were collected in pH 7 MTCN buffer under conditions of 200 μM ATP [200 μM = 20 × K_m(ATP) (Cook et al., 1982)], 10 mM free divalent metal ion, 0.030−1.2 μM catalytic subunit, and varying concentrations of PKS(14−22) (10−200 μM) using the coupled enzyme assay. The catalytic subunit was preequilibrated with ATP for several minutes before initiation of the reaction with peptide. Increasing the concentration of ATP to 1 mM at a fixed level of free Mg^{2+} or Mn^{2+} (10 mM) caused no increase in the initial velocity, indicating that 200 μM ATP is sufficiently saturating (data not shown). The kinetic parameters were also measured using varying pH buffers (MTCN) from 6−9.7. MTCN is a multicomponent buffer system that maintains a constant ionic strength over a large pH range (Ellis & Morrison, 1982). The effects of pH on k_{cat} are presented in Figure 1 for both divalent metal ions. No change in k_{cat} was observed over this pH range in the presence of Mg^{2+}. A small pH dependence in k_{cat} was observed in the presence of Mn^{2+} (Figure 1), and the data were fit well by eq 2. Since no large change in initial velocity was measured over the entire substrate concentration range (10−200 μM), the K_m for PKS(14−22) must be less than the lowest concentration of 10 μM. Consequently, only a lower limit of approximately 2 μM^{-1} s^{-1} can be placed on k_{cat}/K_m. Previously published studies on PKA indicate that the enzyme is sufficiently stable at pH extremes of 6 and 9 so that no loss in enzyme activity due to denaturation is

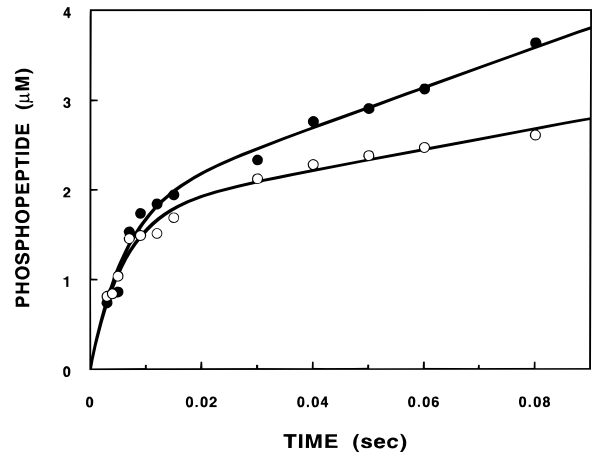

FIGURE 2: Time-dependent phosphorylation of PKS(14−22) in the presence of Mg^{2+} in H_2O (●) or 88% D_2O (○). Both transients were collected in pH 7 MTCN using 2.2 μM catalytic subunit, 100 μM PKS(14−22), 10 mM free Mg^{2+}, and 200 μM [γ-^{32}P]ATP.

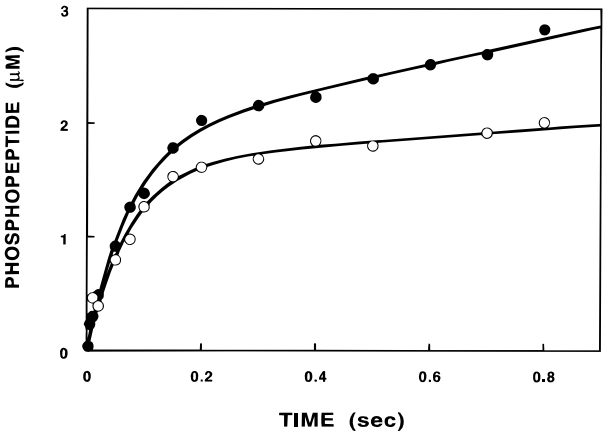

FIGURE 3: Time-dependent phosphorylation of PKS(14−22) in the presence of Mn^{2+} in H_2O (●) or 89% D_2O (○). Both transients were collected in pH 7 MTCN using 2.0 μM catalytic subunit, 100 μM PKS(14−22), 10 mM free Mn^{2+}, and 200 μM [γ-^{32}P]ATP.

detected during the time course of the preequilibration and assay (Adams & Taylor, 1993b; Yoon & Cook, 1987). All steady-state kinetic parameters are summarized in Table 1.

Pre-Steady-State Kinetics. Pre-steady-state kinetic parameters were extracted from plots of phosphopeptide concentration versus time in MTCN buffer using 200 μM ATP, 10 mM free divalent metal ion, 100 μM PKS(14−22), and 2.0−2.2 μM catalytic subunit in the rapid quench flow instrument. The data were collected from 2−80 ms and 2−800 ms using Mg^{2+} and Mn^{2+}, respectively. Two typical transients in pH 7 MTCN buffer in the presence of Mg^{2+} and Mn^{2+} are shown in Figures 2 and 3. For both metals, the production of phosphopeptide is characterized by a rapid exponential ("burst") phase followed by a slower, linear phase and is best fit by eq 3. The fitted values for the "burst" phases (k_b) and the linear phases ($L = k_L \times [E]$) in pH 7 MTCN are presented in Table 2. Increasing or decreasing the concentration of PKS(14−22) from 50 to 200 μM had no effect on any fitted parameters for the pre-steady-state kinetic traces, indicating that the substrate concentration was saturating and the rate of formation of the ternary E·ATP·S complex greatly exceeded the phosphoryl transfer rate. The pre-steady-state kinetic transients were also measured as a function of pH, and similar biphasic kinetic behavior was observed (data not shown). Fitting of these kinetic traces to eq 3 in the presence of Mg^{2+} and Mn^{2+} provided values for k_b that are plotted in Figure 1. No pH dependence on k_b was observed in the presence of either divalent metal ion. No pH dependence in the linear phase was observed in the presence of Mg^{2+} while a small pH dependence in this phase was observed in the presence of Mn^{2+} (Table 1). This latter response followed a similar pH dependence as observed in the steady-state kinetic measurements of k_{cat} (Table 1).

Table 2: Pre-Steady-State Kinetic Parameters for the Phosphorylation of PKS(14−22) in Mg^{2+} and Mn^{2+} [a]

	Mg^{2+}		Mn^{2+}	
parameter	H_2O	D_2O	H_2O	D_2O
k_b (s^{-1})	170 ± 20	165 ± 22	13 ± 1.0	12 ± 1.6
$\alpha/[E]^{exp}$	0.82 ± 0.060	0.80 ± 0.050	0.92 ± 0.040	0.82 ± 0.051
$\alpha/[E]^{calc}$	0.82	0.90	0.92	0.98
k_L (s^{-1})	16 ± 4.0	8.4 ± 1.6	0.40 ± 0.03	0.12 ± 0.040
k_3 (s^{-1})[b]	154 ± 20	157 ± 22	12 ± 1.0	12 ± 1.6
k_4 (s^{-1})[b]	16 ± 4.0	8.4 ± 1.6	0.40 ± 0.03	0.12 ± 0.040

[a] All reactions were performed in pH 7 MTCN buffer with 10 mM free divalent metal ion, 200 μM ATP, and 100 μM PKS(14−22), 27 °C. Concentrations of 2.2 and 2.0 μM catalytic subunit were used for pre-steady-state kinetic transients in the presence of Mg^{2+} and Mn^{2+}, respectively. [b] These rate constants were determined from the relationships: $k_3 = k_b - k_4$ and $k_4 = k_L$ (Grant & Adams, 1996).

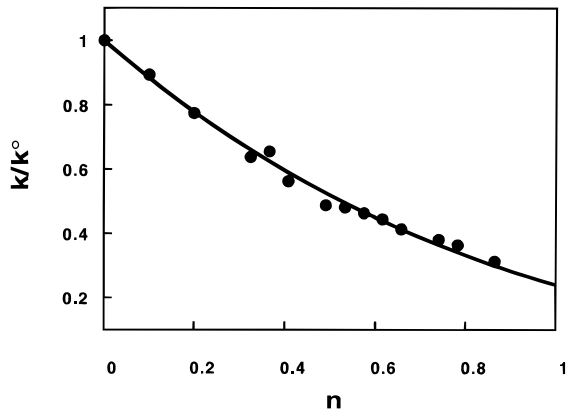

FIGURE 4: Proton inventory plot of k_{cat} for the phosphorylation of PKS(14−22) in the presence of Mg^{2+}. k/k^o is the ratio of k_{cat} in the presence and absence of varying atom fractions (n) of solvent deuterium.

Solvent Isotope Effects. The effects of solvent deuterium were measured on both the steady-state and pre-steady-state kinetic parameters. Rapid quench flow kinetic transients were measured in 88 and 89% D_2O in the presence of Mg^{2+} and Mn^{2+}, respectively, and plotted alongside the transients in the absence of D_2O in Figures 2 and 3. For each divalent metal ion, identical concentrations of catalytic subunit, ATP, and PKS(14−22) were used so that the direct influence of solvent deuterium could be assessed. The kinetic transients in the presence of D_2O are fitted to eq 3 and the experimental values of α, k_b, and k_L are listed in Table 2. No SIEs were detected on the "burst" phases in the presence of both metal ions while large SIEs were detected on the linear phases. In the presence of Mg^{2+} and Mn^{2+}, SIEs on k_L of 1.9 and 3.3 were measured. The influence of varying atom fractions of solvent deuterium on k_{cat} was measured carefully in a proton inventory plot. The ratios of k_{cat} (k_n/k_o) in the presence and absence of varying atom fractions (n) of D_2O are shown in Figure 4. Since doubling the concentration of catalytic subunit at 88% D_2O leads to a doubling of the observed initial velocity (data not shown), solvent deuterium does not

reduce the ability of the coupling enzymes to convert ADP to ATP.

Viscosity Effects on Turnover. The steady-state kinetic parameter, k_{cat}, was measured in the presence and absence of varying amounts of sucrose and the data were interpreted according to previously published procedures (Adams & Taylor, 1992). The ratio of k_{cat} [$(k_{cat})^o/k_{cat}$] in the absence (superpostscript "o") and presence (no superpostscript) of sucrose was plotted against the relative solvent viscosity (η^{rel}). This plot is linear and gives a slope value designated $(k_{cat})^\eta$. As shown in Table 1, this value is close to unity, indicating that added viscosogens have maximal effects on this parameter. This dependence has been observed previously for the phosphorylation of Kemptide by PKA in MTCN buffer (Adams & Taylor, 1993b). The data can be interpreted according to a simple three-step mechanism as shown in Scheme 1.

Scheme 1

$$E\cdot ATP + S \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} E\cdot ATP\cdot S \xrightarrow{k_3} E\cdot ADP\cdot P \xrightarrow{k_4}$$

In this scheme, substrate binds the E·ATP binary complex by the association and dissociation rate constants, k_2 and k_{-2}, respectively. The catalytic step, k_3, describes the favorable, unimolecular rate constant for the transfer of the γ-phosphoryl group of ATP to the hydroxyl of serine and includes any conformational change associated with this transfer. The final step in Scheme 1 (k_4) describes the net bimolecular rate constant for the release of both products. Since the viscosity measurements cannot distinguish between the dissociation rate constants for the phosphorylated peptide and ADP, it is possible that k_4 may be limited by either product or partially limited by both. Finally, it is also possible that reversible, conformational changes contribute to this step. The maximal effects of viscosogen on the kinetic parameters imply that the phosphoryl transfer step is the fastest step in the scheme. The slope of near 1 for $(k_{cat})^\eta$ (Table 1) indicates that k_{cat} is limited by product release ($k_3 > k_4$) (Adams & Taylor, 1993b).

DISCUSSION

The mechanism of phosphoryl transfer in protein kinases is still poorly understood despite the insights provided by the recent X-ray solutions of several of these enzymes. This cavity in our understanding is deepened by the ineffective use of steady-state kinetic approaches for the study of this enzyme class. Recently, the application of a pre-steady-steady kinetic approach to PKA has helped to fill this cavity through the isolation and characterization of the phosphoryl transfer step. Rapid quench flow techniques demonstrated that the rate constant for transfer is 500 s^{-1} using the substrate Kemptide, a value that is 25-fold higher than k_{cat} (Grant & Adams, 1996). This rapid rate of chemistry in PKA accounts for the high catalytic efficiency of this enzyme as measured by the second-order rate constant, *V*/*K*. The X-ray structures for PKA in several ligand forms are sources for many exciting proposals explaining this rapid phosphoryl transfer step (Madhusudan et al., 1994). In particular, the proximity of the conserved residue, Asp-166, to the hydroxyl of the peptide substrate (2.7 Å) entertains a compelling hypothesis for its role in phosphoryl transfer. This ground state hydrogen bond suggests that the carboxyl side chain may serve as a general-base catalyst abstracting a proton for improved nucleophilic attack of the γ-phosphate of ATP in an associative transition state.

The possibility of general-base catalysis in the bimolecular reaction of a phosphate monoester is exciting since no convincing evidence for such a mechanism has been found in either a solution-catalyzed or an enzyme-catalyzed process. The reactions of substituted aryl phosphate dianions and pyridine phosphate monoanions, for example, are thought to proceed through a metaphosphate-like transition state. Large negative values for β_{lg} and small values for β_{nuc} in linear free energy relationships (LFERs) are suggestive of advanced bond breaking and little bond formation in the transition state for these model reactions (Benkovic & Schray, 1973; Kirby & Jencks, 1965a,b; Skoog & Jencks, 1984). Solution studies suggest that an enzyme may facilitate such a transition state by a general-acid rather than a general-base. Indeed, there is convincing evidence that PTPases use general-acid catalysis to support the dephosphorylation of substrates. For example, LFERs on the human dual-specific PTPase VHR are consistent with general-acid-catalyzed phosphoryl transfer (Zhang et al., 1995). The hydrolysis of substituted aryl phosphates by this enzyme shows little dependence on the basicity of the leaving group compared to the solution-catalyzed reaction. Large primary isotope effects on the bridging oxygen of the substrate, *p*-nitrophenyl phosphate, are consistent with advanced bond cleavage to the leaving group, suggesting that this latter effect is not the result of a change to a more associative transition state in the active site (Hengge et al., 1996). Furthermore, removal of Asp-92, the putative acid catalyst, eliminates the basic limb of the *V*/*K* pH−rate profile and causes a 100-fold decrease in turnover (Denu et al., 1995). In light of these findings, strong evidence for a general-base mechanism in a protein kinase would be a significant discovery.

Aside from the persuasive evidence for general-acid catalysis in the PTPases, there is speculation that the transition state for phosphoryl group transfer in an enzyme's active site supports a general-base catalyst [e.g., see (Bossemeyer et al. (1993), Madhusudan et al. (1994), and Yoon and Cook (1987)]. A prerequisite for such a mechanism supposes that the transition states for the reactions of phosphate monoesters in the active site are more associative than in solution. Inverted stereochemistry around phosphorus has been used as evidence for direct, in-line attack of the hydroxyl nucleophile for a number of enzyme-catalyzed reactions [for a review, see Knowles (1980)] including PKA (Ho et al., 1988). However, stereochemical inversion does not preclude a concerted, dissociative mechanism particularly for reactions with weak nucleophiles such as water. The crystal structure of the ternary PKA complex, E·ATP·inhibitor, reveals that the γ-phosphoryl group is 4.2 Å away from the β-methylene carbon at the P-site (Zheng et al., 1993a). Also, a comparison of this complex with the ternary complex, E·ADP-substrate, demonstrates that the γ-phosphoryl group must travel only 1.5 Å to meet the hydroxyl oxygen (Madhusudan et al., 1994). These distances are proposed to be too short to favor an exploded, metaphosphate-like transition state (Granot et al., 1980; Mildvan et al., 1976). These assertions make predictions of transition state structure based on X-ray analysis highly suspect and the need for a kinetic approach imperative. Nonetheless, Asp-166 in PKA is only 2.7 Å away from the hydroxyl, suggesting that if an associative, general-base mechanism is

operative then it is the likely candidate. The strong prejudice for such a mechanism explains why Asp-166 (and its corresponding residue in other protein kinases) is referred to frequently as the "catalytic base" [e.g., see Bossemeyer et al. (1993), Hubbard et al. (1994), Lindberg et al. (1992), and Taylor et al. (1995)]. We tested this hypothesis using detailed kinetic analyses and set two criteria to judge whether or not the phosphoryl transfer reaction for PKA employs general-acid−base catalysis. First, if the hydroxyl proton is removed in the reaction transition state then the rate of the phosphoryl transfer step, is expected to increase with the ionization of Asp-166 through the physiological pH range. Second, the rate of the phosphoryl transfer step may decrease with increasing amounts of deuterium oxide so that a normal kinetic solvent isotope effect (SIE) would be measured.

Influence of pH and Viscosity on Turnover. Although a steady-state kinetic approach to PKA is not expected to provide direct mechanistic information on the phosphoryl transfer step, the sensitivity of k_{cat} and k_{cat}/K_m to pH and solvent viscosity does offer valuable information regarding the rate-determining step in the overall reaction. Careful measurements of the steady-state kinetic parameters show that k_{cat} (Figure 1) is pH independent in the presence of Mg^{2+}. This pH insensitivity has also been observed for Kemptide phosphorylation (Yoon & Cook, 1987) and, given the close values of the turnover numbers, implies that a common rate-determining step is likely to control turnover for both substrates. This interpretation is supported by viscosity studies. At high ATP concentrations, k_{cat} is highly sensitive to solvent viscosity, and a large value of $(k_{cat})^{\eta}$ is observed (Table 1). These data imply that k_{cat} is limited by the release of one or both products ($k_{cat} \approx k_4$). An accurate measurement of the K_m and, thus, the true value of k_{cat}/K_m and any pH response cannot be made using the spectrophotometric assay. This latter problem arises from small time-dependent changes in absorbance at low peptide concentrations (<10 μM). In fact, another laboratory reported a K_m of 0.11 μM for this peptide using a more sensitive radiochemical assay (Mitchell et al., 1995).

Pre-Steady-State Kinetics. The phosphorylation of Kemptide over short time frames (<100 ms) has been shown to exhibit "burst" kinetics consistent with a kinetic mechanism in which the substrate is phosphorylated rapidly (500 s^{-1}) and product(s) is (are) released slowly (20 s^{-1}) in the rate-determining step of turnover (Grant & Adams, 1996). This was the first observation of phosphoryl transfer in the active site of a protein kinase and now offers a powerful approach for rigorous mechanistic studies. The observed "burst" rate is substrate-dependent, reflecting the weak affinity of Kemptide for the active site (K_d = 200 μM) (Grant & Adams, 1996). This presents a problem for further detailed studies since the true "burst" rate constant must be obtained from an extrapolated plot of "burst" rate versus substrate concentration (Grant & Adams, 1996), thereby introducing error into the measurement of the chemical event. This problem was circumvented by using a nine residue peptide substrate based on an inhibitory fragment of PKI, the natural, physiological inhibitor of PKA. The major inhibitory portion of PKI lies within residues 5−24, and further truncation of this peptide fragment impairs inhibitor binding (Walsh et al., 1990). A peptide was designed based on residues 14−22 in PKI, and the nonphosphorylatable alanine residue was substituted with serine to make it a substrate [PKS(14−22)]. Although we cannot accurately measure K_m, the "burst" rate constant is independent of substrate at and above 50 μM, indicating that the active site is fully occupied at concentrations above this.

PKS(14−22) was phosphorylated rapidly by PKA in the first 10 ms of the reaction by an observed rate constant (k_b) of 170 s^{-1} (Figure 2 and Table 2). This rapid "burst" phase attenuates to a slower, linear phase of 16 s^{-1} (L/[E]). At high concentrations of PKS(14−22), the phosphoryl transfer rate constant (k_3) can be obtained from the relationship, $k_b = k_3 + k_4$, where $k_4 = k_L$ (Grant & Adams, 1996). Owing to instrument dead time, approximately 30% of the "burst" amplitude is unavailable for curve analysis. To avoid this fitting problem, the "burst" kinetics were repeated in the presence of Mn^{2+} (Figure 3). A significant reduction in the "burst" rate constant was observed, allowing complete detection of the transient. The reduction in the "burst" rate constant of approximately 13-fold (154 versus 12 s^{-1}) in the presence of Mn^{2+} compared to Mg^{2+} (Table 2) can be interpreted in two ways. First, the phosphoryl transfer step is fully rate-limiting in the "burst" phase in the presence of either metal ion, and the slower value in the presence of Mn^{2+} reflects subtle differences in the metal−oxygen interaction. Second, metal ion substitution may reduce the rate of a slow, cation-dependent conformational change preceding phosphoryl transfer. Although the former interpretation is most plausible, both interpretations will be considered.

Influence of pH on the "Burst" Phase. A general-acid−base catalyst supports the movement of a proton in the reaction transition state. The rate constant for the "burst" phase was measured between pH 6 and 9 in the presence of either Mg^{2+} or Mn^{2+}, and no change in the observed rate was detected (Figure 1). If a general-base catalyst assists the phosphoryl transfer step and the "burst" selectively monitors this catalytic step, then the pK_a of this residue must be lower than 6 or greater than 9. If a 10% change in the observed "burst" rates occurred at pH 6 or 9 and was not detected in the kinetic traces owing to experimental error, then the pK_a would be either 5 or 10. Assuming that the former case were true (p$K_a \approx 5$), it is highly unlikely that Asp-166 would have the basicity to abstract the hydroxyl proton in the ground state complex. However, the transition state pK_a of the oxygen nucleophile will be intermediate between its substrate and product values owing to the movement of the terminal phosphate in a concerted, general-base catalytic mechanism (Jencks, 1972). The synchronous movement of the phosphoryl group, therefore, could lessen the pK_a difference between the substrate hydroxyl and the putative base favoring proton transfer. It is conceivable that the pK_a value of this catalytic base could be higher than 5 although this would imply that a slow, metal ion-dependent conformational change precedes the phosphoryl transfer step and controls the rate of the "burst" phase so that our methods cannot report on this ionization.

Influence of Solvent Deuterium on the "Burst" Phase. If the hydroxyl proton is abstracted in the transition state for phosphoryl transfer, then the rate of the "burst" phase may be sensitive to solvent deuterium. Although SIEs on model reactions of phosphoesters are relatively small, they are measurable. For example, reactions of various nucleophiles with 2-aryloxy-2-oxo-1,3,2-dioxaphosphorinans are subject to SIEs ranging from 1.1 to 2.0 depending on the contribution from general-base catalysis (Khan & Kirby, 1970). If similar

rate reductions in the phosphoryl transfer reaction in PKA occur, the rapid quench technique can easily monitor this SIE, particularly in the presence of Mn^{2+}. The detection of a kinetic SIE on a chemical reaction involving exchangeable hydrogen is dependent on the fraction of deuterium in the transition state compared to the composition of the solvent (Schowen, 1978). A maximal SIE is expected when the proton is equidistant from the donor and acceptor pair (Bell & Goodal, 1966). The "burst" phases for peptide phosphorylation were measured in the presence and absence of Mg^{2+} or Mn^{2+} and 0 or 90% D_2O (Figures 2 and 3). No SIE was detected on either "burst" phase. Since this kinetic transient is pH independent (Figure 1) for both Mg^{2+} and Mn^{2+}, the lack of a SIE on the "burst" phases is not caused by an equilibrium effect on the buffer or pH electrode that would conceal the true SIE. The lack of a SIE on the "burst" phase must be interpreted cautiously. If the "burst" phase is limited by a slow, metal ion-dependent conformational change preceding the phosphoryl transfer reaction, the observed SIE would be greatly diminished. Also, there is reason to suspect that the absence of a SIE may not preclude a general-base catalytic mechanism. The aminolysis of phenyl acetate proceeds with general-base catalysis but displays only a minor rate reduction in deuterium oxide (Jencks & Carriuolo, 1960). There is no explanation for such a phenomenon although the small difference in zero point vibrational energies between hydrogen and deuterium may account for this (Jencks, 1969).

When peptide concentrations are high, the amplitude of the "burst" phase is related to the enzyme concentration by the magnitudes of k_3 and k_4 according to eq 4:

$$\alpha/[E] = \left\{\frac{k_3}{k_3 + k_4}\right\}^2 \qquad (4)$$

where α is the observed "burst" amplitude and [E] is the total enzyme concentration. In H_2O, the predicted ratios of α /[E] ($\alpha/[E]^{calc}$) based on the fitted values of k_3 and k_4 from Table 2 are identical to the experimental values ($\alpha/[E]^{exp}$). This correspondence supports the interpretation of the rapid quench data. In D_2O, the predicted ratios of $\alpha/[E]^{calc}$ are slightly higher than the predicted values in H_2O owing to the large SIE on k_{cat} and the lower observed values for k_4. This small improvement in amplitude, though, is not observed in the experimental data in the presence of D_2O. This small discrepancy is likely due to the small population of an unproductive form of the enzyme in the presence of D_2O.

Proton Inventory Techniques. Although solvent deuterium has no effect on the "burst" phase, the rate-limiting step for turnover is very sensitive to D_2O. A proton inventory technique was used to determine the role of solvent deuterium on k_{cat} in the presence of Mg^{2+} (Figure 4) . The response of k_{cat} to the atom fraction of deuterium (n) exhibits classic "bowed"-down behavior (Quinn, 1987; Schowen & Schowen, 1982; Schowen, 1978). The data were best fit to a modified form of the Gross−Butler equation which incorporates a transition state contribution from solvent. The data in Figure 4 were fit to eq 5:

$$k/k^o = (1 - n + n\phi)^{\nu} \qquad (5)$$

where k and k^o are k_{cat} in the presence and absence of varying atom fractions of deuterium (n), ϕ is the fractionation factor for deuterium in the transition state, and ν is the number of protons transferred in the transition state. The data were best fit when $\nu \geq 2$ and $\phi = 0.45 \pm 0.020$ (when $\nu = 2$). The data imply that two or more protons are transferred in the transition state for product release. Since no chemistry is rate-limiting in k_{cat}, we presume that this SIE reflects a conformational change in the protein that is associated with product release, a proposal that is consistent with the conformational flexibility observed in a number of protein kinase structures (Jeffrey et al., 1995; Zheng et al., 1993b).

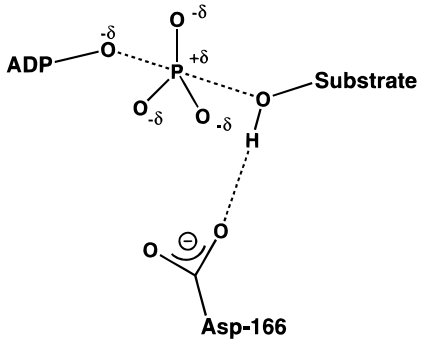

FIGURE 5: Possible transition state structure for proton transfer in the phosphorylation reaction of PKS(14−22) catalyzed by PKA. The structure assumes that cleavage of the $\beta\gamma$ bridging oxygen is advanced relative to bond formation with the nucleophile.

Proton Transfer in PKA. The study of phosphoryl transfer catalyzed by PKA is linked inescapably to the study of its proton transfer mechanism. Based on chemical principle, the hydroxyl proton must be removed to form products. The timing of this transfer, however, is still debated, and three mechanisms are possible. First, the active site may promote the ionization of the substrate in the ground state and the negatively charged oxygen can attack the γ-phosphate of ATP. This mechanism is not likely, though, owing to the weak basicity of the nearby Asp-166 compared to the hydroxyl (compare solvent pK_a's of 4 and 14) and the absence of local countercharges to stabilize the ionized serine. Second, the proton may be delivered after the transfer of the γ-phosphate. No special driving force for this proton transfer is required since the pK_a of the β-methylene oxygen of phosphoserine is presumed to be very low. Third, the proton may be transferred in a reaction transition state assisted by general-acid−base catalysis. It is this last possibility that has received the most attention owing to the proximity of Asp-166 and the hydroxyl, but the second mechanism is also feasible. Although the data presented in this paper do not definitively dispute the viability of the third mechanism, they establish constraints on the ionization constant for the catalytic base if it exists. The pK_a must be either lower than 5 or higher than 10 and can be between these limits if a slow, metal ion-dependent conformational change precedes phosphoryl transfer. Given the strong precedence for a metaphosphate-like transition state in both model (Admiraal & Herschlag, 1995; Benkovic & Schray, 1973) and enzyme (Hengge et al., 1996; Hollfelder & Herschlag, 1995; Zhang et al., 1995) reactions, there is little requirement for general-base catalysis. For PKA, this may define a transition state such as the one in Figure 5 where the proton resides predominantly on the hydroxyl oxygen. In this mechanism, solvent would absorb the proton from the oxygen after phosphoryl transfer.

If PKA does not require a general-base catalyst, two questions arise. What is the function of Asp-166 in PKA,

and will this function be conserved in all protein kinases? Although the data presented herein do not provide evidence for a general-base catalytic process, mutagenesis data clearly show that this conserved residue is important for catalysis. Removal of this residue in yeast PKA yields a mutant enzyme with a 300-fold reduced turnover number (Gibbs & Zoller, 1991) while a subtle glutamate replacement in the tyrosine protein kinase v-Fps leads to an inactive enzyme (Weinmaster et al., 1984). We propose that Asp-166 does not have the basicity to catalyze proton abstraction in the ground state but rather is a good hydrogen bond acceptor. This hydrogen bond facilitates phosphoryl transfer by limiting the number of hydroxyl rotamers, thereby positioning the nucleophile for productive attack of the γ-phosphate of ATP. Whether this carboxyl group serves the same function in other protein kinases is still unclear. The change in the acidity of the substrate nucleophile suggests that a mechanism variation for kinases that phosphorylate tyrosine is possible. The pK_a of the tyrosine hydroxyl is approximately 4 pH units lower than that for serine and may be more readily ionized by the conserved aspartate. In keeping with this argument, a large SIE in excess of 3 is observed on a step preceding product release in v-Fps (Adams, 1996).

REFERENCES

Adams, J. A. (1996) *Biochemistry 35*, 10949−10956.
Adams, J. A., & Taylor, S. S. (1992) *Biochemistry 31*, 8516−8522.
Adams, J. A., & Taylor, S. S. (1993a) *Protein Sci. 2*, 2177−2186.
Adams, J. A., & Taylor, S. S. (1993b) *J. Biol. Chem. 268*, 7747−7752.
Admiraal, S. J., & Herschlag, D. (1995) *Chem. Biol. 2*, 729−739.
Bell, R. P., & Goodal, D. M. (1966) *Proc. R. Soc. London, Ser. A 294A*, 273−297.
Benkovic, S. J., & Schray, K. J. (1973) in *The Enzymes* (Boyer, P. D., Ed.) pp 201−238, Academic Press, New York.
Bhatnagar, D., Roskoski, R. J., Rosendahl, M. S., & Leonard, N. J. (1983) *Biochemistry 22*, 6310−6317.
Bossemeyer, D., Engh, R. A., Kinzel, V., Ponstingl, H., & Huber, R. (1993) *EMBO J. 12*, 849−859.
Cook, P. F., Neville, M. E., Vrana, K. E., Hartl, F. T., & Roskoski, J. R. (1982) *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.
Denu, J. M., Zhou, G., Guo, Y., & Dixon, J. E. (1995) *Biochemistry 34*, 3396−3403.
Ellis, K., & Morrison, J. F. (1982) *Methods Enzymol. 87*, 405−426.
Gibbs, C. S., & Zoller, M. J. (1991) *J. Biol. Chem. 266*, 8923−8931.
Granot, J., Mildvan, A. S., Hiyama, K., Kondo, H., & Kaiser, E. T. (1980) *J. Biol. Chem. 255*, 4569−4573.
Grant, B., & Adams, J. A. (1996) *Biochemistry 35*, 2022−2029.
Hengge, A. C., Denu, J. M., & Dixon, J. E. (1996) *Biochemistry 35*, 7084−7092.
Ho, M.-f., Bramson, H. N., Hansen, D. E., Knowles, J. R., & Kaiser, E. T. (1988) *J. Am. Chem. Soc. 110*, 2680−2681.
Hollfelder, F., & Herschlag, D. (1995) *Biochemistry 34*, 12255−12264.
Hu, S. H., Parker, M. W., Lei, J. Y., Wilce, M. C., Benian, G. M., & Kemp, B. E. (1994) *Nature 369*, 581−584.
Hubbard, S. R., Wei, L., Ellis, L., & Hendrickson, W. A. (1994) *Nature 372*, 746−754.
Jeffrey, P. D., Russo, A. A., Polyak, K., Gibbs, E., Hurwitz, J., Massague, J., & Pavletich, N. P. (1995) *Nature 376*, 313−320.
Jencks, W. P. (1972) *J. Am. Chem. Soc. 94*, 4731−4732.
Jencks, W. P., & Carriuolo, J. (1960) *J. Am. Chem. Soc. 82*, 675−681.
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.
Khan, S. A., & Kirby, A. J. (1970) *J. Chem. Soc. B* 1172−1182.
Kirby, A. J., & Jencks, W. P. (1965a) *J. Am. Chem. Soc. 87*, 3217−3224.
Kirby, A. J., & Jencks, W. P. (1965b) *J. Am. Chem. Soc. 87*, 3209−3216.
Knighton, D. R., Zheng, J., Ten Eyck, L. F., Ashford, V. A., Xuong, N.-h., Taylor, S. S., & Sowadski, J. M. (1991) *Science 253*, 407−414.
Knowles, J. R. (1980) *Annu. Rev. Biochem. 49*, 877−919.
Kong, C.-T., & Cook, P. F. (1988) *Biochemistry 27*, 4795−4799.
Lindberg, R. A., Quinn, A. M., & Hunter, T. (1992) *Trends Biochem. Sci. 17*, 114−119.
Madhusudan, Trafny, E. A., Xuong, N.-h., Adams, J. A., Ten Eyck, L. F., Taylor, S. S., & Sowadski, J. M. (1994) *Protein Sci. 3*, 176−187.
Mildvan, A. S., Sloan, D. L., Fung, C. H., Gupta, R. K., & Melamud, E. (1976) *J. Biol. Chem. 251*, 2431−2434.
Mitchell, R. D., Glass, D. B., Wong, C., Angelos, K. L., & Walsh, D. A. (1995) *Biochemistry 34*, 528−534.
Quinn, D. M. (1987) *Chem. Rev. 87*, 955−979.
Schowen, K. B., & Schowen, R. L. (1982) *Methods Enzymol. 87*, 551−605.
Schowen, K. B. J. (1978) in *Transition States of Biochemical Processes* (Gandour, R. D., & Schowen, R. L., Eds.) pp 225−283, Plenum Press, New York.
Shoemaker, D. P., & Garland, C. W. (1962) *Experiments in Physical Chemistry*, 2nd ed., McGraw-Hill, New York.
Skoog, M. T., & Jencks, W. P. (1984) *J. Am. Chem. Soc. 106*, 7597−7606.
Taylor, S. S., Radzio-Andzelm, E., & Hunter, T. (1995) *FASEB J. 9*, 1255−1265.
Walsh, D. A., Angelos, K. L., van Patten, S. M., Glass, D. B., & Garetto, L. P. (1990) in *Peptides and Protein Phosphorylation* (Kemp, B. E., Ed.) pp 43−84, CRC Press, Boca Raton, FL.
Weinmaster, G., Zoller, M. J., Smith, M., Hinze, E., & Pawson, T. (1984) *Cell 37*, 559−568.
Whitehouse, S., & Walsh, D. A. (1983) *J. Biol. Chem. 258*, 3682−3692.
Whitehouse, S., Feramisco, J. R., Casnellie, J. E., Krebs, E. G., & Walsh, D. A. (1983) *J. Biol. Chem. 258*, 3693−3701.
Xu, R.-M., Carmel, G., Sweet, R. M., Kuret, J., & Cheng, X. (1995) *EMBO J. 14*, 1015−1023.
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.
Yoon, M.-Y., & Cook, P. F. (1987) *Biochemistry 26*, 4118−4125.
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, FL.
Zhang, F., Strand, A., Robbins, D., Cobbs, M. H., & Goldsmith, E. J. (1994) *Nature 367*, 704−711.
Zhang, Z., Wu, L., & Chen, L. (1995) *Biochemistry 34*, 16088−16096.
Zheng, J., Knighton, D. R., Ten Eyck, L. F., Karlsson, R., Xuong, N.-h., Taylor, S. S., & Sowadski, J. M. (1993a) *Biochemistry 32*, 2154−2161.
Zheng, J., Knighton, D. R., Xuong, N.-h., Taylor, S. S., Sowadski, J. M., & Ten Eyck, L. F. (1993b) *Protein Sci. 2*, 1559−1573.
Zheng, J., Trafny, E. A., Knighton, D. R., Xuong, N.-h., Taylor, S. S., Ten Eyck, L. F., & Sowadski, J. M. (1993c) *Acta Crystallogr., Sect. D D49*, 362−365.

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