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New aspects of the phosphatase VHZ revealed by a highresolution structure with vanadate and substrate screening

Vyacheslav I. Kuznetsov, **Alvan C. Hengge**, and **Sean J. Johnson**Department of Chemistry and Biochemistry, Utah State University, Logan, UT 84322-0300

Abstract

The recently discovered 150-residue human VHZ (VH1 related protein, Z member) is one of the smallest protein tyrosine phosphatases (PTPs) known, and contains only the minimal structural elements common to all PTPs. We report a substrate screening analysis and a crystal structure of the VHZ complex with vanadate at 1.1 Å resolution, with a detailed structural comparison with other members of the protein tyrosine phosphatase family, including classical tyrosine-specific protein tyrosine phosphatases (PTPs) and dual specific phosphatases (DSPs). A screen with 360 phosphorylated peptides shows VHZ efficiently catalyzes the hydrolysis of phospho-tyrosine(pY)-containing peptides, but exhibits no activity toward phospho-serine (pS) or phospho-threonine (pT) peptides. The new structure reveals a deep and narrow active site more typical of the classical tyrosine specific PTPs. Despite the high structural and sequence similarities between VHZ and classical PTPs, its general acid IPD-loop is most likely conformationally rigid, in contrast to the flexible WPD counterpart of classical PTPs. VHZ also lacks substrate recognition domains and other domains typically found on classical PTPs. It is therefore proposed that VHZ is more properly classified as an atypical PTP rather than an atypical DSP, as has been suggested.

Keywords

Protein-tyrosine phosphatase; dual-specificity phosphatase; PTP; DSP; VHZ

Protein tyrosine phosphatases (PTPs) are involved in multiple physiological processes including cell growth and proliferation, control of the cell cycle, cytokine activation and cytoskeletal integrity (1, 2). PTPs share the conserved signature motif HCXXGXXR(S/T) that binds the substrate and provides transition state stabilization, and a two-step catalytic mechanism with a cysteinyl-phosphate intermediate (3-5). PTP family members are further subdivided into several subfamilies based on substrate specificity and sequence similarity. In-vivo protein phosphorylation most commonly occurs on hydroxyl containing aliphatic (serine/threonine) or aromatic (tyrosine) side chains, and the classical PTPs (often referred to as tyrosine specific PTPs) hydrolyze only phosphotyrosine containing polypeptides. The dual-specificity phosphatases (DSPs) constitute a subset of (PTPs) that are characterized by the ability to dephosphorylate phosphoserine and phosphothreonine residues in addition to phosphotyrosine (6, 7).

To whom correspondence should be addressed: Alvan C. Hengge: alvan.hengge@usu.edu. Phone: (435) 797-3442. Fax: (435) 797-3390. Sean J. Johnson: sean.johnson@usu.edu. Tel.: (435) 797-2089. Fax: (435)797-3390.

Supporting Information. Complete list of phosphorylated peptides in the substrate screen; phosphopeptides that showed the highest percentage of phosphate release by VHZ; partial sequence alignment of residues 63-138 of VHZ with classic PTPs, DSPs, and other PTPs containing the XXDXXXP sequence of the general acid loop and structurally related to VHZ; a structural alignment of the VHZ-vanadate complex with YopH. This material is available free of charge via the Internet at http://pubs.acs.org.

The active sites of all PTP family members are structurally similar and utilize an invariant triad of amino acids to catalyze phosphate monoester hydrolysis, which includes a nucleophilic cysteine; an arginine that, along with backbone amides, binds substrate and provides transition state stabilization; and an aspartic acid that protonates the leaving group. In the classical, tyrosine-specific PTPs the latter catalytic residue resides on a flexible loop referred to as the WPD loop (8). In the DSPs, the general acid is believed to reside on a structurally rigid element.

Despite the high structural similarities of catalytic domains, superimposable active sites, and common mechanism, PTPs are selective for particular biological targets. The accurate identification of substrate preference and the correct assignment of these enzymes into the various PTP subfamilies associated with different biological pathways and pathological states is one of the most important goals of human phosphatome research. The recently discovered 150-residue human VHZ (also designated as atypical DSP 23) is one of the smallest PTP family members known to date, which contains only the minimal set of secondary structural elements common to all PTPs (9-11). Because of its small size and the absence of substrate targeting domains, it has been classified as an atypical VH-1 related DSP (11)- a subfamily of cysteine based PTPs that regulates mitogenic signaling pathways and cell cycle process (6, 12). Atypical DSPs have been linked to cancer, but despite numerous studies knowledge about these enzymes still remains contradictory and incomplete (13, 14). The attempts to identify the physiological substrates of VHZ have provided conflicting and inconsistent evidence (9, 15, 16). It is known, however, that VHZ is highly expressed in fetal tissues, and recent reports associated VHZ with breast cancer (17).

The only crystal structure of VHZ published to date is a complex with malate trapped in the active site (18). Malate ion is an important part of VHZ crystallization conditions, but represents neither a substrate nor a product analogue. Potential resulting distortions on the local geometry of the active site motivated us to seek a structure with a physiologically relevant reaction analogue. We report a high-resolution X-ray structure of VHZ complexed with the physiologically relevant transition state analogue metavanadate which allows us to compare VHZ structurally with a number of classical PTP and atypical DSP protein-ligand complexes. This analysis suggests that VHZ bears more resemblance to the classical PTPs rather than DSPs, but probably lacks a movable loop. These conclusions from the crystal structure support the results of a phosphopeptide substrate screen, which revealed that phosphotyrosine, but not phosphoserine or phosphothreonine-containing peptides, are substrates for VHZ. The collected results strongly suggest that VHZ is more related to the classical PTPs rather than the atypical DSP subfamily, and in order to more properly guide future research in the biological roles of VHZ, its current classification should be reconsidered.

Experimental Procedures (Materials and Methods)

Materials and methods

All buffer solids, EDTA, malic acid, ammonium sulfate and sodium orthovanadate were obtained from Fisher. Chromatography columns were purchased from GE Healthcare. Protease K was from Sigma. Polyethyleneimine (Mw 50000) was from Acros Organics. A set of standard FastDigest restriction enzymes, DNA Pfu Turbo Polymerase, dNTP master mix were obtained from Fermentas. Custom primer oligonucleotides were synthesized by IDT. The phosphatase substrate screen was from GPT Innovative Peptide Solution. DTT, antibiotics and protease inhibitors were from GoldBio.

Protein cloning—The human VHZ WT gene was amplified using a two-step PCR strategy from a GST-tagged version on a plasmid that was a generous gift of Dr. Subramanyam Swaminathan (18). In the first step the gene was amplified using the following primers: Fwd1: GAAAACCTGTATTTCAGGGCGTGCAGCCCCCC, Rev1: GGAGAGCTCTTATTTCGTTCGCTGGTAGA to incorporate TEV cleavage site (in bold) immediately upstream of the first N-terminal GGC codon (methionine excluded). At the same time SacI restriction site (GAGCTC) was added at the end of the sequence. In the second PCR step, the product of the first step was used as a template and a KpnI restriction site was added upstream of the TEV protease cleavage sequence using the following set of primers: Fwd2:CGGGGTACCGAAAACCTGTAT, Rev1: GGAGA GCTCTTATTTCGTTCGCTGGTAGA. The PCR product was digested with KpnI and SacI restriction enzymes (Fermentas) and inserted into the pet-45(B+) vector (Novagen). The obtained pet 45B+TEV-VHZ construct was successfully expressed in BL21-codon+ (DE3)-RIL cells and purified with Ni-Fast Flow High affinity resin (GE Healthcare). Unfortunately, the resulting fusion protein was resistant to TEV cleavage of the polyhistidine tag and the tagged protein was unstable at low pH. Insertion of a short spacer arm following the histidine tag did not improve TEV cleavage (19). A tagless enzyme construct was prepared by removal of the histidine tag and TEV-cleavage site from the pET 45b-TEV-VHZ construct using one-step overlap-extension PCR (20) using the following primers: Fwd: TATACCATGGGCGTGCAGCCC; Rev:CGTGCACGCCCATGGTATATCT. The resulting construct was used to express tagless VHZ WT in BL-21 DE-3 (codon+) cells.

Protein preparation—Tagless VHZ WT was expressed in BL21-codon+(DE3)-RIL cells. The cells were grown at 37°C to an $OD_{600} = 1.2-1.5$. Protein expression was induced with IPTG (100mg/L) and cell culture flasks were transferred to room temperature shaker for 20 hours before harvesting. The cells were harvested by centrifugation for fifteen minutes and re-suspended in the lysis buffer containing 50mM HEPES, 150 mm NaCl, 1mM EDTA, 10 mM DTT, 5% glycerol pH 7.4 (4°C). Cells were disrupted by sonication on ice and the pellet was separated by centrifugation. The supernatant was transferred into a separate beaker and a 10 % w/v solution of polyethyleneimine (PEI, Mw50000, pH 8.0) was added dropwise to the supernatant at 4°C to reach a final PEI concentration of 0.5% (w/v). White precipitate formed immediately and was separated by centrifugation. Ammonium sulfate was added to the supernatant (to 65% saturation), precipitated protein was collected and resuspended in 150 mL of SP-loading buffer containing 100 mM sodium acetate, 1mM DTT, 1mM EDTA, 10 % glycerol, pH 5.6. The solution was filtered and 15 mL of Hi-Trap SP-HP cation exchange resin (GE-Healthcare) pre-equilibrated with SP-loading buffer were added to the protein (4°C). The resin was transferred into a column and washed with SP-loading buffer (2ml/min) to reach a stable baseline. Protein was eluted using 120 mL linear gradient with elution buffer containing 100 mM sodium acetate, 1mm DTT, 1mM EDTA, 600 mM sodium chloride, 10 % glycerol, pH 5.6. Fractions containing phosphatase activity toward para-nitrophenylphosphate (pNPP) were collected, concentrated to a volume of 3-5 mL and loaded on a Superdex 75 26/60 gel filtration column (GE-Healthcare) pre-equilibrated with buffer containing 10 mM HEPES, 150 mM NaCl, 1 mM methionine, 3 mm DTT, 10 % glycerol pH 7.5 (4 °C, 0.3 ml/min). The single peak correspondent to VHZ WT was collected and estimated to be >98% pure based on the SDS PAGE analysis. Protein was concentrated to 20 mg/mL as determined by UV using an $A^{280}_{1 \text{ mg/ml}} = 0.68$.

Determination of substrate specificity—The commercial phosphopeptide screen from JPT Innovative Peptide Technologies (product code PhSS-360-250) was used. The full list of the phosphopeptides comprising this screen is available at the manufacturer web site (http://jpt.com) and is included in Supporting Information as Table S1. 25 μ L of buffer containing 100 mM sodium acetate 0.5 mM DTT, 150 mM NaCl (pH5.5) and 0.25 units of

VHZ WT (0.55mg/ml) were added in each well of 384-well microtiter plate containing 360 phosphopeptides (250 pmol per well) derived from human phosphorylation sites together with 10 calibration standards. After incubation for two hours at 25 °C, 5 μL of protease K (Sigma, 200 $\mu g/ml$) was added to digest the enzyme and prevent enzyme precipitation after addition of the malachite green reagent. After 15 minutes, 30 μL of the malachite green solution (Promega) were added and color developed for 10 minutes. The absorbance at 600 nm was measured to quantify the amount of free inorganic phosphate released by the enzyme using a standard curve. This was converted to a % of total hydrolysis using the supplier-provided information for amounts of substrate in each well. Subsequently, $k_{\rm cat}$ and $K_{\rm M}$ were measured for two peptide substrates, one that exhibited among the highest percent of hydrolysis (QREAEpYEPETV) and one from the group exhibiting the least (DADEpYLIPQQG). In a control experiment, incubation of pY-containing peptides under the assay conditions without enzyme exhibited no measurable hydrolysis after 48 hours.

Co-crystallization with sodium vanadate—All crystallization experiments were carried out at room temperature using the sitting drop vapor diffusion method. VHZ WT concentrated to 20 mg/mL in the crystallization buffer was mixed with 50 mM solution of sodium vanadate (final vanadate concentration 10mM, K_i =2.7 μ M) prepared as previously described (21). One μ L of protein-vanadate complex was mixed with an equal volume of well solution containing 150 mM L-Malate, pH 7.0 and PEG3350 (15-20 %). 1M stock solution of sodium malate was prepared by titration of malic acid with sodium hydroxide. Crystals grew within one week.

Data collection and processing—The crystal in the droplet was transferred to a cryosolution containing the well solution supplemented with 10% v/v glycerol and 10mM sodium vanadate for 10 min and flash-frozen in liquid nitrogen. Diffraction data were collected at the Stanford Synchrotron Radiation Lightsource (SSRL) on the beam line 9-2. The collection and refinement statistics are shown in Table 1. Molecular replacement was performed with Phenix (22) using the previously published VHZ structure (PDBID 2img) as a search model with malate ion in the active site removed. Coot (23) and MolProbity (24) were used for model building and validation. Phenix was used for model refinement. PyMol (25) was used to prepare figures and superimpose structures. Structure factors and coordinates have been deposited in the Protein Data Bank under accession code 4ERC.

Results

Substrate specificity of VHZ

The substrate specificity of VHZ was assessed using a phosphatase substrate set containing 360 peptides, correspondent to natural *in vivo* phosphorylation sites monophosphorylated on serine, threonine, or tyrosine. Amino acids adjacent to the phosphorylation site play an important role in the substrate recognition by different PTPs (26). The structural diversity of JPT screen eliminates the bias resulting from adverse binding effects caused by unfavorable amino acids proximal to the phosphorylated site, which allows a reliable assessment of the comparative effectiveness of VHZ in catalyzing the hydrolysis of pS, pT and pY containing peptides. Because the goal of this experiment was to determine whether VHZ was a DSP or a PTP, reactions were given a sufficiently long time to incubate before assay to insure that even substrates yielding very low activity would be detected. After two hours, enzymatic activity was observed in every well containing a pY-phosphorylated peptide, with an average hydrolysis completion of 50-60%. No activity was observed in wells containing either pS or pT-phosphorylated peptides. In particular, no activity was observed in the wells containing serine/threonine monophosphorylated peptides corresponding to the previously tested MBP (myelin binding protein) and common DSP substrates containing TXY

activation loop of MAPK 8—MAPK11. The peptide sequences that resulted in hydrolysis yielding greater than the average are presented in Table S2 of Supporting Information. In subsequent experiments, k_{cat} and K_{M} were determined for the substrate QREAEpYEPETV, one of the substrates exhibiting the highest percent of hydrolysis, and for DADEpYLIPQQG, which was among those that exhibited the lowest activity. These kinetic results are shown in Table 2.

General structure description

The crystal structure of VHZ complexed with metavanadate was solved to 1.1 Å resolution (Figure 1). The structure was refined to an R_{work}/R_{free} of 12.8/14.5% (Table 1). Two molecules of VHZ are contained in the asymmetric unit. The final model includes the protein residues 1-150 and a metavanadate (VO3) ligand present in each active site. VHZ is a single domain protein consisting of a central, twisted, five-stranded β sheet surrounded by six α helices, two on one side and four on the other side (Figure 1). The conserved phosphate-binding loop (P-loop) connects the $\beta 5$ strand and the $\alpha 4$ helix, and general acid loop (referred as IPD-loop (11) by analogy to the conserved WPD loop of the classical PTPs) connects $\alpha 3$ and $\beta 4$ elements. The D residue of the IPD motif (D65) acts as the general acid during catalysis. The Q-loop (connecting $\alpha 6$ and $\alpha 5$) further defines the active site cleft. The overall VHZ structure represents a typical for the PTP family α/β domain. Both molecules in the asymmetric unit adopt a similar protein conformation. The guanidinium side chains of R60 and R62 from the symmetry related VHZ molecule is observed in the area of the active sites of molecule A and molecule B, respectively (Figure 2A,C).

Comparison with VHZ-Malate structure

The structure of the VHZ-VO₃ transition state complex differs in several important details from the previously published VHZ-malate complex. In the VHZ-Mal structure, the malate ion makes four hydrogen bonds to the backbone of the P-loop, two hydrogen bonds to the side chain of R101, and one hydrogen bond to the backbone of the IPD-loop. The catalytic acid D65 (in the IPD loop) and E134 (in the Q-loop) are turned away from the active site (presumably repelled by the negative charge of the malate carboxylate groups). As a result, the IPD-loop adopts a catalytically unfavorable conformation in which D65 hydrogen bonds to the side chain of the conserved phosphate-binding residue R101. Additionally, this catalytically unviable conformation imparted by malate distorts the VHZ active site into a shallow and broad crevice reminiscent of DSPs. In the VHZ-Mal complex the side chain of Y136 from the symmetry related molecule is trapped in the active site, which, together with the rest of the molecule, makes five additional crystal contacts in the area of the active site, further complicating the interpretation of this structure.

In the VHZ- VO₃ structure, the active site contains a region of distinct electon density corresponding to a low B-factor water molecule. The water is located whithin hydrogen bond distance from the equatorial oxygens of VO₃, the side chains of E134 (Q-loop), Q138 (Q-loop), and D65 (IPD-loop), and the backbone amide nitrogen of F66 (Figure 1). It is absent in the VHZ-Mal structure due to interactions of the malate ion, but a water in this position is consistently observed in X-ray structures of classical PTPs such as *Yersinia* PTP and PTP1B. It represents an important node coordinating the primary active site elements in classical PTPs but not in VH1 related DSPs (Figure 4, A and B).

The $VHZ-VO_3$ complex (Figure 2) reveals the active site arrangement observed in many classical PTP structures where all of the catalytic elements are properly positioned for catalysis. The electron density in the active site region clearly reveals the $[VO_3]^-$ ion is coordinated in each active site to the P-loop amide backbone as well as to the side chain of

R101. The sulfur of C95 is 2.4 Å from the central vanadium atom. The electron density indicates 100% occupancy of the $[VO_3]^-$ ion bound to one of the VHZ molecules (chain A) but only 86% occupancy in the second molecule (chain B). The guanidinium moiety of R101 makes bidentate hydrogen bonds with the bound metavanadate, which is a transition state analog of the transferring phosphoryl group. The general acid D65 is located in a position suitable for protonation of the scissile oxygen atom, as is commonly observed in similar ligand-containing PTP structures. The nucleophilic cysteine sulfur atom is in an apical position relative to the central VO₃ moiety.

Interestingly, a gap in electron density exists between the C95 and the vanadate in both the 2Fo-Fc difference map and in an unbiased composite omit map (Figure 3 A, C). Modeling and refinement of a covalently bound cysteinyl-vanadate adduct resulted in a large negative electron density peak in the region between the sulfur and vanadium (Figure 3 B), whereas relaxation of the geometry constraints during refinement yielded the original model with a non-covalent sulfur-vanadium interaction. This suggests a primarily electrostatic interaction rather than a covalent bond between cysteine 95 and the vanadium atom, which has been confirmed by a computational analysis (27).

Functional analysis of VHZ active site

The active site of classical PTPs is formed by the phosphate binding loop (P-loop), a glutamine-bearing loop (Q-loop), general acid loop (WPD-loop), and substrate binding (recognition) loop. VHZ lacks a substrate recognition loop, which is normally located at the N-terminus of classical PTPs. These loop regions in VHZ, compared with PTPs and DSPs, are discussed separately.

P-loop interactions (VHZ residues 95-102)—The P-loop interactions with bound VO₃ in VHZ are presented in Figure 1. The backbone amides of five P-loop residues make hydrogen bonds to the [VO₃] ion. The amide of conserved G98 makes a catalytically important hydrogen bond to the side chain of the nucleophilic C95 (28). The side chain of T102 also donates a hydrogen bond to the C95 sulfur, assisting the breakdown of the phosphoenzyme intermediate in the second step of the overall reaction, another conserved feature of the PTP family (29). The carbonyl oxygen of G98 makes a hydrogen bond to the side chain of R129, which, together with the side chain of N7, provides conformational stability to the P-loop. This arginine is highly conserved in all PTPs, and mutation leads to the loss of enzymatic activity (30). In atypical DSPs, in addition to the structural role, this residue is important for recognition of bisphosphorylated peptide substrates, providing a positive charge in the second docking site on the protein surface (30). In classical PTPs this arginine is shielded from the surface by protein structural elements and plays no role in the substrate recognition. In VHZ, as in classical PTPs, R129 is shielded from the surface by the side chain of N7 (Figure 1). The guanidinium R101 side chain, which binds the phosphate moiety of the substrate, makes two hydrogen bonds to the metavanadate equatorial oxygens, the carbonyl oxygen of I63 (in the IPD-loop) and to the carboxyl oxygen of E44.

Q-loop interactions (residues 127-138)—In classical PTPs this region is characterized by two conserved Q residues and participates in catalysis by activation and orientation of the nucleophilic water in the second step (31, 32). Important Q-loop interactions are presented in Figure 1. The Q-loop motif in VHZ is identical to that in classical PTPs with exception of the substitution of the first Q by E, which, however, resulted in no functional changes in YopH (32). The side chains of E134 and Q138 are directed toward the active site, and together with D65 (from the IPD-loop) serve to position the incoming nucleophilic water. The analogous N-loop region in atypical DSPs (Figure 4) is positioned such that it provides

a broad and shallow active site opening required for dual specificity that has no conserved glutamines.

IPD-loop interactions (residues 61-71)—The general acid loop in VHZ has been termed the IPD loop, by analogy with the conserved WPD loop in the classical PTPs (18). This loop carries the D65 general acid that protonates the leaving group in the first step, and acts as a general base in the second step activating the nucleophilic water (16, 33, 34). The interactions involving IPD loop residues are shown in Figure 1 and Figure 2. The IPD loop position corresponds with the closed, catalytically active position of the WPD loops in classical PTPs. The side chain of I63 docks in a hydrophobic pocket created by the aliphatic part of R101 and side chains of M105, V93 and P69, providing hydrophobic interactions similar to those involving the conserved W in classical PTP (35). Similar to clasical PTPs, the carbonyl oxygen of I63 makes a hydrogen bond to R101 retaining its side chain in the catalyticaly favourable substrate binding conformation. Additional hydrogen bonds occur between the carboxyl oxygen of D65 and structural water W_S as well as between carbonyl oxygen of C67 and side chain nitrogen of Q138. Such interactions are characteristic in the WPD-loop stabilization in classical PTPs (Figure 4A) but not of atypical DSPs (Figure 4B) (36). The structural water molecule is absent in all atypical DSP structures reported to date. Instead, general acid positioning is provided by the side chain of a conserved asparagine from the N-loop motif and the conserved serine from the P-loop. As the result, the general acid position in relation to the leaving group differs in PTPs compared to DSPs and protonation occurs from different angles (Figure 4 A and B).

Sequence analysis of VHZ

With the goal of ascertaining where VHZ fits in the PTP-DSP continuum, we carried out sequence and structural comparisons between VHZ and several classical PTPs and VH1-related DSPs. Because of significant size differences and little conservation outside of the following regions, alignments were confined to the general acid-loop, P-loop, and Q-loop as the three most important catalytic regions. The results, shown in Supplementary Information Figure 1S A and B, reveal both significant resemblance of VHZ to the classical PTPs, and important distinctions from atypical DSPs.

Sequence analysis of the P-loop—The general PTP family signature motif (P-loop motif) contains the HCXXGXXR(S/T) sequence (1, 37). As can be seen from the sequence alignments in Figure 1S (A and B), the conserved motif in classical PTPs is HCXXGXGRT whereas in atypical DSPs this sequence is HCXXGXSRS. The **H+6** position (in bold) is exclusively occupied by glycine in classical PTPs and by serine in atypical DSPs. The serine at this position in atypical DSPs donates a hydrogen bond to the general acid (Figure 4B) contributing to its correct positioning for catalysis. Mutation of S129 to G in VHR abolishes general acid catalysis by D92 (data not shown). In classical PTPs, glycine occupies the analogous position (Figure 4 A), which permits conserved Q-loop glutamine residues (Q446 and Q450 in YopH) to access the active site and participate in the second step of the reaction by positioning the nucleophilic water (32, 38). VHZ (Figure 4C) resembles classical PTPs in having glycine (G100) rather than serine in this position, which allows the E134 side chain to access the active site.

The sequence analysis of the Q-loop—In classical PTPs (Figure 1S-A) this region contains the motif (V/I)Q(R/T)XXQ. The region is named after two catalytically important Q residues that serve to orient and activate the nucleophilic water molecule in the second step (32, 38). The corresponding N-loop in atypical DSPs characterized by the highly conserved PNXXFXXQL sequence contains a conserved asparagine residue (N163 in VHR), which together with the conserved S residue in the P-loop (S129 in VHR), donates a

hydrogen bond to the general acid and stabilizes its catalytically favorable conformation (Figure 4B).

The sequence analysis of the IPD-loop—In the atypical DSPs the general acidbearing loop has no sequence conservation (Figure 1S-B) with exception of the catalytic aspartic acid, and unlike the WPD-loop, does not undergo conformational changes (36). The sequence alignment of VHZ with classical PTPs (Figure 1S-A) shows its IPD-loop is highly analogous to the WPD-loop in classical PTPs with the exception of I63 in place of the conserved W. The MOTIF server was used to search the RSCB database for structures that simultaneously contain the PTP family HCXXXXR[S/T] motif and a variable general acid XXDXXP loop region (Figure 1S C) but not the highly conserved PNXXXF N-loop sequence, thus allowing us to eliminate atypical DSPs. The goal was to identify proteins that, like VHZ, are related to classical PTPs but with variability of the general acid loop region. Alignment of the retrieved structures (e.g. VSP (3AWF), PTEN (1D5R), KAP1 (1FPZ), PhyA (3F41), SsoPTP (2I6I), CDC14B(1OHC) revealed the common PTP α/β-fold with general acid XXD-loops superimposable with the IPD-loop in VHZ and WPD-loop in classical PTPs (Figure 1S-C). These proteins share the active site architecture of classical PTPs with substitutions for W in the general acid loop. Based on the sequence alignment analysis and structural and functional information of the retrieved proteins, VHZ is most closely related to S. solfataricus rather than VSP, PTEN, KAP1, PhyA or CDC14B.

Discussion

Identification of VHZ Substrate specificity

The substrate specificity of VHZ has been the subject of several conflicting studies. In one study VHZ was found to effectively dephosphorylate ERK, but not JNK or p38 (10). In another study, VHZ was found to effect JNK and p38 with no apparent effect on ERK (9). These substrates are common ones among DSPs. The substrate screen used in the present study contained several sequences corresponding to activation loops of MAPK 8, MAPK 9, MAPK 10 and ERK1 monophosphorylated on pT or pS. No activity was detected in these wells, indicating that if VHZ targets the reported proteins, it may dephosphorylate only the phosphotyrosine of the pTXpY activation loops, unlike the DSP VHR, which targets both sites (30). The observed dephosphorylation pattern is characteristic of classical tyrosinespecific PTPs, which result in monophosphorylated MAPK forms and retain reduced but significant activity compared to the bi-phosphorylated proteins (39). DSPs have a preference, but not specificity for, bisphosphorylated substrates, and have the ability to act on singly phosphorylated pS and pT-peptides. As described above, because R129 is shielded by the side chain of N7, it is unlikely that VHZ has a preference for bisphosphorylated substrates (30, 40). These results suggest that VHZ is phosphotyrosine specific PTP rather than a DSP.

Two substrates, one from among those showing the highest hydrolysis percentage and one from the lowest, were chosen for more detailed kinetic analysis. Table 2 shows the $k_{\rm cat}$, $K_{\rm M}$, and $k_{\rm cat}/K_{\rm M}$ forthe substrates QREAEpYEPETV and DADEpYLIPQQG. The substrates differ in $K_{\rm M}$ but have similar $k_{\rm cat}$ values. The $k_{\rm cat}$ values are higher than a reported value of $k_{\rm cat}$ of 0.009 with pNPP (11). Related enzymes (VH1, VHX, and VHY) show $k_{\rm cat}$ values in the range 0.2 to 0.9 s⁻¹ with pNPP (11). The substrate DADEpYLIPQQG has also been tested with YopH and PTP1B. At pH 6.6 and 30 C with YopH, $k_{\rm cat} = 1314 \pm 18 \, {\rm s}^{-1}$ and $K_{\rm M} = 59 \pm 4 \, {\rm \mu M}$; with PTP1B, $k_{\rm cat} = 75.7 \pm 1.0 \, {\rm s}^{-1}$ and $K_{\rm M} = 2.63 \pm 0.37 \, {\rm \mu M}$ (41). Both YopH and PTP1B exhibit very similar $k_{\rm cat}$ values for a range of phosphopeptide substrates regardless of sequence. The same trend is apparent in these two VHZ substrates, where the difference in hydrolysis arises from $K_{\rm M}$ rather than $k_{\rm cat}$. Such consistency in turnover

number is a common feature of PTPs, although $k_{\rm cat}$ varies over several orders of magnitude among enzymes within the PTP family. The lower $k_{\rm cat}$ observed for the peptide substrates in this study for VHZ relative to YopH and PTP1B parallels the lower rate reported for VHZ catalysis of pNPP (11). A thorough search for substrate sequences with particularly strong affinity for VHZ is beyond the scope of this study.

The apparent disagreement between our assay results and those of previous reports of VHZ as a DSP led us to consider potential explanations. In one of the previous reports, a commercial kit was used to estimate the phosphatase activity (10). Our previous experiments with commercial kits based on malachite green assays have shown a tendency to give false positives with some proteins, depending on the quantity of protein used. In these cases addition of the acidic malachite green solution causes protein precipitation, forming turbidity that is not always visible to the naked eye in the darkly colored solution. The resulting light scattering can register as absorbance over the blank where no protein is added. We avoided this problem by digesting the enzyme with a phosphate-free protease prior to adding the malachite green solution.

In another study, MBP (myelin basic protein) phosphorylated by protein kinase A (PKA) was used in a dephosphorylation assay that followed radioactively labeled phosphate release (9). The reported pS/pT dephosphorylation rate (0.0073 pmol/min) was 1500 times lower than pY hydrolysis (1.1 pmol/min). Despite the fact that serine and threonine are the predominant phosphorylation sites, the PKA phosphorylation of MBP is not 100% specific, and produces detectable quantities of pY in addition to pS and pT (42). A small incorporation of phosphotyrosine into MBP may explain the low activity of VHZ detected with this substrate.

Finally, the VHZ used in these previous reports contained an N-terminal GST tag. The N-terminal region in classical PTPs contains an important substrate recognition loop. The GST tag alone showed no phosphatase activity, but its proximity to the active site may affect substrate specificity. In order to avoid potential complications with N-terminal tags, all experiments in the present study used untagged VHZ.

Is the IPD loop of VHZ mobile?

The mobility of the WPD loop in classical PTPs has been extensively documented (8, 43-47). In solution the loop is dynamic, and substrate/oxyanion binding shifts the equilibrium to favor the closed conformation that brings the general acid into position favorable for catalysis (8, 48). The crystal structure of the VHZ-VO₃ complex shows the IPD-loop is properly positioned for catalysis, and superimposes with the closed WPD-loop conformation observed in classical PTPs. Because the highly conserved W residue is important for WPD-loop dynamics (49), we searched for related proteins containing substitutions for W in this position. Like VHZ, most of the sequences resulting from this search (Figure 1S-C) contain isoleucine. Two of these proteins, the S. solfataricus PTP and the CDC14 B PTP (containing an IPD-loop and FAD-loop, respectively) have been crystallized in both apo (PDBID 2I6I and 1OHC) and tungstate bound forms (PDB ID 2I6M and 1OHD). These structures reveal no difference in the loop position in ligand-free and ligand-bound forms, which contrasts with classical PTPs. Additionally, the crystal structures of Phytase (PDB IDs 2PS2 and 3D1H) obtained with no ligand bound has the identical closed loop position. These findings suggest the general acid loop is not a mobile element in these enzymes. To seek a further rationale for the apparent single conformation of the IPDloop in VHZ, we aligned the VHZ-VO₃ complex with the YopH WT ligand-free structure (PDB ID1YPT) where the loop occupies its open conformation. The alignment revealed that, were the IPD loop of VHZ to adopt an open conformation, D65 would clash with the anionic side chain of E44, which corresponds to the smaller and neutral S287 in YopH

(Figure 2S). The *S. solfataricus* PTP and Phytase contain analogous E37 and E132 residues in this location. In all, there is no evidence or likelihood that these enzymes undergo conformational changes in their general acid loop. We conclude that, although VHZ is likely related to the classical PTPs in other structural aspects and in substrate preference, its IPD loop probably does not undergo conformational changes in the process of catalysis.

The shape of the active site

The active site width and depth are primary factors defining substrate specificity in the PTP family. In Figure 5 the active sites of two DSPs (CDC14B (A) and VHR (B)), the classical PTP YopH (C), and VHZ(D) are rendered as surface models. Two peptide substrates containing pY and pS are modeled in the catalytically favorable orientation. In this position the equatorial oxygens are stabilized by hydrogen bonds from the P-loop, and the scissile phosphate monoester oxygen is located within hydrogen bond distance of the general acid. These common determinants allow one to model substrates into the active sites of PTPs and DSPs with little ambiguity. Atypical DSPs are characterized by shallow and/or broad active sites that allow them to accommodate the aliphatic side chains of pS (2.63Å distance between phosphorus and the alpha-carbon) and pT (3.76 Å), in addition to longer pY (7.44Å) residues (Figure 5 A and B). In contrast, classical PTPs have a deep and narrow active site pocket that accommodates pY-containing peptides (Figure 5 C and D). Modeling shows that the pT and pS side chains of a polypeptide substrate are too short to reach the nucleophilic cysteine at the bottom of the active site, consistent with their lack of activity by classical PTPs, although low activity with monomeric alkyl phosphate esters has been reported (50). With its shallower active site depth of 6 Å, the DSP VHR already displays a 2000-fold preference for pY over pS/pT-containing substrates. The active site of VHZ is deeper than that of VHR and is close to those of classical tyrosine specific PTPs (Figure 5 B, C, D). The depth of the active site is a required but not exclusive factor that provides PTPs with the ability to discriminate between aliphatic and aromatic substrates. For example, CDC14B reveals a deep 8Å active site crevice but specifically targets cyclin-dependent kinases phosphorylated on serine (51). This ability is provided by the greater width of its active site, which renders dual specificity to some enzymes in the family. The active site of VHZ is both narrow and deep, similar to classical PTPs. This provides a structural rationale for the absence of activity toward pS and pT-containing peptides observed in the substrate screen.

In conclusion, a new structure of VHZ complexed with the physiologically relevant inhibitor vanadate has been solved. This structure reveals important structural evidence that, together with the results of broad phosphopeptide screening, allows the following conclusions to be made:

- **a.** VHZ is a viable catalyst for the hydrolysis of pY-containing peptides. In contrast, no activity toward pS or pT residues was observed. This tendency is that of a classical PTP rather than a DSP.
- **b.** Classical PTPs have a deep and narrow active site cavity consistent with the observed specificity for pY-containing substrates. Atypical DSPs have a broader and shallower active site in order to accommodate phosphoserine/threonine side chains. The active site of VHZ more closely resembles those of classical PTPs.
- c. VHZ lacks the secondary positively charged binding site characteristic of atypical DSPs that confers a preference for bis-phosphorylated substrates (30). A conserved arginine residue characterizes this binding site. A correspondent R129 residue in VHZ is in an analogous position as in the secondary binding pockets of atypical DSPs (30), but it is blocked from the surface by the N7 side chain.

d. In classical PTPs, a structural water molecule seen in crystal structures that stabilizes the position of the general acid loop. No such water molecule is observed in DSP structures, where this structural role is fulfilled by the asparagine residue from the N-loop and a serine residue from the P-loop. As a result of differences in the positioning of the general acid loop, protonation of the leaving group occurs from different angles. In both respects VHZ resembles PTPs rather than DSPs.

e. The Q-loop motif containing two catalytically important glutamines (QXXXQ) is present in all classical PTPs. Atypical DSPs contain the N-loop in the analogous position. Its conserved PNXXF motif stabilizes the general acid and defines the wide and shallow active site of atypical DSPs. Analogous to the classical PTPs, the Q-loop in VHZ contains E134 and Q138 residues superimposable with Q446 and Q450 in YopH. It is concluded that the Q-loop in VHZ operates as in classical PTPs.

While sharing a number of similarities, VHZ also bears several important distinctions from the classical PTPs:

- a. In addition to the catalytic domain, classical PTPs contain multiple domains defining substrate specificity and subcellular localization. The VHZ protein is comprised of only a catalytic domain, and lacks the N-terminal extension containing a substrate recognition loop found in all classical PTPs (26, 52).
- b. The general acid IPD-loop in VHZ, unlike the WPD-loop in classical PTPs, is not mobile.

On total, the data suggest that VHZ does not fit the profile of an atypical DSP, but rather is structurally and functionally more closely related to tyrosine-specific PTPs. However, the absence of a substrate recognition loop and additional domains is not consistent with its placement as either a receptor or non-receptor type classical PTP. It is similar in size to the Class II low-molecular weight (LMW) phosphatases which display the same phosphotyrosine specificity; however, significant sequence, functional and structural distinctions exist between the LMW family and VHZ (53, 54). We propose that a separate category designated "atypical PTPs", shown in the shaded box in Figure 6, is more appropriate for VHZ. Other analogous phosphatases, including that from *S. solfataricus*, and others identified in section C of Figure 1S, may also prove to belong in this category.

PTPs are classified based on the basis of sequence similarity and substrate specificity, as well as protein size and subcellular localization. Structural and functional distinction between classical PTPs and atypical PTPs can be used to discriminate between the two subfamilies. The identification and proper classification of VHZ serves will assist subsequent research into its biological role and its physiological substrates.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

DSP dual specificity phosphatase
PTP protein tyrosine phosphatase
VHZ VH1-related member Z
VHR VH1-related member R
YopH Yersinia outer protein H

VSP voltage sensor (sensing) protein

PTEN phosphatase and tensin homologue

KAP1 kinase associated phosphatase

PhyA phytase A

SsoPTP Sulfolobus solfataricus protein tyrosine phosphatase

TEV protease tobacco etch virus protease

MBP myelin basic proteinPKA protein kinase A

MAPK mitogen activated protein kinase

ERK Extracellular signal-regulated kinase

JNK c-Jun N-terminal

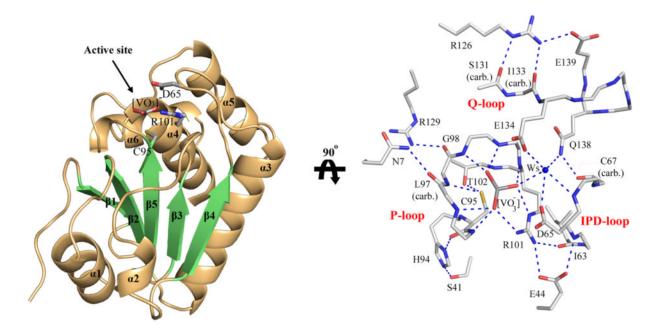


Figure 1. Structure of the VHZ-VO $_3$ complex. Cartoon representation of the VHZ-VO $_3$ structure reveals a classical PTP α/β fold with central region composed of β sheet (green) surrounded by α helices (brown). The important active site residues and hydrogen bond network (dashed lines) surrounding the VO $_3$ oxyanion are shown.

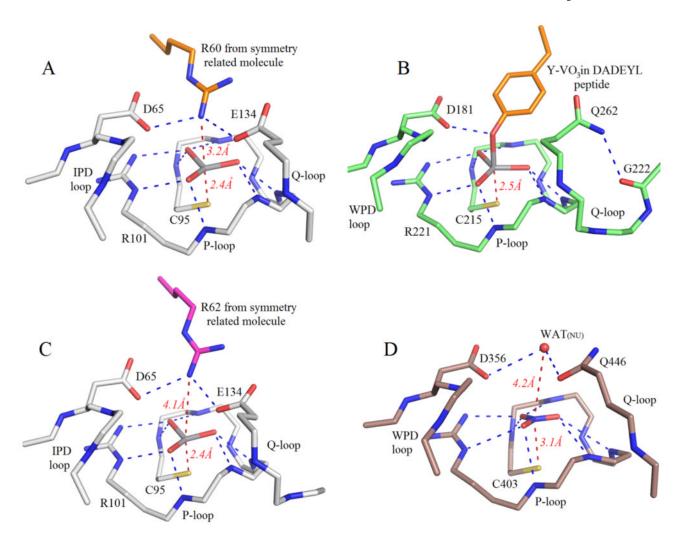


Figure 2

Comparison of the VHZ-VO₃ complex with analogous PTP1B structures. (A) The VO₃ molecule at the VHZ active site in Chain A coordinated by P-loop hydrogen bonds. The structural water Ws shown in Figure 1 has been omitted for the sake of clarity. Hydrogen bond distances (in *red*) are in Å. The vanadium-sulfur distance is similar to that observed in PTP1B structure PDBID 3I7Z (shown in B) with a bound vanadyl peptide. The R60 side chain belongs to the symmetry related VHZ molecule trapped near the active site. (B) A transition state analog structure for the first catalytic step of PTP1B (PDBID 3I7Z), consisting of the enzyme bound to the tyrosyl vanadyl ester of the peptide DADEYL; (C) The VO₃ moiety at the VHZ active site in Chain B. (D) A crystal structure of YopH with bound nitrate, taken as a transition state analogue for the second catalytic step of the PTP mechanism (PDBID 1YPT). The R62 side chain nitrogen atom from a symmetry related VHZ molecule near the active site in Chain B in (C) assumes a position that is analogous to the nucleophilic water molecule (WAT_{NII}).

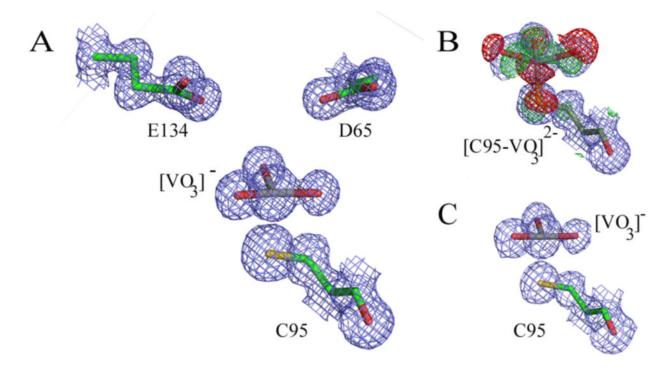


Figure 3. The electron density at the VHZ active site reveals a non-covalently bound vanadate molecule. (A) 2Fo-Fc (blue) contoured at 1.5 σ around catalytic C95, metavanadate, and neighboring residues in the refined model. The arginine from the symmetry related molecule was omitted for the sake of clarity. (B) 2Fo-Fc (1.5 σ) (blue) and Fo-Fc ($\pm 3.0 \sigma$) maps (green is positive; red is negative) produced after refining with a covalently bound cysteinyl-vanadate model. (C) An unbiased composite omit map contoured at 1.5 σ in the same orientation as (A).

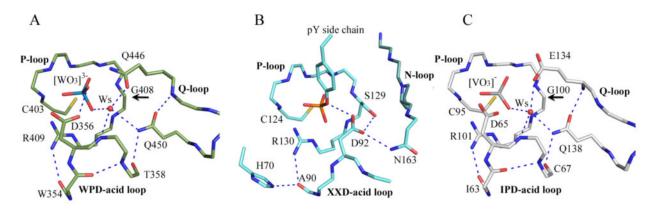


Figure 4.

Important distinctions between atypical DSPs and classical PTPs. The catalytic general acids are D356, D92 and D65 in YopH, VHR and VHZ respectively; all structures are viewed at the same angle in relation to the structurally superimposable P-loop. Topology and hydrogen bond network of the general acid loop and Q-loop in YopH-1YTW (A), VHR-1J4X (B) and VHZ-VO₃ (C) reveal important distinctions between classical PTPs (represented by YopH) and atypical DSPs (represented by VHR): 1) Stabilization of general acid WPD-loop in classical PTPs (Panel A) and atypical DSPs (Panel B) is obviously different. In particular, the structural water (Ws) provides stabilization to the general acid loop in YopH and VHZ, but is never observed in atypical DSPs. In VHR (and other atypical DSPs) the general acid loop is stabilized by the cluster of highly conserved serine/asparagine residues (\$129/N163 in VHR, Panel B). As a result, the position of the general aspartic acid in PTPs and DSPs is different and the leaving group protonation occurs at different angles; 2) Classical PTPs use invariant Q-loop residues (Q446 and Q450, Panel A) to orient the incoming nucleophilic water (not shown in the figures). The analogous E134 and Q138 residues are present in VHZ (Panel C). The analogous region in VHR, named N-loop, has no analogous residues and bears the invariant structural asparagine (N163, Panel B); 3) Glycine residue in YopH (G408, Panel A) and VHZ (G100, Panel C) provides the important access of Q446 and E134 into the active site, whereas the invariant structural serine is present in VHR (S129, Panel B) and other atypical DSPs.

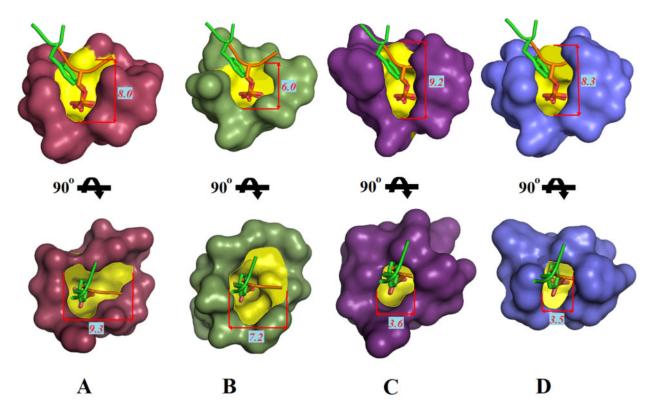


Figure 5.

The effect of active site width and depth, which explains the substrate selectivity observed in different PTP subfamilies exemplified by: A) CDC14B (DSP), B) VHR(DSP), C) YopH (PTP) and D) VHZ (blue). The active site dimensions are presented in angstroms. Phosphoserine (orange) and phosphotyrosine (green) containing peptides are modeled in the active sites such as the P-loop stabilized phosphate moiety is in the catalytic proximity to the nucleophilic cysteine, and the scissile bridging oxygen on the leaving group is within hydrogen bond distance from the catalytic general acid. The importance of the active site depth on the substrate selectivity is illustrated in the top row, whereas the bottom row reveals the effect of the width. The backbone of phospho serine - containing peptide in C and D is buried below the protein surface indicating it clashes with several residues on the edge of the active site. At the same time the binding of the phospho tyrosine containing peptide reveals no such problem due to the longer side chain. The importance of this clash for the substrate selectivity is consistent with the reported ability of classical enzymes to catalyze monomeric alkyl phosphate esters but not when a part of a peptide (50).

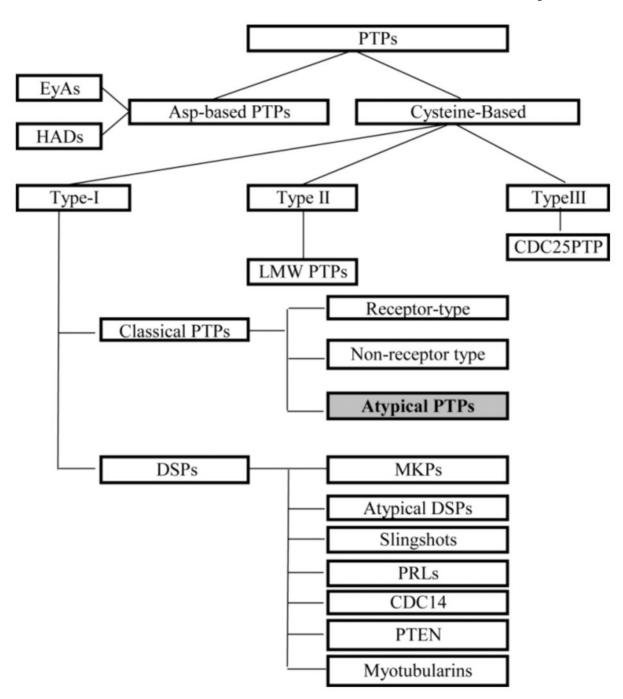


Figure 6.

A classification of PTP family members (1). On the basis of early reports VHZ has been suggested to belong to the atypical DSP group. The evidence reported here suggests VHZ is structurally and functionally more closely related to classical tyrosine-specific PTPs. However, the absence of a substrate recognition loop and additional domains is not consistent with its placement as either a receptor or non-receptor type classical PTP. We propose that a separate category designated "atypical PTPs", shown in the shaded box, is more appropriate for VHZ and probably for similar enzymes in Figure 1S.

Table 1

Data collection and refinement statistics

	VHZ-VO3		
Data collection	SSRL		
Beamline	9-2		
Wavelength (Å)	0.97946		
Resolution range (outer shell) (Å)	50.00-1.15 (1.19 -1.15)		
No. of reflections	20.00 1112 (1115 1112)		
Unique	94,449		
Total	1,237,514		
Average redundancy	13.1 (12.2)		
Mean $I/\sigma(I)$	31.3 (3.8)		
Completeness (%)	99.6 (99.4)		
R _{svm} (%)	6.8 (66.6)		
Space group	P 2 ₁ 2 ₁ 2 ₁		
Unit cell dimensions (a, b, c (Å); $\alpha = \beta = \gamma 90^\circ$)	33.0, 79.9, 99.7		
Refinement	33.0, 73.0, 73.7		
R _{work} /R _{free} (%)	12.8/14.5		
Atoms in the structure	5312		
Protein	4838		
Waters	466		
Ligands/ions	8		
Average <i>B</i> -factors (Å ²)			
Protein	13.6		
Water	26.6		
R.M.S.D. bond (Å)/angle (degrees)	0.008/1.245		
Protein geometry			
Ramachandran favored (%)	96.07%		
Ramachandran outliers (%)	0.66		
Rotamer outliers (%)	1.57%		
Protein Data Bank entry	4ERC		

Table 2

Kinetic constants for hydrolysis of two substrates selected from the substrate screen of differing percentages of hydrolysis. The last column shows the percent of hydrolysis observed in the long time course general substrate screen.

Peptide Sequence	$k_{\rm cat}$ (s ⁻¹)	K _M (mM)	$10^{-3}k_{\rm cat}/{\rm K_M}~({\rm M}^{-1}{\rm s}^{-1})$	% hydrolysis
QREAEpYEPETV	0.56 ± 0.02	1.9 ± 0.2	0.3	100%
DADEpYLIPQQG	0.53 ± 0.06	4.2 ± 0.4	0.12	50%