Kinetic Mechanism for Human Rho-Kinase II (ROCK-II)

John W. Trauger, Fen-Fen Lin, Mary S. Turner, Jeffrey Stephens, and Philip V. LoGrasso*

Department of Molecular Neuroscience, Merck Research Laboratories, 3535 General Atomics Court, San Diego, California 92121

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ABSTRACT: Rho-Kinase is a serine/threonine kinase that is involved in the regulation of smooth muscle contraction and cytoskeletal reorganization of nonmuscle cells. While the signal transduction pathway in which Rho-Kinase participates has been and continues to be extensively studied, the kinetic mechanism of Rho-Kinase-catalyzed phosphorylation has not been investigated. We report here elucidation of the kinetic mechanism for Rho-Kinase by using steady-state kinetic studies. These studies used the kinase domain of human Rho-Kinase II (ROCK-II 1−534) with S6 peptide (biotin-AKRRRLSSLRA-NH_2) as the phosphorylatable substrate. Double-reciprocal plots for two-substrate kinetic data yielded intersecting line patterns with either ATP or S6 peptide as the varied substrate, indicating that Rho-Kinase utilized a ternary complex (sequential) kinetic mechanism. Dead-end inhibition studies were used to investigate the order of binding for ATP and the peptide substrate. The ATP-competitive inhibitors AMP-PCP and Y-27632 were noncompetitive inhibitors versus S6 peptide, and the S6 peptide analogue S6-AA (acetyl-AKRRRLAALRA-NH_2) was a competitive inhibitor versus S6 peptide and a noncompetitive inhibitor versus ATP. These results indicated a random order of binding for ATP and S6 peptide.

Rho-Kinase plays a key role in Ca^{2+}-independent smooth muscle contraction (*1*, *2*). The contraction of smooth muscle is regulated primarily by the concentration of free Ca^{2+} in the cytosol ($[Ca^{2+}]_{free}$), which in turn regulates phosphorylation of the 20 kDa myosin light chain (MLC)[1] by the Ca^{2+}-calmodulin-dependent myosin light chain kinase (MLCK). However, increased phosphorylation of MLC relative to $[Ca^{2+}]_{free}$, which is referred to as increased Ca^{2+} sensitivity, can occur following stimulation of smooth muscle cells with various agonists. This Ca^{2+} independent mechanism of smooth muscle contraction has been shown to result from activation of the GTPase Rho, subsequent activation of Rho-Kinase by activated Rho, and then inhibition of myosin phosphatase (MP) by Rho-Kinase-catalyzed phosphorylation (*3*−*7*). Inhibition of MP results in increased MLC phosphorylation and muscle contraction. Direct phosphorylation of MLC by Rho-Kinase may also play a role in Ca^{2+}-independent smooth muscle contraction (*8*, *9*). Small-molecule inhibitors of Rho-Kinase, which promote smooth muscle relaxation, are effective for treatment of cereberal vasospasm (*10*) and potentially effective for treatment of angina (*11*), hypertension (*12*), arteriosclerosis (*13*), and erectile dysfunction (*14*−*16*).

In addition to regulating smooth muscle contraction, the Rho/Rho-Kinase pathway also regulates the contraction of nonmuscle myosin by phosphorylation of MLC, which is important for regulation of a variety of nonmuscle cell functions including stress fiber and focal adhesion formation (*6*, *17*, *18*), neurite retraction (*19*, *20*), and tumor cell invasion (*21*). In addition to MP, Rho-Kinase phosphorylates other proteins including (1) ERM (ezrin, radixin, moesin) family proteins, to regulate microvilli formation (*22*), (2) glial fibrillary acidic protein, to regulate cytokinesis (*23*), (3) collapsin response mediator protein, to regulate neuronal growth cone collapse by lysophosphatidic acid (*24*), and (4) LIM kinase, to regulate actin cytoskeleton rearrangement (*25*). Inhibitors of Rho-Kinase have been shown to promote axon outgrowth and axon regeneration in mammalian CNS neurons (*26*, *27*), and showed promise of effectiveness in promoting neurological recovery after traumatic spinal cord injury in a rat model (*28*), suggesting an approach to the treatment of spinal cord injury.

Two isoforms of Rho-Kinase have been identified, ROCK-I (also called p160ROCK and ROKβ) and ROCK-II (also called ROKα) (*29*−*32*). The two isoforms are 60% identical overall, and their kinase domains are 86% identical. Rho-Kinase is a 160 kDa multidomain protein containing an N-terminal kinase domain, a coiled-coil (Rho binding) domain, and a C-terminal pleckstrin homology (PH) domain with an inserted Cys-rich Zn finger motif. In the absence of the C-terminal PH domain or the coiled-coil and PH domains, the kinase domain of Rho-Kinase is constitutively active (*19*, *33*, *34*).

We set out to determine the kinetic mechanism of Rho-Kinase II (ROCK-II) using recombinant ROCK-II kinase

* To whom correspondence should be addressed at the Department of Molecular Neuroscience, Merck Research Laboratories, 3535 General Atomics Court, San Diego, CA 92121. Telephone: 858-202-5443, FAX: 858-202-5813, E-mail: lograsso@merck.com.

[1] Abbreviations: AMP-PCP, adenylyl methylenediphosphonate; LCB, long-chain biotin; MLC, myosin light chain; MLCK, myosin light chain kinase; MP, myosin phosphatase; ROCK-II 1−543, kinase domain of the ROCK-II isoform of human Rho-Kinase; S6-AA, acetyl-AKRRRLAALRA-NH_2; S6 peptide, LCB-AKRRRLSSLRA-NH_2; Y-27632, (+)-(*R*)-*trans*-4-(1-aminoethyl)-*N*-(4-pyridyl)cyclohexanecarboxamide dihydrochloride.

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domain (ROCK-II 1−543) with S6 peptide (biotin-AKRRRL-SSLRA-NH_2) as substrate. Our results from two-substrate kinetic analysis and inhibition studies with inhibitors competitive with either ATP or the peptide substrate indicated that Rho kinase-catalyzed phosphorylation involved formation of a ternary complex with a random order of binding for ATP and S6 peptide.

MATERIALS AND METHODS

Materials. Adenylyl methylenediphosphonate (AMP-PCP), bovine serum albumin (BSA), dithiothreitol (DTT), and Tris-HCl (1 M, pH 7.4) were purchased from Sigma, and guanidine hydrochloride (8 M) was from Pierce. ATP and [γ-^{33}P]ATP (>2500 Ci/mmol) were from Amersham Biosciences. S6 peptide and the inhibitor peptide S6-AA were purchased from Peptidogenic Research, Inc.

Expression and Purification of ROCK-II 1−543. The kinase domain (residues 1−543) of human ROCK-II was amplified from a human brain cDNA library, expressed with an N-terminal FLAG tag in Sf21 cells, and purified by anti-FLAG affinity chromatography as reported (*35*).

Steady-State Kinetics. Initial velocity experiments were carried out in a total volume of 50 μL, with the following final concentrations: 25 mM Tris-HCl, pH 7.4, 5 mM $MgCl_2$, 2 mM DTT, 1 mg/mL BSA, 2.5−100 μM ATP, 3 μCi of [γ-^{33}P]ATP, 0.2−10 μM S6 peptide (LCB-AKRRRLSSLRA-NH_2, where LCB = long-chain biotin), and 24−72 pM ROCK-II 1−543. Reactions were initiated by the addition of either ATP or S6 peptide and incubated for 60 min at 30 °C. Under these conditions, less than 10% of either substrate was converted to product. A time course experiment demonstrated that product formation was linear with time at 15, 30, 45, and 60 min time points. Reactions were quenched by the addition of 25 μL of 8 M guanidine hydrochloride, and 15 μL of the quenched reaction was spotted onto a streptavidin-coated membrane (SAM^2 biotin capture membrane, Promega). The membrane was washed twice with 1 M NaCl, twice with 1 M NaCl/1% phosphoric acid, once with water, and once with ethanol (3 min per wash). The washed membrane was dried, transferred to a scintillation vial containing 4 mL of scintillation fluid (Packard Ultima Gold), and analyzed by using a liquid scintillation counter (Beckman-Coulter).

Steady-state kinetic parameters were determined by a nonlinear least-squares fit of the initial velocity data to the equation for a ternary complex (i.e., sequential) kinetic mechanism (*36*):

$$v/[E]_o = \frac{k_{cat}[S6][ATP]}{K_{ia}^{S6}K_M^{ATP} + K_M^{S6}[ATP] + K_M^{ATP}[S6] + [S6][ATP]} \quad (1)$$

where v is the initial velocity, k_{cat} is the turnover number, $[E]_o$, [S6], and [ATP] are the concentrations of the enzyme, S6 peptide, and ATP, respectively, K_{ia}^{S6} is the dissociation constant for S6 peptide, and K_M^{S6} and K_M^{ATP} are the K_M values for S6 peptide and ATP, respectively. Nonlinear least-squares fitting was carried out using GraFit software version 5.0 (Erithacus Software, Staines, U.K.). Reported kinetic parameters are the average values from four independent experiments.

Enzyme Inhibition. Enzyme inhibition experiments were carried out as described above with the addition of either adenylyl methylenediphosphonate (AMP-PCP), Y-27632, or S6-AA (acetyl-AKRRRLAALRA-NH_2). When ATP was the varied substrate, S6 peptide was used at a fixed concentration of 2.5 μM, and when S6 peptide was the varied substrate, ATP was used at a fixed concentration of 40 μM. For the concentration ranges of the inhibitors used in these experiments, see Figure 2. Inhibition constants were determined by nonlinear least-squares fitting of the initial velocity data for each substrate/inhibitor pair to the equations for competitive (eq 2), noncompetitive ("mixed-type") (eq 3), or uncompetitive (eq 4) inhibition:

$$v/[E]_o = \frac{k_{cat}[S]}{K_M(1 + [I]/K_{is}) + [S]} \quad (2)$$

$$v/[E]_o = \frac{k_{cat}[S]}{K_M(1 + [I]/K_{is}) + [S](1 + [I]/K_{ii})} \quad (3)$$

$$v/[E]_o = \frac{k_{cat}[S]}{K_M + [S](1 + [I]/K_{ii})} \quad (4)$$

where [S] and [I] are the concentrations of the varied substrate and the inhibitor, respectively, and K_{is} and K_{ii} are the dissociation constants for dissociation of the inhibitor to yield the free enzyme and the enzyme−substrate complex, respectively. Nonlinear least-squares fitting was carried out using GraFit software. Inhibition experiments were performed twice for AMP-PCP and S6-AA and once for Y-27632.

RESULTS

Two-Substrate Kinetics. To determine the kinetic mechanism of Rho-Kinase, we initially screened several peptides as substrates for the recombinant, purified 66 kDa kinase domain of ROCK-II (ROCK-II 1−543). S6 peptide (biotin-AKRRRLSSLRA-NH_2) was found to be a suitable substrate for determination of the kinetic mechanism and for inhibitor screening based on its relatively low K_M, high solubility, and efficient turnover.

Double-reciprocal plots of initial rate data obtained over a range of concentrations for both substrates ($1/v$ vs 1/[S6 peptide] and $1/v$ vs 1/[ATP]) revealed a pattern of intersecting lines (Figure 1), indicating that ROCK-II catalysis follows a ternary complex (i.e., sequential) kinetic mechanism (*36*). These results rule out a ping-pong mechansim, for which a pattern of parallel lines would be expected. Furthermore, the lines on both plots intersect to the left of the $1/v$ axis, indicating that the mechanism is not rapid-equilibrium-ordered (*36*). Nonlinear fitting of the data to the equation for a ternary complex kinetic mechanism (eq 1) yielded values for the parameters k_{cat}, K_M^{ATP}, K_M^{S6}, and K_{ia}^{S6} (Table 1). Once we established a random addition of substrate (see inhibition kinetics below), K_{ia}^{ATP} was calculated by alternatively fitting the data to a variation of eq 1 where K_{ia}^{ATP} is the dissociation constant for ATP. ROCK-II-catalyzed phosphorylation of S6 peptide proceeds efficiently, with k_{cat} = 91 min^{-1} and K_M = 0.9 μM.

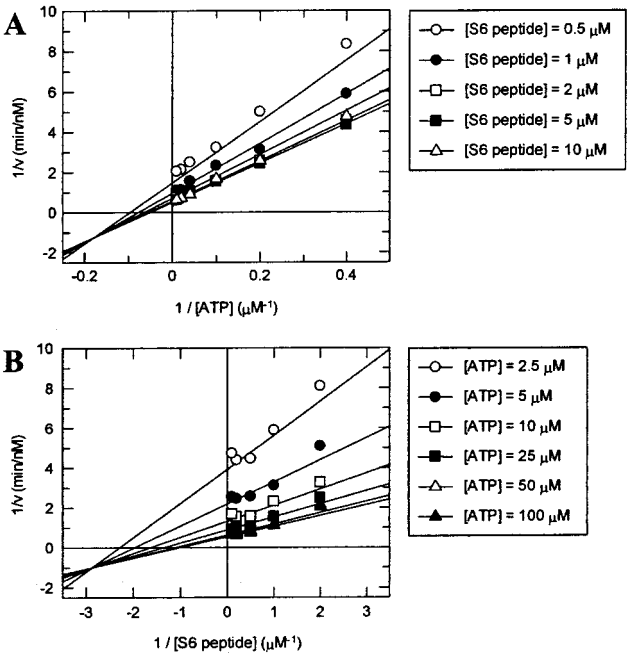

FIGURE 1: Two-substrate profile of ROCK-II-catalyzed S6 peptide phosphorylation. Double-reciprocal plots of (A) $1/v$ (min/nM) vs 1/[ATP] (μM^{-1}) obtained at five fixed S6 peptide concentrations, and (B) $1/v$ (min/nM) vs 1/[S6 peptide] (μM^{-1}) obtained at six fixed ATP concentrations. The data were fit to the equation for a ternary complex mechanism (eq 1). All reactions contained 25 mM Tris-HCl, 5 mM $MgCl_2$, 1 mg/mL BSA, 2.5−100 μM ATP, 0.5−10 μM S6 peptide, and 24 pM ROCK-II, at 30 °C and pH 7.4.

Table 1: Kinetic Constants for ROCK-II[a]

substrate	K_M (μM)	k_{cat} (min^{-1})	K_{ia} (μM)
S6 peptide	0.9 ± 0.2	91 ± 9	0.2 ± 0.1
ATP	21 ± 1		5.0 ± 0.9

[a] The kinetic parameters were determined by fitting the data to the equation for a ternary complex (eq 1). Reported parameters are the average values from four independent experiments, with the standard deviation indicated.

Inhibition Kinetics. To probe if the order of addition of substrates was random or ordered, we investigated the modes of inhibition for the inhibitors AMP-PCP, Y-27632, and S6-AA versus ATP and S6 peptide (Figure 2). Modes of inhibition and inhibition constants were determined by fitting initial rate data obtained using various concentrations of one substrate and a fixed concentration of the second substrate to the equations for competitive (eq 2), noncompetitive ("mixed-type") (eq 3), and uncompetitive (eq 4) inhibition. For AMP-PCP and Y-27632 inhibition with varied ATP concentrations, and for S6-AA inhibition with varied S6 peptide concentrations, the data were clearly best-fit to the equation for competitive inhibition. For S6-AA inhibition with varied ATP concentration, and for AMP-PCP and Y-27632 inhibition with varied S6 peptide concentration, the data were better fit to the equation for noncompetitive inhibition than the equations for uncompetitive and competitive inhibition as verified by F tests ($P \leq 0.04$). The modes of inhibition and inhibition constants are shown in Table 2.

When AMP-PCP was used as inhibitor with varied S6 peptide concentration, inhibitor-dependent substrate inhibition was observed at higher S6 peptide concentrations (5−10 μM). In this case, the initial rates obtained in the presence of AMP-PCP with 5−10 μM S6 peptide concentrations were not used for fitting to eqs 2−4.

Table 2: Inhibition Constants and Mode of Inhibition for ROCK-II Inhibitors[a]

inhibitor	varied substrate	mode of inhibition	K_{is} (μM)	K_{ii} (μM)
AMP-PCP	ATP	competitive	96 ± 12	
Y-27632	ATP	competitive	0.098 ± 0.005	
S6-AA	ATP	noncompetitive	35 ± 15	15 ± 2
AMP-PCP	S6 peptide	noncompetitive	1100 ± 370	840 ± 270
Y-27632	S6 peptide	noncompetitive	1.4 ± 0.7	0.37 ± 0.03
S6-AA	S6 peptide	competitive	3.0 ± 0.5	

[a] Inhibition constants were determined by fitting the data to the equations for either competitive (eq 2) or noncompetitive (eq 3) inhibition. Reported values are from a single experiment, with standard errors indicated.

DISCUSSION

A large number of recent studies have implicated Rho-Kinase as a key player in regulation of Ca^{2+}-independent smooth muscle contraction ("Ca^{2+} sensitization") and in regulation of cytoskeletal rearrangements in nonmuscle cells (*1, 2*). While Rho-Kinase is expressed throughout the body and regulates a variety of cellular responses, studies in animals and humans indicate that specific Rho-Kinase inhibitors can be well tolerated (*10*−*12*). Fasudil has been used clinically for treatment of cerebral vasospasm (*10*) and is approved in Japan for this use, making Rho-Kinase one of two kinases that are clinically validated drug targets. The only other clinically approved kinase inhibitor of which we are aware is the Bcr-Abl tyrosine kinase inhibitor Gleevec (*37*). Rho-Kinase inhibitors are also potentially useful for treatment of other conditions including angina (*11*), arteriosclerosis (*13*), erectile dysfunction (*14*−*16*), and spinal cord injury (*26*−*28*). Despite the wealth of information about the biological role of Rho-Kinase, and its importance as a drug target, relatively little biochemical characterization of the enzyme has been reported. Further, the kinetic mechanism of Rho-Kinase has not been studied, nor has that of other Rho effectors such as protein kinase N (PKN, PRK1) and citron kinase. An understanding of the kinetic mechanism of Rho-Kinase is important for the identification and characterization of potent and specific small-molecule inhibitors.

Determination of the kinetic mechanism for an enzyme is usually carried out by using steady-state kinetics (*36, 38*−*40*). We investigated the kinetic mechanism of Rho-Kinase II (ROCK-II 1−543)-catalyzed phosphorylation using S6 peptide (LCB-AKRRRLSSLRA-NH_2, where LCB = long-chain biotin) as the phosphorylatable substrate. Two-substrate steady-state kinetics indicated that Rho-Kinase catalysis followed a ternary complex, or sequential, kinetic mechanism. In addition, the K_M values for S6 peptide and ATP differ by only ~4-fold from the corresponding dissociation constants K_{ia}^{S6} and K_{ia}^{ATP}, respectively, suggesting that the substrates do not interact substantially and that their order of binding to the enzyme during catalysis was random.

To more fully define the kinetic mechanism of the enzyme, we used inhibition studies with the inhibitors AMP-PCP, Y-27632, and S6-AA to address the order of binding of ATP and S6 peptide (*36, 40*). AMP-PCP is a nonhydrolyzable

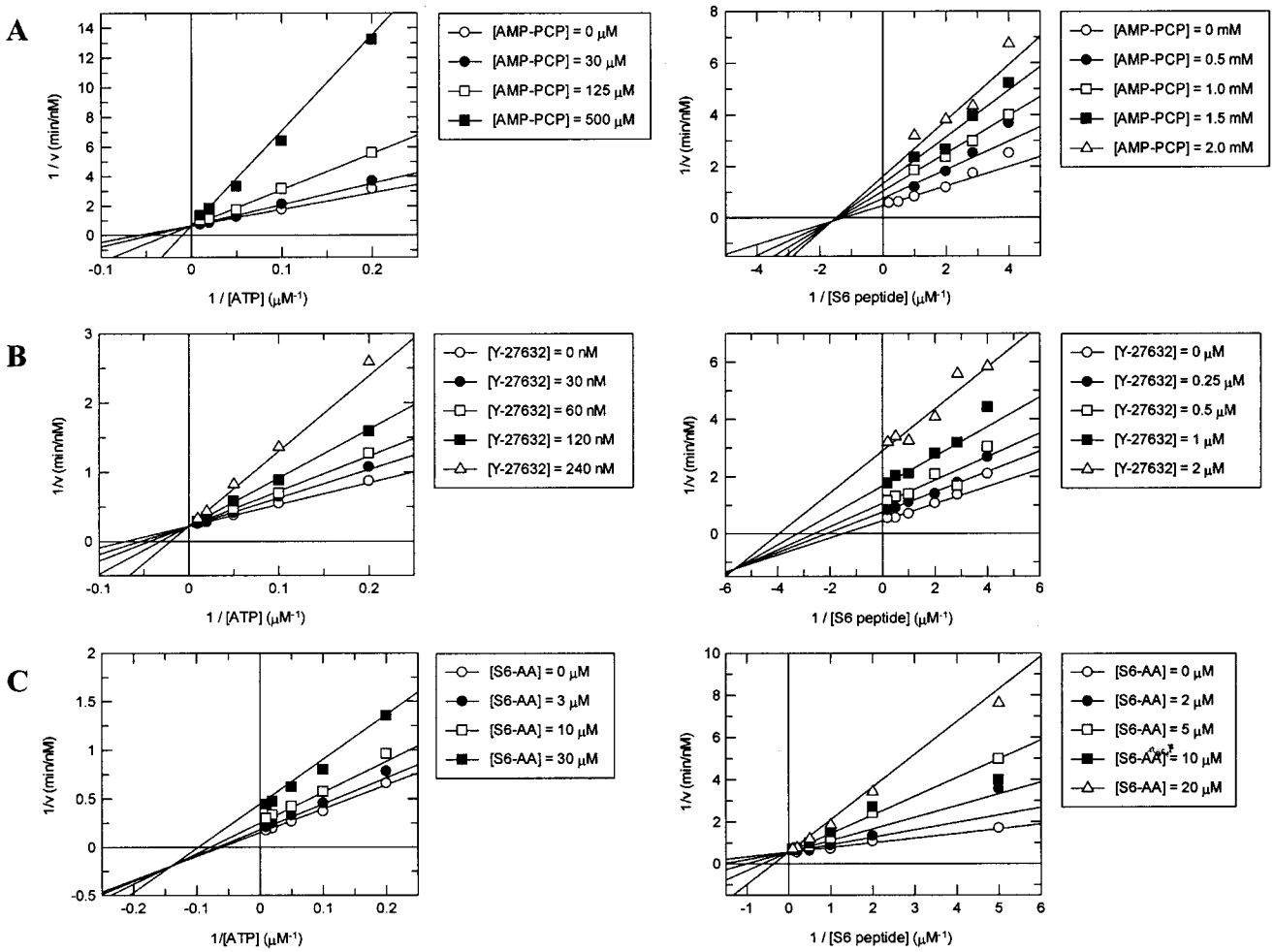

FIGURE 2: Inhibition of ROCK-II by (A) AMP-PCP, (B) Y-27632, and (C) S6-AA. (Left) Double-reciprocal plots of $1/v$ (min/nM) vs 1/[ATP] (μM^{-1}) with [S6 peptide] fixed at 2.5 μM and various inhibitor concentrations. (Right) Double-reciprocal plots of $1/v$ (min/nM) vs 1/[S6 peptide] (μM^{-1}) with [ATP] fixed at 40 μM and various inhibitor concentrations. The data were fit to the equation either for competitive inhibition (eq 2) or for noncompetitive inhibition (eq 3).

ATP analogue which has been shown to be a dead-end, ATP-competitive kinase inhibitor. As expected, we found AMP-PCP was a competitive inhibitor of ROCK-II 1−543 versus ATP, with $K_{is} = 96$ μM. In agreement with a previous report (*41*), we found that Y-27632 was a competitive inhibitor of ROCK-II 1−543 versus ATP, with $K_{is} = 98$ nM. These ATP-competitive inhibitors were found to be noncompetitive inhibitors versus S6 peptide. These results were consistent with either a random order of binding of ATP and S6 peptide or an ordered steady-state mechanism with ATP binding first. To distinguish between these possibilities, inhibition studies were carried out using the peptide S6-AA (acetyl-AKRRR-LAALRA-NH_2), in which the two serine residues in S6 peptide are replaced with alanine. S6-AA was found to be a competitive inhibitor of ROCK-II 1−543 versus S6 peptide, with $K_{is} = 3$ μM. When ATP was the varied substrate, a noncompetitive inhibition pattern was observed. These data were consistent with a random order of binding for ATP and S6 peptide (*36*). For an ordered mechanism with ATP binding first, an uncompetitive pattern of inhibition for S6-AA versus ATP is required (*36*).

The kinetic mechanisms of several Ser-Thr kinases have been investigated. The most well-studied is protein kinase A (PKA; also referred to as cAMP-dependent protein kinase, cAPK). Two kinetic mechanisms for this enzyme have been proposed. One study using the heptapeptide substrate LR-RASLG concluded that the mechanism was random sequential (*42*), while a second study using the same peptide substrate concluded that the mechanism was ordered sequential with ATP binding first (*43*, *44*). These results have been reconciled by a later study that suggests a random sequential mechanism with a preference for ATP binding first at higher Mg^{2+} concentrations (*45*). Studies of the Ser/Thr kinase p38 MAP kinase have also led to two proposed kinetic mechanisms. A study using the physiological protein substrate ATF2 indicated that the kinetic mechanism was ordered sequential with protein substrate binding first (*46*), while a different study using a peptide substrate derived from epidermal growth factor concluded that the kinetic mechanism was ordered sequential with ATP binding first (*47*). A suggested explanation for these results was that p38 MAP kinase allows random binding of substrates, with the preferred order of binding strongly dependent on the phosphorylatable substrate (*47*).

Studies of the kinetic mechanisms of several tyrosine kinases have been reported. Studies of pp60-src tyrosine kinase (src TK) (*48*), Csk tyrosine kinase (*49*, *50*), and the insulin receptor tyrosine kinase (*51*) found that the mechanisms were random sequential. In contrast, the vascular endothelial growth factor receptor-2 tyrosine kinase (VEG-

FR2 TK) (*52*) and v-src tyrosine kinase (*53*) were reported to follow ordered sequential mechanisms with ATP binding first. For the epidermal growth factor receptor tyrosine kinase, one study reported a random sequential mechanism (*54*) while a second study reported an ordered mechanism with peptide substrate binding first (*55*).

The studies mentioned above indicate that both Ser/Thr and tyrosine kinases always utilize sequential (i.e., ternary complex) kinetic mechanisms, but with either random or ordered substrate binding. In cases where ordered binding is observed, while ATP is generally the substrate that binds first, cases in which the phosphorylatable substrate binds first have also been reported for both Ser/Thr and tyrosine kinases. Thus, it is necessary to determine the kinetic mechanism for a kinase of interest.

In the present study of Rho-Kinase, we used as phosphorylatable substrate the nonphysiological substrate S6 peptide (LCB-AKRRRLSSLRA-NH_2, where LCB = long-chain biotin). This peptide was chosen for determination of the kinetic mechanism and for inhibitor screening based on its solubility and high turnover, and because the availability of phospo-S6 peptide-specific antibodies facilitated the development of antibody-based assays for inhibitor screening. Consistent with its efficiency as a substrate for Rho-Kinase, S6 peptide is similar to a peptide (LCB-DRKKRYTWGNPY-NH_2) derived from the physiological Rho-Kinase substrate LIM kinase, which was found to be a substrate for ROCK-II 1−543 with a catalytic efficiency similar to that of S6 peptide (*35*). The relatively low K_M value for S6 peptide (0.9 μM) was consistent with specific recognition by Rho-Kinase. However, as with the majority of studies which use peptide substrates rather than a physiological protein substrate to determine the kinetic mechanism of a kinase, the possibility that our choice of S6 peptide as substrate may affect the observed kinetic mechanism for Rho-Kinase cannot be ruled out without further studies using physiological Rho-Kinase substrates such as MP and LIM kinase.

Our conclusion that Rho-Kinase followed a random sequential kinetic mechanism depended on the use of S6-AA, which differed from S6 peptide by the replacement of the two serine residues with alanine, as an S6 peptide competitive inhibitor. We found that S6-AA was an efficient competitive inhibitor of Rho-Kinase, with K_{is} = 3 μM. Comparison of K_{is} for S6-AA with K_{ia}^{S6}, the dissociation constant for S6 peptide determined from two-substrate kinetics, indicated that removal of the two hydroxyl groups in S6 peptide reduced binding affinity by only ~15-fold. This observation was similar to that seen in a study of the tyrosine kinase pp60-src kinase, in which the dissociation constant of an 18-mer peptide substrate was 6-fold lower than the K_{is} of an analogue in which the phosphorylatable tyrosine residue was changed to phenylalanine (*48*). Thus, replacement of phosphorylatable serine and tyrosine residues with alanine and phenylalanine, respectively, can provide efficient peptide substrate-competitive inhibitors with K_{is} values relatively close to the dissociation constant of the peptide substrate.

Y-27632 is a specific Rho-Kinase inhibitor that has been used extensively to elucidate the Rho/Rho-Kinase pathway and the therapeutic potential of Rho-Kinase inhibitors (*1*, *2*, *41*). Consistent with an earlier report, we found that Y-27632 was a competitive inhibitor of Rho-Kinase versus ATP (*41*). Noncompetitive inhibition of ROCK-II 1−543 was observed with Y-27632 when S6 peptide was the varied substrate, indicating that Y-27632 does not interfere with binding of S6 peptide to the enzyme. Further medicinal chemistry efforts might elaborate on the Y-27632 scaffold to convert it into a bi-substrate inhibitor that is competitive with both ATP and S6 peptide, potentially providing more potent inhibition and higher specificity.

Understanding the kinetic mechanism of an enzyme is important for the identification and characterization of potent and specific inhibitors. For example, since Rho-Kinase-catalyzed phosphorylation followed a random sequential kinetic mechanism in which both substrates bind essentially independently, a binding assay could be used to identify compounds that bind competitively with both ATP and the protein/peptide substrate. Chemical linkage of ATP-competitive compounds to protein/peptide substrate-competitive compounds may then provide bi-substrate inhibitors with high binding affinity and specificity (*56*). In conclusion, we found that Rho-Kinase-II catalysis involves a ternary complex (i.e., sequential) kinetic mechanism with a random order of binding of ATP and S6 peptide. This study lays the groundwork for further mechanistic study of Rho-Kinase as well as for the identification of potent and specific inhibitors of this enzyme.

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