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Activation Loop Phosphorylation and Catalysis in Protein Kinases: Is There Functional Evidence for the Autoinhibitor Model?†

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ABSTRACT: Many protein kinases are activated strongly by the phosphorylation of a polypeptide region (activation loop) that lies outside the active-site cleft. Analysis of the X-ray crystallographic structures of the insulin receptor with the activation loop in the phosphorylated and dephosphorylated forms offers a testable model for the mechanism of activity regulation by the loop. In this model, the dephosphorylated activation loop can act as an autoinhibitor by blocking substrate access to the active site. Phosphorylation of the loop could then release the autoinhibitor from the active site, allowing substrate binding and catalysis. While this model has been widely invoked, it was not clear if solution studies would support an autoinhibitory model for kinase regulation, in general. We review the results of solution studies on six protein kinases that test the role of the activation loop in controlling active-site access. While loop phosphorylation enhances substrate binding in two cases, four protein kinases display little or no effect on substrate dissociation constants. By comparison, phosphorylation increases catalysis by 2−4 orders of magnitude in all cases. These findings can be used to place the phosphorylatable activation loops into two broad, functional subcategories. (i) Gated activation loops exhibit bifunctional properties restricting substrate access and controlling catalysis. (ii) Nongated activation loops allow free movement of the substrate in and out of the active site irrespective of phosphorylation state but potently modulate the phosphoryl transfer step. Thus, while activation loop phosphorylation greatly modulates catalytic potential, it does not necessarily affect substrate binding, as once widely believed.

Protein phosphorylation is, by far, the most significant posttranslational modification affecting cellular and organismal function. For example, intermediary metabolism, cell cycle control, DNA transcription, RNA splicing, growth, muscle contraction, and development are just some of the many biological processes that are controlled by protein phosphorylation at the level of the individual cell. The enzymes that catalyze protein phosphorylation, the protein kinases, are avidly studied for their ability to control all of these processes. While sequence alignment data were originally used to predict that all protein kinases contain a conserved catalytic domain (*1*), the pioneering X-ray studies of Knighton, Sowadski, and Taylor in 1991 (*2*, *3*) defined, for the first time, the three-dimensional structure of this domain. In general, the kinase core possesses elemental components for substrate binding and phosphate delivery and also serves as an essential, minimum frame upon which more complex protein kinases are attached (*4*). While protein kinases regulate the function of target proteins in the cell through the phosphorylation of serine, threonine, and tyrosine side chains, they are also regulated through phosphorylation by other protein kinases in the core structure. In this article,

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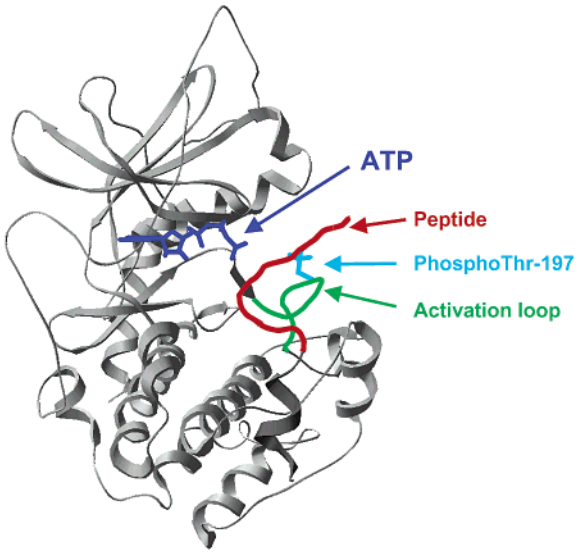

FIGURE 1: Ribbon diagram of PKA cocrystallized with ATP and an inhibitor peptide. The activation loop and its phosphorylated residue, phosphoThr-197, are colored green and light blue, respectively. ATP is shown in dark blue and a portion of the inhibitor peptide in the active site is shown in red. This structure was taken from (*49*).

we will discuss how phosphorylation of a critical polypeptide segment known as the activation loop in the kinase core influences catalytic function. While the structural features of these loops revealed through X-ray crystallographic modeling have been extensively reviewed elsewhere (*5*, *6*), our emphasis in this review will lie in how the expectations of these models compare with kinetic findings.

PHOSPHORYLATION OF THE ACTIVATION LOOP—A MODEL DERIVED FROM X-RAY STUDIES

The structure of the first protein kinase to be solved by X-ray diffraction, methods, cAMP-dependent protein kinase [PKA],[1] is shown in Figure 1. This structure typifies the basic fold within the enzyme family and is worth noting here. The PKA core is composed of two domains—a small ATP binding domain and a larger substrate binding domain. The active-site cleft lies between these two lobes. While ATP binds into a deep pocket lined with hydrophobic residues, the substrate peptide (an inhibitor peptide is shown in the PKA structure) binds toward the lip of the pocket and is held in position largely by electrostatic contacts. The activation loop in PKA, phosphorylated in the X-ray structure at Thr-197, makes several key contacts with charged residues from the large and small lobes. While not all protein kinases have a phosphorylatable residue in this loop segment, many require phosphorylation at this position for catalytic activity. In PKA, activation loop phosphorylation enhances catalytic activity by approximately 3 orders of magnitude (*7*, *8*).

[1] Abbreviations: c-Src, cellular form of the Rous Sarcoma virus nonreceptor PTK; CAK, cyclin activating kinase; cdk2, cyclin-dependent kinase 2; ERK2, extracellular regulated kinase-2; FGFR, fibroblast growth factor receptor; InRK, tyrosine kinase domain of the insulin receptor; Kemptide, peptide sequence LRRASLG; PDK-1, phosphoinositide- dependent protein kinase-1; PKA, cAMP-dependent protein kinase; PKC, protein kinase C; PTK, protein tyrosine kinase; SPK, protein serine kinase; v-Fps, viral form of the Fujinami Sarcoma virus nonreceptor PTK.

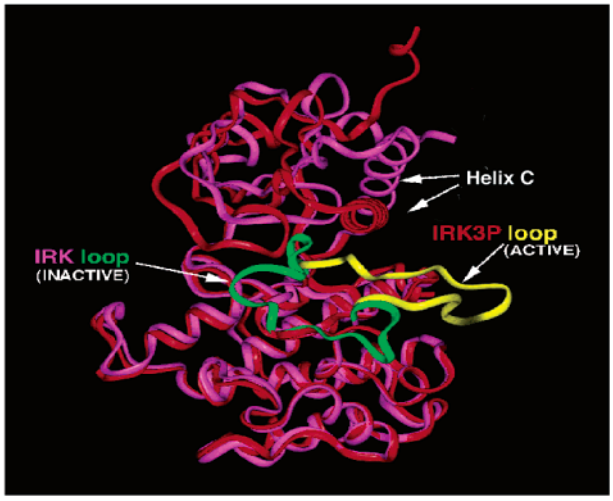

FIGURE 2: X-ray structures of the InRK in the unphosphorylated (red) and phosphorylated (mageneta) forms. The trisphosphorylated and nonphosphorylated activation loops are shown in yellow and green, respectively. These structures are taken from ref *21*.

Phosphorylation of the analogous residues in cdk2, ERK2, InRK, PKC, c-Src, and v-Fps enhance catalytic activity by 2−5 orders of magnitude (*9*−*14*). While most kinases that are up-regulated through loop modification are monophosphorylated, there are examples where 2 or 3 phosphates are incorporated (*13*, *15*−*17*). While phosphorylation can occur autocatalytically in many cases, specific roles for heterologous protein kinases have been defined and may be the principal activating enzymes in vivo. For example, in cdk2, the cyclin activating kinase, CAK, phosphorylates Thr-160, a prerequisite for cell cycle control (*18*, *19*). Activation loop phosphorylation in PKC is conducted by PDK-1 (*20*).

Given the extraordinary catalytic effects of activation loop phosphorylation, special attention has been given to solving the X-ray structures of protein kinases in both inactive (dephosphorylated) and active (phosphorylated) states. While it has been difficult to crystallize both species in many cases, one protein kinase whose structure has been elucidated in both states is shown in Figure 2. In the phosphorylated state of the tyrosine kinase domain of the insulin receptor [InRK], the activation loop is well-ordered and makes several important electrostatic contacts with positively charged side chains. In this configuration, the active site is open and accessible to substrate. In the dephosphorylated state, however, the loop moves considerable distances and occupies the active site. It has been suggested that this loop movement controls catalytic activity in the InRK through an autoinhibitor mechanism (*21*). Upon phosphorylation, the loop departs from the active site allowing ready access of ligands to the pocket. Thus, the activation loop functions as a "phosphorylation-sensitive switch" for controlling substrate binding. Similar loop movements have also been detected for cdk2, but here disengagement of the activation loop is mediated primarily by cyclin binding and secondarily by phosphorylation (*22*, *23*). The X-ray structure of the dephosphorylated form of the FGFR kinase domain also reveals an activation loop embedded in the substrate pocket (*24*). Overall, these structures offer a very compelling model for describing the enormous activity changes seen across a wide range of protein kinases. Indeed, the simplicity of the autoinhibitor model conveys well to general audiences and,

Scheme 1

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

not surprisingly, is often cited as a general regulatory mechanism for this enzyme family.

KINETIC REDUCTIONISM AND INDIVIDUAL RATE CONSTANTS

Since the discovery of the first protein kinase some four decades ago (*25*, *26*), kinetic investigations have been used to understand catalysis in this enzyme family. However, the vast majority of these studies have been limited to the determination of simple steady-state kinetic parameters (e.g., K_m, V_{max}). While valuable, these parameters do not provide direct information on the molecular steps associated with protein phosphorylation. However, to correlate the structural features of these enzymes with their regulatory properties, it is essential to dissect these parameters to understand their true meaning. To illustrate this point, we will consider the simple kinase mechanism in Scheme 1 at high ATP concentrations where S is the substrate and P is the phosphoproduct. For this mechanism, the K_m for the substrate is given by the relationship in eq 1:

$$K_m = \frac{k_4}{k_2}\left(\frac{k_{-2} + k_3}{k_3 + k_4}\right) \quad (1)$$

This equation underscores the dilemma of relying on initial velocity measurements to interpret enzymatic function. For example, it is difficult to assess true substrate affinity from steady-state kinetic measurements since K_m contains added kinetic terms not present in K_d.

To understand the complex interplay between activity measurements and the microscopic steps in Scheme 1, it is useful to consider various conditions where only subsets of the individual rate constants in eq 1 control the steady-state kinetic parameters. Figure 3 outlines the free energy reaction profiles for substrate phosphorylation under four limiting conditions (1 → 4) in Scheme 1. These conditions are defined using two relationships: the substrate commitment factor[2] and the rate-limiting step for turnover.[3] Using these conditions, the K_d for the substrate is equivalent to the K_m in only one example (case 1; weak substrate commitment and rate-limiting transfer). In all other cases, the K_m may be greater or less than K_d, depending on the magnitude of the other steps in the kinetic pathway. Thus, to understand how ligands bind in the active site of a protein kinase, it is necessary to attain detailed information on the individual steps in the kinetic mechanism. We term this process "kinetic reductionism" since it deconstructs complex catalytic pathways

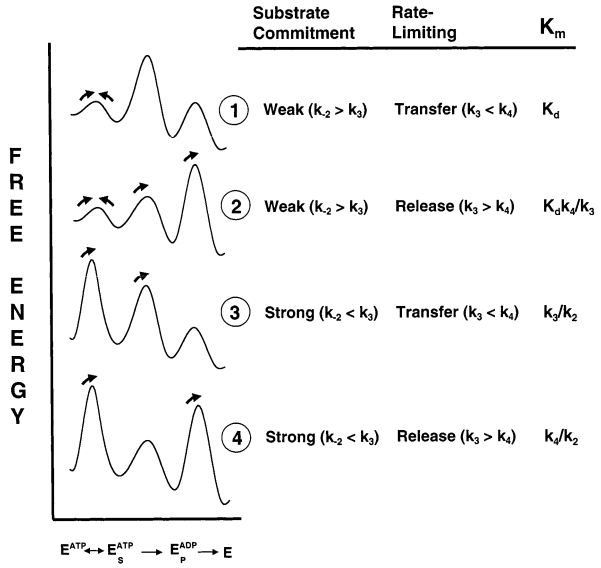

FIGURE 3: Free energy reaction profiles for the protein kinase reaction. K_m is expressed relative to individual rate constants in Scheme 1 using four limiting conditions (1 → 4). The arrows on the free energy profiles designate transitions through steps important for evaluating K_m at each limiting condition. For simplicity, the ground state of the ternary complex, E·ATP·S, is commonly fixed in each case through adjustments in the substrate concentration.

in protein kinases exposing the simple uni- and bimolecular processes comprising the pathways.

METHODOLOGIES FOR ESTABLISHING KINETIC PATHWAYS IN PROTEIN KINASES

Kinetic methods have been applied to the study of protein kinases since their early discovery in the 1950's (*26*). These methods have been used successfully to address key issues regarding substrate and nucleotide selectivity, binding order and metal ion preference [e.g., refs *27*−*33*]. These and many other kinetic investigations have nicely contributed to our general understanding of kinase catalysis, but all have been limited in their ability to establish the individual rate constants in the general mechanism in Scheme 1. Two experimental methodologies, applied in the past decade, have effectively bridged this gap in our understanding of protein kinases:

(1) Viscosity Methods: The viscosity method relies on the ability of viscosogenic agents such as sucrose and glycerol to influence the bimolecular steps in a kinetic sequence. This approach has a rich history with applications to a highly diverse group of enzymes (*34*−*39*). In principle, if any of the steady-state kinetic parameters are limited by the diffusion of the substrate (k_2, k_{-2}) or product (k_4) (i.e., bimolecular processes; Scheme 1), then a large viscosity effect will be measured on that parameter. Conversely, if a steady-state kinetic parameter is limited by a unimolecular step such as the phosphoryl transfer step (k_3), the viscosity of the medium is not expected to influence this parameter. The magnitude of these effects is readily assessed using the Smulchowski equation so that the rates of individual steps in the enzyme mechanism may be estimated (*34*, *37*, *40*).

For determining the role of phosphoryl transfer in controlling substrate turnover, k_{cat} is typically expressed as a ratio in the absence and presence of a solvent viscosogen and plotted as a function of relative solvent viscosity (Figure 4).

[2] The substrate "commitment" factor refers to the ratio of the rate constant for phosphoryl transfer and the substrate dissociation rate constant (k_3/k_{-2}). A substrate with a high commitment factor is more likely to be modified by the enzyme rather than to dissociate from the active site (i.e., $k_3/k_{-2} > 1$). A substrate with a low commitment factor dissociates faster from the active site than is phosphorylated (i.e., $k_3/k_{-2} < 1$).

[3] Substrate turnover (k_{cat}) is a function of two rate constants as defined in the following equation: $k_{cat} = k_3k_4/(k_3+k_4)$. The rate-limiting step(s) for turnover may be k_3 or k_4 or a combination of k_3 and k_4.

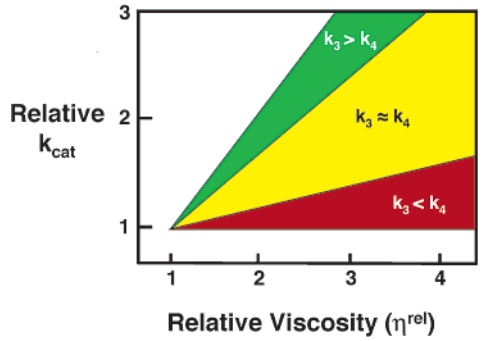

FIGURE 4: Use of viscosity methods to place limits on the phosphoryl transfer rate in a protein kinase. The turnover number, k_{cat}, is expressed as a ratio in the absence and presence of an added viscosogenic agent. Slope values greater than 0.8 imply that $k_3 > k_4$, whereas slope values less than 0.2 imply that $k_3 < k_4$. Intermediate slope values between 0.2 and 0.8 imply that the phosphoryl transfer rate partially limits k_{cat} ($k_3 \approx k_4$). The exact value for k_3 can be evaluated when the slope is less than 0.8 using the following relationship: $k_3 = k_{cat}/(1 - \text{slope})$. Above this slope value, only a lower limit can be placed on k_3.

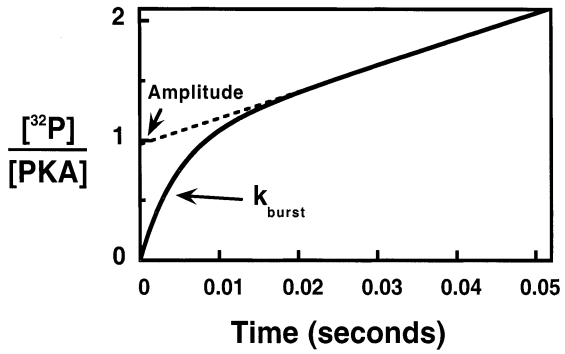

FIGURE 5: Evaluation of the phosphoryl transfer rate using pre-steady-state kinetic methods. The phosphorylation of Kemptide by PKA is conducted in a rapid quench flow instrument (*37*). The rate of the exponential 'burst' phase (k_{burst}) can be used to evaluate k_3 at high substrate concentrations using the following relationship: $k_3 = k_{burst} - k_4$.

Slope values close to 1 (>0.8) imply that the phosphoryl transfer step is fast and not rate-limiting ($k_3 > k_4$), whereas slope values close to 0 (<0.2) imply that the phosphoryl transfer step is slow and limits substrate turnover ($k_3 < k_4$). For intermediate slope values (0.2−0.8), phosphoryl transfer is partially rate-limiting ($k_3 \approx k_4$). The steady-state kinetic parameter k_{cat}/K_m can also be analyzed using viscosity methods, but here information on the substrate commitment factor rather than the phosphoryl transfer step is typically derived.[4] We first applied the viscosity method to PKA in 1991 and found that k_{cat} for the phosphorylation of a short substrate peptide (Kemptide, LRRASLG) is maximally affected by added viscosogens (slope ≈ 1), a result consistent with fast phosphoryl transfer ($k_3 > k_4$) (*37*). These studies established for the first time that the chemical transfer step is not rate-limiting in this protein kinase.

(2) Pre-Steady-State Kinetic Methods: The second advance to impact mechanistic studies on protein kinases came with the application of pre-steady-state kinetic experiments. These studies, originally applied to PKA in our group, were used to isolate the phosphoryl transfer step and place a discrete rate to this event (*41*). In these studies, PKA, preequilbrated with ^{32}P-ATP, is mixed with Kemptide in a rapid quench flow instrument and then rapidly mixed with a quenching agent to stop the reaction (acetic acid or EDTA). The instrument permits the quantitation of radiolabeled phosphate into the substrate in the early millisecond time frame, a window encompassing the first enzyme turnover. As shown in Figure 5, the phosphorylation of Kemptide occurs in two distinct phases: a rapid, exponential phase ("burst" phase, k_{burst}), followed by a slow, linear phase. The latter phase is identical in value to k_{cat}, indicating that this portion of the phosphorylation curve corresponds to steady-state turnover. The burst phase corresponds to the phosphorylation of the substrate in the active site. The true rate of the phosphoryl transfer step is then evaluated by extrapolating the observed burst rate constant (k_{burst}) to high substrate concentrations (*41*). For PKA, it was shown that Kemptide is rapidly phosphorylated in the active site (k_3 = 500 s^{-1}) but subsequent phosphorylation events must await slow, net product release (k_4 = 20 s^{-1}). While the viscosity studies indicate that the phosphoryl transfer step is fast, the rapid mixing methods directly evaluate the rate of this step. The fast mixing methods have recently been applied to two other protein kinases, Her-2 and Csk, revealing that phosphoryl transfer in these enzymes is not rate-limiting (*42*, *43*).

The amplitude of the burst phase in a pre-steady-state kinetic experiment provides information regarding the active concentration of the protein kinase (*44*). For PKA, the enzyme-normalized burst amplitude, obtained from an extrapolation of the linear phase to zero time, is 1 (Figure 4), implying that all the available active sites are competent to bind and phosphorylate substrate. In Csk and Her-2 this amplitude term is much lower accounting for only 20% of the total enzyme in both cases (*42*, *43*). These findings suggest that all the active sites are not available for catalysis. Owing to the difficulty in synthesizing active forms of protein kinases in host cells, there is concern about whether recombinant kinases are produced in native forms. The amplitude of the burst phase provides a unique tool for determining the percent of actively expressed enzyme.

The pre-steady-state kinetic studies not only provide useful mechanistic information on protein phosphorylation in the active site but also can be used to establish the real affinity of the substrate. Since it is known from viscosity studies that Kemptide exchanges rapidly in the active site (*37*, *45*), eq 2 can be used to establish the K_d for this substrate from the ratio of the rate constants of phosphoryl transfer and the net dissociation of products (k_3/k_4). Equation 2 is essentially a limiting case for eq 1 when

$$K_d = K_m\left(\frac{k_3}{k_4} + 1\right) \qquad (2)$$

$k_3 < k_{-2}$ (i.e., case 2 in Figure 3). For PKA, k_3/k_4 is 25 based on rapid quench flow experiments, making the K_m for Kemptide 26-fold lower than the K_d. Prior to the application of these kinetic methods, a measurement of the true affinity of Kemptide in an active ternary complex with ATP was unattainable owing to the inability to establish an exact number for k_3/k_4. In the next section, we will discuss how this term can be used to evaluate the autoinhibitor model.

[4] A plot of relative k_{cat}/K_m as a function of relative viscosity yields a slope term which can be related to the commitment factor by the following equation: k_3/k_{-2} = slope/(1 − slope).

Table 1: Effects of Removing the Activation Loop Phosphorylation Site in PKA[a]

parameter	wild-type	T197D	T197A
k_{cat} (sec^{-1})	20	18	1.4
K_m (μM)	30	660	1100
k_3 (s^{-1})	500[b]	27	3.6
k_4 (s^{-1})	20	53	18
k_3/k_4	25	0.51	0.20
K_d (μM)	800	1000	1300

[a] These data are taken from ref *7*. [b] This value was determined using rapid quench flow methods (*41*).

APPLYING KINETIC REDUCTIONISM TO PKA

The notion that phosphorylation induces large motions in the activation loop of the InRK provides a very simple model for activity regulation. If this autoinhibitor model accounts for the large activity increases in the protein kinases, then it follows that substrate binding will be significantly impaired when the activation loop is dephosphorylated. We first tested this model on PKA in 1995 using site-directed mutagenesis (*7*). Upon replacement of Thr-197 with aspartate and alanine, several of the steady-state kinetic parameters were significantly altered (Table 1). Most notably, the K_m for Kemptide increases by approximately 20−40-fold. These findings appear to verify the autoinhibitor model derived from the X-ray data on the InRK. However, these data alone cannot be used to ascertain the true affinity of the substrate owing to the inherent inability of steady-state kinetics to provide a value for k_3/k_4 in eq 2. Without measurements of the substrate K_d's in the phosphorylated and dephosphorylated enzymes forms, functional evidence for the autoinhibitor model is unachievable.

To interpret the effects of Thr-197 mutation on the regulation of PKA, direct information on the rates of the phosphoryl transfer and product release steps are required. As mentioned previously, k_3/k_4 for wild-type PKA is 25, a value which accounts for the large viscosity effect on k_{cat} (*37*) and the large burst in product formation in the first 10 ms of the reaction (Figure 5). When the phosphorylation mutants are interpreted using the viscosity approach, large decreases in k_3/k_4 are observed relative to wild-type owing to substantial decreases in the phosphoryl transfer rate (Table 1). Since the increases in K_m for the mutants are compensated by proportional decreases in k_3/k_4, no significant changes in K_d are observed. Thus, the observed increases in K_m do not reflect changes in real binding affinity. These results not only indicate that PKA does not conform to the autoinhibitor model but also provide a strong case for the general application of reductionist methods for understanding kinase regulation.

EXPLORING OTHER FAMILY MEMBERS

Our kinetic investigation on the PKA activation loop offers a useful paradigm for the analysis of protein kinase function. However, we were concerned that the results derived from the PKA mutants might represent an anomaly and that other kinases might conform to the autoinhibitor model. Accordingly, we investigated activation loop phosphorylation in the nonreceptor PTK, v-Fps, an enzyme distinct from PKA in its substrate specificity, overall size, and tissue expression (*46*). While removal of the phosphorylatable residue, Tyr-1073, in the v-Fps loop leads to large decreases in the rate

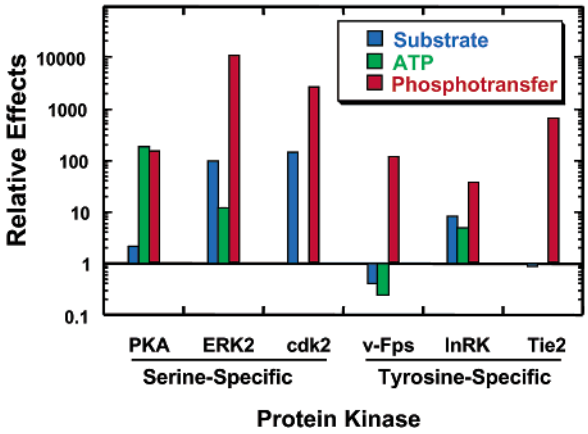

FIGURE 6: Effects of activation loop phosphorylation on substrate (blue) and ATP (green) binding and the phosphoryl transfer rate (red) for several protein kinases. Values greater than one imply that loop phosphorylation enhances substrate or ATP binding or enhances the rate of phosphoryl transfer. The magnitude of the effects is expressed in relative values. The data for this plot were taken from (*7*) for PKA, (*13*) for ERK2, (*12*) for cdk2, (*10*) for v-Fps, (*14*) for the InRK, and (*48*) for Tie2.

of phosphoryl transfer, we detected no significant changes in substrate binding affinity (Figure 6). Indeed, phosphorylation appears to have a small but real negative impact on substrate binding. Since these initial investigations into PKA and v-Fps (*7*, *10*), several other protein kinases from both the serine and tyrosine families have been studied using detailed kinetic methods. Unlike PKA and v-Fps, Lew and co-workers, focusing on the proline-directed SPKs, showed that phosphorylation of the activation loops in ERK2 and cdk2 enhance substrate binding by approximately 2 orders of magnitude (*12*, *47*) (Figure 6). In stark contrast, though, Murray and co-workers showed that activation loop phosphorylation in the receptor PTK, Tie-2, has no influence on substrate binding (*48*). Finally, Kohanski and co-workers showed that phosphorylation of the InRK has only a small effect on substrate binding (8-fold) (*14*). Therefore, of the six protein kinases studied, to date, only $^1/_3$ display clear evidence for the autoinhibitor model.

MODULATION THROUGH THE NUCLEOTIDE POCKET

It has been proposed that the unphosphorylated activation loops from some protein kinases may also interfere with ATP binding (*21*). In accordance with this idea, loop phosphorylation improves ATP affinity to PKA by approximately 2 orders of magnitude (Figure 6). For ERK2, modification also enhances ATP binding but to a lesser degree. While this appears encouraging, activity control through ATP binding modulation presents a more difficult problem than the substrate autoinhibitor paradigm. Present in low millimolar concentrations in the cell, ATP is expected to be a potent competitor for the activation loop and may easily displace it from the nucleotide pocket. An autoinhibitor model operating through the nucleotide pocket would necessarily require large phosphorylation-driven changes in ligand affinity to compensate for the high intracellular ATP levels. For example, the large reduction in ATP affinity for PKA (Figure 6) would not be expected to lower enzyme activity by more than 2-fold since the available ATP in the cell is still at or above the K_m for the nonphosphorylated form

(Table 1). Irrespective of these considerations, modulation through the nucleotide pocket is a moot point in other cases. In v-Fps, cdk2, and Tie-2, loop phosphorylation leads to no improvements in ATP binding. Given these observations, any changes in ATP affinity upon activation loop phosphorylation do not appear to possess the power to greatly regulate protein kinase function.

EFFECTS ON PHOSPHORYL TRANSFER RATES

While the present survey of protein kinases reveals that activation loop phosphorylation does not consistently lead to improved substrate and/or ATP binding affinities, in every case studied, thus far, phosphorylation significantly increases the rate of the phosphoryl transfer step (Figure 6). On the basis of the expectations of competitive inhibitors, the rate of phosphoryl transfer should be unaffected by the phosphorylation state of the activation loop since excess substrate would effectively "compete away" the dephosphorylated loop. The inability to accomplish this, however, should not be used to refute the validity of the autoinhibitor model. As evidenced in the X-ray studies, activation loop phosphorylation alters other structural components in the kinase domain. As shown in Figure 2, phosphorylation of the InRK causes large movements in helix αC. The latter structure contains a conserved glutamate that contacts a conserved lysine important for interacting with the α phosphate of ATP. Dephosphorylation causes a disruption in this electrostatic dyad that may misalign ATP and lead to the observed low rates of phosphoryl transfer. Similar motions are also elicited in helix αC in cdk2 upon cyclin binding and phosphorylation (*22*, *23*). In this case, the cocrystallized nucleotide displays large movements in the triphosphate region of ATP, supporting this model.

SUMMARY

The model derived from X-ray structures posits that activation loop phosphorylation provides catalytic activation through the modulation of substrate binding, the traditional autoinhibitor model. In this review, we outlined how kinetic reductionist techniques (pre-steady-state kinetic and viscosometric) can be used to address the occurrence of this model in the protein kinase family. These methods permit the careful dissection of the individual steps in the kinase pathway such as the substrate binding, product release, and phosphoryl transfer steps. The data show explicitly that the original paradigm forged from the InRK and cdk2 models cannot explain all the available kinetic data. Indeed, the autoinhibitor hypothesis has failed to account for the empirical data in more than 50% of the cases. These discrepancies can be used to classify activation loops regulated by phosphorylation into two primary, functional groups. Gated activation loops synchronously modulate substrate binding and the phosphoryl transfer step, whereas nongated activation loops modulate solely the phosphoryl transfer step. Thus, gated activation loops are bifunctional, while the nongated activation loops exhibit a single function. As shown in Figure 7, the distinction between these groups can be made largely on the observed effects of phosphorylation on substrate affinity. Here, we define a steric factor for all the activation loops and rank the protein kinases based on this scale. A steric factor close to zero (nongated) represents an activation loop that does not block substrate binding in the dephosphorylated state. In comparison, a steric factor in large excess of 10 (gated) represents an activation loop that impedes the binding of the substrate. Many protein kinases are regulated through activation loop phosphorylation but we are only now beginning to understand the molecular causes underlying these activity changes. The kinetic methods and nomenclature presented here provide a means for quickly evaluating the role of these loops in solution.

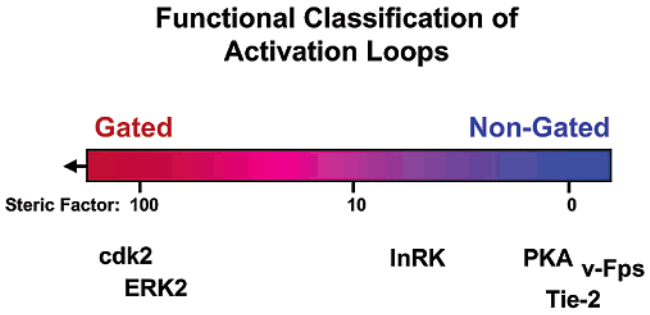

FIGURE 7: Functional classification of activation loops regulated by phosphorylation. The steric factor (SF) is defined using the following equation, $SF = K_d(nonP)/K_d(P) - 1$, where $K_d(nonP)$ and $K_d(P)$ are the dissociation constants for substrate in the nonphosphorylated and phosphorylated forms of the activation loop. Gated activation loops (red) enhance substrate binding using steric factors in excess of 10, whereas nongated activation loops (blue) have no influence on binding and have steric factors near zero. The protein kinases, ranked based on their steric factors, are placed appropriately along this scale.

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