cAMP-dependent protein kinase: Crystallographic insights into substrate recognition and phosphotransfer


Protein Sci. 1994 3: 176-187

Supplementary data
"Data Supplement"
http://www.proteinscience.org/cgi/content/full/3/2/176/DC1

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cAMP-dependent protein kinase: Crystallographic insights into substrate recognition and phosphotransfer

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Abstract

The crystal structure of ternary and binary substrate complexes of the catalytic subunit of cAMP-dependent protein kinase has been refined at 2.2 and 2.25 Å resolution, respectively. The ternary complex contains ADP and a 20-residue substrate peptide, whereas the binary complex contains the phosphorylated substrate peptide. These structures were refined to crystallographic R-factors of 17.5 and 18.1%, respectively. In the ternary complex, the hydroxyl oxygen OG of the serine at the P-site is 2.7 Å from the OD1 atom of Asp 166. This is the first crystallographic evidence showing the direct interaction of this invariant carboxylate with a peptide substrate, and supports the predicted role of Asp 166 as a catalytic base and as an agent to position the serine -OH for nucleophilic attack. A comparison of the substrate and inhibitor ternary complexes places the hydroxyl oxygen of the serine 2.7 Å from the γ-phosphate of ATP and supports a direct in-line mechanism for phosphotransfer. In the binary complex, the phosphate on the Ser interacts directly with the EN of Lys 168, another conserved residue. In the ternary complex containing ATP and the inhibitor peptide, Lys 168 interacts electrostatically with the γ-phosphate of ATP (Zheng J, Knighton DR, Ten Eyck LF, Karlsson R, Xuong NH, Taylor SS, Sowadski JM, 1993, Biochemistry 32:2154-2161). Thus, Lys 168 remains closely associated with the phosphate in both complexes. A comparison of this binary complex structure with the recently solved structure of the ternary complex containing ATP and inhibitor peptide also reveals that the phosphate atom traverses a distance of about 1.5 Å following nucleophilic attack by serine and transfer to the peptide. No major conformational changes of active site residues are seen when the substrate and product complexes are compared, although the binary complex with the phosphopeptide reveals localized changes in conformation in the region corresponding to the glycine-rich loop. The high B-factors for this loop support the conclusion that this structural motif is a highly mobile segment of the protein.

Keywords: crystal structure; glycine-rich loop; phosphotransfer; protein kinases; substrate recognition

The catalytic (C) subunit of cAMP-dependent protein kinase (cAPK) catalyzes the phosphorylation of proteins having several arginines preceding the site of phosphotransfer (Kemp, 1990; Zetterqvist et al., 1990). The kinetics established that there is a preferred order for substrate binding, with MgATP preceding the peptide substrate (Cook et al., 1982; Whitehouse et al., 1983). Once both substrates bind, catalysis is rapid and the phosphorylated peptide dissociates quickly. The rate-limiting step is the release of ADP (Cook et al., 1982; Adams & Taylor, 1992). The C-subunit of cAPK forms a tight inhibitor complex with two proteins: the regulatory (R) subunit and the heat-stable protein kinase inhibitor (PKI). In the case of PKI, the inhibitor region was localized to a 20-residue sequence near the N-terminus. By characterizing numerous analogs of this peptide, the residues that were important for high-affinity binding were identified (Cheng et al., 1986; Scott et al., 1986; Walsh et al., 1990). Many features of this inhibitor peptide are also shared by substrates of this enzyme. Specifically, a common consensus sequence of

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either Arg-Arg-X-Ser/Thr-Y or Arg-X-Arg-X-Ser/Thr-Y is found in most protein substrates (where Y is a large hydrophobic residue and X is any residue; Zetterqvist et al., 1990).

Two recently solved crystal structures of the C-subunit complex with PKI(5-24) provide not only a template for the folding of the polypeptide chain but also a molecular explanation for inhibition of the C-subunit by PKI. The binary complex containing PKI(5-24) described the overall architecture of the enzyme and the peptide recognition site (Knighton et al., 1991a, 1991b, 1993a). Briefly, it consists of 2 lobes, with the small lobe constituting an ATP binding domain. The large lobe provides the substrate binding site and also contains those residues that are required for phosphotransfer. The structure of a ternary complex containing MgATP as well as PKI(5-24) allowed us to understand more clearly the precise role of all the highly conserved residues that are clustered around the active site in the cleft between the 2 lobes (Zheng et al., 1993a, 1993b,c). These structures are also consistent with the extensive chemical and genetic information that preceded the structure solutions (Taylor et al., 1993).

Although these inhibitor complexes also provide enormous insights to aid our understanding of general peptide and ATP recognition as well as the mechanism of phosphotransfer, this inhibitor complex is distinct from a catalytic complex in several ways. First is the absence of an acceptor site. Instead of a Ser or Thr at the phosphorylation (P) site, PKI(5-24) contains an Ala. Second is the role of MgATP in this inhibitor complex. When MgATP serves as a substrate for small peptides such as kemptide, it has a $K_m$ of approximately 10 $\mu$M, and this is the same as the $K_a$ measured by fluorescence displacement (Bhatnagar et al., 1983) or equilibrium dialysis (Cook et al., 1982; Whitehouse et al., 1983). In this inhibitor complex, however, MgATP binds with a high affinity (30-200 nM), and MgATP is essential for stabilizing the complex (Whitehouse & Walsh, 1983; Van Patten et al., 1991). The high-affinity binding of PKI and MgATP is synergistic. Furthermore, nonhydrolyzable analogs of ATP do not completely mimic ATP in stabilizing the PKI-C-subunit complex. The $K_m$ for ATP in the phosphorylation reaction of the substrate peptide SP20 (see Table 1) is 7.3 $\pm$ 1.7 $\mu$M in pH 7 MES, Tris, CAPS, NACl (MTCN; see Table 1, note b). For this extended substrate, either the binding synergism is disconnected or $K_m$ no longer reflects $K_d$. The latter possibility needs to be tested with further kinetic experiments.

In an effort to understand the molecular differences between an inhibitor complex and a substrate complex, the recombinant C-subunit was co-crystallized with a substrate analog of PKI(5-24) in which Asn 20 at the P - 1 site was replaced with Ala and Ala 21 at the P-site was replaced with Ser. Based on the analog studies of PKI (Walsh, 1990), replacing the P - 1 Asn with Ala should reduce its affinity, and this would potentially facilitate kinetic analysis of the peptide. In addition, the residue at the P - 1 position may influence the relative ATPase activity, as opposed to the peptide transfer reaction (Mendelow et al., 1993). Two complexes were crystallized (see Table 1). In one case, the C-subunit was co-crystallized with the substrate peptide and MgADP. In addition, the C-subunit was co-crystallized with a phosphorylated form of this peptide. A comparison of these 2 structures with the binary and ternary complexes containing the inhibitor peptide provides for the first time crystallographic insights into the mechanism of substrate recognition and phosphotransfer.

### Results

**Description of the ternary complex**

The ternary complex was refined with both X-PLOR (Brünger et al., 1987) and TNT (Tronrud et al., 1987) to reasonable crystallographic R-factors and geometries (Table 2). All of the main chain dihedral angles for the ternary complex with the exception of Glu 13 were within either the allowed or near allowed region of the Ramachandran plot. During TNT refinement, 84 atoms hit the upper limit (99.99 $\AA^2$) of $B$-values. All 168 solvent molecules had $B$-values lower than the upper limit. In this structure, the first 8 amino acids of the enzyme are disordered and not seen. The peptide had well-defined density, with the exception of the last 2 residues, corresponding to the P + 2 and P + 3 sites, which showed some disorder. The ADP had well-defined density except for the $\beta$-phosphate. The B-factor for the $\beta$-phosphorus hit the upper limit (99.99 $\AA^2$) (see Kinemage 1).

The overall structure of this ternary complex is very similar to that of the ternary complex of the mouse Ca-subunit containing the inhibitor peptide (Zheng et al., 1993a, 1993c), with the exception of the P (where Ala is replaced with Ser) and the P - 1 (where Asn is replaced with Ala) sites. Figure 1A, a difference Fourier map of the ternary complex in which the entire

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**Table 1. Inhibitor and substrate peptides for the catalytic subunit**

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequence</th>
<th>$K_m$ (µM)</th>
<th>$K_c$ (nM)</th>
<th>$k_{cat}$ (s⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IP20</td>
<td>TTYADFIASGTRRNASID</td>
<td>2.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SP20</td>
<td>TTYADFIASGTRRASID</td>
<td>&lt;5</td>
<td>1.8 ± 10</td>
<td></td>
</tr>
<tr>
<td>Kemptide</td>
<td>LRRASLG</td>
<td>28 ± 5</td>
<td>22 ± 2</td>
<td></td>
</tr>
</tbody>
</table>

*IP20 corresponds to residues 5–24 in the heat-stable protein kinase inhibitor (Cheng et al., 1986). The altered residues in the substrate peptide, SP20, are shown in bold. For comparison, a small heptapeptide substrate, kemptide, is also shown. The consensus site for all 3 peptides is enclosed in the box. In the crystal structures, SP20 is numbered as 361–380, with 377 correspondence to the P-site Ser.*

*The $K_m$ and $K_c$ were measured in 50 mM MES [2-(N-morpholino)ethanesulfonic acid], 25 mM Tris, 25 mM CAPS [3-(cyclohexylamino)propanesulfonic acid], 50 mM NaCl buffer, pH 8.0, 2 mM ATP, 12 mM MgCl₂.*

* $K_c$ is from Cheng et al. (1986).*
Table 2. Geometries of the final models for both binary and ternary complexes

<table>
<thead>
<tr>
<th>RMS deviation from target values</th>
<th>Binary complex</th>
<th>Ternary complex</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bonds (Å)</td>
<td>0.012</td>
<td>0.013</td>
</tr>
<tr>
<td>Angles (deg)</td>
<td>2.295</td>
<td>2.463</td>
</tr>
<tr>
<td>Planarity (Å)</td>
<td>0.009</td>
<td>0.009</td>
</tr>
</tbody>
</table>

substrate peptide and ADP were omitted, shows well-defined density for the substrate peptide, including the Ser hydroxyl at the P-site. Figure 2A shows the network of hydrogen bonding that exists between the substrate peptide and numerous conserved residues at the active site. In this ternary complex, the hydroxyl group of the P-site Ser is 2.7 Å from the carboxylate of Asp 166, thus providing direct confirmation that this conserved Asp is functioning as a catalytic base to promote nucleophilic attack. The Ser side chain also comes close enough (2.8 Å) to hydrogen bond to its own backbone amide. Lys 168, another conserved residue at the active site, also stabilizes binding of the peptide and is important for anchoring the peptide to the surface of the large lobe. Two prominent interactions are hydrogen bonding to the P – 2 backbone carbonyl and hydrogen bonding to the side chain hydroxyl of Thr 201. This Thr is conserved as either Ser or Thr in all serine-specific protein kinases, although it is not conserved in the protein tyrosine kinases. In this complex, when ADP, not ATP, is present, Lys 168 is also close (3.0 Å) to the side chain oxygen of the P-site Ser. The density for the β-phosphate is weak, so its specific interactions with the protein are unclear. The positions of the adenine ring and the α-phosphate are, however, unambiguous.

Description of binary complex

As seen in Table 2, the binary complex also refined to a reasonable crystallographic R-factor with good geometry. With the ex-
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Fig. 2. Stereo views of the active site in the substrate and product complexes. Important hydrogen bonding distances and electrostatic interactions are indicated by dashed lines. The actual distances are given in Table 3. A: Active site region of the ternary complex containing ADP and SP20. B: Active site residues in the product binary complex.

Fig. 3. Stereo view of the superimposed α-carbon backbones of the binary complex with the product peptide (thick line) and the ternary complex containing MnATP and IP20 (thin line). The peptide is shown in red.

ception of Asp 286, all residues were within or near the allowed regions of the Ramachandran plot. During refinement, 109 atoms hit the upper limit (99.99 Å²) for B-values, and most of these were surface side chains. In the binary complex, the first 11 residues at the N-terminus were disordered and thus are not included in the model (see Kinemage 2).

As seen in Figure 1B, when the P - 1, P, and P + 1 sites are omitted, a difference Fourier map of the binary complex shows good density for these 3 residues. Overall, as seen in Figure 3, the structure of this binary complex is very similar to what was seen for the inhibitor binary complex with the mouse Ca-subunit (Knighton et al., 1991a, 1991b, 1993a). There are, however, some specific differences in the active site region. These differences are in the immediate region surrounding the P-site phosphate and in the glycine-rich loop between β-strands 1 and 2 in the small lobe. The phosphate is anchored in this complex by
ion pairing with Lys 168. The distance between these 2 residues is 3.0 Å. The phosphate also is close enough (2.8 Å) to hydro- 
gen bond to its own backbone amide. Surprisingly, the side chain 
of Asp 166 is also only 2.4 Å from the phosphate. Lys 168 still 
makes additional hydrogen bonding contacts with the P - 2 
α-carbonyl and with the side chain of Thr 201.

In contrast to the ternary substrate complex and the 2 previ-
sely discussed inhibitor complexes of the recombinant enzyme, 
the glycine-rich loop is clearly displaced away from the active 
site cleft in this binary complex. When the α-carbon backbone 
of the inhibitor complex is compared to that of the binary com-
plex (Fig. 3), the glycine-rich loop is the only region where sig-
ificant differences are seen.

Comparison with other structures

Although ideally one would like to look at a complex that re-
sembles a transition state intermediate, these 2 complexes, one 
containing a substrate and the other a product, do shed some 
light on the process of phosphate transfer. A specific com-
parison of each of these complexes with the ternary complex of 
the enzyme with the inhibitor peptide and MnATP allows us to 
delineate the beginning and end stages of the reaction and to search 
for potential conformational changes that might occur during 
catalysis. In order to compare these structures, both complexes 
were superimposed on the inhibitor ternary structure of the 
C-subunit, PKI(5-24), and MnATP using the Ca atoms of resi-
dues 150-250 of the larger lobe with the INSIGHT program (Bi-
osym). This method of superposition involving the residues of 
only the larger lobe is intended to highlight relative displace-
ments of the 2 lobes and is consistent with movements seen in 
previously described “open” and “closed” conformational states 
(Karlsson et al., 1993; Zheng et al., 1993b). The RMS difference 
in Ca atoms of the C-subunit, residues 15-350, and the peptide 
(residues 361-378) of the substrate ternary complex and the tern-
ary complex of the C-subunit with MnATP and inhibitor pep-
tide is 0.43 Å. The RMS difference in Ca atoms of the product 
substrate complex and inhibitor ternary complex is 0.48 Å.

In Figure 4A, the active site of the refined ternary complex 
of the C-subunit, MnATP, and PKI is compared with that of the 
ternary substrate complex. For reference, the hydrogen 
bonding network between active site residues, Ala at the P-site, 
and ATP in the inhibitor complex is shown in Figure 4B. Dis-
tances between active site residues in the different complexes are 
also summarized in Table 3. The first observation from the su-
perimposition of these 2 structures is that there are no signif-
ificant changes in the positions of key residues at the active site 
or are there any changes in the backbone of the peptide. The 
backbone and the Cα of the 2 residues that differ in the sub-
strate peptide, the P - 1 Ala and the P-site Ser, are superim-
posable with the backbone and β-carbons of the corresponding Asn 
and Ala in the inhibitor peptide. This comparison of the 2 struc-
tures thus places the Ser -OH 2.7 Å from the γ-phosphate of 
ATP, ideally positioned for an in-line transfer of the phosphate. 
This comparison also shows that the side chain of Lys 168 can 
readily interact with the γ-phosphate of ATP without having to 
undergo any major conformational change. There is a slight 
displacement of the glycine-rich loop, but because of the high 
B-factors for the ternary substrate complex, the significance of 
this difference cannot be evaluated.

When the inhibitor complex is compared in a similar way to 
the product binary complex (Fig. 4C), once again one can see 
that there are no major changes in the active site residues that 
flank the site of phosphotransfer. This indicates that there are 
no major changes in the positions of the active site residues be-
fore and after the transfer of the phosphate. The position of the 
γ-phosphate of ATP in the inhibitor complex has moved slightly 
(1.5 Å) from its position in the phosphopeptide complex, but 
the relationship of this phosphate to the side chain of Lys 168 
is essentially unchanged in these 2 structures. The major change 
when these 2 structures are compared is the movement of the 
glycine-rich loop (Gly 50-Gly 55). In the product complex, the 
loop has rotated away from the phosphopeptide. The exact con-
formation of the loop in the binary complex is not known, since 
the main chain of residues 52-55 has high B-values, indicating 
that this crucial conserved structural motif is rather mobile.

Discussion

Several conserved charged residues at the active site clearly play 
an essential role in catalysis (see Table 4). A schematic diagram 
for the active site and most of the essential interactions is shown 
in Figure 5A, based on the ternary complex with the IP20 in-
hibitor peptide and ATP (Zheng et al., 1993a). The small lobe 
consists of residues that are essential for fixing the nontransfer-
able α- and β-phosphates of ATP. The glycine-rich loop between 
β-strands 1 and 2 fixes the pho-phosphate by hydrogen bonding 
to several backbone amides. Lys 72 forms a salt bridge with the 
α- and β-phosphates, thereby fixing their position and also help-
ing to neutralize the charge on the phosphates. Glu 91 positions 
Lys 72 so it is poised to interact with the phosphates. Several 
residues in the large lobe are essential for phosphotransfer. 
Asp 184 binds to the activating Mg2+ ion that bridges the 
β- and γ-phosphates. The 2 remaining conserved charged resi-
dues at the active site are Asp 166 and Lys 168. Schematic rep-
resentations of the interaction of these 2 residues with serine and 
phosphoserine at the P-site are shown in Figure 5B and C, re-
spectively. A comparison of the ternary inhibitor complex with 
the 2 substrate complexes described here allows us to better un-
derstand what roles these various active site residues play in ca-
talyis. Distances between some of the key residues in these 
complexes are summarized in Table 3. In particular, we can be-
gin to understand the specific role of Asp 166 and Lys 168 in 
the phosphotransfer process.

Role of Asp 166

Asp 166, conserved in all protein kinases, is the nearest ioniz-
able residue to the hydroxyl group of the substrate. In the in-
hibitor ternary complex, Asp 166 is less than 4.0 Å from the 
substrate group of the P-site Ala (Zheng et al., 1993c) and, on this 
basis, was predicted to be a catalytic base. In the substrate ter-
nary complex described here, Asp 166 is only 2.7 Å from the serine 
-OH. This proximity implies a key role for this carboxyl side 
chain in catalysis.

The existence of a catalytic base was predicted prior to the 
structure solution from the pH dependency studies of Yoon and 
Cool (1987), although recent pH dependency studies have es-
established that the ionizable group with a pKα of 6.1 does not
correspond to Asp166 but rather to another carboxyl group associated with recognition of the P - 2 Arg (Adams & Taylor, 1993). This clarification of the steady-state kinetic parameters does not preclude a general base role for this residue. Asp166 is clearly within hydrogen bonding distance of the serine -OH. However, the structure alone cannot distinguish whether Asp166 actually removes the proton to enhance the nucleophilic character of the substrate, thus functioning as a general base catalyst, or whether Asp166 simply isolates a productive rotamer of the side chain via hydrogen bonding.

In the yeast C-subunit, replacement of the equivalent of Asp166 with Ala reduced the $V_{\text{max}}$ approximately 300-fold without greatly affecting the $K_m$’s for either ATP or kemptide (Gibbs & Zoller, 1991). This result suggests that Asp166 is important for catalytic efficiency and not for overall ligand affinity. The fact that adenosine has a $K_p$ of 30 $\mu$M as opposed to 10 $\mu$M for ATP also confirms that the energy for binding the nucleotide does not come from the conserved residues that are clustered around the active site. Replacement of the Lys 72 equivalent with Ala in the yeast enzyme leads to an 800-fold decrease in $V_{\text{max}}$ with only a 5-fold increase in $K_m$(ATP). Lys 72 is another invariant residue that interacts with only the nontransferable phosphates of ATP and cannot directly influence phosphotransfer, provided ADP is an adequate leaving group. The comparable effects of these 2 active site residues on catalysis suggest that carboxyl-assisted proton transfer does not play a larger
Table 3. Comparison of distances between key active site residues in the various complexes

<table>
<thead>
<tr>
<th></th>
<th>Product/substrate complexes</th>
<th>Inhibitor complexes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Binary</td>
<td>Ternary</td>
</tr>
<tr>
<td>Asp 166 to substrate (P-site)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ala CH3 in PKI(5-24)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ser -OH in substrate</td>
<td>2.7</td>
<td></td>
</tr>
<tr>
<td>P-Ser</td>
<td>&lt;3.0</td>
<td></td>
</tr>
<tr>
<td>Lys 168 to phosphate oxygen</td>
<td></td>
<td></td>
</tr>
<tr>
<td>To Thr 201</td>
<td>3.1</td>
<td>2.6</td>
</tr>
<tr>
<td>To P = 2 a-carbonyl</td>
<td>2.9</td>
<td>2.9</td>
</tr>
<tr>
<td>Lys 72 to a-phosphate</td>
<td></td>
<td></td>
</tr>
<tr>
<td>To Glu 91 (OE1)</td>
<td>2.9</td>
<td>2.6</td>
</tr>
<tr>
<td>To Asp 184 (OD1)</td>
<td>3.4</td>
<td>3.3</td>
</tr>
<tr>
<td>Arg 165 to P-Thr 197</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(NH1) - (OE1)</td>
<td>2.9</td>
<td>2.7</td>
</tr>
<tr>
<td>(NH2) - (OE2)</td>
<td>2.9</td>
<td>2.7</td>
</tr>
</tbody>
</table>

Role in transition state stabilization than do positioning effects of the phosphate donor.

Although Asp 166 is certainly essential for phosphotransfer, it may assume other functions. The phosphorylation of kemptide (LRRASLG) occurs rapidly, with the release of ADP being rate-limiting (Kong & Cook, 1988; Adams & Taylor, 1992). The dissociation rate constant of the phosphorylated peptide is presumed to be fast, because its measured Kd exceeds that for ADP by more than 2 orders of magnitude (Whitehouse et al., 1983) and kcat is mostly insensitive to changes in the short peptide structure (Järn & Ragnarsson, 1991). Furthermore, the dissociation constant of the phosphorylated peptide exceeds that for the nonphosphorylated substrate by more than an order of magnitude, suggesting that the phosphate group is destabilized by at least 2 kcal/mol at the active site. The proximity of the carbonyl of Asp 166 and the phosphate group may provide a mechanism for destabilization so that kcat is not limited by the nature of the substrate. In this regard, Asp 166 may serve to both facilitate chemical transfer and raise the ground state energy of the product complex.

Role of Lys 168

Lys 168 also plays a key role at the active site (Bossemeyer et al., 1993; Zheng et al., 1993c). It is conserved in all Ser/Thr kinases and is replaced by an Arg in members of the protein tyrosine kinases (Hanks & Quinn, 1991; Knighton et al., 1993b). This is perhaps the most critical residue at the active site because it has the potential to interact with components of both the small and large lobes. In the inhibitor complex, it binds to the γ-phosphate of ATP. Therefore, in the ternary complex, it can help to pull the equilibrium in the direction of the closed conformation by its contact with the γ-phosphate. Once transfer occurred, however, Lys 168 would no longer make any contact with components from the small lobe. In the phosphopeptide binary complex, Lys 168 continues to bind to the phosphate, although now the phosphate is part of the peptide that is firmly anchored in the large lobe.

Both before and after transfer of the phosphate, Lys 168 also helps to anchor the residue to the surface of the large lobe by hydrogen bonding the a-carbonyl of the P = 2 residue in the peptide and by hydrogen bonding to the side chain hydroxyl group of Thr 201. The fact that replacement of Lys 168 with Ala has a 300-fold effect on the Kcat (pep) emphasizes the importance of this residue for substrate binding and probably reflects the importance of its interaction with the a-carbonyl of the residue preceding the P-site Ser and also with Thr 201. The kcat and Km values for Lys 168 Ala and wild-type yeast C-subunits are 0.345 s⁻¹ and 2.149 μM and 16.9 s⁻¹ and 67 μM (Gibbs & Zoller, 1991). In all cases, the side chain of Lys 168 hydrogen bond to the hydroxyl of Thr 201 and, thus, bridges the interactions of the peptide with the large lobe. Thr 201 is conserved as either a Ser or Thr in all of the protein kinases that are specific for Ser/Thr-containing substrates. In the tyrosine-specific

Table 4. Conserved residues at the active site and their role in catalysis

<table>
<thead>
<tr>
<th>Conserved residue</th>
<th>Role</th>
</tr>
</thead>
<tbody>
<tr>
<td>Small lobe</td>
<td></td>
</tr>
<tr>
<td>Gly 50-Thr-Gly Ser-Phe-Gly (glycine-rich loop)</td>
<td>Anchors β-phosphate of ATP by hydrogen bonding to backbone amides of Phe 54 and Gly 55</td>
</tr>
<tr>
<td>Lys 72</td>
<td>Anchors ATP and neutralizes charges by electrostatic interactions with α- and β-phosphate</td>
</tr>
<tr>
<td>Glu 91</td>
<td>Positions Lys 72 by electrostatic interaction</td>
</tr>
<tr>
<td>Large lobe</td>
<td></td>
</tr>
<tr>
<td>Arg 165</td>
<td>Hydrogen bonds to phosphorylated Thr 197</td>
</tr>
<tr>
<td>Asp 166</td>
<td>Hydrogen bonds to hydroxyl group of serine at P-site</td>
</tr>
<tr>
<td>Lys 168</td>
<td>Base catalyst and/or positions –OH for nucleophilic attack</td>
</tr>
<tr>
<td></td>
<td>Binds to phosphate before and after transfer</td>
</tr>
<tr>
<td>Asn 171</td>
<td>Stabilizes peptide binding to large lobe</td>
</tr>
<tr>
<td>Asp 184</td>
<td>Hydrogen bonds to the α-carbonyl of Asp 166</td>
</tr>
<tr>
<td></td>
<td>Stabilizes catalytic loop by hydrogen bonding to the α-carbonyl of Asp 166</td>
</tr>
<tr>
<td></td>
<td>Electrostatic interaction with activating Mg2+ (Zheng et al., 1993a) that bridges the β- and γ-phosphate. Also bridges inhibitory Mn2+ (Zheng et al., 1993c)</td>
</tr>
</tbody>
</table>
Crystallographic insights into phosphotransfer in cAPK

A

![Diagram of essential residues that contribute to nucleotide binding and catalysis. A: Inhibitor ternary complex. Distances are taken from the ternary complex of C:IP20:ATP (Zheng et al., 1993c). The crystals were soaked in Mn2+, and both the inhibitor and the activating metals are shown (++). The activating metal bridges the β- and γ-phosphates, whereas the inhibitory metal bridges the α- and γ-phosphates. The arrow bridges the methyl side chain of the P-site Ala and the γ-phosphate of ATP. B: Substrate ternary complex. C: Phosphorylated substrate binary complex.](image)

B

C

Fig. 5. Diagram of essential residues that contribute to nucleotide binding and catalysis. A: Inhibitor ternary complex. Distances are taken from the ternary complex of C:IP20:ATP (Zheng et al., 1993c). The crystals were soaked in Mn2+, and both the inhibitor and the activating metals are shown (++). The activating metal bridges the β- and γ-phosphates, whereas the inhibitory metal bridges the α- and γ-phosphates. The arrow bridges the methyl side chain of the P-site Ala and the γ-phosphate of ATP. B: Substrate ternary complex. C: Phosphorylated substrate binary complex.

protein kinases, this residue is replaced by Pro. We predict, therefore, that Lys 168 will bridge the phosphate to the protein via an -OH at position 201 in all of the Ser/Thr-specific protein kinases. Only in one case, the dual specificity protein kinase weel, is the equivalent of Thr 201 replaced by an Asp (Takeya et al., 1982; Hanks & Quinn, 1991).

Mechanism of catalysis

Kinetic experiments predict the general, overall mechanism for catalysis shown in Figure 6. According to this preferred ordered scheme for substrate binding, MgATP binding typically precedes peptide binding (Cook, 1982; Whitehouse & Walsh, 1983). The chemical reaction is fast, as is the release of the peptide when a small heptapeptide substrate is used. The rate-limiting step is the release of ADP (Cook et al., 1982; Adams & Taylor, 1992). Based on the crystal structures of the various inhibitor, substrate, and product complexes, it is now possible to predict with reasonable precision the geometry of the substrate:ATP complex and of the product complex. When the substrate:ADP complex is compared with the inhibitor:ATP complex, the distance of the Ser -OH to the γ-phosphate of ATP is 2.7 Å. On the basis of this comparison, the active substrate complex prior to the phosphate transfer shown in Figure 7A can be predicted. This
geometry is consistent with the direct, in-line transfer mechanisms predicted by Ho et al. (1988).

The interactions of the phosphopeptide with active site residues following transfer of the phosphate are shown in Figure 7B. Although the release of phosphopeptide is typically fast when a small substrate is used, the situation with the tight binding SP20 substrate peptide is different. We presume that the release of the phosphorylated SP20 peptide limits $k_{cat}$ since this parameter is diffusion controlled. In a separate experiment, $k_{cat}$ varied with the relative viscosity of buffer in a manner predicted by the Stokes–Einstein relationship (unpubl. data). Because the measured $k_{cat}$ for SP20 phosphorylation is 10-fold lower than the $k_{cat}$ for kemptide phosphorylation, and this parameter is limited by ADP release, the rate constant for phosphorylated SP20 dissociation must control $k_{cat}$. A mechanism that incorporates the initial loss of peptide prior to ADP release would give a higher $k_{cat}$. In this case, release of the phosphopeptide is rate-limiting, thus enabling the enzyme:product complex to be captured in the crystal as a stable intermediate. It is possible that the stable, trapped intermediate is favored by crystal contacts in the orthorhombic space group. In a true enzyme:ADP:phosphopeptide complex, one would predict that the fast off-rate of the phosphopeptide is due in part to charge repulsion between Asp 166 and the phosphopeptide as well as the enhanced charge repulsion between the $\beta$-phosphate of ADP and the phosphopeptide. This charge repulsion may also contribute in part to the rotation of the glycine-rich loop away from the phosphopeptide, which results in an opening of the cleft.

**Flexibility of the glycine-rich loop**

The various conformational states of the small lobe and, in particular, of the glycine-rich loop in the different crystal forms of the C-subunit are compared in Figure 8 and Kinemage 3. For this comparison, the large lobes were superimposed. Because all significant differences were localized to the small lobe, only this lobe is shown. The open conformation seen in the cubic crystals of the mammalian enzyme reveals a rigid motion of the small lobe that results in a shift of the entire $\beta$-sheet relative to the closed conformation seen in all of the ternary complexes (Karlsson et al., 1993; Zheng et al., 1993b). The binary complex with the phosphopeptide shown here reveals a partial movement of only the glycine-rich loop. The remainder of the small lobe does not move. The high $B$-factors associated with residues 52–55 indicate that this loop has a high degree of flexibility. Comparison of the $B$-factors for residues 52–55 indicates that the

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**Fig. 6.** Reaction pathway for catalysis. The kinetic parameters for this pathway were obtained using a heptapeptide substrate, LRRASLG (kemptide) (Kemp et al., 1977).

**Fig. 7.** Predicted substrate and product complexes. On the left is the predicted substrate complex prior to phosphate transfer, with the mechanism of nucleophilic attack. On the right is the product complex showing the interactions of the phosphopeptide with active site residues prior to its release.
lowest values are observed for the inhibitor ternary complex with MnATP (Zheng et al., 1993c) and the highest for the binary complex containing the phosphorylated peptide. Whether movement of this loop is a prerequisite step to the release of ADP in the normal pathway for the enzyme remains to be established.

Materials and methods

Preparation of substrate peptide

The substrate peptide shown in Table 1 was synthesized at the University of California at San Diego (UCSD) Peptide and Oligonucleotide Core Facility and purified by HPLC. This peptide is an analog of the inhibitor peptide PKI(5-24) from the heat-stable protein kinase inhibitor (Cheng et al., 1986). For simplicity, the inhibitor peptide is designated as IP20 and the substrate peptide as SP20. The peptide was enzymatically phosphorylated by cAPK and separated from the enzyme by HPLC. The reaction mixture (5 mL) contained MOPS (pH 7.0) with 80 mM MgCl2, 3 mM peptide, 15 mM ATP, and 4 U C-subunit. After 1 h, the mixture was applied in 2 batches to a Vydac C18 column (1.0 x 25 cm), and the peptide was eluted with a 10-40% gradient for 60 min (solvent A: 0.1% trifluoroacetic acid [TFA] milli-treated [MQ] water; solvent B: 0.8% TFA in acetonitrile:water [95:5]). In the crystal structure, the peptide is designated as residues 361-380, with 377 corresponding to the P-site Ser.

Protein purification

The mouse Cα-subunit of cAPK was expressed in Escherichia coli and purified as described by Herberg et al. (1993). Isoform II was used for crystallization.

C-subunit crystallization

The C-subunit was concentrated to 10 mg/mL in N,N-bis(2-hydroxy ethyl)glycine (bicine) buffer at pH 8.0 and crystallized using the hanging-drop vapor-diffusion method as described previously (Zheng et al., 1992), but with 0.4% (w/v) of the detergent MEGA-8 (octanoyl N-methyl glucamide) present in the hanging drop (Knighton et al., 1993a). To obtain ternary complex crystals, the Cα-subunit was mixed with the substrate peptide and magnesium ADP using molar ratios of 1:3:5:20 of protein:peptide:Mg2+:ADP. Binary crystals were grown from a solution containing the substrate peptide and C-subunit in a molar ratio of 3:1. Both complexes crystallized in a P21212, space group. The ternary complex diffracted to 2.3 Å and the binary complex to 2.2 Å resolution. The unit cell dimensions of the binary and ternary complexes were: a = 73.87, b = 75.58, c = 80.70 Å and a = 73.96, b = 76.11, c = 81.00 Å, respectively.

Data collection

Diffraction data from both the binary and ternary crystals were collected at 4 °C on a Xupham Hamlin multiwire area detector diffractometer mounted on a Rigaku RU-200 rotating anode operated at 100 mA and 50 kV. Two data sets were processed and reduced using programs developed at UCSD (Howard et al., 1985). Statistics for the 2 data sets are shown in Table 5.

Structure solution and refinement

Both the binary and ternary crystals were isomorphous with the other binary and ternary complexes of the recombinant mouse Cα-subunit (Knighton et al., 1991b, 1993a; Zheng et al., 1993a). The recently solved ternary complex of the recombinant C-subunit, MnATP, and PKI(5-24) was taken as the starting model (Zheng et al., 1993c). The Asn at the P-1 site was replaced by Ala, and ATP and Mn2+ ions were omitted from the model. The temperature factors of all the atoms were set to 17 Å2, and all the occupancies were set to unity.

Table 5. Data collection statistics

<table>
<thead>
<tr>
<th></th>
<th>Resolution</th>
<th>No. of crystals used</th>
<th>Rsyma (%)</th>
<th>No. of reflections of completion (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Binary complex</td>
<td>2.25</td>
<td>1</td>
<td>4.3</td>
<td>19,924 (90%)</td>
</tr>
<tr>
<td>Ternary complex</td>
<td>2.20</td>
<td>2</td>
<td>5.5</td>
<td>21,131 (89%)</td>
</tr>
</tbody>
</table>

\[ R_{sym} = \frac{\sum_{hkl} |I_{obs} - I_{avg}|}{\sum_{hkl} I_{avg}} \]
Refinement of the binary complex

The model was refined with X-PLOR (brünger et al., 1987). In the first step, rigid body refinement for 8.0-2.4-Å-resolution data with F > 2σ lowered the R-factor from 0.42 to 0.31. Further refinement was carried out using simulated annealing (SA) and conjugate gradient (CG) positional refinement. At an R-factor of 2.3. The next step was restrained temperature factor refinement of all residues in the protein and peptide inhibitor. This reduced the R-factor to 0.21. At this stage, 2Fo − Fc and Fo − Fc difference maps were computed and examined with TOM/FRODO (Jones, 1978; Camblin & Horjales, 1987), and from these 2 maps it was possible to model the phosphoserine at the P-site. In addition, several peaks were evident in difference Fourier maps that could be modeled as solvent molecules. From this set of maps, 55 water molecules were included. The model was again subjected to coordinate and restrained B-refinement, which lowered the R-factor to 0.20. At this stage, resolution was extended to 2.3 Å. Fifty-one more waters were added from difference Fourier maps in the next 2 stages. After every stage the model was refined. Examination of these maps showed that, for 29 residues, density was rather weak beyond their Cβ atoms, and all those residues were treated as Ala for subsequent refinements. This X-PLOR refined model had an R-factor of 0.19 for 16,895 reflections between 10.0 and 2.3 Å resolution (F > 2σ), with RMS deviations of 0.022 Å and 3.4° from ideal bond lengths and bond angles, respectively.

To improve the geometry of the model and for a better estimate of the B-values, it was refined using the TNT program (Tronrud et al., 1987) using unrestrained B and bulk solvent correction options for all the data between 30.0 and 2.25 Å resolution. The TNT-refined structure gave an R-factor of 0.181 for 19,924 observed reflections. The final refined model consisted of C-subunit residues 12-350, peptide 1-20, MEGA-8 modeled as n-octane, and 116 water molecules.

Refinement of the ternary complex

The same starting model was used for refinement of the ternary complex. Refinement for this structure was carried out in a manner similar to that for the binary complex, with X-PLOR followed by TNT. ADP and serine at the P-site were modeled during the refinement. In this structure, 29 residues were treated as Ala owing to weak density for their atoms beyond Cβ. This structure refined to a crystallographic R-factor of 0.175 for all the 21,349 observed reflections between 30.0 and 2.20 Å resolution. The final model consisted of C-subunit residues 9-350, peptide 1-20, ADP, MEGA-8 modeled as n-octane, and 168 water molecules.

Acknowledgments

This work was supported by the Lucille P. Markey Charitable Trust, by PHS grants GM 19301 (S.S.T.) and GM 37674 (J.M.S.), by NSF grant DIR 88-22385 (S.S.T. and L.T.E.), and by PHS research fellowship GM 14528-02 (J.A.A.).

We thank the following individuals and resources for their contributions: Gene Hasagawa for help in preparation of the manuscript, D. Appley for preparation of the drawings, the NIH National Resource at UCSD ( RR 01644) and staff members Chris Nielsen and Don Sullivan for data collection facilities, and the San Diego Supercomputer Center for use of the Advanced Scientific Visualization Laboratory and the Cray Y-MP8/864.

Coordinates will be deposited in the Brookhaven Protein Data Bank coincident with publication of this paper.

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Crystallographic insights into phosphotransfer in cAPK


