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cAMP-Dependent Protein Kinase Defines a Family of Enzymes

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cAMP-dependent protein kinase defines a family of enzymes

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SUMMARY

The structure of the recombinant mouse catalytic subunit of cAMP-dependent protein kinase is reviewed with particular emphasis on the overall features and specific amino acids that are shared by all members of the eukaryotic protein kinase family. The crystal structure of a ternary complex containing both MgATP and a twenty-residue inhibitor peptide defines the precise role of the conserved residues that are clustered at the active site. In addition to catalysing the post-translational modification of other proteins, the catalytic subunit is itself subject to covalent modifications. It is a phosphoprotein and is also myristylated at its amino terminus. The enzyme when crystallized in the presence of detergent shows a detergent molecule bound to an acyl pocket that is presumably occupied by the myristyl moiety in the mammalian enzyme. When expressed in *E. coli*, the catalytic subunit is autophosphorylated at four sites. Two stable phosphates at Ser338 and Thr197 interact with multiple protein side chains thus explaining why they are inaccessible to phosphatases. Although all substrates and inhibitors of the catalytic subunit share a general minimum consensus sequence, the high affinity binding of protein inhibitors such as the regulatory subunits and the heat stable protein kinase inhibitors require additional determinants that lie beyond the consensus site. These two physiological inhibitors of the catalytic subunit appear to use different sites to achieve high-affinity binding.

1. INTRODUCTION

The first protein kinase to be purified was phosphorylase kinase in 1955 (Fischer & Krebs 1955) and this was followed a decade later by phosphorylase kinase kinase, later renamed cAMP-dependent protein kinase (cAPK) when its broader specificity was appreciated (Walsh *et al.* 1968). Other protein kinases followed, but it was not until the end of the next decade that we began to appreciate the full diversity of this family of enzymes (Hunter 1987). Three findings were critical: (i) Collett & Erickson (1978) established that the transforming protein from Rous sarcoma virus, $pp60^{v\text{-}src}$, was a protein kinase; (ii) Hunter demonstrated that $pp60^{v\text{-}src}$ phosphorylated tyrosine rather than threonine or serine (Hunter & Sefton 1980); and (iii) Barker & Dayhoff (1982) found that the sequence of $pp60^{v\text{-}src}$ showed extensive similarities to cAPK. We now recognize that the protein kinases constitute a very large family of diverse but related enzymes that serve as critical switches for all phases of growth and development in the eukaryotic cell. One has only to look at how many genetically altered protein kinases are oncogenic to appreciate the essential nature of these on–off switches (Cooper 1990).

Although over 300 enzymes are now recognized as belonging to the protein kinase family (Hanks *et al.* 1988; Hanks 1991), cAPK remains as one of the simplest of these enzymes that catalyse the transfer of the γ-phosphate from ATP to a substrate protein (for review see Beebe & Corbin (1986); Taylor *et al.* (1990*b*)). It is simple because it is relatively small and also because the major regulatory component is released as part of the activation mechanism. Most protein kinases are sequestered in the inactive state in the absence of the appropriate signal. In the case of cAPK, this inactive state is an aggregate of regulatory (R) and catalytic (C) subunits. cAMP activates this R_2C_2 holoenzyme complex by binding to the R-subunit, thus promoting dissociation of the complex into an $R_2(cAMP)_4$ dimer and two active C-subunits. Unlike phosphorylase kinase, a highly specific protein kinase that only recognizes phosphorylase as a substrate, cAPK is a broad specificity protein kinase. However, although it recognizes many different

† $1\text{Å} = 10^{-10}\ \text{m} = 10^{-1}\ \text{nm}$.
‡ The coordinates for the binary and ternary complexes are available from the Brookhaven Protein Data Bank as 1APM and 1ATP, respectively.

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protein substrates, all have the general consensus sequence of RRXS/TY or RXXRXXS/TY where Y is a hydrophobic residue (Zetterqvist *et al.* 1990). The R-subunits also contain a substrate-like sequence, and this segment of the R-subunit occupies the peptide binding site in the holoenzyme complex thus preventing access of other substrates and rendering the C-subunit inactive (Corbin *et al.* 1978; Granot *et al.* 1980; Taylor *et al.* 1990*b*).

cAPK was the first protein kinase to be sequenced (Shoji *et al.* 1983), and it was also the first to have functional sites mapped by procedures such as affinity labeling and group-specific labeling (Bramson *et al.* 1984; Taylor *et al.* 1990*a*). These chemical approaches identified a number of residues such as Lys72 and Asp184 that were associated with the ATP binding site (Zoller & Taylor 1979; Buechler & Taylor 1988). Crosslinking subsequently established that these two particular side chains were in close proximity to one another at the active site (Buechler & Taylor 1989). Differential labeling of solvent accessible lysines confirmed that ATP binding was confined to the N-terminal portion of the protein (Buechler *et al.* 1989). The peptide binding site was first localized in the middle of the protein near Cys199 by affinity labeling with a peptide analogue (Bramson *et al.* 1982). In addition to the chemical evidence, alignment of the various protein kinase sequences revealed that there are nearly a dozen residues scattered throughout the polypeptide chain that are conserved throughout the family (Hanks *et al.* 1988). Thus the alignments independently highlighted a set of residues that were presumably important for function. Many of these, such as Lys72 and Asp184, overlapped with those that were identified earlier by chemical methods.

cAPK was also the first protein kinase to be crystallized, and this crystal structure now puts all of the previous chemical and sequence alignment information into a true molecular context (Taylor *et al.* 1992*a*,*b*). Two crystal structures of the mouse recombinant Cα-subunit have been solved so far. The first structure to be solved was a binary complex containing a bound 20-residue inhibitor peptide, PKI(5-24) (Knighton *et al.* 1991*a*,*b*, 1993*a*). The inhibitor peptide constitutes the primary inhibitor segment of the heat stable protein kinase inhibitor (Scott *et al.* 1985*a*; Cheng *et al.* 1986; Walsh *et al.* 1990). The second structure was a ternary complex that contained ATP as well as PKI(5-24) (Zheng *et al.* 1993*a*,*b*). The binary complex described the general folding of the polypeptide chain, localized the active site at a cleft between two lobes, and defined the features that were important for peptide recognition. The ternary complex, on the other hand, defines a role for most of the conserved residues and provides a better basis for understanding the mechanism of phosphotransfer. The binary structure has been refined now to a resolution of 2.0 Å† due to improved purification methods and to the inclusion of detergent in the crystallization solution (Knighton *et al.* 1993*a*). The ternary complex has been refined to 2.2 Å (Zheng *et al.* 1993*b*).‡

† 1 Å = 10^{-10} m = 10^{-1} nm.

‡ The coordinates for the binary and ternary complexes are available from the Brookhaven Protein Data Bank as 1APM and 1ATP, respectively.

2. RESULTS AND DISCUSSION

(*a*) *General protein kinase structure*

The general folding of the C-subunit polypeptide chain is shown in figure 1*a*. The smaller lobe contains mostly residues from the amino-terminus and is dominated by an antiparallel β-sheet. The A-helix near the amino-terminus covers the surface of both lobes. The larger lobe consists mostly of the C-terminal portion of the polypeptide chain and is dominated by α-helices. The last 50 residues wrap as an extended chain around the surface of both lobes. Catalysis occurs at the cleft between the lobes.

The inhibitor peptide, shown in table 1, consists of two segments. The consensus region (P − 3 to P + 1) is shared in general by all substrates and inhibitors of the enzyme. This consensus region is shaded in figure 1*a*. It forms an extended chain that lies along the cleft on the surface of the large lobe. The unique high-affinity binding of this peptide is attributed to the N-terminal segment with the P − 11 Phe and P − 6 Arg being particularly important (Cheng *et al.* 1986; Walsh *et al.* 1990; Knighton *et al.* 1991*b*). This region forms an amphipathic helix that binds to a hydrophobic patch on the surface of the large lobe.

(*b*) *Location of conserved residues*

The conserved core, shared by all members of the protein kinase family, extends from residues 40 through 300. The general location of conserved residues in the catalytic core relative to the overall folding and ATP binding is shown in figure 1*b*. Most of the conserved residues lie at the active site in the cleft and are associated with ATP binding or catalysis. These residues are also summarized in table 2. Two exceptions are Glu208 and Arg280 that form a buried ion pair in the large lobe. In general, the conserved features in the small lobe serve to anchor the ATP, leaving the γ-phosphate poised for transfer. In contrast, most of the conserved residues in the small lobe appear to be important for phosphotransfer and in general lie in loops connecting the β-strands (Zheng *et al.* 1993*a*).

The nucleotide binding fold found in the protein kinases is unique (Knighton *et al.* 1991*a*). Although there are some common features such as a glycine-rich loop that helps to anchor the non-transferable phosphates, the general folding of the motif, the location of conserved residues throughout that motif, and the orientation of the nucleotide relative to the overall folding is quite distinct from the motifs found in the dehydrogenases (Rossmann *et al.* 1974), in p21 ras (Saraste *et al.* 1990), and in HSP70 (Flaherty *et al.* 1990). The protein kinase nucleotide fold is dominated by an antiparallel β-sheet with the only helical segment lying between β-strands three and four. Three

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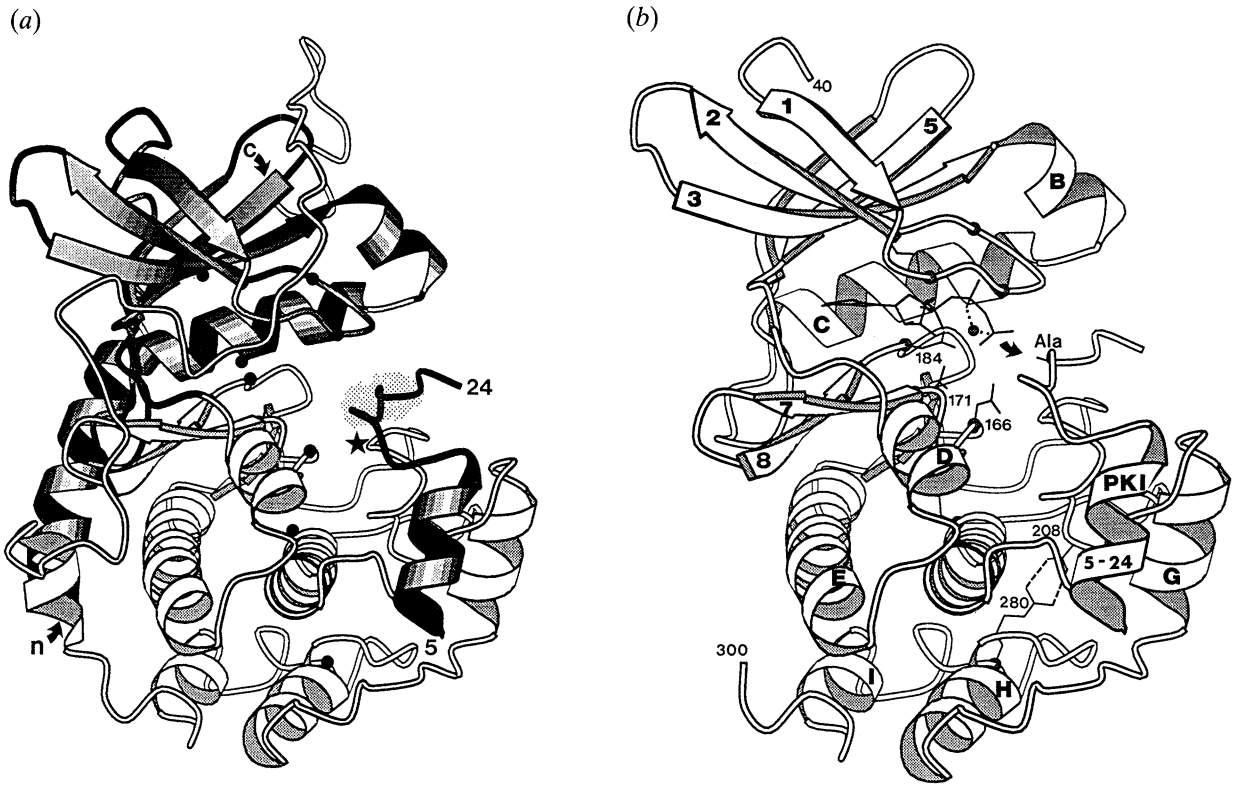

Figure 1. Ribbon diagrams of the C-subunit of cAMP-dependent protein kinase. (*a*) The binary complex of the C-subunit and PKI (5-24). Residues 15–126 are shaded and constitute most of the small lobe. The inhibitor peptide is also shaded. The N-terminus (n) and C-terminus (c) of the C-subunit are indicated with arrows and the termini of the PKI peptide are designated as 5 and 24. (*b*) The core region only of the ternary complex containing C-subunit, PKI(5-24), and MgATP. The conserved core consists of residues 40 through 300 as indicated. Conserved residues scattered throughout the core are indicated by dots (Gly50, Glu52, Gly55, Lys72, Glu91, Asp166, Asn171, Asp184, Glu208, Asp220, and Arg280). Several of the side chains of those residues are indicated. The salt bridge between Arg280 and Glu208 is indicated by dashed lines. The numbering and lettering of the β-strands and helices is based on Knighton *et al.* (1991*a*). Asp166, near the site of phosphotransfer, is positioned to serve as a catalytic base. The Ala side chain at the P-site in the inhibitor is also indicated with an arrow indicating where phosphotransfer would take place if a Ser was located at this P-site. The dotted line bridging the β and γ-phosphates indicates the position of the activating Mg^{2+} ion.

Table 1. *Sequence of PKI(5-24) and the homologous regions from the R-subunits of cAPK*

(The inhibitor peptide that was co-crystallized with the C-subunit, PKI(5-24) is shown at the top. Beneath it is indicated the P-site nomenclature that has been adopted for substrates and inhibitors of the protein kinases. The consensus site and the high-affinity binding site are also indicated. The sequences of the consensus site and the flanking regions of the R-subunits is also shown as well as a commonly used heptapeptide substrate and inhibitor. The sequences for these proteins were determined as follows: bovine R^I (Lee *et al.* 1983; Clegg *et al.* 1988); bovine R^{II} (Jahnsen *et al.* 1986; Scott, *et al.* 1987); PKI (Walsh *et al.* 1971; Scott *et al.* 1985*b*; Van Patten *et al.* 1991). The kinetic data were determined as follows: PKI(5-24) (Scott *et al.* 1985*a*; Cheng *et al.* 1986); R-subunits (Hofmann 1980); Kemptide (Kemp *et al.* 1977); PKI (Whitehouse & Walsh 1982).)

high-affinity site / consensus site		
T T Y A D F I A S G R T G R R N A I H D	PKI (5-24):	$K_i = 2.3$ nM
-11 -6 -3-2 P 1		
5 10 ↓ 25		
T T Y A D F I A S G R T G R R N A I H D I L V S S A S G N S	PKI	$K_i = 0.2$ nM
L R R A S L G	Kemptide:	$K_m = 16$ μM
L R R A A L G	Ala-Kemptide:	$K_i = 200$ μM
90 105		
S P P P P N P V V K G R R R R G A I S A E V Y T E E D A A S	RI-Subunit:	app$K = 0.2$–0.3 nM
E E D L D V P I P G R F D R R V S V C A E T Y N P D E E E E	RII-Subunit:	app$K = 0.2$–0.3 nM
90 ↑ 100		
P		

Table 2. *Conserved sequence motifs at the active site*
(Highly conserved residues are shown in bold.)

motif	location	residue	function
	small lobe		
glycine-rich loop	between β-strands 1 and 2	Gly50	
		Thr51	
		Gly52	
		Scr53	α-NH H-bonds to β-PO_4
		Phe54	
		Gly55	
		Arg56	
		Val57	side chain lies over ribose ring
phosphate anchor	β-strand 3	Lys72	ion-pairs to α-phosphate and Glu91
(non-loop)	C-helix	Glu91	ion-pairs with Lys72
	large lobe		
catalytic loop	between β-strands 6 and 7	Arg165	ion pairs with (P)Thr197
		Asp166	catalytic base
		Leu167	
		Lys168	ion pairs with γ-PO_4
		Pro169	
		Glu170	ion pairs with P-2 Arg
		Asn171	ligand to inhibitory Mg^{2+} H bonds to α-carbonyl of Glu166
metal binding loop	between β-strands 8 and 9	Asp184	ligands to 1° and 2° Mg^{2+} ions
		Phe185	
		Gly186	
		Phe187	α-C=O H-bonds to ε N of Arg165

conserved sequence motifs are found in this nucleotide binding fold: (i) a glycine-rich loop located between β-strands 1 and 2; (ii) Lys72 in the middle of β-strand 3; and (iii) Glu91 in the middle of the C-helix. The glycine-rich loop anchors the β-phosphate by hydrogen bonding through the backbone amides of Ser53, Phe54, and Gly55. In the ternary complex Lys72 is within hydrogen bonding distance of both the α- and β-phosphates of ATP. Thus both the glycine-rich loop and Lys72 anchor the two phosphates that are not involved in phosphotransfer. Glu91 does not interact directly with the nucleotide but instead forms an ion pair with Lys72 thus helping to position it for its interaction with the phosphates of ATP.

The adenine base lies beneath β-strands 1 and 2 and is quite buried in this structure relative to other nucleotide binding proteins. The C-terminal tail (residues 300–350) that wraps as an extended chain along the surface of both lobes further shields the adenine and ribose rings from solvent. These last 50 residues lie outside the conserved core. The adenine ring lies in a hydrophobic pocket with the N6 nitrogen hydrogen bonding to the backbone carbonyl of Asn121. Val55 in β-strand 2 lies over the ribose ring and provides steric constraints for this site.

In contrast to the small lobe, the conserved residues in the large lobe at the active site are located in flexible loops that lie between four β-strands that form two small ribbons at the surface of the cleft. There are two such loops. Asp184 is in the DFG loop connecting β-strands 8 and 9, and this conserved residue chelates the activating Mg^{2+} ion that bridges the β and γ-phosphate. The other conserved active site residues lie in a loop connecting β-strands 6 and 7, and this loop is referred to as the catalytic loop. This loop is a hub for interacting with many different parts of the molecule. It begins with R165, conserved in most but not all protein kinases. In cAPK, this Arg binds to the essential phosphorylation site, Thr197, that lies on the surface at the edge of the cleft. Asp166 is positioned to serve as a catalytic base. Lys168 interacts with the γ-phosphate in this inhibitor complex and also hydrogen bonds to the backbone carbonyl of the P−2 Arg in the inhibitor peptide. Glu170 participates in recognition of the P−2 Arg in the inhibitor peptide. Asn171 comes close to Asp184 in the binary complex. In the ternary complex in the presence of excess Mg^{2+} Asn171 chelates a second inhibitory Mg^{2+} ion. In both structures Asn171 also fixes the loop by forming a hydrogen bond with the α-carbonyl of Asp166. Figure 2 summarizes the disposition of conserved residues in loops at the active site of the enzyme and shows how these residues relate to the binding of ATP and catalysis.

Three other residues in the large lobe are highly conserved but are removed from the active site. Arg280 and Glu208 form a buried ion-pair as indicated in figure 1*b* and this can be seen in figure 2. In addition, Asp220 at the end of helix E is conserved. This side chain points towards the catalytic loop and appears to stabilize it by forming hydrogen bonds with the backbone amides of Arg165 and Tyr164.

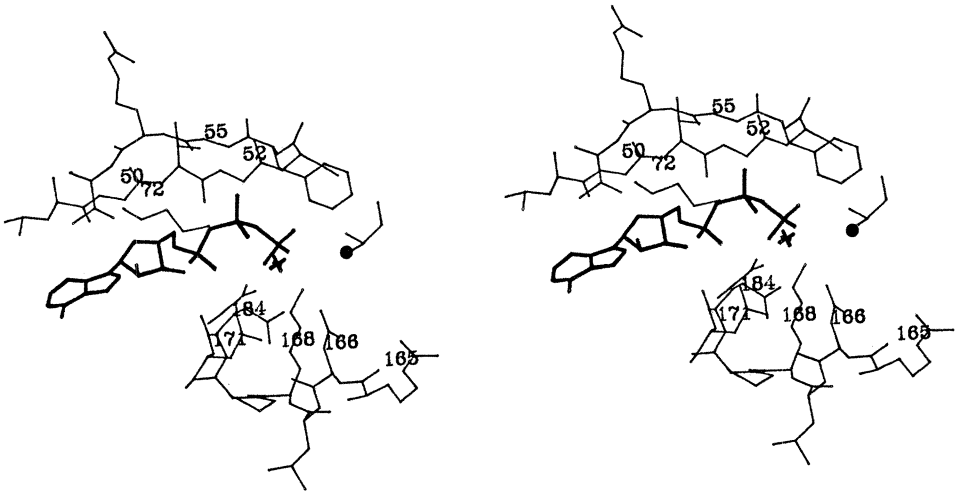

Figure 2. Conserved residues at the active site. Two of the conserved loops are indicated: the glycine-rich loop in the small lobe and the catalytic loop in the large lobe. The activating Mg^{2+} is indicated by a cross and the Cβ atom of the P-site Ala by a solid dot.

(*c*) *Modifications associated with the catalytic subunit*

The C-subunit is an enzyme that catalyses the post-translational modification of other proteins; however, it is also subject to post-translational modifications itself. For example, it was the first protein shown to be myristylated at its N-terminus (Carr *et al.* 1982). In addition, it has at least two sites of phosphorylation (Shoji *et al.* 1979). The crystal structure also allows us to understand how these post-translational modifications interact with the protein and provides a basis for understanding how a particular phosphorylation can be important for structure.

Although farnesylation and palmitylation typically convey membrane-associating properties to the acyl-protein, many myristylated proteins appear to be quite soluble (Towler *et al.* 1988). The C-subunit is one such example. The recombinant C-subunit is not myristylated since *E. coli* does not code for N-myristyl transferase, the enzyme necessary for carrying out this post-translational modification. The kinetic properties of the recombinant C-subunit are basically indistinguishable from the myristylated mammalian enzyme; however, it is more sensitive to thermal denaturation (Slice & Taylor, 1989). When the C-subunit is co-expressed in *E. coli* with yeast N-myristyl transferase, it is fully myristylated, and the thermal stability of this myristylated recombinant enzyme now closely resembles the mammalian enzyme (Yonemoto *et al.* 1992). Hence, in the case of the C-subunit, the N-terminal myristyl moiety appears to convey structural stability. Whether it has additional functional significance is still unclear. It is not required for translocation of the C-subunit to the nucleus, a process that takes place once the C-subunit dissociates from the holoenzyme complex (Meinkoth *et al.* 1990). However, the steps involved in the processing of the mature enzyme are still unclear (Steinberg 1991). Myristylation could play a role in this process.

In the original 2.7 Å structure of the C-subunit, the first 14 residues could not be visualized suggesting that they were not locked into a stable conformation. To improve the resolution of these crystals, two steps were taken. First, Mono S chromatography was used to resolve the protein into three distinct isoforms (Herberg *et al.* 1993). Attempts were also made to improve the quality of the crystals by including detergent, MEGA-8, in the crystallization buffer. These improved crystals diffracted to 2.0 Å (Knighton *et al.* 1993*a*). With these crystals, five additional residues could be visualized at the N-terminus and a molecule of detergent was bound at a hydrophobic site near the N-terminus. The detergent occupied a hydrophobic pocket between the A-helix and the E-helix on the surface of the large lobe. It is quite sequestered from the solvent by its interactions with the protein. Preliminary comparisons with the crystal structure of the mammalian enzyme confirmed that the fatty acid occupies the site that is filled by the detergent in these crystals of the recombinant enzyme (Zheng 1993*c*). This localization of the acyl binding pocket provides an explanation for the enhanced stability of the myristylated enzyme. The general location of the detergent molecule is shown in figure 3.

Unlike many protein kinases such as MAP kinase and cdc2, where phosphorylation serves as a critical switch for turning the enzyme either on or off (Haystead *et al.* 1992), the C-subunit appears to have at least two 'stable' phosphorylation sites. These sites, Thr197 and Ser338, do not turn over, but instead appear to be quite resistant to removal by protein phosphatases (Chiu & Tao 1978; Shoji *et al.* 1979; Toner-Webb *et al.* 1992).

The recombinant C-subunit can be resolved into three distinct isoforms using Mono S chromatography, and these isoforms differ by a single phosphate moiety based on mass spectrometry (Herberg *et al.* 1993). In the non-myristylated recombinant enzyme, there are a maximum of four phosphorylation sites: Ser10, Ser139, Thr197, and Ser338 (Yonemoto *et al.* 1993). Ser338 lies near the C-terminus outside the conserved catalytic core. It is anchored by two interactions. It ion pairs with the side chain of Lys342, and it hydrogen bonds to the backbone amide of Ile339. Thus the phosphorylation of Ser338 seems to tether

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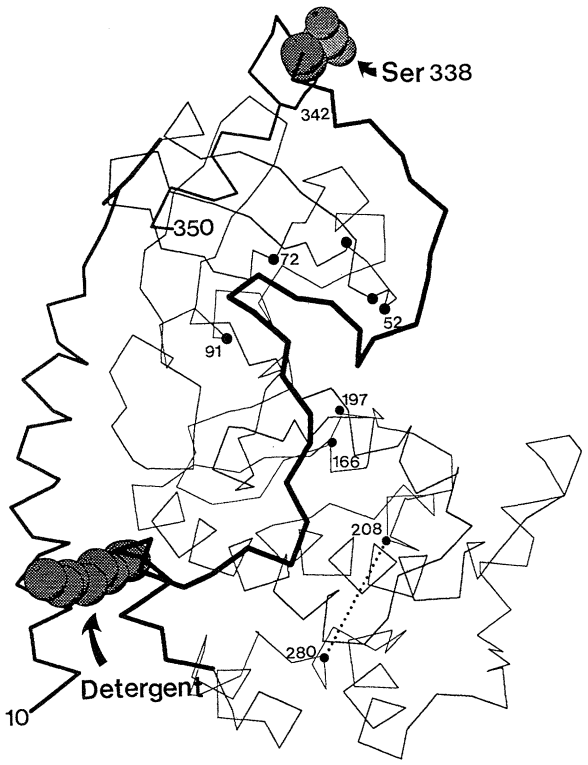

Figure 3. α-Carbon backbone of the C-subunit showing the detergent molecule and P-Ser338. The portions of the polypeptide chain that extend beyond the core (residues 10–39 and 301–350) are indicated in bold. The position of various conserved residues in the core is also indicated. The side chain of Lys342 that interacts with the phosphate of Ser338 is also shown.

the C-terminal end of the protein while acylation stabilizes the N-terminus. Both of these modifications lie outside the conserved catalytic core. Their location relative to the core can be seen in figure 3.

Unlike Ser338, Thr197 is located in the middle of the catalytic core at the cleft, just adjacent to the active site. Its resistance to phosphatases can be explained by multiple interactions with nearby side chains. These interactions are summarized in figure 4. Most of its contacts are with residues in the large lobe: Arg165, Lys189 and Thr195. One interaction is with His87 in the C-helix of the small lobe. Based on preliminary site-directed mutagenesis, we believe that the phosphorylation of Thr197 is essential for kinase function.

These phosphorylations found in the recombinant enzyme are not catalyzed by *E. coli* protein kinases. For example, when mutant forms of the C-subunit, such as Lys72Arg where the essential Lys in the ATP binding site it replaced, are expressed the resulting protein is present at levels comparable to what is found for the native subunit. However, the protein is not phosphorylated (Yonemoto *et al.* 1993). These phosphates, therefore, must be introduced by an autocatalytic mechanism in *E. coli*. Whether this occurs as an intramolecular or intermolecular event is not known. This also does not imply that phosphorylation of the C-subunit in the eukaryotic cell occurs autocatalytically. Some recent evidence suggests that phosphorylation of the C-subunit *in vivo* in eukaryotic cells occurs post-translationally as a terminal step in the assembly of the active enzyme (Steinberg 1991). Other protein kinases such as cdc2, the insulin receptor, and MAP kinase contain phosphorylation sites in a region of the protein that is close to Thr197 in the C-subunit (Tornqvist & Avruch 1988; Ducommun *et al.* 1991; Zhang *et al.* 1991; Haystead *et al.* 1992). Other protein kinases such as protein kinase C have potential sites of phosphorylation in this region. Some of these phosphates turn over as part of the regulatory mechanism. Cdc2, for example, is phosphorylated at Thr161 as part of the activation that follows cyclin binding. Other protein kinases, however, may resemble the C-subunit of cAPK and have stable phosphates that seem to be an integral part of the folded structure. If these phosphorylations are catalyzed by a heterologous protein kinase, it may pose serious problems for expression in *E. coli* where the normal milieu of kinases found in the eukaryotic cell is missing.

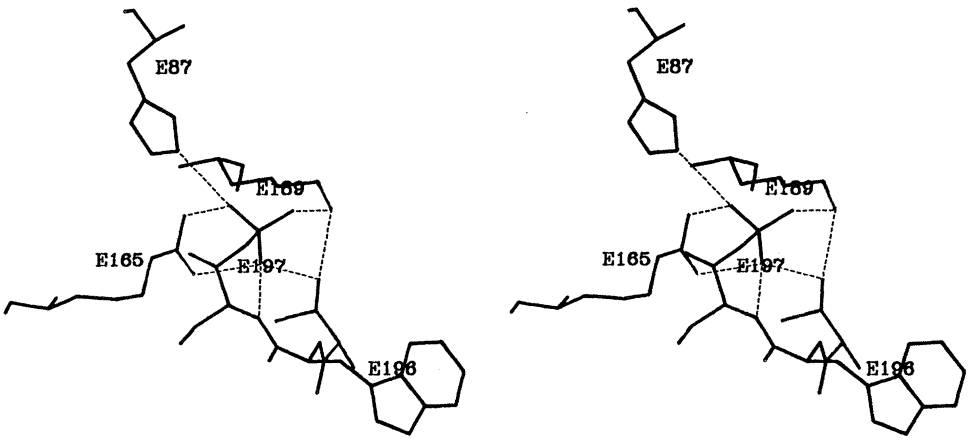

Figure 4. Stereo view of the environment surrounding phospho-Thr197. Thr197 is located near the edge of the cleft on the large lobe (see figure 1). In the ternary complex, the phosphate binds to the three residues in the large lobe, Arg165, Lys189, and Thr195 as well as to His87 from the small lobe. The side chain of Trp196 is also a prominent feature of the enzyme surface that flanks Thr197.

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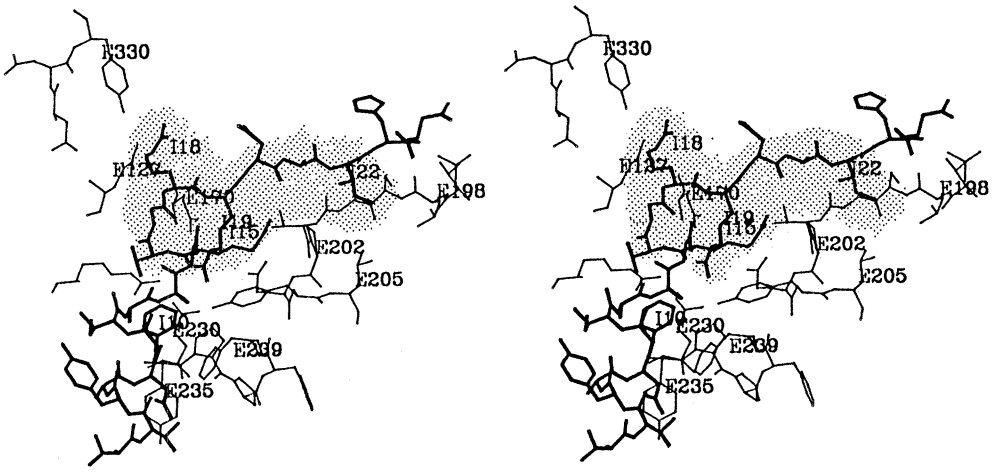

Figure 5. Features of the C-subunit critical for recognition of PKI(5-24). The shaded region shows the region that corresponds to the consensus site, and this will be shared in general by most substrates and inhibitors of PKI. The three glutamates, Glu127, Glu170, and Glu230 that provide recognition sites for the P−3 and P−2 Args are indicated. The loop that extends from residue 198 to 205 provides a hydrophobic pocket for the P+1 Ile and also contains Glu203 that binds the P−6 Arg. The region on the surface that recognizes the amphipathic helix of PKI(5-24) lies N-terminal to the consensus site and this site contributes to the unique high-affinity binding of PKI. The R-subunits will share the consensus site but presumably require recognition sites on the surface of the enzyme that complements regions that are on the C-terminal side of the consensus site near Thr197.

(*d*) *Peptide recognition*

As stated earlier, the C-subunit recognizes peptides having basic residues preceding the phosphorylation site (Zetterqvist *et al.* 1990). The minimum consensus sequence, based on the sequencing of many physiological cAPK phosphorylation sites, consists of an Arg at the P−3 and P−2 positions and a large hydrophobic residue at the P+1 position. The amino acids that contribute to recognition of this consensus sequence are shown in the shaded region of figure 5. Peptides having this minimum consensus sequence have K_ms in the range of 10–20 μM. Additional sequence requirements are necessary to achieve high affinity binding, and high affinity binding is essential for the physiological inhibitors of the C-subunit. Both PKI and the R-subunits, the two classes of known physiological inhibitors of the C-subunit, bind with appK_ds in the subnanomolar range (Hofmann 1980; Whitehouse & Walsh 1983).

In the case of PKI, high affinity binding is attributed primarily to the region that lies N-terminal to the consensus site (table 1). This conclusion was based on extensive analog studies. Glass and Walsh established the importance of the P−11 and Phe and P−6 Arg (Walsh *et al.* 1990), and the crystal structure of the PKI(5-24) binary complex is totally consistent with the analogue results. This region of PKI forms an amphipathic helix that binds to a hydrophobic surface of residues 235–239 (Y-P-P-F-F) with the P−11 Phe complementing this surface (figure 5). The P−6 Arg forms a salt bridge with Glu203.

Although the R-subunits also bind with a high affinity, they appear to have critical recognition sites that are distinct from PKI. As seen in table 1, the R-subunits share the common consensus site found in PKI and in most protein substrates. However, cleavage of the R-subunit after the P−4 residue yields an R-subunit that still binds to the C-subunit with a high affinity indicating that for the R-subunit, the requirements for high affinity binding lie C-terminal to the consensus site (Weldon & Taylor 1985). This is confirmed by genetic analysis of the yeast cAPK. Gibbs & Zoller (1991) used charged-to-alanine scanning mutagenesis to probe the importance of charged residues in the yeast C-subunit, TPK1. In this approach each of the charged residues is selectively changed to alanine. With this family of mutants, Gibbs *et al.* (1992) specifically searched for mutants that were catalytically competent but defective in their capacity to be regulated by the R-subunit, BCY1. Several such mutants were identified, and their location on the surface of the C-subunit was then mapped based on the crystal structure of the mouse C-subunit. One site, Arg133,134 mapped to a region that lies just N-terminal to the heptapeptide substrate that was used for this screen. All of the other mutations, however, mapped to the surface that surrounds the phosphorylation site at Thr197 (Gibbs *et al.* 1992). Mutation of the residues that interact with this phosphate all gave unregulated phenotypes as did Lys213Ala and Lys217Ala. Replacement of Thr241, the equivalent of Thr197 in the mammalian C-subunit, also leads to an unregulated phenotype (Levin *et al.* 1988). Orellana and McKnight independently identified two mutations in the mouse Cα-subunit that have unregulated phenotypes (Orellana & McKnight 1992). One of these, His87Gln, is a residue that binds directly to the phosphoThr in the binary and ternary complex. The other, Trp196Arg, immediately precedes the phosphorylation site and is a dominant feature of the surface surrounding this phosphorylation site (Figure 5).

Therefore, although the specific contact points between the R- and C-subunits that lie beyond the consensus site are still not identified, it is nevertheless

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clear that the R-subunit interacts with a region on the surface of the C-subunit that is different from PKI. Whether this type of flexibility will be a characteristic feature of all protein kinases remains to be established.

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