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Active and Inactive Protein Kinases: Structural Basis for Regulation

Review

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Introduction

Protein kinases and phosphatases play pivotal roles in regulating and coordinating aspects of metabolism, gene expression, cell growth, cell motility, cell differentiation, and cell division. As a result, if cellular life is to function in an orderly manner, the switching on and off of protein kinases and phosphatases is as crucial for their function as their catalytic activity.

The total number of distinct kinase domain sequences available is now approaching 400 (Hardie and Hanks, 1995). Multiple sequence alignments have indicated that all protein kinases should have similar structures, and this has been confirmed by recent crystal structure determinations. Conserved features have been identified in 12 subdomain regions of all protein kinases, and residues from these subdomains have been implicated in essential roles in enzyme structure and function. Protein kinases exhibit variability in other parts of the kinase domain, and different kinases may contain additional domains, additional subunits, or both. These features allow several different mechanisms for control.

Control mechanisms that have been recognized to date include the following: control by additional subunits or domains that may function in response to second messengers (e.g., cyclic AMP binding to the regulatory subunit of cyclic AMP–dependent protein kinase [cAPK], Ca^{2+}/calmodulin binding to calmodulin-dependent protein kinases, and Ca^{2+} and diacyl glyerol binding to N-terminal domains of protein kinase C); control by additional subunits whose level of expression varies depending on the functional state of the cell (e.g., cyclin regulation of the cyclin-dependent protein kinases [CDKs]); control by additional domains that target the kinase to different molecules or subcellular localizations (e.g., the SH2 and SH3 domains of the Src kinases); control by additional domains that inhibit the kinase activity by an autoregulatory process (e.g., myosin light chain kinase [MLCK]); and control by phosphorylation and dephosphorylation by kinases and phosphatases. Phosphorylation of specific threonine, serine, or tyrosine residues may occur at a number of sites. Some of these are located in the N-terminal or C-terminal portions of the polypeptide chain, which lie outside the kinase domain (e.g., in Src kinase and calmodulin-dependent kinase II [CaMKII]) or on other subunits (e.g., in phosphorylase kinase [PhK]). A key aspect of regulation recognized in recent years is that many protein kinases are phosphorylated on a residue(s) located in a particular segment in the center of the kinase domain, which is termed the "activation segment."

This review will focus on the role of the activation segment in the mediation of these different aspects of control. The activation segment is defined as the region spanning conserved sequences DFG and APE and corresponds to residues 184–208 in cAPK (Taylor and Radzio-Andzelm, 1994). The activation segment includes Thr-197, one of two autophosphorylation sites in cAPK. The conversion of an inactive kinase to an active kinase involves conformational changes in the protein that lead to the correct disposition of substrate binding and catalytic groups and relief of steric blocking to allow access of substrates to the catalytic site. The activation segment and the control of its conformation via phosphorylation plays a key role in these transformations. It can be involved in recognition of regulatory subunits, in autoinhibition of substrate binding, and in promotion of the correct orientation of domains and catalytic site residues. This review summarizes our current understanding of control by the activation segment based on recent structure determinations of active and inactive kinases.

The first observation of Thr-197 phosphorylation in the activation segment of cAPK was reported in 1979 (Shoji et al., 1979). Although it was speculated that phosphorylation at discrete sites might be of physiological importance in the regulation of enzyme activity, it was not until 1990 that mutagenesis studies indicated the significance of this site for the recognition of the regulatory subunit (Levin and Zoller, 1990), and not until 1993 that it was definitively shown that phosphorylation is promoted by an autocatalytic event that is crucial for activation (Steinberg et al., 1993). The crystal structure determination of cAPK in 1991 (Knighton et al., 1991a, 1991b) showed the structural importance of Thr-197 phosphorylation and demonstrated possible roles of phosphorylation in promotion of activation. The structure provided a definitive model to which other kinases could be related. Also in 1991, both the fission yeast cell division control kinase (cdc2) (Gould et al., 1991) and the microtubule-associated protein kinase (MAPK) (Payne et al., 1991) were found to be activated by phosphorylation on residues that mapped to a position similar to Thr-197 in cAPK. These results showed the importance of this site not only as an autophosphorylation site, as in cAPK, but also as a site involved in kinase cascade activation mechanisms. For the tyrosine kinases, autophosphorylation of pp60$^{v\text{-}src}$ at position Tyr-416 (now known to be in the activation segment) had been shown in the early 1980s, and its significance for control in the cellular counterpart of Src kinase was established by 1987 (reviewed by Hunter, 1987). The following year, *trans*-autophosphorylation of the insulin receptor tyrosine kinase (IRK) was elaborated, and the similarity in sequence location of some of these sites to that in Src kinase and its relatives and in cAPK was noted (reviewed by White and Kahn, 1994). As more and more kinases have been discovered and sequenced and further kinase cascades established, it is recognized that control by phosphorylation in the activation segment is a property of most, but not all, protein kinases (Table 1).

Table 1. Examples of Protein Kinases Phosphorylated and Not Phosphorylated in the Activation Segment

Kinases Phosphorylated in the Activation Segment	Kinases Not Phosphorylated in the activation Segment
RD kinases	RD kinases
Cyclic AMP–dependent kinase (cAPK)[a,b]	Phosphorylase kinase (PhK)[a]
Cyclin-dependent kinases (p34*cdc2*, cdc2, and CDK2[a], CDK7)	Casein kinase 1 (CK1)[a]
Microtubule-associated kinase (MAPK/ERK-2[a])	Ca^{2+}/calmodulin-dependent kinase II (CaMII)
MAP kinase kinase (Mek1)	Epidermal growth factor receptor tyrosine kinase
Raf1 kinase	C-terminal Src kinase (Csk)
Protein kinase C-βII (PKC)	
Ca^{2+}/calmodulin-dependent kinase I (CaMKI)[a]	Non-RD kinases
Insulin-stimulated protein kinase 1 (ISPK-1, rsk^{mo-2})	Twitchin kinase[a]
Glycogen synthase kinase 3 (GSK3)	Myosin light chain kinase (MLCK)
Insulin receptor tyrosine kinase (IRK)[a,b]	Wee1 kinase
Platelet-derived growth factor (PDGF) receptor tyrosine kinase	
c-Src tyrosine kinase[b] (and other kinases in the Src family, e.g., Yes, Fyn, Fgr, Lyn, Mck, Lck, and Blk)	

RD kinases are defined as those kinases in which the conserved catalytic aspartate is preceded by an arginine residue. Details are taken from Hardie and Hanks (1995).
[a]Kinases for which crystal structure is available.
[b]Kinases phosphorylated by autophosphorylation mechanism.

What are the structural determinants by which activation segments are recognized by kinases? Whether autophosphorylation may be possible or not is governed by the sequence specificity of the kinase and the sequence in the activation segment that surrounds the phosphorylated residue. The activation segment of cAPK contains the sequence RTWT*L surrounding the phosphorylated threonine (T*). This sequence is consistent with the consensus recognition sequence for cAPK (RXXT*/S*Hy) (Hardie and Hanks, 1995) (in single letter code in which X denotes any residue and Hy denotes a hydrophobic residue). Hence, autophosphorylation with cAPK is possible. The activation segment of CDK2 has the sequence RTYT*HE, and that of MAPK has the sequence GFLT*EY*V. These sequences are not compatible with the consensus sequences recognized by these kinases (XS*/T*PX for CDK2 and PXS*/T*P for MAPK). Hence, phosphorylation by other kinases is required for activation of these enzymes.

In this review, we seek a structural explanation of why some kinases require activation by phosphorylation and others do not. Sequence comparisons provide some clues. All protein kinases contain an aspartate residue (Asp-166 in cAPK), which has been implicated in the catalytic mechanism, most likely as a base that activates the incoming substrate hydroxyl. Most Ser/Thr and all tyrosine protein kinases have an arginine immediately preceding this catalytic aspartate. Knowledge of the kinase structures so far suggests that these kinases, which we term "RD" kinases, require some ionic interactions of the arginine with a phosphate or a carboxylate group. Kinases that are known to be activated by phosphorylation on the activation segment have an RD sequence at the catalytic aspartate (e.g., all those listed in the left column of Table 1). The structural examples suggest that these kinases require charge neutralization of a cluster of basic residues, including the arginine of the RD sequence, by the phosphoamino acid. These interactions may promote a number of other effects, such as relief of autoinhibition by the activation segment (IRK), promotion of the correct domain orientation (MAPK), and correct alignment of ATP-binding residues (CDK2). For CDK2, it appears that access to the threonine for phosphorylation is only possible when the kinase has been partially activated by complex formation with the rigid cyclin molecule. Not all RD kinases are regulated by phosphorylation (e.g., some of those listed in the right column of Table 1). In these kinases, charge neutralization of the arginine can be achieved by a glutamate (PhK and probably epidermal growth factor receptor kinase) or by binding of a dianion (casein kinase 1 [CK1]). However, the mammalian CK1 structure has shown that the arginine of the RD sequence does not necessarily interact with a charged group: in this case it interacts with three carbonyl oxygen groups. Finally the non-RD kinases (i.e., those kinases in which the catalytic aspartate is not preceded by an arginine residue) do not appear to be regulated by phosphorylation in the activation segment (Table 1, right column). In twitchin kinase and MLCK, there is a nonpolar residue preceding the catalytic aspartate and a nonpolar residue corresponding to the threonine of cAPK in the activation segment. The following descriptions of the kinase structures that are known to date provides some details of these interactions and their significance for activation.

Kinase Structures

The crystal structures of eight unique protein kinases have been reported (Table 1). Three of the protein kinases have been crystallized in active conformations (cAPK, PhK, and CK1), one in a partially active conformation (CDK2 in complex with cyclin A), and five in inactive conformations (CDK2, MAPK, IRK, twitchin kinase, and CaMKI). Five of these kinases are regulated by phosphorylation on residues in the activation segment (cAPK, CDK2, MAPK, IRK, and CaMKI) and three are not (PhK, CK1, and twitchin kinase).

Protein Kinases Crystallized in Active Conformations: cAPK, PhK, and CK1

The first X-ray study of a protein kinase, cAPK, revealed the basic architecture that has been observed in all

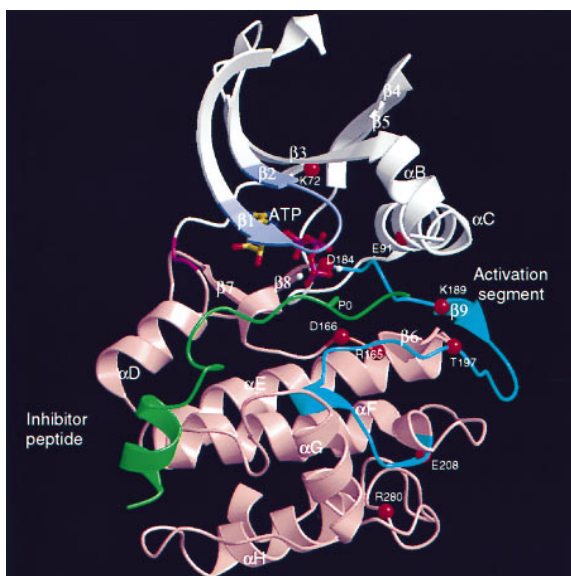

Figure 1. The Catalytic Core of cAPK

The N-terminal lobe is colored white, and the C-terminal lobe is pink. The hinge region between the lobes is magenta. The inhibitor peptide is shown in green with the position of the serine that is the target for phosphorylation shown as P0. ATP is in ball and stick representation. The glycine-rich region that is important for localization of the phosphates of ATP is shown colored light purple. α Helices and β strands are labeled. The diagram is rotated about 45° to the conventional view to display the activation segment (residues 184–208), which is colored cyan and includes strand β9. The Cα positions of other important residues are labeled and are referred to in the text.

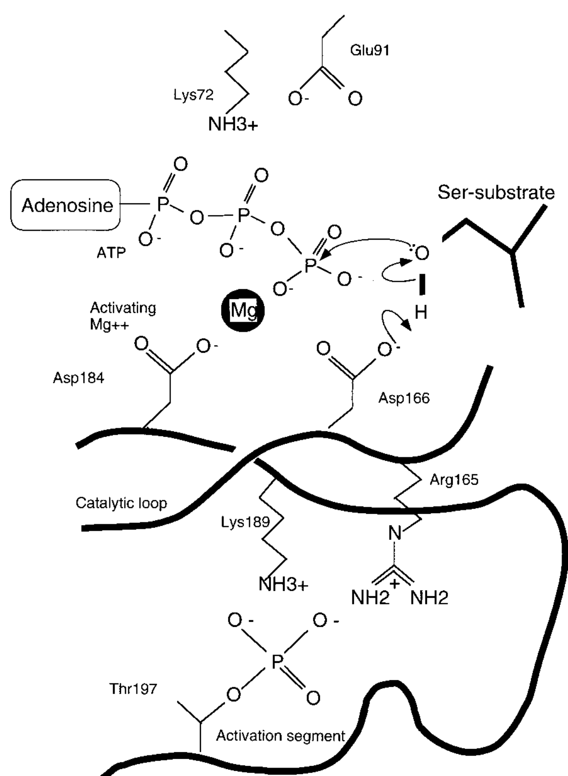

Figure 2. A Schematic Representation of ATP and Some of the Key Residues at the Catalytic Site and Activation Segment of cAPK

A possible mechanism is shown in which base-catalyzed attack by Asp-166 on the substrate serine promotes the formation of an alcoholate ion, which can then attack the γ phosphate of ATP. The catalytic mechanism is not definitively established.

subsequent kinase domain structures (Knighton et al., 1991a, 1991b; Bossmeyer et al., 1993; Taylor and Radzio-Andzelm, 1994). cAPK is a key player in cellular responses to the second messenger cyclic AMP. The inactive form is a heterotetramer of two regulatory and two catalytic subunits. Activation is mediated by binding of cyclic AMP to the regulatory subunits, which causes the release of the catalytic subunits. cAPK is primarily a cytoplasmic protein, but upon activation it can migrate to the nucleus, where it phosphorylates proteins important for gene regulation.

The first cAPK structure was a binary complex with a 20 amino acid pseudosubstrate inhibitor peptide. Later, structures of the ternary complex of cAPK with inhibitor and either Mg^{2+}/ATP or Mn^{2+}/AMP-PNP were also solved. The kinase core comprises a bilobal scaffold that has an N-terminal lobe composed almost entirely of β sheet (colored white in Figure 1) and a C-terminal lobe in which α helices dominate (colored pink in Figure 1). The two lobes in the core are joined by a polypeptide chain, which functions as a hinge (magenta in Figure 1), allowing the two lobes to articulate. In the catalytic subunit of cAPK, there are additional residues at the N-terminus and C-terminus that are important for stability, but which show variation in other protein kinases (not shown in Figure 1). The catalytic site is at the interface of the two lobes. The ATP-binding site spans both lobes. The inhibitor peptide (colored green in Figure 1) is associated mostly with the C-terminal lobe. In the binary complex with inhibitor peptide or in the ternary complexes, the lobes adopt a closed conformation. However, in the structure of apo cAPK or cAPK in a binary complex with an iodinated inhibitor (Cox et al., 1994), a more open lobe orientation was observed, supporting results from solution studies that had also identified open and closed conformations (Olah et al., 1993). Such transitions seem likely to be important: the open form is necessary to allow access of ATP to the catalytic site and release of product ADP; the closed form is necessary to bring residues into the correct conformation to promote catalysis.

The positions of some of the residues important for binding ATP in cAPK are shown as red circles in Figure 1, and their roles are illustrated in Figure 2. These are conserved in other kinases. The correct alignment of the ATP with respect to the catalytic residues appears crucial for catalysis. The adenine is located in a hydrophobic pocket, and the ribose is stabilized by hydrogen bonds. The phosphates of ATP are aligned for catalysis by interactions with the main chain nitrogens of a glycine-rich loop (light purple in Figure 1), by interactions with Lys-72 (which is localized by a salt bridge to Glu-91), and by interactions with Mg^{2+} ions (white spheres). One Mg^{2+}, identified as the activatory Mg^{2+} (discussed in Adams and Taylor [1993]), binds to the β and γ phosphates of ATP and to Asp-184 (Figure 2). The catalytic aspartate, Asp-166, which is invariant in all kinases, is located in the C-terminal lobe in a loop, termed the

catalytic loop (orange in Figure 1). The aspartate is presumed to act as a base to remove a proton from the protein substrate hydroxyl group (Figure 2), although the catalytic mechanism is not definitively established. The resulting alcoholate or phenolate ion is positioned to attack the γ phosphate of ATP.

The activation segment (cyan in Figure 1) begins at the highly conserved DFG motif with Asp-184. The segment continues with a region that is remarkably different in the active and inactive kinase structures. In cAPK there is a short β strand, $\beta 9$, which is followed by a loop containing the autophosphorylation site Thr-197. The segment ends in the region that has a 3_{10} helix structure and includes residues that are important for the peptide substrate hydrophobic recognition site C-terminal to the phosphorylated serine (labeled P0 in Figure 1). The final residue, Glu-208, is part of the APE motif, which is conserved in most kinases, and is hydrogen bonded to the conserved arginine Arg-280.

As shown in Figure 3a and summarized in Table 2, the phosphothreonine Thr-197-P contacts His-87 from the N-terminal lobe, Arg-165, which precedes the catalytic base (Asp-166), Lys-189, and Thr-195. The phosphate is placed to compensate the cluster of positively charged residues (Taylor and Radzio-Andzelm, 1994). Steady-state kinetic analysis combined with viscosometric measurements of cAPK mutants, in which the threonine was changed to an aspartate or an alanine, showed reductions in catalytic efficiency that were due to the specific effects of weakened ATP affinity and reduced rates of phosphoryl transfer (Adams et al., 1995). There was little effect on peptide substrate binding affinities. These results imply that the Thr-197-P group is important for modulating the catalytic flux of cAPK.

The structural results suggest several possible roles for the phosphothreonine in activation. First, the phosphothreonine stabilizes the positively charged cluster of Arg-165, Lys-189, and His-87 in a way that can only be promoted by a dianionic group. Second, the interactions, especially those through Arg-165, may help promote the correct orientation and electrostatic environment for the catalytic base Asp-166. Third, the location of the Thr-197-P may determine the correct conformation for residues in the activation segment that are important for peptide substrate recognition. However, the mutants Thr-197→Asp and Thr-197→Ala showed little change in peptide substrate affinity, indicating that substrate specificity promoted by the activation segment may not be an important factor for this kinase (Adams et al., 1995). Fourth, interactions between the phosphothreonine and Lys-189 may help promote the correct conformation for Asp-184, which contacts the activatory Mg^{2+}, and for residues 185–189, which interact with the N-terminal lobe. Fifth, the contact between His-87 and Thr-197-P may promote the correct domain–domain orientation, which is also critical for ATP binding. However, open conformations of phosphorylated cAPK are observed, and hence phosphorylation is not sufficient for domain closure. Kinetic studies on the His-87→Ala mutant of cAPK have shown that, although the mutant is impaired in its recognition of peptide substrate, it exhibits a 2- to 3-fold higher k_{cat} compared with the native enzyme (Cox and Taylor, 1995). Thus, this contact may not be important for the catalytic function in cAPK, but it may be important for other functions, such as recognition of the regulatory subunit, and equivalent contacts may be important in other kinases.

We now compare these results with a kinase in which the catalytic core is constitutively active and requires no phosphorylation on the activation segment for activity. PhK is one of the largest and most complex of the known protein kinases and is central to the regulation of glycogen metabolism. The PhK holoenzyme is hexadecameric, with subunit stoichiometry $(\alpha\beta\gamma\delta)_4$. The α and β subunits are regulatory and transmit information via their state of phosphorylation in response to cAPK and other kinases. The δ subunit is an integral calmodulin subunit and provides the Ca^{2+} sensitivity. The γ subunit is the catalytic subunit (M_r 45 kDa) and contains an N-terminal kinase catalytic domain and a C-terminal regulatory domain. Peptides from two distinct regions in the regulatory domain have been shown to exhibit a high affinity for calmodulin and to inhibit the Ca^{2+}-activated holoenzyme (Dasgupta et al., 1989; Dasgupta and Blumenthal, 1995). The truncated γ subunit (residues 1–298), which lacks the regulatory domain, has similar activity to the activated holoenzyme but, as expected, exhibits no regulation by Ca^{2+} or phosphorylation (Cox and Johnson, 1992).

The structure of the active PhK truncated γ subunit (Owen et al., 1995) is similar to that of cAPK. The equivalent residue to Thr-197 is a glutamate, Glu-182 in PhK (Table 2). The Glu-182 side chain is turned in to form an ionic link with Arg-148, the arginine that precedes the catalytic base (Figure 3b). In PhK the residues equivalent to His-87 and Lys-189 are Ala-69 and Cys-172, respectively. Thus, in both kinase structures, the activation segments contribute a stabilizing ionic interaction with the conserved arginine preceding the catalytic aspartate; this interaction is mediated by Thr-197-P in cAPK and by Glu-182 in PhK. The sequence changes indicate that in PhK there is no cluster of basic residues that need a dianionic group for stabilization and the role of an ionic interaction with the arginine can be accomplished by a glutamate.

In another kinase, CK1, a cluster of basic charged residues exists, but phosphorylation is not required for activation. CK1 phosphorylates a number of important physiological substrates, including glycogen synthase, the simian virus 40 large T antigen, the tumor suppressor p53, and the cyclic AMP response element modulator, CREM. The structures of the Schizosaccharomyces pombe CK1 (Xu et al., 1995) and a mammalian CK1 (Longenecker et al., 1995) have been reported. Both structures lack the C-terminal domain, which has been implicated in subcellular targeting and regulation of activity (Carmel et al., 1994; Longenecker et al., 1995)

In their topology, the two CK1 structures (56% sequence identity) are similar to each other and to the structure of cAPK. The activation segment conformation is similar to that in cAPK except for a ten residue insertion in CK1. S. pombe CK1 was crystallized in the presence of ammonium sulfate, and in the crystal structure there is a sulfate ion bound close to the phosphate position of the phosphothreonine in cAPK. The ion is bound by two basic residues that are spatially equivalent

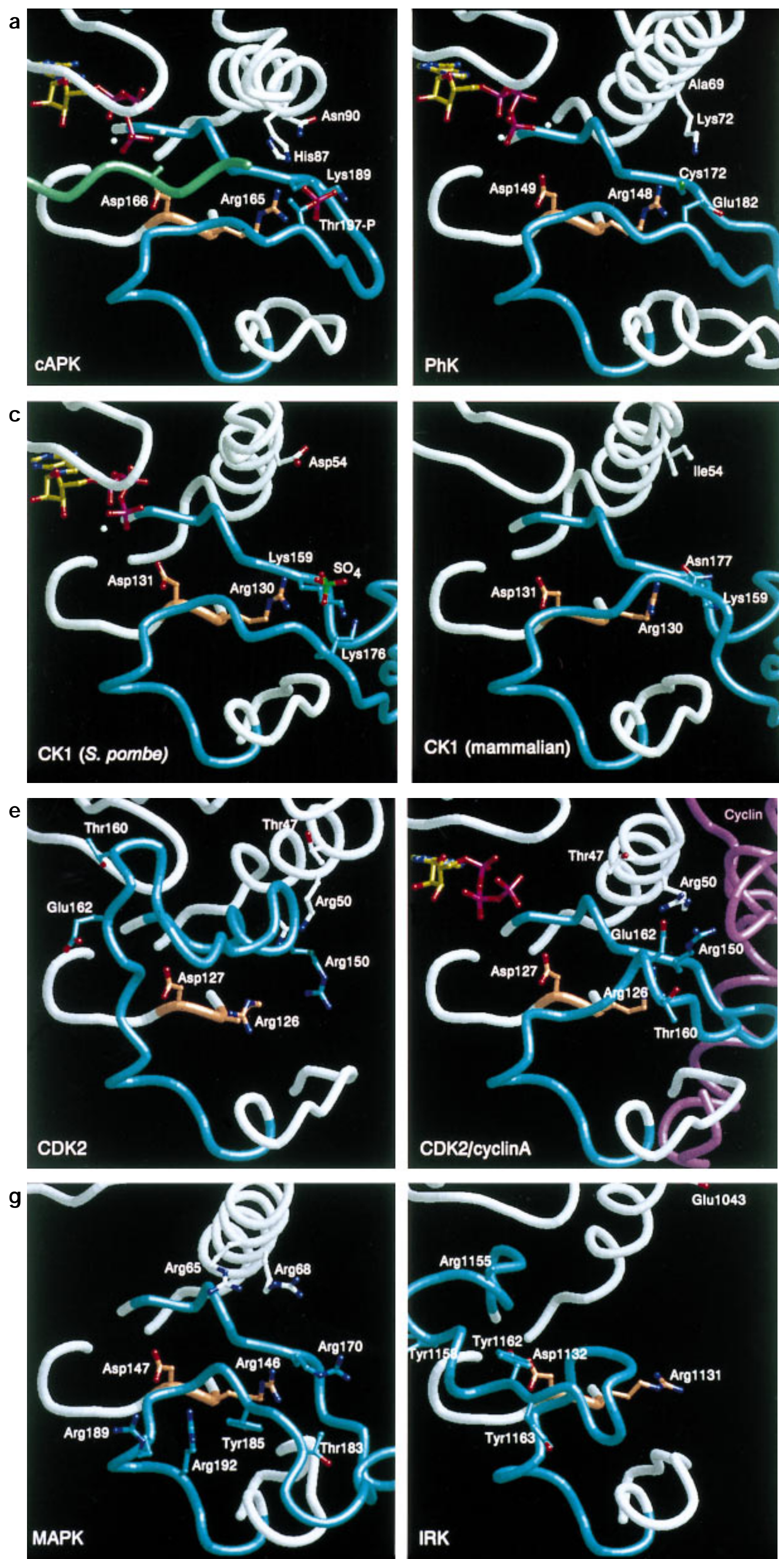

Figure 3. The Activation Segments and Their Relationship to the Catalytic Loop for Different Kinases

The view is similar to that in Figure 1. The activation segment is colored cyan, the catalytic loop orange, and the remainder of the polypeptide chain white. The inhibitor peptide in (a) for cAPK is shown in green. Important residues given in Table 2 are displayed. The kinases were superimposed using coordinates for the C-terminal lobe corresponding to residues 128–253 in cAPK. The fragments displayed correspond to cAPK residues 43–100 (including the glycine loop that interacts with ATP and the C helix), residues 164–171 (the catalytic loop), and residues 183–218 (including 184–208, the activation segment). (a)–(d) represent four active kinase structures; (e), (g), and (h) represent inactive kinase structures, and (f) is a partially active kinase.

(a) cAPK in the active, closed, and phosphorylated state. Thr-197-P interacts with His-87, Lys-189, and Arg-165. Arg-165 precedes Asp-166, the presumed catalytic base.

(b) PhK in the constitutively active state. Glu-182 occupies a similar position to Thr-197-P in cAPK and interacts with the arginine, Arg-148, adjacent to the catalytic base. Lys-72 is over 5 Å from the glutamate.

(c) CK1 (S. pombe) in the active state. A sulfate ion is bound in the crystalline protein and contacts Lys-159, Lys-176, and Arg-130.

(d) CK1 (mammalian) in the active state. Asn-177 occupies a similar position to Thr-197-P in cAPK and interacts with Arg-130. Arg-130 also interacts with main chain carbonyl oxygens from the activation segment.

(e) CDK2 in the inactive and closed state. Thr-160 is close to the glycine loop at the ATP-binding site.

(f) CDK2–cyclin A in a partially active conformation. Cyclin A is shown in magenta. Both the C helix (containing the PSTAIRE motif, residues 45–51) and the activation loop shift significantly from their positions in inactive CDK2. Glu-162 occupies a position approximately equivalent to His-87 in cAPK and hydrogen bonds to Arg-126, the arginine adjacent to the catalytic base, and Arg-150. Arg-50 and Arg-150 also interact with cyclin A.

(g) MAPK in the inactive and open conformation. Thr-183 is exposed, and Tyr-185 is buried. Arg-65, Arg-68, Arg-170, and Arg-146 could form a recognition site for a phosphorylated residue in the active state. Arg-189 and Arg-192 form a second dianion recognition site.

(h) IRK in inactive and open conformation. The activation segment blocks the catalytic site, and Tyr-1162 is hydrogen bonded to Asp-1132, the catalytic aspartate. Arg-1131 and Arg-1155 could form a potential phosphate recognition site in the active state after substantial conformational change.

to Arg-165 and Lys-189 in cAPK and by a lysine from the activation loop (Figure 3c; Table 2). The residue equivalent to His-87 in cAPK is deleted in CK1. The stabilization of the charged cluster by a dianion is consistent with the notion that this stabilization is important for maintaining the activation loop in the correct conformation for catalysis. In the crystals of mammalian CK1, there are two molecules per crystallographic asymmetric unit, providing two independent views of the enzyme. The activation segment of one molecule is similar to the

Table 2. Possible Contact Residues to Phosphorylated Amino Acids and Corresponding Nonphosphorylated Amino Acids in the Activation Segment of Protein Kinases

			Contact Residues to Thr-197-P in cAPK and Equivalent Residues in Other Kinases			
Kinase	Activation State in Crystal	Phosphoamino Acid(s) or Equivalent	Residue Preceding Catalytic Aspartate	Residue in DFG Motif Loop	Contact from N-Terminal Domain	Residue in Vicinity but Not in contact in cAPK
Kinases controlled by phosphorylation on residues in the activation segment						
cAPK	Active Closed Phosphorylated	Thr-197	Arg-165	Lys-189	His-87	Asn-90
CDK2	Inactive Closed Not phosphorylated	Thr-160	Arg-126	Arg-150	Thr-47	Arg-50
CDK2—cyclin A	Partially active Partially open Not phosphorylated	Glu-162 mimics Thr-160-P	Arg-126	Arg-150	Thr-47	Arg-50
MAPK	Inactive Closed Not phosphorylated	Thr-183 Tyr-185	Arg-146	Arg-170	Arg-65	Arg-68
IRK	Inactive Open Not phosphorylated	Tyr-1158 Tyr-1162 Tyr-1163	Arg-1131	Arg-1155	Glu-1043[a]	Asn-1046[a]
CaMKI	Inactive Open Not phosphorylated	Thr-177	Arg-140	Lys-167	Ser-62[a]	Asn-65[a]
Kinases that are not controlled by phosphorylation on residues in the activation segment						
PhK	Active Closed	Glu-182	Arg-148	Cys-162	Ala-69	Lys-72
CK1	Active Closed	Asn-177 or Sulfate or tungstate	Arg-130	Lys-159	Deletion	Asp-54
Twitchin	Inactive Open	Val-6098	Leu-6062	Ala-6088	Thr-5983	Lys-5986

[a]Tentative assignment.

S. pombe structure and binds a tungstate ion. However, in the other molecule in the asymmetric unit of the mammalian enzyme, the activation loop exhibits a different conformation and an asparagine (Asn-177), equivalent to Thr-197 of cAPK, is swung in and occupies a position identical to the phosphate site. The arginine adjacent to the catalytic base hydrogen bonds to the asparagine and to two main chain carbonyl oxygens from residues equivalent to 187 and 198 in cAPK numbering (Figure 3d). These latter interactions appear to help orientate two important parts of the activation segment and reinforce the asparagine interactions. The lysine side chain (Lys-159, equivalent to Lys-189 in cAPK) is turned away from the site. Here it seems that the correct constellation of groups can be stabilized without the need for ionic interactions.

Comparison of Inactive and Partially Active Kinase: CDK2 and CDK2–Cyclin A Complex

The determination of the crystal structures of the inactive human CDK2 and the partially activated human CDK2–cyclin A complex has provided remarkable insights into the activation mechanism of the CDKs. The CDKs are regulatory enzymes that initiate and coordinate events of the eukaryotic cell cycle (Morgan, 1995). Their role in cell proliferation is determined by elaborate control mechanisms. The activity of CDKs is dependent upon their association with a cognate cyclin, whose concentration varies during the cell cycle. The CDK–cyclin complex may exhibit some kinase activity, but requires phosphorylation of a threonine residue (Thr-160 in human CDK2) for full activity. The crystal structure of CDK2 in its inactive state provided an image of an inactive kinase (De Bondt et al., 1993). The basic closed bilobal architecture is similar to cAPK, but there are several major differences in structure between inactive CDK2 and active cAPK. The activation segment, containing the phosphorylatable threonine Thr-160, is folded so that it blocks the substrate recognition site (Figure 3e). The helix corresponding to helix C in cAPK (see Figure 1) contains the PSTAIRE sequence (residues 45–51) of CDK2 and is known to be important for recognition of cyclin A. This helix is displaced in CDK2, and residues important for binding ATP are wrongly disposed.

The crystal structure of the CDK2–cyclin A complex

(Jeffrey et al., 1995) has revealed the conformational changes that result in partial activation. Cyclin A (purple) binds to one side of the catalytic cleft of CDK2, inducing large conformational changes in its activation segment and PSTAIRE helix (Figure 3f). These changes activate the kinase by realigning active site residues and relieving the steric blockade at the entrance to the catalytic site. The recent crystal structure determination of cyclin A in its unbound form (Brown et al., 1995) has shown that there is no conformational change in cyclin A upon forming the complex.

CDK2 in the complex with cyclin A exhibits a partially open structure. The activation segment shifts from its inactive conformation, with movements of some atoms up to 21 Å, and adopts a conformation similar to, but not identical with, the activation segment of cAPK (Figure 3f). The N-terminal portion of this segment in CDK2 interacts with helices from cyclin A. Arg-50 and Arg-150 from CDK2 (equivalent to His-87 and Lys-189 in cAPK, respectively) hydrogen bond to cyclin A backbone carbonyl oxygens (residue Phe-267 and residues Glu-269 and Ile-270, respectively). Thr-160 is exposed and available for phosphorylation. The pocket, which in cAPK contains the basic residues Arg-165, Lys-189, and His-87, is lined with equivalent basic residues in CDK2 (Table 2). This cluster of positively charged residues is stabilized by interactions with the side chain of Glu-162, which is directed into the pocket, and by interactions with cyclin A (Figure 3f). It is speculated that when Thr-160 is phosphorylated, it turns into the pocket to compensate the charged residues and promote domain closure. The phosphorylated CDK2–cyclin A complex has been reported to have a 17-fold increase in activity compared with the nonphosphorylated complex (Jeffrey et al., 1995).

Inactive Kinase Structures: MAPK, IRK, Twitchin Kinase, and CaMKI

The inactive kinase structures show four very different conformations of the activation segment, indicating that there are a variety of conformations accessible to different kinases in the inactive state.

MAPK is phosphorylated on two residues in the activation segment, Thr-183 and Tyr-185. Following the discovery of MAPK as a crucial component in the response to insulin (Sturgill and Ray, 1986), subsequent work showed that several protein kinases phosphorylated on tyrosine residues in response to mitogens and certain transforming agents were identical or closely related to MAPK and established a central role for this kinase in signal transduction pathways (Ahn et al., 1991).

The crystal structure of MAPK (ERK2) in its inactive, nonphosphorylated form (Zhang et al., 1994) has shown an open conformation, in which the N-terminal lobe is both rotated (17°) and translated with respect to the N-terminal lobe of the active cAPK structure. Individual lobes superimpose well with their counterparts in cAPK, but they assume different relative conformations and result in incorrect alignment of catalytic residues.

The activation segment includes a six residue insertion. The conformation is similar to the activation segment in cAPK at the two ends, but is different for the major part that contains the two target sites for phosphorylation in MAPK (Figure 3g); Thr-183 is exposed, while Tyr-185 is buried. Glu-184, the intervening residue, does not make major contacts with the rest of the protein. Activation requires both local and global conformational changes. Residues in MAPK that are equivalent to those that line the Thr-197-P pocket in cAPK could contribute a similar grouping of positive charge when aligned correctly (Figure 3g; Table 2). There is also a second external dianion-binding site created by Arg-189 and Arg-192 from the MAPK activation segment (Figure 3g). This site is occupied by a sulfate ion in the crystal and is close to the main chain atoms of Tyr-185. Kinetic analysis indicates that Tyr-185 is phosphorylated first. The mutant Tyr-185→Glu exhibits disorder in the activation segment, indicating that the stability of the loop region is easily perturbed by a charged residue. The authors (Zhang et al., 1995) have argued on structural grounds that the likely changes upon the double phosphorylation (Thr-183-P and Tyr-185-P) result in an interchange of Tyr-185-P side chain with its main chain position, so that the phosphate of Tyr-185-P is placed in the external (sulfate) recognition site, and a shift of the side chain of Thr-183-P to the interior, so that the phosphate of Thr-183-P is placed in the internal dianion site. There it may draw together the charge cluster promoting the correct relative orientation of the two domains, which appears to be the major change required to activate this enzyme.

IRK exhibits a dramatic example of autoinhibition by the activation segment. The insulin receptor is an $\alpha_2\beta_2$ heterodimer. The extracellular α chain is responsible for binding insulin, and the intracellular part of the β chain contains the tyrosine kinase domain, which is flanked by a juxtamembrane region and a C-terminal tail, both of which are targets for phosphorylation. The primary substrate of the insulin receptor is the receptor itself (White and Kahn, 1994). The autophosphorylation of three tyrosines (Tyr-1158, Tyr-1162, and Tyr-1163) in the activation segment of the kinase domain results in activation.

The crystal structure determination of IRK in the inactive form (Hubbard et al., 1994) has provided the first illustration of a tyrosine kinase. As expected from the sequence similarity between IRK and cAPK, the two kinases share the same fold. IRK structure is an open structure with the N-terminal lobe rotated 26° with respect to the C-terminal lobe, compared with the active closed form of cAPK. The catalytic loop and the catalytic aspartate, Asp-1132, are in similar positions to their counterparts in cAPK.

The activation segment (residues 1150–1179) has five extra residues and adopts a very different conformation to the segment in cAPK. The DFG motif is displaced so that it is close to the glycine-rich nucleotide-binding loop. The ATP adenine recognition site is blocked by Phe-1151, from the DFG motif, and the α and β ATP phosphate sites are blocked by Gly-1152 and Met-1153. Tyr-1162, which is equivalent to Thr-197 in cAPK, is directed into the catalytic site, and its phenolic hydroxyl group is hydrogen bonded to the catalytic base (Figure 3h). Hubbard et al. (1994) argue that the binding of this

residue mimics substrate binding. However, *cis*-autophosphorylation is prevented because the ATP site is blocked in the inactive open conformation.

Phosphorylation of Tyr-1162 (probably by a *trans*-autophosphorylation mechanism) results in activation. Tyr-1158 and Tyr-1163 are both external, and the structural roles of phosphorylation at these sites are less clear. Like cAPK, IRK contains an arginine preceding the catalytic base and a further arginine corresponding to Lys-189 of cAPK (Table 2). In the apo structure, both these arginines exhibit alternative conformations. It seems likely that upon phosphorylation there is a massive change in the activation segment so that Tyr-1162-P adopts a position similar to the Thr-197-P in cAPK in which it can draw together and compensate the basic cluster. It also seems likely that there should be contacts from the N-terminal lobe that may promote domain closure, but the candidates are less obvious.

Twitchin kinase reveals a further twist in interactions that stabilize the activation segment. Twitchin, the *unc-22* product that is involved in muscle function, is a 753 kDa protein composed of multiple copies of both fibronectin type III–like domains and immunoglobulin-like domains and a Ser/Thr protein kinase, which is located near the C-terminus. The kinase portion exhibits 52% identity to smooth muscle MLCK, and, like MLCK, twitchin kinase is regulated by autoinhibition by 60 residues C-terminal to the kinase core (Kemp and Pearson, 1991).

The crystal structure of twitchin kinase (Hu et al., 1994) has revealed important features of this autoinhibitory mechanism. The crystallized protein comprises the catalytic core and 60 residues C-terminal to the core. Overall, the structure is similar to cAPK. The structure is an open conformation with the N-terminal domain rotated approximately 30° with respect to the C-terminal domain. The C-terminal 63 residues (6197–6260) extend into the catalytic site and make extensive interactions, many of which, by analogy with the structure of the inhibitor peptide with cAPK, mimic the substrate interactions. The activation segment exhibits a different conformation to the activation segment in cAPK. The residues corresponding to Arg-165 and Thr-197 in cAPK are valine and leucine, respectively, in twitchin kinase (Table 2). Thus, in twitchin kinase there appears to be no need for activation by a phosphate group in the activation segment, and release of the C-terminal tail by proteolytic cleavage is sufficient to activate.

The recently determined structure of CaMKI (Goldberg et al., 1996) has provided a further elaboration of the autoinhibitory mechanism. CaMKI phosphorylates the synaptic vesicle–associated proteins synapsin 1 and 2, thereby modulating interactions of synapsin with actin filaments and neurofilaments. The structure of CaMKI in the absence of Ca^{2+}/calmodulin reveals an open conformation in which there are extensive interactions between the C-terminal autoinhibitory sequence and the kinase core. However, the autoinhibitory region does not enter the ATP-binding site (as it does in twitchin kinase), but interacts on the outside of the ATP-binding domain, leading to conformational changes at the ATP-binding site. The activation segment is disordered. Conversion to the fully active CaMKI requires both binding of Ca^{2+}/calmodulin and phosphorylation on a threonine in the activation segment by a specific kinase.

Discussion

The structures of the kinases in the active conformation all show equivalent positions for essential catalytic site residues Lys-72, Asp-166, and Asp-184 in cAPK. Their positions and the correct orientation of the Mg^{2+}/ATP and the protein substrate appear crucial for catalysis and are dependent upon the tertiary structure of both lobes and the correct relative orientation of the lobes. Both CDK2 and MAPK bind ATP in their inactive conformations in the crystal, but the γ phosphate is either in the wrong position to promote in-line transfer to the substrate or not located, thus demonstrating the subtle dependence of activity on structure. In the inactive IRK and CaMKI structures, the ATP site is blocked or destroyed.

The position of the activation segment appears important in aligning the catalytic residues. Despite the variation in sequence of this region among the different kinases and in the number of residues, the active kinase structures all show a similar role for this segment. The contact from the phosphothreonine (or equivalent residue) of the activation segment to the conserved arginine, adjacent to the catalytic base, appears especially important. The contact provides a direct link from the phosphorylated or charged residue to the catalytic site. In both cAPK and CK1, the phosphothreonine and the dianion, respectively, also interact with two other basic groups, and the dianionic group is therefore important in holding this cluster of positively charged groups together. Other kinases that require activation by phosphorylation on a residue(s) in the activation loop (CDK2, MAPK, and IRK) exhibit the possibility of a cluster of charged residues similar to those noted for cAPK (Table 2). It seems likely, therefore, that these enzymes also demand a dianionic phosphate group for successful charge compensation and that without a dianion the positively charged groups would repel each other so that they could not be brought into their correct alignment.

In the partially active CDK2–cyclin A complex, the charge grouping is compensated by a glutamate, Glu-162, (two residues removed from the phosphorylatable threonine Thr-160) and by interactions of arginyl residues with the main chain carbonyl groups from cyclin A. In PhK, there is only one basic group, the arginine adjacent to the catalytic base, and charge compensation can be satisfactorily accomplished by a carboxylate group.

The extent to which kinases that are normally activated by phosphorylation on the activation segment can be activated by mutagenesis of the phosphorylated residue to glutamate or aspartate varies. Both cAPK and MAPK are not appreciably activated by the mutations of Thr-197→Asp in cAPK (Adams et al., 1995) or Thr-183→Glu or Tyr-185→Glu in MAPK, although the MAPK mutant Thr-183→Glu can achieve 7% of native activity when phosphorylated on Tyr-185 (Zhang et al., 1995). In S. pombe cells, a mutant $p34^{cdc2}$ kinase in which a glutamate replaces the phosphorylatable threonine Thr-167 gives rise to cells that arrest in a pseudomitotic

state. This suggests that the kinase may have been active enough to drive the cells into mitosis, but that dephosphorylation is important for exit (Gould et al., 1991). Significant biological activity has been reported for the MEK1 mutants Ser-218→Asp and Ser-222→Glu (Huang and Erickson, 1994) and for the protein kinase C mutant Thr-500→Glu (Orr and Newton, 1994). These observations suggest some partial compensation could be achieved by a carboxylate in the right position, similar to that achieved by the glutamate in PhK. In protein kinase C, there was no activity with the Thr-500→Asp mutant, demonstrating the importance of the correct position of the carboxylate. The epidermal growth factor receptor kinase, although it contains a tyrosine in the activation segment, is not regulated by phosphorylation. Mutagenesis experiments and modeling (Timms et al., 1995) suggest that a glutamate, Glu-848, in the activation segment may play a role similar to the glutamate in PhK.

The activation segments of protein kinases vary in length (up to 10 amino acids). The variability in sequence may allow the kinase to be constitutively active, or it may allow control by autophosphorylation, if the segment has a sequence corresponding to the substrate specificity of the kinase itself, or control by phosphorylation directed by other kinases that function as part of a cascade. The structural studies provide examples of each of these mechanisms, but there are other possibilities. For example, it is possible that a phosphate recognition site generated and normally recognized by a phosphorylated residue from the activation segment could also bind a phosphoresidue from elsewhere in the molecule, and this could provide a further inhibitory mechanism. More definite information is required to understand the exquisite regulatory properties of this enormous family of enzymes, for which phosphorylation on the activation segment is only one aspect of a number of diverse schemes for regulation.

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