Crystal structures of the myristylated catalytic subunit of cAMP-dependent protein kinase reveal open and closed conformations

JIANHUA ZHENG,^{1,5} DANIEL R. KNIGHTON,^{1,6} NGUYEN-HUU XUONG,²
SUSAN S. TAYLOR,¹ JANUSZ M. SOWADSKI³, AND LYNN F. TEN EYCK^{1,4}

¹ Department of Chemistry, 0654, ² Departments of Biology, Chemistry, and Physics, 0317, and

³ Department of Medicine, 0654, University of California at San Diego, La Jolla, California 92093

⁴ San Diego Supercomputer Center, P.O. Box 85608, San Diego, California 92186-9784

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Abstract

Three crystal structures, representing two distinct conformational states, of the mammalian catalytic subunit of cAMP-dependent protein kinase were solved using molecular replacement methods starting from the refined structure of the recombinant catalytic subunit ternary complex (Zheng, J., et al., 1993a, *Biochemistry* 32, 2154–2161). These structures correspond to the free apoenzyme, a binary complex with an iodinated inhibitor peptide, and a ternary complex with both ATP and the unmodified inhibitor peptide. The apoenzyme and the binary complex crystallized in an open conformation, whereas the ternary complex crystallized in a closed conformation similar to the ternary complex of the recombinant enzyme. The model of the binary complex, refined at 2.9 Å resolution, shows the conformational changes associated with the open conformation. These can be described by a rotation of the small lobe and a displacement of the C-terminal 30 residues. This rotation of the small lobe alters the cleft interface in the active-site region surrounding the glycine-rich loop and Thr 197, a critical phosphorylation site. In addition to the conformational changes, the myristylation site, absent in the recombinant enzyme, was clearly defined in the binary complex. The myristic acid binds in a deep hydrophobic pocket formed by four segments of the protein that are widely dispersed in the linear sequence. The N-terminal 40 residues that lie outside the conserved catalytic core are anchored by the N-terminal myristylate plus an amphipathic helix that spans both lobes and is capped by Trp 30. Both posttranslational modifications, phosphorylation and myristylation, contribute directly to the stable structure of this enzyme.

Keywords: conformational changes; crystal structure; myristylation site; posttranslational modifications; protein kinases

Protein kinases are enzymes that catalyze the transfer of phosphate from a nucleotide to protein substrates, typically from ATP to Ser, Thr, or Tyr. The cAMP-dependent protein kinase (cAPK) was one of the first protein kinases to be discovered and purified (Walsh et al., 1968). It was also the first protein kinase for which an X-ray crystal structure was determined (Knighton et al., 1991b,c). The crystal structure of this recombinant catalytic (C)

subunit⁷ not only disclosed the general architecture including its unique nucleotide-binding fold, but also defined many structural features of substrate–peptide interaction (Knighton et al., 1991b,c) and the role of many conserved residues that are clustered near the active site (Zheng et al., 1993a). The architecture and C-subunit–nucleotide

Reprint requests to: Lynn F. Ten Eyck, Department of Chemistry, 0654, University of California at San Diego, 9500 Gilman Drive, La Jolla, California 92093-0654.

⁵ Present address: Immunopharmaceutics, Inc., 11011 Via Frontera, San Diego, California 92127.

⁶ Present address: Agouron Pharmaceuticals, Inc., 3565 General Atomics Court, San Diego, California 92121.

⁷ The recombinant C-subunit corresponds to the mouse C α -subunit expressed in *E. coli* (Slice & Taylor, 1989). The mammalian C α -subunit is purified from porcine heart (Nelson & Taylor, 1981). The amino acid sequence of the porcine heart C-subunit differs from the amino acid sequence of mouse recombinant C-subunit at the following positions: Asn(Thr) 32, Ala(Ser) 34, His(Gln) 39, Glu(Asp) 44, Thr(Ser) 65, Phe(Tyr) 69, Tyr(Phe) 108, Pro(Ala) 124, and Ser(Thr) 348, where the sequence of mouse enzyme is indicated in parentheses. In addition, the porcine heart C-subunit, in contrast to the mouse recombinant C-subunit, contains a myristic acid at its N-terminus.

interactions, in general, are conserved throughout the entire protein kinase family (Hanks et al., 1988). Two structures of the mouse recombinant C α -subunit have been solved. In the binary complex, the C-subunit was complexed with a high-affinity 20-residue inhibitor peptide derived from the amino-terminal portion of a naturally occurring heat-stable protein kinase inhibitor (PKI) (Knighton et al., 1991b,c). The subsequent refinement of the ternary complex model containing ATP revealed the detailed interactions between protein, ATP, and metal ions (Zheng et al., 1993a). The binary and ternary crystal structures have been refined at 2.0 Å (Knighton et al., 1993) and 2.2 Å resolution, respectively (Zheng et al., 1993b).⁸ In contrast to the mammalian enzyme, the recombinant enzyme lacks a myristyl moiety at its amino-terminus, and in the binary and ternary crystals the first 9 and 14 residues, respectively, are not visible.

Catalysis requires binding of both MgATP and a protein or peptide substrate. Kinetic evidence suggests a preferred order of substrate binding with ATP preceding the peptide (Cook et al., 1982; Whitehouse et al., 1983). Catalysis is then rapid and follows a direct in-line transfer mechanism (Ho et al., 1988). The rate-limiting step is the release of ADP (Cook et al., 1982; Adams & Taylor, 1992). Several lines of evidence suggest that significant conformational changes are associated with substrate binding. The circular dichroism studies of Reed et al. (1985), for example, indicated not only that changes are associated with peptide binding, but also that the changes in secondary structure differ depending on whether a substrate or an inhibitor peptide is bound. Indications of substrate-induced conformational changes were shown independently using low-angle neutron scattering. Parello et al. (1993) established that the radius of gyration (R_g) is reduced when the C-subunit forms a complex with MgATP and the high-affinity inhibitor peptide derived from PKI. These studies showed, furthermore, that the binding of inhibitor peptide alone, but not ATP alone, was sufficient to cause the reduction in R_g .

Crystal structures of the binary (C:PKI(5-24)) and ternary (C:PKI(5-24):ATP) complexes of the recombinant mouse C α -subunit showed no major conformational differences (Knighton et al., 1993; Zheng et al., 1993a). These structures represent a "closed" conformation of the enzyme, with the inhibitor peptide binding in the cleft between the two lobes. The small lobe participates in nucleotide binding and, in particular, holds the portion of the nucleotide that is not involved in phosphotransfer. The large lobe, on the other hand, provides interaction sites for the inhibitor peptide and also contributes residues that participate in catalysis.

The mammalian porcine C-subunit crystallized in several different forms. The free enzyme yielded cubic crystals, whereas the ternary complex, co-crystallized in the presence of ATP and PKI(5-24), yielded hexagonal crystals (Knighton et al., 1991a; Zheng et al., 1991). A binary complex with an iodinated form of PKI(5-24) also crystallized in a cubic space group. In order to better understand the various conformational states of this enzyme, these cubic and hexagonal crystal structures were solved using molecular replacement methods. A comparison of these three structures, the apoenzyme, the binary complex containing di-iodinated PKI(5-24), and the ternary complex, reveals not only open and closed conformations but also describes the location of the myristyl group that is bound to the N-terminus of the mammalian C-subunit and absent in the recombinant enzyme.

Results

Models of the mammalian catalytic subunit

The best data for the mammalian enzyme crystals were obtained for the binary complex containing di-iodinated PKI(5-24) (Table 1). The mammalian C-subunit consists of residues 1–350, a complete carbon myristylate, a 20-residue inhibitor peptide, two iodine atoms that are covalently bound to Tyr 7 of the inhibitor, and two phosphorylated residues, Ser 338 and Thr 197. The total number of non-hydrogen atoms was 3,010. The very high symmetry space group (24 equivalent positions) and high solvent content (>67%) combined to produce a large number of reflections for this relatively low resolution, but with quite weak data. The overall R_{sym} is 9.7% despite an average of 6.7 measurements per reflection. We believe this to be the primary reason for a relatively poor R -factor of 23.3%.

Crystals were also obtained for the ternary complex of the mammalian C-subunit, even though data were collected only to 3.0 Å. The model for this structure was not exhaustively refined due to a poor observation-to-parameter ratio, but clearly establishes that the conformation is nearly identical with that reported earlier for the recombinant mouse enzyme ternary complex (Table 2).

The free mammalian enzyme, like the binary complex, crystallized in a cubic space group. Despite the low resolution of the data (3.9 Å), a comparison of this model of the apoenzyme with the model of the binary mammalian complex showed that both models adopt very similar conformations (Table 2). Thus, for the mammalian enzyme, two distinct conformational states are observed. The hexagonal crystals obtained for the ternary complex represent a closed conformation, whereas the cubic crystals obtained for the free enzyme and for the binary complex with the iodinated peptide both correspond to a more open conformation.

⁸ The coordinates for these structures are available from the Brookhaven Data Bank as 1APM and 1ATP, respectively, for the 2.0-Å binary and 2.2-Å ternary complexes.

Table 1. Statistics for the structure solutions of the three complexes of the mammalian catalytic subunit of cAMP-dependent protein kinase

	Apo enzyme	Binary complex of the mammalian catalytic subunit with di-iodinated PKI(5-24)	Ternary complex of the mammalian catalytic subunit with MgATP and PKI(5-24)
Diffraction data			
Resolution (Å)	3.9	2.9	3.0
No. of reflections	6,678	20,940	9,934
R_{sym} (%)	10.2	9.7	5.4
Unit cell (Å)	$a = 169.13$	$a = 171.52$	$a = b = 80.2, c = 295.0$
Space group	$p4_132(p4_332)$	$p4_132(p4_332)$	$p6_122(p6_522)$
Rotation function and Patterson correlation (PC) refinement			
Resolution limits (Å)	10–4.0	10–4.0	10–4.0
No. of reflections, $F > 2\sigma$	6,164	7,173	4,791
Rotation solution	$\theta 1 = 54.578^\circ$ $\theta 2 = 60.000^\circ$ $\theta 3 = 354.58^\circ$	$\theta 1 = 54.578^\circ$ $\theta 2 = 60.000^\circ$ $\theta 3 = 354.58^\circ$	$\theta 1 = 357.69^\circ$ $\theta 2 = 87.500^\circ$ $\theta 3 = 327.69^\circ$
Peak heights before PC refinement ^a	4.2746/4.3797 22nd peak	4.5333/4.5135 1st peak	1.7999/1.9000 6th peak
Rotation solution after PC refinement	$\theta 1 = 55.274^\circ$ $\theta 2 = 59.459^\circ$ $\theta 3 = 350.91^\circ$	$\theta 1 = 54.576^\circ$ $\theta 2 = 58.081^\circ$ $\theta 3 = 352.51^\circ$	$\theta 1 = 355.02^\circ$ $\theta 2 = 89.457^\circ$ $\theta 3 = 330.09^\circ$
Peak heights after PC refinement ^a	0.025/0.0154 1st peak	0.0272/0.016 1st peak	0.067/0.033 1st peak
Translation function			
Resolution limits (Å)	8–3.9	10–4.0	10–4.0
No. of reflections, $F > 2\sigma$	5,403	7,173	4,791
xyz position of the center of gravity (fractional coordinates)	$x = 0.395$ $y = 0.250$ $z = 0.267$	$x = 0.383$ $y = 0.250$ $z = 0.258$	$x = 0.233$ $y = 0.167$ $z = 0.336$
Peak heights and R -factors	11 σ 46%	13.2 σ (6.5 σ) 48.3% (53.5%)	24 σ (9.85 σ) 41.2% (54.2%)
Rigid body refinement			
Rotation and translation of small domain	$\theta 1 = 2.44^\circ$ T1 = 0.95 $\theta 2 = 15.76^\circ$ T2 = –0.48 $\theta 3 = 1.01^\circ$ T3 = –0.70	$\theta 1 = 5.25^\circ$ T1 = 1.16 $\theta 2 = 5.44^\circ$ T2 = 0.68 $\theta 3 = 5.00^\circ$ T3 = –2.42	$\theta 1 = 0.09^\circ$ T1 = 0.00 $\theta 2 = 0.13^\circ$ T2 = 0.00 $\theta 3 = -0.01^\circ$ T3 = 0.05

^a First number is peak height of correct solution; second number is height of largest nonsolution peaks; both are in σ units.

Comparison of models from the mammalian and recombinant catalytic subunits

The refined model of the binary complex of the mammalian enzyme is superposed in Figure 1 and Kinemage 1 on the ternary complex of the recombinant enzyme, revealing the structural features that define an open and a closed conformation. Whereas the large lobe (corresponding to residues 128–300) shows few changes, the small lobe (residues 40–127) rotates a substantial distance. A preliminary report of this binary structure, solved by an independent method, described the displacement of this small lobe (Karlsson et al., 1993). This concerted shift of the small

lobe leads to an opening of the cleft. In addition to major conformational change of the small lobe, significant changes are also seen in a portion of the C-terminal tail (residues 301–350) that wraps around the surface of both lobes. Superposing these two structures also shows the importance of the cAPK myristylation motif, which includes not only the myristyl moiety but also the first 14 residues that were not visible in the recombinant enzyme. Each of these features, the acylation site, the conformational changes in the core, and the changes in the C-terminal region, will be considered in detail.

When the structures of the hexagonal mammalian and orthorhombic recombinant ternary complexes are super-

Table 2. Comparison of the three models of the porcine catalytic subunit^a

Residue range	rms deviations (Å) ^b				Structure features
	m-Bin @ 2.9 Å/ m-Apo @ 3.9 Å	m-Bin @ 2.9 Å/ r-Ter @ 2.2 Å	m-Bin @ 2.9 Å/ m-Ter @ 3.0 Å	r-Ter – 2.2 Å/ m-Ter @ 3.0 Å	
128–300	0.55	0.43	0.62	0.55	Core of big domain
15–127	0.66	3.14	3.11	0.71	Small domain
15–350	0.67	2.54	2.59	0.65	Whole cAPK
40–127	0.66	3.46	3.40	0.66	Core of small domain
301–350	1.01	4.50	4.67	0.79	C-terminal tail
50–58	0.67	5.50	5.69	0.51	Glycine-rich loop
40–300	0.59	2.04	2.01	0.59	Core of cAPK
361–380		1.83	1.77	0.73	Inhibitor
15–380		2.50	2.55	0.65	cAPK + inhibitor

^a The comparisons of mammalian binary with mammalian apoenzyme clearly show that these structures have the same conformation. Similarly, recombinant ternary complex and mammalian ternary complex are the same. The comparison of mammalian binary complex with either ternary complex shows large changes in the conformation of the small domain and the C-terminal tail.

^b These values represent rms distances (Å). All comparisons are based on the superimposition of the C α atoms of residues 128–300.

posed (residues 15–350), no major differences are seen in the folding of the polypeptide chain, in the position of ATP and its interactions with the protein, or in the conformation of the bound peptide. Likewise, no major conformational changes are seen between the orthorhombic binary and ternary structures.

Binding site for the N-terminal myristyl moiety

The surface of the enzyme surrounding the myristyl moiety attached to the N-terminal glycine is very hydrophobic,

and when the nonmyristylated recombinant enzyme was co-crystallized in the presence of octanoyl-*N*-methylglucamide (MEGA-8) (Knighton et al., 1993), a detergent molecule occupied a portion of this acyl binding pocket (Fig. 6). As indicated earlier, the first 14 residues of the polypeptide chain are not visible in crystals of the recombinant enzyme grown in the absence of detergent (Knighton et al., 1991b,c). Even with detergent, nine residues were still not visible. In contrast, in the crystals of the mammalian enzyme these residues, with the exception of Ala 3, can all be seen clearly, as can the myristyl group (Fig. 2A). In the mammalian enzyme structure, the helix at the

Fig. 1. Stereo view of the C α -backbones of the binary complex of the mammalian C-subunit and PKI(5-24)-I₂ superimposed with the ternary complex of the recombinant C-subunit, ATP, and PKI(5-24). The mammalian enzyme (residues 15–350) and PKI(5-24)-I₂ are shown in red. The myristylation site (residues 1–14 plus the myristylate moiety) is shown in green. The position of the iodinated Tyr in PKI(5-24)-I₂ is indicated by a black dot. The ternary complex of the recombinant C-subunit is shown in black.

Fig. 2. Myristylation site in the mammalian C-subunit. **A:** Stereo view of the $F_o - F_c$ difference map showing a part of the N-terminal region of A-helix (residues 1–15) and the myristylate moiety (green). The map was contoured at 1.5σ and calculated using refined phases of the binary complex of the mammalian enzyme complexed with PKI(5–24)-I₂. Residues 1–15 and the fatty acid were omitted from the model for the calculation of F_c . **B:** Stereo view of the acyl-binding pocket. The hydrophobic residues contributing to this site (shown in black) derive from four regions that are widely dispersed in the linear sequence. These regions, the A-helix, the loop connecting the C-helix and β -strand 4 (residues 98–104), the E-helix (residues 139–160), and the boundary between the conserved core and the C-terminal tail, are shown as red ribbons. The myristylated motif defined as residues 1–14 plus the myristic acid are shown in green. The van der Waals surface of the acyl group is also shown in green. Residues involved in anchoring the A-helix to the core, Trp 30, Arg 93, and Arg 190, are shown in black.

N-terminus is extended by two turns and the chain then folds inward with the acyl group occupying a deep hydrophobic pocket. As seen in Figure 2B, this hydrophobic pocket is formed by residues from four different regions

of the molecule. (1) The hydrophobic surface of the A-helix contributes Val 15, Phe 18, and Leu 19. (2) Phe 100 and Phe 102 lie at the apex of a loop in the small lobe that connects the C-helix with β -strand 4. (3) The E-helix, an

unusual buried hydrophobic helix, contributes Leu 152, the β - and γ -carbons of Glu 155 and Tyr 156 to the acyl-binding site. (4) The end of the conserved core and the beginning of the C-terminal tail, Lys 292, Trp 302, Ile 303, and Tyr 306, form the final surface of this acyl pocket. The myristic acid is thus firmly anchored to the enzyme by multiple hydrophobic contacts.

Conformational changes in the active site

As seen in Figure 1, the movement of the small lobe is substantial and reflects a concerted movement of the β -sheet. The nature of this motion is described by Karlsson et al. (1993). This rotation alters the disposition of several conserved residues at the active site. The protein kinases contain highly conserved residues that are clustered around the cleft near the site of catalysis. The glycine-rich loop between β -strands 1 and 2, Lys 72 in β -strand 3, and Glu 91 in the C-helix are all in the small lobe and contribute to ATP binding (Zheng et al., 1993a). The catalytic loop between β -strands 6 and 7 in the large lobe provides most of the residues that are important for the transfer of the phosphate from ATP to the peptide. In addition, the loop connecting β -strands 8 and 9 contains the conserved residue Asp 184, which binds to the activating Mg^{2+} ion (Zheng et al., 1993a). Another prominent feature of the surface of the cleft on the large lobe is the phosphate at Thr 197. This phosphate interacts with Arg 165, Lys 189, and Thr 195 in the large lobe (Knighton et al., 1993), and these interactions render it inaccessible to removal by phosphatases (Shoji et al., 1979). All of these key residues on the surface of the large lobe are essentially unchanged in the two structures (Figs. 3A, 6).

The distances between the conserved features in the small lobe, in particular, the glycine-rich loop, Lys 72 and Glu 91, also do not change significantly in the binary cubic structure relative to each other. The distances of these conserved residues in the small lobe relative to the conserved residues in the large lobe, however, change dramatically. The most significant changes are indicated in Figure 3B. In the orthorhombic crystals, for example, Asp 184 is only 6.5 Å from Gly 52 in the glycine-rich loop and 3.7 Å from Lys 72. In the cubic crystals, Gly 52 has moved 4 Å further away from Asp 184, whereas Lys 72 has moved nearly 3 Å. In the orthorhombic crystals, His 87 forms a salt bridge with P-Thr 197, whereas in the cubic crystals, His 87 is nearly 6 Å away from the phosphate and is too far to interact.

Rigid features of the small lobe

The opening of the active site is accomplished by a rotation of the antiparallel β -sheet through 15° (Karlsson et al.,

1993). In understanding this movement it is also important to consider the portions of the molecule that do not change. As indicated above, no significant changes are seen in the conformation of the entire large lobe. There are also stable segments within the amino-terminal portion of the molecule (Fig. 4A; Kinemage 1). One such segment is the long A-helix that abuts both lobes. This amphipathic helix is anchored at the N-terminal end by the myristic acid attached to Gly 1. The other end of this helix is anchored to the core by Trp 30, which binds in a hydrophobic pocket between the small and large lobe (Fig. 2B). Neither this A-helix nor the acyl group pocket change in the two structures. The other segment that does not change in the small lobe is the loop that connects the C-helix with β -strand 4. As seen in Figure 4B, these rigid portions in the N-terminal segment complement the surface of the large lobe. Specifically, the loop connecting the C-helix and β -strand 4 binds between the surface of the large lobe and the hydrophobic surface of the A-helix. Phe 100 and Phe 102 at the apex of this loop also contribute to the acyl-binding site for the fatty acid. The A-helix not only interacts with this loop but also complements a portion of the surface of the large lobe.

C-terminal tail

In addition to the conformational changes in the small lobe, significant differences are also observed when the C-terminal tail is compared in the orthorhombic and cubic crystals. This C-terminal segment, residues 301–350, is not conserved in the protein kinase family and wraps as an extended chain around the surface of both lobes. As seen in Figure 5A, the initial portion of this extended chain, residues 301–316, does not move. However, the remainder of the chain shows considerable change. Only at the C-terminal Phe do the two structures converge again. This Phe is located in a hydrophobic pocket, and these hydrophobic interactions may help to anchor the C-terminus (Fig. 5B). One of the stable phosphates, Ser 338, is also located in this segment. The phosphate appears to stabilize the local conformation by forming a salt bridge with Lys 342. This interaction is present in both structures even though the main chain itself is displaced.

Discussion

The structure solution of the cubic crystals of the mammalian catalytic subunit reveals several important features of this enzyme that were not apparent in the orthorhombic crystals of the recombinant enzyme. Of particular importance for this enzyme is the myristylation site and an understanding of how this posttranslational modification can contribute structural stability. The other important feature revealed in this structure is the concerted move-

Fig. 3. Active-site region in the open conformational state. **A:** The active site region of the binary complex from the mammalian enzyme (red) is superimposed in stereo with the corresponding region from the ternary complex of the recombinant enzyme (blue). The ATP in the ternary complex is shown in black. **B:** Some of the distances that change most going to the open conformation of the mammalian binary complex are indicated. Distances in Å between several key residues in the two structures are as follows (numbers in parentheses correspond to the closed conformation): Asp 184 OD2 to Gly 52 C α , 10.5 (6.5); Asp 184 to Lys 72 NZ, 6.6 (3.7); Phe 54 CZ to His 87 ND1, 7.4 (6.4); His 87 NE2 to P-Thr 197 OE2, 6.0 (2.7); Glu 91 OE1 to Lys 72 HZ, 4.1 (3.5).

ment of the small lobe and the consequence of this movement at the active site at the cleft between the lobes. Although the significance of this movement for catalysis must still be demonstrated, this structure indicates very clearly some of the conformational changes that this enzyme is capable of undergoing. The conformational changes also demonstrate the importance of phosphorylation and how the phosphorylation state of a protein can influence its conformation.

Myristylation

What role does myristylation play in the C-subunit? Myristylated proteins can be separated into two categories, those that are soluble and those that associate with membranes (Towler et al., 1988). In the case of the transforming

protein of Rous sarcoma virus (pp60^{v-src}), the myristyl group is clearly necessary, although perhaps not sufficient, to convey membrane-associating properties (Kaplan et al., 1990). In the case of the poliovirus capsid protein, VP4, the myristyl group appears to provide a hydrophobic anchor between subunits on the coat surface and plays a structural role in capsid assembly (Chow et al., 1987). The structure of the mammalian C-subunit provides the first example of how a myristyl group can contribute to the stability of a soluble protein. When the C-subunit was first expressed in *Escherichia coli*, it was not myristylated because *E. coli* lacks *N*-myristyl transferase (NMT). The recombinant enzyme is kinetically indistinguishable from the mammalian enzyme; however, it is more labile to heat denaturation, both in the monomeric state and in the holoenzyme complex (Slice & Taylor, 1989; Yonemoto et al.,

Fig. 4. Flexible and fixed segments in the N-terminal region. **A:** Stereo view of the N-terminal region of the mammalian binary and recombinant ternary complexes. Residues 1–127 are shown in red for the mammalian enzyme and residues 15–127 in blue for the recombinant enzyme. The fatty acid is shown in green; ATP is shown in blue. **B:** Superposed models rotated approx. 180° from the view of Figure 1A. The interactions between the rigid portions of the N-terminal sequence and the large lobe are highlighted.

1993). In the crystals of this recombinant enzyme, the first 14 residues were not visible, suggesting that this segment is unstructured when the N-terminal Gly is not myristylated (Duronio et al., 1990). When the C-subunit and NMT are coexpressed in *E. coli*, the recombinant enzyme is fully myristylated. The stability of this myristylated rC-subunit (myr-rC) closely resembles the mammalian en-

zyme, confirming that one role for the acyl group is to convey structural stability (Yonemoto et al., 1993).

The crystal structure explains how this is accomplished. Instead of associating with a membrane, the acyl group folds into the protein, making important contacts with several distinct regions of the polypeptide chain. It attaches the N-terminal portion of the A-helix to the E-helix

Fig. 5. Stereo view comparison of the nonconserved C-terminal tail in the mammalian binary complex and the recombinant ternary complex. **A:** Comparison of the C α -backbones, with the position of Ser 338, Lys 342, and Phe 350 indicated. The series of acidic residues (Asp 328 through Glu 335) is shown by black dots. The *B*-factors for this region are high in both structures, suggesting considerable flexibility. **B:** The hydrophobic binding site for the Phe 350 at the C-terminus.

on the surface of the large lobe. The loop that extends into this pocket from the small lobe also contributes to the acyl-binding pocket. The junction between the large lobe of the conserved core and the nonconserved tail also converges at this acyl site. Whether the myristyl group is added cotranslationally or posttranslationally is not known, but it clearly has the potential to facilitate proper folding if it is added cotranslationally. This small segment, consisting of residues 1–14, corresponds to a single intron in the C-subunit (Chrivia et al., 1988). For the proteins where the myristyl group conveys membrane-associating capabilities, such as the *src* family of protein kinases, it is not clear whether there exists in the membrane a specific receptor for the myristylate or whether the acyl group binds directly to *src* and then exposes another hydrophobic region that interacts with the membrane.

Phosphorylation

Posttranslational modifications are clearly very important for assembling the proper conformational state of this enzyme. The structural importance of the two stable phosphorylation sites in the C-subunit was apparent from the orthorhombic crystals of the recombinant enzyme. Ser 338 interacts with Lys 342 and stabilizes a loop near the end of the C-terminal tail (Knighton et al., 1993). The structure thus explains not only why the phosphate at Ser 338 is resistant to phosphatases (Shoji et al., 1979), but also why Lys 342 does not react with acetic anhydride (Buechler et al., 1989).

The resistance of P-Thr 197 to phosphatases was also explained by the structure of the recombinant enzyme (Knighton et al., 1991b,c, 1993). Its interactions with

Arg 165, Lys 189, His 87, and Thr 195 anchor it firmly on the surface of the large lobe at the edge of the cleft. Genetic evidence in the yeast enzyme (Gibbs et al., 1992) indicates the importance of this surface for recognition by the R-subunit. This was confirmed independently by Orellana and McKnight (1992), who showed that mutation of Trp 196 to Arg and His 87 to Gln yielded an unregulated phenotype. Recent work by Steinberg (1991) suggests that phosphorylation at this site may be a critical step in the final maturation of the C-subunit in eukaryotic cells.

This structure of the mammalian enzyme not only reveals a novel open conformation relative to the orthorhombic crystals, but also highlights the prominence of P-Thr 197 at the cleft interface (Fig. 6; Kinemage 2). Specifically, the two conformational states of the C-subunit show important changes in the environment of P-Thr 197. Perhaps the most significant consequence of the conformational changes at the cleft interface is the shift of His 87. In the closed conformation, it interacts directly with

P-Thr 197, whereas in the open conformation, it is nearly 6 Å away from the phosphate. Thus the phosphate can serve as an important link between the lobes. This phosphate is, in fact, the major direct contact site between the two lobes in the mammalian binary complex.

A phosphorylation site exists in this region in many protein kinases. For example, Thr 161 in human cdc2 and Thr 160 in human cdk2 are analogous to Thr 197 in the C-subunit, and their phosphorylation by a heterologous protein kinase following association with cyclin is essential for activation (Solomon et al., 1992; Connell-Crowley et al., 1993). The insulin receptor and pp60^{src} also have a phosphorylation site in this region (Hunter, 1987; Tornqvist & Avruch, 1988; Zhang et al., 1991). These phosphates appear to turn over, whereas Thr 197 in the C-subunit seems to be a stable phosphorylation site. Based on sequence alignments, protein kinase C is another good candidate for having a stable phosphorylation site in this region (Coussens et al., 1986). The mitogen-activated protein kinases (MAP kinases) are regulated by the consec-

Fig. 6. The C α -backbone of the closed and open conformations, highlighting critical changes at the cleft interface. The binary complex of the recombinant C-subunit, representing the closed conformation, is shown on the right. The open conformation associated with the mammalian binary complex is shown on the left. The N-terminal regions (1–127 in the mammalian C-subunit and 9–127 in the recombinant enzyme) are shown in red. The C-terminal regions (residues 128–350) are shown in blue. The peptides are shown in black. The fatty acid in the mammalian complex and the MEGA-8 detergent in the recombinant complex are shown in green. Key residues at the cleft interface (His 87, Asn 90, Thr 197), whose environment changes as a consequence of the cleft opening, are also indicated in green.

utive phosphorylation of Tyr 183 and Thr 185 in this region by MAP kinase kinase (Haystead et al., 1992). Based on the cAPK structure, we predict that phosphorylations in this region will result in localized conformational changes that are important for generating the active conformational state of many protein kinases.

Flexible tail

The region extending from residues 317 to 340 exhibits high backbone *B*-factors in both orthorhombic and cubic binary structures relative to the rest of the molecule, suggesting that there may be considerable flexibility in this region. Chemical evidence also supports flexibility in this region. The acidic residues comprising the segment from 328 through 334 (Asp-Asp-Tyr-Glu-Glu-Glu-Glu), for example, react readily with a soluble carbodiimide in the absence of ATP and peptide inhibitor, whereas in the ternary complex they are all protected (Buechler & Taylor, 1990). In the ternary orthorhombic crystals, Asp 328 and Tyr 330 come close to the P-3 Arg in the inhibitor peptide, whereas Asp 329 forms a salt bridge with Lys 47 (Zheng et al., 1993a). Lys 47 also reacts readily with acetic anhydride in the apoenzyme but is protected in the presence of ATP (Buechler et al., 1989). This flexible anionic site could serve as an initial docking site for basic peptides as suggested by CD studies (Reed et al., 1985). Shaltiel and coworkers showed that the catalytic subunit undergoes salt-induced conformational changes in this region based on the effects of salt on the reactivity of Cys 199 and Cys 343 (Kupfer et al., 1982). They indicated, furthermore, that this intrinsic malleability might be important for peptide recognition. Their observations would be consistent with the types of conformational changes observed in this C-terminal segment.

Ordered binding of substrates

Kinetic studies predict a preferred, but not obligatory, ordered binding of substrates for the C-subunit with ATP binding preceding substrate binding (Cook et al., 1982; Whitehouse et al., 1983). ATP binds in a deep pocket, and in the ternary complex, closed structure is completely buried by the inhibitor peptide, β -strands 1 and 2, and the C-terminal tail (Zheng et al., 1993a). The ATP site appears to be more accessible in the open binary conformation, suggesting that an open apo-structure could bind either ATP or peptide, but that the closed conformation cannot bind ATP after binding peptide.

Equilibrium between different conformational states

The binary complex of the mammalian C-subunit, with an iodinated peptide inhibitor, crystallizes in an open conformation, whereas the binary complex of the recombinant C-subunit with the noniodinated peptide crystallized

under identical conditions in a closed conformation. What explains this difference? There are two differences between the mammalian and recombinant enzyme complexes other than the nine mostly conservative changes in primary sequence. First is the presence of the myristic acid at the N-terminus in the mammalian enzyme, and second is the presence of two iodines on Tyr 7 in the inhibitor peptide. At present, we have no evidence about the role of the myristic acid in contributing to conformational changes in the enzyme. Whether the acylated enzyme is more or less flexible than the unmodified enzyme is not known.

Does iodination of the Tyr influence peptide interaction with the enzyme? Kinetic analysis of the mono- and di-iodinated peptide and the noniodinated PKI(5-24) indicated no differences (Yap, 1984; Yap & Kemp, pers. comm.). Phosphorylation of Tyr 7 was shown to increase the K_i of PKI by 10-fold (Van Patten et al., 1987). Although this is a relatively small shift, it could nevertheless be significant for the binary complex. Furthermore, all C-subunit kinetic studies are carried out under assay conditions that include ATP. In the presence of ATP, the equilibrium strongly favors the ternary complex. The K_d of ATP is 60 nM and the K_i for PKI(5-24) is 2.3 nM (Whitehouse & Walsh, 1983; Van Patten et al., 1986). For the C:PKI complex, the apparent K_d for protein interaction increases four orders of magnitude to 2.3 μ M in the absence of ATP (Herberg & Taylor, 1993). A corresponding effect on the C:PKI(5-24) complex would give an apparent K_d of 23 μ M for the unmodified peptide. Modification of the Tyr, either by iodination or phosphorylation, might increase this further so that both conformational states probably exist in solution under the crystallization conditions. Thus, it may simply be crystal contacts that determine which conformational state is selected for the formation of a stable lattice. The fact that the low resolution cubic crystal structure of the apoenzyme shows a conformation that is very similar to that of the binary cubic crystal structure indicates that this open conformation is not an artifact. In addition, small-angle neutron scattering showed conformation changes in solution that are consistent with what is observed for the open and closed conformations in the two crystal structures (Parello et al., 1993). Further crystallographic and solution structure studies obviously are required before we can answer why this binary complex crystallized in an open conformation and how these open and closed conformations relate to the normal functioning of the enzyme during catalysis.⁹

⁹ In the final stages of preparing this manuscript, a report appeared of a ternary complex of the porcine catalytic subunit (Bossemeyer et al., 1993). This monoclinic crystal structure confirms that the ternary complex of the mammalian enzyme has a conformation that is identical to the ternary complex of the recombinant enzyme (Zheng et al., 1993a). In this structure, the authors were unable to see the N-terminal seven residues and the complete myristic acid.

Materials and methods

Preparation of proteins and peptides

The inhibitor peptide PKI(5-24) was synthesized by the UCSD Peptide and Oligonucleotide Core facility. The same peptide, 3,5-di-iodinated at Tyr 7 (PKI(5-24)-I₂), was synthesized at the La Jolla Cancer Research Foundation. Both peptides were purified to homogeneity by reverse-phase HPLC. The C-subunit was purified from porcine heart according to Nelson and Taylor (1981). The sequence of this porcine C-subunit differs at nine positions from the recombinant mouse C α (rC)-subunit. The mouse rC-subunit also lacks myristic acid at its N-terminus (Slice & Taylor, 1989).

Crystallization

The purified protein was concentrated to 10 mg/mL in pH 8.0–8.3 Bicine (*N,N*-bis(2-hydroxyethyl)glycine) buffer and crystallized using the hanging drop vapor diffusion method as described previously (Knighton et al., 1991a). The binary complex crystals were obtained by mixing protein with di-iodinated peptide in a molar ratio of 1:6, whereas ternary complex crystals were obtained with molar ratios of 1:1:5:20 of protein:peptide:Mg²⁺:ATP.

The free mammalian C-subunit crystallized in a cubic form; however, this crystal only diffracted to 3.9 Å. In contrast, the ternary complex crystallized in a hexagonal form and showed diffraction to 3.0 Å, as described previously. When the protein was co-crystallized with PKI(5-24)-I₂, it crystallized in a cubic form. These crystals diffracted to 2.9 Å and were of better quality than the apoenzyme and ternary complex crystals. The data for all of these crystals are summarized in Table 1. Under identical crystallization conditions, the recombinant mouse C α -subunit, both as a binary complex with PKI(5-24) and as a ternary complex, crystallized in an orthorhombic space group (Knighton et al., 1991a).

Data collection

All of the mammalian cAPK crystal diffraction data were collected at 4 °C with graphite monochromated CuK α X-rays from a Rigaku Ru-200 rotating anode diffractometer at the UCSD Research Resource equipped with two Xuong–Hamlin multiwire area detectors (Hamlin et al., 1981; Xuong et al., 1985). Statistics for the three data sets are summarized in Table 1.

Structure solution

A generalized molecular replacement technique implemented in XPLOR was used in this study (Brunger, 1990). The refined ternary complex model of the recombinant C-subunit (Zheng et al., 1993a) was used as the search

model. Calculations were performed at the San Diego Supercomputer Center on the Cray Y-MP8/864.

The conventional real space rotation search method of Huber (1985) was conducted with the recombinant ternary model omitting ATP and searching against the hexagonal and cubic crystal data at 15–4 and 10–4 Å, respectively. For the apoenzyme, PKI was omitted from the search model as well as ATP. Patterson peaks corresponding to vectors with length 30–5 Å were used. All results are summarized in Table 1.

In order to select and refine the correct orientations from the rotation search, a Patterson correlation (PC) coefficient refinement technique (Brunger, 1990) was used. After PC refinement of the rotation search orientations, peak 6 in the ternary hexagonal crystal form and peak 22 in the apo-cubic crystal form became the highest peaks. The highest binary cubic rotation peak also had the highest PC coefficient (Table 1).

Assuming that the highest PC peaks corresponded to the correct orientations, the model was oriented according to these angles, and the translation search was done in space groups P6₁22, P4₁32, and their corresponding enantiomer space groups P6₅22 and P4₃32. The translation function (TF) values of the space groups P6₁22 and P4₁32 were at least twice as high as those for their enantiomers (Table 1).

Rigid body refinement and conformational changes

For rigid body refinement, the molecule was divided into two parts. Based on the recombinant binary structure, the small domain was defined as residues 31–127 and 328–350, whereas the larger domain was defined as residues 15–30 and 128–327. PKI(5-24) was defined as a part of the large domain. The rigid body refinement strategy proceeded by refining the total C-subunit first, followed by refinement of the individual domains.

The *R*-factor of the unrefined hexagonal model using 8–3 Å data was 43%. Rigid body refinement of the whole model reduced the *R* to 39.6%. Subsequent domain refinement did not significantly reduce the *R*-factor. There was essentially no change in relative orientation or position of the domains of the hexagonal mammalian ternary complex compared to the starting recombinant ternary complex model.

In contrast to the hexagonal crystal form, the C-subunit of the mammalian cAPK in the two cubic crystal forms showed large conformational changes as indicated by interdomain movement. The *R*-factor of the unrefined cubic binary model using 8–2.9 Å data was 47.6%. Rigid body refinement reduced the *R*-factor to 46.3%. A significant decrease in *R* value to 40.9% was observed after small domain refinement. There was little change in the *R*-factor or orientation after subsequent large domain refinement. Reversal of the order of the refinement did not change the results, and the major *R*-factor decrease was

always due to the model improvement of the small domain. This decrease of the *R*-factor by 5.4–5.5% indicates a different conformation of the small domain in the binary complex cubic crystal form, which can be roughly described as a 10° rotation about Z and a 2.8-Å translation. Similar results were obtained from rigid body refinement of the apo-model using apoenzyme cubic crystal data. The *R*-factor decreased from 46.2 to 43.3% when the whole model was refined against 8–3.9-Å data; large domain refinement resulted in a 0.3% decrease of the *R*-factor (43.3% to 43.0%), whereas small domain refinement resulted in a 5.2% decrease of the *R*-factor (43.0% to 37.8%). As shown in Table 1, in the two cubic crystal forms, the small domain is rotated 10–15° after the rigid body refinement. The apparent differences in rotation angles are due to the complexity of comparing Eulerian transformations. The overall conformations are very similar for the two cubic crystal structures. The root mean square (rms) distance between the α -carbons in the binary and apoenzyme models is 0.67 Å, whereas a comparison of the small domain alone shows an rms distance of 0.66 Å.

Simulated annealing (SA) refinement

The SA refinements were performed using the recommended slow cooling protocol (Brunger et al., 1990). The major refinement effort focused on the binary cubic crystal form because of its better resolution and data completeness.

Refinement of the binary cubic crystal form

Data for this crystal form are 98.7% complete to 2.9 Å resolution, but are quite weak due to the high solvent content of the unit cell. This is reflected in an $R_{sym} = [\Sigma(I - I_{av})/\Sigma I_{av}]$ of 9.7%, which compares very unfavorably with the R_{sym} of 5.6% obtained in a recent study of the orthorhombic crystal form of 2.0 Å. Each reflection was measured an average of 6.7 times.

The starting model for refinement was the model produced by rigid body refinement, consisting of residues 15–350 and the inhibitor peptide without iodine. A constant *B*-value was used. One refinement cycle of the cubic binary complex reduced the *R*-factor from 48% to 26.5%. An $F_o - F_c$ map using phases with small domain and iodine atoms omitted was used to refit the glycine loop (residues 49–58) and to position the iodine atoms. The second SA refinement and the subsequent $F_o - F_c$ map using the large domain phases and including the iodines showed clear density that fit the entire small domain model well. In subsequent map calculations, inclusion of the small domain with the exception of the glycine loop region in phasing showed the positions of residues 7–14, which were missing in the starting model. In addition, strong density located between helices A, E, and I indicated a

possible location for the myristyl group. Simultaneous iterative building/rebuilding in difference maps of this N-terminal region and of C-terminal residues 317–340 allowed the remaining N-terminal residues and the complete myristic acid to be modeled. The final model contained 3,010 non-hydrogen atoms and had an *R*-factor of 23.3% with rms deviations from ideal values of 0.012 Å for bond lengths and 3.9° for bond angles. A Ramachandran plot showed seven residues outside allowed conformations.¹⁰

Refinement of the ternary hexagonal crystal form

For the hexagonal crystal, 9,934 reflections were measured in the range 10–3 Å. For the partial model of the C-subunit containing residues 15–350, PKI(5-24)-I₂, and MgATP, there are a total of 2,971 non-hydrogen atoms. The low reflection:parameter ratio prohibited extensive model refinement. One round of SA refinement was carried out with the refined rigid body model of the ternary recombinant C-subunit, given the porcine sequence, against 10–3 Å hexagonal crystal data. The resulting model with restrained individual isotropic *B*-factors had an *R*-factor of 22.1% and had the same conformation as the recombinant ternary complex model. The rms deviations from ideal values were 0.022 Å for bond lengths and 4.3° for bond angles.

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¹⁰ Residues 323, 324, and 328 are in poor density in the C-terminal tail, and residue H23 is one of the disordered pair at the C-terminus of the inhibitor peptide. Residue 5 is also in poor density. Residues 38 and 165 are outside but still close to allowed regions.

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