Kinetic Mechanism of the p38-α MAP Kinase: Phosphoryl Transfer to Synthetic Peptides

Guanjing Chen, Margaret D. Porter, John R. Bristol, Matthew J. Fitzgibbon, and S. Pazhanisamy*

Vertex Pharmaceuticals Inc., 130 Waverly Street, Cambridge, Massachusetts 02139-4242

Received August 20, 1999; Revised Manuscript Received December 6, 1999

ABSTRACT: p38 is a member of the mitogen-activated protein (MAP) kinase family. Activation (phosphorylation) of p38 acts as a switch for the transcriptional and translational regulation of a number of proteins, including the proinflammatory cytokines. Investigation of a set of small peptides revealed that, as with protein substrates, p38-α behaves as a proline-directed Ser/Thr MAP kinase for a peptide substrate, peptide **4** (IPTSPITTTYFFFKKK). We investigated the steady-state kinetic mechanism of the p38-α-catalyzed kinase reaction with EGF receptor peptide, peptide **1**, as a substrate. Lineweaver−Burk analysis of the substrate kinetics yielded a family of lines intersecting to the left of the ordinate, with either ATP or peptide **1** as the varied substrate. Kinetic analysis in the presence of ADP yielded a competitive inhibition pattern when ATP was the varied substrate and a noncompetitive pattern if peptide **1** was the varied substrate. At saturating peptide substrate concentrations, inhibition by phosphopeptide product yielded an uncompetitive pattern when ATP was the varied substrate. These data are consistent with ordered binding with ATP as the initial substrate. We provide further evidence of the existence of a productive p38·ATP binary complex in that (a) activated p38-α has intrinsic ATPase activity, (b) ATPase and kinase activities are coupled, and (c) inhibitors of ATPase activity also inhibit the kinase activity with a similar inhibition constant. The k_{cat} for the kinase reaction was lowered by 1.8-fold when ATP-γ-S was used. Microviscosity linearly affected the k_{cat} values of both the ATP and ATP-γ-S reactions with a slope of about 0.8. These observations were interpreted to mean that the phosphoryl transfer step is not rate-limiting and that the release of product and/or enzyme isomerization is a possible rate-limiting step(s).

p38 is a member of the mitogen-activated protein (MAP) kinase family of intracellular enzymes which are part of the signal transduction cascade transmitting signals from an extracellular stimulus to the nucleus of the cell (*1*−*4*). Three groups of enzymes belong to the MAP kinase family: extracellular signal-regulated kinases (ERKs), c-Jun N-terminal kinases (JNKs), and p38s. The MAP kinases are distinguished by their unique phosphorylation sites on a triad of amino acids, Thr-X-Tyr. Amino acid X in the triad is Glu, Pro, and Gly in ERKs, JNKs, and p38s, respectively. Both Thr and Tyr must be phosphorylated for full activity, and the dual specificity kinase which phosphorylates MAP kinase is called MAP kinase kinase (MKK). Among the MAP kinases, ERKs are primarily activated in response to growth factors and hormones while JNKs and p38s are activated in response to environmental and cellular stresses such as osmotic shock, UV radiation, cytotoxic chemicals, and proinflammatory cytokines. Hence, JNKs and p38s are also called stress-activated protein kinases (SAPKs). Activated p38 enters the nucleus chaperoned by transporter proteins to activate specific transcription factors which in turn modulate the synthesis of various proteins, including the proinflammatory cytokines. It has been well documented, in vitro, that the concentrations of proinflammatory cytokines, IL-1 β and TNF-α, are elevated in concert with the activation of p38 and that inhibitors of p38 activity decrease the level of the proinflammatory cytokines dramatically (*5*−*7*). Hence, p38 is a target for the design of drugs for autoimmune indications such as rheumatoid arthritis and inflammatory bowel disease.

To date, there are four isoforms of p38 known, namely, p38-α, -β, -γ, and -δ (*8*−*12*). The α- and β-isoforms are expressed in most tissues, whereas the γ-isoform is expressed primarily in the skeletal muscles. p38-δ, on the other hand, appears to be predominantly expressed in the lung, testis, kidney, and small intestine (*1*, *11*). The sequence alignments showed that p38-δ is approximately 61, 59, and 65% identical to p38-α, -β, and -γ, respectively. Pyridinylimidazole inhibitors such as SB202190 and SB203580 are very selective for p38, potently inhibit the α- and β-isoforms, and do not inhibit the γ- and δ-isoforms (*13*−*15*). A number of crystal structures of p38-α have been determined, with and without inhibitor bound at the ATP pocket (*13*, *16*−*18*), to aid in the drug discovery process. A crystallographic study of VK-19911, another pyridinylimidazole inhibitor, in complex with p38-α predicted that Thr106 may be the specificity determinant (residue 106 is Thr in p38-α and -β, while it is Met in the γ- and δ-isoforms) for binding pyridinylimidazole inhibitors. Mutation of Thr106 to Met in p38-α indeed eliminated the affinity for VK-19911 and other pyridinylimidazole inhibitors (*13*). In monocytes, macrophages, and

* Corresponding author. E-mail: pazhanisamy@vpharm.com. Telephone: (617) 577-6769. Fax: (617) 577-6713.

10.1021/bi9919495 CCC: $19.00
Published on Web 02/02/2000

neutrophils, p38-α is abundantly expressed while p38-β is expressed at a low level (*12*). Both in cell assays and in in vivo animal studies, pyridinylimidazole inhibitors were able to block the synthesis of IL-1 β and TNF-α (*19*). Hence, p38-α has been the primary focus of anti-inflammatory drug discovery.

The kinetic mechanisms of a number of protein kinase reactions have been investigated extensively. PKA is one of the most thoroughly studied Ser/Thr protein kinases (*20*−*22*). It follows an ordered binding mechanism with ATP as the first substrate to bind. KDR, a receptor tyrosine kinase, experiences rapid equilibrium ordered binding with ATP as the first substrate (*23*). On the other hand, for p60c-src tyrosine kinase, the kinetic mechanism is rapid equilibrium random binding (*24*). LoGrasso et al. reported a sequential order of binding for the p38-α-catalyzed kinase reaction using ATF2 as the phosphoryl acceptor. They found that the protein substrate was the first substrate to bind (*25*). This conclusion seemed inconsistent with our finding that highly purified activated recombinant p38-α has significant ATPase activity. We, therefore, investigated the kinetic mechanism of the kinase reaction catalyzed by p38-α. A peptide based on the EGF receptor phosphorylation site (Thr669) was used as a substrate instead of the protein substrate. The kinase kinetics were monitored either by a spectrophotometric coupled-enzyme assay or by HPLC analysis of quenched samples. From the substrate kinetics and kinetics of inhibition by substrate analogues and products, we conclude that phosphoryl transfer to the synthetic peptide follows a sequential ordered mechanism with ATP as the initial substrate. We used viscosity and ATP-γ-S effects on rates to further understand the nature of the rate-limiting step for the p38-catalyzed kinase reaction. MAP kinases are known to phosphorylate proteins only on a Ser or Thr that is directly linked to proline on its C-terminus. We report here the proline directedness of phosphorylation by p38-α MAP kinase using synthetic substrates.

MATERIALS AND METHODS

Materials. Pyruvate kinase, lactate dehydrogenase, ATP-γ-S, phosphoenolpyruvate (PEP), and NADH were purchased from Boehringer Mannheim. ADP, ATP, and its analogues AMPPNP[1] and AMPPCP were obtained from Sigma Chemical Co. EGF receptor peptide (peptide **1**, KRELVEPLTPSGEAPNQALLR) and other peptide substrates were purchased from either AnaSpec or American Peptide Co. EnzChek (E-6646), a phosphate assay kit, was purchased from Molecular Probes.

Activated p38. $(His)_6$-p38 was expressed in baculovirus-infected Sf9 cells, and the unactivated $(His)_6$-p38 was purified as described previously (*16*) by omitting the final size-exclusion chromatography step. About 10−20 mg of dialyzed Q-Sepharose-eluted p38-α was diluted to a concentration of 0.5 mg/mL in buffer A [50 mM HEPES (pH 7.5), 2 mM DTT, and 5% (v/v) glycerol containing 10 mM $MgCl_2$]. A constitutively active double mutant of MKK6, (DD)MKK6, was added to a final concentration of 10 μg/mL, and the activation was initiated by the addition of 2 mM ATP at 20 °C. After reaction for 30 min, the activation mixture was applied to a 1 cm × 10 cm Mono-Q HR 10/10 column (Pharmacia) preequilibrated with buffer A and eluted with a 20 column volume gradient to buffer A containing 0.5 M NaCl. The activated p38 peak was pooled and dialyzed at 4 °C versus buffer A. Next, hydrophobic interaction chromatography was performed at room temperature. Typically, 3−5 mg of Mono-Q-purified p38 at a concentration of 0.5−1 mg/mL was adjusted to 1.1 M KH_2PO_4 (pH 7.4) by the addition of a 4 M stock solution. The sample was filtered (0.2 μm) and applied at a rate of 1 mL/min to a 1 cm × 10 cm Hydropore HIC (Rainin) column preequilibrated with 1.1 M KH_2PO_4 (pH 7.4), 2 mM DTT, and 10% (v/v) glycerol and eluted with a 20 column volume gradient to 0.6 M KH_2PO_4 (pH 7.4), 2 mM DTT, and 10% (v/v) glycerol. The bis-phosphorylated p38, eluting as the main peak, was dialyzed against buffer A and stored at −70 °C until it was used.

ATPase Kinetics. Activated p38 has intrinsic ATPase activity. The ATPase activity of activated p38 was characterized in 0.1 M HEPES buffer (pH 7.6) containing 10 mM $MgCl_2$ and 10% glycerol. The kinetics were followed by one of the following two methods. In method A, the ADP that was generated was quantified using pyruvate kinase and lactate dehydrogenase coupled-enzyme assay as described by Fox et al. (*26*). In method B, the phosphate that was generated by the hydrolysis reaction was quantitated by the nucleoside phosphorylation reaction using the EnzChek phosphate assay kit from Molecular Probes. In this method, 1 unit/mL nucleoside phosphorylase and 200 μM nucleoside substrate (MESG) were used in the reaction. Unless otherwise indicated, the p38 concentration was maintained at 100 nM. The kinetic analyses were carried out in a 96-well plate, at 30 °C, on a Molecular Devices spectrophotometer.

Kinase Kinetics. The kinase rates were followed either spectrophotometrically, on a Molecular Devices plate reader, by monitoring the ADP generated during the reaction by the above-described coupled-enzyme assay, or by monitoring the peptide and its phosphorylation product by HPLC. The reactions were carried out at 30 °C in 0.1 M HEPES buffer (pH 7.6) containing 10 mM $MgCl_2$ and 10% glycerol. The concentration of p38 was in the range of 5−40 nM. For the HPLC analysis, the reactions were quenched after a specified time with 125 mM EDTA (final concentration) at pH 8.0. The samples were run on a Gilson HPLC system to separate the peptide substrate and product. A C18 reversed phase column from Phenomenex (catalog no. 00B-4108-E0, 5 μm, 100 Å, 50 mm × 4.6 mm) was used. Baseline separation was accomplished by a linear increase in the level of mobile phase B (20 mM phosphoric acid and 100 mM sodium perchlorate in a 1:1 water/acetonitrile solvent) from 30 to 70% over the course of 10 min at a rate of 1 mL/min. Mobile phase A was 20 mM aqueous phosphoric acid (pH 2.0) containing 100 mM sodium perchlorate.

DATA ANALYSIS

Substrate Kinetics. Peptide **1**, based on the EGF receptor phosphorylation site, was used for the investigation of the

[1] Abbreviations: AMPPNP, β,γ-imidoadenosine 5′-triphosphate; AMPPCP, β,γ-methyleneadenosine 5′-triphosphate; PKA, protein kinase A; cAPK, cyclic AMP-dependent protein kinase; EGF, epidermal growth factor; ATF2, activating transcription factor-2; MAPKAP, mitogen-activated protein kinase-activated protein; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; DTT, dithiothreitol; MESG, 2-amino-6-mercapto-7-methylpurine ribonucleoside.

Table 1: Peptide Substrate Specificity for the p38-α-Catalyzed Kinase Reaction

peptide	sequence	substrate	inhibitor	IC_{50} (mM)	K_m (μM)[a]	k_{cat} (s^{-1})	k_{cat}/K_m (mM^{-1} s^{-1})
1	KRELVEPLTPSGEAPNQALLR	yes	−	−	840 ± 80	22.6 ± 0.8	27 ± 3
2	DSVIVADQTPTPTRFLKNEEE	no	−	−			
3	WIMQSTKVPQTPLHTSRVLK	yes	−	−	>500	10 ± 2	
4	IPTSPITTTYFFFKKK	yes	−	−	103 ± 16	22.8 ± 1.2	221 ± 36
5	IPTTPITTTYFFFKKK	yes	−	−	49 ± 20	8.4 ± 0.8	171 ± 72
6	IPTYPITTTYFFFKKK	no	yes	0.7[b]			
7	IPTAPITTTYFFFKKK	yes	−	−	250 ± 50	3.8 ± 0.4	15 ± 3
8	IPAAPITTTYFFFKKK	no	yes	1.0[b]			
9	IPTSAITTTYFFFKKK	yes	yes[c]	−			
10	IATSPITTTYFFFKKK	yes	−	−	58 ± 8	2.4 ± 0.2	41 ± 7
11	IPSTPITTTYFFFKKK	yes	yes[c]	−			

[a] The ATP concentration was maintained at 1 mM. [b] Obtained by a spectrophotometric coupled-enzyme assay in which the concentrations of ATP and peptide **1** were maintained at 100 and 200 μM, respectively. [c] At low peptide concentrations (<200 μM), there was a small increase in the rate in the coupled-enzyme assay. At higher concentrations, the rate decreased to well below the ATPase rate observed in the absence of peptide.

binding order in the p38-catalyzed kinase reaction. The initial rates obtained at various fixed concentrations of peptide **1** and ATP were fitted to eq 1

$$v/[E_o] = \frac{k_{cat}[A][B]}{K_{ia}K_b + K_a[B] + K_b[A] + [A][B]} \quad (1)$$

where $[E_o]$, [A], and [B] are the enzyme, ATP, and peptide **1** concentrations, respectively, k_{cat} is the turnover number, K_{ia} is the dissociation constant for ATP, K_a and K_b are the K_m values for ATP and peptide **1**, respectively.

Inhibition Kinetics. The initial rates of kinase reaction were obtained either as a function of dead-end inhibitors such as ATP analogues or as a function of ADP or phosphopeptide, products of the kinase reaction. In these studies, one of the kinase substrates (ATP or peptide) is varied keeping the other constant. The data are fitted to the noncompetitive inhibition model (eq 2), an uncompetitive inhibition model (eq 3), or to a competitive inhibition model (eq 4).

$$v/[E_o] = \frac{k_{cat}[S]}{K_m(1 + [I]/K_{is}) + [S](1 + [I]/K_{ii})} \quad (2)$$

$$v/[E_o] = \frac{k_{cat}[S]}{K_m + [S](1 + [I]/K_{ii})} \quad (3)$$

$$v/[E_o] = \frac{k_{cat}[S]}{K_m(1 + [I]/K_{is}) + [S]} \quad (4)$$

where K_{is} and K_{ii} are the dissociation constants for dissociation of the inhibitors from the complex to yield either the free enzyme or the substrate bound enzyme, respectively. The data were analyzed on a Macintosh computer using the software KinetAsyst, from Intellikinetics (State College, PA).

RESULTS

ATPase Activity of Activated p38. Phosphorylated p38 had significant ATP hydrolyzing activity in the absence of any added peptide substrate for which its kinetic parameters can be obtained with a catalytic amount of p38 (100 nM in spectrophotometric assays and 50 nM in HPLC assays). The K_m and k_{cat} values for the ATP[2] hydrolyzing activity were 170 μM and 0.4 s^{-1}, respectively, at pH 7.6. ADP is a common product of kinase and ATPase reactions. Phosphate and phosphorylated peptide, on the other hand, are unique products of the ATPase and kinase reactions, respectively. To characterize the ATPase activity of p38, we monitored the activated p38-catalyzed hydrolysis of ATP in the presence of various concentrations of the added peptide. Peptide **1** is an excellent substrate for the kinase reaction (Table 1). In the absence of any added peptide, there is a considerable ATP hydrolysis activity as demonstrated from the significant phosphate release rate (Figure 1). Progressive addition of peptide **1** resulted in the decline of the rate of phosphate production with a concomitant increase in the rate of ADP production. Since the rate of ADP release from the ATPase reaction should be stoichiometric with respect to the phosphate release, it is obvious that the net kinase rate is several-fold higher than the ATPase rate. The observation that there was significant ATPase activity in the absence of peptide substrate and that this activity is in competition with the peptide kinase activity suggests that the ATPase and kinase activities of p38-α are coupled.

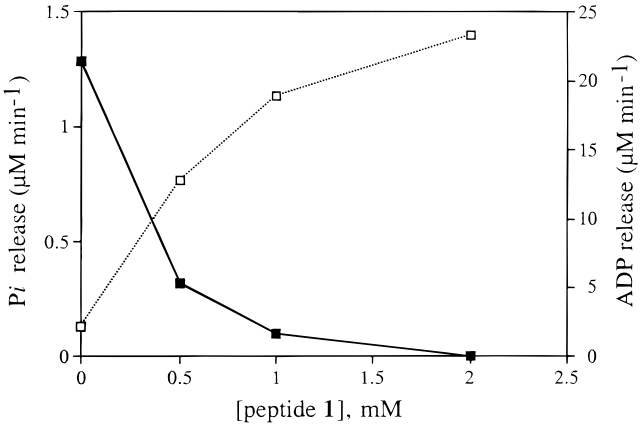


FIGURE 1: ATPase and kinase activities of p38-α are coupled. The ATP concentration was fixed (200 μM) and the peptide **1** concentration varied from 0 to 2 mM. As the peptide **1** concentration increased, the p38-α-catalyzed rate of phosphate release [ATPase activity (■)] decreased while the net rate of ADP release [ATPase plus kinase activities (□)] increased.

Peptide Substrate Specificity. ATF2, MAPKAP kinase-2, EGF receptor, and myelin basic protein are some of the in

[2] Since the Mg^{2+} concentration is kept high (10 mM) compared to ATP and ADP concentrations (=2 mM), the nucleotides will exist predominantly as $[Mg{\cdot}ATP]^{2-}$ and $[Mg{\cdot}ADP]^{-}$, respectively.

vitro substrates of p38 (*25*−*27*). We were interested in designing a small peptide substrate suitable for a spectrophotometric assay for detailed kinetic studies. A fragment of EGF receptor containing the phosphorylation site Thr669 was reported to be a substrate for p38 in a filter binding assay (*27*). We found the EGF receptor peptide, peptide **1** (Table 1), to be a good substrate in a filter binding assay as well as in the spectrophotometric coupled-enzyme assay. However, similar efforts in designing a small peptide substrate for p38 from ATF2, peptide **2**, were not successful, and the peptide derived from MAPKAP K-2, peptide **3**, was only modestly active (Table 1). Peptide **4** was suggested by L. C. Cantley of Harvard Medical School (Cambridge, MA) as a substrate for p38. Peptide **4** had a k_{cat} value identical to that of peptide **1**, and the K_m value was about 8-fold lower than that for peptide **1**.

MAP kinases are called proline-directed Ser/Thr kinases since these enzymes phosphorylate their respective protein substrates on a Ser or Thr that is directly linked to the Pro on its carboxyl terminus (*31*, *32*). To test if this generalization will hold true for small peptide substrates, we replaced the Ser next to the Pro in peptide **4** with Thr and Tyr in peptides **5** and **6**, respectively. From the results shown in Table 1, it is clear that Ser and Thr at amino acid −1 of Pro are equally preferred for phosphorylation while Tyr is not accepted. However, peptide **6** inhibited the phosphorylation of peptide **1** with an IC_{50} of 0.7 mM. Unexpectedly, substitution of Ser with Ala, peptide **7**, yielded a kinase substrate, but the specific activity (k_{cat}/K_m) went down by a factor of 14. Sequencing of the phosphopeptide from the kinase reaction of **7** revealed that the Thr next to the Ala was phosphorylated. Substitution of both −2 and −1 amino acids on the N-terminus of Pro with Ala, peptide **8**, resulted in the complete elimination of kinase activity for the peptide. Peptide **8** inhibited the p38-catalyzed kinase reaction of peptide **1** with an IC_{50} of 1 mM. This suggests that peptide **8** may still bind at the peptide binding site on p38. The binding of peptide **6** and **8** could not be characterized further due to low solubility. The extent to which Pro is necessary for the peptide to behave as a kinase substrate for p38 was examined with peptide **9** where Pro was substituted with Ala. This peptide behaved as a substrate at low concentrations (<200 μM), whereas it is clearly an inhibitor at high concentrations. There is one other proline in peptide **4** which is the second amino acid from the N-terminus. Replacement of this proline with Ala, peptide **10**, reduced the specific activity by a factor of 5, but it did not alter the K_m for the substrate significantly. Finally, interchanging Thr and Ser in peptide **4** yielded peptide **11** (peptide **11** can also be thought of as resulting from the substitution of Thr in peptide **5** with Ser) which exhibited no significant kinase activity. This peptide behaved like peptide **9** by inhibiting the kinase and ATPase activities at high concentrations.

Substrate Kinetics. Peptide **1** was used to investigate the binding order mechanism for the kinase reaction catalyzed by the activated p38. The spectrophotometric coupled-enzyme assay was used to obtain the initial rates of the reaction. The initial rates were obtained at a range of peptide and ATP[2] concentrations to cover the K_m values for both. Under these conditions, the contributions of ATPase rates were not significant compared to the net rate of ADP generated by p38 catalysis. Lineweaver−Burk analysis

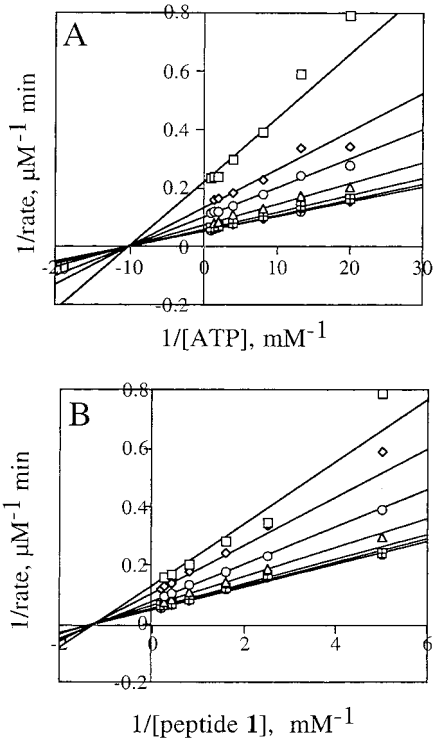


FIGURE 2: Substrate kinetics for the p38-α-catalyzed kinase reaction. Peptide **1** was used as the phosphoryl acceptor. Initial rates were obtained for various combinations of ATP (0.05, 0.075, 0.125, 0.25, 0.5, 0.75, and 1.0 mM) and peptide **1** (0.2, 0.4, 0.625, 1.25, 2.5, 3.75, and 5.0 mM) at 30 °C in 0.1 M HEPES buffer (pH 7.6) containing 10 mM $MgCl_2$ and 10% glycerol. The concentration of activated p38-α was 20 nM. Plots of 1/rate vs either 1/[ATP] (A) or 1/[peptide **1**] (B) show an intersecting pattern of straight lines with a point of intersection to the left of the ordinate. The solid lines represent the best fit of the data to eq 1.

Table 2: Kinetic Parameters for the Inhibition of the p38-α-Catalyzed Kinase Reaction

varied substrate	inhibitor	inhibition pattern	K_{is} (μM)	K_{ii} (μM)
ATP	SB203580	C	0.10 ± 0.02	
ATP	AMPPNP	C	830 ± 200	
ATP	AMPPCP	C	200 ± 30	
ATP[a]	ADP	C	570 ± 90	
peptide **1**[a]	ADP	NC	1100 ± 200	2000 ± 600
peptide **1**	AMPPCP	NC	340 ± 100	90 ± 10
peptide **1**	SB203580	NC	0.076 ± 0.011	0.052 ± 0.004
ATP[b]	phosphopeptide **1**	UC		3300 ± 130

[a] The initial rates for these experiments were obtained by HPLC analysis of quenched samples. Initial rates for all other experiments were obtained by the spectrophotometric coupled-enzyme assay. The data were fitted to either eq 2 (noncompetitive pattern, NC), eq 3 (uncompetitive pattern, UC), or eq 4 (competitive pattern). See Materials and Methods for more details. [b] The concentration of peptide **1**, the fixed substrate, was kept at 4 mM ($5K_m$). In all other experiments, the fixed substrate concentration was below saturation.

(Figure 2) yielded an intersecting pattern for both $1/v$ versus 1/[ATP] (Figure 2A) and $1/v$ versus 1/[peptide **1**] (Figure 2B).

Inhibition by ATP Analogues and SB203580. ATP analogues AMPPNP and AMPPCP are competitive inhibitors of ATP with K_i values of 800 and 200 μM, respectively (Table 2). When the ATP concentration was held constant and that of peptide **1** was varied in the presence of various fixed concentrations of AMPPCP, the double-reciprocal plots yielded a noncompetitive pattern (Figure 3A). Analysis of

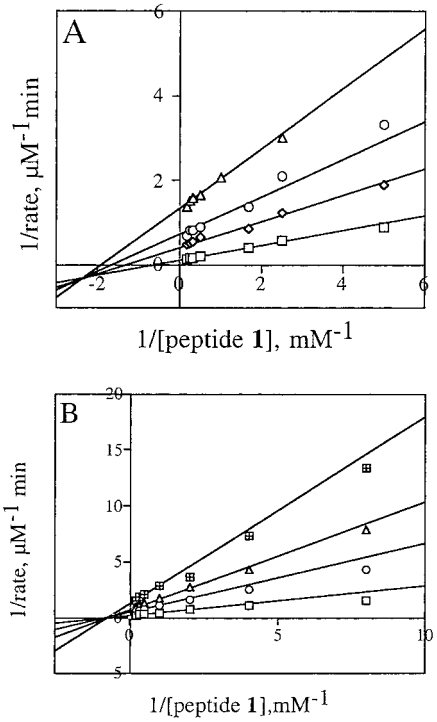


FIGURE 3: Inhibition kinetics with AMPPCP and SB203580. The kinetic determinations were carried out at 100 μM ATP. The concentration of peptide **1** was varied (0.2−5.0 mM) in the presence of various fixed concentrations of either AMPPCP (0, 0.25, 0.5, and 1.0 mM) (A) or SB203580 (0, 0.1, 0.2, and 0.4 μM) (B). The theoretical lines were generated using the parmeters of the data best fit to eq 2.

the data, fitted to eq 2, yielded K_{is} and K_{ii} values of 340 and 100 μM, respectively. SB203580 belongs to the pyridinylimidazole class of inhibitors which bind at the ATP site (*18*). Inhibition kinetics also yielded a competitive pattern for SB203580 when ATP was the varied substrate (K_i = 100 nM) and a noncompetitive pattern when peptide **1** was the varied substrate (Figure 3B).

Kinetics in the Presence of Product. The kinetics of the p38-catalyzed kinase reaction were examined in the presence of various fixed concentrations of ADP, one of the products of the kinase reaction. The initial rates were obtained from the phosphopeptide peak in HPLC analysis. When ATP was the varied substrate, the double-reciprocal plots yielded a simple competitive pattern with a K_i of 0.57 mM (Figure 4A). A noncompetitive pattern with K_{is} and K_{ii} values of 1.1 and 2.0 mM, respectively, was obtained when peptide **1** was the varied substrate (Figure 4B). The effect of phosphopeptide product on the kinetics of phosphorylation of peptide **1** was also examined.[3] The rates were measured as a function of ATP concentration keeping the peptide **1** concentration at 4 mM (5K_m). Under these conditions, the phosphopeptide exhibited an uncompetitive inhibition pattern (Figure 5) with a K_{ii} of 3.3 mM.

Kinetics with ATP-γ-S. ATP-γ-S is not hydrolyzed by p38-α; however, ATP-γ-S inhibited the hydrolysis of ATP by p38-α with a K_i of 240 μM. On the other hand, ATP-γ-S was a substrate for the kinase reaction catalyzed by p38. The generation of thiophosphopeptide was confirmed by the appearance of a unique peak in HPLC as well as by mass spectral analysis. Kinetic analyses were performed at saturating peptide **1** (5 mM) and ATP (2 mM) or ATP-γ-S (2 mM) concentrations. The initial rates of reaction were computed from the peak areas of phospho- and thiophosphopeptide peaks by HPLC analysis. The k_{cat} for the kinase reaction is lowered by a factor of 1.8 for ATP-γ-S.

[3] Suggested by one of the reviewers.

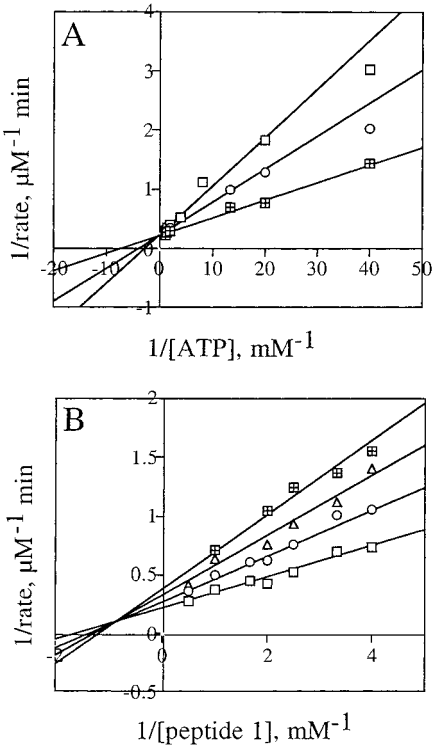


FIGURE 4: Inhibition by ADP. In panel A, the inverses of kinase rates obtained at various fixed concentrations of ADP (0, 0.5, and 1.0 mM) were plotted against the inverses of ATP concentrations (0.025−1.0 mM). Peptide **1** was kept constant at 200 μM. The solid lines represent the best fit of the data to competitive inhibition kinetics (eq 4). In panel B, the inverses of rates obtained as a function of peptide **1** concentration (0.25−2.0 mM) at various fixed concentrations of ADP (0, 0.5, 1.0, and 1.5 mM) were plotted against the inverses of the peptide concentrations. In this experiment, the ATP concentration was fixed at 50 μM. The solid lines represent the best fit of the data to noncompetitive inhibition kinetics (eq 2).

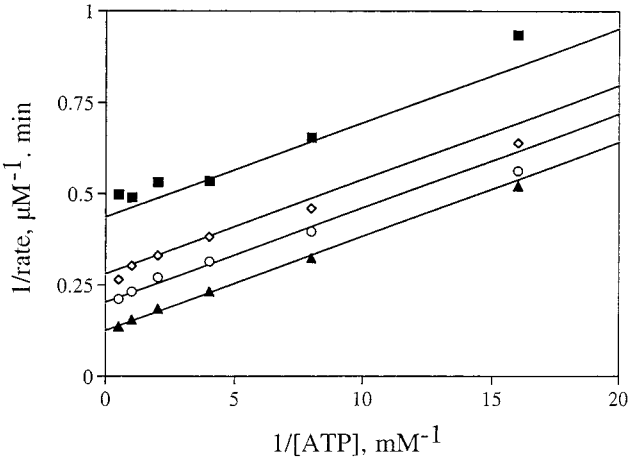


FIGURE 5: Inhibition by phosphopeptide product. The peptide **1** concentration was kept at 4 mM in all the kinetic experiments. The initial rates were measured as a function of ATP concentration (0.062−2.0 mM) keeping the phosphopeptide at 0 (▲), 2 (○), 4 (◇), and 8 mM (■). The solid lines represent the best fit of the data to eq 3 for uncompetitive inhibition kinetics.

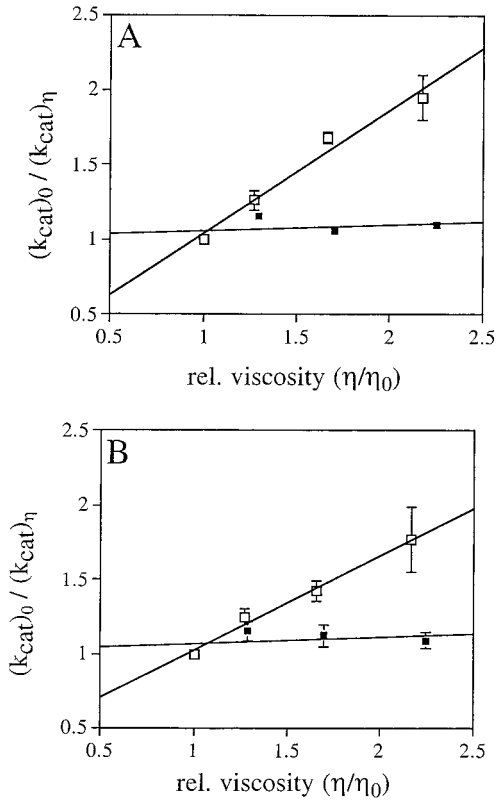


FIGURE 6: Effect of viscosity on p38-α-catalyzed kinase reactions. Panels A and B are for ATP and ATP-γ-S reactions, respectively. The kinase rates at k_{cat} conditions ([peptide **1**] = 5 mM and [ATP] or [ATP-γ-S] = 2 mM) were measured as a function of sucrose [microviscosity (□)] and PEG-8000 [macroviscosity (■)] concentrations. The ratios of rates obtained in the absence of viscogen [$(k_{cat})_0$] to that obtained in the presence of viscogen [$(k_{cat})_\eta$] were plotted against the relative viscosity (η/η_0) of the medium.

Viscosity Effect on k_{cat}. The effect of viscosity on rate constant is often used to infer the nature of the rate-limiting step (*33*). We prepared buffers containing various concentrations of viscogen (sucrose or PEG-8000) to yield different relative viscosities. An Ostwald viscometer was used to measure the relative viscosity of the solutions. Sucrose, being a small molecule, contributes microviscosity to the solution, while PEG-8000 contributes macroviscosity to the solution. Rates were measured under k_{cat} conditions[4] for the kinase reaction ([peptide **1**] = 4 mM, [ATP] or [ATP-γ-S] = 2 mM). The ratios of the rates were plotted as a function of the relative viscosity for ATP and ATP-γ-S reactions. As shown in Figure 6, both the ATP and ATP-γ-S reactions were significantly affected by the microviscosity but very little by the macroviscosity of the medium. The slopes are 0.8 and 0.7 for ATP and ATP-γ-S reactions, respectively, in the microviscosity medium and <0.1 for both reactions in the macroviscosity medium.

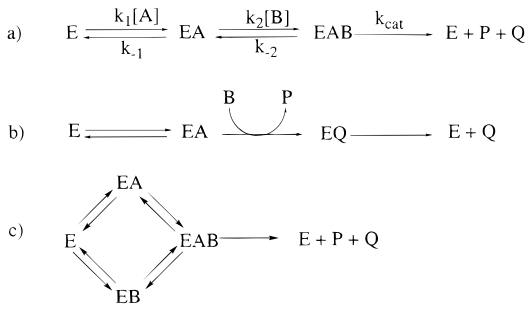
DISCUSSION

We investigated a variety of small peptides as potential substrates for p38 MAP kinase for designing a rapid and robust assay for the investigation of kinetic mechanism as well as for inhibitor screening. Among the peptides designed from the known phosphorylation sites of protein substrates for p38, peptide **1** (Table 1), based on the EGF receptor phosphorylation site, was selected for the investigation of the kinetic mechanism as it had good aqueous solubility allowing variation over a wide range of substrate concentrations. Peptide **4**, on the other hand, was used in the investigation of the substrate−p38 interaction as the peptide is small enough to allow redesign of the peptide for exploring the contribution of a specific amino acid residue, and it had the lowest K_m value (Table 1). Sequencing of the phosphopeptide product from the p38-catalyzed reaction of peptide **4** identified Ser as the site of phosphorylation. A number of peptides were designed specifically around peptide **4** to address the following questions. (1) Is p38 a true Ser/Thr kinase? (2) Is p38 a proline-directed MAP kinase? That is, should proline necessarily be present one amino acid upstream of the site of phosphorylation for efficient kinase activity? From the investigation of peptides **4**−**6**, it is clear that p38 indeed phosphorylates Ser and Thr very specifically and rejects Tyr for phosphorylation (Table 1). Substitution of serine with alanine (peptide **7**) results in the phosphorylation of an adjacent Thr. However, the efficiency of phosphorylation was decreased 14-fold. Substitution of the proline upstream of the Ser with Ala, peptide **9**, virtually eliminated the phosphoryl group accepting ability of the peptide. This confirms the proline directedness of p38-α with small peptide substrates as was previously observed in protein substrates (*31*, *32*).

The kinetic mechanism of the kinase reaction catalyzed by p38-α was investigated with peptide **1** as the phosphoryl acceptor. The double-reciprocal plots resulting from the substrate kinetics are shown in Figure 2. Both panels A and B display intersecting patterns with a point of intersection to the left of the ordinate. This is a clear indication that neither a ping-pong mechanism nor a rapid-equilibrium ordered binding is operative. Also, fitting the data to eq 1 yielded non-zero values for all of the parameters in the denominator which again is consistent with the above conclusion. AMPPNP and AMPPCP are nonhydrolyzable analogues of ATP and are competitive with ATP binding, as expected, with K_i values of 800 and 200 μM, respectively (Table 2). Kinetic analyses in the presence of AMPPCP yielded a noncompetitive pattern when peptide **1** was the varied substrate (Figure 3A). Analysis of inhibition kinetics with SB203580, shown to bind at the ATP site by X-ray crystallographic studies, also yielded a similar pattern (Figure 3B). These observations are consistent with either a random binding mechanism or a steady-state binding mechanism with ATP[2] as the initial susbtrate. Also, the fact that (a) highly purified phosphorylated recombinant p38 had ATPase activity, (b) SB203580 inhibits ATPase and kinase activities with similar inhibition constants (K_i = 50 ± 10 and 100 ± 30 nM, respectively), and (c) the ATPase and kinase activities are coupled requires the formation of a productive p38·ATP binary complex. Our attempts to obtain a useful dead-end inhibitor of the peptide site were not successful. Peptides **6** and **9**, which were based on peptide **4**, do inhibit the kinase reaction but with K_I values in the millimolar range (Table 1). Poor solubility of these peptides rendered them useless as probes of the kinetic mechanism. Instead, we studied

[4] We obtained the K_m for ATP, ATP-γ-S, and peptide **1** at 0% as well as at 30% sucrose containing buffers. For ATP, the K_m did not change with sucrose concentration, whereas for ATP-γ-S and peptide **1**, the K_m decreased <2-fold in 30% sucrose.

Scheme 1: Sequential Binding Mechanisms[a]



[a] (a) Ordered binding, (b) Theorell−Chance mechanism with no accumulation of the EAB ternary complex, and (c) random binding of substrates, where E, A, B, P, and Q represent activated p38, Mg·ATP, peptide **1**, phosphopeptide **1**, and Mg·ADP, respectively.

Table 3: Kinetic Parameters for the p38-α-Catalyzed Kinase Reaction with Peptide **1**[a]

k_{cat}	$19 \pm 0.3\ s^{-1}$
$K_{ia,\ ATP}$ $(=k_{-1}/k_1)$	$100 \pm 14\ \mu M$
$K_{m,\ ATP}$ $(=k_{cat}/k_1)$	$100 \pm 6\ \mu M$
$K_{m,peptide}$ $[=(k_{cat} + k_4)/k_3]$	$812 \pm 44\ \mu M$
k_1	$0.19 \pm 0.01\ \mu M^{-1}\ s^{-1}$
k_{-1}	$19 \pm 3\ s^{-1}$

[a] Initial rates obtained as a function of ATP and peptide **1** concentrations by spectrophotometric coupled-enzyme assay were fitted to eq 1 to obtain the best fit values for the parameters. See Materials and Methods for details.

product inhibition kinetics to further probe the kinetic mechanism.

The kinetics in the presence of ADP yielded a competitive pattern when ATP was the varied substrate and a noncompetitive pattern when peptide was the varied substrate (Figure 4). Phosphopeptide **1** product, on the other hand, gave an uncompetitive inhibition pattern (Figure 5) when ATP was varied, keeping the enzyme saturated with the peptide substrate ($5-6K_m$). The product inhibition data are clearly consistent with an ordered binding mechanism with ATP as the initial substrate, and eliminate the random binding as well as Theorell−Chance mechanisms (Scheme 1). Under the experimental conditions, the phosphopeptide product should not inhibit if any of the latter mechanisms were operative. The data in Figure 2 were consequently fitted to eq 1 for the sequential ordered binding mechanism, and the kinetic parameters yielding the best fit are shown in Table 3. From the macroscopic parameters K_{ia} and K_m of ATP, the microscopic rate constants for ATP binding to the free enzyme and dissociation from the p38·ATP binary complex (k_1 and k_{-1}, respectively) were calculated using the relations shown in Table 3. It is obvious that the dissociation constant for dissociation of ATP from the binary complex, p38·ATP, is comparable to k_{cat} (identical by coincidence!), a requisite condition for observing steady-state ordered binding of substrates. LoGrasso et al. (*25*), on the other hand, observed an ordered binding mechanism for p38 kinase but with the phosphoryl acceptor as the first substrate. A key difference between the two studies is that LoGrasso et al. used a protein substrate, ATF2 ($K_m = 6\ \mu M$), whereas a peptide substrate ($K_m = 800\ \mu M$) is used in the work presented here. An intriguing possibility which reconciles these apparent inconsistencies is that p38 allows random binding of substrates and the preferred order of binding may be dependent upon the nature of the phosphoryl acceptor. Similar observations have been made with the glycerokinase-catalyzed phosphoryl transfer reactions. The kinetics are consistent with an ordered binding mechanism, glycerol binding before MgATP; however, the order of addition was reversed when 1-aminopropanediol was the phosphoryl acceptor (*34*). It is interesting to note that glycerokinase also exhibited ATPase activity.

Next, we were interested in investigating the nature of the rate-limiting step for k_{cat}. The effect of viscosity and thio substitution (replacing a terminal oxygen on the reactive phosphorus center with sulfur) on the observed rate constants have often been used to explore the nature of the rate-limiting step in enzyme-catalyzed phosphoryl transfer reactions (*33*, *35*). The thio effect can be defined as the ratio of the rate constant for the oxo analogue to that for the sulfur-substituted analogue. Thio effects on nonenzymic phosphoryl transfer reactions are well studied and serve as the basis of probing whether the chemical step is the slow step in the enzyme-catalyzed reaction (*35*). Although the nature of the nucleophile will influence the extent of bond making and bond breaking at the transition state, nonenzymic phosphate monoester reactions follow a dissociative mechanism whereas triester reactions undergo an associative (addition and elimination) mechanism. The thio effect in uncatalyzed reactions is very diagnostic of the type of the mechanism, varying from 0.1 to 0.3 (i.e., rates accelerated by the presence of S) for monoesters, from 4 to 11 for diesters, and from 10 to 160 for triesters (*35*−*37*). Comparison of the thio effect between an enzyme-catalyzed reaction with its uncatalyzed reaction could, therefore, reveal if the chemical step is rate-limiting in the enzymic reaction. For example, in DNA polymerase β-catalyzed duplex elongation (*38*) (a phosphodiester reaction) under pre-steady-state conditions, a thio effect of 4.3 was observed for the correct incorporation of the base and a thio effect of 9 was observed for misincorporation. From these studies, it was concluded that the chemical step was at least partially rate-limiting for the normal reaction.

The ATPase and kinase reactions occur at the terminal phosphorus of ATP and hence are monoester reactions. The thio substitution, therefore, should speed the phosphoryl transfer step. Hence, the specific rate constant for enzyme-catalyzed reactions with ATP-γ-S, be it k_{cat} or k_{cat}/K_m, should either increase (thio effect of <1) if the chemical step was rate-limiting or remain the same (thio effect of 1) if a nonchemical step was rate-limiting. In the case of p38, a thio effect of 1.8 was observed on k_{cat} which is unexpected. In fact, thio effects of 2−20 have been observed for other kinase-catalyzed reactions (*38*−*40*). In a detailed study, Hollfelder and Herschlag (*41*) have found that for the alkaline phosphatase-catalyzed hydrolysis of *p*-nitrophenyl phosphate (PNPP), k_{cat}/K_m decreased several-hundred-fold upon thio substitution of PNPP (the exact magnitude depends on the pK_a of the leaving group), yet the $\beta_{leaving-group}$ was -0.77, suggesting that the mechanism is largely dissociative, i.e., going through a metaphosphate-like intermediate. At least in this instance, the mechanism did not change between the enzymatic and nonenzymatic phosphoryl group transfer reactions. Some of the factors that may contribute to this unexpectedly lowered enzymatic rate for the O to S substitution are, as summarized by Hollfelder and Herschlag (*41*), the differences in size, bond length, charge distribution,

ability to coordinate with metal ion, etc. Some of these factors may also affect the "nonchemical" step(s). In fact, in the case of protein tyrosine kinase Csk (*42*), the thio effect was shown to be extremely dependent upon the metal ion ranging from 2 (Ni^{2+}) to 136 (Mg^{2+}). Therefore, the thio effect of >1 in enzyme-catalyzed ATPase and kinase reactions *by itself* is insufficient to infer the nature of the rate-limiting step.

Viscosity effects on rate constants can be a valuable tool in identifying whether a nonchemical step such as diffusion of a ligand in and out of the enzyme's active site is contributing to the rate-limiting step (*33*). We measured the effect of viscosity on the k_{cat} parameter for the p38-α-catalyzed phosphoryl as well as thiophosphoryl transfer to peptide **1**. The k_{cat} decreased linearly with increasing microviscosity for both the oxo- and thiophosphoryl transfer reactions, whereas the macroviscosity had very little effect on the rates. The lack of a macroviscosity effect ensures that the nonspecific effect of the solvent on the measured rates is insignificant. A plot of the ratio of k_{cat} obtained in the absence of microviscogen [$(k_{cat})_0$] to that obtained in its presence [$(k_{cat})_\eta$] as a function of the relative viscosity of the medium (η/η_0) yielded a slope of 0.7−0.8 for both the ATP and ATP-γ-S reactions (Figure 6). The high sensitivity of k_{cat} to the viscosity of the medium (limiting value of the slope = 1) suggests that the rate-limiting barrier under these conditions is predominantly from a nonchemical step(s) such as product diffusion or enzyme isomerization. It is interesting to note here that for PKA (also called cAPK), another Ser/Thr protein kinase, the k_{cat} was also found to be sensitive to the viscosity of the medium (*21*) (slope ≈ 1.0). Analysis of quench-flow and other pre-steady-state kinetics confirmed that the chemical step is not rate-limiting and that enzyme isomerization prior to ADP release from PKA is the slowest step (*43*).

In summary, the presence of ATPase activity in highly purified bis-phosphorylated p38 led us to reinvestigate the kinase kinetic mechanism reported earlier (25). The steady-state substrate kinetics and the various inhibition kinetics of the p38-catalyzed phosphorylation of the EGFR peptide reported in this paper are fully consistent with a steady-state ordered mechanism with ATP as the first bound substrate. LoGrasso et al., on the other hand, deduced from their kinetic studies that the protein substrate, ATF2, binds first. Therefore, we propose that p38 indeed presents the random binding opportunity for the susbtrates and the preferred order is highly dependent on the phosphoryl acceptor. Since the kinetic investigation of compound inhibition is often an integral part of the drug discovery process, knowing the steady-state kinetics of substrate binding using a homogeneous preparation of the enzyme in question is essential. As with PKA, phosphoryl transfer is not the rate-limiting step for p38 catalysis. However, with the data presented here, we cannot pinpoint whether an enzyme isomerization step or a product release step limits the rate of the p38-catalyzed kinase reaction. Finally, one should exercise caution in interpreting a thio effect of greater than 1 in kinase reactions as the predicted thio effect based upon nonenzymic precedent of phosphoryl transfer from monoesters is less than 1.

ACKNOWLEDGMENT

Thanks to Drs. Ted Fox, Ursula Germann, Scott Raybuck, and John Thomson for their comments on the manuscript. We especially want to acknowledge Dr. Scott Raybuck for his many suggestions which improved the manuscript. Mark Fleming and Dr. Ursula Germann initially demonstrated the presence of ATPase activity for phosphorylated p38. We appreciate the peptide sequencing help from Mark Fleming and HPLC method development by Paul Taslimi. We thank Drs. Michael B. Yaffe and Lewis C. Cantley of Harvard Medical School for suggesting peptide **4** as a substrate for p38. Our sincere thanks to Drs. Michael Su, David J. Livingston, and Vicki Sato for the support, guidance, and encouragement during this research.

REFERENCES

1. New, L., and Han, J. (1998) *Trends Cardiovasc. Med. 8*, 220.
2. Cano, E., and Mahadevan, L. C. (1995) *Trends Biochem. Sci. 20*, 117.
3. Davis, R. J. (1994) *Trends Biochem. Sci. 19*, 470.
4. Moriguchi, T., Gotoh, Y., and Nishida, E. (1996) *Adv. Pharmacol. 36*, 121.
5. Gallagher, T. F., Fier-Thompson, S. M., Garigipati, R. S., Sorenson, M. E., Smietana, J. M., Lee, D., Bender, P. E., Lee, J. C., Laydon, J. T., Griswold, D. E., Chabot-Fletcher, M. C., Breton, J. J., and Adams, J. (1995) *Biorg. Med. Chem. Lett. 5*, 1171.
6. Young, P. R., McLaughlin, M. M., Kumar, S., Kassis, S., Doyle, M. L., McNutty, D., Gallagher, T. F., Fisher, S., McDonnell, P. C., Carr, S. A., Huddleston, M. J., Seibel, G., Porter, T. G., Livi, G. P., Adams, J. L., and Lee, J. C. (1997) *J. Biol. Chem. 272*, 12121.
7. Lee, J. C., and Young, P. R. (1996) *J. Leukocyte Biol. 59*, 152.
8. Jiang, Y. J., Chen, C., Li, Z., Guo, W., Gegner, J. A., Lin, S., and Han, J. (1996) *J. Biol. Chem. 271*, 17920.
9. Stein, B., Yang, M. X., Young, D. B., Janknecht, R., Hunter, T., Murray, B. W., and Barbosa, M. S. (1997) *J. Biol. Chem. 272*, 19509.
10. Li, Z., Jiang, Y., Ulevitch, R. J., and Han, J. (1996) *Biochem. Biophys. Res. Commun. 228*, 334.
11. Jiang, Y., Gram, H., Zhao, M., New, L., Gu, J., Feng, L., Di Padova, F., Ulevitch, R. J., and Han, J. (1997) *J. Biol. Chem. 272*, 30122.
12. Hale, K. K., Trollinger, D., Rihanek, M., and Manthey, C. L. (1999) *J. Immun.*, 4246.
13. Wilson, K. P., McCaffery, P. G., Hsiao, K., Pazhanisamy, S., Galullo, V., Bemis, G. W., Fitzgibbon, M. J., Caron, P. R., Murcko, M. A., and Su, M. S.-S. (1997) *Chem. Biol. 4*, 423.
14. Cohen, P., and Goedert, M. (1998) *Chem. Biol. 5*, R161.
15. Eyers, P. A., Craxton, M., Morrice, N., Cohen, P., and Goedert, M. (1998) *Chem. Biol. 5*, 321.
16. Wilson, K. P., Fitzgibbon, M. J., Caron, P. R., Griffith, J. P., Chen, W., McCafferey, P. G., Chambers, S. P., and Su, M. S.-S. (1996) *J. Biol. Chem. 271*, 27696.
17. Wang, Z., Harkins, P. C., Ulevitch, R. J., Han, J., Cobb, M. H., and Goldsmith, E. J. (1997) *Proc. Natl. Acad. Sci. U.S.A. 94*, 2327.
18. Tong, L., Pav, S., White, D. M., Rogers, S., Crane, K. M., Cywin, C. L., Brown, M. L., and Pargellis, C. A. (1997) *Nat. Struct. Biol. 4*, 311.
19. Badger, A. M., Bradbeer, J. N., Votta, B., Lee, J. C., Adams, J. L., and Griswald, D. E. (1996) *J. Pharmacol. Exp. Ther. 279*, 1453.
20. Shaffer, J., and Adams, J. A. (1999) *Biochemistry 38*, 5572.
21. Adams, J. A., and Taylor, S. S. (1992) *Biochemistry 31*, 8516.
22. Qamar, R., Yoon, M.-Y., and Cook, P. (1992) *Biochemistry 31*, 9986.
23. Parast, C. V., Mroczkowski, B., Pinko, C., Misialek, S., Khambatta, G., and Appelt, K. (1998) *Biochemistry 37*, 16788.
24. Boerner, R. J., Barker, S. C., and Knight, W. B. (1995) *Biochemistry 34*, 16419.
25. LoGrasso, P. V., Frantz, B., Rolando, A. M., O'Keefe, S. J., Hermes, J. D., and O'Neill, E. A. (1997) *Biochemistry 36*, 10422.

26. Fox, T., Coll, J. T., Xie, X., Ford, P. J., Germann, U. A., Porter, M. D., Pazhanisamy, S., Fleming, M., Galullo, V., Su, M. S.-S., and Wilson, K. P. (1998) *Protein Sci. 7*, 2249.
27. Kramer, R. M., Roberts, E. F., Strifler, B. A., and Johnstone, E. M. (1995) *J. Biol. Chem. 270*, 27395.
28. Stokoe, D., Campbell, D. G., Nakielny, S., Hidaka, H., Leevers, S. J., Marshall, C., and Cohen, P. (1992) *EMBO J. 11*, 3985.
29. Raingeaud, J., Gupta, S., Rogers, J. S., Dickens, M., Han, J., Ulevitch, R. J., and Davis, R. J. (1995) *J. Biol. Chem. 270*, 7420.
30. Songyang, Z., Blechner, S., Hoagland, N., Hoekstra, M. F., Piwnica-Worms, H., and Cantley, L. C. (1994) *Curr. Biol. 4*, 973.
31. Takishima, K., Griswald-Prenner, I., Ingebritsen, T., and Rosner, M. R. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 2520.
32. Gonzalez, F. A., Raden, D. L., and Davis, R. J. (1991) *J. Biol. Chem. 266*, 22159.
33. Blacklow, S. C., Raines, R. T., Lim, W. A., Zamore, P. D., and Knowles, J. R. (1988) *Biochemistry 27*, 1158.
34. Knight, W. B., and Cleland, W. W. (1989) *Biochemistry 28*, 5728.
35. Herschlag, D., Piccirilli, J. A., and Cech, T. R. (1991) *Biochemistry 30*, 4844−4854.
36. Yang, Y.-S., and Frey, P. A. (1990) *Bioorg. Chem. 18*, 373.
37. Catrina, I. E., and Hengge, A. C. (1999) *J. Am. Chem. Soc. 121*, 2156.
38. Gratecos, D., and Fischer, E. H. (1974) *Biochem. Biophys. Res. Commun. 58*, 960.
39. McKay, G. A., and Wright, G. D. (1996) *Biochemistry 35*, 8680.
40. Cole, P. A., Burn, P., Takacs, B., and Walsh, C. T. (1994) *J. Biol. Chem. 269*, 30880.
41. Hollfelder, F., and Herschlag, D. (1995) *Biochemistry 34*, 12255.
42. Grace, M. R., Walsh, C. T., and Cole, P. A. (1997) *Biochemistry 36*, 1874.
43. Grant, B. D., and Adams, J. A. (1996) *Biochemistry 35*, 2022.

BI9919495