The Structure of Dimeric ROCK I Reveals the Mechanism for Ligand Selectivity*

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ROCK or Rho-associated kinase, a serine/threonine kinase, is an effector of Rho-dependent signaling and is involved in actin-cytoskeleton assembly and cell motility and contraction. The ROCK protein consists of several domains: an N-terminal region, a kinase catalytic domain, a coiled-coil domain containing a RhoA binding site, and a pleckstrin homology domain. The C-terminal region of ROCK binds to and inhibits the kinase catalytic domains, and this inhibition is reversed by binding RhoA, a small GTPase. Here we present the structure of the N-terminal region and the kinase domain. In our structure, two N-terminal regions interact to form a dimerization domain linking two kinase domains together. This spatial arrangement presents the kinase active sites and regulatory sequences on a common face affording the possibility of both kinases simultaneously interacting with a dimeric inhibitory domain or with a dimeric substrate. The kinase domain adopts a catalytically competent conformation; however, no phosphorylation of active site residues is observed in the structure. We also determined the structures of ROCK bound to four different ATP-competitive small molecule inhibitors (Y-27632, fasudil, hydroxyfasudil, and H-1152P). Each of these compounds binds with reduced affinity to cAMP-dependent kinase (PKA), a highly homologous kinase. Subtle differences exist between the ROCK- and PKA-bound conformations of the inhibitors that suggest that interactions with a single amino acid of the active site (Ala215 in ROCK and Thr216 in PKA) determine the relative selectivity of these compounds. Hydroxyfasudil, a metabolite of fasudil, may be selective for ROCK over PKA through a reversed binding orientation.

Because desired therapeutic outcomes would likely result from a reduction of ROCK activity, much research has focused on designing small molecule inhibitors of ROCK.

There are two isoforms of ROCK, known as ROCK I and II, or Rho-kinase β and α, respectively (5). These two kinases regulate the activity of muscle myosin regulatory light chain (RLC)2 proteins by direct phosphorylation, (6, 7) and by phosphorylation and inhibition of the myosin binding subunit of myosin phosphatase. This leads to increased levels of phosphorylated myosin light chain and subsequent muscle contraction (8). The Rho/ROCK pathway is also involved in nonmuscle myosin regulation and has been implicated in stress fiber and focal adhesion formation (9), neurite retraction (10), and tumor cell invasion (11).

The importance of ROCK in regulating such key processes has been recognized, and considerable effort has been expended on delineating the details of its mechanism of action. ROCK is composed of a catalytic kinase domain (residues 73–405), a coiled-coil region (residues 425–1100), and a C-terminal pleckstrin homology domain (residues 1103–1230). A similar domain organization is shared with three closely related kinases: myotonic dystrophy kinase (DMPK) (12), myotonic dystrophy kinase-related Cdc42-binding kinase (MRCK) (13), and citron kinase (CRIK) (14). The work described herein represents the first structure of a DMPK family member.

Full-length ROCK, in the absence of effector molecules, exists as an autoinhibited structure. The C-terminal region of ROCK (coiled-coil and pleckstrin homology domains) has been shown to partially inhibit the kinase catalytic activity by binding directly to the kinase domain (15). When GTP-bound RhoA binds to the Rho-binding region of the coiled-coil domain, the interactions between the catalytic kinase domain and the C-terminal region are disrupted, relieving the inhibition (16). Cleavage of the C-terminal inhibitory domain from the catalytic domain by caspase-3 during apoptosis also activates the enzyme (17, 18).

We have previously characterized the oligomerization state of full-length ROCK and four truncated constructs using light scattering and analytical ultracentrifugation methods (19). Protein constructs containing residues 6–415 exist as dimers, whereas smaller constructs are predominantly monomeric. ROCK (6–415) proved to be highly homogeneous in solution and yielded diffraction quality crystals suitable for x-ray structure determination. This construct lacks the C-terminal inhibitory domains and is similar to the caspase-3-activated form of ROCK. An analogous form of truncated CRIK has also been observed in vivo (14).

Here we report the x-ray crystal structure of ROCK bound to ATP-competitive inhibitors. The structure reveals two kinase domains linked by an N-terminal dimerization domain comprised of five α-helices from each monomer. In this arrangement, the active sites of the two kinases share a common face, possibly facilitating interactions with dimeric...
substrates or inhibitory domains. Each kinase domain appears to have a catalytically competent conformation in the absence of phosphorylation. In full-length ROCK, interactions with the C-terminal domains may change this arrangement of the kinase domains or alter the structure of a regulatory element such as the activation loop.

Crystal structures of four ROCK protein-ligand complexes were determined containing the inhibitors Y-27632, fasudil (HA-1077), hydroxyfasudil (HA-1100), and a dimethylated analog of fasudil (H-1152P). Y-27632 is a pyridine compound that is chemically distinct from the other three isoquinoline based ligands. In a prior study, Breitenlechner et al. (20) determined the structures of Y-27632, fasudil, and H-1152P bound to PKA. To allow comparisons among all four ligands in both ROCK and PKA, we also determined the structure of PKA bound to hydroxyfasudil. All four compounds are competitive inhibitors at the ATP binding site. Several previous studies have established that ROCK 6–415 construct was purified using an identical procedure except that the clostripain proteolysis step was no longer required.

**EXPERIMENTAL PROCEDURES**

Cloning and Expression—Rock I (Swiss-Prot code Q13464) was isolated from a human leukocyte cDNA library. The cDNA encoding the ROCK protein comprising residues 6–553 and 6–415 were cloned into a baculoviral transfer vector, pBEV10, and expressed in insect cells as described (29). The proteins were engineered to include a hexahistidine tag (His6) at the N terminus to facilitate purification.

Protein Purification—The protein used for crystallization was first produced by proteolytic cleavage of a larger construct and later through construction of a DNA expression vector containing sequences corresponding to the desired proteolytic product. The ROCK Ser6–Leu553 construct was metal affinity-purified as described (19). After thrombin cleavage of the His tag, the sample was treated with clostripain (Washingtion Chemicals) at a ratio of 1:100 (w/w) and 10 mM CaCl2 for 3 h at room temperature followed by overnight incubation at 4 °C. This yielded a C-terminal truncation whose limits were determined to be 6–415 by N-terminal sequencing (Applied Biosystems Procise STR Biospectrometry work station).

The protein was then diluted 10-fold with 20 mM HEPES, pH 7.4, loaded onto a MonoQ HR 5/5 ion exchange column (Amersham Biosciences), and eluted with a 100–400 mM NaCl gradient over 40 column volumes. The sample was then loaded onto a HiLoad 16/60 Superdex 200 column (Amersham Biosciences) equilibrated with 20 mM HEPES, pH 7.4, 200 mM NaCl, and 2 mM β-mercaptoethanol. The main peak was pooled and appeared to be 99% pure as judged by SDS-PAGE analysis. The ROCK 6–415 construct was purified using an identical procedure except that the clostripain proteolysis step was no longer required.

Ligand Inhibition Measurements—The inhibition constant, $K_i$, for inhibiting PKA and ROCK were obtained from dose-response titration curves. The residual enzyme activity remaining was determined by the spectrophotometric coupled enzyme assay as previously described (30). In this assay, every mole of ADP generated in the kinase reaction is coupled to the generation of NAD from NADH using pyruvate kinase and lactate dehydrogenase. The final concentration of the assay components were as follows: 0.1 M HEPES, pH 7.6, 10 mM MgCl2, 1 mM dithiothreitol, 2.5 mM phosphoenolpyruvate, 200 μM NADH, 2.5% Me2SO, 50 μg/ml pyruvate kinase, and 10 μg/ml lactate dehydrogenase. The ATP and peptide concentrations were 10 and 15 μM (LRRASLG) for PKA and 15 μM and 45 μM (KKRRNTLTSV) for ROCK I assays, respectively. Assay plates were incubated at 30 °C for 10 min, and the absorbance change at 340 nm was monitored (Table 1).

**Crystallization and X-ray Analysis**—ROCK crystals were grown by the vapor diffusion method at 22 °C. Equal volumes of protein stock solution (20 mg/ml protein, 20 mM HEPES, pH 7.8, 100 mM NaCl, 2 mM 2-mercaptoethanol) and well solution (3–8% polyethylene glycol 3350, 100 mM MES, pH 5.5, 2.5 mM CaCl2, 10 mM dithiothreitol) were mixed and suspended over 1 ml of well solution. Over 4 days, the crystals reached a final size of ~200 μm. The crystals were harvested and flash-frozen in a solution composed of the well solution with 25–30% (v/v) glycerol. The Y-27632 (BIOMOL International L.P.) complex with ROCK was made by adding the ligand (2 mM) to the clostripain treated 6–553 construct prior to crystallization. Subsequent complexes with fasudil (Toronto Research Chemicals), hydroxyfasudil (prepared as in Ref. 31), and H-1152P (Calbiochem) were made by soaking unliganded crystals (ROCK 6–415) with 500 μM compound and 5% Me2SO (final concentration) for 48 h at room temperature.

The Y-27632/ROCK diffraction data were recorded at the COMCAT (32-ID) beamline at the Advanced Photon Source (Argonne National Laboratories and Emerald Biosciences). All other diffraction data were recorded at Beamline 5.0.2 at the Advanced Light Source (Lawrence Berkeley Laboratories). Intensities were integrated and scaled using the programs DENZO and SCALEPACK (32) and CrystalClear (33).

The structure was determined by molecular replacement using homology models based upon PKA (Protein Data Bank code 1ATP). The molecular replacement solution was determined using AMORE (34, 35). The crystals belong to the space group P3121. The asymmetric unit contained one trimer.

**TABLE 1 Inhibition constants determined for ROCK and PKA**

| Compound         | Structure          | ROCK $K_i$ (μM) | PKA $K_i$ (μM) | PKA $K_i$/ROCK $K_i$ |
|------------------|--------------------|----------------|---------------|---------------------|
| Y-27632         | ![Structure](image) | 0.15           | > 5           | > 33                |
| fasudil (HA-1077) | ![Structure](image) | 0.53           | 0.46          | 0.9                |
| hydroxyfasudil (HA-1100) | ![Structure](image) | 0.15           | 2.2           | 15                  |
| H-1152P         | ![Structure](image) | 0.006          | 0.34          | 57                  |

![Structure](image)
Structure of Rho-associated Kinase

### RESULTS

**Two Kinase Domains Interact via a Dimerization Domain**—The protein used in this study consists of the N-terminal region comprising approximately one-third of the full-length sequence of ROCK. The crystal structure reveals two protein molecules in a head-to-head arrangement, related by a pseudo-2-fold rotation noncrystallographic symmetry (Fig. 1A). Each monomer consists of an N-terminal helical domain (residues 5–72), a bilobed kinase domain (residues 73–356), and a kinase tail (residues 357–405). Helices from the two N-terminal domains interact with each other to form a single structure (Fig. 1, B and D). This region will be referred to hereafter as the dimerization domain. The kinase tail of each molecule lies across its own kinase domain and interacts with the dimerization domain.

The kinase domain of ROCK has a global fold typical of serine/threonine kinases, consisting of two lobes linked by a hinge region. The smaller, N-terminal lobe (residues 73–153) contains a twisted five-helix sandwich and a kinase tail (residues 357–405). Helices from the two N-terminal domains interact with each other to form a single structure (residues 383–405). The kinase tail lies roughly parallel to helix 2 and packs between its own kinase domain and helix 2 and also helix 4 of the noncrystallographic symmetry-related monomer (Fig. 1). Most of the contact surface area is comprised of the helices of one chain interacting with the helices of the other monomer. The kinase tails do not interact with each other except via contacts between Phe387 on each chain. The extensive interface between the two chains is largely hydrophobic and buried −4120 Å2 solvent-accessible surface area/dimer. For comparison, the buried surface area in complexes between a protein antigen and an antibody are typically less than 2000 Å2 (39). These dimer interactions orient the two monomers such that the active sites and regulatory elements of the kinase domains appear on a single face of the dimer (Fig. 1B).

**Active Conformation Independent of Phosphorylation**—ROCK is activated by disrupting the interactions between the kinase domain and regulatory elements in the C terminus, either through RhoA binding or cleavage of the C terminus by caspase-3. Because the crystallization construct lacks these inhibitory C-terminal domains, we would expect to observe a catalytically competent conformation. In particular, the residues of the active site should be aligned as in other catalytically active kinase structures, and the activation loop should not occlude the peptide substrate binding groove. The catalytic residues of the kinase domain were compared with the PKA structure to determine whether the ROCK structure represents a catalytically active conformation (40).

### Table 2

**Data collection and refinement statistics**

|                      | ROCK             | Fasudil          | Hydroxysfudil | H-1152P | PKA hydroxysfudil |
|----------------------|------------------|------------------|---------------|---------|------------------|
| X-ray source         | APS              | ALS              | ALS           | ALS     | Rigaku Baxis     |
| Space group          | P3,21            | P3,21            | P3,21         | P3,21   | P2,2,2           |
| Unit cell parameters (Å) | a = b = 180.9, c = 91.5 | a = b = 181.0, c = 89.4 | a = b = 181.8, c = 91.7 | a = b = 183.6, c = 91.7 | a = 72.8, b = 75.8, c = 80.0 |
| Resolution (Å)       | 20–2.6           | 20–3.2           | 20–2.95       | 20–3.3  | 20–2.2           |
| Redundancy           | 6.7              | 4.4              | 7.3           | 6.1     | 4.9              |
| Completeness (%)     | 90.8 (53.6)      | 92.2 (90.2)      | 93.3 (76.2)   | 99.4 (99.4) | 96.6 (84.5)     |
| Rmerge (%)           | 0.086 (0.40)     | 0.12 (0.38)      | 0.062 (0.34)  | 0.12 (0.37) | 0.075 (0.34)    |
| Dihedral angles (°)  | 22.6             | 22.2             | 22.7          | 22.4    | 22.3             |

Values for the highest resolution shell are shown in parentheses. $R_{merge} = \frac{\sum_{hkl} \sum_{i} (|I_{obs}(hkl) - |I_{calc}(hkl)|^2) \sum_{i} |I_{obs}(hkl)|^2}$ over i observations of reflection hkl.

R factor = $\frac{\sum_{hkl} (|I_{calc}(hkl)|^2 - |I_{calc}(hkl)|^2)}{\sum_{hkl} |I_{calc}(hkl)|^2}$ where $I_{calc}$ and $F_{calc}$ are the observed and calculated structure factors, respectively. Free R factor is calculated from a randomly chosen subset of reflections not used for refinement. The same reflection subset was used for all four ROCK data sets.
Among protein kinases, the conformation of the activation loop varies widely (reviewed in Ref. 41). In inactive kinase conformations, the activation loop often blocks the active site, interfering with peptide and ATP substrate binding. In active kinases, this loop lies parallel to helices E and F, and the active site is accessible. Although the sequence and conformation of the activation loop vary among active kinase conformations, they all share the same global conformation. In the ROCK structure, even though no phosphorylation is observed in the structure, the position of the activation loop resembles an active conformation. The activation loops of the two ROCK molecules in the asymmetric unit are very similar (0.3 Å root mean square difference over main chain atoms), diverging only at the region between the activation loop and the EF helix (Asp232–Pro238), where each of the two monomers makes different crystal lattice contacts. In this region, one ROCK monomer (chain A) most closely resembles the corresponding region in PKA, whereas these residues in the other monomer (chain B) move away from the active site by about 5 Å. These two different conformations indicate that these loop regions are likely to be flexible.

In many kinases, a catalytically competent conformation is stabilized by interactions with a phosphorylated residue on the activation loop. In the ROCK structure no phosphorylation is observed in the electron density map. Furthermore, the residues that typically interact with the phosphate group are not conserved in ROCK. In PKA for example, the active conformation of the loop is stabilized by interactions between a phosphothreonine (Thr197) and three basic residues (His87, Arg165, and Lys189) and one polar residue (Thr195). In ROCK, the corresponding residue (Thr233) appears unmodified and lies about 4 Å outside the active site relative to PKA. Also three of the four corresponding residues that are in a position to interact with the phosphothreonine in ROCK are hydrophobic (Phe120, Met221, and Cys231) (Fig. 3).

In the absence of interactions with a phosphorylated residue, we would expect other interactions to stabilize the loop conformation. Indeed, the interactions between the activation loop and the αEF/αF loop are more extensive in ROCK than in PKA. Both the αEF/αF loop and the activation loop are each four residues longer than the corresponding sequences in PKA (Fig. 2). These insertions almost double the buried surface between the two loops (437 Å² in PKA Ala188–Cys199/Ser212–Lys217, and 857 Å² in ROCK Cys220–Val235/Ser248–Arg257). The increased contact surface area in ROCK may be sufficient to stabilize the position of the activation loop in the absence of the phospho-threonine salt bridges observed in PKA and other kinases. Stabilization of the activation loop conformation through additional residues in the activation and αEF/αF loops has been observed in other kinases. For example, in phosphorylase kinase (Protein Data Bank code 1PHK) (42) additional residues in the αEF/αF loop stabilize the activation loop conformation, and in casein kinase 1 (Protein Data Bank code 1CK) (43) the activation loop is 10 residues longer than that found in PKA and forms additional contacts with the αEF/αF loop and the E helix (41).

Structures of ATP Site Inhibitor Complexes—The x-ray structures of ROCK bound to four small molecule inhibitors were determined: Y-27632, fasudil, hydroxyfasudil, and H-1152P. These compounds differ in their ability to inhibit ROCK kinase activity and in the selectivity of this inhibition in ROCK relative to PKA. Of these compounds, Y-27632 and H-1152P are more than 30-fold selective for ROCK over PKA, whereas hydroxyfasudil is 15-fold selective, and fasudil inhibits both enzymes with equal potency (Table 1).

The structure of Y-27632 bound to ROCK is shown in Fig. 4A. The pyridine ring occupies the same space as the adenine six-member ring in other kinase structures. The pyridine nitrogen accepts a hydrogen bond from the amide nitrogen of Met186, and an aromatic carbon donates a
hydrogen bond to the Glu\textsuperscript{154} main chain carbonyl (44). The amide carbonyl of Y-27632 interacts with the protein via a bound water molecule that in turn forms a hydrogen bond to the main chain amide nitrogen of Asp\textsuperscript{216}. Primary amines are quite basic (pK\textsubscript{a} \approx 10), so at neutral pH (or the acidic pH of the crystallization experiment), the compound has a \( +1 \) charge. This amine interacts with the side chains of Asn\textsuperscript{203} and Asp\textsuperscript{216}, residues that normally coordinate Mg\textsuperscript{2+} ions in kinase structures with bound ATP or ATP analogs.

In the fasudil-ROCK complex, the isoquinoline occupies the same space as the adenine of ATP occupies (Fig. 4\textsuperscript{B}). The sulfonamide and homopiperazine ring are located in the ribose-binding region. The isoquinoline nitrogen accepts a hydrogen bond from the amide nitrogen of Met\textsuperscript{156}, whereas the C-1 donates a hydrogen bond to the amide carbonyl of Glu\textsuperscript{154}. The homopiperazine secondary amine interacts with the side chain of Asp\textsuperscript{160}. Because of the low diffraction resolution, we cannot determine the ring pucker of the aliphatic homopiperazine ring of this ligand or the related two compounds (hydroxyfasudil and H-1152P). Fasudil is metabolized to hydroxyfasudil through the addition of an oxygen to the isoquinoline C-1 position. In a polar solvent such as water, the equilibrium between the tautomers isoquinolinone and isoquinoline is shifted toward isoquinolinone (45). In comparison to fasudil, where the isoquinoline N accepts and the C-1 donates a hydrogen bond, the interactions are reversed; the protonated N is now a hydrogen bond donor, whereas the carbonyl can only accept a hydrogen bond. We would therefore expect to observe a new binding mode for this compound. Indeed, the orientation of the isoquinolinone is reversed relative to the isoquinoline (Fig. 4, compare \( A \) and \( B \)). The structures are drawn such that the hinge is shown on the left, and the glycine-rich loop would lie above the plane of the page. The \( F_{o} - F_{c} \) electron density map is drawn around the compound at the 2.5 \( \sigma \) level. Active site water molecules are drawn as a red sphere, and the hydrogen bonds are drawn in purple.
The compound H-1152P differs from fasudil because of the addition of two methyl groups, one at the isoquinoline and the other on the homopiperazine ring. As in the ROCK/fasudil structure, the isoquinoline N accepts a hydrogen bond from the main chain amide nitrogen of Met156, and the aromatic C-1 donates a hydrogen bond to the main chain carboxyl of Glu154 (Fig. 4D). The C-4 methyl of isoquinoline sterically restricts conformations about the sulfur-carbon bond, such that the homopiperazine ring is rotated by about 110° relative to that in the ROCK/fasudil structure.

H-1152P inhibits ROCK with a much lower Ki than the other three compounds (Table 1). Because the isoquinoline methyl group limits the number of low energy conformations available to the ligand, the entropic cost of immobilizing the rotatable bonds upon protein binding should be low. Furthermore, the two methyl groups make additional van der Waals contacts with the protein; the methyl on the isoquinoline ring contacts the side chains of Phe368 and Ile82, whereas the homopiperazine methyl contacts the side chains of Ala215 and Asp216.

**Comparison of Inhibitor Complexes in ROCK and PKA**—To understand the origin of the compound selectivity, the ligand conformations and protein-ligand interactions in ROCK and PKA were compared. The active sites of ROCK and PKA are quite similar, both in sequence and in structure. There are several sequence differences in the ATP-binding site; however, most of these differences are likely to play a small role in selectivity because only conserved main chain contacts are observed (20, 46).

One residue that varies between ROCK and PKA, with a side chain in contact with the inhibitors, is Ile82 (Leu49 in PKA). Position 82 in ROCK is on the β-strand before the glycine-rich loop. In each of the structures, the closest contact between the ligand and Ile82 in ROCK is 0.2–0.4 Å shorter than the corresponding distance to Leu49 in PKA. The closer van der Waals contacts in ROCK may increase the potency of the compounds described herein. A larger inhibitor moiety near the hinge could favor PKA binding over ROCK by causing a steric clash with Ile82. There is not, however, a simple correlation between the contact distances or areas and the potency or selectivity.

Of the four compounds examined in this study, fasudil is the only one that binds to ROCK and PKA with equal potency. In the PKA/fasudil structure (Protein Data Bank code 1Q8W), the isoquinoline makes the same interactions with the hinge region; however, the sulfonamide and the homopiperazine are rotated ~90° (Fig. 5B). The difference in conformations is due to a longer side chain interacting with the homopiperazine; the Asp160 in ROCK is replaced by a glutamic acid in PKA (Glu127). The Ki for fasudil in ROCK and PKA may be similar because ligand conformational changes can accommodate active site differences. This sequence variation is likely to only affect fasudil binding; none of the other three inhibitors contact this residue.

Y-27632, H-1152P, and hydroxyfasudil have the same conformation in both the ROCK and PKA structures. Of these, Y-27632 and H-1152P bind each of the two proteins in a slightly different orientation. In the Y-27632 structures for instance, pyridine nitrogen in both the ROCK and PKA (Protein Data Bank code 1Q8T) structures occupy the same position; however, the rest of the compound is rotated by about 7° such that the methyl and amine groups are shifted by 1.3 Å (Fig. 5A). This rotation may be due to close contacts between the cyclohexane and the side chain of Thr183 in PKA (3.25 Å), limiting the extent to which the compound can enter the active site. In ROCK, the inhibitor can bind further into the interior of the active site because the smaller side chain of Ala155 occupies this position. The consequence of this shift in position is that the Y-27632 cyclohexane atoms in ROCK can form van der Waals contacts with the CB of Asp216, whereas the same atoms in PKA lack these interactions. A similar effect is observed when comparing H-1152P bound to ROCK and PKA (Protein Data Bank code 1Q8U) (Fig. 5D). Contacts between the homopiperazine methyl and Thr183 in PKA cause a rotation of about 9° relative to the ROCK structure and thus reduce the extent of van der Waals contacts between the homopiperazine and the protein in the vicinity of Asp184.

In the PKA-hydroxyfasudil structure, the position of the ligand is very similar to that observed in ROCK (Fig. 5C), and the small differences in the ligand-protein contacts cannot be easily quantified because of the diffraction resolution of the ROCK-hydroxyfasudil crystals. In ROCK, hydroxyfasudil occupies the same space as Y-27632, and it is likely that

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**FIGURE 5. Comparison of ROCK and PKA inhibitor-bound structures.** The structures of Y-27632 (A), fasudil (B), hydroxyfasudil (C), and H-1152P (D) are shown with the carbon atoms of the ROCK green and PKA in gray. The structures are in the same orientation as Fig. 4. Alternate conformations of Thr183 are shown in the PKA structures in A and D. The ROCK and PKA structures were aligned by superimposing main chain atoms from residues 154, 156, 160, 203, and 215 in ROCK with residues 121, 123, 127, 171, and 183 in PKA.
the interactions that determine selectivity for Y-27632 have a similar but lesser effect on hydroxyfasudil. A higher resolution structure may be necessary to observe subtle differences in the binding interactions of hydroxyfasudil in ROCK and PKA.

**DISCUSSION**

**N-terminal Dimerization Domain** —The full-length ROCK contains two dimerization domains: one at the N terminus as shown herein and one in the C-terminal region of the protein that has a coiled-coil structure (47, 48). Three related kinases, CRIK, MRCK and DMPK, share similar sequences N-terminal to the kinase domain and are likely to adopt a similar dimeric structure (12, 13, 49). The complementary shape and the extensive contact surface between the two molecules suggests that the crystallization construct (residues 6–415) of ROCK should exist as a dimer in solution. Indeed, earlier studies demonstrated that the crystallization construct (residues 571–379) of ROCK contains an inhibitory domain that resembles the dimerization domain (50). Analytical ultracentrifugation measurements showed that the self-association constant of the crystallization protein construct is 0.4 μM (19). In the ROCK crystal structure, no contacts are made between the kinase domains, and therefore, protein constructs including only the kinase domain (residues 71–379) are monomeric in solution. Furthermore, deletion of only the kinase tail, leaving the helical N-terminal region intact (residues 6–379), resulted in a mixture of dimers and monomers. Thus, whereas the kinase tail is not necessary for dimer formation, it does appear to stabilize the interaction. This is consistent with the observation that the two kinase tails do not interact significantly with each other, but there are extensive contacts between the kinase tail and the N-terminal helical region.

Several studies have shown that ROCK activity is inhibited through interactions with the Rho binding and pleckstrin homology domains at the C terminus of the protein, and truncation of these regions yields constitutively active protein. When activated Rho binds to ROCK, the interaction with the C-terminal domains is disrupted, relieving the inhibition. Inhibition could occur through two mechanisms: blocking the active site of the kinase domain or stabilizing a catalytically incompetent conformation. Because of the structure of the ROCK dimer, the kinase domains are arranged in such a manner as to present many of the important regions of the active site on a common face. In this dimer, the two activation loops, glycine-rich loops, peptide substrate pockets, and ATP substrate pockets are all presented on one side of the protein surface (Fig. 1). Therefore an inhibitory binding protein or domain could block or alter important catalytic regions of both kinase molecules. The structures of the Rho-binding domain in the presence and absence of RhoA have been determined (Protein Data Bank codes 1UIX and 1S1C) (47, 48). In these structures, the region of residues 945–1015 of ROCK is a dimer organized as a parallel coiled-coil domain. If the inhibitory domains are organized as a dimer, then the kinase domains would have to be similarly organized to ensure that both could interact with the C terminus at the same time.

The dimeric arrangement of the kinase domains may also play a role in substrate recognition. Myosin II, like ROCK, has a ROCK substrate, is phosphorylated near the N terminus (Ser19) and is a component in smooth muscle and nonmuscle myosin complexes. Such complexes consist of six proteins: two dimers each containing an actin-binding motor domain, essential light chain, and RLC. Two methods have been employed to elucidate the arrangement of these proteins in the complex: cryo-electron microscopy (51, 52) and chemical cross-linking (53, 54). The models proposed in these studies differ from each other with respect to the arrangement of the subunits in the complex. One common feature, however, is that RLC proteins are closely associated and are related to one another by a pseudo-2-fold rotation. For instance, in the model proposed by Wahlstrom et al. (54), the phosphorylation site on each of the two RLCs in the dimer appears on a common face. It is possible then that N-terminal regions from two RLCs could bind in the substrate clefts of two ROCK kinase domains concurrently. Interestingly, myosin light chain kinase, another kinase that phosphorylates RLC at Ser19, also forms dimeric and oligomeric structures in solution; however, myosin light chain kinase has a very different domain organization from ROCK. Because the two RLCs directly interact with one another, dual phosphorylation of both chains may be necessary for proper regulation of myosin function (55).

Measurements of the kinase enzymatic activity of full-length and truncated ROCK proteins have shown that residues that form the dimerization domain influence catalytic activity (19). Protein constructs where only the kinase tail is deleted (residues 6–379) or where both the N- and C-terminal dimerization regions are deleted (residues 71–379) have an 80-fold higher $K_m$ for ATP and a >20-fold higher $K_i$ for Y-27632 when compared with the crystallization construct. In these truncations, however, the $K_m$ for RLC and the $K_i$ for ATP are unaffected. Although it is possible that the formation of the monomeric state has a direct catalytic effect, it seems more likely that interactions between the kinase tail and the ATP pocket account for the differences observed. In the ROCK structure, part of the kinase tail (residues 371–374) makes direct contact with and lies over the glycine-rich loop. In both truncations, residues past Glu379 were deleted, and this may remove an anchor for the kinase tail, disrupt interactions between the tail and the glycine-rich loop, and affect ATP and Y-27632 binding. A construct that includes the kinase tail but lacks the N-terminal helical region could be used to test this hypothesis.

**Activation Loop Conformation** —It has been suggested that trans-autophosphorylation of both the activation loop and the C-terminal tail is essential for activation of ROCK and MRCK (13, 50). For instance, ROCK II and MRCK mutations, in positions corresponding to Thr233 in ROCK I, have decreased kinase activity and have been proposed to be sites of phosphorylation. The ROCK structure suggests that autophosphorylation of the activation loop may not be essential, however, because of 1) the orientation of the kinase domains, 2) the conformation of the activation loop in the absence of phosphorylation, and 3) the hydrophobic rather than positively charged residues near the phospho-receptor.

Although the dimerization domain holds the two kinase domains in close proximity, the active sites of each of the two monomers are facing the same direction and neither of the activation loops are in a position to be readily phosphorylated by the other monomer. So, the dimer observed in this crystal structure would have to be disrupted for the activation loop of one kinase monomer to enter the active site of the other. This suggests that either trans-autophosphorylation is not an essential activation step for ROCK or that the N-terminal dimer must readily dissociate to allow one monomer to phosphorylate the other.

The crystal structure of unphosphorylated ROCK suggests that phosphorylation of the activation loop is not necessary for a catalytically competent conformation. The activation loop of the two ROCK molecules of the asymmetric unit have different conformations because of crystal packing, indicating conformational flexibility in this region. One of these two conformations closely resembles that observed for the PKA-ATP-peptide complex and is consistent with peptide ligand binding. In the absence of phosphorylation, the conformation appears to be stabilized through extensive contacts with the αEF-αF loop. Furthermore, the binding site for a phosphothreonine residue in ROCK is
absent. In PKA, the phosphothreonine interacts with one polar and three positively charged residues; however, in ROCK, three of these four are hydrophobic residues. Kinases with neutral residues at these positions do not require phosphorylation (41, 56). So although phosphorylation in the activation loop may occur, it is not necessary for the protein to adopt a conformation competent for ligand binding and catalysis. Instead, activation through RhoA binding may be the primary mechanism for ROCK activity regulation. The absence of two of the basic residues surrounding the predicted phospho-threonine site is common to DMPK, MRCK, and CRIK (57), and it is possible that these related kinases share similar mechanisms for regulation.

**Ligand Selectivity**—The differences in the positions of Y-27632, H-1152P, and hydroxyfasudil in ROCK and PKA correlate with the compounds' selectivity; the less selective compounds have more similar structures in the two kinases. The degree to which each compound is a more potent inhibitor of ROCK over PKA depends upon the extent to which Thr\(^{183}\) in PKA alters the ligand position relative to ROCK. Y-27632 and H-1152P, for instance, both make close contacts with Thr\(^{183}\) in PKA and rotate away from the interior of the active site relative to their positions in ROCK.

The contribution of these contacts in the case of fasudil are harder to estimate because the conformations of the ligands differ in the ROCK and PKA structures. Although fasudil does contact Thr\(^{183}\) in PKA, the position of the isoquinoline remains fixed relative to ROCK. In this orientation, however, the bond between the isoquinoline and the sulfonamide is free to rotate and thus accommodate the Asp/Glu variation at position 160 in ROCK. So, although the compound contacts a residue that differs between ROCK and PKA, conformational flexibility of the ligand appears to compensate for the difference, and no selectivity is observed.

In the design of protein kinase inhibitors, selectivity presents a significant issue because of the similarity of kinase active sites and the diversity of signal transduction pathways regulated. The ROCK active site is very similar to that of PKA, demonstrated both by their structural similarity and the ability of certain inhibitors to bind both proteins. Because PKA is involved in many pathways, avoiding inhibition of PKA could be essential in a ROCK inhibitor designed for therapeutic use. Fasudil is noteworthy in this regard because it inhibits both ROCK and PKA with equal potency. Hydroxyfasudil, the metabolite of fasudil, is 15-fold selective for ROCK over PKA. This selectivity comes from a new binding mode caused by the hydrogen bonding pattern between the isoquinoline and the hinge, which in turn contacts Ala\(^{215}\), a position of sequence variation with PKA. Thus, it is through the altered binding mode of hydroxyfasudil that relative selectivity for ROCK is observed upon administration of fasudil. The pattern of selectivity observed here suggests that in the design of selective ROCK inhibitors, close contacts with Ala\(^{215}\) would be beneficial, because the same interactions with the larger side chain in PKA would adversely affect ligand binding.

**Conclusions**—In this study we found that ROCK has two dimerization domains: an N-terminal helical dimerization domain in addition to the C-terminal coiled-coil region previously described. The N-terminal dimerization region orients the two kinase domains such that they could simultaneously interact with the C-terminal inhibitory regions. Also, this dimeric arrangement may facilitate phosphorylation of dimeric substrates such as myosin light chain. The observation that the unphosphorylated kinase domains have a conformation that is catalytically competent implies that RhoA binding is sufficient to activate the protein. Finally, sequence differences in the active sites of ROCK and PKA can be exploited in structure-based drug design toward making a selective ROCK inhibitor. Such a compound could be used to validate ROCK as a therapeutic target in diverse indications such as hypertension and immunosuppression.

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