Purification and Kinetic Characterization of the Mitogen-activated Protein Kinase Phosphatase rVH6*

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(Received for publication, July 3, 1996, and in revised form, August 21, 1996)

The dual specificity protein-tyrosine phosphatase rVH6 belongs to a subfamily of enzymes that have in vivo and in vitro catalytic activity against mitogen-activated protein kinases. A method was developed for the expression and efficient purification of recombinant rVH6 in quantities sufficient for physical and kinetic characterization of the enzyme. Matrix-assisted laser desorption mass spectrometry verified the mass of purified rVH6 to be 43,500 ± 150, and NH2-terminal sequence analysis confirmed the predicted amino acid sequence. Kinetic characterization of full-length rVH6 identified the critical ionizations involved in the $K_{cat}/K_m$ parameter (apparent $pK_a$ values 5.1 and 6.6) and revealed a pH-independent $K_{cat}$ value of 0.014 s⁻¹. In an attempt to define the essential catalytic core of this enzyme, amino acids 134–381 of rVH6 were expressed, purified, and characterized enzymatically. Kinetic analysis revealed that the truncated enzyme exhibited a turnover value similar to that of the full-length enzyme ($K_{cat} = 0.017$ s⁻¹), with p-nitrophenyl phosphate as substrate. Secondary structure prediction and molecular modeling of rVH6 based on the x-ray structure of the dual specificity protein tyrosine phosphatase, VHR, further supported the assignment of residues 134–381 to the core catalytic domain of rVH6. These results demonstrate that the NH2-terminus of rVH6 (residues 1–133) is not required for full enzyme activity and comprises a separate domain that may play a distinct physiological function.

Phosphorylation of specific tyrosine residues in proteins regulates many cellular functions, including cell growth and proliferation, cell cycle progression, and receptor-mediated responses to external stimuli (1). The extent of protein-tyrosine phosphorylation within the cell is regulated by the coordinated activity of protein-tyrosine kinases and phosphatases (PTPases) (reviewed in Ref. 2). The vaccinia virus protein, VH1, was identified as the first member of a growing subclass of PTPases, called dual specificity protein-tyrosine phosphatases (dsPTPases), which possess phosphatase activity directed against both phosphotyrosine (Tyr(P)) and phosphoserine (Ser(P)) containing peptides (3). Additional dsPTPases have been identified based on their sequence similarity to VH1 and their ability to hydrolyze Tyr(P), Ser(P), and Thr(P) phosphate monoesters (reviewed in Ref. 4). DsPTPases contain an extended PTPase active site signature sequence, VXVHCGG-XXRSXXXAY(L/I)M, which includes the catalytic cysteine that is essential for the formation of the phosphoenzyme reaction intermediate (5). Outside of the conserved active site, dsPTPases share little sequence identity with other members of the PTPase family. Recent elucidation of the crystal structure of the human dsPTPase, VHR, has revealed that VHR is composed of structural elements shared by the catalytic domains of other PTPases (6). This finding, along with the well established catalytic mechanism of VHR (7), provides a prototype which aids in the analysis of the biochemical properties of other dsPTPases.

DsPTPases have been shown to play key roles in several cellular signaling processes. The cell cycle regulator p80cdc25 has been shown to dephosphorylate both Tyr(P) and Thr(P) in p34cdc2 of fission yeast, thereby activating the maturation promoting factor complex and allowing the cell to enter into mitosis (reviewed in Ref. 8). Other dsPTPases, including CL100 (MKP-1), PAC-1, HVH2 (MKP-2), B23 (hVH3), hVH5, and rVH6 (MKP-3) (9–14), contain an extended amino- and/or carboxyl-terminal sequence, along with the catalytic domain that is shared by VHR and other PTPases. CL100, B23, and PAC-1 are immediate early gene products, which are induced by a variety of external stimuli including growth factors, oxidative stress, heat shock, and mitogens (4, 9, 10, 12). Several laboratories have identified mitogen-activated protein kinases (MAPK) as substrates for these enzymes (11, 12).

Previously, a novel dsPTPase was isolated and cloned from a rat hippocampus brain library, termed rVH6 (rat VH1-like phosphatase 6) (14). RVH6 was shown to exhibit phosphatase activity in vitro against MAPK, contain an NH2-terminal extension (~133 amino acids), and was the first dsPTPase to be localized exclusively in the cytoplasm. Interestingly, it has proven to be very difficult to express and purify the entire coding sequence of several dsPTPases that contain extensive NH2- or COOH-terminal extensions (i.e. B23/hVH3; Ref. 12), including rVH6. In this study, we outline a method for expression and purification of recombinant full-length rVH6 that may be applicable to other dsPTPases. Availability of the purified protein has enabled us to physically and kinetically characterize the enzyme and define the residues that comprise the catalytic core of rVH6. This paper indicates that there are specific

* This work was supported in part by grants from the Walther Cancer Institute and National Institutes of Health Grant NIDDKD 18024 (to J. E. D.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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1 The abbreviations used are: PTPases, protein-tyrosine phosphatases; dsPTPases, dual specificity protein-tyrosine phosphatase; pNPP, p-nitrophenyl phosphate; gNPP, γ-nitrophenyl phosphate; GST, glutathione S-transferase; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; HPLC, high performance liquid chromatography; MKPs, MAPK phosphatases; CD, catalytic domain.

2 CL100 is also known as MAP kinase phosphatase 1 or MKP-1 (4); HVH2 is also termed MKP-2 (36); B23 is also called hVH3 (37); and rVH6 is also known as MKP-3 (23).
functional roles for individual structural domains of rVH6 that are generally applicable to other MAPK phosphatases.

**MATERIALS AND METHODS**

**Overexpression and Purification of Full-length rVH6—**The coding sequence of rVH6 was isolated as described previously (14). RVH6 coding sequence, modified to encode a carboxyl-terminal polyhistidine tag, was ligated into the NdeI/EcoRI sites of pT7-7 vector. pT7-7 rVH6(His)₆ was used to transform BL21/DE3 (Novagen) Escherichia coli. Colonies selected and grown on agarose slurry for every 5 ml of supernatant. The supernatant was incubated batchwise with the Ni-NTA-agarose at 4°C for 1 h. The cells were harvested by centrifugation at 4000 g for 30 min, and the supernatant decanted into a 50 ml conical tube, to which was added 1 ml of 50% Ni-NTA-agarose (Qiagen) slurry for every 5 ml of supernatant. The supernatant was incubated batchwise with the Ni-NTA-agarose at 4°C for 1 h. Following the incubation, the supernatant/agarose mixture was poured into a 100 ml column and washed with 20 bed volumes of lysis buffer, followed by 20 bed volumes of wash buffer (400 mM NaCl, 50 mM Tris, 10 mM β-ME, 10 mM EDTA, 10 mg/ml ampicillin) over-night with shaking at 37°C. The 50 ml overnight culture was used to inoculate 1 liter of 2 x YT medium supplemented with 100 μg/ml ampicillin over-night with shaking at 37°C. The cultures were grown to an OD₆₀₀ of 0.8-1.0 and induced with 1 mM IPTG for 3 h. The cells were harvested by centrifugation at 4000 g for 30 min, and the supernatant decanted into a 50 ml conical tube, to which was added 1 ml of 50% Ni-NTA-agarose (Qiagen) slurry for every 5 ml of supernatant. The supernatant was incubated batchwise with the Ni-NTA-agarose at 4°C for 1 h. Following the incubation, the supernatant/agarose mixture was poured into a 100 ml column and washed with 20 bed volumes of lysis buffer, followed by 20 bed volumes of wash buffer (400 mM NaCl, 50 mM Tris, 10 mM β-ME, 10% glycerol, final pH 6.0). The enzyme was eluted from the column with a 100 ml 0-0.5 M imidazole gradient in wash buffer. Fractions containing enzymatic activity against p-nitrophenyl phosphate (pNPP) were pooled and concentrated with a Cen-triprep 30 filtration column (Amicon), applied onto a Sephadex G-75 gel filtration column (2.5 x 100 cm), and eluted with 200 ml of buffer containing 250 mM NaCl, 25 mM Tris, pH 8.0, 10 mM β- ME, 1 mM EDTA. Fractions exhibiting phosphatase activity were analyzed by SDS-polyacrylamide gel electrophoresis. Fractions containing a single protein band corresponding to the size of rVH6, as determined by Coomassie staining, were pooled and stored at 4°C.

**Protein Characterization: Mass Spectroscopy, and Amino-terminal Sequencing—**The mass of the pure protein was determined by matrix-assisted laser desorption mass spectroscopy on a VESTEC-2000 instrument. The amino acid sequence of the NH₂ terminus of the enzyme was analyzed by an Applied Biosystems 473A sequenator at the University of Michigan Protein Core Facility.

**Expression and Purification of rVH6 Catalytic Domain—**The catalytic domain of rVH6, comprised of amino acids 134-281, was expressed as a glutathione S-transferase (GST) fusion protein. The 700-base pair insert was generated by polymerase chain reaction using the rVH6 coding sequence (14) with 5 ’ primer 5’-CTTGGGATCCGCGGTCT-TCGTAAGAT-3’ and 3’ primer 5’-CTTGGGATCCACATGGATCTGAGG-3’. The polymerase chain reaction product was ligated into BamHI/EcoRI-digested pGEX-ktText (15) expression vector to produce pGEX-rVH6-CD (Catalytic Domain). pGEX-rVH6-CD was used to transform BL21/DE3 E. coli, and the cells were grown and induced as described for full-length rVH6. The harvested bacterial pellets were resuspended in 17 ml of modified lysis buffer containing 10 mM β- ME, 0.8 M phenylmethanesulfonyl fluoride, 10 mg/ml N′-tosyl-L-phenylalnine chloromethyl ketone/liter of culture, and the cells lysed by passage through a French press. The lysed cell suspension was cleared of cellular debris by centrifugation at 35,000 x g for 30 min at 4°C. For every 10 ml of clarified supernatant, 3 ml of 50% glutathione-ONE-agarose (in phosphate-buffered saline) was added and the suspension rotated at 4°C for 1 h. The agarose was washed in batch four times with 25 ml of lysis buffer. GST-rVH6-CD was eluted from the agarose by incubation in 20 ml of modified lysis buffer containing 20 mM glutathione for 30 min at 4°C. The sample was supplemented with 1 mM Tris, pH 7.4, 80 mM EDTA, 5 mM NaCl to a final concentration of 150 mM Tris, pH 7.4, 200 mM NaCl, 8 mM EDTA, 1 mM β- ME. The eluant was assayed for phosphatase activity as described below. SDS-polyacrylamide gel electrophoresis and Coomassie Blue staining were used to confirm the purity of the fusion protein.

**Kinetic Assays—**The assays were performed as described previously for human dsPTPase VHR (16). Artificial PTPase substrate p-nitrophenyl phosphate was used for all assays. The final concentration of enzyme in the reactions ranged between 0.53 and 2.4 μM. Initial velocities were fitted directly to the Michaelis-Menten equation (Equation 1) using the nonlinear least squares program KinetAsyst (IntelliKinetics, State College, PA). Data analysis and construction of a pH profile curve for the kinetic parameter kcat/Km (Equation 3) (17) were performed using KaleidaGraph (Synergy Software, Reading, PA). In Equations 1-4, C is the pH-independent value of either kcat or kcat/Km; H is the proton concentration; Kᵡ and Kᵢ are the ionization constants of the residues involved in the enzymatic reaction; and S is substrate concentration.

\[
v = \frac{k_{\text{cat}}[S]}{K_c + [S]} \quad (\text{Eq. 1})
\]

\[
v = \frac{C(1 + H/K_C)}{K_H + (1 + H/K_C)} \quad (\text{Eq. 2})
\]

\[
v = \frac{C(1 + H/K_C + H^2/K_{K_C})}{K_H + (1 + H/K_C)} \quad (\text{Eq. 3})
\]

\[
v = \frac{C(1 + H/K_C)}{K_H + (1 + H/K_C)} \quad (\text{Eq. 4})
\]

Experimental procedures for rapid reaction assays were performed as described previously (5), using a Hi-Tech SP-61 stopped-flow spectrophotometer (Salisbury, UK). Oxyanions vanadate, tungstate, and phosphate were tested to verify their ability to inhibit rVH6 phosphatase activity. The concentration of each inhibitor was held constant while the substrate concentration was varied to allow for the calculation of Kᵡ using KinetAsyst (IntelliKinetics, State College, PA).

**Reverse phase HPLC was employed for the separation and quantitation of the substrates and products from an rVH6-catalyzed reaction with a diphosphorylated MAP kinase peptide, as described previously by Denu et al. (18). The peptide sequence corresponded to the putative activation sites on MAP(177–189) kinase (DHTGFLpTEpYVATR). A continuous spectrophotometric assay previously described by Zheng et al. (19) was used to follow the dephosphorylation of the Tyr(P) residue in the diphosphorylated peptide by taking advantage of the difference in absorbance at 282 nm between Tyr(P) and Tyr. The difference in absorbance can be used to follow the complete time course of the enzyme-catalyzed hydrolysis of Tyr(P) in the peptide. Initial concentrations of peptide ranged between 100 and 500 mM, and the final enzyme concentration in the reactions was 7.2 μM. The data were directly fit to Equation 3, and an initial rate (kcat/Km) was calculated.

**Molecular Modeling of rVH6 with X-ray Structure of VHR—** RVH6 primary sequence was aligned with dsPTPase CL100 and PAC-1, and subsequent secondary structure predictions were performed as described (20). A model of rVH6 was constructed, using the crystal structure backbone of VHR (amino acids 11–185) (6) as a template. VHR amino acid residues were mutated to generate the rVH6 structure using graphics program O (21). Small insertions and deletions in the backbone of the model were modified using the lego-loop option of O. The model was placed in a box of 900 water molecules and subjected to energy minimization and simulated molecular dynamics using XPLOR (22).

**RESULTS**

**Purification and Physical Characterization of Full-length rVH6—** Recombinant histidine-tagged rVH6 (rVH6(His)₆) was expressed and purified, with approximately 17 mg of pure enzyme recovered from 4 liters of bacterial culture (Table 1).

| Step            | Volume | Protein | Total protein | Activity* | Specific activity |
|-----------------|--------|---------|---------------|-----------|------------------|
| French press    | 65     | 5.05    | 328.3         | 99        | 6430             |
| Ni-NTA-agarose  | 42     | 1.38    | 57.9          | 36        | 1520             |
| Sephadex G-75   | 14     | 1.24    | 17.3          | 22        | 310              |

*This is a typical preparation from 4 liters of bacterial culture, as described under “Materials and Methods.” One milliliter is 1 mmol of pNP forms per min at 30°C.
The protein eluted at 250 mM imidazole from the Ni-NTA agarose and was ~70% pure, as shown by Coomassie staining (Fig. 1, lane 2). Subsequent purification by Sephadex G-75 gel filtration led to >98% pure enzyme (Fig. 1, lane 3). An apparent decline in specific enzyme activity following G-75 Sephadex purification was attributed to the removal of bacterial contaminants capable of pNPP hydrolysis, and this loss was consistently observed with each preparation of the protein. Purified rVH6(His)6 could be stored for several weeks at 4 °C in the presence of 10 mM β-ME and 250 mM NaCl with no loss of activity.

SDS-polyacrylamide gel electrophoresis analysis of rVH6 indicated an apparent mass of ~43 kDa (Fig. 1). This was consistent with the elution time estimated for the protein when subjected to size exclusion chromatography (not shown). Matrix-assisted laser desorption mass spectrometry confirmed the molecular mass of the enzyme to be 43,500 ± 150, which agreed with the predicted mass of ~42,315 Da for a 381-amino acid protein. Amino-terminal sequencing verified that the expressed protein starts with the predicted amino acid sequence Met-Ile-Asp-Thr-Leu-Arg.

Kinetic Parameters of Full-length rVH6—The values for \( k_{cat} \) and \( K_m \) for rVH6 were determined as a function of pH with pNPP as substrate. The \( \frac{k_{cat}}{K_m} \) parameter for rVH6 decreased with a slope of ~2 below pH 6.6 (Fig. 2), indicating that two ionizable groups (pKₐ values of 5.66 ± 0.22) must be unprotonated for catalysis. The \( \frac{k_{cat}}{K_m} \) profile for VHR (16) has been included for comparison to show that rVH6 displayed a distinct profile, particularly on the basic limb of the curve. The \( \frac{k_{cat}}{K_m} \) parameter for rVH6 displayed a two-ionization curve, which is in contrast to the three ionizations observed for VHR (Fig. 2). R VH6 lacks a critical third ionization that is present in the curve exhibited by VHR (pKₐ = 5.7), which must be protonated for catalysis.

The \( k_{cat} \) parameter of rVH6 displayed less than 2-fold difference over a pH range of 5.4 to 9.0 (Fig. 3). Again, the \( k_{cat} \) value for VHR has been included for comparison (16), to show the extreme differences in the \( k_{cat} \) parameter between the two enzymes. R VH6 displayed a linear, pH-independent profile, with an average \( k_{cat} \) value of 0.01 ± 0.0005 s⁻¹, in contrast to the pH-dependent bell-shaped profile exhibited by VHR.

It has been previously demonstrated that mitogen-activated protein kinase (MAPK) is a substrate for rVH6 in vitro (14, 23). We tested the dual-specific catalytic nature of rVH6, against the diposphorylated MAP (177–189) kinase peptide, DHTGEPTEpYVATR (18). Diposphorylation of Tyr(P) by full-length rVH6 could be followed using a continuous spectrophotometric assay, as described under “Materials and Methods.” By varying the peptide concentration in each reaction, an initial rate of Tyr(P) diposphorylation (\( k_{cat}/K_m \)) of 5.97 ± 0.13 s⁻¹ was calculated (Equation 3). The Tyr(P) residue in the peptide is approximately a 3.5-fold better substrate for rVH6 than pNPP; however, due to the extremely slow reactivity of rVH6 and the inability to saturate the enzyme with peptide, we were unable to calculate the kinetic parameters \( k_{cat} \) and \( K_m \) for rVH6 with this substrate. To visualize and quantitate dephosphorylation of the Thr(P) residue of the peptide, we employed an HPLC-based assay (18) which allowed us to monitor all possible phosphorylation states of the peptide occurring in the reaction. As evidenced from the HPLC elution profiles (Fig. 5), Tyr(P) was rapidly hydrolyzed from the diposphorylated (Thr(P)/Tyr(P)) peptide (~35% within 60 min), whereas no Thr(P) dephosphorylation was observed in significant amounts from the monophosphorylated (Thr(P)) peptide after 23 h (Fig. 5).

Rapid Reaction Kinetics—rVH6 was rapidly reacted with pNPP in a stopped-flow spectrophotometer, and the appearance of product, p-nitrophenol, was followed at 405 nm. The kinetic traces displayed no discernible biphasic characteristics (i.e. no burst of p-nitrophenol formation); however, linear rates were observed that were consistent with steady-state \( k_{cat} \) values. At pH 7 and 30 °C, the linear rate of 0.2 min⁻¹ (average of seven traces) was obtained when 11.3 μM rVH6 was rapidly mixed with 10 μM pNPP. Steady-state initial velocities were 0.3 min⁻¹ under identical conditions.

Inhibition of rVH6 with Oxyanions—Inhibition constants, \( K_i \), were calculated for three known phosphatase inhibitors, phosphate, tungstate, and vanadate. The \( K_i \) values for phosphate, tungstate, and vanadate for rVH6 were 50 μM, 29 μM and 41 μM, respectively, when pNPP was used as substrate. Both phosphate and tungstate exhibited competitive inhibition, whereas vanadate displayed uncompetitive inhibition, when the data were fitted by KinetAsyst. Vanadate is a potent inhibitor of PTPases and has been shown to covalently bind to the catalytic cysteine of the Yersinia phosphatase (7). Vanadate...
forms a complex with the active site cysteine which adopts a distorted trigonal bipyramidal geometry that mimics the catalytic transition state of the enzyme-substrate complex. Formation of a covalent adduct between VO₄ and rVH₆ is consistent with the primary structure predictions of VHR (20), with the exception of the helix between cdc25 (blue) and cdc25 (magenta) (Fig. 6). This helix, termed CH2B (blue) region of the VHR structure (Fig. 6). The NH₂ terminus of the loop between CDC25 helix and the phosphate binding loop (Fig. 6, magenta) represents the catalytic domain (amino acids 134–381) model, including the general acid loop (Fig. 6, yellow), the phosphate binding loop (Fig. 6, red). As anticipated from our kinetic studies and primary structure alignment, amino acids 1–133, which contain the two CH2 regions, cannot be accommodated in the rVH6 catalytic domain and are therefore depicted as a separate structural domain (Fig. 6, white dotted line containing green and blue boxes). Consistent with the similar kinetics of the full-length and the truncated form of rVH6, the predicted three-dimensional model supports our proposal that amino acids 134–381 are all that are structurally required to form the catalytic domain of rVH6.

**DISCUSSION**

Although extensive kinetic analyses have been performed with VHR, PTP1, and Yersinia PTPase, the catalytic properties of dsPTPases have not been fully elucidated. Because many MAPK phosphatases (MKPs) have extensive NH₂-terminal sequences of unknown function, we set out to define the role of these residues in enzyme catalysis. The ability to purify large amounts of stable, full-length rVH6 made it possible to purify large amounts of stable, full-length rVH6 made it possible to determine the necessary residues for enzyme catalysis. The amino terminus of rVH6, as well as other MAPK phosphatases, contains an extended amino acid sequence, with identical amino acid sequence, with residues that are homologous to the dsPTPase, p80cdc25, termed cdc25 homology 2 (CH2) regions. While the CH2 regions in p80cdc25 flank the active site of the enzyme, these regions in rVH6, CL100, and PAC-1 are located amino-terminal to the catalytic core sequence (Fig. 4, shown in green and blue). The functional role of the CH2 regions in p80cdc25 and the dsPTPases is unknown. In an effort to determine the necessity of these residues in enzyme catalysis, we removed the first 133 amino acids of rVH6, which contain the CH2 regions, and expressed the proposed catalytic domain (CD) (amino acids 134–381) as a glutathione S-transferase fusion protein (GST-rVH6-CD). Kinetic experiments were conducted with the truncated version of rVH6. Full-length rVH6 and rVH6-CD had nearly identical kcat, K_m, and kcat/K_m kinetic parameters at both pH 7 and 8 (Table II). rVH6-CD catalytic activity directed against diphasphorylated MAPK (177–189) peptide (DHTGFLpTEpYVATR) was comparable with that observed with full-length rVH6, with the truncated protein preferentially dephosphorylating Tyr(P) (data not shown). Mouney et al. (14) have previously demonstrated that full-length rVH6 can inactivate ERK1 in vitro. Our experiments with the catalytic domain of rVH6 showed levels of ERK1 inactivation that were comparable with those reported for the full-length protein (not shown, (14)).

**Molecular Modeling**—To test the proposal that amino acids 134–381 form the catalytic core of rVH6, we constructed a structural model (Fig. 6) of rVH6, based on the x-ray crystallographic model of the dual specificity phosphatase VHR (6). rVH6 shares 30% sequence identity with VHR within the catalytic domain, and it has been demonstrated that the structure of VHR defines a minimal structural requirement for a PTPase domain (6). When the rVH6 and VHR primary sequences were compared, the secondary structural elements predicted for rVH6 aligned with the corresponding structural features of VHR (20), with the exception of the helix between β3 and β4 in the VHR structure (α3 of VHR) (Fig. 6). This helix, along with three residues adjacent to it, resulted in a gap in the sequence alignment (CH2) regions. While the CH2 regions in p80cdc25 and the dsPTPases are identical, amino acid residues in these regions are LLXDCR(X)XH and (X)5,LLXGAX(X)FXX, respectively. Red boxes represent the conserved amino acid sequence of the catalytic motif of dsPTPases, VX1VH1C(X)0X1R(S/T). Based on primary sequence homology, the location of VHR secondary structural elements (6) was predicted in the other dsPTPases. The first structural element of VHR, α1, corresponds to the amino acid sequence that follows the CH2B (blue) region of the other three dsPTPases. The remaining secondary structure components are predicted to lie within the catalytic domains of the dsPTPases.

**TABLE II**

**Summary of kinetic analysis with pNPP as substrate**

| Enzyme* | pH | kcat | kcat/K_m | K_m |
|---------|----|------|----------|-----|
| rVH6 (full length) | 7 | 0.014 ± 0.0005 | 1.58 ± 0.1 | 9.85 ± 0.97 |
| rVH6-CD⁶ | 7 | 0.017 ± 0.0013 | 1.80 ± 0.1 | 9.45 ± 1.60 |
| rVH6 (full length) | 8 | 0.013 ± 0.0003 | 1.40 ± 0.1 | 9.00 ± 1.22 |
| rVH6-CD⁶ | 8 | 0.011 ± 0.0003 | 1.45 ± 0.1 | 7.23 ± 1.11 |

* Enzymes were assayed at 30 °C in the same buffer system (see "Materials and Methods").

⁶ CD, catalytic domain (amino acids 134–381).

**Fig. 4. Schematic representation of primary sequence and secondary structure alignment of rVH6, CL100, and PAC-1 with VHR.**

The boxes represent primary amino acid sequence alignment of the three CH2-domain-containing dsPTPases, rVH6, PAC-1, and CL100, with VHR by PILE-UP command (Genetics Computing Group, Madison, WI) (14). Green and blue boxes represent the two CH2 regions, CH2A and B, and identical amino acid residues in these regions are LLXDCR(X)XH and (X)5,LLXGAX(X)FXX, respectively. Red boxes represent the conserved amino acid sequence of the catalytic motif of dsPTPases, VX1VH1C(X)0X1R(S/T). Based on primary sequence homology, the location of VHR secondary structural elements (6) was predicted in the other dsPTPases. The first structural element of VHR, α1, corresponds to the amino acid sequence that follows the CH2B (blue) region of the other three dsPTPases. The remaining secondary structure components are predicted to lie within the catalytic domains of the dsPTPases.
it possible to employ rVH6 as a prototype of the MKPs. A number of studies have suggested a common catalytic mechanism for all PTPases and dsPTPases (5, 25). Chemical and mutagenic experiments have shown that this mechanism involves several key residues that are conserved in the consensus active site sequence, HCXGXXRS(T). The two-step mechanism illustrated in Scheme I has been defined for the well-characterized enzymes, Yersinia PTPase and VHR.

The first step of the reaction depicted in Scheme I is the formation of a thiol-phosphate enzyme intermediate ($k_3$). The essential active site cysteine acts as the thiolate anion and is the catalytic nucleophile that attacks the phosphoryl group of the substrate, thus forming the intermediate ($k_3$). A second enzyme ionization involved in intermediate formation has also been identified in the Yersinia PTPase and VHR (16, 26). This ionization represents a conserved aspartic acid, which has been proposed to play the role of a general acid in catalysis by donating a proton to the bridge oxygen of the leaving group (16, 26, 27). The second step in the reaction is the hydrolysis of the intermediate ($k_3$), which involves a water molecule that attacks the intermediate, resulting in the release of free phosphate and enzyme (5, 25). To gain insights into the catalytic mechanism of rVH6, values for the kinetic parameters $k_{cat}$ and $K_m$ were calculated, using $p$-nitrophenyl phosphate as an artificial Tyr(P) substrate.

The apparent second order rate constant, $k_{cat}/K_m$, plotted as a function of pH (Fig. 2), generates a curve reflecting the ionizations of residues involved in both substrate binding and catalysis, accounting for both free enzyme and free substrate. In Scheme I, the $k_{cat}/K_m$ parameter includes the reaction rates for reversible substrate binding ($k_1$ and $k_2$) and formation of the enzyme intermediate ($k_3$). With rVH6, the $k_{cat}/K_m$ curve indicated that two groups with $K_m$ values of 5.1 and 6.6 must be unprotonated for substrate binding and/or catalysis. The $K_m$ value 5.1 has been previously shown to represent the phosphate group of the dianion substrate, pNPP, and the apparent $K_m$ value of 6.6 falls within the range of values (4.7–8.3) shown to represent the catalytic thiolate (16, 28). It is interesting to note that with rVH6, there are only two ionizations observed in the $k_{cat}/K_m$ curve, whereas in VHR, Yersinia, and PTP1, there is a third ionization present which must be protonated for catalysis (16, 26, 29). This ionization has been identified as a conserved aspartic acid residue, which acts as a general acid in catalysis (16, 26). In Yersinia PTPase and PTP1B, the aspartic acid residue has been shown to be located on a flexible loop which undergoes a conformational change when ligand is bound to the active site pocket (24, 27, 30). In rVH6, Asp262 is predicted to function as the general acid, but its ionization is not observed in the $k_{cat}/K_m$ profile. It is not yet clear why the Asp262 ionization is not seen; however, it is striking that both the magnitude and the pH profiles of $k_{cat}/K_m$ for rVH6 closely resemble those observed for the aspartic acid mutants of VHR and Yersinia PTPase (16, 26). An explanation as to why this ionization is not seen in the rVH6 $k_{cat}/K_m$ profile may be that Asp262 is not positioned properly to aid in catalysis. It has been demonstrated with PTP1B that movement of the Asp-containing loop is dependent upon the nature of the ligand. Only when a phosphotyrosine peptide was bound to the active site of PTP1B was loop movement observed (24). Therefore, with pNPP as substrate, our observed kinetics with rVH6 may be explained by a lack of sufficient loop movement bringing Asp262 into proper position for proton donation. Future mutagenic studies should reveal the role of Asp262 in rVH6 catalysis.

The first order rate constant $k_{cat}$ was determined for rVH6, and the apparent $K_m$ values derived from the pH dependence of this parameter should also reflect the critical ionizations of the amino acid residues within the enzyme-substrate complex. For rVH6, $k_{cat}$ plotted as a function of pH displayed a pH-independent value with less than 2-fold difference over the pH range investigated (Fig. 3). This observation is in distinct contrast with the pH-dependent $k_{cat}$ value of VHR and other PTPases (16, 25, 29). The lack of pH dependence of the rVH6 $k_{cat}$ value suggests that the rate-limiting step of the reaction is not phosphoryl-transfer, since the pH-dependent $K_m$ value for the dsPTPase VHR has been shown to reflect the ionizations involved in intermediate formation (16). Therefore, we employed rapid-reaction kinetics to test whether breakdown of the intermediate was rate-limiting for rVH6. Interestingly, rVH6 did not exhibit burst kinetics, indicating that intermediate hydrolysis was not rate-limiting. Based on the kinetic data, it was determined that a non-chemical step, prior to phosphoryl-transfer, limits turnover. Precedent for a substrate-induced conformational change being the slowest step in a phosphoryl-transfer reaction mechanism has been set by experimental evidence involving other phosphoryl-transfer enzymes (31, 32). More importantly, conformational change brought about by substrate binding has been reported in the PTPase family, specifically Yersinia PTPase and PTP1B (24, 30). Therefore, the data suggest a modified kinetic mechanism for rVH6, involving a rate-limiting step prior to $k_1$ (Scheme I) that differs from the nature of the rate-determining steps previously proposed for other PTPases (5, 25, 29). However, we cannot rule out other possible explanations for the observed kinetic behavior of rVH6.

Based on sequence homology to other VH1-like phosphatases and its ability to inactivate MAP kinase in vitro (14, 23), rVH6 was classified as a dual specificity protein tyrosine phosphatase. With this in mind, we attempted to kinetically characterize rVH6 using a diphosphorylated MAP$_{177-189}$, kinase peptide, DHTGFLpTEpYVATR. Full-length rVH6 was capable of hydrolyzing phosphate from the Tyr(P) residue in the peptide, with a $k_{cat}/K_m$ value that was approximately three times higher than that observed with pNPP, indicating that the peptide was a better substrate for the enzyme than pNPP. However, no appreciable dephosphorylation of Thr(P) was observed (Fig. 5). The apparent preference of rVH6 for Tyr(P) hydrolysis has also been observed in other dsPTPases, such as p80$^{\text{cdc}25}$ (8), and may reflect rVH6 substrate preference in vivo.
The crystal structures of several PTPases have been solved (6, 27, 33), and despite the limited sequence homology among PTPases, the three-dimensional structure of these proteins are remarkably similar. A structure-based sequence alignment of the Yersinia PTPase, PTP1B, and VHR (6) revealed the common secondary structural elements that define the catalytic domain of all three enzymes. This finding prompted us to construct a primary sequence alignment of VHR with rVH6 and other dsPTPases that contain an extended NH2 terminus, CL100 and PAC-1 (Fig. 4). Incorporating VHR secondary structure into the alignment clearly demonstrates that the catalytic domains of all three enzymes are located in the carboxyl terminus (Fig. 4).

Using the alignment to define the residues that comprise the catalytic domain of rVH6 (amino acids 134–381), we set out to establish the role of the NH2-terminal residues (amino acids 1–133) in enzyme catalysis. We demonstrated that the first 133 residues of rVH6 were not critical for enzymatic activity directed against pNPP, as evidenced by the similar kinetic parameters displayed by full-length and truncated rVH6 (Table II). Furthermore, there were no appreciable differences in the catalytic abilities of full-length rVH6 and rVH6-CD in dephosphorylating the MAPK177–189 peptide or inactivating ERK1 in vitro. These findings show that NH2-terminal residues 1–133 are not essential for rVH6 catalysis of either an artificial or physiologically relevant substrate and that residues 134–381 define the catalytic PTPase core within rVH6. These results are important because many PTPases and dsPTPases contain both NH2- and COOH-terminal extensions outside of the catalytic domain, and based on our results, it is probable that these regions do not contribute to the catalytic activity of the enzymes.

Corroborating our kinetic analysis, secondary structure prediction and molecular modeling of rVH6 confirmed that the COOH-terminal amino acids (134–381) define the catalytic domain for this dsPTPase (Fig. 6). The loop between α1 and β1, proposed to be involved in substrate recognition, is highlighted in magenta, with the first 24 residues of the loop illustrated as a white dashed line, due to their absence in the VHR sequence. For comparison purposes, a diagram for the x-ray structure of VHR is displayed in the same orientation and color scheme as the rVH6 model. Due to the lack of sequence homology between the NH2 terminus of rVH6 and VHR, along with the undetermined conformation of the α1–β1 loop, we cannot confirm the correct orientation of the predicted NH2-terminal helix, α1.
site of action. Since rVH6 was modeled using VHR as the template, the corresponding loop of rVH6 is shown in the closed form (Fig. 6, yellow). However, the lack of any observed general-acid catalysis in the $k_{cat}/K_m$ pH profile suggested that, when pNPP is the substrate, this loop may not close completely, preventing Asp$^{262}$ from assisting in catalysis.

Collectively, secondary structure predictions and the kinetic data have allowed us to define the catalytic core of rVH6. The high degree of sequence homology between rVH6 and CL100 and PAC-1 suggests that the latter two dsPTPases share this catalytic core structure. These results suggest that the NH$_2$-terminal extensions of these enzymes constitute a separate domain, and analyses of the gene structures of PAC-1 and CL100 (34, 35) further support the idea that the extended NH$_2$-terminal may be of distinct evolutionary origin. In CL100 and PAC-1, the NH$_2$-terminal extensions are encoded by different exons than the catalytic domain residues, implying that these exons may have converged with a VHR-like gene to create a subfamily of dual specificity protein tyrosine phosphatases. Future studies involving the NH$_2$-terminal extension of rVH6 should shed light on the role of these amino acids in cellular processes.

Acknowledgments—We thank J. A. Stuckey for assistance in the molecular modeling and M. J. Wishart and J. C. Clemens for critical reading of the manuscript and helpful advice. We also thank Dr. Phil Andrews and the Protein Core Facility at the University of Michigan Medical School for NH$_2$-terminal sequencing and matrix-assisted laser desorption mass spectroscopy; Drs. Elizabeth Butch and Kun-Liang Guan for providing ERK1 and MEK; and Dr. Dave Ballou for use of the stopped-flow spectrophotometer.

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