Mechanistic Basis for Catalytic Activation of Mitogen-activated Protein Kinase Phosphatase 3 by Extracellular Signal-regulated Kinase*

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The dual specificity mitogen-activated protein kinase phosphatase MKP3 has been shown to down-regulate mitogenic signaling through dephosphorylation of extracellular signal-regulated kinase (ERK). Camps et al. (Camps, M., Nichols, A., Gillieron, C., Antonsson, B., Muda, M., Chabert, C., Bosichert, U., and Arkinstall, S. (1998) Science 280, 1262–1265) had demonstrated that ERK binding to the noncatalytic amino-terminal domain of MKP3 can dramatically activate the phosphatase catalytic domain. The physical basis for this activation has not been established. Here, we provide detailed biochemical evidence that ERK activates MKP3 through the stabilization of the active phosphatase conformation, inducing closure of the catalytic “general acid” loop. In the closed conformation, this loop structure can participate efficiently in general acid/base catalysis, substrate binding, and transition-state stabilization. The pH activity profiles of ERK-activated MKP3 clearly indicated the involvement of general acid catalysis, a hallmark of protein-tyrosine phosphatase catalysis. In contrast, unactivated MKP3 did not display this enzymatic group as critical for the low activity form of the enzyme. Using a combination of Brønsted analyses, pre-steady-state and steady-state kinetics, we have isolated all catalytic steps in the reaction and have quantified the specific rate enhancement. Through protonation of the leaving group and transition-state stabilization, activated MKP3 catalyzes formation of the phosphoenzyme intermediate ~100-fold faster than unactivated enzyme. In addition, ERK-activated MKP3 catalyzes intermediate hydrolysis 5–6-fold more efficiently and binds ligands up to 19-fold more tightly. Consistent with ERK stabilizing the active conformation of MKP3, the chemical chaperone dimethyl sulfoxide was able to mimic this activation. A general protein-tyrosine phosphatase regulatory mechanism involving the flexible general acid loop is discussed.

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‡ The abbreviations used are: DSP(s), dual specificity phosphatase(s); PTP, protein-tyrosine phosphatase; MAP, mitogen-activated protein kinase; MKK, MAP kinase kinase; ERK, extracellular signal-regulated kinase; MKP, MAP kinase phosphatase; bis-Tris, bis(2-hydroxyethyl)iminom-tris(hydroxymethyl)methane; pNPP, para-nitrophenyl phosphate; pNP, para-nitrophenol; oMFP, 3-0-methylfluorescein phosphate; VHR, vaccinia H1 related.
and structural studies (15, 17–21), and the deduced catalytic mechanism has served as a model for all DSPs. The thiolate of cysteine 124 in the signature motif HCXCGXXRS(T) is the nucleophile that attacks the phosphorus of substrate, transferring the phosphate group to the enzyme, forming a cysteinyl-phosphate enzyme intermediate. Catalysis is enhanced by conserved aspartic acid 92, which is positioned to act as a general acid by protonating the leaving group oxygen. Aspartic acid 92 is found on a separate loop structure that was shown to be flexible in several PTPs. In these PTPs, ligand binding induces the movement of the “general acid loop” toward the active site such that the conserved aspartic acid is in position to transfer a proton to the leaving group. In the recent Pyst1 structure, the proposed general acid loop was flipped 20 Å away from the active-site cleft. In this conformation, the proposed general acid (Asp-262) would not be expected to contribute to catalysis. Consistent with this idea, mutation of Asp to Asn (D262N) resulted in an enzyme that displayed activity similar to that of the wild type enzyme (22). More interestingly, the D262N mutant could no longer be activated by ERK protein. Collectively, these data suggested that ERK binding to MKP3/Pyst1rVH6 induces general acid loop closure and the subsequent involvement of Asp-262 acting as the general acid catalyst.

To elucidate the mechanistic basis for the dramatic catalytic enhancement and provide direct evidence that ERK binding induces general acid catalysis by wild type MKP3, we have performed an extensive enzymatic comparison of the activated and unactivated form of MKP3. Using steady-state and rapid-reaction kinetics, pH studies, and Brønsted analysis, we demonstrated that ERK-activated MKP3 utilizes general acid catalysis, enhancing the rate of P-O bond cleavage by 100-fold. Also, the catalytic activation on all reaction steps is evaluated.

MATERIALS AND METHODS

Reagents and Enzymes—All chemicals were of the highest grade commercially available. MKP3 was expressed and purified as described in (16), and ERK was purified as described previously in (23). The 8-fluoro-4-methylumbelliferol phosphate substrate (24) was from Molecular Probes (Eugene, OR).

Assays—The assay buffer was a three-component system consisting of 0.1 M acetate, 0.05 M Tris, and 0.05 M bis-Tris. This buffer system maintains a constant ionic strength of 0.1 M throughout the entire pH range. To determine the kinetic parameters $k_{cat}$ and $K_m$, the initial velocities were measured at various substrate concentrations, and the data were fitted to Equation 1. For the construction of the pH profiles, $k_{cat}$ and $K_m$ values were determined at various pH values. The pH data were fitted to Equations 2–5 depending upon the shape of the profile. Data were fitted to Equation 1 using KinetAsyst (IntelliKinetics, State College, PA). Fitting of the pH-dependent data to Equations 2–5 was accomplished with nonlinear least squares fitting using the computer program KALEIDAGRAPH (Abelbeck Software) for Macintosh. In Equations 2–5, $C_1$ (or $C_2$, $C_3$) is the pH-independent value of either $k_{cat}$ or $k_{cat}/K_m$; $H$ is the proton concentration; $K_a$, $K_b$, and $K_c$ are the ionization constants.

$$v = \frac{V_{max} \cdot S}{(K_m + [S]) + S}$$  
(Eq. 1)

$$v = \frac{C_1}{[1 + H/K_a] \cdot [1 + K_b/H]}$$  
(Eq. 2)

$$v = \frac{(C_2 \cdot (1 + H/K_a) \cdot (1 + K_b/H))}{(C_2 \cdot (1 + K_b/H))}$$  
(Eq. 3)

$$v = \frac{C_2}{[1 + H/K_a] \cdot (1 + K_b/H)}$$  
(Eq. 4)

$$v = \frac{(C_3 \cdot (1 + H/K_a) \cdot (1 + K_b/H))}{(C_3 \cdot (1 + K_b/H))}$$  
(Eq. 5)

Inhibition by Phosphate—The inhibition constant for phosphate, $K_i$, was determined for MKP3 in the following manner. At various fixed concentrations of inhibitor, the initial velocity at different pNPP concentrations was measured as described previously (18). The inhibition was competitive with respect to substrate, and the data were fit using to Equation 6 to yield the inhibition constant.

$$v = \frac{V_{max} \cdot S}{(K_m + [I] + [K_i])}$$  
(Eq. 6)

Leaving Group Dependence—Substrates 3-o-methylfluorescein phosphate (oMFP; $pK_a$ = 4.6), 6,8-difluoro-4-methylumbelliferyl phosphate ($pK_a$ = 4.7), 8-fluoro-4-methylumbelliferyl phosphate ($pK_a$ = 6.4), pNPP ($pK_a$ = 7.1), 4-methylumbelliferol phosphate ($pK_a$ = 7.8), β-naphthyl phosphate ($pK_a$ = 9.38), and phenylphosphate ($pK_a$ = 9.39) were employed and assayed as described previously (22). The $k_{cat}$ and $k_{cat}/K_m$ values were determined as described above.

Rapid-reaction Kinetics—Enzyme and substrate were rapidly mixed at 25 °C in a temperature-controlled SF-61 Hi-Tech Scientific (Hi-Tech Labs., Salisbury, U. K.) stop-flow spectrophotometer. Product formation of p-nitrophenol was monitored at 410 nm, 3-o-methylfluorescein at 477 nm, 6,8-difluoro-4-methylumbelliferone at 360 nm, 8-fluoro-4-methylumbelliferone at 360 nm, and 4-methylumbelliferone at 370 nm. The data were fitted to Equation 7 using the nonlinear least squares fitting capability of the kinetics software (KinetAsyst), where A is the amplitude of the burst, $h$ is the first-order rate constant of the burst, $B$ is the slope of the linear portion of the curve, $C$ is the intercept of the line, and $t$ is time.

$$A = \frac{E}{h} \cdot (k_b + h) \cdot S$$  
(Eq. 7)

The burst rate and the linear rate of the stopped-flow trace were determined as a function of substrate concentration. Each kinetic trace was fitted to Equation 7. The resulting burst rate and slope rate were then fitted to Equations 8 and 1, respectively. The resulting $(k_b + h)$ value from Equation 8 is essentially the maximal rate constant obtained from the plots of $k_{cat}/K_m$ versus [S]. Using the maximal $(k_b + h)$ value, the fit to Equation 9 yields the actual $k_b$ value. Equation 9 describes the observed amplitude (B) of the burst phase as a function of $[E]_0$. $h$, $(k_b + h)$, $K_m$, and varied $[S]$

$$B = \frac{[E]_0 \cdot (k_b + h) \cdot S}{1 + (K_m/[S])^2}$$  
(Eq. 8)

RESULTS

pH Rate Analysis—In initial experiments, the amount of ERK required to activate MK3 maximally was determined by measuring the initial velocities at increasing concentrations of ERK and varying concentrations of the phosphatase substrate oMFP. Consistent with previous observations (14), we found that maximal activation resulted from an approximate 1:1 stoichiometry of MKP3 to ERK concentration (data not shown). To ensure maximal activation, the concentration of ERK in all experiments was 2-fold higher than the MKP3 concentration. To begin to probe the mechanism of activation, the $k_{cat}$ and $k_{cat}/K_m$ pH profiles were generated for MKP3 (both in the presence and absence of the activator ERK), and the critical ionizations were determined. The apparent second-order rate constant $k_{cat}/K_m$ describes the reaction between free enzyme and free substrate. Reflecting both substrate binding and catalysis, the $k_{cat}/K_m$ pH profile yields the intrinsic $pK_a$ values of critical ionizations. The $k_{cat}$ parameter describes the unimolecular catalytic steps subsequent to enzyme-substrate complex formation and will therefore yield apparent $pK_a$ values of the complex.

Previously, pH studies of unactivated MKP3 had not revealed an ionization that must be protonated for activity, suggesting the lack of general acid catalysis (16). Based upon the precedent for general acid catalysis by the DSPs (20), we predicted that the $pK_a$ profiles of activated MKP3 would display the ionization for general acid catalysis. The commonly employed phosphatase substrate pNPP was used to generate the $pK_a$ profiles (Fig. 1), allowing direct comparison with previously published data using this substrate (14, 16).

Two dramatic features distinguished the $pK_a$ profiles of activated and unactivated MKP3 (Fig. 1). The $k_{cat}/K_m$ $pK_a$ profile of activated MKP3 displayed an optimum value that was 48-fold higher than the optimum value observed in the absence of ERK.
value was 10-fold higher at the optimum and displayed an ionization with a \( pK_a \) value of 5.8, which must be unprotonated for activity, and an ionization with a \( pK_a \) value of 8.1, which must be protonated for activity (Table I). Although the group that must be unprotonated (\( pK_a \) value = 6.0 ± 0.1) was still observed, unactivated MKP3 displayed no steep drop in activity at high pH due to a critical ionization. Instead, the \( k_{cat} \) values fell only slightly at high pH, and therefore the data were fitted to Equation 5. As concluded previously (16), the group with a \( pK_a \) value of 6—6 is the catalytic Cys-293. In activated MKP3, the group that must be protonated is consistent with the involvement of a general acid catalyst.

**Leaving Group Effects: Brønsted Analysis**—To provide evidence for general acid involvement induced by the binding of ERK to MKP3, a Brønsted analysis was carried out. It has been shown previously that the \( pK_a \) value of the substrate leaving group (R-O\(^-\)) has little effect on the ability of wild type PTPs to catalyze the first chemical step, P-O bond cleavage and concomitant formation of the thiol-phosphate intermediate (Scheme 1). Because expulsion of the leaving group is facilitated by proton donation from the conserved aspartic acid to the leaving group oxygen, the leaving group \( pK_a \) has little effect on catalysis (26–28). However, when aspartic acid mutants were analyzed, large linear free energy relationships between leaving group \( pK_a \) values and the rates of catalysis were observed (28).

If general acid catalysis is not employed, one would predict that activity will decrease as the \( pK_a \) of the leaving group increases. On the other hand, if MKP3 utilizes general acid catalysis, the leaving group dependence would be greatly diminished. The \( k_{cat} \) and \( k_{cat}/K_m \) values were determined for substrates whose leaving group \( pK_a \) values ranged from 4.6 to 10. The \( k_{cat} \) data are plotted as the log of \( k_{cat} \) versus the \( pK_a \) value of the leaving group (Fig. 2a). In the absence of activation by ERK, the \( k_{cat} \) value displays a small dependence on the leaving group \( pK_a \) value, with a slope of −0.08 ± 0.03. In contrast, the \( k_{cat} \) value of activated MKP3 displays no significant \( pK_a \) effect (slope = 0.013 ± 0.017). It should be noted that leaving group dependence on \( k_{cat} \) values can be kinetically masked because \( k_{cat} \) values are a function of all first-order steps in the reaction. For DS-PTPs and PTPs, \( k_{cat} \) is composed of the individual rate constants of intermediate formation, \( k_3 \) and intermediate hydrolysis, \( k_5 \) (Scheme 1), and is equal to \( k_5 k_5 / (k_3 + k_5) \). The lack of a substantial \( pK_a \) effect in the activated enzyme could be caused by a change in the rate-limiting step, from intermediate formation to intermediate hydrolysis. Although this scenario would still require that the rate of intermediate formation (\( k_3 \)) be greatly enhanced relative to intermediate hydrolysis (\( k_5 \)), the magnitude of the rate acceleration on individual catalytic steps cannot be gleaned directly from the steady-state \( k_{cat} \) values alone. Fortunately, the \( k_{cat}/K_m \) values do not suffer from this problem because the rate of intermediate hydrolysis, \( k_5 \), does not contribute to the \( k_{cat}/K_m \) values. The \( k_{cat}/K_m \) value is a direct measure of sub-

**TABLE I**

| pH-independent value | Steady-state parameters |
|----------------------|-------------------------|
| Unactivated \( k_{cat}/K_m \) | Activated by ERK \( k_{cat}/K_m \) | Unactivated \( k_{cat} \) | Activated by ERK \( k_{cat} \) |
| \( M^{-1} s^{-1} \) | \( s^{-1} \) | \( s^{-1} \) | \( s^{-1} \) |
| \( C \) | 6.38 ± 2.6 | 305 ± 103 | 0.045 ± 0.008 | 0.45 ± 0.08 |
| \( pK_a \) | 6.56 ± 0.23 | 6.39 ± 0.20 | 6.20 ± 0.22 | 5.8 ± 0.2 |
| \( pK_a \) | 7.58 ± 0.45 | 7.59 ± 0.19 | 7.62 ± 0.74 | 8.1 ± 0.1 |

**FIG. 1.** Effect of pH on the \( k_{cat}/K_m \) and \( k_{cat} \) parameters for activated and unactivated MKP3. At each indicated pH value, substrate saturation curves were generated, and the data were fitted to Equation 1. To activate MKP3, ERK protein was added at 2 \times \) the concentration of MKP3. The unactivated pH profiles (open circles) of \( k_{cat}/K_m \) and \( k_{cat} \) were fitted to Equations 3 and 5, respectively. Activated pH profiles (open diamonds) of \( k_{cat}/K_m \) and \( k_{cat} \) were fitted to Equations 2 and 4, respectively. Results from these fits are summarized in Table I. Conditions: 0.1 M sodium acetate, 0.05 M Tris, and 0.05 M bis-Tris, 25 °C.

(Fig. 1a and Table I). Second, activated MKP3 displayed a critical ionization (\( pK_a \) = 7.6) from an amino acid residue that must be protonated for activity. In the \( k_{cat}/K_m \) profile generated by MKP3 alone, a critical ionization was not observed (Fig. 1a). Instead, there was a small drop in rate at high pH caused by a noncritical ionization with an apparent \( pK_a \) value of 7.6. This ionization did not decrease the rate to zero, but rather only dropped the rate to a new plateau value. These data were fitted to Equation 3, which describes this profile. Below the pH optimum of ~7, the shape of the two \( k_{cat}/K_m \) profiles was nearly identical. Both \( k_{cat}/K_m \) profiles displayed two ionizations (\( pK_a \) values of 5.1 and ~6.5) that must be unprotonated for activity. The acidic limb of these pH profiles was consistent with the results reported by Wiland et al. for MKP3 (16) and by Denu et al. for the DS-PTP VHR (20). The \( pK_a \) value of 5.1 was assigned to the ionization of the substrate NPP, which reacts with DSPs and PTPs as the dianion (26, 27). The \( pK_a \) of 6.5 represents the \( pK_a \) of the cysteine nucleophile Cys-293 of MKP3 and is consistent with the low \( pK_a \) value observed for the catalytic Cys-124 of VHR (20).

The \( k_{cat} \) pH profiles (Fig. 1b) displayed the same general trend as the \( k_{cat}/K_m \) profiles; however, the magnitude of the activation was less pronounced. With activated MKP3, the \( k_{cat} \)
induced activation results in only ~6-fold catalytic rate enhancement. However, with poorer leaving groups, activated MKP3 catalyzed P-O bond cleavage 2 orders of magnitude more efficiently than unactivated enzyme. These data suggest that ERK functions to enhance the rate of phospho-enzyme intermediate formation and that this may be the direct result of general acid catalysis. To elucidate the individual rate enhancement on each discrete step in catalysis and to provide evidence that ERK induces general acid involvement during the event of P-O bond cleavage, rapid-reaction kinetic analyses were performed.

Rapid-reaction Kinetics—With the DSPs VHR and CDC25, rapid-reaction kinetic experiments have proven extremely useful in resolving the rates for both intermediate formation and hydrolysis (19, 29). When rapid biphasic kinetics were observed, it was demonstrated that the rapid exponential rate (burst phase) corresponds to intermediate formation (k3 in Scheme 1), whereas the slower linear phase corresponds to intermediate hydrolysis (k5). Here, kinetic isolation of these steps in MKP3 catalysis would provide a direct measure of the intrinsic rate enhancement upon each chemical step in catalysis. Therefore, MKP3 and substrate pNPP were mixed rapidly in a stopped-flow spectrophotometer, and the production of pNP was monitored at 410 nm. The resulting kinetic traces displayed biphasic characteristics, although the two phases were not well resolved (Fig. 3a). A slow exponential burst phase was followed by a slower linear steady-state rate. The kinetic traces were fitted to Equation 7 to yield the rate constants for the first-order burst and the steady-state phase. Because the two rates were not well resolved, both phases are a function of both individual rate constants k3 and k5. To obtain a complete complement of individual rate constants, the rapid-reaction rate constants were determined as a function of substrate concentration (Fig. 3b). The linear portion (steady state) of the traces were fitted to Equation 1 to yield the steady-state parameters kcat, kcat/Km, and Km. These values were in excellent agreement with those values obtained from the steady-state analysis (Figs. 1 and 2). The calculated kburst values were fitted to Equation 8 to yield (k3 + k5) and k3 values. When k3 >> k5, then the (k3 + k5) term simplifies to k3, the rate constant for intermediate formation. The results are listed in Table II. From the analysis, k3 + k5 = 0.08 ± 0.01 s⁻¹, and the k3 value for pNPP was 34 ± 10 mm. Using the (k3 + k5) value, a fit to Equation 9 yielded the actual k3 value of 0.07 s⁻¹ (Table II). Equation 9 describes the observed amplitude (B) of the burst phase as a function of [E]0, k3, (k3 + k5), Km, and varied [S].

The rapid kinetics of MKP3 and pNPP were repeated in the presence of ERK protein. In these experiments, a solution of MKP3 and ERK was mixed rapidly with pNPP, and product formation was followed as discussed above. The resulting kinetic traces displayed a rapid exponential burst phase followed by a well resolved slower linear phase (Fig. 3a). To obtain a complete set of kinetic constants, the kinetic traces were determined as a function of pNPP concentration (Fig. 3b). The subsequent fitting analysis yielded a first-order rate constant of 4.46 s⁻¹ and a kcat value of 0.45 s⁻¹. Amazingly, the first-order rate was 57-fold higher, and the linear rate was 14-fold higher when MKP3 was activated by ERK. Because the kbursts and the kcat values were well resolved with activated MKP3, kburst = k3 and the kcat = k5 at saturating levels of substrate (Table II). The calculated kcat values were in good agreement with the kcat values obtained in the steady-state analysis (Table I).

We predicted that if general acid catalysis is employed by the activated phosphatase, then substrates with very low leaving group pKₐ values should display more comparable P-O cleavage (k3) rates between activated and unactivated enzyme. To
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In excellent agreement between the two substrates pNPP and oMFP, the activated \( k_3 \) values of 0.72 s\(^{-1}\) and 0.66 s\(^{-1}\), respectively, are consistent with this substrate-independent step. It should be noted that this slow step \( (k_3) \) in catalysis may not reflect intermediate hydrolysis, but rather may reflect the release of enzyme-bound phosphate. Although we do not have direct evidence, two observations suggest that intermediate hydrolysis is rate-limiting in MKP3. First, the \( K_a \) for phosphate (competitive versus substrate) inhibition of MKP3 ranges from 1.5 to 28 mM. This high value indicates that MKP3 has a low affinity for phosphate and therefore suggests a rapid dissociation of phosphate from the enzyme. Also, in the related enzyme VHR, \( k_3 \) was shown to correspond to the slow hydrolysis of the intermediate (28). Because the rate of intermediate hydrolysis is independent of substrate, we could use the unactivated \( k_3 \) value of 0.13 s\(^{-1}\) from oMFP and the unactivated \( k_{cat} \) value from pNPP to solve for the unactivated \( k_3 \) value for pNPP (using Equation 10).

\[
k_{cat} = k_3^o k_2^o / (k_3^o + k_3)
\]

With pNPP, the unactivated value for \( k_3 \) was calculated to be 0.044 s\(^{-1}\). Compared with the ERK-activated \( k_3 \) value of 4.46 s\(^{-1}\), this represents a rate enhancement of 101-fold.

Utilizing the rapid-reaction approach to obtain the rate of P-O bond cleavage \( (k_3) \) for several different substrates, the \( k_3 \) value was determined as a function of leaving group \( pK_a \) value (Fig. 5). Consistent with the steady-state Brønsted analysis of \( k_{cat}/K_m \) values, the \( k_3 \) value displayed a large dependence (slope = \(-0.58 \pm 0.14\)), whereas the \( k_3 \) value of activated MKP3 displayed no significant effect (slope = \(-0.13 \pm 0.13\)) on leaving group \( pK_a \) value. Because of the lack of significant absorbance at pH 7, substrates with leaving group \( pK_a \) values above 7.8 could not be analyzed by this stopped-flow approach.

Collectively, the results are fully consistent with activated MKP3 utilizing general acid catalysis during P-O bond cleavage. In several PTP x-ray structures, it has been observed that the general acid loop \( (\text{i.e. the loop structure harboring the general acid}) \) can occupy two distinct conformations. Ligand binding is thought to induce the "closed" or active conformation, bringing the conserved aspartic acid into position to act as the general acid. In the "open" conformation (inactive), the loop is flipped away from the active-site cleft, and the aspartic acid cannot participate in the reaction. Interestingly, the x-ray structure of the catalytic domain of Pyst1 (ortholog to MKP3) revealed a markedly open conformation. Presumably, this conformation would render the enzyme incapable of general acid catalysis. Logically, we proposed that ERK induces (or stabilizes) general acid loop closure, thereby permitting efficient general acid catalysis. To test this hypothesis further, we attempted to mimic artificially the effect of ERK activation by stabilizing the closed conformation. This was accomplished by the addition of the organic solvent dimethyl sulfoxide (Me\(_2\)SO). Reagents such as Me\(_2\)SO have been referred to as "chemical chaperones" because of their ability to stabilize certain protein conformational states.

Substrate saturation curves were generated in increasing amounts of Me\(_2\)SO (Fig. 6). Me\(_2\)SO had a dramatic stimulatory effect on the kinetics of pNPP hydrolysis, with a maximal effect occurring by 25% Me\(_2\)SO. The \( k_{cat}/K_m \) and \( k_3 \) values were 42-fold and 2.2-fold higher, respectively, than those values obtained in the absence of Me\(_2\)SO. This level of activation was very similar to that observed with ERK. To examine whether the same mechanism of activation was utilized by both ERK and Me\(_2\)SO, ERK was added to samples containing either par-
| Table II | Kinetic constants for the activation of MKP3 by ERK, using pNPP as substrate, pH 7 and 25 °C |
|----------|--------------------------------------------------------------------------------------------------|
| $k_3$ | $k_5$ | $(k_3 + k_5)$ | $K_m$ | $K_d$ |
| s$^{-1}$ | s$^{-1}$ | s$^{-1}$ | mM | mM |
| pNPP | 0.044 ± 0.005 | 0.13 ± 0.01 | 0.078 ± 0.011 | 10.4 ± 1.9 | 34 ± 10 |
| pNPP + ERK | 4.46 ± 0.36 | 0.72 ± 0.05 | 7.0 ± 1.9 | 12.7 ± 3.0 |

To provide additional evidence for this proposal, the ability of MKP3 to bind the competitive inhibitor phosphate was assessed. In the closed conformation observed in the VHR crystal structure, the general acid loop makes two key interactions that allow efficient oxyanion binding in the active-site cleft. First, the general acid Asp-92 hydrogen bonds to one of the oxygens of ligand. Second, the backbone carbonyl of Ala-90 hydrogen bonds to the catalytic Arg-130, positioning this critical arginine toward direct electrostatic interactions with two of oxygens of phosphate. Therefore, we predicted that induced loop closure by ERK or Me$_2$SO would optimize the active site for binding oxyanions, such as the competitive inhibitor phosphate. The inhibition constant $K_i$ of phosphate was determined for MKP3 alone, ERK-activated MKP3, and Me$_2$SO-activated MKP3. With MKP3 alone, the $K_i$ value was 28 ± 2 mM. The $K_i$ values with ERK-activated MKP3 and Me$_2$SO-activated MKP3 were 1.5 ± 0.1 mM and 4.8 ± 0.7 mM, respectively. Both ERK and Me$_2$SO significantly increased the affinity for ligand binding to MKP3. Consistent with these findings, both $K_m$ and $K_d$ values for substrates were significantly lower in ERK-activated MKP3 (Tables II and III).

**DISCUSSION**

When the enzymatic activity of MKP3 was first characterized (16), it was quite surprising that the rates of catalysis were almost 3 orders of magnitude lower than the related DSP VHR. It was also curious that MKP3 lacked the critical ionization for a group that must be protonated for activity (Ref. 16 and this paper). The pH profiles of unactivated and activated MKP3 bore a remarkable resemblance to the pH profiles of wild type VHR and the general acid mutant VHR D92N (20), respectively, both in the magnitude of the general acid rate enhancement and the loss of this ionization in the pH profiles. The D92N mutant of VHR was ~100-fold less active than native enzyme and exhibited the loss of the basic limb in the pH profiles. We had suggested that MKP3 was not employing general acid catalysis by the proposed general acid Asp-262 (16). We reasoned that Asp-262 was not positioned properly to aid in catalysis; however, why this was the case was not clear. In 1998, Camps et al. (14) discovered that MKP3 could be activated catalytically by binding to ERK, MKP3's proposed authentic substrate. The noncatalytic amino-terminal domain was believed to play a role in this activation because this domain could bind ERK independently and was necessary to induce the dramatic activation of MKP3. The recent x-ray structure of the catalytic domain of Pyst1 (MKP3) revealed an overall structure (22) that was very similar to VHR (15). However, the proposed general acid loop was flipped 20 Å away from the active-site cleft. In this conformation, the proposed general acid Asp-262 would not be expected to contribute to catalysis. Consistent with this idea, mutation of Asp to Asn (D262N) resulted in an enzyme that displayed activity similar to that of the wild type enzyme (22). Moreover, the D262N mutant could no longer be activated by ERK protein. Collectively, these observations suggested that ERK binding to MKP3 activate MKP3 through the same mechanism of inducing the general acid loop closure.

![Rapid kinetics between activated and unactivated MKP3 reacting with oMFP](image)

**Fig. 4.** Rapid kinetics between activated and unactivated MKP3 reacting with oMFP. Panel a, representative reaction traces of activated (top trace) and unactivated (bottom trace) MKP3 reacting with oMFP. Each trace was fitted to Equation 7. Panel b, concentration dependence of $k_{	ext{burst}}$ for activated (circles) and unactivated (diamonds) MKP3. Resulting saturation curves were fitted using Equation 8. The theoretical fits are displayed as the solid line curves, and the results are summarized in Table III. Conditions: pH 7, 25 °C, [ERK] = 18 μM, and either [unactivated MKP3] = 3.6 μM or [activated MKP3] = 2.6 μM.

Taken together, these data strongly suggest that Me$_2$SO and

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* C. C. Fjeld, A. E. Rice, Y. Kim, K. R. Gee, and J. M. Denu, unpublished data.
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### Results in parentheses are those resulting from a fit to Equation 9.

| Substrate | $k_{cat}$ | $K_m$ | $K_{act}$ | $k_3$ | $k_4$ | $K_d$ |
|-----------|-----------|-------|-----------|-------|-------|-------|
| 0.152 ± 0.010 | 968 ± 45 | 0.157 ± 0.025 | 1.14 ± 0.13 | 0.13 ± 0.01 | 0.580 ± 0.121 |
| 0.52 ± 0.04 | 7430 ± 670 | 0.070 ± 0.023 | 2.51 ± 0.36 | 0.66 ± 0.11 | 0.216 ± 0.083 |

**Fig. 5.** Effect of leaving group pK$_a$ value on the rate of intermediate formation ($k_3$) for activated and unactivated MKP3. The $k_3$ value was determined as a function of leaving group pK$_a$. *Open diamonds* represent activated MKP3; *closed circles* represent unactivated MKP3. For each substrate, the exponential burst rate was determined at an increasing concentration of substrate, and the data were fitted to Equation 1 to obtain the maximum $k_3$ value. Altarnatively, the $k_3$ value was obtained from a fit to Equation 7 at saturating concentrations of substrate. The drawn lines are best fits of linear least squares regression. Conditions: pH 7 and 25°C.

**Fig. 6.** Me$_2$SO mimics the ERK-induced activation of MKP3. Substrate ([pNPP]) saturation curves were generated in increasing amounts of Me$_2$SO. *Filled circles*, 0% Me$_2$SO; *filled squares*, 5% Me$_2$SO; *open circles*, 10% Me$_2$SO; *filled triangles*, 15% Me$_2$SO; *open triangles*, 20% Me$_2$SO; *filled diamonds*, 15% Me$_2$SO + 120 mM ERK; *open triangles*, 30% Me$_2$SO + 120 mM ERK. *Framed curves* are the best fit to Equation 1. Conditions: 63 nM MKP3, pH 7, and 25°C.

MKP3 may induce general acid loop closure and the subsequent involvement of Asp-262 acting as general acid catalyst. The rationale for the present study was to pinpoint which steps in catalysis undergo activation and to provide evidence for direct general acid catalysis by wild type activated MKP3.

We have not only provided direct evidence that ERK induces efficient general acid catalysis during phosphoenzyme intermediate formation, but we have identified and quantified the activation on all major catalytic steps in the reaction. The largest catalytic activation occurs on intermediate formation ($k_3$, Scheme 1), where ERK induces 2 orders of magnitude rate enhancement. ERK also induces 5–6-fold activation on intermediate hydrolysis ($k_5$, Scheme 1). The apparent $K_m$ values are 2–3-fold lower in the presence of ERK. Consistent with higher affinity for substrate, the oxyanion phosphate binds 19-fold tighter to ERK-activated MKP3 than to MKP3.

The pH dependence of activated MKP3 clearly established the involvement of general acid catalysis (Fig. 1). In contrast, unactivated MKP3 did not display this ionization as critical for the low activity form of the enzyme. Instead, only slight decreases in rates were observed when Asp-262 is deprotonated. These results can be explained by a model in which ERK binding induces the movement of the general acid loop into its catalytically competent position. In the active conformation, the Asp must be protonated to act as a general acid. In its ionized form, Asp-262 is incapable of stabilizing the developing negative charge on the leaving group; and, if the negative charge on unprotonated Asp-262 is positioned at the active site, this will generate charge repulsion between the diion of the binding substrate. Consistent with the conclusion that ERK binding correctly positions the general acid loop and Asp-262, there is a greater increase in $K_m$ values (with increasing pH above the pK$_a$ for Asp-262) for activated versus unactivated MKP3 (Fig. 1). In the unactivated enzyme, the loop would be predominantly flipped out of the active site, resulting in only a small relative change in activity and binding when Asp-262 becomes ionized. However, because there is a slight change in activity caused by this ionization, Asp may be partially or inefficiently contributing to binding/catalysis in the unactivated enzyme.

With excellent leaving groups such as 3-0-methylfluorescein and 6,8-difluoro-4-methylumbelliferone, the rate of intermediate formation is similar between activated and unactivated MKP3 (Fig. 2). However, as the pK$_a$ of the leaving group increases, there is a large dependence on leaving group pK$_a$ for unactivated enzyme compared with activated enzyme (Fig. 2a). This result was observed in both the pre-steady-state (Fig. 5) and steady-state (Fig. 2) analyses and strongly suggests that unactivated MKP3 lacks efficient general acid catalysis. In contrast, ERK-activated enzyme is able to utilize general acid catalysis, transferring a proton to the leaving group oxygen such that no significant negative charge is built up during the transition state of P-O bond cleavage. Curiously though, in k$_{cat}$/K$_m$ activation by ERK reaches a plateau value of ~70-fold with increasing leaving group pK$_a$ value (Fig. 2b). Unfortunately, the pre-steady-state analysis could not be extended to substrates whose leaving groups were greater than 7.8 (Fig. 5). The plateau in k$_{cat}$/K$_m$ activation would not necessarily be predicted if the unactivated enzyme were completely devoid of any general acid catalysis. One might expect that the magnitude of activation would become increasing larger as the pK$_a$ value increases. These observations suggest that the rate of loop closure in unactivated MKP3 may contribute to the observed rates of intermediate formation. The data can be explained in the following manner. With good leaving groups, unactivated MKP3...


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structure revealed a severely open general acid loop conformation, and the corresponding Arg-299 is positioned incorrectly to bind the oxyanion of substrate efficiently (22). Although there is no structure available for the closed conformation of Pst1, we would predict that the carboxyl of Ile-262 will interact with and position Arg-299, creating an efficient pocket for phosphate binding. Consistent with the idea of the general acid loop positioning Arg-299, activated MKP3 displays higher affinities for oxyanions such as phosphate as well as for substrates.

We have demonstrated previously that the conserved Asp of VHR and PTP1 plays a role during intermediate hydrolysis (28). The Asp may function as a general base during intermediate hydrolysis, activating a water molecule to facilitate nucleophilic attack. Consistent with this mechanism and the involvement of Asp-262, intermediate hydrolysis is 5–6-fold faster for ERK-activated MKP3 (Tables II and III). Because the rate enhancement is significantly smaller than that observed for intermediate formation, this may indicate that the general acid loop is better able to adopt a closed conformation in the phosphoenzyme intermediate.

We provide detailed biochemical evidence that ERK activates MKP3 through the stabilization of the active phosphate conformation, where the general acid loop efficiently participates in general acid/base catalysis, substrate binding, and transition-state stabilization. ERK binding acts as a switch in converting the low activity form to the activated form of MKP3.

Regulation of PTPs through the utilization of this flexible general acid loop may be a general mechanism. Two inhibitory mechanisms have been proposed from observations of the crystal structures of SHP-2 (an SH2-domain PTP) (37) and RPTPα (a receptor-like PTP) (38). With SHP-2, an intramolecular interaction with its SH2 domain sterically blocks and inhibits the active site in the unliganded form. For RPTPα, an inhibitory dimer is thought to form upon binding extracellular ligand. The structure revealed that the amino-terminal helix-turn-helix of one monomer is wedged into the active site of the dyad-related monomer. In both cases, these interactions lock the PTP domain into the open conformation by preventing closure of the general acid loop.

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