Kinetic and Catalytic Mechanisms of Protein Kinases

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I. Introduction

A. Protein Phosphorylation and Protein Kinases

The reversible phosphorylation of proteins ranks among the most important posttranslational modifications that occur in the cell. In the last several decades, protein phosphorylation has shifted from a singular curiosity uncovered during the study of glycogen metabolism[1,2] to a universal mechanism for cellular control and adaptation (see refs 3 and 4). Today, it is difficult to discuss any biochemical event in the living cell that is not touched directly or indirectly by protein phosphorylation. The discovery that a large class of enzymes known as the protein kinases catalyze the phosphorylation of target proteins and enzymes, thereby regulating biological output, is now a cornerstone of the signal transduction field. The protein kinases can regulate a wide range of processes including carbohydrate and lipid metabolism, neurotransmitter biosynthesis, DNA transcription and replication, organelle trafficking, smooth muscle contraction, and cell differentiation. Given this diversity, it is not surprising that the vertebrate genome is estimated to contain as many as 2000 protein kinases to carry out these numerous biochemical processes.[5] The scientific community has invested significant effort in discovering new and interesting protein kinases, evaluating complex signaling pathways which include these enzymes, assessing the structural outcome of phosphorylation on substrate proteins, determining the three-dimensional structures of protein kinases, and developing potential drugs directed at specific protein kinases that can interdict cell function. Great strides have been made in all of these areas, but the extraordinary advances in establishing the molecular structure of simple and complex protein kinases has been particularly encouraging for the study of their catalytic and regulatory mechanisms. We now understand the basic fold of the catalytic core of a protein kinase,[6−10] and more recently, we have been able to understand how some noncatalytic regulatory elements attach to this core.[11,12]

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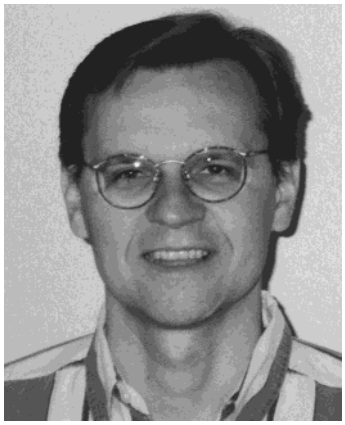


Joseph Adams was raised in Secaucus, NJ, a small town several miles outside of New York City. As an undergraduate majoring in Chemistry at Rutgers University in nearby Newark, the author got his first experience working with enzymes in the laboratory of Frank Jordan. Here, he studied the thiamin-dependent enzyme, pyruvate decarboxylase, developing suicide-based inactivators. He attained his Ph.D. degree at Penn State University studying the folate-dependent enzyme, dihydrofolate reductase, and catalytic antibodies. The author performed his postdoctoral studies at the University of California at San Diego with Susan Taylor, where he developed an interest in the enzymes that catalyze protein phosphorylation. He started his first faculty position in the Chemistry Department at San Diego State University in 1994 and then moved to the University of California at San Diego Medical School in 1999, where he is currently an Assistant Professor in the Pharmacology Department. The author is married with two small children, Trevor (b. 1995) and Kayleigh (b. 1997). His wife, Patricia A. Jennings, who he met in graduate school, is an Associate Professor in the Chemistry and Biochemistry Department at the University of California at San Diego.

B. Scope of Review

Protein kinases are ATP-dependent phosphotransferases that deliver a single phosphoryl group from the γ position of ATP to the hydroxyls of serine, threonine, and tyrosine in protein substrates. They require an essential divalent metal ion, usually Mg^{2+}, to facilitate the phosphoryl transfer reaction and assist in ATP binding. While the phosphoryl transfer mechanism is chemically simple, we are learning that the phosphorylation of protein substrates in the active site of a protein kinase is complex and involves structural changes. While the simple core structure is shared within the protein kinase family, the core frequently interacts with regulatory proteins or domains that either enhance or repress catalytic function. This structural hierarchy offers an extraordinary level of complexity to the enzyme family and makes functional studies on catalysis and regulation intricate. To understand catalysis in these complex systems, it will be important to evaluate how events sometimes far from the active site influence the kinetic mechanism. Knowledge of the individual steps associated with substrate recognition, product release, and phosphoryl transfer are essential for an effective evaluation of these enzymes and the manners in which they are regulated. This review will focus on approaches taken to assess the catalytic and kinetic mechanisms of protein phosphorylation catalyzed by protein kinases. Attention will be concentrated on the roles of active-site residues, metal ions, conformational changes, and regulatory phosphorylation on the processing of peptide and protein substrates.

While data will be reviewed from many different enzymes within the protein kinase family, one member, cAMP-dependent protein kinase [PKA], has taken center stage and is, by far, the best understood protein kinase from a mechanistic viewpoint. PKA is a heterotetramer composed of a regulatory dimer (R_2) and two catalytic subunits (2C). The C-subunits are active when cAMP binds R_2 and induces dissociation of the tetramer.[13−15] While PKA is the best studied, substantial progress has been made with other members of the protein kinase family. Table 1 provides a representative list of other protein kinases for which mechanistic studies have been performed. For the majority of protein kinases listed in this table, X-ray diffraction data is available on either the kinase core and/or the entire protein complex. For example, several X-ray structures for the C-subunit of PKA are available without the R_2 dimer[8,16−20] but the inactive, tetrameric complex has not been solved. Cdk2, an enzyme involved in control of the cell cycle, requires a cyclin subunit for activity, and X-ray structures for cdk2 with and without cyclin have been solved.[10,11,21] In some cases, the structures of multidomain protein kinases have been solved. The X-ray structure of c-Src which includes the kinase core in addition to two noncatalytic portions, SH2 and SH3 domains, has been solved recently.[12,22−24] In some cases, such as PhK, a multisubunit protein kinase with a molecular weight in excess of one million, only the crystallographic structure of the catalytic portion has been solved.[7] The number of available protein kinase structures will certainly continue to grow and provide an essential foundation for future studies on catalysis and its regulation. For this review, the

Table 1. Several Protein Kinases and Their Substrate Specificities[a]

	name	consensus sequence	ref
	serine protein kinases		
PKA	cAMP-dependent protein kinase	-R−R-X-**S/T**-hyd-	173
PhK	phosphorylase kinase	-R−X-X-**S/T**-F−F-	38
cdk2	cyclin-dependent kinase-2	-**S/T**-P−X-R/K	32
ERK2	extracellular-regulated kinase-2	-P−X-**S/T**-P-	36−38
	tyrosine protein kinases		
c-Src	cellular form of the transforming agent of the Rous sarcoma virus	-E−E-I-**Y**-E/G−X-F-	30
v-Fps	transforming agent of the Fujinami sarcoma virus	-E−I-**Y**-E−X-I/V-	30, 174
Csk	C-terminal Src kinase	-I-**Y**-M−F−F−F-	175
InRK	Insulin receptor kinase	-**Y**-M−M-M-	30
EGFR	Epidermal growth factor receptor	-E−E-E-**Y**-F-	30

[a] Phosphorylation sites (P-sites) are shown in bold type.

analyses of the protein kinases in Table 1 along with other enzymes in the family will be discussed and compared to PKA as a model structure and from this comparison some general features for catalysis will be put forth. While noncatalytic domains and subunits affect kinase function, this review will focus largely on the core structure for the interest of brevity.

II. Substrate Recognition

A. Local Sequence Elements

The majority of the protein kinases fall into two large classes based on their ability to phosphorylate serine, threonine, or tyrosine side chains. Protein kinases that phosphorylate serine or threonine are termed serine protein kinases [SPKs], and those that phosphorylate tyrosine are called tyrosine protein kinases [TPKs]. There is a third class of kinases that can phosphorylate both serine and tyrosine side chains.[25,26] This group, termed dual-specific protein kinases, is small in number and is not well characterized from a mechanistic standpoint. Protein kinases poorly phosphorylate free amino acids and rely partly on local residues for high affinity. In general, they phosphorylate peptide regions based on the residues immediately flanking the site of phosphorylation. This site is called the P-site, and residues N-terminal to this site are sequentially numbered P-1, P-2, P-3, etc. Residues C-terminal to the P-site are termed P+1, P+2, P+3, etc. Consensus sequences for optimal phosphorylation have been determined using peptide variants in either a manual screening [e.g., refs 27−29] or random library approach [e.g., refs 30−34]. In the former approach, phosphorylation sites in proteins are identified and short substrate peptides, based on the local sequence surrounding this site, are synthesized. Replacements in individual residues are frequently made, and the effects on the steady-state kinetic parameters are measured to evaluate the significance of the residues. This approach, while effective, can be time-consuming. The search for good substrate peptides has been streamlined by the application of random peptide libraries. In this technique, all possible amino acids with the exclusion of the P-site are inserted at each position in a short peptide sequence. Optimal peptide substrates are then selected from this library using peptide sequencing and statistical methods.

The application of substrate specificity studies to protein kinases has provided considerable data on the role of local residues for efficient phosphorylation. These local residues define a consensus sequence for substrate phosphorylation. Table 1 lists the consensus sequences for several protein kinases. In general, amino acids that are approximately four residues or less from the phosphorylation site in either the N- or C-terminal directions are the most significant for directing phosphoryl transfer. For example, PKA prefers to phosphorylate serine or threonine side chains with positively charged residues in the P-2 and P-3 positions and a small hydrophobic residue in the P+1 position. A standard substrate peptide for PKA, based on this consensus sequence, is Kemptide (LRRASLG). Substitution of either the P-2 or P-3 arginine in Kemptide with uncharged residues leads to poor substrates.[35] The nature of the consensus sequence can vary depending on the type of protein kinase. For example, ERK2, a member of the MAPK (mitogen-activated protein kinase) family, and cdk2, a member of the cyclin-dependent kinase family, prefer to phosphorylate serine peptides where proline is in the P+1 position. Owing to this strict substrate specificity, these two protein kinases and their family members are termed "proline-directed" kinases.[36−39] Many nonreceptor TPKs, such as c-Src, Csk, and v-Fps, demonstrate a strong preference for hydrophobic residues (e.g., isoleucine, valine) in the P-1 position. In addition, many nonreceptor TPKs such as c-Src and v-Fps prefer negatively charged residues in the P+1 position but there are exceptions (e.g., Csk). In some cases, protein kinases may display overlapping substrate specificities based on in vitro assays. For example, PKA and PKC will phosphorylate similar peptides on serine.[31,32]

B. Distal Recognition Elements

While many factors influence substrate specificity in vivo, the local sequences flanking the P-site can certainly impart strong binding affinity.[40] Nonetheless, it has become apparent that in many cases short peptide sequences may not exploit the complete binding capacity offered by the protein kinase. For example, based on k_{cat}/K_m, p38 MAPK phosphorylates the protein substrate MAPKAP2 150-fold more efficiently than the corresponding 14-residue substrate peptide that contains the target's phosphorylation site and the recognition motif, PXTP.[41] The natural substrate is, thus, likely to utilize additional binding determinants not present in the immediate environment of the active site. In general, the MAPK family of which p38 is a member utilizes one or more regions outside the consensus region for substrate recognition and specificity (see ref 42). For example, many of the transcription factor substrates phosphorylated by MAPK including c-Jun, ATF-2, and MEF2A contain one or more docking domains between 50 and 100 amino acids N-terminal to the phosphorylation sites.[43−46] These distal recognition elements are likely to be very important for efficient phosphorylation. For example, the transcription factor ATF2 is phosphorylated 1300-fold more efficiently than the corresponding 14-residue substrate peptide which contains the phosphorylation site.[41] While a detailed molecular description of long-range substrate contacts in protein kinases is not available in all cases, the binding of a protein substrate to cdk2 has provided new insights into substrate recognition. As a member of the cyclin-dependent kinases, cdk2 requires a bound cyclin for maximum activity. The efficient phosphorylation of the protein substrate p107 by the cyclinA-cdk2 complex requires a small hydrophobic patch, RXL, known as a recruitment peptide.[47] The X-ray structure of cyclinA-cdk2 has been solved with a short peptide fragment from p107 containing the RXL motif (residues 658−668), and a complementary pocket in the cyclin molecule has been established.[48] Surprisingly, this pocket is 40 Å away from the site

of phosphoryl transfer, a distance that is likely to reflect a lower limit upon consideration of the complete protein surface. While it is still unclear whether binding of the P-site region of p107 occurs synchronously with the recruitment segment, the data extend the scope of the available substrate binding surface from the immediate consensus sequence to regions far outside the active site. As in the case for cdk2, this surface extends to the bound cyclin regulatory protein.

III. Reaction Order

A. Kinetic Studies on PKA

The reactions catalyzed by protein kinases require both ATP and a substrate protein and, thus, can be viewed as bisubstrate kinetic mechanisms. Accordingly, protein phosphorylation could conform potentially to a random or ordered pathway with respect to substrate and ATP binding. For many enzyme systems, distinguishing between random and ordered reactions is suited to traditional steady-state kinetic approaches.[49] PKA has been shown to incorporate a random kinetic mechanism using Kemptide as a substrate. These conclusions are largely derived from two kinetic outcomes. First, noncompetitive inhibition patterns are observed either using a serine peptide analogue, guanethidine, with respect to ATP or using ADP with respect to Kemptide.[50] Both results indicate that ATP and Kemptide can bind PKA independently as expected in a strictly random kinetic mechanism. Second, in pulse-chase experiments, radiolabeled phosphokemptide is generated from tritiated Kemptide that is preequilibrated with PKA prior to a cold chase, indicating that the substrate peptide can bind prior to ATP.[51] Likewise, ^{32}P is incorporated into phosphokemptide when PKA is preequilibrated with [$^{32}P\gamma$] ATP prior to addition of Kemptide and cold ATP.[51] These results are not possible unless ATP and Kemptide have unrestricted access to the active site, and the binding of one does not exclude the binding of the other. Despite this unambiguous evidence for random binding, PKA has a preference for binding ATP prior to substrate based on thermodynamic grounds.[50,52] Longer substrate peptides have been designed that bind significantly better than ATP, but no studies have been performed to determine whether the reaction ordered is affected.[40]

B. Other Protein Kinases

To determine whether a common reaction order is shared among the protein kinases, many research groups have analyzed the steady-state kinetic parameters in the presence of inhibitors. For example, c-Src, Csk, PhK, and the InRK have been shown to utilize random kinetic mechanisms.[53−57] These conclusions emanate largely from the observation of noncompetitive inhibition plots using substrate analogues. In these cases, peptide or random copolymer substrates were used as phosphoryl acceptors, but there is some concern whether these artificial substrates adequately mimic the real in vivo substrate proteins. For large protein substrates, ATP access could be limited or the substrate protein could induce a conformation in the kinase core that favors ATP binding. In either scenario, the mechanism would become ordered with either ATP or substrate protein binding first. Two kinetic investigations suggest that the reaction order can, indeed, be influenced by the nature of the substrate. For p38α, a member of the MAPK family, ATP binds prior to a peptide substrate that is derived from a phosphorylation site in the EGFR.[58] In contrast, another research group found that this enzyme binds the larger protein substrate ATF2, a transcription factor, prior to ATP binding.[59]

C. Is There a Consensus?

In the majority of cases, protein kinases incorporate random kinetic mechanisms. Nonetheless, there are a number of examples where ordered processes are observed, warranting some concern about the assignment of a general kinetic mechanism for the enzyme family.[59−61] In addition to reports where larger substrates precede ATP binding, there are also examples where smaller substrates exclude ATP binding. For example, in one study, the EGFR was found to observe an ordered reaction pathway with the peptide substrate, LEDAEYAARRRG, binding prior to ATP.[61] Surprisingly, the same enzyme is reported to utilize a random kinetic mechanism using the larger poly Glu_6Ala_3Tyr as a substrate.[62] These results appear to conflict with the notion that larger substrates may enforce an ordered mechanism owing to steric exclusion of the nucleotide. In these cases, the kinetic studies on protein kinases have led to some confusion regarding reaction order. The determination of mechanism relies upon the intersection of double reciprocal plots of substrate and ATP and/or the inhibition patterns of dead-end substrate or product analogues. Whether these analogues ideally represent the desired substrate or not may limit data interpretation. The pulse-chase experiment, brilliantly applied more than two decades ago to hexokinase, does not rely upon analogues.[32] This technique showed definitively that PKA observes a random kinetic pathway[51] when prior reports supported other mechanisms.[63,64] Nonetheless, protein kinases bind large proteins with intricate tertiary and quaternary structure that may not be mimicked adequately, in all cases, by small peptides or random copolymers. Given these issues, it is worthwhile to bring all kinetic means of investigation to each protein kinase and include a sufficient range of model and, if possible, real substrates so that a comprehensive position can be established.

IV. Protein Kinase Structural Elements

A. Conserved Core

Sequence comparisons of members of the protein kinase family reveal that they share a conserved region of approximately 200−250 amino acids that confers kinase activity.[65,66] From these studies, several homologous regions and strictly conserved residues have been detected within the protein kinase

family. In 1991, the first protein kinase structure, the C-subunit of PKA, was elucidated by X-ray diffraction methods.[16,17] This structure put finally into complete context the three-dimensional arrangement of amino acids that constitute an active protein kinase. In three short years after this discovery, the X-ray crystallographic solutions of two more protein kinases, cdk2[10] and ERK2,[9] made it abundantly clear that all protein kinases would share a common kinase domain.[67] Owing to the highly conserved nature of the core structure, a description of PKA encompasses the main features of all members of the family and is worth noting here. The C-subunit of PKA is comprised of two domains—a small ATP binding domain composed primarily of α helices and a larger substrate binding domain composed mostly of β strands (Figure 1). The two domains, connected

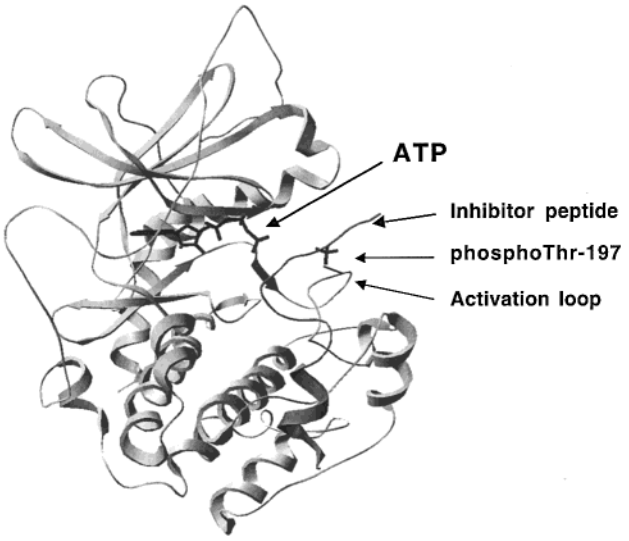


Figure 1. Ribbon diagram of PKA cocrystallized with ATP and a peptide inhibitor, PKI(5−24).[18] Arrows point to the activation loop and the phosphorylation site, pThr-197, in the activation loop.

by a small linker region, generate a binding pocket for ATP and the substrate. While ATP is nestled deep between the domains, the inhibitor peptide resides on the periphery of the pocket. The structural database on protein kinases is expanding rapidly, but the basic fold revealed in PKA is present in all these structures.

B. Active Site and Mutations

Several conserved residues converge near the site of phosphoryl transfer and appear to play important catalytic functions based on the available X-ray data. Figure 2 shows the active site of PKA and highlights several key residue interactions. Asp-184, a strictly conserved residue, interacts with the essential Mg^{2+} (Mg1), which chelates the β and γ phosphates of ATP. The chelation of this metal may position the terminal phosphate for direct transfer to the hydroxyl acceptor or mask the charge of the γ phosphate, thereby reducing charge development in the reaction transition state or limiting electrostatic repulsion for an incoming nucleophile. It is difficult to discern which function predominates, and it is possible that all

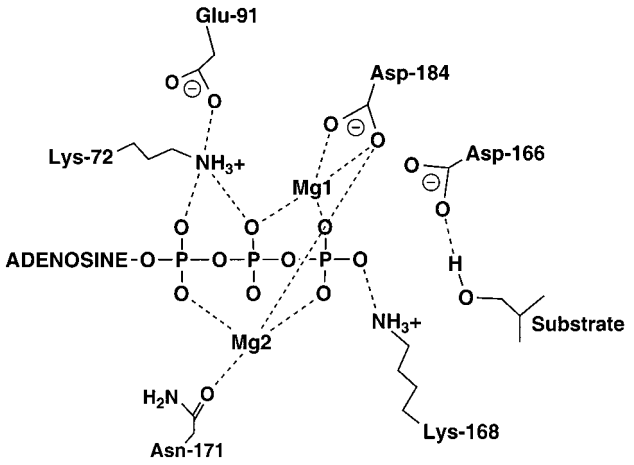


Figure 2. Key residue interactions in the active site of PKA. Mg1 and Mg2 represent the activating and inhibitory divalent metal ions, Mg^{2+}, respectively. This drawing is based on the PKA ternary complex with ATP and a peptide inhibitor.[18] The dotted lines indicate close contacts of less than 2.6 Å but are not drawn to scale. The hydroxyl of the substrate is shown based on a binary complex of PKA with a substrate peptide.[19]

participate in facilitating phosphoryl transfer. Whatever the role, Asp-184 is extremely important for catalytic function. Yeast expressing a mutant PKA where aspartate is replaced with alanine are not viable.[68] Additional stabilization of the phosphates may emanate from the interaction of Lys-72 with the α and β phosphates of ATP. Unlike the role of Asp-184, the function of Lys-72 is better understood. Mutagenesis studies on this conserved lysine indicate that its primary function is to facilitate phosphoryl group transfer without influencing ATP binding. Arginine substitution in Lck, a nonreceptor TPK expressed in T-lymphocytes, and ERK2 lead to mutant proteins with extremely low turnover numbers, but in both cases, no changes in the binding of ATP are detected.[69,70] A more nonconservative alanine replacement in this residue in yeast PKA results in only a 6-fold increase in K_m for ATP compared to an 840-fold reduction in k_{cat}.[68] These findings are consistent with prior structure−function studies on PKA. The affinities of ATP, ADP, and adenosine are very similar in PKA,[71] suggesting that any electrostatic contacts between the phosphates and the active site are not solely committed to lowering the ground-state energy of the nucleotide−enzyme complex but rather subtly position the γ phosphate for nucleophilic attack. Lys-72 is specifically labeled and inactivated by the irreversible inhibitor, *p*-fluorosulfonylbenzoyl 5′-adenosine.[72,73] This modification is common and has been used as confirmatory evidence for the presence of a protein kinase [e.g., refs 72 and 74−76].

In PKA, the conserved Asp-166 (Figure 2) is near the alanine of the inhibitor peptide, and in crystal structures of the ADP−substrate complex (where the P site is serine), a direct hydrogen bond can be assigned between the carboxyl and the hydroxyl groups.[19] This hydrogen bond has also been observed in the ternary complex for the InRK and PhK, suggesting that the conserved aspartate is likely to serve a general function in all protein kinases. Owing

to its proximity, Asp-166 may direct the hydroxyl for attack on the γ phosphate of ATP. The potential function of this conserved residue is ardently debated. Further consideration of its function will be offered in section VIII. Lys-168 in PKA makes an electrostatic contact with the γ phosphate, but this interaction is not fully conserved throughout the enzyme family. For example, many TPKs have an arginine or alanine in this position.[66] In PKA, Lys-168 also makes a contact with Thr-201, a residue that forms part of the P+1 binding cleft. In yeast PKA, replacement of this lysine with alanine leads to a mutant protein (K212A) with a 30-fold elevated K_m for Kemptide and 50-fold reduced k_{cat}.[68] This is consistent with a role for lysine in supporting both the phosphoryl transfer step and possibly peptide binding. The comparatively mild effect of alanine substitution on turnover is also compatible with the variability of this residue in other protein kinases.

V. Activation Loop Phosphorylation

A. Autoinhibition—Crystallographic Insights

The activities of the protein kinases are regulated through an assortment of mechanisms involving regulatory subunits and domains [see refs 77−80], fatty acid acylation [see refs 81 and 82], isoprenylation [see refs 83 and 84], second messengers [see refs 85−87], subcellular localization [see refs 88−90], and phosphorylation [see refs 3 and 91]. While an adequate summary of these mechanisms is outside the scope of this review, the phosphorylation of protein kinases is germane since this posttranslational modification may occur within the kinase core and can be correlated directly with the catalytic mechanism. The essential phosphorylation site in PKA, Thr-197, is part of a loop segment known as the activation loop (Figure 1). This phosphorylation enhances catalytic activity by approximately 3 orders of magnitude in PKA and is, consequently, an essential posttranslational modification.[92,93] Many protein kinases are also phosphorylated in the activation loop, but for some that are not, a negatively charged residue is substituted for the phosphoamino acid.[94] In cases where X-ray diffraction data is available, this residue is far from the site of phosphoryl transfer in active protein kinases so that its role in activity regulation must involve interstitial structure. The crystallographic models of phosphorylated and nonphosphorylated protein kinases have been used to put forth an autoregulatory role for the activation loop. In cdk2, FGFR kinase, and the InRK, the B factors of residues in the activation loops are high when the loops are not phosphorylated. Under these circumstances, best modeling places them partly in the active site.[11,21,95−97] Phosphorylation then lowers the B factors and causes large movements in the activation loop, allowing better substrate access to the active site. In this manner, the activation loop may function as a "door" to the substrate pocket, a phenomenon which could explain the large rate enhancements observed upon activation loop phosphorylation [e.g., refs 93 and 98−101]. This is an exciting possibility since it has the capacity to unify the regulatory mechanisms of a broad array of protein kinases whose activities are largely controlled by phosphorylation.

B. Opening the "Door"

The data from several X-ray structures have been used to posit that the activation loop limits access of substrates to the active site.[11,96] If this hypothesis is correct, then it follows that substrates either cannot gain access or have very limited access to the active site in the dephosphorylated enzyme. In essence, this model predicts that the loop serves as a competitor for the substrate when the enzyme is repressed. As such, loop dephosphorylation is expected to impair substrate and/or ATP binding but have little or no effect on the rate of the phosphoryl transfer step. Attempts to test this hypothesis using dephosphorylated enzymes have unfortunately provided mixed results. Figure 3 shows the relative effects of activation loop phosphorylation on the kinetic mechanisms of four protein kinases. The bar graph in this figure compares three parameters—the dissociation constants of ATP and substrate and the rates of phosphoryl transfer. Effects greater than one in this graph imply that phosphorylation enhances ligand binding or increases the rate of the phosphoryl transfer step. Conversely, effects less than one imply that phosphorylation impairs ligand affinity or reduces the rate of phosphoryl transfer. These studies show that activation loop phosphorylation enhances substrate binding for ERK2 and cyclinA-cdk2 by about 2 orders of magnitude but has no effect for PKA and v-Fps. Surprisingly, the binding affinity of ATP is impaired significantly in only one example, PKA. Given these results on ligand affinity, it is not clear that the activation loop serves the universal function of an autoinhibitor. Loop phosphorylation appears to generally increase the rate of phosphoryl transfer by 2−4 orders of magnitude in all examples, an effect larger than those on substrate binding. Given the nature of these findings, the activation loop is not behaving as a pure competitor for either ATP or substrate. In

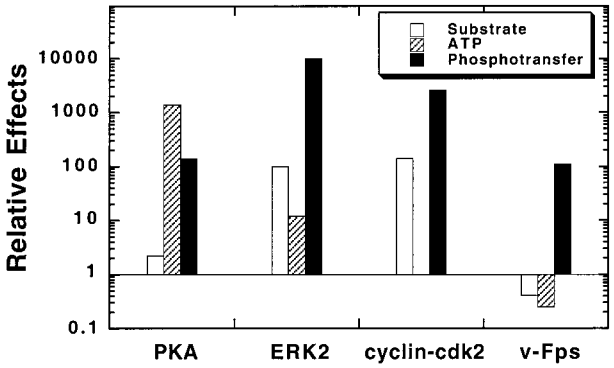


Figure 3. Effects of activation loop phosphorylation on the dissociation constants for ATP and substrate and the rate of phosphoryl transfer for four protein kinases. Relative effects larger than one imply that ligand affinity or the rate of phosphoryl transfer is enhanced by activation loop phosphorylation. Relative effects less than one imply that ligand affinity or the rate of phosphoryl transfer is decreased by activation loop phosphorylation. The data for PKA, ERK2, cyclinA-cdk2, and v-Fps were taken from refs 92, 98, 100, and 178.

fact, the greatest role of the activation loop is in facilitating the phosphoryl transfer step through other stabilizing phenomena.

C. Is There a Common Activation Mechanism?

While phosphorylation may affect the flexibility of the activation loop, the nature of the movement does not appear to be the same in all protein kinases. A comparison of the phosphorylated and nonphosphorylated forms of the InRK and FGFR kinase suggest that kinase activation causes different movements in the activation loop.[102] Whereas the loop in the InRK appears to occlude the substrate pocket and part of the ATP pocket,[96] the loop in the FGFR displays more localized conformational changes upon phosphorylation that are not predicted to influence ATP binding.[97] In addition to shifts in B factors in the crystalline state, phosphorylation certainly affects the structure of some protein kinases in solution. In the InRK, activation loop phosphorylation destabilizes the protein toward guanidine-induced denaturation.[103] On the basis of hydrogen/deuterium exchange data, the N-terminal lobe of MKK1 (MAPK kinase-1), the protein kinase that phosphorylates and activates ERK1 and ERK2 in the MAPK cascade, becomes *more* flexible upon activation loop phosphorylation.[104] Crystallographic studies of the repressed and activated ERK2 reveal more subtle changes in the activation loop than those witnessed for the InRK and cdk2. Upon dual phosphorylation of ERK2, movements in the activation loop are localized to a specific region important for recognizing the P+1 site in peptide substrates.[105] While other long-range structural changes occur due to phosphorylation, the overall shape of the substrate pocket does not change significantly.[105]

The data garnered from structural and kinetic studies do not agree upon a general mode of regulation through the activation loops of protein kinases. Indeed, there may be no structural cause for proposing such a universal mechanism. There is little sequence conservation within the loops, and the requirement for phosphorylation is not universal. Some activation loops require multiple phosphorylations such as the InRK and ERK2, while some require single phosphorylation such as PKA and cdk2. Still others, such as PhK, do not have a phosphorylation site in this loop region.[7] Furthermore, activation loop size does not appear to be largely restricted with lengths ranging from 19 to 32 residues.[106] As evidenced in the available X-ray crystallographic structures, phosphorylation-driven movements of the loop may vary from kinase to kinase but it is becoming evident that regulation via this loop incorporates movements in other specific structural elements in the core. For example, phosphorylation of the InRK, ERK2, and cdk2 causes large movements in αC so that the conserved glutamate (Glu-91 in PKA) in this helix is positioned appropriately to interact with and stabilize the conserved lysine in the active site (Lys-72 in PKA) (Figure 2). The creation of this important dyad may be one of the underlying causes for the extraordinary enhancements in phosphoryl transfer rate upon activation loop phosphorylation (Figure 3).

VI. Phosphoryl Transfer Mechanism

A. Phosphoenzyme as a Reaction Intermediate?

Unlike some of the protein phosphatases,[107,108] there is no convincing evidence that protein kinases utilize a phosphoenzyme intermediate to facilitate protein phosphorylation. PKA transfers the γ phosphate of ATP to a substrate peptide with inversion of stereochemical configuration.[109] The simplest interpretation of this result is that the hydroxyl of the substrate directly displaces the phosphate in a single step. Of course, more complex mechanisms involving multiple covalent intermediates are possible theoretically, but the active site does not appear to present the appropriate nucleophiles for such a mechanism. In fact, the crystal structures of the protein kinases do not position a good nucleophile such as a cysteine near the terminal phosphate of ATP. For the tyrosine protein phosphatases, a cysteine residue is positioned near the phosphoryl group and forms a transient thiophosphoryl enzyme adduct. This intermediate has been supported by many kinetic experiments including the physical isolation of the chemical intermediate.[107] While stereochemical studies on a sufficient number of protein kinases are lacking, it is reasonable to assume that, based on the PKA data, they incorporate a single displacement of the γ phosphate by the substrate without the incorporation of any intermediates. This assumption is bolstered by the high level of sequence similarity within the protein kinase superfamily. By comparison, not all protein phosphatases incorporate a phosphoenzyme intermediate, but this enzyme family is composed of three unique gene families with distinctive three-dimensional structures and active-site configurations.[107,108]

B. Associative vs Dissociative Mechanisms

The mechanisms whereby phosphoryl groups are transferred from donors to acceptors in solution reactions have been studied extensively for decades and are adequately reviewed elsewhere.[110,111] The transition states for these mechanisms have been characterized as either dissociative or associative. Figure 4 presents both transition states for the phosphoryl transfer reaction catalyzed by protein kinases. In the dissociative transition state, bond breaking between the phosphorus center and the leaving group atom is more advanced than bond making with the atom of the in-coming nucleophile. This contrasts with an associative transition state where bond formation between the nucleophile and the phosphorus center are advanced. Of course, these putative structures reflect extreme cases and it is possible for a given phosphoryl transition state to incorporate aspects of both. While the transfer mechanisms appear to be distinct for mono-, di-, and triesters of phosphate in model solution reactions, the data garnered from the monoesters are particularly relevant for the reactions catalyzed by protein kinases. In general, the reactions involving phosphoryl monoesters proceed through dissociative-like transition states. Several lines of kinetic evidence have been used to draw this conclusion. The attack of

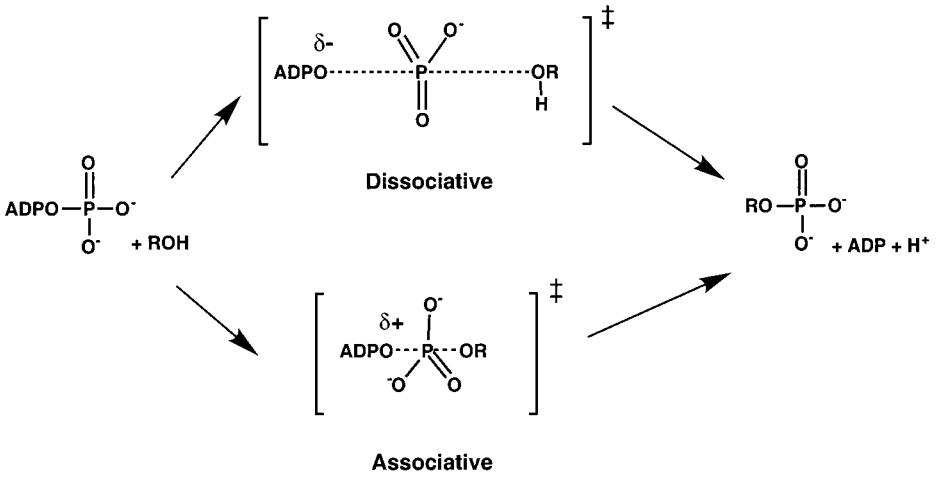


Figure 4. Associative and dissociative reaction transition states for protein kinases. The phosphoacceptor, ROH, represents the side chain of serine, threonine, or tyrosine.

nucleophiles on aryl phosphate dianions and pyridine phosphate monoanions are accompanied by low values for β_{nuc} and large negative values of β_{lg}.[112−114] A large negative β_{lg} denotes a strong dependence between the reaction rates and the pK_a's of the leaving group and implies that a large amount of bond breaking between the phosphorus center and the leaving group atom occurs in the transition state. The large value of this coefficient also implies appreciable development of charge on the leaving group. The low β_{nuc} denotes insensitivity between the reaction rates and the pK_a's of the attacking nucleophiles. This is consistent with little bond formation between the nucleophile and phosphorus atom in the transition state. These two coefficients are complementary and consistent with a transition state that has low associative character.

It has been demonstrated in a number of cases that enzymatic and nonenzymatic reactions of phosphoryl esters occur through similar transition states that differ only by the extent of stabilization.[115−118] If the active site of an enzyme alters the energy without affecting the nature of the transition state, then it is expected that the protein kinases will adopt dissociative-like transition states akin to the solution reactions.[118] This possibility has been explored experimentally for the nonreceptor TPK, Csk. This enzyme can phosphorylate fluorotyrosine peptides so that the acidity of the nucleophile can be altered and detailed linear free energy relationships can be derived. The Bronsted plots of k_{cat} and k_{cat}/K_m versus the pK_a's of various fluorotyrosine peptides have very low values for β_{nuc} (∼0.08). This is consistent with a transition state for phosphoryl transfer that possesses a high degree of dissociative character. In other studies, it was demonstrated that k_{cat} reflects the phosphoryl transfer step for the substrate peptide series,[56] an important prerequisite for the interpretation of structure−function studies. As shown in Figure 4, the phosphorylation of tyrosine by Csk adopts a pathway that is closer to a dissociative mechanism (upper pathway) where a large amount of bond breaking between the β and γ phosphates of ATP occurs in the transition state. It is still unclear whether a dissociative mechanism also applies to the SPKs. Detailed structure−reactivity plots as those applied to Csk cannot be applied to these protein kinases owing to limitations in the structure of the substrate. Nonetheless, given the results on Csk and the model reaction studies, it is likely that the SPKs also incorporate a dissociative-like transition state as well.

C. Insights from Structural Studies

Further support for a dissociative-like transition state for the protein kinases has come from X-ray crystallographic and NMR data. PKA has been cocrystallized in a ternary complex with ADP and a 20-residue phosphopeptide so that distance geometries for phosphoryl transfer can be estimated.[19] If this structure corresponds with a viable enzyme species populated under catalytic turnover conditions and the transition state for phosphoryl transfer is assumed to be symmetric, then the distance between the attacking nucleophile (serine oxygen) and the leaving group ($\beta\gamma$ bridging oxygen of ATP) can be used to calculate the bond order for the incoming oxygen. Such calculations predict that the transition state for phosphoryl group transfer in PKA is 8.4% associative and 91.6% dissociative.[119] Substitution inert Co(III) and Cr(III) complexes of PKA, AMPPCP (an ATP analog), and Kemptide have been studied using NMR methods to derive interatomic distances.[120] Such studies indicate a longer distance between the attacking oxygen and the γ phosphate of the nucleotide than that detected in the X-ray models. While some of this discrepancy could be due to the use of an inactive nucleotide analogue, the combined data from NMR and X-ray methods suggest that the amount of associative character in the transition state is very small.[119]

VII. Metal Ion Catalysis

A. Structure and Chelation

Protein kinases require an essential Mg^{2+} ion for catalysis. As shown in Figure 2, this metal ion [Mg(1)] chelates the $\beta\gamma$ phosphates of ATP and Asp-184 in PKA. From a structural perspective, Mg(1) is considered the primary metal since it is visible in the

crystal at low concentrations and is likely to gain entrance to the active site as a Mg·ATP complex. A second metal ion is also observed in the active site of this enzyme and chelates the $\alpha\gamma$ phosphates of ATP, Asp-184, and Asn-171, another conserved residue. This metal site is visible when the crystal is soaked with high concentrations of Mg^{2+}. Two metals are also present in the X-ray structures of other protein kinases. PhK binds two Mg^{2+} and utilizes the same phosphates on ATP and residue side chains as PKA to position these metals. The InRK also binds two Mg^{2+} in similar positions to those in PKA, but the chelation is subtly altered. In this protein kinase, the second metal [Mg(2)] interacts with only the β phosphate rather than the $\alpha\gamma$ phosphates of ATP.[96] It is not easy to extract a relevant meaning to these differences at this point since the crystallization conditions for the InRK included an ATP analogue which may not exactly reflect the true nucleotide. In some cases, protein kinases have been cocrystallized with Mn^{2+} rather than Mg^{2+}. For PKA, Mn^{2+} occupies the same two positions as Mg^{2+} in the active site and interacts with the same residues and phosphate groups.[18,121] In all examples where an active protein kinase has been cocrystallized with ATP or an analogue, the γ phosphate forms an electrostatic pair with one of the metal ions.

B. Kinetic Parameters

Prior to the solution of the first protein kinase structure, kinetic studies showed that these enzymes are capable of binding two metals. The activity of PKA was shown to increase up to a stoichiometry of 1 metal per ATP.[122] Above this level, the activity declines to a nonzero value.[50,122] The apparent affinity constant for the binding of the first metal is similar to that for the metal−ATP complex in solution (~10 μM), implying that Mg−ATP is the essential nucleotide complex that binds in the active site and supports phosphorylation. The dissociation constant of the second metal binding event (~2 mM) is about 2 orders of magnitude higher than that for the first, so that this secondary site is only partially occupied under physiological metal concentrations. These kinetic observations are consistent with the X-ray diffraction data. The second metal is observed only at high concentrations in the crystalline state. Owing to the shift in activity above stoichiometric metal concentrations, the first metal is called activating while the second metal is termed inhibitory. The latter inhibition by excess amounts of Mg^{2+} is the result of a reduced k_{cat},[50,123] but the steady-state kinetic parameters reveal a more complex role for this secondary metal. In PKA, the reaction velocity actually increases when the second metal binds if ATP concentrations are limiting. This occurs because the lower k_{cat} values are offset by larger decreases in the K_m for ATP at high metal concentrations. In this sense, the term inhibitory metal is somewhat misleading.

While all protein kinases appear to bind two metal ions which surround the triphosphates of ATP, the function of these ions on a kinetic level does not appear to be conserved. Table 2 shows the effects of the second Mg^{2+} on the steady-state kinetic parameters for several protein kinases. Although the number of protein kinases analyzed, to date, is limited, it is already clear that there is no consistency within the enzyme family. While the second metal lowers V_{max} in PKA, it enhances this parameter in others.[124,125] There are currently no examples where the second Mg^{2+} negatively influences ATP affinity, but there are cases where it has no apparent effect. In some cases, the increase in apparent ATP affinity is large and significantly affects catalytic function. For v-Fps, the binding of the second metal increases ATP affinity by as much as 80-fold.[126] This enormous increase accounts for the large activation observed for this enzyme under normal assay conditions.[126] While most protein kinases appear to be active in the presence of a single Mg^{2+}, Csk has been reported to require two metals for basal activity.[125] A plot of the reaction velocity for this enzyme as a function of free Mg^{2+} provides an apparent zero intercept on the *y*-axis, indicating that the primary metal site does not provide adequate stabilization for phosphoryl transfer.[125] In addition to these effects, the second metal ion can influence the binding of substrates. For example, the K_m for the random copolymer, poly-Glu_4-Tyr, increases with increasing free Mg^{2+} concentration for Csk, Src, and FGFR.[125] Clearly, the overall role of metal ions in the active sites of protein kinases is the facilitation of the phosphorylation reaction, but the discrete mechanism of involvement for each metal is curiously not shared throughout the enzyme family and, in some cases, is kinetically complex.

Table 2. Changes in the Steady-State Kinetic Parameters for Several Protein Kinases upon Binding of the Second Mg^{2+} in the Active Site

enzyme	substrate	V_{Max}	K_m (ATP)	K_m (substrate)
PKA [a]	LRRASLG	↓	↓	none
v-Fps [b]	EAEIYEAIE	none	↓	none
Csk [c]	poly-Glu_{4-Tyr}	↑	none	↑
Src [c]	poly-Glu_{4-Tyr}	↑	none	↑
FGFR [c]	poly-Glu_{4-Tyr}	↑	↓	↑

[a] Data taken from refs 50 and 123. [b] Data taken from ref 126. [c] Data taken from ref 125.

C. Nonphysiological Metal Ions

Owing to its high concentration in the cell compared to other divalent metal ions,[127] Mg^{2+} is considered the physiological activator of protein kinases. Nonetheless, other divalent metal ions can replace Mg^{2+} in the active site and, in some cases, support catalysis in vitro. The ability of a diverse group of divalent metal ions to support binding of lin-benzo-ADP, a fluorescent analogue of ADP, and catalysis has been thoroughly tested.[128] While a number of metal ions will support nucleotide binding (Mg^{2+}, Mn^{2+}, Co^{2+}, Ca^{2+}, Sr^{2+}, and Cd^{2+}), catalytic activity is detected in only four (Mg^{2+}, Mn^{2+}, Co^{2+}, and Cd^{2+}). In crude activity assays, initial velocities are 2−10 times higher in the presence of Mg^{2+} compared to the others, supporting a dominant role for this metal as the true activator. While the changes in catalytic power have not been rigorously analyzed in all cases, the low activities in the presence of Mn^{2+} and Co^{2+}

are due mostly to attenuations in k_{cat} for PKA.[129] PhK will also utilize Mn^{2+} as an alternative metal activator, but as in the case for PKA, k_{cat} is about 20-fold lower.[130] Co^{2+} and Ni^{2+} bind to Csk in the secondary site with high affinity compared to Mg^{2+}, but activities are lower.[124]

The TPKs appear to behave uniquely with Mn^{2+} as an activator. Not only do Csk and the InRK utilize this divalent metal ion, but, under certain concentrations, the activities are higher in the presence of Mn^{2+} compared to Mg^{2+}.[124,131,132] The source of this rate enhancement is not yet fully understood. Divalent metal ions have been shown to influence substrate binding and selectivity. For example, the K_m for poly-Glu_4Tyr is 14-fold lower in the presence of Mn^{2+} compared to Mg^{2+} for Csk.[132] While this is an apparent effect on binding, there is some evidence that changes in K_m for substrates may be correlated with changes in true binding affinities. For example, the K_I for the PKA inhibitor LRRNAI is 2-fold lower in the presence of Mn^{2+} compared to Mg^{2+}.[129] A dramatic effect on substrate selectivity has been observed for PhK where Mn^{2+} substitution converts the enzyme's specificity from serine to tyrosine.[130] While PhK does not phosphorylate angiotensin on tyrosine in the presence of Mg^{2+}, this substrate is phosphorylated in the presence of Mn^{2+}.[130]

VIII. Acid–Base Catalysis

A. Structural and Kinetic Observations

Protein kinases possess extraordinary catalytic power, accelerating phosphorylation rates by 9−11 orders of magnitude.[119] This enormous rate enhancement is likely to stem from a number of factors, but the participation of a general-base catalyst has been given special consideration in the literature.[8,133,134] The major impetus for the proposal of such a mechanism is likely to be grounded in the presumption that the alkoxide of serine or threonine or the phenolate of tyrosine should be a better general nucleophile than the alcohol or phenol form and, thus, would enhance phosphoryl group donation. Figure 5A depicts a transition state for phosphoryl group transfer in PKA that incorporates general-base catalysis via Asp-166. Two experimental findings have been used as evidence to support a general-base catalyst. First, the second-order rate constant, k_{cat}/K_m, for the phosphorylation of Kemptide by PKA is pH sensitive. A plot of this parameter as a function of pH is bell-shaped with the acidic limb ($pK_a = 6$) being ascribed to the ionization of a general-base catalyst.[133] The elevation of the carboxyl pK_a from its intrinsic value of 4 to 6 is thought to reflect a hydrophobic environment surrounding the ionizable residue. An acidic limb is also observed in the pH−rate profile for the EGFR tyrosine kinase.[135] In this example, a detailed substrate dependence was not performed so that it is unclear which steady-state kinetic parameter is pH sensitive. Second, the conserved aspartate in the catalytic loop of PKA forms a hydrogen bond with the substrate peptide so that it is ideally poised for proton abstraction.[19] The conservation of this residue and the observation of this hydrogen bond in other protein kinase structures intuitively supports a general role for the carboxyl.[19,65]

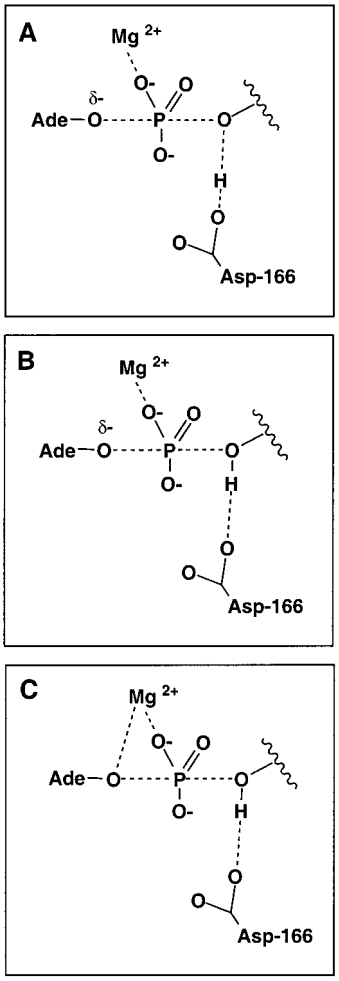


Figure 5. Potential roles for Asp-166 and Mg^{2+} in the active site of PKA. (A) The carboxyl of Asp-166 acts as a general-base catalyst abstracting the hydroxyl proton of the substrate in the transition state for phosphoryl transfer. (B) Asp-166 forms a hydrogen bond with the hydroxyl of the substrate for correct positioning of the nucleophile but does not remove the proton in the transition state. (C) Asp-166 serves the same function as in panel B, but Mg^{2+} stabilizes the developing negative charge on the leaving group.

B. Low pH and Low Activity

If protein kinases utilize a general-base catalyst, it is reasonable to suspect that protonation of this residue would lower the activity of an enzyme provided the chemical step is rate-limiting in the steady-state time frame. While substrate phosphorylation in PKA is pH dependent, several kinetic studies challenge the origins of the acidic pK_a in the k_{cat}/K_m pH−rate profile. Replacement of the P-2 arginine in the substrate peptide, LRRNSI, with alanine produces a substrate (LRANSI) that does not display an acidic pK_a.[136] In contrast, removal of the P-3 arginine generates a substrate (LARNSI) with a pH−rate profile similar in shape to that for Kemptide and LRRNSI. These data suggest that an ionizable residue interacting with a single arginine in the substrate must be correctly ionized for binding. Two

glutamates interact with the P-2 arginine, but both residues are considerably removed from the site of phosphoryl transfer. Other data suggest that the acidic limb is involved in substrate binding and does not correspond to the ionization of Asp-166. The K_I for the inhibitor peptide, LRRAALG (Ala-Kemptide), is pH-dependent with the lower pK_a identical to the lower pK_a in the k_{cat}/K_m plot.[133] While this alone does not rule out the possibility of Asp-166 ionization, Kemptide and Ala-Kemptide bind with identical affinities, an outcome which implies that the ionization state of the carboxyl does not influence substrate affinity.[52,64] If the ionization of Asp-166 supports phosphoryl transfer and substrate binding as suggested by the pH-dependent K_I and k_{cat}/K_m data, it is not clear why removal of the hydroxyl does not impair affinity or alter the pH dependence of the K_I. Finally, it has been demonstrated using pre-steady-state kinetic methods that the rate of phosphoryl transfer in PKA is invariant over a wide pH range of 6−9 and is not influenced by solvent deuterium, lending further support that the pH−rate profile for k_{cat}/K_m does not reflect the ionization of a general-base catalyst.[137]

C. Active Form of the Substrate

To shed light on the role of a potential general-base catalyst in protein kinases, several studies have focused on determining the ionization state of the hydroxyl nucleophile. Fluoridation of the tyrosine side chain of the substrate peptides for Csk has provided some interesting insights into the phosphoryl transfer mechanism for TPKs. The second pK_a in the bell-shaped plots of k_{cat}/K_m vs pH for Csk decreases linearly with the solvent pK_a's of the fluorotyrosine substituents.[138] These results indicate that the second pK_a in the pH−rate profiles reflects the ionization of the tyrosine hydroxyl. Since activity is lost at higher pH, the data imply that the neutral form of the phenol is the active substrate species. In a general-base-catalyzed mechanism, the ionized substrate is expected to be more reactive than the uncharged form. Applying this presumption, the higher activity associated with the neutral phenol species does not provide support for a general-base-catalyzed process for this enzyme. In a related study, the neutral form of the tetrafluorotyrosine substrate peptide for the InRK was shown to be more reactive than the ionized form.[139] Overall, the data from both Csk and the InRK are not strictly consistent with a general-base mechanism but are instead supportive of a dissociative-like transition state as shown in Figure 4.

D. Computational Approaches

The possibility of a catalytic base in protein kinases has sparked intense investigation. In addition to experimental approaches, the viability of Asp-166 in PKA as a general-base catalyst has been challenged using computational methods. The phosphoryl transfer mechanism for PKA has been explored using hybrid quantum mechanical/molecular mechanical (QM/MM) calculations.[140,141] In these studies, the active site of PKA was represented by 46 atoms that mimic Asp-166, Lys-168, the triphosphate group of ATP, the serine substrate, and the two magnesium ions. The lowest energy transition state was attained in these calculations when the catalytic aspartate was protonated. Similar results were obtained by another group that used semiempirical AM1 molecular orbital calculations and a larger ensemble of atoms for the active site.[142] In this study, the authors mutated the catalytic aspartate to alanine but surprisingly observed no effect on the free energy profile for phosphoryl transfer. The identical computational approach on the Lys-168-to-alanine mutation results in a greatly altered reaction surface with larger free energy barriers for phosphoryl transfer,[142] in keeping with the observed experimental results.[68] These unusual findings have been interpreted to imply that Asp-166 is important for maintaining the structure of the active site, not for proton abstraction.[142] Moreover, the two computational approaches applied to PKA suggest that Asp-166 does not abstract the hydroxyl proton from the substrate in the transition state.

E. What Is the Role of the Conserved Aspartate?

On the basis of available primary and tertiary structural comparisons,[65−67,94,106] it is likely that all protein kinases position a conserved aspartate residue in hydrogen-bonding distance to the hydroxyl of the substrate as depicted in Figure 2. Despite the attractiveness of an acid−base role for this residue, the data garnered from simple organic reaction mechanisms and from kinetic studies on Csk, PKA, and the InRK do not provide convincing evidence for a general-base catalyst.[111,118,136−139,143] In fact, it is conceivable that ionization of the hydroxyl acceptor via a catalytic base could have adverse effects on the phosphoryl transfer step. As pointed out in one study, high negative charge on the hydroxyl could inhibit the reaction if charge repulsion with the γ phosphate is considered.[138] Given these results, the mechanism in Figure 5A may not provide a catalytic advantage to the protein kinase. Nonetheless, the conserved aspartate is clearly important for efficient catalysis. Replacement of this residue with alanine in PKA and PhK results in decreases in k_{cat} of 2−3 orders of magnitude.[68,144] If there is no need for a base catalyst for the phosphoryl transfer step, what role does this conserved residue play in catalysis? It has been proposed that the carboxyl group may position the hydroxyl for productive attack of the γ phosphate of ATP.[137] This mechanistic alternative, shown in Figure 5B, is more in line with the structure−reactivity studies. The proton would not be transferred to the aspartate in the transition state as expected in general-base catalysis, but the hydrogen bond would "freeze out" a productive rotamer of the hydroxyl group so that appropriate attack geometry could be attained. Alternatively, the aspartate could serve to repel the phosphoproduct and facilitate dissociation.[19] Mutations at this position would then be expected to improve the affinity or reduce the release rate of the phosphoproduct. For cases where the conserved aspartate has been removed by mutagenesis,[68,144]

analysis of the effects on phosphoproduct binding were not performed, so that this hypothesis cannot be currently substantiated.

While dissociative mechanisms are not expected to be accelerated by general-base catalysts, they are affected by general-acid catalysis.[111] The protonation of the $\beta\gamma$ bridging oxygen could promote bond cleavage and formation of the metaphosphate-like transition state. As shown in Figure 4, a considerable amount of negative charge develops on the leaving-group oxygen in the dissociative pathway. It is not clear which residue in the active site would serve to stabilize this charge. Asp-166 in PKA is not near the bridging oxygen, and the neighboring Lys-168 is not a conserved residue.[65,66] The phosphoryl transfer step in PKA has been directly measured using pre-steady-state kinetic techniques (see section IX), but this step is insensitive to a wide range of pH values (6−10) and unaffected by deuterium oxide.[137] An acid−base catalyst could ionize outside this pH range, but this would limit its catalytic power.[145] While these and other observations do not support the general-base mechanism, a significant amount of negative charge is expected to develop on the bridging oxygen and it may be the function of the metal ions in the active site to stabilize this charge.[118] As shown in Figure 5C, one of the magnesium ions could stabilize the developing charge on the bridging oxygen, thereby facilitating departure of the leaving group, ADP. Taking all the evidence together, the reactions catalyzed by the protein kinases do not appear to require a general-base catalyst for proton abstraction. Consequently, alternative mechanisms that incorporate a positioning function for the aspartate either with or without participation of a metal ion (Figure 5B and C) may be considered.

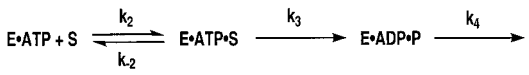
IX. Rate-Determining Steps in Protein Kinases

A. Historical Perspective

While the importance of protein kinases has been appreciated for decades, the inner workings of the kinetic mechanisms of these enzymes have only been revealed in recent years. One controversy that has prevailed and limited the detailed analysis of catalytic and regulatory phenomena is the velocity at which protein kinases can deliver the γ phosphate of ATP to protein substrates in the active site. Such a quantity is essential for establishing the rate-determining step in the overall reaction, a key acquisition for meaningful structure−function studies on this or any enzyme family. Two contrasting views could be put forth historically based on the interpretation of steady-state kinetic data. PKA has an intrinsic ATPase activity (water as a substrate) that is approximately 3 orders of magnitude lower in rate than k_{cat} (Kemptide as substrate).[50,146] This extraordinary difference in rate suggests that water phosphorylation ($k_{cat} = 0.01\ s^{-1}$) must be limited by the chemical delivery of the phosphate as opposed to nucleotide binding steps. Replacement of the essential, activating Mg^{2+} with Mn^{2+} causes 20-fold decreases in both ATPase rate and the k_{cat} for Kemptide phosphorylation.[146] The similarity in these rate reductions could result from rate-limiting phosphoryl transfer for both the good (LRRASLG) and poor (water) substrates.

The effects of divalent metal ion concentrations on PKA activity can also be used to propose an alternative kinetic mechanism. Cook and co-workers posited that phosphoryl transfer is fast and that ADP release is the rate-determining step based on the effects of varying Mg^{2+} concentration on V_{max} and nucleotide binding using only Kemptide as a substrate.[50] Increasing free Mg^{2+} from 0.5 to 10 mM leads to a decrease in V_{max} and an increase in the affinity of ADP. Cook and co-workers argued that the coordinate reduction in ADP affinity and increase in k_{cat} is due to rate-limiting ADP release and fast phosphoryl transfer. This mechanism is attractive since it explains why K_m is more than an order of magnitude lower than K_d for the substrate peptide.[50,64] Scheme 1 depicts a simple phosphorylation mechanism for a

Scheme 1



protein kinase at high ATP concentrations.

In this reaction scheme, K_m for the substrate can be related to the individual steps by eq 1

$$K_m = \frac{k_4}{k_3} \times \frac{k_{-2} + k_3}{k_3 + k_4} \quad (1)$$

If the phosphoryl transfer step is fast ($k_3 > k_4$), K_m can be lower than K_d by the term k_4/k_3 [$K_m = K_d k_4/k_3$] if the substrate is in rapid exchange with the enzyme ($k_3 < k_{-2}$). If the substrate is phosphorylated faster than it can dissociate from the enzyme ($k_3 > k_{-2}$), K_m may still be lower than K_d if product release is slower than substrate dissociation ($k_{-2} > k_4$). By comparison, K_m can never be less than K_d when phosphoryl transfer is rate-limiting. Regardless of the dissociation rate of the substrate, K_m will either equal or exceed the K_d value. Despite the logic of this argument, neither interpretation of the steady-state kinetic parameters could dismiss the other. As a result, the controversy regarding the nature of the rate-determining step persisted for some time since both interpretations relied upon indirect measurements of the phosphoryl transfer step. Two kinetic approaches in the past decade, however, shed new light on this problem. The application of viscosometric and pre-steady-state kinetic techniques have been used together to isolate the phosphoryl transfer step in PKA and unequivocally establish its rate compared to turnover.[52,147]

B. Viscosity Effects on Turnover

In the viscosity approach, the effects of added viscosogens (e.g., glycerol and sucrose) on the steady-state kinetic parameters are monitored. In principle, bimolecular events such as product dissociation are expected to be sensitive to solvent viscosity while the phosphoryl transfer step, a unimolecular event, is expected to impart no viscosity dependence.[147−150] If turnover is limited by a product release step, a large

Table 3. Viscosity Effects on Turnover for Several Protein Kinases

protein kinase	k^{η}_{cat}	mechanism	$K_{d\,(\mu M)}$	substrate	ref
PKA	1	$k_3 \gg k_4$	200	LRRASLG	52, 147
PhK	1	$k_3 \gg k_4$	6	GPb	144
cyclinA-cdk2	0.7	$k_3 = 2 \times k_4$	25	PKTPKKAKKL	98
ERK2	0.2	$k_3 = 0.25 \times k_4$	≤0.5	MBP	176
p38α	0.8	$k_3 = 4 \times k_4$	?	KRELVEPLTPSGEAPNQALLR	58
Csk	0.4	$k_3 = 0.7 \times k_4$	?	poly-Glu_{4Tyr}	56
v-Fps	0.7	$k_3 = 2 \times k_4$	1,200	EAEIYEAEI	177

viscosity effect is expected on k_{cat}. In contrast, if the phosphoryl transfer step is slow, k_{cat} should be unaffected by the viscosity of the buffer. To verify the assumption that the unimolecular phosphoryl transfer step is viscosity independent, the phosphorylation of good and poor substrates are compared. If a viscosity effect is observed on k_{cat} or k_{cat}/K_m for a good substrate, none should be detected for a poor substrate. For PKA, a large viscosity effect is observed on k_{cat} for Kemptide phosphorylation but the ATPase rate (water as a substrate) is unaffected by highly viscous buffers.[147]

The effects of solvent viscosity on turnover can be quantified using the Stokes−Einstein equation, which relates diffusion coefficients and intrinsic viscosity. For a simple bimolecular reaction with association and dissociation rate constants, k_1 and k_{-1}, an inverse relationship between observed rate constants and intrinsic viscosity (η) is observed. Accordingly, the description of the ratio of the rate constants in the absence (k°_1, k°_{-1}) and presence (k_1, k_{-1}) of added viscosogens can then be expressed in terms of the relative viscosity of the medium ($\eta^{rel} = \eta/\eta^{\circ}$). In effect, the ratio of k°_1/k_1 or k°_{-1}/k_{-1} is then equal to η^{rel} at constant temperature ($k^{\circ}_1/k_{-1} = k^{\circ}_{-1}/k_{-1} = \eta^{rel}$). This relationship can be applied to the kinetic mechanism in Scheme 1 to derive a linear function of relative turnover (k°_{cat}/k_{cat}) versus η^{rel}. The slope of this linear equation [$k^{\eta}_{cat} = k_3/(k_3+k_4)$] lies within the theoretical limits of 0 and 1. From the values for k_{cat} and k^{η}_{cat}, k_3 and k_4 can be estimated using eqs 2 and 3

$$k_3 = \frac{k_{cat}}{1 - k^{\eta}_{cat}} \tag{2}$$

$$k_4 = \frac{k_{cat}}{k^{\eta}_{cat}} \tag{3}$$

These two equations illustrate which step will be rate-determining depending on the viscosity sensitivity of k_{cat}. If k_{cat} is viscosity independent (i.e., $k^{\eta}_{cat} = 0$), then the rate-determining step in k_{cat} is phosphoryl transfer, k_3. Conversely, if k_{cat} is maximally affected by solvent viscosity (i.e., $k^{\eta}_{cat} = 1$), then the rate-determining step in k_{cat} is product release, k_4, and phosphoryl transfer is fast. For PKA, a maximal viscosity effect is observed using Kemptide as a substrate, implying that the phosphoryl transfer step is fast relative to turnover (Table 3).

Since the initial analysis of PKA, other protein kinases have been studied using the viscosity approach. To date, the analysis of seven protein kinases have demonstrated that the effects of added viscosogens on k_{cat} fall into three general categories—full, intermediate, and no viscosity effect. Figure 6A shows a plot of relative k_{cat} as a function of relative solvent viscosity. The slope values for this plot (k^{η}_{cat}) fall between the theoretical limits of 0 and 1, where the former implies that the phosphoryl transfer step (k_3) is rate-determining and the latter implies that net product release (k_4) is rate-determining. Intermediate values can also be measured, implying that phosphoryl transfer and net product release are partially rate-determining. A value of k^{η}_{cat} between 0.2 and 0.8 can be considered intermediate, although k_3 would be 4-fold greater or less than k_4 at one of these extremes [see eqs 2 and 3]. At these slope limits, the faster of the two steps still reduces k_{cat} by 20% compared to the slower of the two steps. Table 3 summarizes the results of all the protein kinases studied thus far. While this table reflects a small subset of protein kinase family members, it is clear

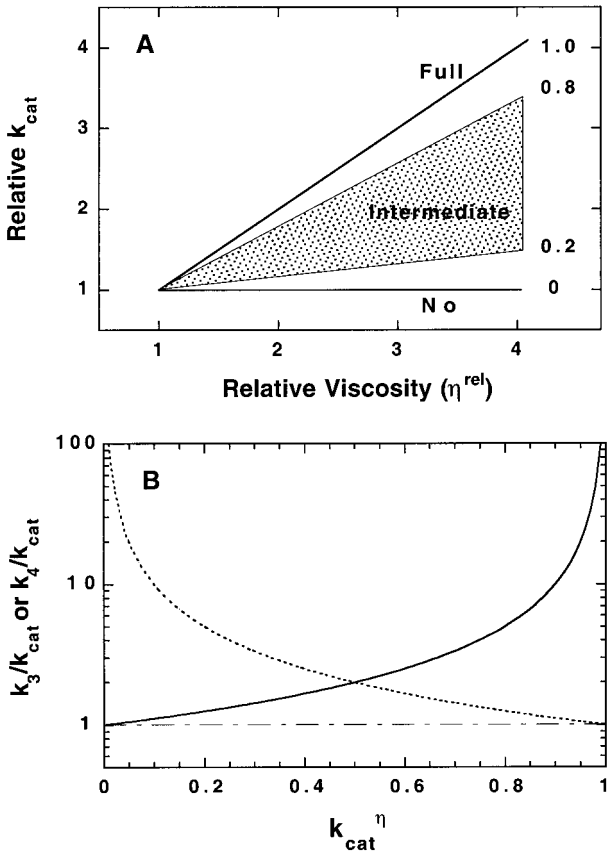


Figure 6. Viscosity effects on turnover. (A) Potential effects of relative solvent viscosity (η^{rel}) on relative k_{cat}. The numbers on the right represent values for the slopes (k^{η}_{cat}). (B) Effects of the viscosity slope term, k^{η}_{cat}, on the rate of phosphoryl transfer (k_3, —) and net product release (k_4, - - -).

that the current viscosity effects span a large range. PKA and PhK incorporate kinetic mechanisms with fast phosphoryl transfer rates ($k_3 \gg k_4$). However, the remaining enzymes incorporate kinetic pathways in which k_3 is partially rate-limiting for k_{cat}. While the substrates used in these studies differ considerably, there does not appear to be any correspondence between the nature of the kinetic mechanism and substrate affinity (Table 3). To further characterize the kinetic mechanisms, more protein kinases must be studied using a range of substrates. Nonetheless, it is likely, based on the available studies, that the role of phosphoryl group transfer in limiting turnover will vary irrespective of the type of protein kinase.

C. Limitations of the Viscosity Approach

The viscosity approach is an efficient method for analyzing the kinetic mechanisms of protein kinases. The protocol is fast and does not require large enzyme concentrations or any specialized mixing instrumentation. For PKA, this approach demonstrated that the phosphoryl transfer rate is not rate-limiting for turnover.[52] Nonetheless, the technique has two limitations that should be considered for data interpretation. First, the technique is restricted in its ability to define the kinetic rate constants when k_{cat}^{η} is near 0 or 1. As shown in Figure 6B, k_3 and k_4 cannot be determined simultaneously when k_{cat}^{η} is either too low or high. For PKA and PhK, only lower limits can be placed on k_3. Ultimately, these rate constants can only be evaluated together when intermediate values for k_{cat}^{η} (0.2−0.8) are measured and the experimental error in the slope is low. Second, the original assumption that viscosity influences only bimolecular steps while unimolecular steps are free of solvent effects may not apply in all cases. This is particularly relevant if different substrates or mutants incorporate distinctive mechanisms. For example, a mutation in the active-site loop (Y208F) of triose phosphate isomerase reduces k_{cat}/K_m by 2000-fold compared to wild-type yet a significant viscosity effect is observed on this parameter.[151] This extraordinary decrease in catalysis and large solvent effect implies that sizable conformational changes limit substrate processing. In section XI, the presence of viscosity-dependent conformational changes in PKA that partially limit turnover will be discussed. In all, the viscosity approach partitions viscosity-sensitive and -insensitive steps and allows a direct measure or estimate of their relative rates, but extreme care must be taken in interpreting the source of their effects.

D. Pre-Steady-State Kinetics

To gain further mechanistic information, direct observation of the phosphoryl transfer step in pre-steady-state kinetic experiments has been performed using rapid quench flow techniques. A schematic representation of this technique is shown in Figure 7. Reactants A and B are pushed together in a mixing chamber by a stepping motor. The reaction is allowed to age in variable length loops before meeting a quenching agent, Q, in the second mixing chamber.

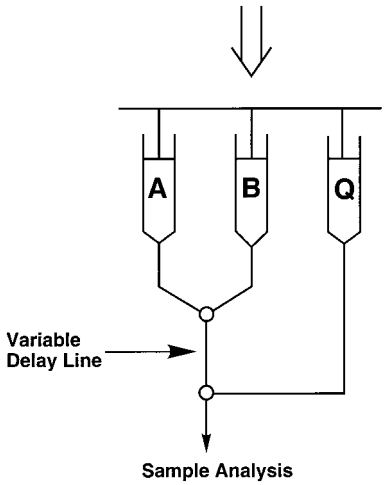


Figure 7. Schematic design of the rapid quench flow experiment. A and B represent reservoirs of enzyme and substrate, while Q represents the quench solution. The large arrow at the top represents the stepping motor and the drive platform which mixes A, B, and Q in the first and second mixing chambers (represented by two small circles).

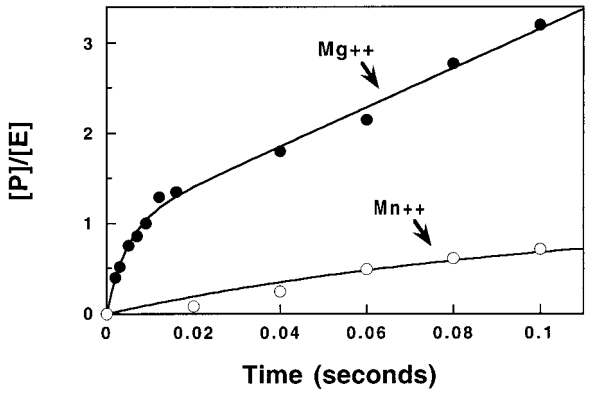


Figure 8. Pre-steady-state kinetic traces for PKA-catalyzed phosphorylation of Kemptide (LRRASLG) in the presence of Mg^{2+} (●) or Mn^{2+} (○). Experiments were performed using the rapid quench flow instrument (Figure 7) where A contains PKA and [$^{32}P\gamma$] ATP, B contains Kemptide, and Q contains 30% acetic acid. The divalent metal ions are placed in both A and B. [P]/[E] represents the enzyme-normalized amount of phosphopeptide. The free concentrations of Mg^{2+} and Mn^{2+} are 10 mM. The data were taken from ref 52.

By altering loop length and stepping motor speed, varying reaction times can be attained. Figure 8 shows a typical pre-steady-state experiment using this instrumentation. PKA is preequilibrated with [$^{32}P\gamma$] ATP (reactant A), and the reaction is initiated with Kemptide (reactant B). The incorporation of ^{32}P into Kemptide is measured as a function of time in the presence of the activating metal Mg^{2+}, and the reaction is quenched with 30% acetic acid (Q). The fast rise in phosphopeptide in the first 10 ms of the reaction ('burst' phase) is consistent with rapid phosphoryl transfer in the active site, and the linear phase (20 s^{-1}) is consistent with steady-state turnover. Repetition of this experiment in the presence of Mn^{2+} produces a 'burst' rate that is approximately 20-fold lower, indicating that the rapid rise in phosphopeptide in the first 10 ms of the reaction in the presence of magnesium is likely to be limited by the

phosphoryl transfer step (Figure 8). The data can be fit analytically to eq 4

$$\frac{[E{\cdot}ADPaP] + [P]}{[E]_t} = \alpha[1 - \exp(-k_b t)] + k_L t \quad (4)$$

where

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

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

and α, k_b, and k_L are the 'burst' amplitude, the rate constant of the 'burst' phase, and the rate constant of the linear phase, respectively. The rapid 'burst' rate constant ($k_b = 250\ s^{-1}$) demonstrates that phosphoryl transfer is not rate-limiting for turnover, and an extrapolation of k_b to infinite substrate concentration provides the phosphoryl transfer rate in the active site ($\sim$500 s^{-1}). The dependence of k_b on substrate concentration also provides a true dissociation constant for the substrate (K_d). This dependence supplies a singular approach for measuring substrate affinity in a real, active ternary complex, a quantity unavailable using standard steady-state kinetic measurements.

In addition to the ability to measure directly the phosphoryl transfer step, the pre-steady-state kinetic studies permit an accurate measurement of the active enzyme concentration or the number of active sites in solution. When substrate is saturating and the observed 'burst' rate is much faster than the linear, steady-state phase ($k_b \gg k_L$), the amplitude of the 'burst' phase [$\alpha = (k_3/(k_3 + k_4))^2$] reflects the number of active sites per enzyme monomer. If there is inactive enzyme, substoichiometric amplitudes will be obtained. For PKA, α is equivalent to the total enzyme concentration [α/[E] = 1] when [S] > K_m, indicating that all the active sites, predicted by standard protein concentration determinations, are viable.[52] Recently, however, pre-steady-state kinetic studies have been performed on the receptor TPK, Her2, an oncogenic form of the natural Erb receptor.[152] In this study, a 'burst' phase amplitude that corresponds with only 10% of the total enzyme concentration is observed. On the basis of estimates of k_{cat} using α, as much as 80% of the enzyme may be inactive and incapable of facilitating productive substrate phosphorylation.[152] While PKA and Her2 rapidly phosphorylate their respective substrates in the active site, a recent pre-steady-state kinetic study on the cyclin-dependent kinase-activating kinase (CaK1p) demonstrates that phosphoryl transfer for this enzyme is slow.[153] No 'burst' phase is observed for this enzyme prior to the conductance of normal steady-state turnover.

E. Application of Pre-Steady-State Kinetics to Mutants

The pre-steady-state kinetic techniques, originally applied to PKA,[52] can provide useful mechanistic insights into the protein kinases. Unlike steady-state kinetic and viscosometric techniques, fast mixing methods allow direct measurements of the phosphoryl transfer step and the dissociation constants of substrates. In section X, an adaptation of this technique will be discussed that allows measurements of the release rates of substrates and products. Among the many applications of this approach for defining wild-type function, this technique is particularly useful for analyzing the function of individual residues using site-directed mutagenesis. In one study, Glu-230, which is important for binding the P-2 arginine in substrates for PKA, was converted to glutamine. On the basis of viscosometric and inhibitor binding studies, this mutation is predicted to reduce substrate affinity by about 1 order of magnitude.[154] The absence of a large 'burst' phase is consistent with a 25-fold decrease in the rate of phosphoryl transfer. These findings are compatible with the viscosity measurements and demonstrate that the rate of phosphoryl transfer is approximately equivalent to the net release rate of the products. In another study, pre-steady-state kinetic measurements were used to determine the rates of phosphoryl transfer for several glycine-rich loop mutants in PKA.[155] Removal of two of the highly conserved glycines in this loop with serine results in dramatic reductions in 'burst' rate and amplitude, demonstrating that the loop stabilizes the transition state for phosphoryl group transfer. Oddly enough, the loop is more important for controlling phosphoryl transfer then binding of either ATP or ADP.

X. Product Release

A. Catalytic Trapping Studies

Despite their considerable importance, the viscosometric and pre-steady-state kinetic methods are not capable of defining the physical nature of the k_4 step in Scheme 1. The rapid quench flow experiment in Figure 8 monitors the transfer of phosphate into the substrate so that it reports directly on the phosphoryl transfer event, but it does not provide any information on the release rates of products. In the viscosity experiments, it can be demonstrated that the phosphoryl transfer step is fast and not affected by solvent additives but the participation of slow, viscosity-dependent conformational changes linked to product release cannot be summarily ruled out. To determine the net dissociation rate constants for products from PKA, a catalytic trapping experiment has been developed.[156] This protocol is a variation on that presented in Figures 7 and 8 using the rapid quench flow instrument. In this new application, PKA is preequilibrated with ADP or phosphopeptide (reactant A) and then mixed rapidly with substrate peptide and ATP (reactant B). If the release of the preequilibrated product is fast compared to the phosphoryl transfer rate, then the presence of the product will not affect the 'burst' kinetics as long as sufficiently high concentrations of substrate peptide and ATP are used to trap the enzyme. Alternatively, if the release of the product is slow compared to phosphoryl transfer, the 'burst' will exhibit a lag

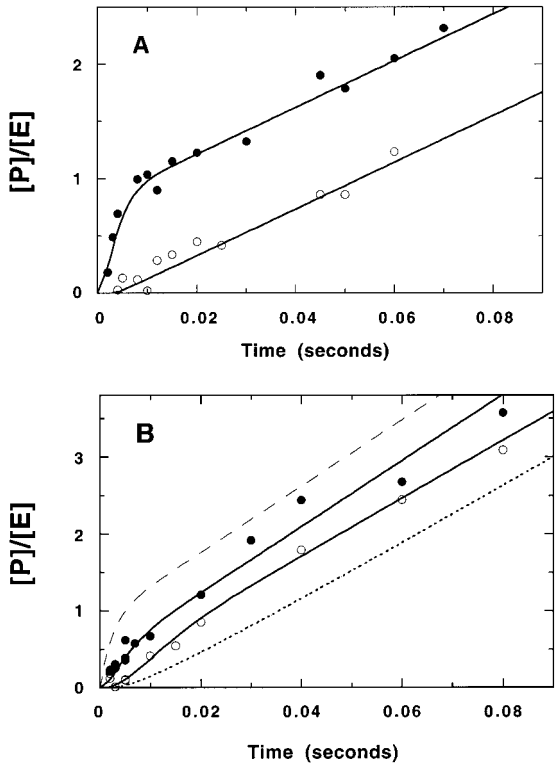


Figure 9. Pre-steady-state kinetic traces for PKA-catalyzed phosphorylation of Kemptide (LRRASLG) in the presence of 10 mM free Mg^{2+} (A) or 0.5 mM free Mg^{2+} (B). In both panels, PKA is mixed with [$^{32}P\gamma$] ATP and Kemptide (●) or PKA is first preequilibrated with ADP prior to mixing with [$^{32}P\gamma$] ATP and Kemptide (○). The dashed line in panel B reflects the kinetic outcome when PKA is first preequilibrated with ATP before reaction initiation with Kemptide (data not shown). The dotted line in panel B reflects the simulated kinetic outcome of ADP preequilibration with PKA if the release of ADP rate limits turnover. These data were taken from ref 123.

phase that can be modeled using numerical integration software.[157] A catalytic trapping experiment for PKA at 10 mM free Mg^{2+} is shown in Figure 9A. Preequilibration of PKA with sufficient ADP to bind all the active sites results in a significant lag in the production of phosphopeptide. The kinetic transient can be modeled to a mechanism which incorporates the dissociation of ADP prior to ATP binding and phosphoryl transfer. These simulations indicate that ADP dissociates from the enzyme at a net rate constant (k_{off} = 23 s^{-1}) that is close in value to k_{cat} (20 s^{-1}). This confirms that the net release of ADP is the rate-determining step in PKA at levels of Mg^{2+} when both sites are mostly occupied (i.e., 10 mM free Mg^{2+}). It is important to recognize that the k_{off} value determined in this technique is an apparent value. If a conformational change is linked to product release and is similar in value to the release rate, the method would not be able to easily separate the two events. However, if the conformational change precedes product release, for example, it is possible to measure a faster release rate. In section XI, this possibility will be considered for PKA.

B. Turnover and Nucleotide Affinity

It is presently unknown whether product release will play a dominant role in limiting turnover in other

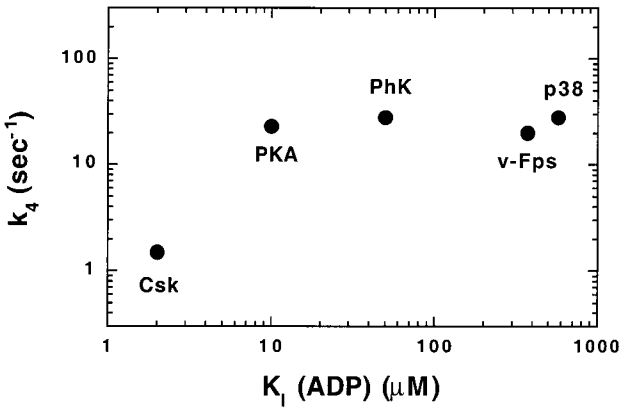


Figure 10. Correlation of the net product release rate, k_4, with the affinity of ADP (K_I) for several protein kinases. The values of k_4 were determined from viscosometric analyses, and the K_I values for ADP were determined from inhibition studies. The data were taken from the references listed in Table 3.

protein kinases. Several protein kinases have been analyzed using viscosometric techniques and shown to exhibit either rapid or partially rate-limiting rates of phosphoryl transfer (Table 3). In these cases, there appears to be no clear relationship between the value of the k_4 step and the affinity of ADP. As shown in Figure 10, for four of the five protein kinases studied, thus far, large changes in the K_I for ADP are not coupled with appreciable changes in k_4. While Csk is an exception since it displays higher affinity for ADP and a lower value for k_4 compared to other enzymes, these studies were performed in the presence of Mn^{2+} rather than Mg^{2+}.[56] While it will be meaningful to see where other protein kinases lie on this plot, the current absence of a satisfactory correlation between rate and affinity suggest that factors other than the physical release of ADP could limit turnover in some protein kinases. As stated previously, the viscosity approach is not capable of distinguishing what physical event comprises the k_4 step. Indeed, it is possible that the release of the phosphoproduct or a slow conformational change in the enzyme could limit this step.

XI. Slow Conformational Changes

A. Diffusion Limits and Conformational Changes

There is indirect kinetic evidence that conformational changes are important for the binding of substrates and ATP. Data derived from viscosometric and isotope partitioning studies indicate that ATP is a sticky substrate for PKA.[51,147] As such, k_{cat}/K_m for ATP must reflect the true, bimolecular association constant for the nucleotide, a value of about 2×10^6 M^{-1} s^{-1}. It is reasonable to assume that this rate constant also governs the association of ADP. Using a K_I of 10 μM[123] and the dissociation rate constant of 23 s^{-1} from the catalytic trapping study (Figure 9A), the calculated association rate constant for ADP is also 2×10^6 M^{-1} s^{-1}. Under some buffer conditions, Kemptide is also a sticky substrate so that k_{cat}/K_m for this substrate peptide reports a bimolecular association rate constant that is close in value to those for the nucleotides.[51,136] In these examples, the

measured association rate constants are well below the expected diffusion-controlled value for the binding of a small ligand to a protein. For example, enzymes such as superoxide dismutase and acetylcholine esterase have values of k_{cat}/K_m between 10^8 and 10^9 M^{-1} s^{-1}.[158,159] The low observed rates for PKA are not likely to result from difficulty in the selection of a productive substrate form. For the substrate peptide, GRTGRRNSI, k_{cat}/K_m is about 2×10^8 M^{-1} s^{-1},[40] a value that is 2 orders of magnitude greater than that for the smaller peptide Kemptide (LRRASLG). These findings suggest that conformational changes that occur after initial association of the ligand influence binding. In PKA, it is expected that these potential conformational changes do not limit turnover at high magnesium concentrations since a rapid 'burst' phase is observed using either GRTGRRNSI or Kemptide.[137] However, it is possible that conformational changes associated with ADP release could limit turnover.

B. Mutant Studies

Several mutagenesis studies suggest that the lack of a direct correlation between the k_4 step (Scheme 1) and the affinity of ADP in the protein kinases (Figure 10) may reflect the participation of slow conformational changes in the catalytic mechanism. Replacement of the conserved aspartate in the catalytic loop of PhK results in a mutant enzyme (E149A) with a turnover number that is reduced by 4 orders of magnitude.[144] Surprisingly, k_{cat} (0.005 s^{-1}) is still maximally affected by solvent viscosity, indicating that k_4 is the rate-determining step in this mutant. Since no changes in nucleotide affinity are reported for this mutant, it is possible that the 4 orders of magnitude reduction in the k_4 step could reflect a bold change in the kinetic mechanism where a viscosity-sensitive conformational change in the enzyme may limit turnover. In another study, removal of the activation loop phosphorylation site in PKA (T197A) results in a mutant with a 2 orders of magnitude reduced affinity for ATP but an unaffected k_4 step.[92] If ATP and ADP affinities are similar in the mutant as they are in the wild-type enzyme, an event other than ADP dissociation may limit turnover in the dephosphorylated mutant. It is important to note that these findings result from the study of mutant proteins with low catalytic activities. Departures from the simple view of slow ADP release comprising the k_4 step may be a natural outcome of the replacement of certain critical catalytic residues. Conformational changes may be a less significant component in the wild-type enzymes.

C. Fluorescent Protein Probes

The application of stopped-flow fluorescence methodologies to protein kinases has provided some insights into the role of conformational changes in governing substrate turnover. A mutant form of PKA where Asn-326 in the C-terminus is converted to cysteine has been labeled specifically with the fluorophore acrylodan.[160] The steady-state kinetic parameters of this labeled mutant, Acr-N326C, are similar to those of the wild-type enzyme with no parameters deviating by more than 2-fold. The fluorescence spectrum of Acr-N326C is quenched differentially by ATP and ADP so that the phosphorylation of a substrate peptide can be monitored directly using stopped-flow technology. The design of the stopped-flow instrument is similar conceptually to the rapid quench flow schematic in Figure 7 except the second mixing chamber is omitted and reactants A and B flow into a cuvette after mixing. The mixing is driven pneumatically rather than with a stepping motor, and the flow is terminated by a stopped syringe. Analysis is spectroscopic rather than chemical in nature so that kinetic observations are continuous. Mixing large amounts of the substrate with PKA that is preequilibrated with ATP produces a double-exponential rise in fluorescence with rate constants of 500 and 60 s^{-1}.[161] The first phase is identical in rate to the phosphoryl transfer rate constant measured by chemical analysis in the rapid quench flow instrument.[52] While the second phase rate is about 2-fold larger than k_{cat} for this mutant, trapping ADP from the active site using ATP provides a release rate for ADP that is about 2-fold higher than turnover.[161] These results demonstrate that ADP release and a conformational change are partially rate-limiting for Acr-N326C. While this mutation could certainly influence protein dynamics making comparisons difficult, the transient state kinetics suggest that conformational changes in the wild-type enzyme could play a role in limiting turnover.

D. Slow Structural Changes in Wild-Type PKA

While the simple kinetic mechanism for protein phosphorylation in Scheme 1 is adequate to describe the kinetic results for wild-type PKA when both metal sites are occupied by Mg^{2+}, other studies illustrate that the kinetic pathway is more complex at physiological Mg^{2+} concentrations of 0.5 mM.[162−165] Under these lower Mg^{2+} concentrations, fewer of the second metal sites are occupied. When the free concentration of Mg^{2+} is lowered from 10 to 0.5 mM, k_{cat} increases 2-fold and K_m for ATP increases 10-fold.[50,123] On the basis of the apparent affinity of the second metal, lowering the free Mg^{2+} concentration from 10 to 0.5 mM causes the occupancy of the second site to shift from 80% to 20%. The presence of a full viscosity effect on k_{cat} at these lower metal concentrations is consistent with fast phosphoryl transfer.[129,166] In agreement with this finding, a rapid and large 'burst' amplitude in the pre-steady-state kinetics is observed when the enzyme is preequilibrated with ATP (Figure 9B; dashed line). However, when the reaction is initiated with ATP (Figure 9B; filled circles), the amplitude and rate of the 'burst' phase is markedly attenuated, implying that a nucleotide-linked conformational change, preceding the phosphoryl transfer step, limits the 'burst' phase. The sigmoidal appearance of the 'burst' phase is not dependent on the concentration of ATP, indicating that the phase is not limited by a ligand binding event. Also, at 0.5 mM free Mg^{2+}, catalytic trapping studies show that ADP release only partially limits turnover (Figure 9B; open circles). The dotted line in this panel represents the expected transient when

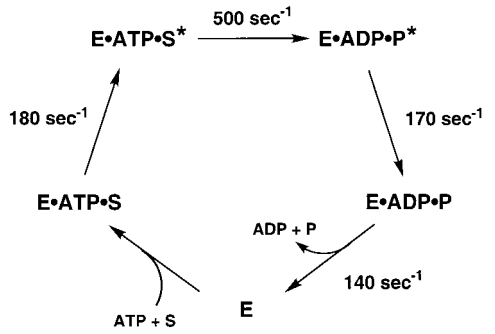


Figure 11. Kinetic mechanism for PKA at 0.5 mM free Mg^{2+}. This mechanism is based on the analysis of the kinetic data presented in Figure 9. The starred ternary complexes E·ATP·S* and E·ADP·P* represent activated species that promote phosphoryl transfer.

ADP release is fully rate-limiting ($k_{off} \approx k_{cat}$). In other experiments, it was shown that preequilibration with phosphopeptide does not affect the 'burst' kinetics, indicating that the release of this product is fast relative to the phosphoryl transfer step.[123] These findings suggest that a conformational change after the phosphoryl transfer step partially limits turnover at physiological Mg^{2+} concentrations.

The results from the kinetic studies in Figure 9 are summarized in the catalytic pathway shown in Figure 11. Unlike the simple kinetic mechanism in Scheme 1, two conformational change steps (170 and 180 s^{-1}) and ADP release (140 s^{-1}) partially control turnover (k_{cat} = 50 s^{-1}) when few of the secondary metal sites are occupied (20%). These conformational changes are not detected at higher metal ion concentrations (Figure 8), but there is no reason to suspect that the physical changes that underlie these steps do not occur at other metal concentrations. Under these conditions, the rates of these steps may be much faster and, therefore, unobservable. Since a full viscosity effect is observed on k_{cat} at the physiological concentration of 0.5 mM free Mg^{2+}, the slow conformational events must be sensitive to media viscosity. As mentioned in the previous section, there is no reason all unimolecular events must be insensitive to solvent viscosity particularly when large changes in protein organization may occur. The data in Figure 9 demonstrate that conformational changes in protein kinases may be slow relative to substrate turnover and may display sensitivity to solvent viscosogens. These kinetic experiments do not provide structural information regarding the nature of the conformational steps, but certainly the viscosity sensitivity of these steps suggests that the changes are significant enough to be affected by solvent friction.

XII. Ligand-Induced Structural Changes

It is evident from X-ray diffraction data that protein kinases are flexible enzymes. Movements in the core have been well documented, and a summation of the data highlight several classes of motion. The relative orientation of the small and large domains of the core is variable, but there may be a loose connection between the extent of domain closure and the activation state of the protein kinase.[106] Phosphorylation of the activation loop (e.g., InRK, ERK2) or the binding of regulatory proteins (e.g., cdk2-cyclinA) alters two regions in the core which include the activation loop and helix C. While it is unclear whether these movements occur in solution and can be linked to the slow conformational changes in catalysis, the present data underscore the structural plasticity of the enzymes and offer some exciting possibilities. Already there is some evidence that structural changes in protein kinases occur upon ligand binding in solution. For example, the binding of PKI causes a reduction in the radius of gyration of the core.[167] These data can be used to promote a model in which PKI induces the closed form of the enzyme. ATP binding alters the extent to which chemical reagents cleave PKA at specific loci in the polypeptide chain, suggesting that the nucleotide may induce conformational changes in the core.[168] Regardless, it should be noted that all structural studies designed to characterize conformational changes in protein kinases on a molecular scale have been performed under equilibrium conditions where the lowest energy state of the ligand-bound complex is populated. Furthermore, in the cases of the crytallographic models, packing artifacts could misrepresent the structure of the enzyme in solution. Whether these structural changes are achieved under turnover conditions is presently unknown. In addition, the motions in the core detected by these methods may be catalytically relevant, but it is not certain if the attainment of these states is energetically difficult and presents an inherent barrier to substrate phosphorylation. Given the observation that rate-limiting conformational changes are essential, slow events in PKA and possibly other protein kinases, understanding the physical nature of these steps is paramount to understanding the mechanisms of regulation for this enzyme family.

XIII. Catalysis and Disease

As prime effectors of signal transduction processes, it is not surprising that mutant forms of protein kinases are oftentimes causative agents in human disease. In many cases, specific mutations in the kinase domain which can be linked to a disorder display elevated catalytic activities.[169] While mutations have been detected throughout the kinase domain in these cases, a significant number have been localized in or near the glycine-rich and activation loops.[169] For example, activating mutations in the glycine-rich loop of MET, a receptor TPK, have been identified in patients diagnosed with multiple papillary renal-cell carcinomas.[170] Furthermore, a single mutation in the activation loop of the receptor TPK FGFR (K650E) is responsible for thanatophoric dysplasia type II, a lethal neonatal disorder.[171,172] While the cause for this activation is unknown, it is possible that this mutation could mimic the phosphorylated, active state of the loop. With the growing evidence for slow conformational changes in protein kinase catalysis, it is meaningful to correlate the kinetic mechanisms of these aberrant protein kinases with structural data. Mutations that enhance or repress catalytic activity may cause changes in the rate and/or nature of these conformational events.

XIV. Acknowledgment

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