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Expression, purification, and enzymatic characterization of the dual specificity mitogen-activated protein kinase phosphatase, MKP-4

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Abstract

Mitogen-activated protein kinase phosphatase-4 (MKP-4) is a dual specificity phosphatase, which acts as a negative regulator of insulin-stimulated pathways [1]. Here, we describe expression, purification, and biochemical characterization of MKP-4. We used the Baculovirus

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expression system and purification with a combination of affinity and gel filtration chromatography to generate pure MKP-4 and MKP-4/p38 complex. Both MKP-4 and the MKP-4/p38 complex exhibited moderate activity toward the surrogate substrates *p*-nitrophenyl phosphate, 6, 8-difluoro-4-methylumbelliferyl phosphate, and 3-*O*-methylfluorescein phosphate. The phosphatase activity could be inhibited by peroxovanate, a potent inhibitor of protein tyrosine phosphatases. We further determined kinetic parameters for the MKP-4 and the MKP-4/p38 by using spectrophotometric and fluorescence intensity methods. The MKP-4/p38 complex was found to provide substantially higher phosphatase activity than MKP-4 alone, similar to what has been shown for MKP-3. Our data allow the configuration of screens for modulators of MKP-4 activity.

Keywords: Dual specificity phosphatase; Protein tyrosine phosphatase superfamily; Type 2 diabetes; MAP kinase; Insulin signaling; Diabetes mellitus

1. Introduction

Reversible protein phosphorylation is a critical means of modulating signal transduction; dysregulation of this pathway has been implicated in several disease states. Prominent and heavily studied examples of phosphorylation state dysregulation can be found throughout the insulin signaling pathway [2–4]. This dysfunction has important ramifications for glucose homeostasis and thus is an important target for amelioration of type 2 diabetes mellitus. Mitogen-activated protein kinase phosphatase-4 (MKP-4),[4] has been described as a negative regulator of insulin-stimulated pathways [5,6]. This is the first dual specificity phosphatase to be associated with insulin resistance, while a distant homolog, PTP1B, is a well-known tyrosine phosphatase that has been a target of drug development efforts for several years [7–9]. The dual specificity phosphatases which dephosphorylate both phosphotyrosine and phosphothreonine residues of the MAP kinases are called the MAP kinase phosphatases (MKPs). To date, at least ten mammalian genes encoding MKPs have been identified and characterized [10,11, references therein]. MKP-4 has been demonstrated in vitro to signal through dephosphorylation of ERK and p38 [5,12]. Tissue distribution analysis showed that human MKP-4 is expressed in kidney, placenta and adipose tissue [6]. The level of MKP-4 is elevated in insulin-responsive tissues in obese/diabetic states, suggesting that inhibition of the MKP-4 may improve insulin sensitivity. In support of this hypothesis, heterologous expression of MKP-4 blocks both insulin action in hepatoma cells (using PEPCK promoter as a readout) and insulin-stimulated glucose uptake in cultured adipocytes [6].

We describe herein the expression and purification of the full length human MKP-4 either alone or together with its substrate p38. Interestingly, the co-expressed MKP-4/p38 complex was found to provide substantially higher phosphatase activity

[4] *Abbreviations used:* MKP, mitogen-activated protein kinase phosphatase; DiFMUP, 6,8-difluoro-4-methylumbelliferyl phosphate; DiFMU, 6,8-difluoro-4-methylumbelliferone; *p*NPP, *p*-nitrophenyl phosphate; OMFP, 3-*O*-methylfluorescein phosphate; OMF, 3-*O*-methylfluorescein; bpV(Hopic), bisperoxo (5-hydroxypyridine-2-carboxyl) oxovanadate (v); MOI, multiplicity of infection.

than MKP-4 expressed as a single entity, and this increase in k_{cat} could not be duplicated through simple titration of the purified individual monomers of MKP-4 and p38. These results may suggest a potential stabilization of MKP-4 secondary or tertiary structure by p38 during the purification process. Alternatively, the co-expression may generate a quaternary complex not attainable through simple titration.

2. Materials and methods

2.1. Materials

6, 8-Difluoro-4-methylumbelliferyl phosphate (DiFMUP) and 6, 8-difluoro-4-methylumbelliferone (DiFMU) were purchased from Molecular Probes (Eugene, OR). *p*-Nitrophenyl phosphate (*p*NPP), 3-*O*-methylfluorescein phosphate (OMFP) and 3-*O*-methylfluorescein (OMF) were purchased from Sigma (St. Louis, MO). Dipotassium bisperoxo(5-hydroxypyridine-2-carboxyl)oxovanadate (V) (bpV(HOpic)) was obtained from CalBiochem (San Diego, CA). All other reagents are purchased from Sigma unless noted otherwise. Corning Costar Brand 384-well black assay plates and Becton Dickinson Falcon Microtest 384-well black polystyrene assay plates were used for continuous spectrophotometric assays and continuous fluorescence assays, respectively.

2.2. Recombinant Baculoviruses encoding MKP-4 and p38 for expression in Sf9 cells

For Baculovirus expression, full length sequence encoding the human MKP-4 [6] or p38α (Accession No. Q16539) was cloned into pFastBac from Invitrogen (Carlsbad, CA) as N-terminal-$(His)_6$ fusions. The p38α clone was generated in pAcSG2 with the BaculoGold System (BD Biosciences Pharmingen, San Diego, CA) [13]. Constructs were confirmed by sequencing. Recombinant Baculoviruses were generated using the Bac-to-Bac Baculovirus expression system (Invitrogen). Briefly, the recombinant pFastBac vector was transformed into DH10Bac and plated onto Luria Agar plates containing 50 μg/mL Kanamycin, 7 μg/mL gentamicin, 10 μg/mL tetracycline, 100 μg/mL Bluo-gal, and 40 μg/mL IPTG. White colonies were picked for inoculation and isolation of recombinant Bacmid. To generate Baculovirus, the recombinant Bacmid was used for transfecting *Sf*9 cells using CellFECTINE (Invitrogen). At fourth day of post transfection, recombinant Baculovirus was harvested, and the cells collected. Cell extract was prepared in lysis buffer containing 20 mM Tris–HCl, pH 8.0, 137 mM NaCl, 10% (v/v) glycerol, 8 mM EDTA, and 1% (v/v) Triton X-100. Total cell extract and soluble fraction were analyzed by western blot, using His-probe (H-15) antibody from Santa Cruz Biotechnology (Santa Cruz, CA).

2.3. Baculovirus expression

For large scale MKP-4 protein production, *Sf*9 cells were infected with the full length N-terminal $(His)_6$-tagged MKP-4 baculoviruses at a multiplicity of infection

(MOI) of 5 with cell density at 2.0×10^6 cells/mL in shake flasks and harvested at 72 h post infection. To prepare activated MKP-4, *Sf*9 cells were co-infected with Baculoviruses expressing N-terminal $(His)_6$-tagged MKP-4 and N-terminal $(His)_6$-tagged p38 at the MOI of 5 and 0.2, respectively.

2.4. Protein purification

The cell pellets from a 10 L cell fermentation were brought to a concentration of 1 g/7.5 mL of buffer A [20 mM Tris–HCl, pH 7.5, 200 mM NaCl, 5 mM BME, 0.1% (v/v) Tween-20, 10% (v/v) glycerol, and Complete-EDTA free Protease Inhibitor Cocktail (1 tablet per 50 mL of buffer, Roche Diagnostics, Indianapolis, IN)], and the suspension was run through the microfluidizer twice. The disrupted cells were spun at 17,000*g* for 40 min at 4 °C. The supernatant was then bound to the Ni^{2+}-NTA-agarose resin from Qiagen (Valencia, CA) that was previously equilibrated in buffer A at 4 °C. The protein was eluted with 20–250 mM imidazole in buffer A. The protein was further purified by fast protein liquid chromatography Superdex S-200 column with a buffer in 20 mM Tris–HCl, pH 7.5, 200 mM NaCl, 5 mM BME, 0.1% (v/v) Tween-20, and 10% (v/v) glycerol. Fractions containing either MKP-4 or MKP-4/p38 were pooled and concentrated by Centriprep 10 from Millipore (Billerica, MA). The purity of each protein was over 95%, as judged by SDS–PAGE (Fig. 1). The full length His-tagged p38 was also expressed in *Sf*9 cells and similarly purified through an Ni-column and a size exclusion column (Superdex S-200), yielding over 95% pure enzyme. N-terminal sequencing and mass spectroscopy confirmed the identities of purified MKP-4 and p38. After the final step, the yield of MKP-4/p38, MKP-4 alone, and p38 were approximately 100 mg, 7 mg, and 42 mg, respectively. The protein concentration was determined by the Pierce BCA Protein Plus assay using bovine serum albumin as a standard. The purified protein was flash frozen with liquid nitrogen and stored at −80 °C.

2.5. Phosphatase assays

The phosphatase activity of MKP-4 was determined using either *p*NPP, DiFMUP, or OMFP as substrates at 25 °C in 50 mM Hepes (pH 7.5), 5 mM DTT, 150 mM NaCl, 1 mM EDTA, and 0.1 mg/mL BSA in a total volume of 100 μL. For the substrate *p*NPP, continuous assays were carried out at 25 °C in a temperature-controlled Molecular Devices SpectraMax Plus^{384} by monitoring the production of *p*-nitrophenol at 400 nm. The initial rates were corrected for non-enzymatic degradation of the *p*NPP by measuring the control without addition of enzyme. For the substrates DiFMUP and OMFP, continuous assays were carried out for 30 min at 25 °C in a BMG POLARstar Galaxy microplate fluorometer by monitoring the release of the fluorogenic product DiFMU from DiFMUP or OMF from OMFP. DiFMU has excitation/emission maxima at 358/455 nm while OMF has excitation/emission maxima at 490/514 nm. DiFMUP substrate stock solution was prepared by dissolving 5.0 mg DiFMUP into 5.0 mL of 50 mM Tris–HCl, pH 8.0/0.1% BSA buffer. OMFP stock solution was prepared according to the published procedure [14]. DiFMUP and OMFP

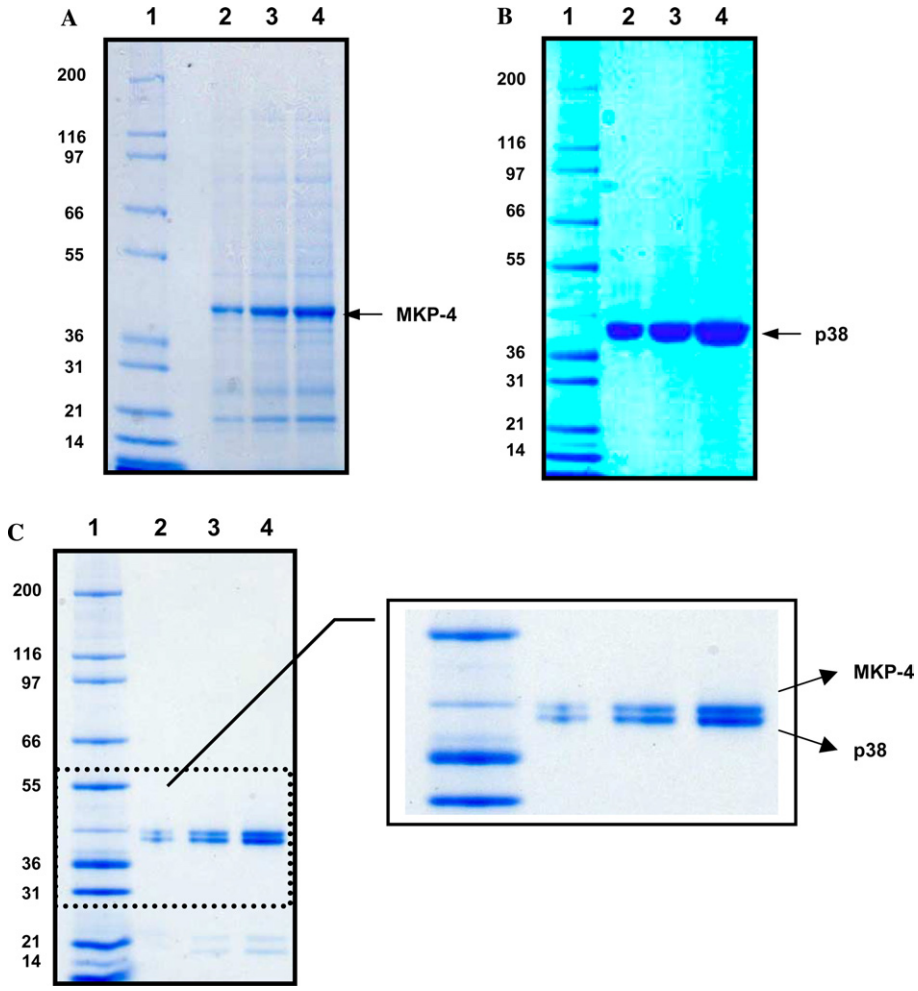

Fig. 1. SDS–PAGE analysis of purified human MKP-4 and MKP-4/p38. (A) MKP-4: lane 1, mark 12 molecular weight standards from Invitrogen (Carlsbad, CA); lane 2, 0.54 μg; lane 3, 1.01 μg; lane 4, 2.02 μg. (B) p38: lane 1, mark 12; lane 2, 9.85 μg; lane 3, 19.7 μg; lane 4, 39.4 μg. (C) MKP-4/p38: lane 1, mark 12; lane 2, 0.52 μg; lane 3, 1.04 μg; lane 4, 2.08 μg.

were stored in their solid forms and stock solution made up just prior to use. For DiFMUP, the reaction was initiated by the addition of the enzyme to a reaction mixture containing various concentrations of substrates in 50 mM Hepes (pH 7.5), 5 mM DTT, 150 mM NaCl, 1 mM EDTA, and 0.1 mg/mL BSA in a total volume of 100 μL. A control in which the DiFMUP was incubated for 2 h at 25 °C in the assay solution without added MKP-4 showed no degradation of the compound, indicating that the compound is very stable during long-term incubation. OMFP reaction was monitored in similar fashion as described above for the DiFMUP assay. Standard curves

Table 1
Kinetic parameters[a] of MKP-4 with *p*NPP, DiFMUP, and OMFP as substrates

Parameters	pNPP	DiFMUP	OMFP
k_{cat} (min^{-1})			
without p38	0.30	0.21	0.27
with p38	1.68	1.68	3.9
k_m (mM)			
without p38	8.1	0.040	0.18
with p38	4.7	0.030	0.15
k_{cat}/k_m (M^{-1} s^{-1})			
without p38	0.62	87.5	25
with p38	6.0	944	433

[a] The values represent the mean of at least three experiments; the standard error is $\leqslant 10\%$ in all cases.

correlating the total fluorescence at pH 7.5 as a function of DiFMU or OMF concentration were first obtained. Then, the standard curve was used to convert the initial rates to specific activity. In general, MKP-4 reactions containing DiFMUP (40 μM) in 50 mM Hepes (pH 7.5), 5 mM DTT, 150 mM NaCl, and 0.1 mg/mL BSA in a total volume of 95 μL were prepared in triplicate and incubated for 10 min. Then, the enzyme was added to initiate the reaction, and the fluorescence was measured over the course of the reaction. In order to see whether p38 had intrinsic endogenous phosphatase activity, p38 purified in house was tested using DiFMUP as a substrate. The p38 expressed in *Sf*9 showed less than 1% phosphatase activity compared to that of MKP4/p38 complex.

The kinetic constants for *p*NPP, DiFMUP, and OMFP were obtained by fitting the data to Eq. (1) using Graph Pad Software Inc.'s Prism (San Diego, CA), where v is the initial velocity, V_{max} is the maximal velocity, S is the substrate concentration, and K_m is the Michaelis constant.

$$v = V_{max}S/(K_m + S). \tag{1}$$

The kinetic parameters are listed in Table 1.

2.6. pH-rate analysis

For the pH–rate profiles, the following buffers were used at a concentration of 50 mM: acetate (pH 5.5), 3,3-dimethylglutarate (pH 6.0–6.5), Tris–HCl (7.0–7.5), Bis–Tris (8.0–9.5), and Borate (pH 10.0–11.0). Each buffer contained constant ionic strength of 150 mM NaCl, 5 mM DTT, and 1 mM EDTA over the pH range investigated. The k_{cat} value at each pH was approximated using a saturating amount of DiFMUP in each assay. The apparent pK values for activated MKP-4 were calculated using Eq. (2)

$$v = C/[(1 + H/K_a) * (1 + K_b/H)], \tag{2}$$

where C is the pH-independent constant, H is the proton concentration, and K_a and K_b are the ionization constants of the group involved in catalysis.

2.7. Determination of the IC_{50} value of peroxovanadate

Peroxyvanadium was tested as an inhibitor of MKP-4/p38. The IC_{50} value (the concentration of compound that inhibits 50% of enzyme activity) of bpV(HOpic) was determined using 12 variable concentrations of the inhibitor from 5.6 nM to 1 mM at 30 μM of DiFMUP (the K_m value in the presence of p38).

3. Results and discussion

3.1. Cloning, overexpression, and purification

The insect cell–Baculovirus expression system was used for overexpression of MKP-4/p38, MKP-4, or p38 proteins. The recombinant N-terminal $(His)_6$-MKP-4 and recombinant *N*-terminal $(His)_6$-p38α were each constructed into Baculoviruses by transforming the recombinant pFastBac vectors into DH10Bac. Over-expression of proteins was achieved through infection of *Sf*9 cells using the recombinant Baculoviruses.

MKP-4/p38, MKP-4, or p38 proteins were purified by successive chromatography on Ni^{2+}-NTA agarose and Superdex S-200. For purification of MKP-4 or p38 proteins, only one major species was visually detected by a Coomassie Blue-stained gel (Figs. 1A and B). Following purification of MKP-4/p38, two proteins were detected in the Coomassie Blue-stained gel (Fig. 1C). The human $(His)_6$-MKP-4 had a molecular weight of 42 kDa, whereas human p38α migrated at a molecular mass of 38 kDa as predicted. As assessed by SDS–PAGE analysis and Superdex S-200 elution profile, MKP-4 was co-eluted with p38 with an approximate 1:1 stoichiometry of MKP-4 to p38. It has previously been observed that MKP-4 binds p38, resulting in activation of MKP-4 [12].

3.2. Analysis of MKP-4 catalytic activity

In order to determine K_m and k_{cat} values of MKP-4 with each substrate, initial velocity measurements were performed over a wide range of substrate concentrations. Fig. 2 shows the dependence of the initial velocity of MKP-4 on DiFMUP substrate concentration. The K_m and k_{cat} values of MKP-4 with or without p38 using *p*NPP, DiFMUP, and OMFP as substrates are listed in Table 1. Values for K_m showed less than two-fold differences when p38 was present, regardless of the substrate, whereas increases in k_{cat} of 6–14-fold were observed. The effect of this was that the specificity constant, k_{cat}/K_m, was 10-fold higher in the presence of p38 than without when *p*NPP or DiFMUP was the substrate, and 17-fold higher when OMFP was the substrate. Based on k_{cat}/K_m values, MKP-4 hydrolyzes DiFMUP approximately 150-fold better than *p*NPP and two to four-fold better than OMFP. It is noteworthy to mention that the higher k_{cat} observed with the co-expressed MKP-4/p38 complex was not duplicated through simple in vitro combination of the purified individual MKP-4 and p38 proteins. For example, in the absence of p38, a k_{cat} of 0.30

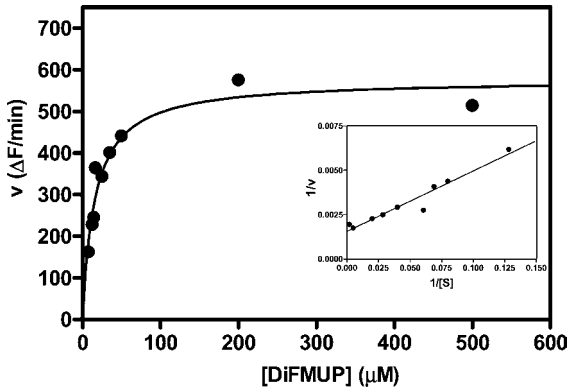

Fig. 2. Dependence of the initial velocity on DiFMUP substrate concentration in the MKP-4 catalyzed dephosphorylation. The experiments were performed in the presence of 25 nM MKP-4 in the presence of p38, and various concentrations of DiFMUP in 50 mM Hepes, pH 7.5, 150 mM NaCl, 1 mM EDTA, 5 mM DTT, and 0.1 mg/mL BSA. Inset represents Lineweaver–Burk plot.

min^{-1} was observed for the MKP-4 catalyzed *p*NPP hydrolysis. Upon addition of p38 to the MKP-4 in vitro, the k_{cat} value for the *p*NPP reaction was 0.38 min^{-1} for the MKP-4 catalyzed hydrolysis. However, for the co-expressed MKP-4/p38 complex, the k_{cat} value for *p*NPP hydrolysis was 1.7 min^{-1}, which is six-fold higher than that for the p38-induced MKP-4 activation in vitro. One plausible explanation is that when co-expressed, p38 and MKP-4 forms a complex that cannot be formed in vitro for some reason. The small substrates can then bind to the complex, which is in an active conformation, leading to an increased k_{cat}. We surmise that p38 mainly functions as a stabilizing molecule that prevents MKP-4 from irreversible unfolding. It has been shown that ERK activates MKP-3 through the stabilization of the active closed conformation that facilitates general acid/base catalysis and substrate binding [15]. Therefore, it is yet to be demonstrated whether the active site conformation of MKP-4 with p38 is different from that when ERK is bound to MKP-4. Zhou and Zhang reported that the bisphosphorylated Erk2/pTpY is a highly specific substrate for MKP-3 due to high affinity binding interactions between the N-terminal domain of MKP-3 and Erk2 [16]. When the reported data are taken altogether, the k_{cat}/K_m value for the hydrolysis of physiological substrate Erk2/pTpY is 10^4–10^5-fold higher than that for the MKP-3-catalyzed hydrolysis of small phosphate molecules, *p*NPP, and OMFP [15–18]. Since nonspecific substrate molecules (e.g., *p*NPP, DiFMUP, and OMFP) are insufficient to induce full activation of both MKP-3 and MKP-4, binding of the native substrate MAP kinase (either Erk2 or p38 kinase) is needed to induce conformational reorientation in the active site to achieve full activity. It was noted that the small molecule substrates exhibited decrease in K_m values significantly as the size of the substrate increases, but displayed approximately the same values of k_{cat}. It is likely that the active site of MKP-4 is better able to accept the large and the bulkier substrate (DiFMUP and OMFP) than simple aryl phosphate such as *p*NPP. The increase in k_{cat}/K_m values for the larger substrates might be

explained by an increase in the rate constant for substrate binding and the chemical step of the phosphoenzyme intermediate formation. The similar results have also been observed previously for MKP-3 and Cdc25 [14–16]. Since the catalytic activity of MKP-4 is substantially different when co-expressed with p38, the inhibitory activity of small molecules may also be different. Thus, the complex may be the more appropriate in vitro target for screening.

3.3. pH–Rate analysis and inhibition of MKP-4

The k_{cat} parameter is the unimolecular constant that describes the rate of an enzyme-catalyzed chemical transformation of substrates to products. Therefore, the k_{cat}–pH profile yields apparent pK_a values of the enzyme–substrate complex. Our pH studies of activated MKP-4 show an ionization with a pK_a value of 6.1 that must be unprotonated for activity, and an ionization with a pK_a value of 9.3, which must be protonated for activity (Fig. 3). It has previously been shown that other dual specificity phosphatases also displayed bell-shaped pH–rate profiles, consistent with the presence of the group that must be unprotonated and one group that must be protonated in the enzyme. The k_{cat}–pH profile of activated MKP-3 has displayed the ionization with pK_a values of 5.8 and 8.1, respectively, whereas the k_{cat}–pH profile of VHR has displayed two critical ionizations with pK_a values of 5.26 and 7.17, respectively [15,16,19]. All the MKPs contain the highly conserved consensus sequence **D**X_{26}(V/L)X(V/I)H**C**X_5**R**(S/T) in the catalytic domain (the boldcase residues in the signature motif). On the basis of sequence analyses of dual specificity protein phosphatases and the MKP-3 model, it has been proposed that the amino acids responsible for MKP-4 catalysis are Cys-290, Arg-296, and Asp-259 [5,18]. In this model, it is speculated that Arg-296 forms hydrogen bonds to a phosphate group of the substrate and stabilize the transition state and Asp-259 serves as the catalytic acid to protonate the leaving group while Cys-290 functions as a nucleophile to form a thiol-phosphate intermediate in the MKP-4 reaction. However, the role of these residues in the MKP-4 reaction mechanism should be addressed by mutational studies.

To establish a standard for quality control, inhibition by peroxovanadate bpV(HOpic), a potent inhibitor of protein tyrosine phosphatases, was evaluated.

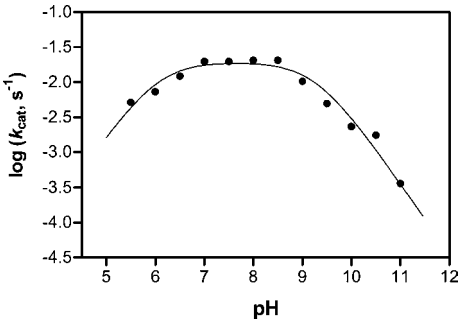

Fig. 3. Effect of pH on k_{cat} parameter for activated MKP-4. The pH data for k_{cat} were fitted using the equation $v = C/[(1 + H/K_a) * (1 + K_b/H)]$, yielding p$K_a$ values of 6.05 ± 0.20 and 9.27 ± 0.83.

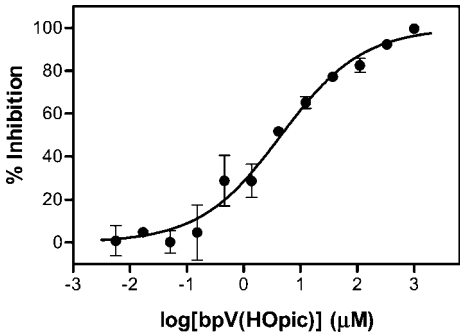

Fig. 4. Determination of IC_{50} value of bpV(HOpic). Peroxovanadate demonstrated dose-dependent inhibition of MKP-4 in the presence of p38 with an IC_{50} of 4.6 μM.

Vanadate tends to adopt pentavalent geometry that mimics the transition state for the PTPase-catalyzed reaction. It has been reported that orthovanadate inhibits MKP-3 with an IC_{50} value of 0.049 μM in the presence of Erk2 when *p*NPP was used as a substrate [16]. Inhibition of MKP-4 in the presence of p38 by bpV(HOpic) has an IC_{50} value of 4.6 μM, $n = 3$, when DiFMUP was used as a substrate (Fig. 4). It has been proposed that Erk2 induces general acid loop closure of MKP-3, resulting in optimizing the active site for binding oxyanions such as vanadate [16]. Our result suggests that the level of p38 activation for MKP-4 is not as effective as Erk2 activation for MKP-3.

In summary, we present the molecular cloning, purification, and characterization of the dual specificity mitogen-activated protein kinase phosphatase, MKP-4. Our results suggest that MKP-4/p38 complex is the more relevant target to screen for modulators of MKP-4 activity and further establish the feasibility of obtaining potent MKP-4 inhibitors for pharmacological development.

Acknowledgments

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