Pim-1 Ligand-bound Structures Reveal the Mechanism of Serine/Threonine Kinase Inhibition by LY294002*

Marc D. Jacobs‡, James Black, Olga Futer, Lora Swenson, Brian Hare, Mark Fleming, and Kumkum Saxena

From Vertex Pharmaceuticals Incorporated, Cambridge, Massachusetts 02139

Pim-1 is an oncogene-encoded serine/threonine kinase primarily expressed in hematopoietic and germ cell lines. Pim-1 kinase was originally identified in Maloney murine leukemia virus-induced T-cell lymphomas and is associated with multiple cellular functions such as proliferation, survival, differentiation, apoptosis, and tumorigenesis (1). Since the initial report of the cloning of Pim-1, the structure of the Pim-1 kinase domain has been determined (2). The crystal structures of Pim-1 complexed with staurosporine and adenosine were determined. Although a typical two-domain serine/threonine protein kinase fold is observed, the inter-domain hinge region is unusual in both sequence and conformation; a two-residue insertion causes the hinge to bulge away from the ATP-binding pocket, and a proline residue in the hinge region is conserved (3). Without this proline insertion, van der Waals interactions with the hinge serve to position the ligand. The hinge region of Pim-1 resembles that of phosphatidylinositol 3-kinase more closely than it does other protein kinases. Although the phosphatidylinositol 3-kinase inhibitor LY294002 also inhibits Pim-1, the structure of the LY294002-Pim-1 complex reveals a new binding mode that may be general for Ser/Thr kinases.

The Pim-1 oncogene was first identified as the preferred site of integration of the slow transforming Maloney murine leukemia virus in lymphoblastic T-cells (1). Direct evidence for the oncogene potential of the Pim-1 gene comes from the study of transgenic mice in which overexpression of Pim-1 produces a low but spontaneous rate of tumor incidence (2). The x-ray structure of Pim-1 was pursued, in part, to determine how an unusual sequence feature in the active site affects ligand binding. In protein kinases, one hydrogen bond between the ATP molecule and the amino acid side chain of a proline residue in the hinge region is conserved (3). Since the initial report of the cloning of mouse Pim-1 gene (4), Pim-1 has been cloned from human, rat, bovine, and zebrafish cDNA libraries (5). In humans, the Pim-1 gene is expressed mainly in the developing fetal liver and spleen (5) and in hematopoietic malignancies (6, 7). Two homologs of the Pim-1 gene, pim-2 (8) and pim-3/kid-1 (9), have also been identified.

The human Pim-1 gene encodes a 313-amino acid serine/threonine kinase (10) and is associated with multiple cellular functions such as proliferation, differentiation, apoptosis, and tumorigenesis (1). Several cellular substrates of Pim-1 have been identified, including the transcription factors cMyb (11) and NFATc1 (12), the transcriptional co-activator of cMyb p100 (13), phosphatases Cdc25A (14) and PTPU2S (15), Pim-1-associated protein 1 (16), cell cycle inhibitor p21/WAF1 (17), heterochromatin protein 1 (18), TRAF2/SNX6 (19), and nuclear mitotic apparatus (20). The consensus sequence for Pim-1 substrate recognition is Lys/Arg-Lys/Arg-Leu-Ser/Thr-Xaa, where Xaa is an amino acid with a small side chain (21). The expression of Pim-1 is induced by a number of cytokines, mitogens, and hormones (reviewed in Ref. 1). The JAK/STAT (22, 23), AKT (24), mitogen-activated protein kinase, and phosphatidylinositol 3-kinase (PI3K) (23) pathways may all mediate Pim-1 expression.

The x-ray structure of Pim-1 was pursued, in part, to determine how an unusual sequence feature in the active site affects ligand binding. In protein kinases, one hydrogen bond between the ATP molecule and the amino acid side chain of a proline residue in the hinge region is conserved (3). Since the initial report of the cloning of mouse Pim-1 gene (4), Pim-1 has been cloned from human, rat, bovine, and zebrafish cDNA libraries (5). In humans, the Pim-1 gene is expressed mainly in the developing fetal liver and spleen (5) and in hematopoietic malignancies (6, 7). Two homologs of the Pim-1 gene, pim-2 (8) and pim-3/kid-1 (9), have also been identified.

The human Pim-1 gene encodes a 313-amino acid serine/threonine kinase (10) and is associated with multiple cellular functions such as proliferation, differentiation, apoptosis, and tumorigenesis (1). Several cellular substrates of Pim-1 have been identified, including the transcription factors cMyb (11) and NFATc1 (12), the transcriptional co-activator of cMyb p100 (13), phosphatases Cdc25A (14) and PTPU2S (15), Pim-1-associated protein 1 (16), cell cycle inhibitor p21/WAF1 (17), heterochromatin protein 1 (18), TRAF2/SNX6 (19), and nuclear mitotic apparatus (20). The consensus sequence for Pim-1 substrate recognition is Lys/Arg-Lys/Arg-Leu-Ser/Thr-Xaa, where Xaa is an amino acid with a small side chain (21). The expression of Pim-1 is induced by a number of cytokines, mitogens, and hormones (reviewed in Ref. 1). The JAK/STAT (22, 23), AKT (24), mitogen-activated protein kinase, and phosphatidylinositol 3-kinase (PI3K) (23) pathways may all mediate Pim-1 expression.

EXPERIMENTAL PROCEDURES

Cloning and Expression of Pim-1—Full-length Pim-1 (residues Met1–Lys125) was cloned in two parts by PCR from a human IMAGE Consor-

* This work was supported in part by the United States Department of Energy under Contract DE-AC03-76SF00098 at Lawrence Berkeley National Laboratory. The costs of publication of this article were de-

‡ To whom correspondence should be addressed: 130 Waverly St., Cambridge, MA 02139. Tel.: 617-444-6451; Fax: 617-444-6566; E-mail: marc_jacobs@vrtx.com.
tium clone (GenBank™ accession number GI 1845036) and from a human bone marrow cDNA library (BD Biosciences, Clontech, Palo Alto, CA). The pieces were fused by PCR and inserted into the NdeI and EcoRI sites of the dual promoter vector pBEV1, encoding a protein with an N-terminal His6 tag and thrombin cleavage site (26). The amino acid sequence of this Pim-1 clone is identical to SwissProt entry P11309.

BL21/DE3 pLysS Escherichia coli cells were transformed with the construct encoding full-length human Pim-1 kinase, using a standard transformation protocol (Stratagene, La Jolla, CA). Freshly transformed cells were grown at 37 °C in brain heart infusion medium (DIFCO Laboratories, Detroit, MI) supplemented with 100 μg/ml carbenicillin and 35 μg/ml chloramphenicol. The cells were grown at 37 °C up to an optical density of 0.75 at 600 nm, and expression was induced at 28 °C with 1 mM isopropyl-β-D-thiogalactopyranoside. The cells were harvested via centrifugation 4 h post-induction and stored at −80 °C prior to purification.

Protein Purification—Frozen cell pellets (~30 g) were thawed in 7 volumes of Buffer A (50 mM HEPES, pH 7.8, 300 mM NaCl, 10% (v/v) glycerol, 3 mM β-mercaptoethanol) containing 0.1% (v/v) Tween 20, 50 μM diisopropyl fluorophosphate, 1 μg/ml E-64, 1 μg/ml leupeptin, and 10 μg/ml pepstatin (Roche Applied Science) and lysed in a microfluidizer (Microfluidics, Newton, MA). The lysate was centrifuged at 54,000 g for 45 min, and the supernatant was incubated with 1 ml of Talon metal affinity resin (BD Biosciences, Clontech)/5 mg of protein overnight at 4 °C. The cells were washed with 20 column volumes of Buffer A and eluted with Buffer A containing 100 mM imidazole. Fractions containing Pim-1 were pooled and concentrated by ultrafiltration using a 30-kDa molecular mass cut-off membrane in an Amicon stirred cell concentrator (Millipore, Billerica, MA). The Pim-1 fraction was loaded onto a Superdex 200 column (90 × 2.6 cm; Amersham Biosciences) equilibrated in Buffer B (50 mM HEPES, pH 7.8, 200 mM NaCl, 10% (v/v) glycerol, and 5 mM β-mercaptoethanol). The fractions were pooled based on SDS-PAGE, diluted to 25 mM NaCl with 50 mM HEPES, pH 7.8, 10% (v/v) glycerol, and 5 mM dithiothreitol (DTT), and loaded onto a 8-ml prepacked MonoQ (HR 10/10) anion exchange column (Amersham Biosciences) equilibrated in Buffer C (50 mM HEPES, pH 7.8, 20 mM NaCl, 10% (v/v) glycerol, 5 mM DTT). Pim-1 was eluted using a gradient of 0–40% Buffer D (buffer C plus 1M NaCl) over 60 column volumes. Peak fractions were collected as four separate pools (I–IV) based on the elution chromatogram. The Pim-1 fraction was dialyzed into 20 mM Tris, pH 8.0 (25 °C), 100 mM NaCl, 5 mM DTT and concentrated to 10 mg/ml using a 10-kDa molecular mass cut-off Vivaspin concentrator (Vivascience, Hanover, Germany). The identity of the purified Pim-1 was confirmed by N-terminal amino acid sequencing.

Typically, preparations contained a mixture of species with 0–5 phosphoryl groups, which were partially resolved by anion exchange chromatography (Fig. 1). Phosphoamino acid analysis showed that Pim-1 purified from E. coli was extensively phosphorylated in the His6 tag (MGSSHHHHHHSSGLVPRGSH), and the four MonoQ pools differed mainly in the degree of phosphorylation in this region. Dephosphorylation of Pim-1 was accomplished by Lambda phosphatase (New England Biolabs) followed by autophosphorylation showed that Pim-1 readily autophosphorylates in the His6 tag region (data not shown). Ser99S was the major phosphorylation site observed in Pools III and IV. Other minor sites, Ser3, Thr32S, and Ser39S, were phosphorylated to varying degrees in each pool. Pim-1 crystallized from different MonoQ pools yielded similar crystal forms.

The phosphorylation state of each of the MonoQ-purified pools I–IV of Pim-1 was determined by electrospray mass spectrometry of thrombin-cleaved Pim-1. Spectra were collected using a Micromass Quattro II triple quadruple mass spectrometer (Waters Corp., Milford, MA) (27). Phosphorylation sites were identified from tryptic digests of the MonoQ-purified pools I–IV to liquid chromatography-tandem mass spectrometry on a QSTAR Pulsar quadrupole time-of-flight tandem mass spectrometer (AB/MDS-Scieix, Toronto, Canada) equipped with a nanoelectrospray ion source (MDS Protea, Odense, Denmark). The data were analyzed using the Mascot search engine (Matrix Science, London, UK).
Kinase Assays—A coupled-enzyme assay (28) was used to quantify the ADP generated in the kinase reaction with S6 peptide (RRLSSSLRRA) (American Peptide Co, Sunnyvale, CA) as the phosphoacceptor substrate. The assay was carried out in a total volume of 100 μl in 0.1 M HEPES, pH 7.6, containing 10 mM MgCl2, 2.5 mM phosphoenolpyruvate, 0.2 mM NADH, 30 μg/ml pyruvate kinase, 10 μg/ml lactate dehydrogenase (Roche Applied Science), and 2 mM DTT in a 96-well plate and read at 340 nm at 30 °C on a Spectramax spectrophotometer.

Enzymatic Activity—Kinase activity assays of the MonoQ pools I–IV were performed to determine whether the observed phosphorylation affected the catalytic activity. All four pools showed very similar kinetic parameters (kcat = 4 ± 0.4 s−1, peptide Km = 51 ± 2 μM, and ATP Km = 120 ± 16 μM). Pim-1 purified from E. coli was phosphorylated at Ser261 as well as multiple sites in the His tag region. Palaty et al. (36) have identified Ser180 in Xenopus Pim-3 as the major autophosphorylation site and showed that S190A and S190E mutants are 7-fold less active than the wild type Pim-3. The equivalent residue in human Pim-1, Ser180, was not phosphorylated in the E. coli purified preparations. The fact that all four MonoQ Pim-1 pools exhibit very similar kinetic parameters indicates that the enzyme is constitutively active and that the phosphorylation state does not affect enzymatic activity. The specific activity (5 ± 0.2 μmol/min/mg) observed here is much higher than previously reported (21, 36–38). It is 60-fold greater than that reported by Friedman et al. (21) for human GST-Pim-1 using a histone H1 peptide (KRRASGP) and over 104-fold greater than that reported by Palaty et al. (38) for GST fusions of human Pim-1 using S6 peptide (AKRRRLSSLRRA). Because both studies utilized GST fusions for expression and purification, it is possible that this large protein tag had a detrimental effect on enzyme activity, either by interfering with substrate access to the active site or with overall protein folding.

| Data set       | Staurosporine | Adenosine | LY294002 |
|---------------|---------------|-----------|----------|
| X-ray source  | Rigaku RU-H3R | ALS 5.0.2 | Rigaku RU-H3R |
| Space group   | P63           | P63       | P63      |
| Unit cell parameters (Å) | a = b = 97.7, c = 80.5 | a = b = 98.3, c = 80.4 | a = b = 97.7 |
| Resolution (Å) | 20–2.4        | 20–2.4    | 20–2.5   |
| Unique reflections | 22615         | 16430     | 14445    |
| Redundancy    | 3.6           | 5.2       | 3.1      |
| Completeness (%) | 94.9 (74.8)   | 94.3 (96.1) | 94.9 (87.6) |
| Rmerge (I/σ(I)) | 0.050 (0.250) | 0.060 (0.361) | 0.072 (0.336) |
| Refinement    | 10.6 (2.3)    | 14.7 (3.9) | 12.0 (2.6) |

Reflections used 22526 1706 1268
Test reflections 16152 1268 1097
R factor 0.205 0.210 0.208
Free R factor (% data) 0.233 (7.6) 0.246 (7.9) 0.259 (7.7)
Root mean square deviation Bond lengths (Å) 0.015 0.007 0.009
Bond angles (°) 1.7 1.3 1.2
Dihedral angles (°) 25.1 22.8 22.2
Protein atoms 2202 2202 2202
Solvent atoms 142 81 136

The refined model consists of the protein kinase catalytic domain. Although full-length protein was used for crystallization (313 residues), 32 residues at the N terminus, 8 residues at the C terminus, and 4 residues in one loop (80–83) were not sufficiently ordered to be built into the electron density. Phosphorylation of Ser261 is clearly visible in the electron density map. The phosphoserine side chain participates in both intramolecular interactions and may be important in formation of the crystal packing interactions. Also, the electron density map reveals additional density adjacent to the sulfur of Cys161, indicating an adduct at this residue. The electron density was large enough to accommodate four nonhydrogen atoms; it was modeled as a β-mercaptoethanol adduct; however, it is also consistent with a partially ordered DTT adduct. Both DTT and β-mercaptoethanol were used in the purification.

RESULTS AND DISCUSSION
A panel of kinase inhibitors was evaluated for their ability to inhibit Pim-1. Staurosporine and structurally similar compounds, such as K-252a and bisindolyl-maleimides I and IX, were found to inhibit Pim-1 with submicromolar potency (Table 1). These compounds are nonspecific inhibitors of Ser/Thr and Tyr kinases (39). Surprisingly, LY294002 was found to be an inhibitor of Pim-1 with IC\textsubscript{50} = 4 \muM. This compound was originally described as a specific inhibitor of PI3K with 1.4 \muM IC\textsubscript{50} (40). Later, Davies et al. (39) reported that LY294002 inhibits PI3K and casein kinase 2 with a similar potency (10 and 6.9 \muM, respectively).

**Protein Structure**—The structure of Pim-1 has a global fold typical of protein serine/threonine kinases, consisting of two domains linked by a hinge region (Fig. 2). The smaller, N-terminal domain (residues 33–121) consists primarily of \( \beta \)-strands with one \( \alpha \)-helix, and the C-terminal domain (residues 128–305) is largely \( \alpha \)-helical. The active site is formed by a groove at the interface between these two domains and is enclosed by the hinge region (residues 122–127), the glycine-rich loop (residues 44–52), and the activation loop (residues 186–210). The Pim-1 structure was compared with several other protein kinases with high sequence homology such as cAMP-dependent kinase (PKA) and phosphorylase kinase. When secondary structural elements are aligned, a root mean square deviation of 1.3 Å for C-\( \alpha \) atom positions is observed between Pim-1 and both PKA or phosphorylase kinase (using 213 residues from phosphorylase kinase (Protein Data Bank code 1PHK) (31) and 220 residues from cAMP-dependent kinase (Protein Data Bank code 1ATP) (41), respectively). The Pim-1 protein structures described here are similar to unliganded Pim-1 (25), with a root mean square deviation of 0.4 Å for C-\( \alpha \) atoms.

The conformation of the glycine-rich loop (residues 45–52) in this structure differs from that of the PKA structures and most closely resembles that of unliganded Pim-1 (25). The Pim-1 glycine-rich loop moves toward the C-terminal domain, and Phe\textsuperscript{49} adopts a rotamer in which the side chain points toward the hinge region, thereby filling the space usually occupied by ATP phosphates (Fig. 2). In the Pim-1-AMP-PNP complex structure, the ligand phosphates displace Phe\textsuperscript{49}, and the loop adopts a more open conformation. This is similar to GSK-3\( \beta \), for instance, where the corresponding phenylalanine residue is observed both within the active site pointing toward the hinge (peptide complex) (42) and, in another structure (unliganded), outside the active site, pointing away from the hinge (43).

The Pim-1 hinge sequence is unusual because of a two-residue insertion relative to kinases such as CDK-2 (44) and JNK-3 (45) and a single residue insertion relative to PKA. A comparison of the hinge regions of Pim-1 and PKA is shown in Fig. 3. Residues before and after the insertion superimpose well; however, at the point of insertion (Pro\textsuperscript{125}), the hinge bulges away from the ATP-binding site by up to 4 Å. Some of the additional space created by the change in main chain position is occupied by the Val\textsuperscript{126} side chain, which is oriented toward the ATP-binding pocket and interacts with Pro\textsuperscript{123}. This unique hinge conformation could be utilized for the design of specific Pim-1 inhibitors and creates a space for substitution at the position corresponding to C-2 of ATP. For instance, polar interactions with the carbonyl oxygen of Pro\textsuperscript{123} or hydrophobic contacts with the side chain of Val\textsuperscript{126} would be unique to PIM. One kinase sharing this two-residue insertion is PI3K (46). Although there is little sequence homology in the hinge region between PI3K and Pim-1, the main chain conformations are remarkably similar (0.86 Å root mean square deviation over 13 C-\( \alpha \) positions). The PI3K and Pim-1 hinge conformation differ most at Pro\textsuperscript{125} (Asp\textsuperscript{884} in PI3K) (Fig. 4).

**Adenosine and Staurosporine Ligand Structures**—The positions of staurosporine and adenosine bound to Pim-1 are similar to those observed in other protein kinase complex structures. Both compounds are sandwiched between hydrophobic residues from the glycine rich loop, the C-terminal domain, and the hinge. In each case, only one hydrogen bond between the ligand and the hinge is observed. In the Pim-1-adenosine complex, a hydrogen bond is observed between the Glu\textsuperscript{121} main chain carbonyl and the N-6 amino group. The pyrrolidinone nitrogen of staurosporine forms a similar hydrogen bond. The presence of proline at position 123 prevents each of the two compounds from forming the second hydrogen bond to the hinge.

The Pim-1 ligand structures were aligned and compared with PKA. Relative to the PKA-adenosine complex (Protein Data Bank code 1FMO) (47), the adenosine in Pim-1 rotates by \( \sim 20^\circ \) toward the hinge into the additional space formed by the proline insertion (rotation axis perpendicular to the plane of the adenine ring; see Fig. 3B). The purine rings of adenosine in the Pim-1 and PKA structures are approximately co-planar. The relative position of the adenine in the two structures is, in part, fixed by the length of the side chain to which the sugar moiety forms a hydrogen bond (Asp\textsuperscript{128} in Pim-1 and Glu\textsuperscript{128} in PKA). Likewise, the aromatic rings of staurosporine in Pim-1 and PKA (Protein Data Bank code 1STC) (48) are approximately co-planar. Staurosporine in Pim-1 rotates toward the hinge by about 10° and also forms a hydrogen bond to Asp\textsuperscript{128} (Fig. 3A).

A comparison of the ligand positions in the Pim-1 and the PI3K complexes (Protein Data Bank codes 1E8Z and 1E8X) (46, 49) reveals a shift and a rotation. In PI3K, two hydrogen bonds are made between the main chain (Val\textsuperscript{882} NH and Glu\textsuperscript{880} O).
and the ligands (adenosine N1 and N6 and staurosporine pyrrolidinone N and O). Adenosine and staurosporine bound to PI3K are shifted toward the outermost edge of the hinge by about 2.5 Å relative to the Pim-1 structure (Fig. 4). Also, the staurosporine is tilted about 30° about an axis parallel to the main chain of the hinge (between Ile879 and Val882), such that the pyrrolidinone ring lies below (toward the C-terminal domain) the same ring in the Pim-1 structure (Fig. 4A).

Although the hinge conformations between Pim-1 and PI3K are very similar, the positions of adenosine and staurosporine differ. In fact, the orientations of the ligands in Pim-1 more closely resemble those found in other protein kinases. The PI3K binding mode, characterized by the shift toward the hinge and tilt toward the C-terminal domain, is sterically hindered in Pim-1. The presence of the C-δ atom of Pro123 and the larger side chain at position 126 (valine instead of alanine) prevent the shift toward the hinge. The tilt toward the C-terminal domain is hindered by the side chain of Ile104 in Pim-1. In the absence of the conserved pair of hydrogen bonds to the adenine ring of ATP, the contacts between the Pim-1 hinge and LY294002 are highly unusual. Typically, protein kinase ligands interact with the hinge via hydrogen bonds, where the donor hydrogen is bonded to either oxygen or nitrogen. In this case, only hydrogens bonded to aromatic carbon atoms interact with the hinge. If indeed these interactions are important for LY294002 binding, we would expect the arrangement of the atoms to be favorable for hydrogen bonding. The ideal (C)H to O distance is ~2.6–2.7 Å, and the distance between the Glu121 carbonyl and the LY294002 hydrogens is 2.6 and 2.9 Å (50). The ideal O-CH angle is between 90 and 180°. The angles observed with LY294002 in Pim-1 are 140 and 130°.

The contacts between the Pim-1 hinge and LY294002 are highly unusual. Typically, protein kinase ligands interact with the hinge via hydrogen bonds, where the donor hydrogen is bonded to either oxygen or nitrogen. In this case, only hydrogens bonded to aromatic carbon atoms interact with the hinge. If indeed these interactions are important for LY294002 binding, we would expect the arrangement of the atoms to be favorable for hydrogen bonding. The ideal (C)H to O distance is ~2.6–2.7 Å, and the distance between the Glu121 carbonyl and the LY294002 hydrogens is 2.6 and 2.9 Å (50). The ideal O-CH angle is between 90 and 180°. The angles observed with LY294002 in Pim-1 are 140 and 130°. Further, the hydrogen and the amide bond should be co-planar, which is the case in the Pim-1-LY294002 complex. It is likely, therefore, that a pair of aromatic CH hydrogen bonds are formed between LY294002 and the Pim-1 hinge.
The fact that LY294002 binds to Pim-1, a protein with a PI3K-like hinge, appears to be coincidental, because the compound orientation is dramatically different in the two proteins. Although the proteins have structural similarities, none of the features in common contribute to the binding of LY294002. In fact, superposition of the two complexes reveals that the PI3K binding mode is sterically hindered by Pro123 in Pim-1. Also, the Pim-1 binding mode is incompatible with the PI3K structure; Trp182 in PI3K packs against the phenyl and the morpholine rings of LY294002 but would collide with the phenyl ring if the compound bound in the Pim-1 orientation.

The compound LY294002 is commonly used to assess the role of PI3K in cell signaling and does not significantly inhibit most protein kinases (39). For instance, PKA activity is reduced by only 9% (±5%) in the presence of 50 μM LY294002, so we would not expect the structure of PKA to easily accommodate LY294002 binding. Indeed, both the Pim-1 and PI3K binding modes are sterically hindered by Thr183 and Val123, respectively, in PKA. One kinase inhibited by LY294002 is casein kinase 2 (CK2) (IC50, 6.9 μM). The structures of CK2 and Pim-1 were aligned to predict how LY294002 might bind to CK2. The PI3K binding mode is blocked by the side chain of Val116 in CK2. However, the CK2 active site will accommodate LY294002 in the Pim-1 binding mode, with a only 0.5 Å translation to avoid a close contact with Ile86.

Interestingly, in addition to kinases, LY294002 has also been observed to bind to proteins with unrelated sequences and functions. In these cases, inhibition by LY294002, but not by wortmannin (another PI3K inhibitor), was observed and indicated a PI3K-independent mechanism. For instance, LY294002 has been shown to alter intracellular calcium concentrations in bronchial smooth muscle cells (51), block the Kv2.1 and Kv1.4 channels (52), and also bind to and inhibit estrogen receptor (53). This promiscuity may be due to the fact that LY294002 is a relatively small, planar, and unelaborated molecule with several hydrogen bonding opportunities. It is possible that there are other as yet unidentified targets of this compound, and therefore LY294002 should be used with caution in cellular assays.

Conclusions—In protein kinases, the hinge conformation and the hydrogen bonds to ATP are highly conserved. The Pim-1 structure reveals how a catalytically competent sub-
strate complex can form even when the hinge is unusual in both sequence and conformation. The structures of the adenosine and staurosporine complexes show van der Waals contacts playing the same role as a conserved hydrogen bond to position the substrate. Although the Pim-1 hinge closely resembles the analogous region in the active site of PI3K, the compound LY294002 interacts with the hinges of the two proteins in very different ways. This new binding mode provides a model to show how LY294002 might inhibit other protein kinases, and this structure can be used to aid in the design of specific inhibitors, utilizing unique features of the Pim-1 active site.

Acknowledgments—We thank H. Siasoa for preparation of DNA constructs, J. Fulghum for protein expression, M. Pitta for mass spectrometry, X. Liu for analytical ultracentrifugation, and G. McDermott and the staff at Beamline 5.0.2 at the Advanced Light Source for assistance.

REFERENCES

1. Wang, Z., Bhattacharya, N., Weaver, M., Petersen, K., Meyer, M., Gapter, L., and Magnuson, N. S. (2003) J. Biol. Chem. 278, 1524–1527
2. Levenson, J. D., Koskinen, P. J., Orrico, F. C., Rainio, E. M., Jalkanen, K. J., Dash, A. B., Eisenman, R. N., and Ness, S. A. (1998) Mol. Cell. 2, 417–425
3. Moehle, K., Tishkova, C., Noguchi, K., Muramatu, T., Asai, A., and Kushino, T. (1999) J. Biol. Chem. 274, 16859–16866
4. Wang, Z., Bhattacharya, N., Boccelli, L., Seimiya, H., Tsuruo, T., Tischer, G., and Ariga, H. (2001) Arch. Biochem. Biophys. 390, 9–18
5. Maita, H., Harada, Y., Nakagubo, D., Kita, K., Ikeda, M., Tani, K., Takahashi, K., Ariga, H., and Ichiguchi-Ariga, S. (2000) J. Biol. Chem. 275, 5618–56178
6. Wang, Z., Bhattacharya, N., Dixkens, P. P., Wei, W., Sedivy, J., and Magnuson, N. S. (2002) Biochem. Biophys. Res. Commun. 293, 45–55
7. Kaike, N., Maita, H., Taira, T., Ariga, H., and Ichiguchi-Ariga, S. (2000) FEBS Lett. 467, 17–21
8. Bhattacharya, N., Maddox, J. D., Bhattacharya, N., Wehr, E., and Magnuson, N. S. (1999) Arch. Biochem. Biophys. 368, 376–385
9. Nagata, Y., Nakagisa, H., Nakagawa, T., and Todokoro, K. (1997) Leukemia 11, 553–556
10. Krieken, J. S., Narang, V. S., Bucker, D. J., and Bucker, A. R. (2001) J. Neuroimmunol. 113, 249–259
11. Krishnam, N., Fan, H., Bucker, D. J., and Bucker, A. R. (2003) Endocrine 20, 123–130
12. Dong, D. J., Noble, M. E., Garman, E. F., Papageorgiou, A. C., and Johnson, L. N. (1995) Structure 3, 477–482
13. Tereshko, V., Teplova, M., Brunzelle, J., Wattersen, D. E., and Eglit, M. (2001) Nat. Struct. Biol. 8, 899–907
14. CCP4 (1994) Acta Crystallogr. Sect. D Biol. Crystallogr. 50, 760–763
15. Rice, L. M., and Brunger, A. T. (1994) Proteins 19, 277–290
16. Roversi, P., Bianci, E., Vonghe, C., Evans, G., and Bricogne, G. (2000) Acta Crystallogr. Sect. D Biol. Crystallogr. 56, 1316–1323
17. Polanyi, C., Clark-Lewis, I., Leung, D., and Pelech, S. L. (1997) Biochem. Cell Biol. 75, 153–162
18. Davies, S. F., Reddy, H., Caivano, M., and Cohen, P. (2000) Biochem. J. 351, 95–105
19. Vlahos, C. J., Matter, W. P., Huss, H., and Brown, R. F. (1994) J. Biol. Chem. 269, 5241–5248
20. Zheng, J., Knight, D. R., ten Eyck, L. F., Karlsson, R., Xunng, N., Taylor, S. S., and Sowadski, J. M. (1993) Biochemistry 32, 1524–1527
21. Cui, B., Carter, P. S., Lewis, D. E., Beitz, H., Rennert, R., Petman, G., Manzur, C., Cubert, A. A., Brown, M. J., Smith, D. G., and Reith, A. D. (2001) Structure 9, 1143–1152
22. ter Haar, C., Coll, J. T., Xie, X., Ford, P. J., Germann, U. A., Porter, M. D., and Fox, T. (1998) Acta Crystallogr. Sect. D Biol. Crystallogr. 54, 599–608
23. Worsley, K., Teplova, M., Brunzelle, J., Wattersen, D. E., and Eglit, M. (2001) Nat. Struct. Biol. 8, 899–907
24. Prade, L., Ehrich, R. A., Aebischer, M., and Pelech, S. L. (1997) Structure 5, 1627–1637
25. Pasapera Limon, A. M., Herrera-Munoz, J., Gutierrez-Sagal, R., and Ulloa-Aguirre, A. (2000) Mol. Cell. Endocrinol. 198, 239–248
26. DeLano, W. L. (2002) The PyMOL Molecular Graphics System, DeLano Scientific, San Carlos, CA
27. Cohen, P. (2002) Nat. Rev. Drug. Discov. 1, 309–315
28. Dumas, J. (2001) J. Exp. Clin. Oncol. 2, 405–429
29. Hashimoto, Y., Nakayama, T., Teramoto, T., Kato, H., Watanabe, T., Kinosita, M., Tsukamoto, K., Tokunaga, K., Kurokawa, K., and Nakashima, S. (1991) Biochem. Biophys. Res. Commun. 181, 432–439
30. Berg, M. M., Sternberg, D. W., Parada, L. F., and Chao, M. V. (1992) J. Biol. Chem. 267, 13–16
31. Harris, T. E., Persaud, S. J., and Jones, P. M. (1996) Biochem. Biophys. Res. Commun. 227, 672–676
32. Tereshko, V., Teplova, M., Brunzelle, J., Wattersen, D. E., and Eglit, M. (2001) Nat. Struct. Biol. 8, 899–907
33. CCP4 (1994) Acta Crystallogr. Sect. D Biol. Crystallogr. 50, 760–763
34. Rice, L. M., and Brunger, A. T. (1994) Proteins 19, 277–290
35. Roversi, P., Bianci, E., Vonghe, C., Evans, G., and Bricogne, G. (2000) Acta Crystallogr. Sect. D Biol. Crystallogr. 56, 1316–1323
36. Palaty, C. K., Kalmar, G., Tai, G., Oh, S., Amankwa, L., Affolter, M., Aebeli, J., and L. Sawyer, N. W. I., and S. Bailey, eds) SERC Daresbury Laboratory, Warrington, UK
37. Ariga, H., and Iguchi-Ariga, S. M. (2001) Biochemistry 40, 1524–1527
38. Kim, S. H. (1993) FEBS Lett. 314, 31–36
39. Davies, S. F., Reddy, H., Caivano, M., and Cohen, P. (2000) Biochem. J. 351, 95–105