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A molecular mechanism of P-loop pliability of Rho-kinase investigated by molecular dynamic simulation

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Abstract Rho-kinase is a leading player in the regulation of cytoskeletal events involving smooth muscle contraction and neurite growth-cone collapse and retraction, and is a promising drug target in the treatment of both vascular and neurological disorders. Recent crystal structure of Rho-kinase complexed with a small-molecule inhibitor fasudil has revealed structural details of the ATP-binding site, which represents the target site for the inhibitor, and showed that the conserved phenylalanine on the P-loop occupies the pocket, resulting in an increase of protein–ligand contacts. Thus, the P-loop pliability is considered to play an important role in inhibitor binding affinity and specificity. In this study, we carried out a molecular dynamic simulation for Rho-kinase–fasudil complexes with two different P-loop conformations, i.e., the extended and folded conformations, in order to understand the P-loop pliability and dynamics at atomic level. A PKA–fasudil complex was also used for comparison. In the MD simulation, the flip-flop movement of the P-loop conformation starting either from the extended or folded conformation was not able to be observed. However, a significant conformational change in a long loop region covering over the P-loop, and also alteration of ionic interaction-manner of fasudil with acidic residues in the ATP binding site were shown only in the Rho-kinase–fasudil complex with the extended P-loop conformation, while Rho-kinase with the folded P-loop conformation and PKA complexes did not show large fluctuations, suggesting that the Rho-kinase–fasudil complex with the extended P-loop conformation represents a meta-stable state. The information of the P-loop pliability at atomic level obtained in this study could provide valuable clues to designing potent and/or selective inhibitors for Rho-kinase.

Keywords Conformational pliability · Fasudil · Molecular dynamics · P-loop · Rho kinase

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Introduction

Rho-kinase (Rho-associated kinase, ROCK 2, ROKα, or ROCK II) [1–5] is a serine/threonine protein kinase (1,388 residues) that comprises an N-terminal extension region, a kinase core domain, a C-terminal extension region, a coiled-coil region with the Rho-binding region, and a C-terminal putative pleckstrin homology (PH) domain including a cysteine-rich subdomain. The catalytic activity of the kinase is thought to be negatively regulated by an autoinhibitory interaction between the catalytic kinase domain and the C-terminal PH-like domain [6, 7]. For activation of the kinase, GTP-bound RhoA needs to bind the Rho-binding region of the coiled-coil domain, resulting in loss of interaction between the kinase domain and the C-terminal region [5].

Rho-kinase directly and indirectly regulates to facilitate myosin light chain phosphorylation, inducing interactions between actin and myosin, and thereby enhances myosin ATPase activity required for contraction [8]. Many

contractile processes in smooth muscle and nonmuscle cells including vascular smooth muscle contraction, stress-fiber formation, and cell migration have been linked to the Rho/Rho-kinase pathway [9, 10]. Abnormal activation of this pathway has been observed in various disorders of the central nervous system [11]. Thus, inhibition of this pathway represents a promising strategy for the treatment of these diseases.

One of the most prominent Rho-kinase inhibitors is fasudil (HA-1077, [1-(5-isoquinolinesulfonyl)homopiperazine], Fig. 1) [12], and is clinically used to treat cerebral vasospasm caused by subarachnoid hemorrhage [13]. Recently, the complex structure of the kinase domain of Rho-kinase with the inhibitor has been determined at the atomic level by the X-ray diffraction method [14]. The overall fold of the kinase core domain shows the canonical protein kinase fold that forms a bilobal structure with a small N-terminal lobe (residues 85–174) and a larger C-terminal lobe (residues 175–350) (Fig. 1). Fasudil is identified in the interlobe cleft that corresponds to the conserved ATP binding site. The planar isoquinoline ring of fasudil is inserted into the canonical adenine binding pocket of protein kinases, whereas the seven-membered homopiperazine ring is placed at the entrance of the cleft, where catalytically active residues are clustered. The inserted isoquinoline ring is fixed in the pocket by hydrogen-bonding between the ring nitrogen atom (N7) and the main chain of Met172 in the conserved hinge region (residues 170–175; EYMPGG) between two lobes. The hydrogen bond between the base ring and the hinge region is conserved across various kinases [15].

One of the most interesting aspects of the binding of fasudil to Rho-kinase compared to the binding of fasudil to cAMP-dependent protein kinase (PKA) is that we observed the plasticity or pliability of the phosphate-binding loop (P-loop, or glycine-rich loop) conformation on inhibitor binding (Fig. 1). In the fasudil complex, two crystallographically independent molecules with similar structures (molecule A and molecule B) are identified in the unit cell; the root-mean square deviation between the two molecules is small (0.65 Å for 321 Cα-carbon atoms). In molecule A, the P-loop adopts a similar conformation to that of PKA, and fasudil is likely to adopt a natural conformation. In contrast to molecule A, the loop of molecule B folds down to increase surface complementarity with the inhibitor. The aromatic ring of Phe103 flips inside the loop to form a number of contacts with the homopiperazine ring of fasudil, capping the hydrophobic pocket to which the inhibitor binds. In molecule B, the inhibitor appears to significantly alter the conformation of the homopiperazine ring to accommodate the Phe103 side-chain. P-loop pliability in Rho-kinase contributes to increase contacts when fasudil binds, something which is not observed in the PKA–fasudil complex [16]. The orientation of Phe103 in the Rho-kinase structure might represent an important part of the inhibitor binding mechanism.

To understand the P-loop pliability at atomic level, a molecular dynamic (MD) simulation is planned in this study. The knowledge of the P-loop pliability will be useful to design potent and/or selective inhibitors for Rho-kinase. For the structures of Rho-kinase, two protein structures of Rho-kinase with two different P-loop conformations were employed: molecule A of the fasudil complex (molA), molecule B of the fasudil complex (molB) [14]. In addition, a protein structure of PKA in complex with the same inhibitor fasudil [16] was used for MD simulation for comparison.

Materials and methods

Computational tools

Manipulation of molecular structures for computation was done using the Maestro molecular modeling package (Schrodinger Inc., Portland, OR). Molecular dynamic simulations were performed by AMBER 9.0 [17] on a single Pentium IV CPU (3.0 GHz) machine with Linux/CentOS4.0. Force-fields applied for proteins and a ligand were ff03 and gaff, respectively [18, 19].

Initial structures

In the X-ray structure of Rho-kinase (PDB entry ID: 2F2U, resolution: 2.40 Å), a part of the loop subsequent to C-terminal was undetermined due to crystallographic disorder: in molA a region from Asp389 to Asp393 and in molB a region from Glu388 to Glu395. For MD simulations, the disordered loop was modeled using the main-chain structure of PKA as a template (PDB entry ID: 1Q8W, resolution: 2.20 Å). The PKA structure of corresponding regions to the disordered regions of Rho-kinase was cut and pasted to the structure of molA or molB after both of PKA and molA/molB were superimposed on Cα-atoms. Then the side-chains of the PKA structure were mutated to those for Rho-kinase. The modeled structures of Rho-kinase were energetically minimized with constraint to the whole structure excluding the modeled loop region using the *sander* module of the AMBER package to cancel the distortions.

Hydrogen atoms with the standard geometry were added to fasudil structure using the Maestro package, and atomic charges of fasudil were derived from semi-quantum chemistry calculation AM1-BCC invoked in the *antechamber* module of the AMBER package. The N4′-atom of the homopiperazine ring was modeled to have a positive protonation. The *LEaP* module of the AMBER package

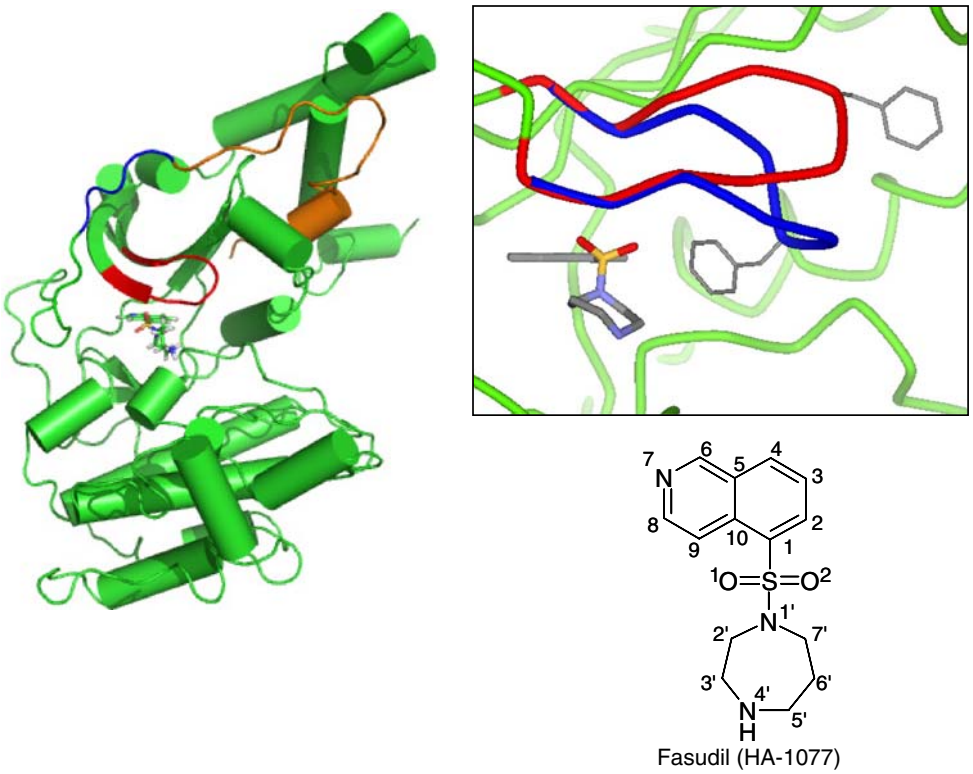

Fig. 1 The crystallographic structure of the Rho-kinase domain (PDB entry ID: 2F2U). Left-hand: Carbon α trace of the kinase domain (molecule A of the fasudil–Rho-kinase complex) with fasudil. The kinase domain is shown in green and the P-loop and C-terminal helix in the domain are represented in red and brown, respectively. The crystallographic disordered loop region which is modeled from the corresponding region of the X-ray structure of PKA (PDB entry ID: 1Q8W) is colored in blue. The structure of fasudil is shown as a stick model. Upper right-hand: Superimposing the P-loop structures of molecule A (red) and molecule B (blue) of the fasudil-bound form. The side-chain atom of Phe103 in the P-loop is shown as a stick model. The binding conformation of fasudil in the ATP binding site of molecule B in the fasudil complex is also represented as a stick model. Lower right-hand: The chemical structure of fasudil

was used to add hydrogen atoms on the protein structures and TIP3P water molecules surrounding the complexes with a 10.0 Å buffering distance between the edges of a solvated box and the protein, and then add five Na^+ ions for the Rho-kinase complexes and eight Cl^- ions for the PKA complex to neutralize the whole system. An octahedral box was used for solvation.

Molecular dynamic simulation

The initial structures in explicit solvent were minimized in a two-stage procedure: a minimization only for water/ions and followed by a minimization for the whole system. Periodic boundary simulations based on the particle mesh Ewald (PME) method was used for MD simulations. The minimized structures were heated from 0 to 300 K in 120 ps. One step of dynamics was 2 fs, and the SHAKE option to constrain bonds with hydrogen atoms was used. PME was employed for calculation of the full electrostatic energy of a periodic box. The cut-off distance for non-bonding interaction was set to be 8.0 Å. All MD simulations were done by the *pmemd* module of the AMBER package. MD simulations at a constant temperature 300 K and a constant pressure 1 atm were performed until 2 ns for each system. A snap shot structure during MD simulations was output by every 5 ps.

Results

Simulation of Rho-kinase molA–fasudil complex

The overall molA structure of Rho-kinase complexed with fasudil was stable in an aqueous MD simulation of a 2-ns run. Figure 2a shows the overlapped structures of snap-shots at 0 and 2 ns. Compared with the two snap-shot structures, the most fluctuated regions were the N-terminal helix, a long loop from αI-helix to C-terminal (colored in brown or magenta in Fig. 2a, hereafter referred to as the "long loop region"), and C-terminal helix. The fluctuated long loop region includes the crystallographic disordered region of the X-ray structures of Rho-kinase. The root mean squared deviation (RMSD) of every-5-ps structure to the 0-ns structure during the MD simulation is plotted in

Fig. 2b. In the early period (ca. 0–800 ps) of the simulation, the RMSD value was small around 1.8 Å, but increased up to around 2.8 Å in the late period (from ca. 800 ps).

During the whole simulation period, the conformational change of the P-loop from extended to folded was not observed. The major differences of the main-chain conformation between the extended and folded P-loops in the X-ray structures are the ϕ angles of Gly101 and Gly104 (Table 1). The ϕ angles of Gly101 and Gly104 in the extended P-loop of molA take *eclipsed* and *trans* (−119.1 and 170.9), but those in the folded P-loop of molB are *trans* and *gauche* (+) (−174.3 and 94.7), respectively. Figure 3 shows the ϕ angles of Gly101 and Gly104 not largely changing in the simulation.

Fasudil was positionally shifted to outside of the ATP binding site in the 2-ns structure. The shift of fasudil was mainly taken place at the seven-membered homopiperazine ring linked by the sulfonyl group (Figs. 4a, 5a). A hydrogen-bond between the ring nitrogen atom (N7) of fasudil and the main-chain amino group of Met172 in the hinge region was retained during the simulation (Fig. 4b) since the position of N7-atom of fasudil was not largely shifted. A distance between fasudil and Ala231, which is considered as a key residue relevant to selectivity between Rho-kinase and PKA [20], was changed by about 2 Å and the shift of fasudil occurred around 800 ps (Fig. 5b). Contrarily, a distance to Ile98, another key residue relevant to selectivity, was constant.

At 0 ns, an ionic interaction was observed between the N4′-atom of fasudil and the side-chain of Asp232. However, according to the shift of the homopiperazine ring, this interaction was broken in the 2-ns structure (Fig. 6a). The disruption of the interaction occurred around 700 ps (Fig. 6b). Instead, in the 2-ns structure, new ionic interactions were made between the N4'-atom of fasudil, and the side-chains of Asp176 and Asp218. The new interaction was formed at a similar timing of the disruption of the interaction with Asp232 (data not shown). Phe384 was a residue consisting of a side wall of the ATP binding site. According to the shift of the homopiperazine ring, the position of Phe384 was shifted to outside of the ATP binding site (Fig. 6a).

Simulation of Rho-kinase molB–fasudil complex

The molB structure of Rho-kinase has the folded P-loop conformation. During a 2-ns MD simulation, the overall

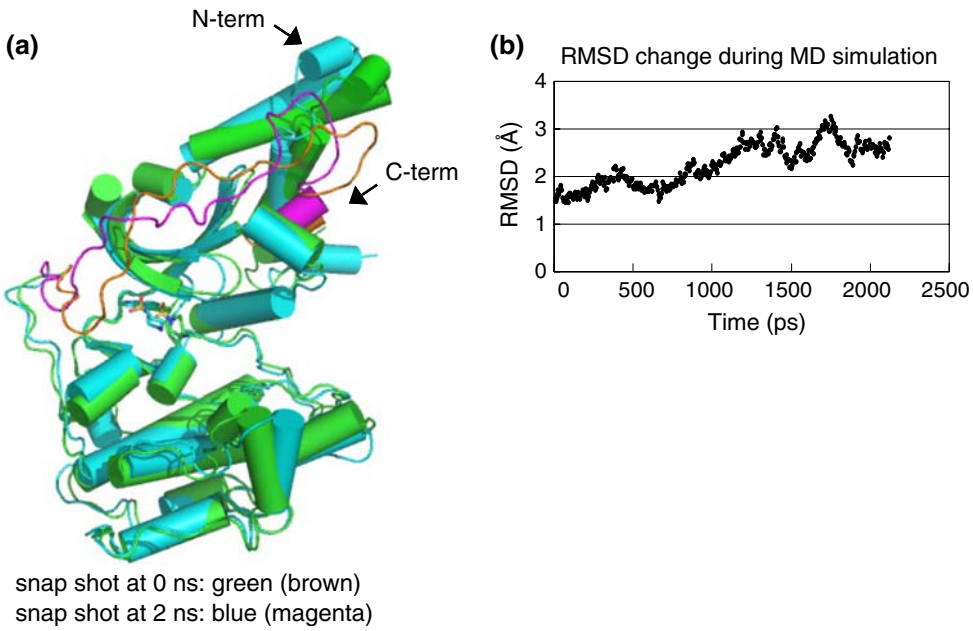

Fig. 2 The results of molecular dynamic simulation of molA–fasudil complex. (**a**) Snap shots at 0 ns (green) and 2 ns (blue) of molA–fasudil complex. The long loop region and C-terminal helix is colored in brown (0 ns) and magenta (2 ns). (**b**) The time-course change of the RMSD value of molA structure during the MD simulation

Table 1 The ϕ and φ angles in the P-loops of Rho-kinase molA and molB in the X-ray structure

	Arg100		Gly101		Ala102		Phe103		Gly104		Glu105	
	ϕ	φ	ϕ	φ	ϕ	φ	ϕ	φ	ϕ	φ	ϕ	φ
molA	−132.5	137.9	**−119.1**	179.6	−76.5	−26.2	−115.6	3.1	**170.9**	−159.8	−123.4	173.0
molB	−158.7	120.0	**−174.3**	−143.1	−66.0	−33.0	−83.1	14.8	**94.7**	175.5	−93.1	116.6

The largest deviations of the angles are shown in bold type

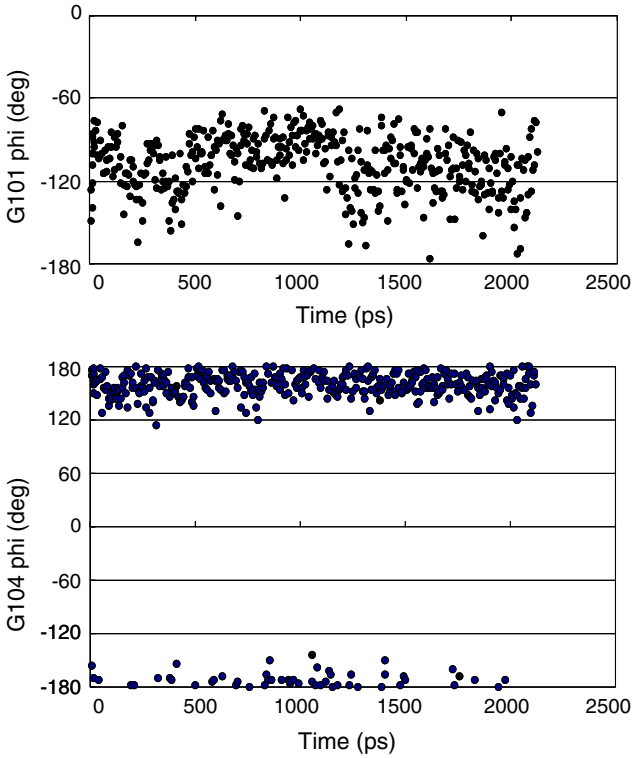

Fig. 3 The time-course change of the ϕ angles of Gly101 and Gly104 of molA structure during the MD simulation

structure and the P-loop conformation were not fluctuated largely (Fig. 7a). The RMSD value of every-5-ps structure to the 0-ns structure was rather constant (Fig. 7b) and the ϕ angles of Gly101 and Gly104 were not deviated during the simulation (data not shown). The conformations of the N-terminal helix, the long loop region where fluctuation was observed in the Rho-kinase molA–fasudil complex, and the C-terminal helix were also fluctuated, however, the range of the fluctuated loop was smaller than that in the molA–fasudil complex. The N-terminal side of the long loop was not largely fluctuated compared with the molA–fasudil complex.

The position of fasudil in the 2-ns structure was slightly shifted to the direction of Ala231, occurred around 1,700 ps (Fig. 8). In the molB complex, the ionic interaction between the N4′-atom of fasudil, and aspartates 176 and 218 was unchanged during the simulation (Fig. 9). The position of Phe384 was not largely changed, either.

Simulation of PKA–fasudil complex

In contrast to the Rho-kinase–fasudil complexes, the structure of PKA–fasudil complex during a 2-ns MD simulation was fairly stable (Fig. 10). The P-loop conformation was also stable in the simulation, retaining an extended conformation. No fluctuation of the long loop region were observed. The binding position of fasudil was kept in the simulation, thereby conserving a distance between Thr183 and fasudil (Fig. 11). A distance between Leu111 and fasudil was retained (data not shown). Both were considered as a key residue for selectivity [20]. Around the binding site of the homopiperazine ring, the interaction-manner of the N4′-atom of fasudil with acidic residues in the ATP binding site was unchanged between the 0-ns and 2-ns structures (Fig. 12). In both structures, the side-chains of Glu127 and Glu170 interacted with the homopiperazine ring. The position of Phe327 was retained either.

Discussion

Here we investigated the dynamic features of Rho-kinase complexed with a Rho-kinase specific inhibitor fasudil by molecular dynamic simulation. Two Rho-kinase structures with the extended or folded P-loop conformation were employed for a 2-ns simulation. PKA complexed with the same inhibitor fasudil was subjected to a 2-ns MD simulation for comparison.

The primary objective of this study is to examine the conformational pliability of the P-loop, especially to understand the flipping down mechanism of the P-loop. As described above, our MD simulation was not able to capture the flip-flop movement of the P-loop conformation

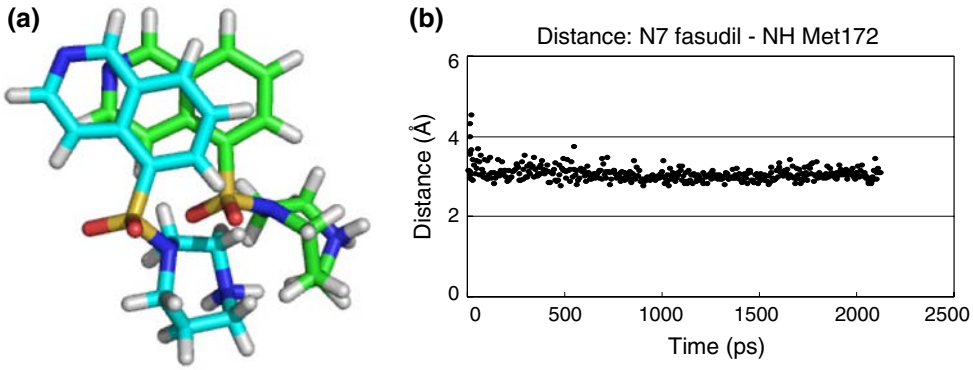

Fig. 4 Conformational change of fasudil in molA–fasudil complex during the MD simulation. (**a**) Comparison of fasudil binding conformation of snap shots between 0 ns (green) and 2 ns (blue). (**b**) The time-course change of a distance between the N7-atom of fasudil and the main-chain amino group of Met172 in molA–fasudil complex

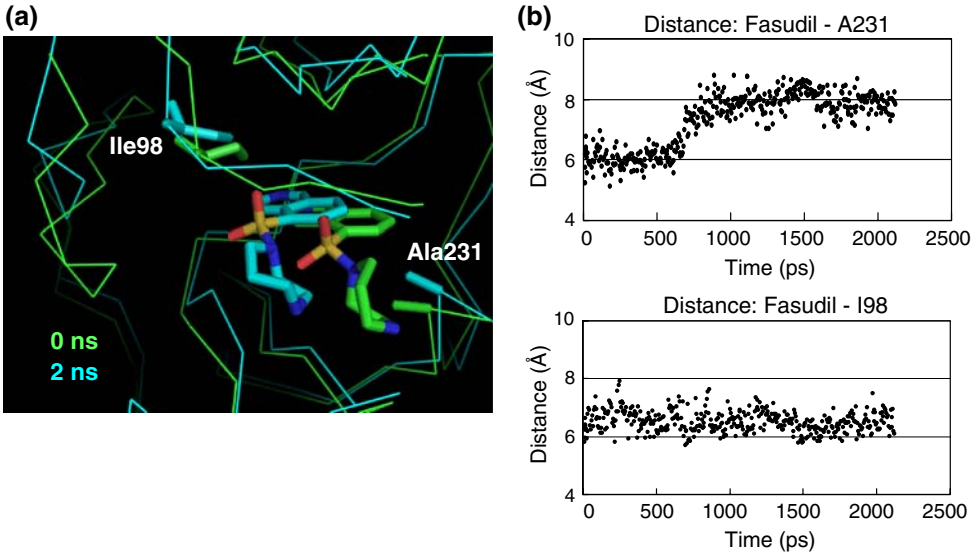

Fig. 5 The results of MD simulation of molA–fasudil complex. (**a**) Close-up view of the ATP binding site at 0 ns (green) and 2 ns (blue). Fasudil and Ile98/Ala231 are shown as a stick model. (**b**) Upper: The time-course change of a distance between fasudil and Ala231. Lower: The time-course change of a distance between fasudil and Ile98

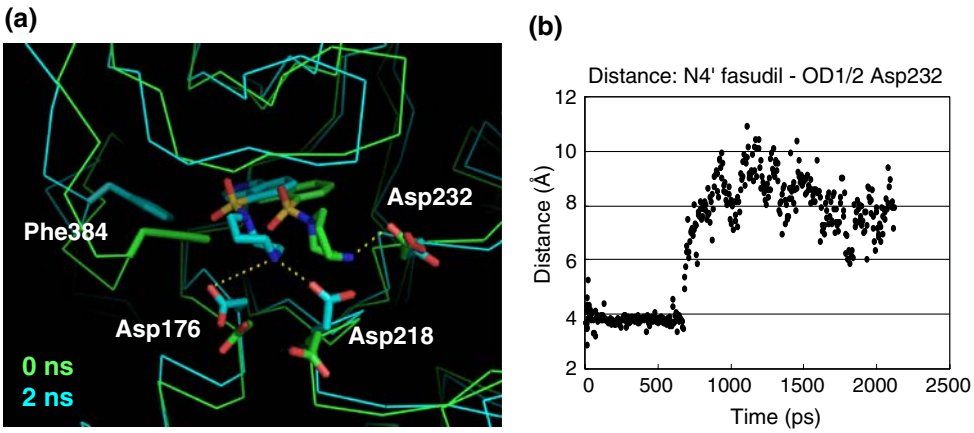

Fig. 6 The results of MD simulation of molA–fasudil complex. (**a**) Close-up view of ionic interactions around the ATP binding site at 0 ns (green) and 2 ns (blue). Fasudil and residues relevant to the interaction are shown as a stick model. Possible ionic interactions are shown as dotted lines. (**b**) The time-course change of a distance between the N4'-atom of fasudil and the $O^{\delta 1}/O^{\delta 2}$-atom of Asp232

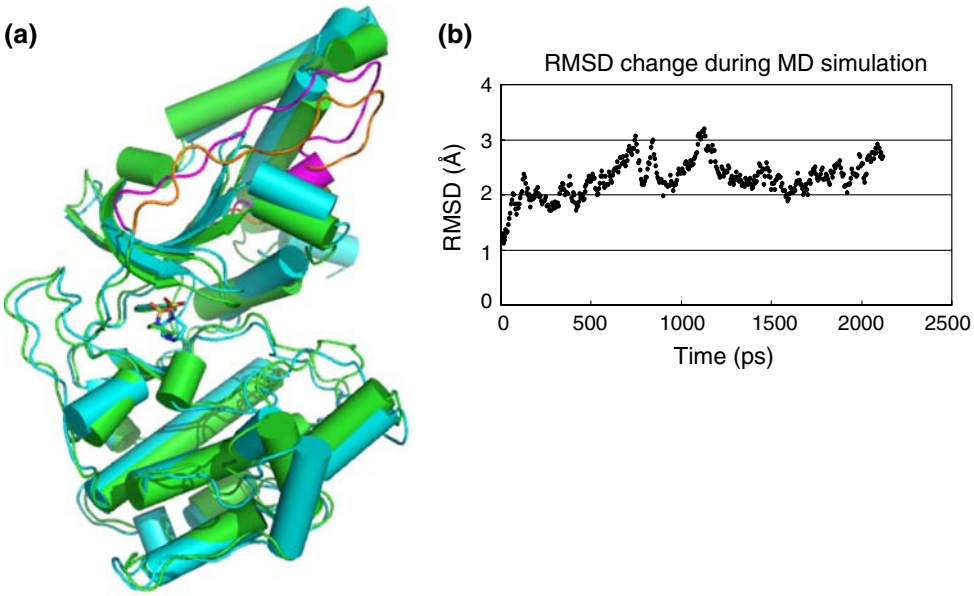

Fig. 7 The results of MD simulation of molB–fasudil complex. (**a**) Snap shots at 0 ns (green) and 2 ns (blue) of molB–fasudil complex. The long loop region and C-terminal helix is colored in brown (0 ns) and magenta (2 ns). (**b**) The time-course change of the RMSD value of molB structure during the MD simulation

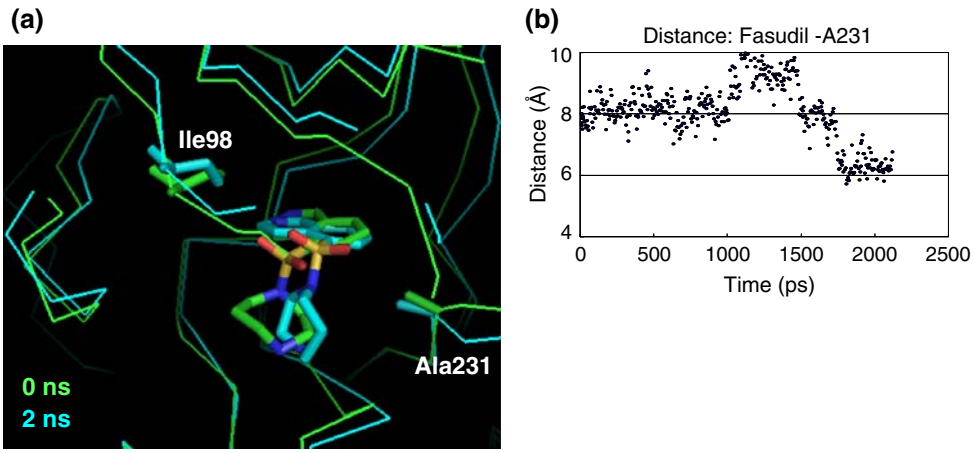

Fig. 8 The results of MD simulation of molB–fasudil complex. (**a**) Close-up view of the ATP binding site at 0 ns (green) and 2 ns (blue). Fasudil and Ile98/Ala231 are shown as a stick model. (**b**) The time-course change of a distance between fasudil and Ala231

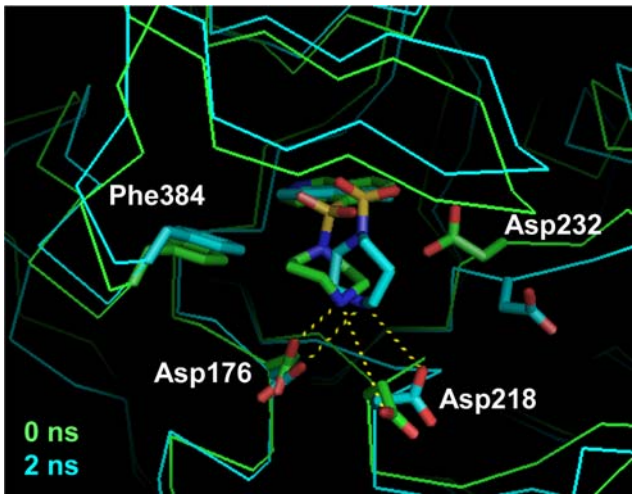

Fig. 9 The results of MD simulation of molB–fasudil complex. Close-up view of ionic interactions around the ATP binding site at 0 ns (green) and 2 ns (blue). Fasudil and residues relevant to the interaction are shown as a stick model. Possible ionic interactions are shown as dotted lines

starting either from the extended conformation in Rho-kinase molA and the folded conformation of Rho-kinase molB. We suppose that the 2-ns time of MD simulation in this study is too short to observe the conformational change of the P-loop. The vicinal structure around Phe103, which is in the P-loop and makes van der Waals contacts to fasudil in the molB complex (Fig. 1), is depicted in Fig. 13. A number of hydrophobic residues including Leu123, Met128, Phe136, and Phe137 surround the side-chain of Phe103, making a hydrophobic cluster. In order to flip down the Phe103 side-chain towards the inside of the P-loop, where is a ligand binding space, the hydrophobic cluster must be disrupted. The P-loop therefore would be lifted up first to relieve the Phe103 side-chain of the hydrophobic cluster at the beginning of the flipping-down process of the P-loop. By looking at the circumstance of the P-loop, the long loop region covers over the P-loop (Fig. 2a). Then, if the long loop region is energetically stable and keeps the conformation as it is, the P-loop

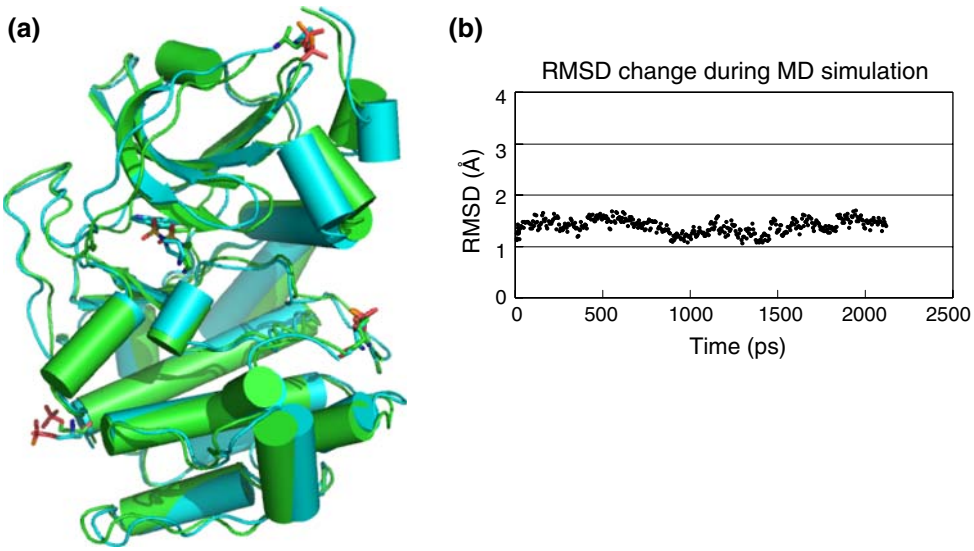

Fig. 10 The results of MD simulation of PKA–fasudil complex. (**a**) Snap shots at 0 ns (green) and 2 ns (blue) of PKA–fasudil complex. (**b**) The time-course change of the RMSD value of PKA structure during the MD simulation

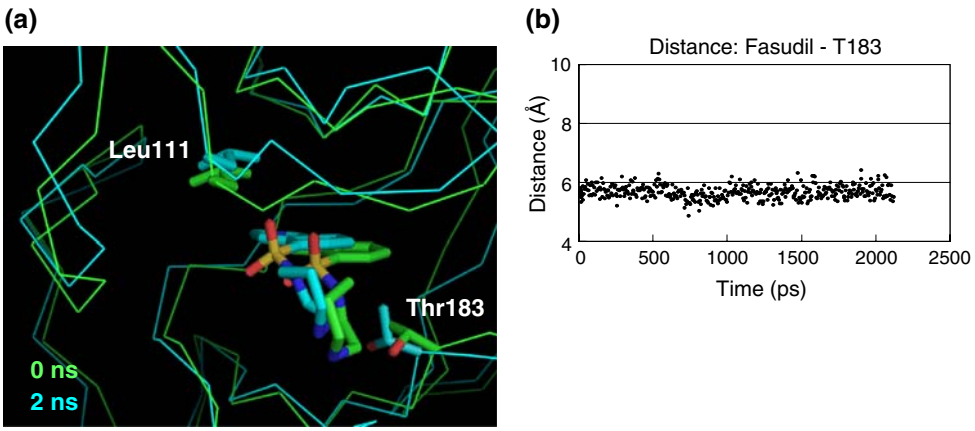

Fig. 11 The results of MD simulation of PKA–fasudil complex. (**a**) Close-up view of the ATP binding site at 0 ns (green) and 2 ns (blue). Fasudil and Leu111/Thr183 are shown as a stick model. (**b**) The time-course change of a distance between fasudil and Thr183

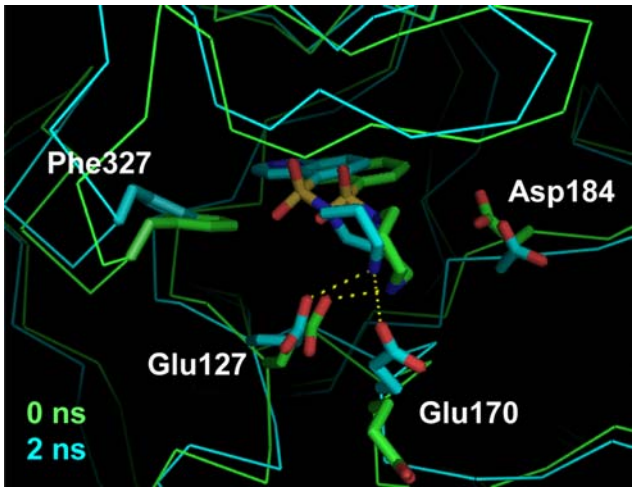

Fig. 12 The results of MD simulation of PKA–fasudil complex. Close-up view of ionic interactions around the ATP binding site at 0 ns (green) and 2 ns (blue). Fasudil and residues relevant to the interaction are shown as a stick model. Possible ionic interactions are shown as dotted lines

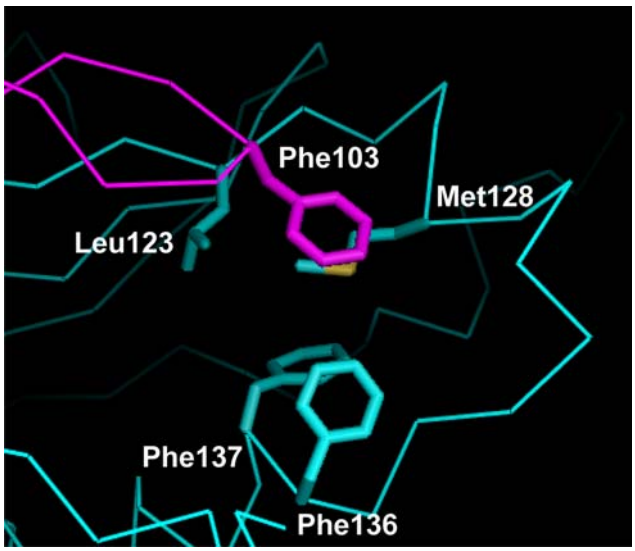

Fig. 13 The vicinal structure of Phe103 in the 2-ns structure of molA–fasudil complex. The P-loop is colored in magenta. Hydrophobic residues are depicted as a stick model

lifting-up would be interfered. In fact, the long loop region includes the crystallographic disordered region of the Rho-kinase X-ray structures, and is most fluctuated in the MD simulations of Rho-kinase, whereas the long loop region in the PKA complex is considered as fairly stable from the experimental and simulation results. Thus, the flexibility of the long loop region may be unique for Rho-kinase–fasudil complex, and could be a necessary feature to lead to the flip-flop movement of the P-loop. Concerning the flexibility of the long loop region between molA and molB, the fluctuation of the loop is occurred in a wider area in molA than in molB.

Compared between the molA and molB X-ray structures of Rho-kinase, the most distinguished structural differences are the conformations of the P-loop and fasudil [14]. After the 2-ns MD simulation of both molecules, the binding conformation of fasudil was significantly changed only in molA (Fig. 4a). The ionic interaction of the N4′-atom of fasudil in the initial structure was re-arranged: from the interaction with Asp232 to the interaction with two aspartates 176 and 218 around 800 ps (Fig. 6a), and these interactions was kept until 2 ns. This ionic interaction-manner is observed in the initial structures of molB of Rho-kinase and PKA complexes, and during the MD simulations no large changes were observed in both complexes. Therefore, the interactions of the N4′-atom of fasudil with two acidic resides may be natural and energetically stable. According to the conformational change of fasudil, Phe384 in molA was moved to avoid a steric clash to fasudil. Since Phe384 is located at the N-terminal side of the long loop region, the positional shift of Phe384 would initiate the conformational change of the long loop region in molA.

Lastly, as a supplemental calculation, a longer MD simulation until 9 ns was done for the molA structure of Rho-kinase complexed with fasudil. No significant conformational change was observed between 2-ns and 9-ns

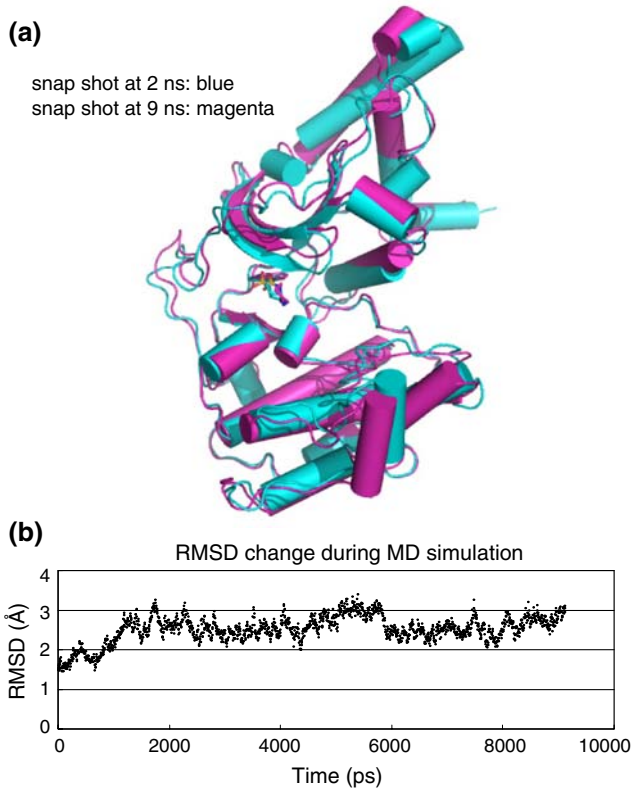

Fig. 14 The results of a 9-ns MD simulation of molA–fasudil complex. (**a**) Snap shots at 2 ns (blue) and 9 ns (magenta) of molA–fasudil complex. (**b**) The time-course change of the RMSD value of molA structure during the MD simulation

strucutures (Fig. 14), indicating that the P-loop conformational change requires an MD simulation in a considerably longer time-scale [21–23].

Conclusion

Rho-kinase is a promising drug target in the treatment of both vascular and neurological disorders. The P-loop pliability is considered to play an important role in inhibitor binding affinity and specificity. In our preliminary MD simulation of Rho-kinase shown in this study, the pliability of the P-loop was not completely captured due to relatively a short time-scale of the simulation. However, a significant conformational change in the long loop region covering over the P-loop, and also alteration of ionic interaction-manner of fasudil with acidic residues in the ATP binding site were shown only in molA with the extended P-loop conformation, while molB with the folded P-loop conformation and PKA complexes did not show large fluctuations, suggesting that the Rho-kinase–fasudil complex with the extended P-loop conformation represents a meta-stable state. The information of the P-loop pliability at atomic level obtained in this study could provide valuable clues to designing potent and/or selective inhibitors for Rho-kinase; for instance, introducing the hydrophobic moiety into an inhibitor to enhance hydrophobic interaction with Phe103.

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