The mitogen-activated protein kinase phosphatase 3 (MKP3)-catalyzed hydrolysis of aryl phosphates in the absence and presence of extracellular signal-regulated kinase 2 (ERK2) was investigated in order to provide insights into the molecular basis of the ERK2-induced MKP3 activation. In the absence of ERK2, the MKP3-catalyzed hydrolysis of simple aryl phosphates does not display any dependence on pH, viscosity, and the nature of the leaving group. Increased catalytic activity and enhanced affinity for oxyanions are observed for MKP3 in the presence of ERK2. In addition, normal bell-shaped pH dependence on the reaction catalyzed by MKP3 is restored in the presence of ERK2. Collectively, these results suggest that the rate-limiting step in the absence of ERK2 for the MKP3 reaction corresponds to a substrate-induced conformational change in MKP3 involving active site rearrangement and general acid loop closure. The binding of ERK2 to the N-terminal domain of MKP3 facilitates the repositioning of active site residues and speeds up the loop closure in MKP3 such that chemistry becomes rate-limiting in the presence of ERK2. Remarkably, it is found that the extent of ERK2-induced MKP3 activation is substrate dependent, with smaller activation observed for bulkier substrates. Unlike simple aryl phosphates, the MKP3-catalyzed hydrolysis of bulky polycyclic substrates exhibits bell-shaped pH rate profiles in the absence of ERK2. Furthermore, it is found that glycerol can also activate the MKP3-catalyzed reaction, increase the affinity of MKP3 for oxyanion, and restore the bell-shaped pH rate profile for the MKP3-catalyzed reaction. Thus, the rate of repositioning of catalytic groups and the reorienting of the electrostatic environment in the MKP3 active site can be enhanced not only by ERK2 but also by high affinity substrates or by glycerol.

A plethora of extracellular stimuli transmit signals into cells through mitogen-activated protein (MAP)1 kinase cascades. Many mammalian MAP kinase cascades have been characterized (1). The three major MAP kinase cascades include the extracellular signal-regulated protein kinase (ERK) pathway, which responds to stimuli that induce proliferation and differentiation, and the c-Jun N-terminal protein kinase pathway and the p38 kinase pathway, both of which are activated in response to environmental stresses. Each cascade is composed of a three-kinase module: a MAP kinase, a MAP kinase/ERK kinase (MEK) that activates the MAP kinase, and a MEK kinase that activates the MEK. After activation, each MAP kinase phosphorylates a distinct spectrum of substrates, which include key regulatory enzymes, cytoskeletal proteins, nuclear receptors, and several transcription factors.

MAP kinase activity is tightly controlled by phosphorylation and dephosphorylation. The activation of the MAP kinase activity requires the dual phosphorylation of the Thr and Tyr residues in the activation loop motif TXY (2–4). Deactivation could occur through the action of serine/threonine protein phosphatases (5), protein-tyrosine phosphatases (5, 6), or dual specificity phosphatases (7, 8). The dual specificity phosphatases are capable of catalyzing the removal of the phosphoryl group from phosphotyrosine as well as phosphoserine/threonine. Interestingly, the dual specificity phosphatases are unrelated to the serine/threonine protein phosphatases but belong to the protein-tyrosine phosphatase (PTPase) superfamily.

The dual specificity phosphatases that specifically inactivate the MAP kinases are called the MAP kinase phosphatases (MKPs). At least nine mammalian MKPs have been identified (9, 10). These MKPs share two common structural features: a conserved catalytic domain containing the PTPase active site sequence (H/V/CX2RX/SFT), and an N-terminal domain containing two short sequences (CH2 motifs), which show homology to the catalytic domain of the cell cycle regulator Cdc25 phosphatase (11). MKP3 (12), also termed Pyst1 (13) or rVH6 (14), is predominantly localized in the cytoplasm. In addition, MKP3 is highly specific for ERK1/2 inactivation, although it is not inducible by either growth factors or stress (12–15). Thus, MKP3 activity must be tightly regulated. Until recently, the mechanism of regulation and the functional role of the N-terminal domain was unknown. An elegant series of studies on MKP3 have shown that the N-terminal domain of MKP3 can physically associate with ERK1/2 (16), and this association can activate the phosphatase activity of MKP3 (17). It has been suggested that the substrate (ERK)-induced MKP3 activation may be responsible for the selective inactivation of ERK1/2 by MKP3. However, the detailed mechanism of this activation has not been described.

The goal of this study is to characterize the MKP3-catalyzed hydrolysis of aryl phosphates and to investigate the molecular and chemical basis of the ERK2-induced MKP3 activation. A detailed kinetic study of the MKP3-catalyzed reaction was carried out. Analysis of the effects associated with different substrates, variation in pH, the nature of the leaving group, the addition of co-solvent, and affinity for oxyanions suggests a working model for the regulation of MKP3 activity.
Mechanism of MKP3 Activation

35527

EXPERIMENTAL PROCEDURES

Chemicals—p-Nitrophenyl phosphate (pNPP) was purchased from Fluka. Phenyl phosphosphate, \( \beta \)-naphthyl phosphate (\( \beta \)NPP), and 3-0-methylfluorescein phosphate (OMFP) were purchased from Sigma. Aryl phosphate monoesters 4-acetylphenyl, 4-(trifluoromethyl) phenyl, 4-chlorophenyl, and 4-fluorophenyl phosphate were synthesized as described (18). Arsenic(V) oxide hydrate (99.999%) and vanadium(V) oxide (99.999%) was obtained from Aldrich. Glycerol (99.95%) was purchased from Fisher.

Protein Expression and Purification—The full-length MKP3 was expressed and purified as a glutathione-S-transferase (GST) fusion protein using a previously described procedure (16) with minor modifications. Briefly, pGEX-4T3-MKP3 (a generous gift from Dr. Marco Muda, University of Michigan) was used to transform Escherichia coli BL21(DE3) by standard methods. Single colonies were selected and was transferred to 1 liter of 2YT medium (supplemented with 100 \( \mu \)M ampicillin) overnight with shaking at 37 °C. A 10-ml overnight culture was transferred to 1 liter of 2YT medium (supplemented with 100 \( \mu \)M ampicillin) and shaken at 37 °C until the absorbance at 600 nm was between 0.6 and 0.8. Following the addition of isopropyl-1-thio-

buffer (150 mM NaCl, 50 mM Tris, pH 7.4, 5 mM dithiothreitol, 2 mM EDTA). The cells were harvested by centrifugation at 5,000 rpm for 5 min, and the supernatant was used for the next step. The cells were lysed by passage through a French pressure cell press at 1,200 p.s.i. twice. Cellular debris was removed by centrifugation at 10,000 rpm for 1 h. The resulting supernatant was dialyzed against 50 mM Tris, pH 7.5, 5 mM dithiothreitol, and 2 mM EDTA, and the supernatant was used for the next step. The unbound material was removed by passage through a Sephadex G-25 column equilibrated with 50 mM Tris, pH 7.5, 5 mM dithiothreitol, and 2 mM EDTA. A 10-ml wash was added. After incubating with gentle agitation at 4 °C for 2 h, the matrix was transferred to a column and washed with 10 bed volumes of wash buffer (150 mM NaCl, 50 mM Tris, pH 7.4, 5 mM dithiothreitol, 2 mM EDTA, 0.1% Triton X-100) and 5 bed volumes of 50 mM Tris, pH 7.5, 5 mM dithiothreitol, 2 mM EDTA, and the fusion protein was eluted by the addition of buffer volume of 10 mM reduced glutathione in 50 mM Tris, pH 8.0. The fusion protein was eluted and collected after incubating the column at 4 °C for 10 min. The elution and collection steps were repeated twice. The elute was pooled and concentrated with a Centriprep-30 filtration unit (Amicon).

Protein Kinase Assays—The relative viscosities of sucrose or glycerol solutions used in this study were measured with a Cannon-Fenske Rotovisor at 30 °C with a solution of 50 mM 3,3-dimethylglutarate, pH 7.0, 1 mM EDTA, \( T = 0.15 \) M used as a reference. Relative viscosities of the sucrose and glycerol solutions are as follows: 5% sucrose, \( \eta_{rel} = 1.18 \); 10% sucrose, \( \eta_{rel} = 1.39 \); 20% sucrose, \( \eta_{rel} = 2.05 \); 25% sucrose, \( \eta_{rel} = 2.56 \); 30% sucrose, \( \eta_{rel} = 3.20 \); 40% sucrose, \( \eta_{rel} = 3.48 \); 10% glycerol, \( \eta_{rel} = 1.31 \); 20% glycerol, \( \eta_{rel} = 1.67 \); 30% glycerol, \( \eta_{rel} = 2.36 \); 40% glycerol, \( \eta_{rel} = 3.50 \); 50% glycerol, \( \eta_{rel} = 4.34 \); and 50% glycerol, \( \eta_{rel} = 4.67 \). For the pH dependence of the MKP3-catalyzed PNP reaction in 50% glycerol, the \( k_{cat}/K_m \) was measured using the initial rate method at low substrate concentrations (\( \leq 5 \) mM) at 30 °C. The reaction was started by introducing 1.7 \( \mu \)M MKP3 into the reaction mixture (1 ml) containing 0.6 mM pNPP and followed continuously at 405 nm. The \( k_{cat}/K_m \) value was obtained from the following equation:

\[
\frac{k_{cat}}{K_m} = \frac{A_{cat}/e \cdot \text{MKP3}[\text{pNPP}]}{[\text{D}]} \text{where } A_{cat} = \text{change of absorbance at 405 nm per second, and } e \text{ is the molar extinction coefficient of p-nitrophenol. The molar extinction coefficient of p-nitrophenol in 50% glycerol solution was determined to be as follows: 770 (5.3), 970 (5.6), 1,800 (6.0), 2,300 (6.3), 4,600 (6.6), 7,800 (7.0), 10,700 (7.3), 13,200 (7.6), 14,600 (8.0), 16,200 (8.6), and 16,500 (9.0) M}^{-1}\text{cm}^{-1}. Numbers in parentheses refer to the pH values of 50% glycerol solutions.

Determinination of Inhibition Constants—The stock solution of sodium orthovanadate was prepared as follows. Vanadium(V) oxide was dissolved in 1 mol eq/vanadium atom of 1.0 M aqueous NaOH. The resulting orange solution (mainly decavanadate) was boiled, allowed to stand overnight, and pH-adjusted to 10. The final solution was colorless, containing mainly orthovanadate. The inhibition constants for phosphate and arsenate were determined for MKP3 at pH 7.0 and 30 °C as follows. The initial rate at various pNPP concentrations was measured following the production of p-nitrophenol. The mode of inhibition and \( K_i \) value were determined in the following manner. At various fixed concentrations of inhibitor (0–3 \( K_i \)), the initial rate at a series of pNPP concentrations was measured, ranging from 0.2- to 5-fold the apparent \( K_m \) value. MKP3 concentration was 0.5 \( \mu \)M in the absence of ERK2 and 0.12 \( \mu \)M in the presence of 1.9 \( \mu \)M ERK2. The data were fitted to appropriate equations using KinetAsyst to obtain the inhibition constant. The inhibition constant \( K_i \) value was measured in the presence and absence of ERK2. All experiments with vanadate were performed in the absence of EDTA, where EDTA forms a very stable 1:1 complex with vanadate. The \( IC_{50} \) for vanadate were determined at the \( K_i \) values of pNPP (12 mM in the absence of ERK2 and 1.3 mM in the presence of ERK2).

RESULTS

Extensive enzymological studies have established a minimal kinetic scheme for the PTPase-catalyzed hydrolysis of aryl phosphates (20). In Scheme 1, substrate \( ROPO_3^- \) binding leads to the formation of an enzyme-substrate complex \( E\text{-ROPO}_3^- \), which is followed by a substrate-induced conformational change in the enzyme that brings the general acid close to the scissile oxygen of the substrate \( E^*\text{-ROPO}_3^- \). Substrate is then cleaved with the departure of the leaving group (ROH) and the phosphoryl transfer to the nucleophilic Cys residue, forming a covalent phosphoethenate intermediate \( E^*\text{-P} \), which is then hydrolyzed by water, generating the noncovalent enzyme phosphate complex \( E^*\text{-P} \). Dissociation of the inorganic phosphate from the enzyme completes the catalytic cycle. In this mechanism, the second order rate constant \( k_{cat} \) monitors the interaction between free enzyme and free substrate to begin with the binding of both substrate and to arrest including the first irreversible step, the loss of the leaving group. The \( k_{cat} \) term describes the rate-limiting unimolecular event subsequent to the formation of the enzyme-substrate complex. To characterize the MKP3 reaction in detail and to provide insights into the activation mechanism by ERK2, we determined the kinetic parameters of MKP3 with several arti-
ficial substrates and determined the pH dependence, leaving group dependence, and the effects of viscosogens on MKP3 activity and the affinity of MKP3 for oxyanions in the presence and absence of ERK2. We also determined the concentration dependence of ERK2 on the kinetic parameters $k_{\text{cat}}/K_m$ and $k_{\text{cat}}$ for the MKP3-catalyzed reaction.

**Kinetic Parameters for pNPP, βNP, and OMFP—**MKP3 is able to physically associate with ERK1/2 and is highly selective for the inactivation of ERK1/2 activity (13, 15, 16). Like many PTPases (21, 22) and dual specificity phosphatases (23, 24), MKP3 can also catalyze the hydrolysis of pNPP. A recent study showed that the hydrolysis of pNPP by GST-MKP3 could be stimulated by purified recombinant GST-ERK2, and this ERK2-induced MKP3 activation resulted from a direct interaction between ERK2 and the N-terminal domain of MKP3 (17). Because the effect of recombinant ERK2 on MKP3 activity was determined at a single pNPP concentration, the overall increase in activity could be a composite effect on both $k_{\text{cat}}$ and $K_m$. To begin to understand the molecular basis of the ERK2-induced MKP3 activation, steady state parameters for the GST-MKP3-catalyzed hydrolysis of pNPP in the presence and absence of ERK2 were determined at pH 7.0 and 30 °C. As shown in Table I, a $k_{\text{cat}}$ of 0.0078 s$^{-1}$ and a $K_m$ of 12 mM were observed for the GST-MKP3-catalyzed pNPP hydrolysis. These values are similar to those determined in a previous study using recombinant histidine-tagged MKP3 (25), indicating that the GST portion of the fusion protein does not affect MKP3 phosphatase activity. Furthermore, we have also found that the activity and the degree of activation by ERK2 are similar for the GST-MKP3, the MKP3 derived from thrombin cleavage of the fusion protein, or the histidine-tagged MKP3. All of the experiments described in this study were performed with GST-MKP3, which we will hereafter refer to as MKP3. We also found that GST-ERK2 and ERK2 derived from thrombin cleavage of the fusion protein behave identically in binding and activating MKP3. The ERK2 used in this study was derived from thrombin-cleaved GST-ERK2 fusion protein. The recombinant ERK2 had no measurable phosphatase activity. However, upon the addition of ERK2 to the reaction, the $k_{\text{cat}}$ increased 9-fold, and the $K_m$ decreased 9-fold for the MKP3-catalyzed hydrolysis, resulting in an 82-fold increase in the second order rate constant, $k_{\text{cat}}/K_m$.

To determine if the ERK2-induced MKP3 activation is unique for pNPP, we also measured the kinetic parameters for the hydrolysis of βNP and OMFP by MKP3. The chemical structures of pNPP, βNP, and OMFP are shown in Fig. 1. As summarized in Table I, ERK2 could also increase the $k_{\text{cat}}$ and decrease the $K_m$ of the MKP3-catalyzed hydrolysis of βNP and OMFP. Interestingly, the magnitude of the increase in $k_{\text{cat}}/K_m$ by ERK2 decreases as the size of the substrate increases. This is primarily due to the lower $K_m$ values for the bulkier substrates yielding higher intrinsic $k_{\text{cat}}/K_m$ values in the absence of ERK2.

### Table I

**Kinetic parameters of MKP3 with pNPP, βNP, and OMFP as substrates**

| Parameters | pNPP | βNP | OMFP |
|------------|------|-----|------|
| $k_{\text{cat}}$ (s$^{-1}$) | | | |
| Without ERK2 | 0.0078 ± 0.0004 | 0.012 ± 0.001 | 0.018 ± 0.001 |
| With ERK2 | 0.071 ± 0.001 | 0.064 ± 0.002 | 0.046 ± 0.002 |
| $K_m$ (mM) | | | |
| Without ERK2 | 12 ± 1.4 | 3.8 ± 0.3 | 0.966 ± 0.007 |
| With ERK2 | 1.3 ± 0.05 | 0.31 ± 0.04 | 0.031 ± 0.004 |
| $k_{\text{cat}}/K_m$ (M$^{-1}$ s$^{-1}$) | | | |
| Without ERK2 | 0.67 ± 0.07 | 3.2 ± 0.17 | 190 ± 10 |
| With ERK2 | 55 ± 1.5 | 210 ± 18 | 1500 ± 110 |

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**Mechanism of MKP3 Activation**

**pH Dependence of the MKP3-catalyzed Reaction—**The pH dependences of $k_{\text{cat}}$ and $k_{\text{cat}}/K_m$ for the MKP3-catalyzed hydrolysis of pNPP were studied from pH 5.3 to 9.0. The results are shown in Fig. 3. In the absence of ERK2, no significant pH dependence of $k_{\text{cat}}$ and $k_{\text{cat}}/K_m$ was observed for the pNPP reaction catalyzed by MKP3. In contrast, a bell-shaped pH rate curve was observed for both $k_{\text{cat}}$ and $k_{\text{cat}}/K_m$ when ERK2 was included in the assay. In fact, the pH rate profiles of the MKP3-catalyzed pNPP reaction in the presence of ERK2 were...
pNPP was 40 mM, and the concentration of MKP3 was 0.10 μM. B, the pH-independent second order rate constant, $k_{\text{cat}}/K_m$, represents the ionization constants of the free enzyme (21).

Previous work have shown that the substrate must be in the diionic form for optimal PTPase catalysis (21, 22, 27). Because of the instability of MKP3 at pH <5 and the low activity toward substrates, it was not possible to observe the expected variation due to substrate ionization (pK$_S$) in the low pH region of the pNPP reaction at pH 7.0 and 30 °C.

For the analysis of $k_{\text{cat}}/K_m$, Equation 1 was used, where ($k_{\text{cat}}/K_m$)$_{\text{lim}}$ represents the pH-independent second order rate constant, $K_{E1}$ represents the second ionization constant of the substrate, and $K_{E2}$ and $K_{E2}$ represent the ionization constants of the free enzyme (21).

$$
\frac{k_{\text{cat}}}{K_m} = \frac{(k_{\text{cat}}/K_m)_{\text{lim}}}{(1 + H^+/K_{E1})(1 + H^+/K_{E2} + K_{E2}/H^+)} \quad \text{(Eq. 1)}
$$

The results are listed in Table III. In the presence of ERK2, the variation of $k_{\text{cat}}/K_m$-pH plot suggests that the MKP3-catalyzed hydrolysis of pNPP requires an ionizing residue that must be deprotonated with a pK$_{E1}$ value of 6.5 and another ionizing residue that must be protonated with a pK$_{E2}$ value of 7.1 for optimal binding and catalysis.

For the analysis of $k_{\text{cat}}$, Equation 2 was used, where $k_{\text{cat}}^{\lim}$ represents the pH-independent turnover number, and $K_{E1}$ and $K_{E2}$ are the apparent ionization constants of the enzyme-substrate complex. The data indicate that in the presence of ERK2, the variation of $k_{\text{cat}}$-pH profile is consistent with the presence of the enzyme-substrate complex of an ionizable residue that must be deprotonated with a pK$_{E1}$ value of 6.2 and another ionizable residue that must be protonated for the catalytic turnover with a pK$_{E2}$ value of 8.0 (Table III).
that relates the logarithm of phate, and phenyl phosphate. Fig. 5 shows the Brønsted plot for the influence of steric and differential binding was minimized. The substitution at the para position were utilized such that the sensitive to the size of the substrate, only aryl phosphates with pMKP3 used was 0.5 mM and 0.12 mM in the presence and absence of ERK2.

We have also examined the pH dependence of the MKP3-catalyzed OMFP hydrolysis. Unlike the pNPP reaction, bell-shaped pH rate profiles were observed both in the presence and absence of ERK2 (Fig. 4), although the two curves are not identical. The data were analyzed similarly as described for pNPP. The ionization parameters and the pH-independent rate constants are summarized in Table III. The catalytically important enzyme ionization constants determined in the presence of ERK2 using pNPP as a substrate are similar to those using OMFP as a substrate.

Leaving Group Dependence of the MKP3-catalyzed Hydrolysis of O-Aryl Phosphates—Aryl phosphates serve as substrates for MKP3, so that leaving group effects can be studied. The leaving group dependence of the hydrolysis of O-aryl phosphates catalyzed by MKP3 was investigated at pH 7.0 and 30 °C using a series of aryl phosphates with leaving group pKa values ranging from 7 to 10. Because the K_m values for MKP3 are found to be sensitive to the size of the substrate, only aryl phosphates with substitution at the para position were utilized such that the influence of steric and differential binding was minimized. The aryl phosphates used in the leaving group study included pNPP, 4-acetylphenyl phosphate, 4-(trifluoromethyl)phenyl phosphate, 4-chlorophenyl phosphate, 4-fluorophenyl phosphate, and phenyl phosphate. Fig. 5 shows the Brønsted plot that relates the logarithm of k_cat/K_m to the pK_a values of the leaving group. Linear least-squares fitting of the Brønsted plot yielded the slope, which corresponds to the β_1p value. The β_1p values for MKP3 in the presence and absence of ERK2 are 0.041 ± 0.042 and −0.037 ± 0.068, respectively, indicating no leaving group dependence for the MKP3-catalyzed reactions.

Effects of Glycerol on MKP3 Reaction—During the course of examination of the viscosity dependence of the MKP3-catalyzed reaction, we discovered that glycerol had a profound stimulating effect on MKP3 activity (Fig. 6). It was found that, in the absence of ERK2, MKP3 activity could be activated by increasing the amount of glycerol. The apparent affinity of glycerol for MKP3 was low, because maximal activation was not observed even at 50% glycerol concentration. Nevertheless, the k_cat/K_m value for the MKP3-catalyzed pNPP reaction in 50% glycerol (relative viscosity = 4.67 at pH 7.0 and 30 °C) was 17-fold higher than that in buffer alone. Glycerol could also stimulate the MKP3 reaction in the presence of ERK2. The maximal level of activation by glycerol was 2-fold, and the glycerol concentration required to reach half-maximal activation was 10%. However, unlike the effect of ERK2 on MKP3 reaction, glycerol does not change the K_m of the reaction. For example, in the absence ERK2, the k_cat/K_m values for the pNPP reaction were 0.031 ± 0.002 s^-1 and 12 ± 1.4 mM in 20% glycerol and 0.085 ± 0.008 s^-1 and 11 ± 2.4 mM in 45% glycerol. In the presence of ERK2, the k_cat and K_m values for the pNPP reaction were 0.10 ± 0.002 s^-1 and 1.3 ± 0.08 mM in 10% glycerol and 0.15 ± 0.01 s^-1 and 1.6 ± 0.3 mM in 45% glycerol. As a control, we also examined the effect of sucrose, another commonly used viscosogen, on the MKP3-catalyzed pNPP hydrolysis at a number of sucrose concentrations (0–40% sucrose, relative viscosity 1–3.48). Although sucrose was also found to have a stimulatory effect on the MKP3 reaction, the effect was very modest in comparison with glycerol. For example, in the presence of 40% sucrose (relative viscosity 3.48), the k_cat/K_m value of the MKP3 reaction was increased by only 25% of that in the absence of sucrose. This result suggests that the stimulatory effect of glycerol was not a general viscosity effect.

To examine the effect of glycerol further, we also studied the pH dependence of the MKP3-catalyzed pNPP reaction in the presence of 50% glycerol. As shown in Fig. 7, unlike the reaction in buffer alone (Fig. 3), the pH-k_cat/K_m profile in 50% glycerol is bell-shaped. The data were analyzed using Equation 1. The pH-independent (k_cat/K_m)_lim value was determined to be 19.7 ± 1.6 M^-1 s^-1, and pK_ES1 and pK_ES2 were 5.6 ± 0.1 and 7.3 ± 0.1, respectively. Finally, the affinity of MKP3 for arsenate was also determined at pH 7.0 and 30 °C in 50% glycerol. The K_i value of MKP3 for arsenate was found to be 19 ± 2 μM, which is 6-fold lower than that in the absence of glycerol (Table II).

**DISCUSSION**

The dual specificity phosphatases and the PTPases share the active site signature motif (H/V(C/R)S/T) and other key structural features that are important for catalysis and are believed to utilize a common mechanism to effect catalysis (20).
The PTPase catalyzed reaction proceeds through a double displacement mechanism in which the phosphoryl group from the substrate is first transferred to the active site Cys residue, leading to the formation of a cysteinyl phosphate intermediate that is subsequently hydrolyzed by water. The guanidinium group of the active site Arg makes two hydrogen bonds with two phosphoryl oxygens in the substrate and plays an important role in substrate binding and transition state stabilization (28, 29). A unique feature of PTPase catalysis is the use of a conserved Asp residue as a general acid (30), which is brought into position for catalysis by a movement of a flexible loop that occurs upon binding of substrate (31–33). The dual specificity phosphatases also employ a similarly positioned Asp residue for catalysis (34). It is likely that a ligand-induced movement is also required to bring the general acid into the active site. The overall catalytic mechanism, including the conformational change involving the flexible loop movement, is shown in Scheme 1.

MKP3, like other members of the MAP kinase phosphatase family, contains a C-terminal phosphatase domain with significant sequence similarity to VHR and an N-terminal domain containing two CH2 sequences. The N-terminal domain was not required for the MKP3-catalyzed pNPP hydrolysis reaction (25). However, a recent study showed that the MKP3-catalyzed pNPP reaction could be stimulated by ERK2, which binds the N-terminal domain of MKP3 (16, 17). To gain further insights into the molecular mechanism of the ERK2-induced MKP3 activation, we have carried out a detailed kinetic analysis of the MKP3-catalyzed reaction. From the results that we obtained, together with published structural and biochemical data, a working model for the molecular basis of MKP3 activation by ERK2 can be proposed.

A Working Model for MKP3 Activation—We propose that like the Yersinia PTPase and PTP1B (31, 32), MKP3 can exist in two conformational distinct states (Fig. 8). In the open conformation, the enzyme is catalytically incompetent, and only when the enzyme is in the closed form can substrate turnover occur. For PTPases, in order for the general acid-containing loop to adopt the closed conformation, an important hydrogen bond between a carbonyl oxygen within the hinge region of the loop and the NH1 atom of the active site Arg residue in the phosphate binding loop must form. This is made possible as a result of the interactions between the phosphoryl moiety and the side chain of the active site Arg (36, 37). A similar mechanism for loop closure is proposed in MKP3 that allows the repositioning of catalytic groups and the reorienting of the electrostatic environment at the active site. It is proposed that in the absence of ERK2, the slowest step in the MKP3-catalyzed pNPP reaction corresponds to the substrate-induced conformational change in the active site that leads to the closure of the general acid-containing loop. The binding of ERK2 to the N-terminal domain of MKP3 accelerates the rate of repositioning of active site residues and the general acid loop closure in MKP3 such that chemistry becomes rate-limiting in the presence of ERK2. Our data indicate that the same conformational change can also be promoted by glycerol or substrates with enhanced affinity with MKP3.
oxygen of the substrate for catalysis upon substrate binding. The loop closure occurs as a result of the interactions between the phosphoryl moiety and the side chain of the active site Arg, which in turn enables the Arg residue to interact with a hinge residue in the general acid loop (36, 37). This conformational change involving active site reorganization occurs much faster than the bond-breaking/making chemical step in the pNPP reaction catalyzed by the Yersinia PTPase (33), protein-tyrosine phosphatase 1B, and VHR, since intrinsic heavy atom kinetic isotope effects are fully expressed (38, 39). A similar mechanism for loop closure could also occur in MKP3 that allows the repositioning of catalytic groups and the reorienting of the electrostatic environment at the active site (Fig. 8). However, our data suggest that in MKP3 this substrate-induced conformational change is slower than the chemical step and determines the overall rate of pNPP hydrolysis in the absence of ERK2. This may be caused by insufficient interactions between MKP3 and simple aryl phosphate to allow optimal alignment of active site residues with respect to the substrate. The binding of ERK2 to the N-terminal domain of MKP3 facilitates the repositioning of active site residues and accelerates the general acid loop closure such that chemistry becomes the rate-limiting step in the presence of ERK2. We present below evidence in support of this model.

**ERK2 Binding to MKP3 Results in a Conformational Change in MKP3 Active Site**—The crystal structures of PTPases and VHR complexed with oxyanions (e.g. tungstate, phosphate, and sulfate) indicate that the major interactions between the oxyanion and the enzyme involve hydrogen bonds with the NH amides of the peptide backbone and the guanidinium group of the invariant Arg making up the active site phosphate-binding loop (31, 32, 34). In the structure of MKP3 solved in the open conformation, residues in the phosphate-binding loop important for oxyanion binding are displaced from the corresponding positions observed in the VHR structure (35). This suggests that MKP3 may bind oxyanions with affinity lower than VHR. Indeed, the $K_\text{cat}$ values of MKP3 with phosphate and arsenate (Table II) are 26- and 5-fold higher than those determined for VHR (40). However, when MKP3 is associated with ERK2, the affinity of MKP3 for phosphate and arsenate increases 10-fold (Table II).

Vanadate binds MKP3 with the highest affinity and is consistent with it acting as a transition state analog for the PTPase-catalyzed reaction due to its tendency to adopt a pentavalent geometry. The structural basis for the high affinity vanadate binding is revealed by the fact that equatorial oxygens in vanadate form shorter, and therefore stronger, hydrogen bonds with the phosphate-binding loop and the active site Arg residue (41, 42). Furthermore, the apical oxygen atom, which resembles the leaving group oxygen or the oxygen atom of the attacking nucleophilic water molecule, forms a hydrogen bond with the carboxyl group of the Asp general acid/base (41, 43, 44). We observed that ERK2 can also enhance the affinity of MKP3 for vanadate 33-fold (Table II). Collectively, these results suggest that the interactions between ERK2 and the N-terminal domain of MKP3 triggers a conformational change in the catalytic domain of MKP3 that better positions the phosphate binding loop and the general acid loop for effective oxyanion binding and transition state stabilization.

**ERK2 Binding to MKP3 Causes a Change in the Rate-limiting Step in the MKP3-catalyzed pNPP Reaction**—In all cases that have been examined in detail, the pH dependence of the hydrolysis of pNPP catalyzed by the PTPases (21, 22) and the dual specificity phosphatase VHR (27) is bell-shaped. The $k_{\text{cat}}/K_m$ for the phosphatase-catalyzed hydrolysis of pNPP is controlled by chemistry (38, 39), and the $k_{\text{cat}}/K_m$-$p$H profile is consistent with the presence in the enzyme of one group that must be unprotonated and one group that must be protonated for catalysis. The group that must be unprotonated is probably the active site Cys residue, and the group that must be protonated corresponds to the general acid (22, 27, 30). Substitution of the general acid Asp to Asn reduces the activity by 2–3 orders of magnitude and eliminates the basic limb of the pH rate profile (27, 30).

An early study showed that the MKP3-catalyzed pNPP hydrolysis retained the acidic limb; however, the basic limb was missing from the the $k_{\text{cat}}/K_m$-$p$H profile (25). This behavior is similar to those observed with the general acid deficient mutant Yersinia PTPase and VHR, in which the ionizations of the active site Cys and the substrate are observable, and chemistry (phosphoryl transfer, i.e. $E^*$-$P$ formation) is rate-limiting for $k_{\text{cat}}/K_m$ (27, 30, 37–39). The inability to observe the ionization due to the general acid was attributed to the incorrect position of Asp$^{302}$ for proper donation (25). If this were the case, one would expect to observe a strong leaving group dependence for the MKP3-catalyzed reaction in the absence of ERK2. When the general acid is functional, no leaving group dependence is observed (21, 23), because P–O bond cleavage and proton transfer from the general acid to the phenolic oxygen in the transition state are concurrent (38, 39). Indeed, we found no leaving group dependence on $k_{\text{cat}}/K_m$ for the MKP3-catalyzed hydrolysis of aryl phosphates in the presence of ERK2 (Fig. 5). When the general acid is removed, a full negative charge accumulates on the phenolic oxygen (38, 39), and a $p$H value of close to 1 is obtained (37, 45). Surprisingly, the MKP3-catalyzed hydrolysis of aryl phosphates exhibits no leaving group dependence on $k_{\text{cat}}/K_m$ when ERK2 is excluded from the assay (Fig. 5). The lack of leaving group dependence on $k_{\text{cat}}/K_m$ in the absence of ERK2 suggests that chemistry cannot be rate-limiting in the MKP3 reaction.

Because in the absence of ERK2 the $k_{\text{cat}}/K_m$ for the MKP3-catalyzed pNPP reaction is extremely low (0.65 m$^{-1}$ s$^{-1}$) and displays no significant viscosity effect in sucrose solution, the binding event between MKP3 and pNPP is unlikely to be rate-limiting. Thus, for MKP3-catalyzed simple aryl phosphates in the absence of ERK2, $k_{\text{cat}}/K_m$ is probably controlled by the substrate-induced general acid loop closure ($E$-$ROPO_3^2$ $\rightarrow$ $E^*$-$ROPO_3^2$ in Scheme 1; also see Fig. 8) that reorganizes the active site and brings the general acid into proximity to the scissile bond. We found no pH dependence on $k_{\text{cat}}/K_m$ in the MKP3-catalyzed pNPP reaction in the absence of ERK2 (Fig. 3). This complete lack of pH dependence in the MKP3 reaction is in accord with a physical step, the loop closure, being the rate-limiting step. In contrast, when ERK2 is present in the reaction, a bell-shaped pH profile similar to those observed with the MKP3 reaction (27, 30) is obtained (37, 39). The reaction catalyzed by the MKP3-catalyzed pNPP reaction due to its tendency to adopt a pentavalent geometry. The structural basis for the high affinity vanadate binding is revealed by the fact that equatorial oxygens in vanadate form shorter, and therefore stronger, hydrogen bonds with the phosphate-binding loop and the active site Arg residue (41, 42). Furthermore, the apical oxygen atom, which resembles the leaving group oxygen or the oxygen atom of the attacking nucleophilic water molecule, forms a hydrogen bond with the carboxyl group of the Asp general acid/base (41, 43, 44). We observed that ERK2 can also enhance the affinity of MKP3 for vanadate 33-fold (Table II). Collectively, these results suggest that the interactions between ERK2 and the N-terminal domain of MKP3 triggers a conformational change in the catalytic domain of MKP3 that better positions the phosphate binding loop and the general acid loop for effective oxyanion binding and transition state stabilization.

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3 It is not clear what causes the discrepancy between the current study and the previous one. However, it is possible that the previously observed pH dependence in the low pH region might be due to enzyme inactivation, since we find MKP3 very sensitive to acid denaturation.
tween MKP3 active site and the substrate and speed up the substrate-induced loop closure such that chemistry becomes rate-limiting in the presence of ERK2. In support of the hypothesis that ERK2 binding causes a change in the rate-limiting step in the MKP3-catalyzed pNPP reaction, kinetic traces from stopped-flow experiments showed no burst of p-nitrophenol production in the absence of ERK2, whereas burst kinetics was observed in the presence of ERK2.4 Below, we provide evidence that the loop closure can also be promoted by larger substrates or co-solvent glycerol.

Substrate with Higher Affinity to MKP3 Accelerates the General Acid Loop Closure—Active site substrate specificity studies have shown that dual specificity phosphatases prefer bulky polycyclic ary1 phosphates (23, 24, 46). This may be the result of the shallower active site pockets of the dual specificity phosphatases, which enable them to accommodate the shorter side chains of phosphoserine/threonine as well as that of phosphotyrosine (11, 34, 35). We found that ERK2 also stimulated the MKP3-catalyzed hydrolysis of β-NP and OMFP, which was accomplished by increasing the $k_\text{cat}$ and by decreasing the $K_m$. However, the extent of ERK2-induced MKP3 activation was substrate-dependent; the magnitude of the activation was smaller as the size of the substrate became larger. It was noted that the $k_\text{cat}/K_m$ values do not differ greatly among the different substrates, but the $K_m$ values decrease drastically as the size of the substrate increases (Table I). It is likely that the bulkier polycyclic substrates interact with MKP3 more favorably than simple ary1 phosphates such as pNPP. The increase in $k_\text{cat}/K_m$ for the larger substrates could be due to an increase in the rate constant for substrate binding, substrate-induced enzyme conformational change, or the chemical step ($E^*P$ formation) at the expense of the additional binding energy. The fact that the MKP3-catalyzed hydrolysis of OMFP displays a bell-shaped pH curve in the absence of ERK2 (Fig. 4) suggests that the rate of active site reorganization and loop closure must have been increased to such an extent that chemistry has become at least partially rate-limiting. Indeed, unlike pNPP, the MKP3-catalyzed hydrolysis of OMFP exhibited burst kinetics even in the absence of ERK2.4 The decrease in the magnitude of activation by ERK2 as the size of the substrate increases is in accord with the hypothesis that the loop closure is more facile for bulkier substrates and less help is needed from ERK2 binding. However, because the MKP3-catalyzed OMFP reaction can be further activated by ERK2 (Table I and Fig. 4), the active site conformation induced by OMFP may not be as optimal as that promoted by ERK2.

Glycerol Stabilizes the Closed Catalytically Competent MKP3 Conformation—Glycerol is a common viscosogen used to determine if a diffusion-controlled binding event limits the overall reaction. If substrate binding is the slowest step, one would expect to see linear viscosity dependence in which $k_\text{cat}/K_m$ will decrease as the solution viscosity increases. No viscosity dependence was observed for the MKP3 reaction in 50% glycerol, whereas the $k_\text{cat}/K_m$ is reduced by more than 2 orders of magnitude (Table III). These results suggest that MKP3 has achieved an activated conformation in 50% glycerol compatible with that in the presence of ERK2. However, the fact that the $k_\text{cat}/K_m$ for the MKP3 reaction in 50% glycerol can be increased further by ERK2 indicates that the active site conformation of MKP3 in 50% glycerol may not be identical to that when ERK2 is bound to MKP3. Glycerol is known to stabilize the structure of native proteins because it interacts unfavorably with nonpolar residues in the interior of a protein when the protein is denatured (47, 48). It may be that glycerol preferentially stabilizes the closed conformation because it has fewer nonpolar residues exposed in comparison with the open conformation. This lowers the kinetic barrier for the substrate-induced loop closure so that chemistry becomes rate-limiting in 50% glycerol.

We have previously shown that unlike tyrosine-specific PTPases, which are unable to catalyze phosphoryl transfer to alcohols, the dual specificity phosphatases are able to catalyze phosphoryl transfer to alcohols from $E^*P$ (49). When the MKP3-catalyzed pNPP reaction was measured in the presence of glycerol using $^{31}$P NMR, phosphoryl transfer from $E^*P$ to glycerol was indeed observed (data not shown). However, it was determined that the rate increase in glycerol was not due to phosphoryl transfer, because both the rate of $E^*-P$ hydrolysis and the rate of transfer to glycerol from $E^*-P$ were accelerated by glycerol. In addition, since $k_\text{cat}/K_m$ monitors the reaction up to the first irreversible step (i.e., the formation of the $E^*-P$ with the expulsion of the leaving group), phosphoryl transfer from $E^*-P$ to glycerol is not expected to affect the $k_\text{cat}/K_m$ value. Thus, the higher $k_\text{cat}/K_m$ in glycerol is the result of glycerol-induced activation, most likely corresponding to the active site reorganization and general acid loop closure. Interestingly, 4–6 M glycerol also accelerated the activity of the full-length SH2 domain-containing PTPase, SHP-1, by 47-fold, which has been suggested to be caused by a glycerol-induced conformational change alleviating the autoinhibition by the SH2 domains (50).

In conclusion, we propose that MKP3 can exist either in a general acid loop open or closed conformation. Normally, MKP3 is in the open state, in which the active site residues are misaligned and the general acid is positioned away from the active site. The rate of the active site rearrangement and general acid loop closure is dependent on the nature of the substrate. It appears that with a simple aryl phosphate (e.g., pNPP) as a substrate, the interaction between the substrate and MKP3 may not be sufficient to allow the attainment of optimal alignment of active site residues with respect to the substrate and to bring down the general acid-containing loop efficiently. Thus, in the absence of activating agents, the MKP3-catalyzed hydrolysis of simple aryl phosphates is rate-limited by the substrate-induced conformational change in the active site that leads to the closure of the general acid-containing loop. Thus, the reaction displays no dependence on pH, viscosity, and the nature of the leaving group. Increased catalytic activity and enhanced affinity for oxyanions are observed for MKP3 in the presence of ERK2. The molecular basis of MKP3 activation by ERK2 is that ERK2 binding to the N-terminal domain of MKP3 facilitates the repositioning of active site residues and speeds up the loop closure in MKP3. In the closed form, the general acid as well as other active site residues in MKP3 are optimally aligned with the substrate, such that chemistry becomes rate-limiting in the presence of ERK2. Indeed, mutation of Asp262 has no effect on the MKP3 reaction in the absence of ERK2, whereas the $k_\text{cat}/K_m$ is reduced by more than 2 orders of mag-

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4 B. Zhou and Z.-Y. Zhang, unpublished observations.
nitude in the presence of ERK2, suggesting that ERK2 binding may be responsible for bring the general acid into the active site (35). Finally, our data suggest that the rate of repositioning of catalytic groups and the reorienting of the electrostatic environment in the MKP3 active site can be enhanced not only by ERK2 but also by high affinity substrates or by glycerol.

Acknowledgments—We thank Marco Muda for providing pGEX4T3-MKP3 and Chris Marshall for providing pGEX-2T-Erk2. We thank Dr. Jiang Wei and members of the Zhang laboratory for helpful comments.

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