

Structure of Type II β Phosphatidylinositol Phosphate Kinase: A Protein Kinase Fold Flattened for Interfacial Phosphorylation

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Summary

Phosphoinositide kinases play central roles in signal transduction by phosphorylating the inositol ring at specific positions. The structure of one such enzyme, type II β phosphatidylinositol phosphate kinase, reveals a protein kinase ATP-binding core and demonstrates that all phosphoinositide kinases belong to one superfamily. The enzyme is a disc-shaped homodimer with a 33×48 Å basic flat face that suggests an electrostatic mechanism for plasma membrane targeting. Conserved basic residues form a putative phosphatidylinositol phosphate specificity site. The substrate-binding site is open on one side, consistent with dual specificity for phosphatidylinositol 3- and 5-phosphates. A modeled complex with membrane-bound substrate and ATP shows how a phosphoinositide kinase can phosphorylate its substrate in situ at the membrane interface.

Introduction

Phosphoinositides are central to signal transduction (Berridge, 1993; Divecha and Irvine, 1995; Nishizuka, 1995; Carpenter and Cantley, 1996) and membrane trafficking (De Camilli et al., 1996) in all eukaryotes. Following the recent discovery of phosphatidylinositol 5-phosphate (PI5P) (Rameh et al., 1997) and phosphatidylinositol (3,5) bisphosphate (PI(3,5)P₂) in vivo (Dove et al., 1997), it has become clear that the D-3, D-4, and D-5 positions can all be phosphorylated separately and in all possible combinations (Hinchliffe and Irvine 1997). Most, if not all, phosphoinositides have distinct biological roles, and their metabolism is stringently regulated. Phosphoinositide kinases catalyze phosphorylation of the D-3, D-4, and D-5 hydroxyl groups of the inositol ring and therefore are the central players in these regulatory processes.

The phosphoinositide kinases have been divided into two distinct families on the basis of substrate specificity and primary sequence. The phosphoinositide 3-kinases (PI3Ks) and phosphatidylinositol 4-kinases (PI4Ks) are homologous in primary sequence to each other and weakly homologous to the protein kinases (Carpenter

and Cantley, 1996; Vanhaesebroeck et al., 1997). The phosphatidylinositol phosphate kinases (PIPKs) lack significant primary sequence homology to other lipid and protein kinases (Boronenkov and Anderson, 1995; Loijens et al., 1996). The PIPKs are distinguished functionally from the PI3Ks and PI4Ks by their preference for phosphatidylinositol phosphate (PIP) substrates over phosphatidylinositol (PI). All PIPKs phosphorylate PIP to phosphatidylinositol bisphosphate (PIP₂), but they do so by different routes. Type I PIPK phosphorylates PI4P on the 5-hydroxyl, and with lesser activity, PI3P on both the 4- and 5-hydroxyls. Type II PIPKs phosphorylate both PI3P or PI5P on the 4-hydroxyl (Rameh et al., 1997; Zhang et al., 1997).

Despite their ubiquitous roles in eukaryotic signal transduction pathways, no three-dimensional structure has been available for any phosphoinositide kinase. This has left several key questions unanswered. The relationship between the PI3Ks and the PI4Ks on one hand, and the PIPKs on the other, has been unclear. The molecular determinants of substrate specificity and of phosphorylation site selectivity are unknown. While substrate recognition by soluble small molecule kinases and by protein kinases is well understood, it is not known how lipid kinases phosphorylate membrane-bound substrates. The cloning of PIPKII β as a newly discovered effector of the p55 TNF receptor revealed a gene coding for a 47 kDa protein (Castellino et al., 1997). PIPKII β is among the smallest phosphoinositide kinases and is not known to heterodimerize with regulatory subunits. The small size and simplicity of the system suggested it might be more tractable to crystallization than other phosphoinositide kinases. Here, we report the structure of PIPKII β at 3.0 Å resolution.

Results and Discussion

Tertiary and Homodimer Structure

The tertiary structure of PIPKII β consists of two $\alpha + \beta$ domains (Figures 1A, 1B, and 2). The larger N-terminal domain contains a seven-stranded antiparallel β sheet and four α helices. The fourth helix contains a 45° kink. The C-terminal domain contains a five-stranded antiparallel β sheet and four α helices. The third of these helices ($\alpha 7$) extends away from the rest of the enzyme and is poorly ordered. Helix $\alpha 7$ corresponds to the first 15 residues of a highly divergent 70 amino acid sequence known as the "insert" (Boronenkov and Anderson, 1995). The β sheets from the two domains face each other across a deep cleft, while the helices form much of the exterior surface of the protein. There are three regions of significant disorder. The N-terminal 33 amino acids are completely disordered. Residues 304–342, which comprise the central portion of the highly variable insert sequence, are disordered. Finally, the Lys- and Ala-rich loop from 373–390 is disordered. This loop connects the C terminus of $\beta 12$ to the N terminus of $\alpha 8$, a distance of 22 Å. In addition to these entirely disordered regions, the 220–250 region of the C-terminal domain has high

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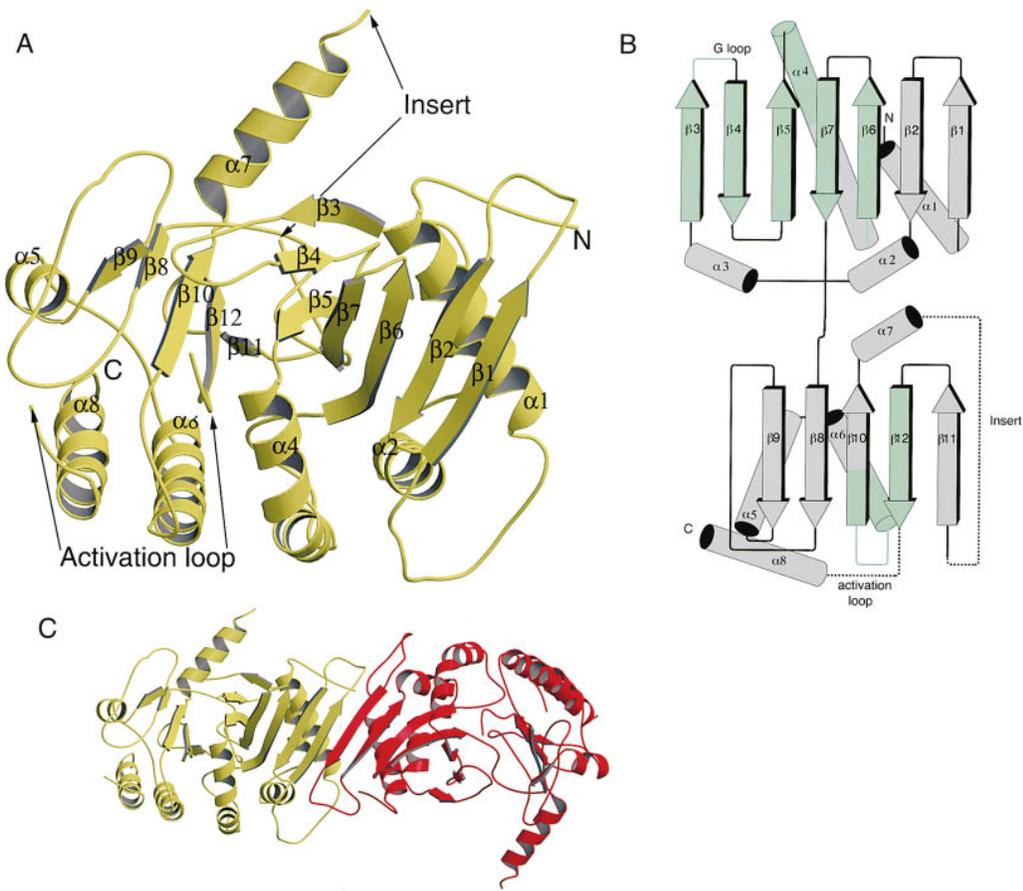


Figure 1. Tertiary and Quaternary Structure of PIPKIIβ

(A) Ribbon drawing of PIPKIIβ monomer prepared with MOLSCRIPT (Kraulis, 1991) and Raster3D (Merritt and Bacon, 1997).

(B) Schematic of the PIPKIIβ fold. The conserved ATP-binding core elements are colored green.

(C) Ribbon representation of the PIPKIIβ dimer. The subunits are colored yellow (molecule A) and red (molecule B).

thermal factors and is more flexible than the rest of the enzyme.

PIPKIIβ is a homodimer in solution on the basis of analytical ultracentrifugation (R. Ghirlando, V. D. R., and J. H. H., unpublished data). The dimer interface is formed

by a subunit-spanning antiparallel β sheet that joins the two β1 strands, and by packing between the two α1 helices (Figure 1C). The clasp-like interface is similar to that exemplified by the α1/α2 interface of class I MHC (Bjorkman et al., 1987) and subsequently observed in a

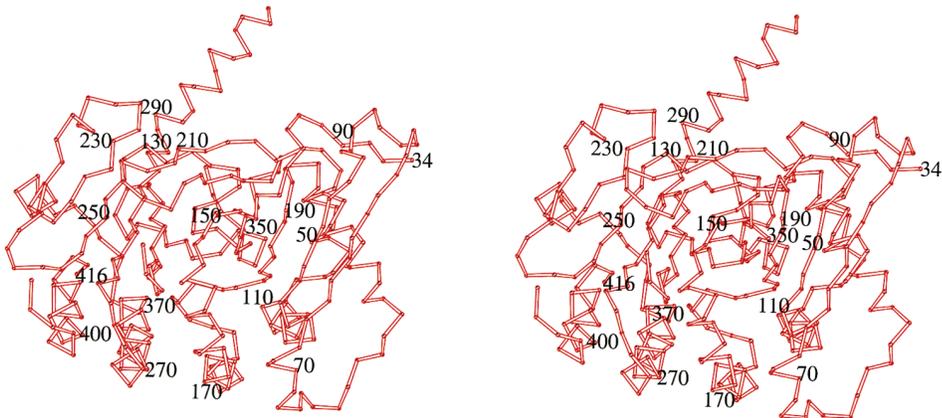


Figure 2. Stereoscopic Cα Trace of PIPKIIβ Monomer

PIPKIIβ is in the same orientation as Figure 1A.

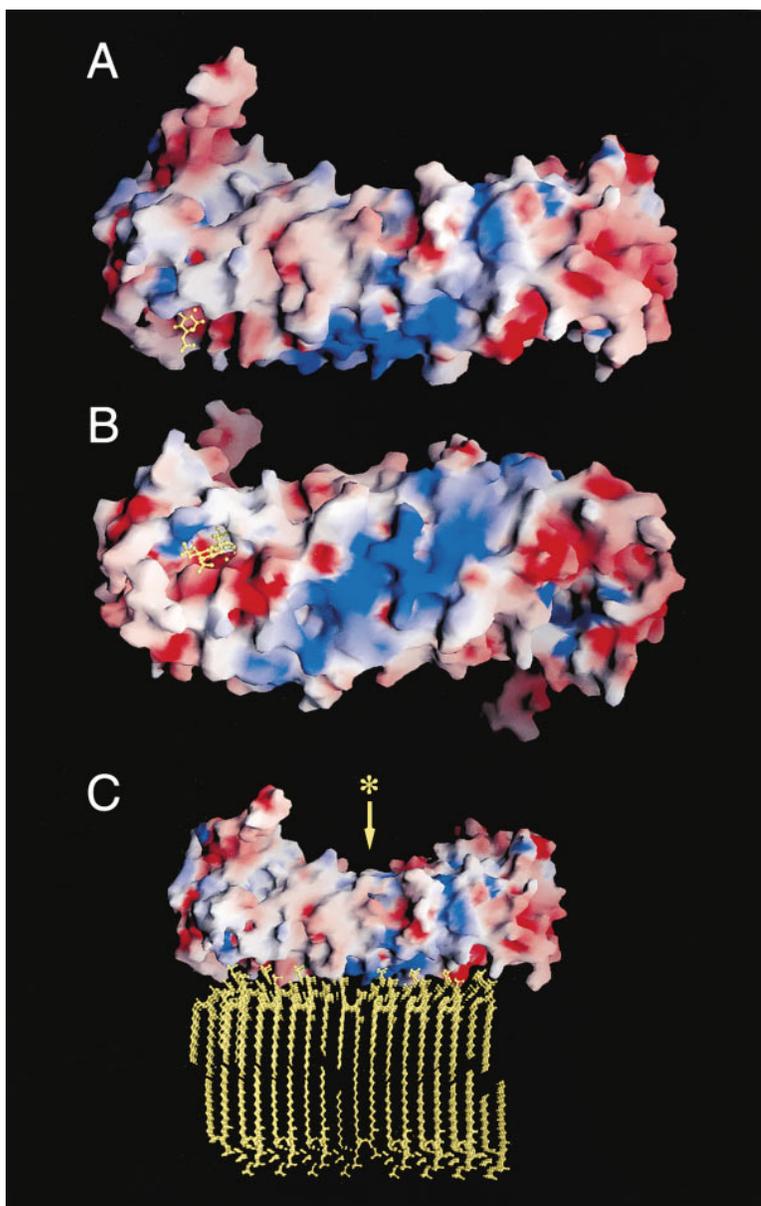


Figure 3. Molecular Surface of the PIPKII β Dimer

The surface is colored according to electrostatic potential using GRASP (Nicholls et al., 1991) in (A) sagittal and (B) normal projections relative to the presumed plane of the membrane. Saturating red indicated $\phi < -10$ kT/e, and saturating blue indicates $\phi > 10$ kT/e, where $T = 293^\circ\text{K}$. ATP and the inositol (1,5) bisphosphate moiety of the PI5P headgroup are shown in yellow bonds docked into one of the two active sites. (C) Docking of PIPKII β dimer onto a membrane surface, using the DMPC crystal structure as a model. The asterisk and arrow indicate the putative TNF receptor-binding site.

number of other proteins. The interface buries 1400 \AA^2 of solvent-accessible surface area (1.4 \AA probe) per monomer and forms β -sheet hydrogen bonds between the two $\beta 1$ strands and adjoining residues over a nine-residue span. There is no other crystal contact that buries a comparable amount of surface area, and there is no doubt that it is the clasp-like interface that mediates the dimerization observed in solution.

While the PIPKII β monomer has a nearly globular shape, the dimer is an elongated disc that is 105 \AA long, 38 \AA across, and 26 \AA thick. The central portion of one face of the disc is highly basic and exceptionally flat (Figures 3A and 3B). The flat surface is 33 \AA by 48 \AA across and extends across the dimer interface. The 2-fold noncrystallographic symmetry axis is normal to the flat face. The two monomers join their flat faces perfectly flush with each other. The flat surface is formed

by the N-terminal half of $\alpha 4$ and part of the N-terminal β sheet consisting of $\beta 1$, $\beta 2$, $\beta 6$, and $\beta 7$. This region of the dimer surface contains 14 Lys, 4 His, and 4 Arg residues. Four acidic residues per monomer lie near the edge of the basic patch can be seen as a red region at the edge of the active site in Figure 3. These acidic residues reduce the net charge on the entire flat surface patch to $+14$. The flatness and positive charge of this region strongly suggest that it functions as the major membrane attachment site. This is the largest flat surface that we are aware of in any known protein structure. The closest analogy is probably to the slightly convex membrane-binding face of the annexins, another class of peripheral membrane protein (Swairjo and Seaton, 1994). Using the crystal structure of dimyristoylphosphatidylcholine (DMPC) (Pascher and Pearson, 1979) as a model, PIPKII β can be docked onto a bilayer surface

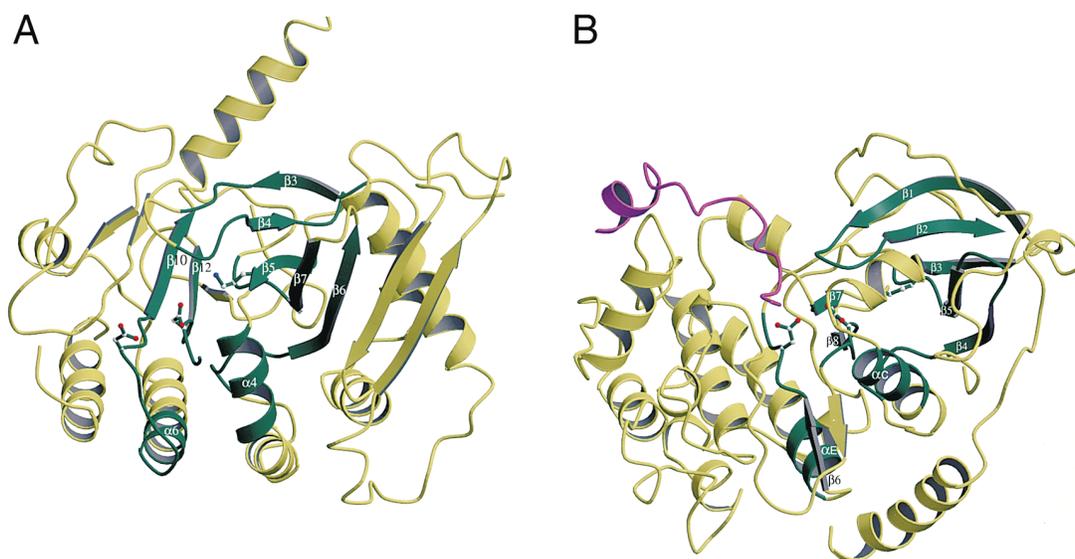


Figure 4. Similarity of PIPKII β and PKA

The shared ATP-binding cores of (A) PIPKII β and (B) PKA are colored dark green, and other protein regions are colored yellow. The ATP-binding core is defined as the region where C α atoms of the two structures can be superimposed within 2.0 Å. The exception is β 10 of PIPKII β , which does not fit this criterion but was judged to correspond to PKA β 7 because it contributes a functionally equivalent Leu to the adenine-binding pocket. The side chains of the conserved catalytic core Asp-278, Asp-369, and Lys-150 (from left to right) are shown. Carbon atoms are white, nitrogen blue, and oxygen red. The PKI inhibitor bound to PKA is magenta.

so that nearly the entire flat surface is in contact with the headgroups but without significant penetration into the membrane (Figure 3C). In contrast to several other lipid signaling proteins that penetrate membranes (Hurley and Grobler, 1997), PIPKII β appears to interact with membranes entirely by means of electrostatic interactions with lipid headgroups.

ATP-Binding Site and Similarity to Protein Kinases

Roughly 80 amino acid residues lining both sides of the central cleft can be superimposed on the ATP-binding and catalytic residues of the cAMP-dependent protein kinase (PKA; Knighton et al., 1991a; Figure 4) with a 2.0 Å cutoff on C α -C α distances. A search of the protein data bank with the program VAST (Gibrat et al., 1996) confirmed that the protein kinases are the only significant structural homologs of PIPKII β . The structural similarity was unexpected, as the PIPKs have no statistically significant primary sequence similarity to the protein kinases (Boronenkov and Anderson, 1995). On the other hand, there are several short motifs within the PIPKs reminiscent of protein kinases (Loijens et al., 1996). The structure of aminoglycoside kinase (Hon et al., 1997) is another example of a protein kinase fold in an enzyme with no apparent sequence homology to the protein kinases.

With only 78 out of 416 residues corresponding closely (2.0 Å cut-off) to PKA, PIPKII β represents the most divergent structure known within the protein kinase fold superfamily. The conserved core suggests the minimal catalytic unit required for ATP binding and substrate phosphorylation comprises a remarkably small portion of the entire structure. It seems reasonable to assume that this minimal catalytic core must also be present in

the PI3K/PI4K family. We have used this concept to extend previous alignments of the PI3K/PI4K family with protein kinases of known structure (Figure 5).

There are three catalytic residues that are absolutely conserved among both the protein kinases and the PIPKs, namely Lys-150, Asp-278, and Asp-369 of PIPKII β (Figures 4 and 5). Their counterparts in PKA are Lys-72, Asp-166, and Asp-184 (Knighton et al., 1991a; Cox et al., 1994; Hanks and Hunter, 1995). Lys-150 corresponds to the Lys that binds the α -phosphate of ATP in the protein kinases. Mutation of the counterpart of Lys-150 in PIPKI α and PIPKII α destroys enzyme activity (Ishihara et al., 1998; I. V. and R. A. A., unpublished data), as also found in protein kinases. Lys-150 is salt-bridged to Asp-156 of α 4. The function of Asp-156 is analogous to that of Glu-91 in PKA, but the C α s of these two residues do not superimpose in the overall best fit between the structures. The main chain of Glu-91 of PKA aligns better with PIPKII β Glu-159, but the PIPK Glu does not interact with Lys-150. The difference in active site structure is probably related to the 45° kink in α 4, which is absent in the PKA α C. This is one of the major differences in the active site configurations of the two enzymes.

Asp-166 of PKA functions as a weak base in kinase catalysis and is part of a "HRDLK" motif that is conserved throughout the protein kinases. Asp-278 appears to play this role in PIPKII β . The main chain at Asp-278 closely superimposes on its counterpart in PKA. There are some differences in the surrounding sequences. A β strand (β 6) immediately precedes Asp-166 of PKA. The corresponding region of PIPKII β is in a similar β conformation but has not been designated a β strand because it does not form β -sheet hydrogen bonds with an ordered partner. In PKA, β 6 and β 9 are hydrogen

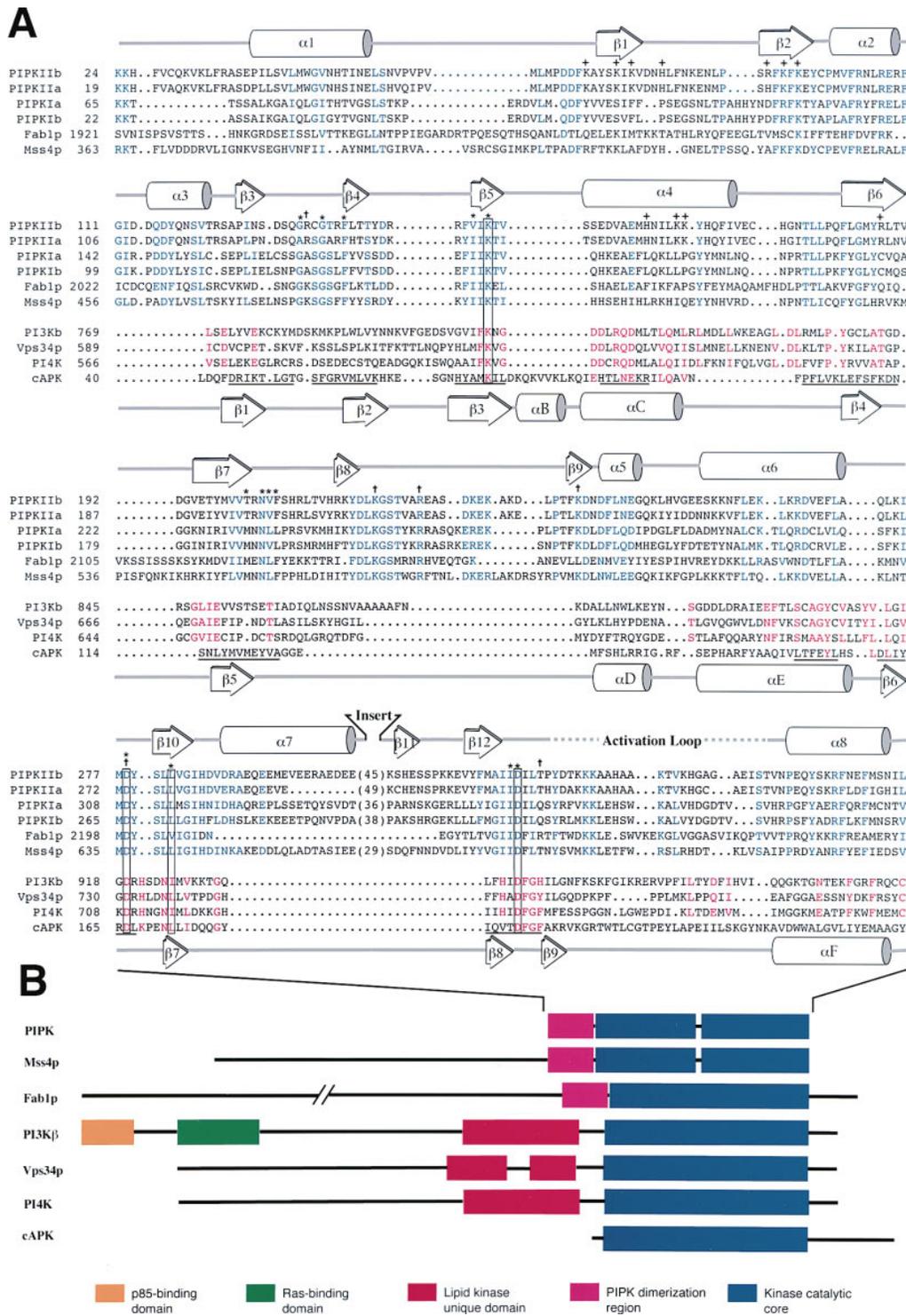


Figure 5. Structure-Based Alignment of the Catalytic Domains of Phosphatidylinositol Kinases and the cAMP-Dependent Protein Kinase

(A) The human type II PIPK and type I PIPK α and β kinases (Borononkov and Anderson, 1995; Loijens and Anderson, 1996; Castellino et al., 1997), and the related yeast Fab1p (Yamamoto et al., 1995) and Mss4p (Yoshida et al., 1994) are grouped together. The human PI-3 kinase 110-kDa β catalytic subunit (Hu et al. 1993), the yeast vacuolar protein Vps34 (Schu et al., 1993), and the human PI-4 kinase (Wong and Cantley, 1994) are grouped together with the sequence of the human cAMP-dependent kinase catalytic subunit (PKA; Maldonado and Hanks, 1988). The PI3Ks and PI4Ks were initially aligned with PKA based on the identification of the catalytic Lys by wortmannin modification (Wymann et al., 1996). The PI3K/PI4K alignment with PKA and the PIPKs proceeded on the assumption that the structural core conserved between the latter two (underlined) represented the minimal unit required for catalysis and must therefore be present in the PI3K/PI4K group. The secondary structure of PKA is shown below the alignment (Knighton et al., 1991a). Conserved and conservatively substituted residues among the PIP kinases are shown in blue; residues conserved or conservatively substituted among the PI3K/PI4K family and PKA are shown in red. Four residues identically conserved between PIPKII β and PKA, Lys-150, Asp-278, Leu-282, and Asp-369 are boxed. PIPKII β residues proposed to participate in membrane binding are designated with a plus sign (+); residues proposed to interact with ATP and PI5P are designated with an asterisk (*) and a dagger (†), respectively. Those parts of the PKA sequence that coincided with PIPKII β within a 2.0 Å cutoff (α) in the structural superposition are underlined. Numbers in parentheses indicate the number of intervening residues not shown.

(B) Domains of the PIPKs and PI3K and PI4Ks. The short break in the PIPK and Mss4p catalytic domains represents the insert loop.

bonded to each other, but the PIPKII β region corresponding to PKA's $\beta 9$ is disordered. The PIPKII β and PKA structures diverge C-terminal to Asp-278. The turn containing the conserved Asn-171 of PKA is missing in the PIPKs due to a two-residue deletion relative to PKA. The structures converge again briefly such that PIPKII β Leu-282 overlays with PKA Leu-172 in the adenine-binding pocket. The protein kinases contain an absolutely conserved DFG motif (Asp-184 of PKA) that binds catalytic Mg²⁺ or Mn²⁺ ions. Asp-369 fills this role in PIPKII β , although the DFG sequence motif is absent.

PIPKII β and other PIPKs have a Gly-containing loop ("G loop;" PIPKII β residues 130–137) that links $\beta 3$ and $\beta 4$ and corresponds topologically to the Gly-rich loop that links $\beta 1$ and $\beta 2$ of the protein kinases. The G loop is very poorly ordered in this structure of ATP-free PIPKII β . The PIPK loop is one residue longer than that of the protein kinases, resulting in a bulge. The two Gly residues do not correspond precisely to the positions of the conserved Gly residues in PKA. However, the Gly residues probably have the same function as those of the protein kinases, to stabilize a conformation in which several consecutive peptide groups are aligned parallel with each other so that their amide NH groups can interact with phosphates of ATP. The disordered loop 373–391 is the topological counterpart to the activation loop of the protein kinases (Cox et al., 1994; Hanks and Hunter, 1995; Johnson et al., 1996). We refer to it as the "activation loop" by analogy to the protein kinases and in order to distinguish this conserved flexible loop from other nonconserved disordered loops. We emphasize that despite this nomenclature the function of the "activation loop" in the PIPKs is unknown. A wide range of activation loop conformations are observed in various structures of inactive protein kinases. The activation loop is disordered in the crystal structures of the inactive c-Src (Xu et al., 1997) and Hck (Sicheri et al., 1997) kinases.

The conservation of catalytic machinery between the PIPKs and protein kinases suggested that it would be possible to model the binding of ATP to PIPKII β based on the known structures of ATP-protein kinase complexes. We have so far been unable to obtain a crystal structure in the presence of ATP because the crystals shatter at ~ 10 mM or greater concentrations of ATP, while they fail to bind ATP or its analogs at lower concentrations. This suggests the present structure represents an inactive conformation. The crystallized enzyme remains competent to bind ATP, although affinity is greatly reduced compared to $K_m = 5 \mu\text{M}$ (Bazenet et al., 1990). This could be due to the 100 mM citrate present in the crystals. We believe this conformation is inactive because it was crystallized in the absence of substrate and nucleotide, rather than because an essential regulatory activation step is lacking. When PKA (PDB entry 1ATP) is superimposed on PIPKII β , physically reasonable contacts are formed between PIPKII β residues and ATP, suggesting this is a sound basis on which to model the ATP-binding site. The only short contacts that are observed are with Phe-139, but these can be relieved by a change of rotamer. Conserved interactions of side chains with the α -phosphate and Mn²⁺ ions are maintained, although Asp-278 probably changes rotamers

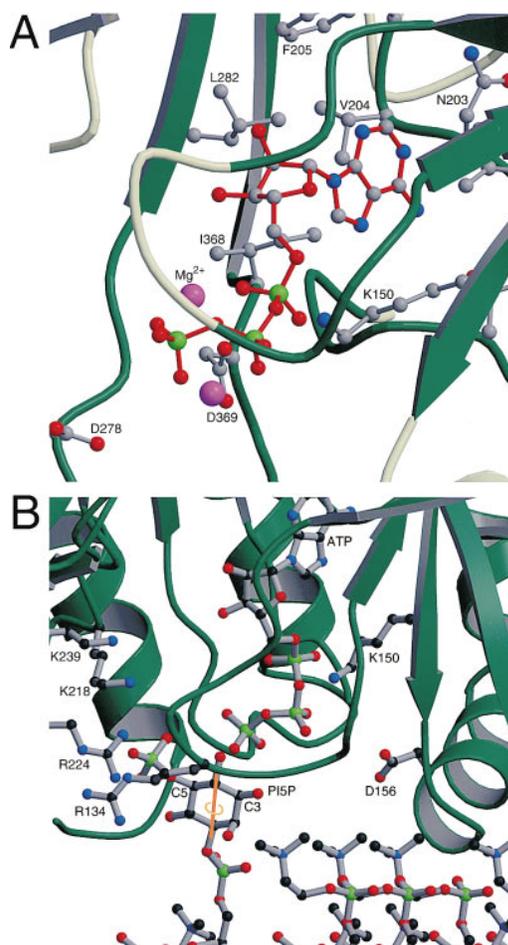


Figure 6. Models for ATP and PI5P Complexes

(A) Model of ATP bound to the crystal structure of apo-PIPKII β derived by superimposing the structure of the PKA-ATP complex on PIPKII β . The conserved kinase core is colored dark green; non-conserved regions are light yellow. Atoms are colored as in Figure 4, except carbon is gray, Mg²⁺ is magenta, and phosphorous is green. ATP bonds are red.

(B) Model of quaternary complex of PIPKII β , ATP, PI5P, and DMPC membrane. The entire protein is colored dark green; atoms are colored as above, except carbon is black. DMPC molecules in front of and to the left of the PI5P have been removed for clarity of viewing.

in the active conformation. The adenine moiety binds in a hydrophobic pocket similar to that of the protein kinases (Figure 6A). Two of the side chains in the pocket, Val-204 and Leu-282, are identical to their counterparts in PKA.

Phosphoinositide Specificity and Interfacial Catalysis

The molecular determinants of specificity for different phosphoinositides are of paramount interest in current signal transduction research. The elements that govern specificity in "downstream" signaling proteins such as phospholipases and pleckstrin-homology (PH) domain-containing proteins are now well understood in a few cases based on crystal structures of their complexes with soluble phosphoinositides (Ferguson et al., 1995;

Essen et al., 1996). Key questions remain about the mechanisms for selective phosphorylation of phosphoinositides. The type II PIPKs are especially intriguing because they have the remarkable ability to phosphorylate the 4-hydroxyl of both PI3P and PI5P. Thus far, we have been unable to detect binding of PI3P(dibutyl) or PI5P(dibutyl) in crystals of PIPKII β at concentrations of up to 5 mM. This is not necessarily surprising because monomeric phospholipids have not been demonstrated to be substrates for PIPKs. Pending crystallization of a PIPKII β complexed with a phosphoinositide, we have obtained some insights into substrate binding by docking PI5P(dimyristoyl) into the active site (Figure 6B). The crystal structure of PKA bound to an inhibitory peptide (Knighton et al., 1991b) that mimics the substrate provides some guidance as to how phosphoacceptors bind to the protein kinase fold. To dock PI5P, the inositol C4 was overlaid with the pseudosubstrate Ala in the structure of the PKI inhibitor peptide complexed with ATP and PKA, and the 4-hydroxyl was positioned for in-line attack on the γ -phosphate of ATP. The phosphatidic acid moiety was positioned to avoid steric conflicts with the protein.

The PIPKs are sharply distinguished from the PI3K/PI4K family by their nearly absolute requirement for a phosphorylated phosphoinositide substrate. A cluster of four basic side chains (Arg-134, Lys-218, Arg-224, and Lys-239) is adjacent to the 5-phosphoinositol moiety in the PIPKII β -PI5P model complex. All of these residues are conserved in the type II PIPKs, and all but the first are conserved among all PIPKs. Given its conservation, the basic cluster is likely to explain their unique requirement for a PIP, rather than a PI, substrate. The origin of the D-4 versus D-5 phosphorylation specificity of the type II versus type I PIPKs is less certain. The PIPK "activation loops" contain significant type-specific but isoform-conserved sequences, suggesting a role for this region in determining the nature of the reaction. The most plausible direct PIP-protein interactions are with Arg-134 and Arg-224. There are no steric collisions between the modeled PI5P and the enzyme. Apart from the putative 5-phosphate-binding pocket, the PI5P-binding site appears to be unusually shallow and open. Virtually no interactions were seen between the protein and the free 2- and 3-hydroxyls of the inositol ring, although it is possible that the "activation loop" becomes ordered and interacts with bound substrate in the membrane-associated state. The openness of the active site suggests a straightforward explanation for the dual specificity of the type II PIPKs. The PI3P could rotate such that the 3-phosphate would occupy the basic phosphate-binding pocket (axis marked in orange, Figure 6B). This would lead to a large movement by the 2-hydroxyl. As there appear to be no interactions with this group in either conformation, there is no apparent reason why both conformations could not be accommodated.

The central mechanistic difference between the lipid kinases and other kinases is that lipid kinases must phosphorylate a substrate that is incorporated into a complex membranous milieu. The presence of the membrane surrounding the substrate poses a potentially severe steric obstacle to substrate binding. Yet the membrane context also provides a way to increase substrate

affinity by coupling substrate binding to favorable protein-membrane interactions. The structure of PIPKII β reveals how the protein kinase scaffold has been modified to meet both of these challenges. PKA was superimposed on the membrane-docked model of PIPKII β in order to understand why the two structures are so divergent. In contrast to the PIPKII β model, which nowhere penetrates the bilayer, four different regions of the PKA main chain would cross below the membrane surface in this orientation. The severe collisions between PKA and the membrane in this hypothetical model show why minor modifications of the PKA scaffold are not enough to create a lipid kinase. Wholesale deletions and replacements of the colliding regions are apparently required to enable a protein kinase fold to interact with membrane-bound substrates. The activation loop of PKA is one of the four regions that has a predicted steric conflict with the membrane. The protein kinase activation loop is replaced by a nonhomologous Lys-rich loop in the PIPKs. This loop is disordered in the present structure and is predicted to be proximal to the membrane. The other three predicted membrane-penetrating regions in the PKA-membrane model, α B and two nonhomologous C-terminal loops, are entirely absent in the PIPK sequences. Aligned sequences (Figure 5) suggest these regions are missing in the PI3Ks and PI4Ks and that the "flattened fold" principle is likely to apply to the broad spectrum of phosphoinositide kinases.

The flatness of the membrane-binding site, the shallowness of the substrate-binding pocket, and its proximity to the membrane (Figure 6B) suggest that PIPKs phosphorylate their substrates in situ in membranes. In modeling the PI5P membrane-PIPK complex, the phosphatidic acid moiety was moved ~ 1 Å above the plane defined by neighboring DMPC molecules. This movement is less than the estimated error of the modeled complex. By phosphorylating its substrate in situ, as opposed to removing it partially or fully from the membrane, the enzyme avoids an energetic penalty for removing the substrate from its low-energy conformation within the membrane. Indeed, catalysis benefits by adding the favorable enzyme-membrane interaction energy term to the energy of the direct interaction between enzyme and substrate. The PIPKs appear to have effectively met the challenge of simultaneous substrate and membrane binding simply by either deleting structures that might clash sterically with the membrane or by replacing them with flexible regions. The PIPKs seem to have recapitulated on molecular dimensions the evolution of the flounder. Their flattened appearance may seem bizarre by comparison to their conventional cousins. Yet this is precisely what makes them so exquisitely adapted to their respective functions of interfacial phosphorylation and bottom feeding.

Regulation and Subcellular Targeting

The activation of phosphoinositide kinases is of profound importance in receptor signaling pathways. Two possible regulatory mechanisms are illustrated by the structure of PIPKII β . The activation loops of the protein kinases play a critical role in most aspects of their regulation (Cox et al., 1994; Johnson et al., 1996). We observe

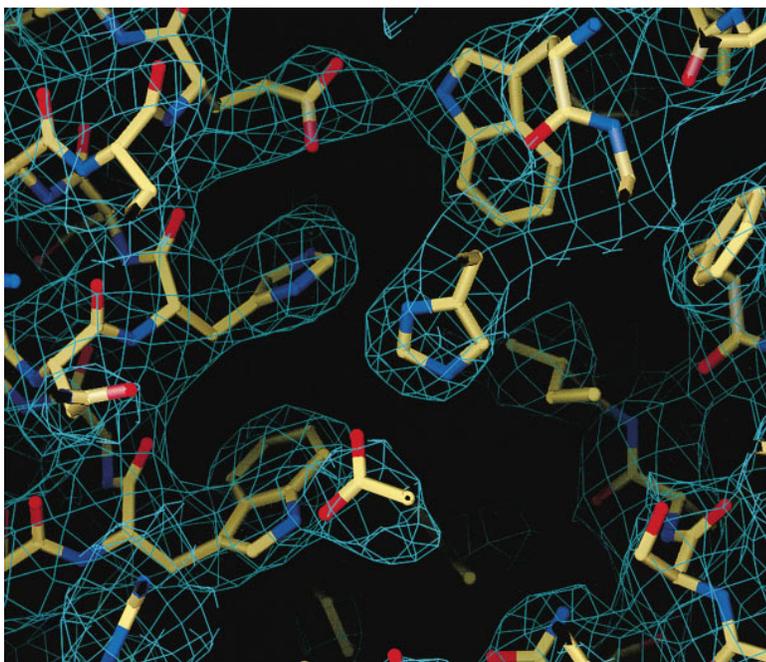


Figure 7. Electron Density from Solvent-Flattened and Symmetry-Averaged SIRAS Electron Density Map

that this loop is replaced in the PIPKs by a nonhomologous flexible, membrane-proximal loop. The conservation of this loop among isoforms of PIPK subtypes suggests that this region has an important type-specific functional role. Interactions between this loop and protein or lipid membrane components might affect the active site structure of the PIPKs by analogy to conformational changes observed in protein kinases (Cox et al., 1994; Johnson et al., 1996). Activation of type I PIPKs by phosphatidic acid (Jenkins et al., 1994) might occur by such a mechanism.

The p55 TNF receptor juxtamembrane fragment comprising residues 204–337 binds to residues 40–241 of PIPKII β (Castellino et al., 1997). We have ascribed functions to most of the N-terminal domain, which contains all of the flat membrane attachment site, all of the dimer interface, and the majority of the conserved ATP-binding core. There is a prominent groove on the membrane-distal surface of the homodimer interface, however, for which no function has been assigned (Figure 3C). Groove residues are generally conserved in PIPKII α , which is

not known to interact with TNF receptor, although Asn-59 and Pro-61 of PIPKII β are replaced by His and Gln. One consequence of PIPK dimerization is to double the size of the membrane attachment site, with a presumably commensurate increase in binding affinity. A second consequence may be the formation of the TNF receptor-binding site.

The TNF receptor region that interacts with PIPKII β is membrane proximal. Targeting of PIPKII β to the plasma membrane is presumably required for the observed direct activation by the TNF receptor. Another PIPK homolog, Mss4p, has been directly observed at the plasma membrane, where it plays a critical role in regulating actin organization in yeast (Homma et al., 1998). It has recently become clear that electrostatic interactions between basic regions of proteins and acidic lipids in the plasma membrane direct the subcellular localization of many signaling proteins, including Src, MARCKS, and K-ras (Murray et al., 1997). These relatively weak interactions may be supplemented by other interactions, such as receptor binding, or reversed by phosphorylation.

Table 1. Summary of Data Collection

Crystal	Resolution (Å)	Total Refs.	Unique Refs.	R _{sym} ^a (%)	<I>/sig(I) (Last Shell ^d)	Complete (Last Shell ^d)	PP ^b (ano)	No. of Sites
Native	3.0	224913	20641	7.0	10.4 (2.0)	93.6 (80.9)		
Native ^e	3.0	297963	21621	5.2	14.7 (4.5)	99.2 (98.3)		
^c Ethgac-a	3.8	165543	18406	9.6	6.2 (2.6)	91.7 (87.6)	1.2 (1.2)	4
^c Ethgac-b	3.0	630711	25970	7.9	12.7 (4.1)	97.4 (97.1)	1.17 (1.3)	4

^a R_{sym} = $\sum_j |I_j - \langle I_j \rangle| / \sum_j I_j$, where $\langle I_j \rangle$ is the average intensity of reflection j for its symmetry equivalents; values in parentheses are for the highest resolution shell.

^b PP = $\langle (|F_{\text{calc}}|) / \text{phase integrated lack of closure} \rangle$.

^c Ethyl mercuric acetate: low resolution data from Ethgac-a and high resolution data from Ethgac-b were combined and used in phasing.

^d Last resolution shell for the native data sets and Ethgac-b was 3.11–3.0 Å and 3.93–3.8 Å for the Ethgac-a data set.

^e Data were collected at NSLS, Brookhaven National Laboratories, Beamline X-9B.

PIPKII β , with its extensive flat basic surface, appears to be superbly designed not only for in situ catalysis, but for regulated subcellular targeting as well.

Experimental Procedures

Protein Expression and Purification

PIPKII β was expressed in *Escherichia coli* BL21(Δ DE3) as a hexahistidine fusion protein (Castellino et al. 1997) and purified over a chelating sepharose column (Pharmacia). The (His)₆-PIPKII β fusion protein was cleaved with 0.1 U of thrombin/mg PIPKII β for 12 hr at 4°C. The cleavage was stopped by adding 2 mM PMSF. The cleaved protein was loaded onto an Affi-gel blue column equilibrated with 50 mM Tris-HCl (pH 7.5), 0.1 mM EDTA, 10 mM DTT, and 100 mM NaCl and eluted with a 0.1 to 1.0 M NaCl gradient using an FPLC system (Pharmacia). The protein was stored at -80°C in elution buffer with 20% glycerol.

Protein Crystallization

Stored PIPKII β was dialyzed into 50 mM Tris-HCl, 500 mM NaCl, 0.1 mM EDTA, and 10 mM DTT and concentrated to 16–17 mg/ml. Small crystals were obtained by hanging drop vapor diffusion at 20°C using the Hampton screen (Jancarik and Kim, 1991). Optimal conditions consisted of 100 mM sodium citrate (pH 5.6), 200 mM magnesium acetate, 100 mM lithium acetate, and 13%–17% PEG 4000 in the reservoir. The drop contained 3 μ l protein at 13–17 mg/ml mixed with 3 μ l reservoir solution. These yielded crystals as large as 0.3 \times 0.6 \times 1 mm³.

Crystallographic Data Collection

Data were collected from frozen crystals using mirror-focused Cu K α radiation from a Rigaku RU-200 rotating anode source at 100 mA and 50 kV and RAXIS II and RAXIS-IV image plate detectors. Crystals were equilibrated in 100 mM sodium citrate (pH 5.6), 100 mM lithium acetate, 20% PEG 1000, and 5% MPD for 10 min and flash frozen in 1.5 ml vials containing liquid propane equilibrated under liquid nitrogen. Data were collected in 1° oscillation frames at 30 min per degree at 95°K. Crystals belong to space group C222₁ and have unit cell dimensions of a = 109.9 Å, b = 182.4 Å, c = 106.4 Å based on autoindexing with HKL (Otwinowski and Minor, 1997). The crystals had 54% solvent and a V_m = 2.7 (Matthews, 1968) based on two molecules in the asymmetric unit. Native data were also collected at beamline X-9B, National Synchrotron Light Source, using 60–120 s exposures and a Mar345 image plate scanned at a radius of 180 mm. Synchrotron data were integrated with HKL.

Structure Determination and Refinement

Two crystals of a mercurial derivative were prepared by soaking in a trace amount and 0.5 mM, respectively, of ethylmercuric acetate for 4 days in 100 mM lithium acetate, 100 mM sodium citrate (pH 5.6), and 20% PEG 4000. SOLVE (Terwilliger and Berendzen, 1996) was used to locate the positions of four bound heavy atoms, which

had the same coordinates in both crystals (Table 1). Positions were confirmed by manual inspection with MAPVIEW (Furey and Swaminathan, 1997). The positions and occupancies were refined and phases were calculated using the maximum likelihood refinement method as incorporated in SHARP (de La Fortelle et al., 1997). The heavy atom positions and a preliminary C α trace were used to calculate the location of the noncrystallographic 2-fold axis and establish a mask for density averaging. The resulting electron density map was subjected to solvent flattening and averaging using DM (CCP4, 1994; Cowtan, 1994) with a solvent fraction of 0.54. The atomic model was built in the modified electron density map (Figure 7) using O (Jones et al., 1991). The structure was refined using X-PLOR, version 3.8 (Brünger, 1996) with harmonic restraints on the two noncrystallographic symmetry related molecules and the parameters of Engh and Huber (1991). The refinement was monitored using the free R factor calculated with 5% of the observed reflections (Brünger, 1992). The structure has been refined against the 6.0 Å \geq d \geq 3.0 Å synchrotron native data with F_{obs} \geq 0. The current model contains 637 residues. The model includes residues 34–131, 137–303, 343–372, and 396–416 of molecule A and 34–303, 342–372, and 397–416 of molecule B. Residues 135–136 of molecule B are not observed in electron density, but their positions have been inferred from the location of the adjacent residues and are included in the model with zero occupancy. Stereochemical quality was assessed using X-PLOR and PROCHECK (Laskowski et al., 1993), and the parameters are shown in Table 2. No nonglycine residues lie in the disallowed regions of the Ramachandran plot.

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Table 2. Refinement Statistics

Parameter	Value
d _{min} (Å)	3.0
R _{free} (%)	29.9
R _{cryst} (%)	22.9
rms Δ bond (Å)	0.009
rms Δ angle (deg)	1.455
rms Δ impropers (deg)	1.339
rms Δ dihedrals (deg)	23.726
rms ncs displacement (Å)	0.282
Protein (Å ²)	48.7
No. of protein atoms/asu	5287
No. of water molecules/asu	20
Water (Å ²)	63.4

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Brookhaven Protein Data Bank Accession Number

The coordinates have been deposited with entry code 1bol. Pre-release coordinates will be made available immediately at <http://www-mslmb.niddk.nih.gov/hurleygroup.html>.