Structural Analysis of Protein Kinase A Mutants with Rho-kinase Inhibitor Specificity*

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Controlling aberrant kinase-mediated cellular signaling is a major strategy in cancer therapy; successful protein kinase inhibitors such as Tarceva and Gleevec verify this approach. Specificity of inhibitors for the targeted kinase(s), however, is a crucial factor for therapeutic success. Based on homology modeling, we previously identified four amino acids in the active site of Rho-kinase that likely determine inhibitor specificities observed for Rho-kinase relative to protein kinase A (PKA) (in PKA numbering: T183A, L49I, V123M, and E127D), and a fifth (Q181K) that played a surprising role in PKA-PKB hybrid proteins. We have systematically mutated these residues in PKA to their counterparts in Rho-kinase, individually and in combination. Using four Rho-kinase-specific, one PKA-specific, and one pan-kinase-specific inhibitor, we measured the inhibitor-binding properties of the mutated proteins and identify the roles of individual residues as specificity determinants. Two combined mutant proteins, containing the combination of mutations T183A and L49I, closely mimic Rho-kinase. Kinetic results corroborate the hypothesis that side-chain identities form the major determinants of selectivity. An unexpected result of the analysis is the consistent contribution of the individual mutations by simple factors. Crystal structures of the surrogate kinase inhibitor complexes provide a detailed basis for an understanding of these selectivity determinant residues. The ability to obtain kinetic and structural data from these PKA mutants, combined with their Rho-kinase-like selectivity profiles, make them valuable for use as surrogate kinases for structure-based inhibitor design.

Phosphorylation via protein kinases is responsible for a large part of cellular signal transduction and is described as a universal regulatory mechanism (1, 2). Perturbation of kinase-mediated signaling pathways results in a number of diseases, including diabetes, cancer, and inflammation (3, 4). Because most protein kinases reside in the cell in an inactive state and are activated by signal transduction processes, many diseases are triggered by overactivation of protein kinases via mutation, overexpression, or malfunctioning cellular inhibition.

The human genome encodes some 518 protein kinases (5) that are notably different in how their catalysis is regulated but share a catalytic domain conserved in sequence and structure (6, 7). The latter consists of 250–300 amino acids, binds substrate and co-substrate, and catalyzes the phosphorylation reaction.

This catalytic domain, together with less conserved surrounding sites, has been the focus of inhibitor design that has exploited differences in kinase structure and pliability to achieve selectivity. Many drugs that target protein kinases are in clinical trials, and some have already been approved, such as the Abl kinase inhibitor Gleevec for therapy against chronic myelogenous leukemia (8) and Tarceva (erlotinib) against non-small cell lung cancer (9). The first protein kinase inhibitor that passed the clinical phase was fasudil (HA1077) in 1995 as a treatment for cerebral vasospasm (10). It has a significant vaso-dilatory effect attributed to its inhibition of Rho-kinase (11) signaling to myosin light-chain kinase (12) and is in clinical trials for the treatment of angina pectoris (13). Apart from its pharmacological role in cerebral vasospasm and angina pectoris, Rho-kinase plays an important role in cell division, differentiation, apoptosis, transformation, and the invasion and migration of cancer cells (12) (for reviews see Riento and Ridley (14), Fukata et al. (15), and Wettcureck and Offermanns (16)).

Selectivity considerations can be critical for inhibitor design, and it is thus of utmost importance to understand the factors that govern the specific binding of kinase inhibitors to the catalytic center of protein kinases (17–21). Accordingly, to address this question with regard to Rho-kinase we recently determined the structure of PKA# in complex with the inhibitors fasudil, H1152P, and Y-27632, demonstrating characteristic binding within the ATP site (22). Homology modeling comparisons

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The atomic coordinates and structure factors (code 2GFC, 2GNQ, 2GNJ, 2GNI.) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

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5 The abbreviations used are: PKA, cAMP-dependent protein kinase catalytic subunit; PAR, mutant PKA with PKA to Rho-kinase amino acid exchanges; HA1077, fasudil; GST, glutathione S-transferase; Maps, 4-morpholinepropanesulfonic acid; Mes, 4-morpholineethanesulfonic acid; BisTris, 2-[bis(2-hydroxyethyl)aminoo]-2-(hydroxymethyl)propane-1,3-diol; r.m.s.d., root mean square deviation; PKB, protein kinase B; AMPNP, adenosine 5‘-(β,γ-imino)triphosphate; PKI(5–24), protein kinase inhibitor residues 5–24.
with Rho-kinase indicated the likely stereochemical basis for the selectivity of the inhibitors and their relatively high affinity for Rho-kinase. From sequence alignment of 491 kinases, and additionally considering an AGC-kinase-specific residue from the non-conserved C-tail, Rho-kinase has a unique combination of residues at the ATP binding site, although individual residues show a considerable degree of conservation in the family. We suggested that the combination of four residues at the ligand-binding site, which generate a uniquely shaped inhibitor binding pocket, confers selectivity of Rho-kinase over closely related kinases like PKA.

Hence we used site-directed mutagenesis to exchange these amino acids in PKA for their counterparts of Rho-kinase, individually and in combination, and evaluated the kinetic binding data of selected inhibitors with the PKA mutants. The data confirm the stepwise introduction of Rho-like inhibitor binding specificity to the PKA mutants. Two exchanges, L49I and T183A, are responsible for most of the selectivity for the inhibitors tested. Crystal structures of the PKA-Rho-kinase surrogate hybrid show the details of the selectivity mechanisms. These results are consistent with results from other kinases that demonstrate importance of a few side chains for protein kinase inhibitor selectivity, for example for RSK by Cohen et al. (17). Use of this PKA-based surrogate Rho-kinase should enable structure based design of therapeutics to treat Rho-kinase mediated diseases.

**EXPERIMENTAL PROCEDURES**

*Site-directed Mutagenesis*—Mutations in the PKA-coding sequence were generated via the QuikChange® site-directed mutagenesis kit (Stratagene), according to the manufacturer’s guidelines. All mutations have been verified by DNA sequencing.

*Protein Expression and Purification*—Recombinant mutated bovine Ca³⁺ catalytic subunit of the cAMP-dependent protein kinase was solubly expressed in *Escherichia coli* BL21(DE3) cells and then purified via affinity chromatography and ion exchange chromatography as previously described (23). Two positions distinguish bovine (Asn-32 and Met-63) from human PKA (Ser-32 and Lys-63). 3-Fold phosphorylated protein was used for crystallization. Mutant proteins used for crystallization were analyzed by liquid chromatography-mass spectrometry to verify their correct mass. Constitutively active GST-Rho-kinase (24) was purified from SF9 cells by use of a baculovirus system (25) by means of a glutathione-Sepharose column.

*Activity Tests*—The determination of enzyme activity was accomplished by an ATP regenerative NADH consuming assay according to Cook (26). After inhibitor and enzyme were added to the assay mixture (100 mM Mops, pH 6.8, 100 mM KCl, 10 mM MgCl₂, 1 mM phosphoenolpyruvate, 0.1 mM Kemptide, 1 mM β-mercaptoethanol, 15 units/ml lactate dehydrogenase (Sigma), 8 units/ml pyruvate kinase (Sigma), 0.21 mM NADH) the reaction was started with ATP. The decrease of NADH was measured as being time-dependent at λ = 340 nm with three independent measurements per data point. K₅₀ values, IC₅₀ values, and standard deviations were calculated using SigmaPlot 8.0 and Origin50 software.

*Crystallization*—The inhibitors Y-27632, KT5720, hydroxyfasudil, and staurosporine were purchased from Merck-Biosciences; the inhibitors HA1077 and H1152P were kindly provided by Hiroyoshi Hidaka.

Inhibitors were co-crystallized with recombinant mutated bovine Ca³⁺ catalytic subunit of the cAMP-dependent protein kinase in the presence of PKI(5–24) using the hanging drop vapor diffusion method. Droplets contained 18 mg/ml protein, 30 mM Mes-BisTris, 75 mM LiCl, 1 mM dithiothreitol, 1 mM PKI(5–24), pH 6.3–6.9, 3 mM MgCl₂, and, except for PKA WT, 1 mM inhibitor, and were equilibrated at 5 °C against 12–18% (v/v) methanol.

*Data Collection and Structure Determination*—Diffraction data were measured from frozen crystals at the beamline BW6, Deutsches Elektronen Synchrotron (DESY), Hamburg, at Angström Quelle Karlsruhe (ANKA) beamline PX (ANKA, Karlsruhe, Germany), or in-house on a Rigaku R300 rotating anode. The data were processed with the programs XDS and XSCALE or MOSFLM and SCALA. The crystals have orthorhombic space group P2₁2₁2₁, a = 72.8 Å, b = 76.0 Å, c = 80.3 Å, with unit cell volume of 12,114 Å³ and atomic resolution of 1.87 Å. The crystals belong to the P2₁2₁2₁ space group and are isomorphous with the starting structure. Temperature factors for the crystals are given in Table 1. The crystals have orthorhombic space group P2₁2₁2₁, a = 72.8 Å, b = 76.0 Å, c = 80.3 Å, with unit cell volume of 12,114 Å³ and atomic resolution of 1.87 Å. The crystals belong to the P2₁2₁2₁ space group and are isomorphous with the starting structure. Temperature factors for the crystals are given in Table 1.
rhombic symmetry (P2₁,2₁,2₁) (Table 1). The structures were determined by molecular replacement using AMoRe from the CCP4 program suite (www.ccp4.ac.uk/main/html). As search model we chose a PKA-PKI(5–24)₆ complex. Calculations of Matthews’s coefficients and solvent contents suggested one molecule in the asymmetric units. Phosphorylation sites were found at Thr-197 and Ser-338. Phosphorylated Ser-10 is resolved in PKAR5–1152, PKAR5–1077, and PKAR5. Water molecules were automatically inserted using the CCP4 programs PEAKMAX and WATPEAK and visually inspected. Finally, the inhibitor molecules were built and the whole complex was further refined. PKAR3–1152 showed a higher $R_{\text{free}}$ to $R_{\text{cryst}}$ difference than the other structures, probably due to lower resolution and data quality from data collection on an in-house rotating anode x-ray generator. Refmac 5.1.24 was used for refinement, and MOLOC (www.moloc.ch) was used for model building and graphical modeling. For data and refinement statistics see Table 1.

**Structural Analysis**—To detect conformational changes between different complexes, the structures were compared pairwise using Protein3Dfit (27), the resulting root mean square deviation (r.m.s.d.) values were graphically displayed. For a visual inspection of conformational changes, structures were aligned between the C-lobe residues 120–281, because this region comprising the conserved C-terminal lobe is conformationally most stable, using the program Pymol (www.pymol.org).

**RESULTS AND DISCUSSION**

**Mutagenesis of PKA Residues toward Rho-kinase**

Homology modeling shows the ATP-binding sites of PKA and Rho-kinase to differ most prominently at four residues (Fig. 1A) that contribute side-chain atoms to the ATP-site binding surface (22). These are Leu-49 (isoleucine in Rho-kinase), Val-123 (M), Glu-127 (D), and Thr-183 (A). Rho-kinase and PKA share AGC kinase characteristic residues, in particular Phe-327 that lies in the ATP pocket adjacent to the adenine ring. The combination of these five residues is unique to Rho-kinase in the human kinome. The structural similarity of AGC kinases with known structures suggests that appropriate mutants of PKA will likely mimic Rho-kinase inhibitor selectivity. This is analogous to our design of PKA mutant mimics of PKB selectivity (28). In this work, we observed that a residue not directly involved in ATP site contacts (Gln-181) can adopt a new conformation and obstruct the ATP site after mutation of Val-123 to the smaller alanine of PKB; an additional PKA to PKB mutation (Q181K) was required to eliminate this effect. Rho-kinase, like PKB, possesses a lysine at the position equivalent to Gln-181, although the PKA to Rho-kinase mutation of V123M does not create a cavity, as does the PKA to PKB mutation of V123A, and T183A does expand the ATP site cavity. Therefore, the exchange Q181K was included in the present mutagenesis analysis as a control. PKA-Cα and PKA mutants with single exchanges or with combinations of

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TABLE 2
Overview of \( K_m \) and \( IC_{50} \) values: comparison of all \( K_m \) and \( IC_{50} \) values measured for PKA-C, eleven PKARs, and Rho-kinase in comparison to literature data for PKA-C and Rho-kinase
All measurements have been conducted at least in triplicate under standard conditions by use of the Cook Assay (26). Inserted mutations into the PKARs are attached in parenthesis; e.g. PKAR(T183A). \( IC_{50} \) values below the detection limit of our assay are given as \(<45 \, \text{nm} \).

| \( K_m \) \( \text{M} \) | HA1077 | H1152P | Y-27632 | KT5720 | Staurosporine |
|-----------------|--------|--------|---------|--------|--------------|
| ATP             |        |        |         |        |              |
| PKA-C-lit       | 13\( ^a \) | \( K_i = 1000 \) \( ^g \) | \( K_i = 630 \) \( ^g \) | \( K_i = 25 \) \( ^{g} \) | \( K_i = 56 \) \( ^{g} \) | 18\( ^{g} \) |
| PKA-C           | 12     | 1494 ± 43 | 394 ± 146 | 42 ± 3 | 56 ± 0 | \(<45 \) |
| PKAR(L49I)      | 10     | 893 ± 243 | 426 ± 41 | 28 ± 0 | 10390 ± 2599 | 47 ± 7 |
| PKAR(V123M)     | 38     | 7605 ± 0  | 1517 ± 186 | 60 ± 16 | 222 ± 38 | \(<45 \) |
| PKAR(T183A)     | 21     | 607 ± 43  | 109 ± 22  | 9 ± 0.6 | \(<45 \) | \(<45 \) |
| PKAR(E127D)     | 28     | 2452 ± 61 | 493 ± 0.16 | 16 ± 0.6 | 1822 ± 437 | \(<45 \) |
| PKAR(Q181K/T183A) | 17    | 541 ± 16  | 109 ± 22  | 9 ± 0.6 | \(<45 \) | \(<45 \) |
| PKAR(L49I/T183A) | 28    | 12 ± 0    | 12 ± 0    | 12 ± 0  | 12 ± 0  | 12 ± 0  |
| PKAR(V123M/T183A) | 39    | 1350      | 293 ± 76  | 31 ± 3  | 31 ± 3  | 31 ± 3  |
| PKAR3(L49I/Q181K/T183A) | 45   | 2280 ± 280 | 149 ± 2   | 6 ± 0   | 1300 ± 722 | \(<45 \) |
| PKAR5(L49I/V123M/E127D/Q181K/T183A) | 29  | 485 ± 90  | \(<45 \) | 0.5     | 1800 ± 541 | 1.2\( ^{f} \) ± 0.3 |
| Rho-kinase-CAT-lit\( ^a \) | 20\( ^{f} \) | \( K_i = 330 \) \( ^{g} \) | \( K_i = 1.6 \) \( ^{g} \) | 0.3±0 | 0.3±0 | 0.3±0 |

\( ^a \) Literature data were collected from Ishizaki et al. (39).

\( ^b \) From Ikenoya et al. (38).

\( ^c \) From Turner et al. (37).

\( ^d \) Rho-kinase-CAT-lit, corresponding values are from the literature.

\( ^e \) From Turner et al. (37).

\( ^f \) Rho-kinase-CAT-lit, constitutively active GST-tagged catalytic domain of bovine ROCKII kinase.

\( ^g \) From Kase et al. (40).

\( ^h \) From Uehata et al. (42).

\( ^i \) From Ikenoya et al. (38).

Rho-kinase was purified after expression in insect cells and subsequently kinetically characterized. Four single point mutants were produced: PKAR(T183A), PKAR(L49I), PKAR(V123M), and PKAR(E127D). Double mutants were made with PKAR(T183A) as a template: PKAR(Q181K/T183A), PKAR(L49I/T183A), and PKAR(V123M/T183A). Triple mutants were made with PKAR(Q181K/T183A) as template: PKAR(L49I/Q181K/T183A) and PKAR(V123M/...
Finally, quadruple and quintuple mutants were made: PKAR(V123M/L49I/Q181K/T183A) and PKAR(L49I/V123M/E127D/Q181K/T183A).

**Rho-kinase and PKA-specific Inhibitors**—The inhibitors used in this study (Fig. 1B) originate from three different chemical classes.

**FIGURE 4.** $K_m$ and IC$_{50}$ values for PKA-C, PKAR3, and GST-Rho-kinase-CAT. A, $K_m$ values for ATP and IC$_{50}$ values with the inhibitors HA1077, H1152P, Y-27632, and staurosporine for PKA-C, PKAR3, and GST-Rho-kinase-CAT in percentage of the corresponding $K_m$ and IC$_{50}$ values for PKA-Cα. B, the corresponding IC$_{50}$ values with the inhibitor KT5720 are shown.

**FIGURE 5.** $K_m$ and IC$_{50}$ values for PKA-C, PKAR5, and GST-Rho-kinase-CAT. A, the $K_m$ values for ATP and IC$_{50}$ values with the inhibitors HA1077, H1152P, Y-27632, and staurosporine for PKA-C, PKAR5, and GST-Rho-kinase-CAT in percentage of the corresponding $K_m$ and IC$_{50}$ values for PKA-Cα. B, corresponding IC$_{50}$ values with the inhibitor KT5720.

**FIGURE 6.** r.m.s.d. values for PKA-C, PKAR3, and PKAR5. A, r.m.s.d. values for co-crystallized PKAR3 mutants compared with the corresponding PKA-Cα co-crystal structures. B, r.m.s.d. values for PKAR5 mutants compared with PKA-Cα. C, r.m.s.d. comparison of PKAR5 bound to HA1077 and PKA-Cα bound to the latter inhibitor.
The Rho-kinase-specific inhibitors fasudil, hydroxyfasudil, and H1152P are isoquinoline sulfonamide derivatives. Relative to fasudil, hydroxyfasudil has an additional hydroxyl group in the position 1 of the isoquinoline ring (29), and H1152P has two additional methyl groups, one at the isoquinoline ring, and the other at the homopiperazine ring that confer higher selectivity and specificity for Rho-kinase. The PKA-specific inhibitor KT5720 is identical to staurosporine in the extended planar portion of the inhibitor but differs especially in its possession of a fatty acid side chain, extending from a furanose instead of a pyranose ring. Y-27632 is from a third chemical class, as a pyridine ring linked via an amide to a para-aminoethylcyclohexane ring.

### Kinetic Data

For the majority of the eleven mutant PKA-Cα kinases (PKARs), as well as for PKA-Cα and GST-Rho-kinase-CAT, we determined $K_m$ values for ATP and IC$_{50}$ values for five inhibitors. To test the extent to which the PKAR mutants mimic Rho-kinase-specificity, IC$_{50}$ values were measured for the three Rho-kinase-specific inhibitors HA1077, H1152P, and Y-27632, for the PKA-specific inhibitor KT5720, and for the pan-kinase inhibitor staurosporine. The kinetic data of the 72 measurements, conducted in triplicate, and corresponding literature values are presented in Table 2.

Additional measurements were made with hydroxyfasudil (HA1100) for PKA-Cα and PKAR3.

At first, we validated our kinetic measurements by comparing PKA-Cα and Rho-kinase $K_m$ and IC$_{50}$ values to the corresponding literature values. IC$_{50}$ values were transformed to $K_i$ values using the Cheng and Prussoff equation (30) to compare our IC$_{50}$ data to literature $K_i$ data. With the exception of the IC$_{50}$ for PKA-Cα with H1152P, which deviated from the literature value by a factor of three, all values were within an acceptable range of the literature data (less than a factor of two) (Table 1). As is the case for PKA-Cα and Rho-kinase, measuring staurosporine inhibition in the Cook assay posed a problem, because staurosporine inhibited almost all of our kinases below the detection limit of the system (<45 nM), and IC$_{50}$ values are marked accordingly as <45 nM. The kinetic data of PKAR(T183A), PKAR(L49I), PKAR3, and PKAR5 will now be described in more detail.

**PKAR(T183A) and PKAR(Q181K/T183A)—**As we have previously analyzed (22), a PKA to Rho mutation of threonine to alanine at position 183 would enlarge the ATP binding pocket and, consequently, favor binding of similarly enlarged inhibitors, such as H1152P. We made the corresponding mutant PKAR(T183A) and measured the inhibitor binding strengths. In addition, we made the double mutant PKAR(Q181K/T183A) to test whether the expansion of the binding pocket by the T183A mutation could influence the conformation of Gln-181, as seen with PKA to PKB hybrid mutants. Fig. 2 shows the results of $K_m$ and IC$_{50}$ measurements for PKAR(T183A) and PKAR(Q181K/T183A). The IC$_{50}$ values for PKAR(T183A) and

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**FIGURE 7.** Conformational changes of the glycine-rich loop (main conformation) in wild-type and mutant structures. Three groups are defined (A, B, and C). The conformation of the wild-type AMP-PNP-bound structure 1CDK is indicated as a gray thin line drawing. All other structures are colored at the carbon atoms, corresponding to the colors of their labels. D, superposition of glycine loop (main conformations), hinge region, and a portion of the C-tail from PKAR5 (color-coded rainbow N to C termini, glycine-rich loop in blue) and PKAWT (gray carbon atoms).

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PKAR(T183A) with HA1077 and H1152P are very close to the corresponding values for Rho-kinase (Fig. 2A). The IC$_{50}$ values for PKAR(T183A) and PKAR(Q181K/T183A) with Y-27632 show considerably enhanced binding of the inhibitor compared with PKA-Cα albeit being intermediate between PKA and Rho-kinase. The $K_m$ values for ATP of the mutant kinases are also intermediate between the values of PKA-Cα and Rho-kinase. No changes in inhibitor binding between the different enzymes could be measured for the inhibitor staurosporine. The IC$_{50}$ values for PKAR(T183A) and PKAR(Q181K/T183A) with KT5720 show no apparent differences compared with the corresponding values of PKA-Cα but are in stark contrast to the corresponding value of Rho-kinase (Fig. 2B). The kinetic data of PKAR(T183A) and PKAR(Q181K/T183A) show no significant differences, indicating that there is no refolding of Gln-181 in PKAR(T183A).

Taken together, these data confirm that most of the specificity of HA1077 and H1152P for Rho-kinase in contrast to PKA arises from the steric hindrance of Thr-183 in PKA. Removal of the steric hindrance does not, however, significantly affect the binding of the PKA-specific KT5720, for which increased Rho-kinase-like behavior would mean lowered binding strength. Binding of the pan-kinase inhibitor staurosporine was not measurably affected by the T183A mutation, because the IC$_{50}$ values for the various proteins remained below 45 nM.

**PKAR(L49I)—**A role for the exchange L49I was, in contrast to T183A, less clear, because there was no net change in side-chain volume. We proposed that an isoleucine residue at position 49 could explain in part Rho-kinase selectivity properties due to the altered shape of the binding surface. As can be seen in Fig. 3A, this may hold true for the binding of the inhibitor HA1077 to PKAR(L49I), because the IC$_{50}$ value for the L49I mutant PKA was intermediate between the corresponding values of PKA-Cα and Rho-kinase. It appears to hold true especially for KT5720, because the IC$_{50}$ value for PKAR(L49I) showed binding to be weakened even beyond that of Rho-kinase (Fig. 3B). It follows that the KT5720-pocket shape mismatch in PKAR(L49I) can be partially compensated for by other PKA mutations.

**PKAR3 (PKAR(L49I/Q181K/T183A))—**As described above, the amino acid residue 183 seems to govern specific binding of inhibitors to Rho-kinase (HA1077, H1152P, and Y-27632) and does not affect specific binding of inhibitors to PKA (KT5720). Amino acid residue 49 displays the contrary effect on inhibitor binding, showing little effect on IC$_{50}$ values for Rho-kinase-specific inhibitors (with the exception of HA1077) and a strong effect on the IC$_{50}$ value for PKA-specific inhibitors. Regarding the effort to create a surrogate kinase for Rho-kinase, it now seemed plausible to us to build a kinase combining those kinetic features by exchanging residue Thr-183 to alanine, Gln-181 to lysine, and Leu-49 to isoleucine in PKA-Cα, thus creating PKAR3.

**Fig. 4 (A and B)** displays the combined kinetic data for PKAR3. The IC$_{50}$ values for PKAR3 with HA1077 and H1152P are indeed very close to the corresponding values for Rho-kinase, and to a lesser degree with Y-27632, all with considerably enhanced binding of the inhibitor compared with PKA-Cα. The effect was confirmed by additional measurements of the binding of hydroxyfasudil (HA1100) with PKAR3 (IC$_{50}$ 1.37 μM) and PKA-Cα (IC$_{50}$ 6 μM), reproducing the ratio of $K_i$ values (Rho-kinase 0.56 μM, PKA 2.5 μM) described in the literature (29). As with the single mutation PKAR(L49I), the IC$_{50}$ value for PKAR3 with KT5720 is greatly increased relative to PKA, and binds more weakly relative to Rho-kinase, although to a lesser extent than the single mutation PKAR(L49I). As it was already the case for PKAR(T183A), the $K_m$ value for ATP of the mutant

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**FIGURE 8. Superposition of PKAR3-Y-27632 (yellow carbon atoms) and 1Q8T (gray carbons) (A) and PKAR5-Y-27632 (blue carbons) and 1Q8T (gray carbons) (B). C, electron density maps (2F$_{o}$ - F, contoured at 1.5 σ) of the inhibitor binding pocket of PKAR3-Y-27632. D, PKAR5-Y-27632.**
kinase occupies an intermediate position between the values of PKA-Cα and Rho-kinase. Binding of staurosporine to PKAR3 seems not to be negatively affected by the introduced mutations, as we again could not detect a measurable increase of the IC_{50} value >45 nM.

Taken together, the kinetic data for PKAR3 demonstrate its suitability as a surrogate for Rho-kinase. It mimics both the Rho-kinase selectivity enhancement that arises from the expansion of the binding site volume due to T183A and the PKA selectivity loss due to the exchange L49I. Binding of staurosporine, an unselective but nanomolar inhibitor of many kinases, to PKAR3 is not noticeably affected. This is consistent with the homology modeling assumptions of equivalent active site geometries of Rho-kinase and PKA such that side-chain identities form the major determinants of selectivity. Very surprising is the fact that selectivity for KT5720 is determined by residue 49, because KT5720 and staurosporine are structurally identical in the part of the molecule which, in the case of staurosporine, is in contact with Leu-49 (31), assuming a principally similar binding mode of KT5720 and staurosporine.

PKAR5 (PKAR(L49I/V123M/E127D/Q181K/T183A))—PKAR5 was derived by introducing the mutations L49I, V123M, E127D, Q181K, and T183A into PKA-Cα. It thus contains all five of the amino acid exchanges proposed to govern the specificity of inhibitor binding to Rho-kinase compared with PKA. Again, as was the case for PKAR3, IC_{50} values for PKAR5 with H1152P and Y-27632 showed strong resemblance to the corresponding IC_{50} values for Rho-kinase (Fig. 5A). The IC_{50} value for PKAR5 with KT5720 approximately equals the IC_{50} value for Rho-kinase with KT5720 (Fig. 5B). Only the IC_{50} value for PKAR5 with HA1077 did not match the corresponding value for Rho-kinase, being similar instead to the corresponding IC_{50} for PKA-Cα (Fig. 5A). Thus, PKAR5 is similar to PKAR3 in its suitability as a surrogate kinase. As with PKAR3, the two single mutations T183A and L49I are most likely the principle selectivity determinants, whereas details of the interactions determine whether PKAR3 or PKAR5 is more suitable for a particular inhibitor (Fig. 5, A and B).

PKAR3 and PKAR5 showed the highest similarity with original Rho-kinase enzyme in their kinetic behavior with respect to inhibition by Rho-kinase-selective inhibitors and were chosen for a structural analysis of inhibitor binding. The proteins were purified into homogeneously phosphorylated isoforms by ion-exchange chromatography and co-crystallized with the inhibitors tested. All structures contained the pseudosubstrate PKI(5–24) inhibitor peptide, which appears to facilitate crystal growth without affecting ATP-site inhibitor binding (except with bisindolylmaleimide in a PKA to PKB surrogate mutant (28)). Structures were solved of complexes of PKAR3 with H1152P (PKAR3–1152, 2.6 Å) and Y-27632 (PKAR3-Y-27632, 2.28 Å), and of complexes of PKAR5 with HA1077 (fasudil) (PKAR5–1077, 2.27 Å), H1152P (PKAR5–1152, 2.05 Å), and Y-27632 (PKAR5-Y-27632, 2.28 Å). Attempts to co-crystallize PKA wild-type and PKA mutants together with KT5720, however, failed. In the presence of KT5720 several crystals of PKA wild type were grown, but no bound inhibitor was detectable in the refined electron density, despite the high affinity of PKA for KT5720. This is evidence of a conformational change associated with binding of KT5720 that is not compatible with either PKI binding or crystal packing.

A comparison of the overall conformation of PKAR5 complexes with PKAR3 or wild-type complexes (1Q8U (PKAWT-H1152P), 1Q8T (PKAWT-Y-27632), and 1Q8W (PKAWT-HA1077) (22) indicated conformational differences in several regions, as discussed below. To distinguish between structural effects of the mutations and inhibitor binding, we also determined the crystal structure of the PKAR5-PKI complex in the absence of an ATP site ligand at 1.85 Å. As a control, we solved the corresponding crystal structure from the bovine wild-type enzyme as a reference (PKAWT, 1.87 Å). (The binary structure of PKAWT available in the protein data bank code 1APM is less suited for comparison, because the mouse PKA of 1APM contains an alanine residue at the hinge position 124, which is proline in the human and bovine sequences. This residue shows
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one of the observed conformational shifts between PKAR5 and WT or PKAR3 enzyme.)

Overall Structure

General Conformational Changes—To identify conformational differences between mutant- and inhibitor-bound PKA enzymes and wild type, the structures were compared pairwise using Proteins3Dfit (27), and the r.m.s.d. values of each residue were listed and graphically displayed (Fig. 6, A–C). Fig. 6A combines two PKAR3 mutants bound to the inhibitors H1152P and Y-27632 compared with PKA-Cα, and Fig. 6B combines three PKAR5 mutants either empty or bound to the inhibitors H1152P and Y-27632 compared with PKA-Cα. The r.m.s.d. comparison of PKAR5 bound to HA1077 and PKA-Cα bound to HA1077 is displayed separately in Fig. 6C due to the generally large r.m.s.d. values. The regions with structural differences are, most prominently, the glycine-rich loop, the region around and including Ala-183, the hinge region around residue Pro-124, and the C-tail mostly around residues 318–339.

Glycine-rich Loop Conformations—Significant conformational variations occur in the region of the glycine flap. In most of the structures, the electron density indicated in addition to one prominent conformation, which was modeled, the presence of minor alternative conformations of residues Gly-52 to Gly-55 (Figs. 6 and 7). With respect to the glycine-rich loop conformations, three groups can be defined (Fig. 7, A–C).

The first group (A) has a glycine-rich loop orientation similar to known closed or intermediate open structures, such as 1CDK. PKAR3–1152 and PKAR5–1152 fall into this first group. The backbone Ψ angle of residue Phe-54 has a moderately negative value around −35° in this group. The second group (B), formed by PKAR5 and PKAR5-Y-27632, has an “upward” (away from the cleft) twist of residues Thr-51 to Ser-53. In contrast to the first group, the Phe-54 Ψ angle is positive around 50°. There are two significant differences in the third group (C) from the other two groups: the region around residues Thr-51 to Ser-53 is bent downward into the catalytic cleft, allowing backbone interactions of the S53 amide to the backbone displacements were observed around residue T183A. Because these changes seem to be related to the binding of the inhibitors, they will be discussed together with inhibitor binding.

Effect of the Mutations on Y-27632 Binding—Y-27632 binds in the structures 1Q8T (PKAWT-Y27632), PKAR3, and PKAR5 similarly, but not identically (Fig. 8). In all structures a hydrogen bond is formed from the pyridine nitrogen to the hinge region V123M backbone amide group. Also, the pucker of the hexane ring is alike in all three inhibitors. In contrast to the wild-type structure 1Q8T,
the aminoethyl group is rotated in both mutants, clearly defined in the electron density, and the amino group can now form two hydrogen bonds, one to the carboxyl of Asp-184, the other to the side-chain carbonyl of Asn-171, both invariant catalytic residues, whereas in the wild-type structure the group is ambiguous, but its prominent conformation supports a hydrogen bond with the backbone of Thr-51 from the glycine-rich loop. In the mutant enzymes, the entire inhibitor is rotated clockwise, when viewed along an axis perpendicular through the plane of the pyridine group from the N-lobe to the C-lobe. At the same time in both mutant structures the inhibitor is parallel translated toward the C-lobe and toward helix C by \( \frac{1}{2} \) Å. This combined movement causes the inhibitor to approach Ala-183. A threonine residue would result in a steric conflict here if placed in the PKAR3-Y-27632 structure; Cys-36 of the cyclohexane ring would be in a distance of \( \frac{2.7}{2} \) Å to the O-\( \gamma \) of a threonine, instead of 3.83 Å to Cβ of Ala-183. The kinetic data indicate a 6-fold improvement of Y-27632 binding in both mutants. This effect appears due to the combination of the positive and negative effects. The T183A mutation has the strongest positive effect. The obvious effect of these mutations in PKAR3 is a change in the van der Waals environment of the inhibitor molecules. The shift of the inhibitor could be a consequence of a combination of both exchanges. Possibly, Ile-49 improves the van der Waals contact area, by 1.3 Å at the Thr-183 Cα atom. The structure of PKAR5–1077 is in a closed conformation and lacks a comparable backbone shift around residue 183 (Fig. 9). The peptide bond between Ala-183 and Asp-184, however, is rotated, clearly indicated in the electron density. This causes a deviation of the backbone around these two residues from the PKAR5 structure (without inhibitor). Otherwise, the PKAR5–1077 structure shows the typical conformational changes of the other 5-fold mutated enzymes in the hinge and C-tail regions.

The isoquinoline group of fasudil binds in PKAR5–1077 in a similar way as in the wild-type enzyme 1Q8W, although its position relative to C-lobe residues such as Leu-173 is shifted by \( \frac{1}{2} \) Å toward the C-lobe and toward the opening of the catalytic cleft. A different orientation and binding pattern is found for the sulfonamide group and the homopiperazine ring, which are rotated by a quarter turn (Fig. 9). The homopiperazine ring nitrogen of fasudil in 1Q8W makes two hydrophilic contacts, one to the backbone of Glu-170, and the other one to the carboxylate group of Glu-127. In PKAR5–1077 only the contact to the Glu-170 carbonyl remains. The shorter side chain of Asp-127 in PKAR5–1077 is oriented away from the inhibitor and is thus too far away to form a contact. This rotamer of Asp-127 allows the observed rotation of the homopiperazine ring, which would clash with a Glu-127 wild-type rotamer. The unusual backbone shift between Lys-181 to Gly-186, notably at Thr-183 (alanine in PKAR5–1077), does not occur in PKA Mutants with Rho-kinase Inhibitor Specificity

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FIGURE 9. A, superposition of PKAR5–1077 (blue carbons) and (1Q8W) PKAWT-HA1077 (gray carbon atoms). B, electron density map (2F\textsuperscript{o} \textendash F\textsuperscript{c}, contoured at 1.5 \( \sigma \)) of the inhibitor binding pocket of PKAR5–1077.
PKAR5–1077. The shift of this region in the wild-type enzyme may be required to allow the homopiperazine ring to form the two hydrogen bonds. Apparently the smaller side chain of Ala-183 of Rho-kinase enables the usual backbone position avoiding steric stress on the inhibitor side chain. This is verified by counting the number of van der Waals contacts to T/A183: 11 to Thr-183 in 1Q8W, and only 5 to Ala-183 in PKAR5–1077. Fasudil binds more weakly to PKAR5 than to PKAR3 and PKA wild type. This is not likely due to the different number of contacts to the smaller Ala-183 side chain, but rather to unfavorable effects of the exchange V123M and E127D. The largest negative contribution with respect to binding comes according to the kinetic data from the Met-123. The negative effect of the Asp-127 introduction may simply be caused by the loss of the hydrogen bond, found between Glu-127 and the homopiperazine ring in 1Q8W.

Comparison of the van der Waals contacts reveals a slightly higher proportion of van der Waals contacts in the PKAR5 enzyme. Six contacts are with Ile-49, instead of three to Leu-49 in 1Q8W. The total number of van der Waals contacts does not necessarily correlate with binding strength, because a larger number of contacts might implicate also a steric conflict, as possibly seen in the wild-type 1Q8W, where a backbone shift out of the normal position is observed, not seen so far in other structures.

Glu-127 is a residue with additional multiple functions in PKA. It binds not only to the 3’OH-group of ATP but has an important role in recognition of the substrate consensus sequence of PKA (33, 34). Glu-127 interacts with Arg-2 (Arg-18 in PKI(5–24)) of the substrate recognition sequence RRX(S/T)Y. Asp-127, conserved in most AGC kinases, and in many others, is a true homologue of Glu-127 in this respect, because it makes a bidentate contact to the guanidinium group of Arg-18 from the bound PKI(5–24) pseudosubstrate peptide.

Binding of H1152P to PKAR3 and PKAR5—The general binding mode of H1152P in the mutants is similar to that in the wild-type structure 1Q8U (PKAWT-H1152P) (Fig. 10). As seen in the Y-27632 complexes, the inhibitors in the mutants are rotated slightly counterclockwise (when viewed from the N-lobe) in the plane of the isoquinoline ring and are lowered by ≈0.5 Å toward the C-lobe. H1152P superimposes well in PKAR3 and PKAR5, despite the PKAR5 typical conformational changes. In contrast to PKAR5–1077, this applies also for the methyl homopiperazine ring, which has the same puckering and orientation in all three structures. Due to the rotation of the inhibitor in the mutant structures, Cys-22 of the homopiperazine ring is by 0.7 (PKAR3–1152) or 0.76 Å (PKAR5–1152) translated toward Ala-183. This closer position is possible only because of the smaller side chain of the alanine residue. In contrast to 1Q8W, where the peptide backbone around Thr-183 is shifted away from the inhibitor, the H1152P wild-type structure 1Q8U superimposes well with other PKA structures, such as 1CDK. In both H1152 mutant complex structures, however, some movement of the Ala-183 backbone is clearly notable. Here, the residue approaches the C2M methyl group at the homopiperazine ring, one of two determinants that distinguish H1152P from fasudil. This appears to indicate an attractive interaction between the two methyl groups (A183:C and H1152P:C2M), another possible reason for the rotation of the inhibitor toward Ala-183 in the mutant structures. We postulated in the previous publication that, in analogy to fasudil in 1Q8W, such an adjustment of the H1152P position would occur, possibly leading to the formation of hydrogen bonds from Asn-24 to Asp-127 and Glu-170-O, when the steric conflict between the C2M methyl group and Thr-183 would be omitted by having an alanine residue in this position. Indeed, both contacts exist, though not directly, but formed via water.

FIGURE 10. Superposition of PKAR3–1152 (copper carbon atoms) and 1Q8U (PKAWT-H1152P) (gray carbons) (A); and PKAR5–1152 (blue carbons) and 1Q8U (gray carbons) (B). C, electron density maps (2Fo − Fc contoured at 1.5σ) of the inhibitor binding pocket of PKAR3–1152. D, PKAR5–1152.
Again, as described for PKAR5–1077, we observed a flip of the peptide link between Ala-183 and Asp-184. This phenomenon is visible in two of the PKAR5 complexes, and the question arises whether the peptide flip could be related to a specific mutation of PKAR5. Notably, we observed this phenomenon before in the PKA staurosporine complex (1STC) and in one of the two molecules in the asymmetric unit of a PKAB3 Bim2 complex (35).

PKAR5–1152 contains a second inhibitor molecule at the surface of the protein, in a region of crystal contacts to a symmetry-related molecule. Two binding sites for H1152P were observed also in the wild-type structure 1Q8U. The second H1152P molecule is clearly defined in the electron density and binds close to the activation loop, making several hydrogen bond contacts with enzyme residues: one contact from a sulfonyl oxygen atom to Lys-189, and two bonds from Asn-24 of the homopiperazine ring to two oxygens from the Thr-197 phosphoryl group. Another contact exists between the isoquinoline amide and the Thr(PKI(5–24))OH group from the symmetry mate. In addition, the molecule makes several van der Waals contacts with the side chain of Glu-86 and Tyr-7 (PKI(5–24)) from the symmetry equivalent. The binding pattern of this molecule in PKAR5–1152 is thus identical to that in 1Q8U. Although this molecule is not inhibitory, because it does not compete with substrate or ATP nor change the activation loop conformation, it may still be of interest, because the site is a binding site for the regulatory subunits of PKA and a binding site for regulatory proteins in other kinases too. Exploring this external binding site may prove useful for interference with cellular pathways or with pathological processes.

CONCLUSIONS

Simulating the unique amino acid composition of the Rho-kinase ATP-binding site in a related protein kinase such as PKA by site-directed mutagenesis generates a useful kinase model, in which the individual effects of each residue on inhibitor selectivity can be studied kinetically and structurally. Two of these residues, Ile-49 and Ala-183, are clearly determinants of Rho-kinase inhibitor binding specificity. Ala-183 enhances binding of Rho-kinase selective inhibitors, Ile-49 acts rather counterselectively against the PKA inhibitor KT5720. The role, if any, of Met-123 in Rho-kinase remains unclear, because this residue appears to reduce binding of Rho-kinase inhibitors in PKA in general; its true effect, however, might be obscured in the PKAR5 mutant by the conformational changes it induces in various regions of the molecule. The region with conformational changes of the C-tail, which is closest to the kinase hinge, has different sequences in Rho-kinase and in PKA; possibly these residues compensate the negative effect on binding of the V123M exchange in PKA. The dual-function residue 127, which participates as glutamic acid or as aspartic acid in substrate recognition, appears to moderately weaken the binding of the isoquinoline inhibitors. Possibly, this is due to the loss of a hydrogen bond, either directly, as with HA1077, or indirectly via water, as in PKAR3–1152. In the case of Y-27632, where no such interaction is indicated, the exchange to Asp-127 is beneficial for binding. Here may be a potential point from which to explore the enhancement of inhibitor binding by rational design. An unexpected result of the kinetic analysis is the consistent contribution of the individual mutations by simple factors, and we did not observe synergistic effects. This corroborates the idea that, despite the flexibility of the kinase domain and mutual interactions of some active site residues, basically the individual residues of the binding site define the selectivity of kinase inhibitor interactions. Our results suggest that the kinase inhibitor binding site is well described by the sum of its parts.
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Addendum—A recent publication (Jacobs et al. (43)) describes the structure of Rock I (Rho-kinase β) in complex with the inhibitors used in the crystallization part of this study. The structures agree very well with our structural findings. They show, besides other things, identical details such as the same rotamers of the mutated residues; the same ~90° rotation of the fasudil side chain; a similar rotation of the inhibitors toward the Ala-183 homologue, and the very well with our structural findings. They show, besides other things, identical details such as the same rotamers of the mutated residues; the same ~90° rotation of the fasudil side chain; a similar rotation of the inhibitors toward the Ala-183 homologue, and the same change in hydrogen bond pattern of Y-27632. Their data thus structurally verify our surrogate approach.

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