PrpZ, a *Salmonella enterica* serovar Typhi serine/threonine protein phosphatase 2C with dual substrate specificity

Sio Mei Lai and Hervé Le Moual

Department of Microbiology and Immunology, McGill University, 3775 University Street, Montréal, Québec, Canada H3A 2B4

Genes encoding eukaryotic-type protein kinases and phosphatases are present in many bacterial genomes. An ORF encoding a polypeptide with homology to protein phosphatases 2C (PP2Cs) was identified in the genomes of *Salmonella enterica* serovar Typhi strains CT18 and Ty2. This protein, termed PrpZ, is the first PP2C to be identified in enterobacteria. Analysis of the amino acid sequence revealed two distinct domains: the N-terminal segment containing motifs of the catalytic domain of PP2Cs and the C-terminal segment with unknown function. PrpZ was expressed in *Escherichia coli* as a histidine-tagged fusion protein (PrpZHis) and the purified protein was analysed for its ability to dephosphorylate various substrates. Using p-nitrophenyl phosphate as a substrate, optimal PrpZHis activity was observed at pH 9–5, with a strong preference for Mn2+ over Mg2+. Activity of PrpZHis was inhibited by EDTA, sodium fluoride, sodium phosphate and sodium pyrophosphate but unaffected by okadaic acid, indicating that PrpZ is a PP2C. Using synthetic phosphopeptides as substrates, PrpZHis could hydrolyse phosphorylated serine, threonine or tyrosine residues, with the highest catalytic efficiency (kcat/Km) for the threonine phosphopeptide. With phosphorylated myelin basic protein (MBP) as the substrate, Mn2+ was only twofold more efficient than Mg2+ in stimulating PrpZHis activity at pH 8–0. The ability of PrpZHis to remove the phosphoryl group from phosphotyrosine residues was confirmed by measuring the release of inorganic phosphate from phospho-Tyr MBP. Together, these data indicate that PrpZ has all the features of a PP2C with dual substrate specificity *in vitro*.

**INTRODUCTION**

Reversible protein phosphorylation through the combined action of kinases and phosphatases is the most common mechanism for regulating protein function in both prokaryotes and eukaryotes (Hunter, 1995). In bacteria, phosphorylation usually occurs on histidine and aspartate residues through two-component regulatory systems (Stock et al., 1989). Phosphorylation on serine, threonine and tyrosine residues, which has long been considered to be specific to eukaryotes, has also been found to occur in bacteria (Wang & Koshland, 1978). Although some bacterial Ser/Thr or Tyr protein kinases or phosphatases, such as the isocitrate dehydrogenase kinase/phosphatase and the Ptk protein tyrosine kinase of *Acinetobacter johnsonii*, have no apparent eukaryotic counterpart (Doublet et al., 1989; LaPorte et al., 1989), others share sequence similarity with their eukaryotic counterparts. The first bacterial gene encoding a eukaryotic-type protein kinase (*pkn1*) was cloned from the Gram-negative soil bacterium *Myxococcus xanthus* (Muñoz-Dorado et al., 1991). Recently, genomics has revealed that most bacterial genomes contain at least a few genes encoding eukaryotic-type protein kinases and phosphatases (Kennelly, 2002, 2003; Shi et al., 1998; Shi, 2004).

Ser/Thr protein phosphatases are divided into two distinct families, the phosphoprotein phosphatases (PPPs) and the Mg2+ or Mn2+-dependent protein phosphatases (PPMs), based on the presence of signature motifs in their amino acid sequences (Barford et al., 1998; Cohen, 1989). Although they lack sequence similarity, PPPs and PPMs share similar protein architecture and contain two metal ions (Mg2+ and/or Mn2+) in their active site. PPMs can be distinguished from PPPs on the basis of their resistance to the inhibitor okadaic acid (Barford et al., 1998). Whereas the PPP family is divided into three distinct subfamilies, protein phosphatases 1, 2A and 2B, the PPM family consists only of protein phosphatases 2C (PP2Cs). The catalytic

**Abbreviations:** BME, β-mercaptoethanol; MBP, myelin basic protein; PNP, p-nitrophenol; PNPP, p-nitrophenyl phosphate; PP2C, protein phosphatase 2C; PPM, Mg2+ or Mn2+-dependent protein phosphatase; PPP, phosphoprotein phosphatase.
domain of PP2Cs consists of 250–300 amino acid residues and contains 11 conserved motifs (Bork et al., 1996). The three-dimensional structure of the human PP2Cx revealed that these motifs contain highly conserved aspartate residues that are involved in the binding of the two metal ions. A metal-bound water molecule has been proposed to act as the nucleophile that attacks the substrate phosphoryl group (Das et al., 1996).

PP2Cs are distributed unevenly among bacterial species. The genomes of Streptomyces coelicolor A3(2) and Streptomyces avermitilis contain 49 and 48 PP2C genes, respectively (Shi & Zhang, 2004). The Bacillus subtilis and Synechocystis sp. strain PCC 6803 genomes contain five and eight PP2C genes, respectively (Kennelly, 2002; Shi, 2004). In contrast, no PP2C gene has been identified in the genomes of Escherichia coli or Salmonella enterica serovar Typhimurium strain LT2. Although some bacterial PP2Cs have been functionally characterized, the function of most of these proteins remains elusive. The SpoIIIA anti-anti-sigma factor (Duncan et al., 1995). In B. subtilis, the general stress response is controlled by two PP2Cs, RsbP and RsbU, that regulate the phosphorylation status of the RsbV anti-anti-sigma factor (Vijay et al., 1995). The SpoIIE PP2C of B. subtilis regulates sporulation by promoting the dephosphorylation of the SpoIIAA anti-anti-sigma factor (Duncan et al., 1995). In B. subtilis, the general stress response is controlled by two PP2Cs, RsbP and RsbU, that regulate the phosphorylation status of the RsbV anti-anti-sigma factor (Vijay et al., 2000). In Moxococcus xanthus, the Pph1 PP2C controls vegetative growth and development by interacting with the Ser/Thr kinase Pkn5 (Treuner-Lange et al., 2001). In Synechocystis sp. strain PCC 6803, the PphA PP2C mediates dephosphorylation of the PII signal transduction protein controlling nitrogen and carbon assimilation (Irmler & Forchhammer, 2001; Ruppert et al., 2002). The Stp1 PP2C of Streptococcus agalactiae has been shown to dephosphorylate an inorganic pyrophosphatase (Rajagopal et al., 2003). In Mycobacterium tuberculosis, the PstP PP2C (also known as MstP) plays a role in regulating cell division by dephosphorylating the Ser/Thr kinases PknA and PknB (Boitel et al., 2003; Chopra et al., 2003). Thus, bacterial PP2Cs control many diverse signalling pathways.

Although no PP2C has been identified in enterobacteria, eukaryotic-type protein phosphatases belonging to other families have been characterized. The PrpA and PrpB proteins of E. coli and S. enterica are PPPs that regulate transcription of the htrA gene through the CpxR/CpxA two-component regulatory system (Missiakas & Raina, 1997; Shi et al., 2001). The S. enterica StpP and Yersinia YopH proteins, which are delivered into host cells by a type III secretion system, possess a C-terminal tyrosine phosphatase domain that modulates the host-cell response to infection (Bliska et al., 1991; Fu & Galan, 1999). The Wzd protein is a low-molecular-mass protein tyrosine phosphatase that dephosphorylates the Wzc protein tyrosine kinase involved in the synthesis and export of exopolysaccharides in E. coli (Vincent et al., 1999).

Recently, the genomes of S. enterica serovar Typhimurium strain LT2, serovar Typhi strain CT18 and serovar Typhi strain Ty2 have been sequenced (Deng et al., 2003; McClelland et al., 2001; Parkhill et al., 2001). Comparative genomics showed that about 600 genes are unique to serovar Typhi (Parkhill et al., 2001). In this study, we identified an ORF (sty4824 or t4521) that is present in both serovar Typhi CT18 and Ty2 but absent from serovar Typhimurium LT2. This ORF encodes a putative protein with sequence similarity to PP2Cs. We designated this gene prpZ and its product PrpZ. We characterized the enzymic properties of the recombinant PrpZHIS protein and showed that it has all the hallmarks of PP2Cs. In addition, we provide evidence that PrpZHIS has broader substrate specificity than most PP2Cs, since it shows unusual reactivity towards phosphotyrosine residues.

### METHODS

#### Bacterial strains and growth conditions.

*E. coli* strain XL-1 Blue (Stratagene) was used for all DNA manipulations. *E. coli* strain BL21(DE3) (Novagen) was used for protein expression. *S. enterica* serovar Typhi strain CT18 was kindly provided by Dr France Daigle, Université de Montréal, Canada. Bacterial cultures were grown at 37 °C with aeration in Luria–Bertani broth medium containing 100 µg ampicillin ml⁻¹, when appropriate.

#### Cloning of the *prpZ* gene.

Chromosomal DNA was isolated from an overnight culture of *S. enterica* serovar Typhi strain CT18 using Genomic-tips (Qiagen). The *prpZ* gene, which corresponds to ORF sty4824 of the *S. enterica* serovar Typhi CT18 genome sequence, was amplified by PCR using an upstream primer carrying an Ndel restriction site (5′-CTCTCCACATGTGGACGAAGCATCTGTCGCTG-3′) and a downstream primer carrying an Xhol site (5′-CCGGCTCGAGATCTTTCATTTTTCGGTCTCATCTCGTGG-3′). The PCR was performed using the Pwo DNA polymerase (Roche Diagnostics), which possesses proofreading activity. The amplified 1200-bp DNA fragment was digested with restriction enzymes Ndel and Xhol and cloned into pET-20b(+) (Novagen) that had been digested with the same enzymes. The insert was sequenced and the translated sequence was identical to ORF sty4824 of *S. enterica* serovar Typhi strain CT18 (Parkhill et al., 2001). The resulting plasmid (pET-PrpZHIS) encodes the PrpZHIS protein harbouring a C-terminal tag of six histidine residues.

#### Expression and purification of the recombinant PrpZHIS protein.

Cultures of *E. coli* BL21(DE3) cells transformed with the pET-PrpZHIS plasmid were induced with 1 mM IPTG at an OD₆₀₀ of 0-8. After 1 h of induction, cells were harvested by centrifugation at 5000 g for 20 min and resuspended in BugBuster protein extraction reagent (Novagen) supplemented with Benzoate (Novagen) according to the manufacturer’s instructions. The suspension was incubated on a shaking platform for 10 min at room temperature and centrifuged at 16 000 g for 20 min. The supernatant, containing PrpZHIS, was applied to a HitTrap chelating column (Amersham Biosciences) charged with Ni²⁺⁺ and equilibrated in binding buffer (20 mM Tris/HCl, pH 8-0, 500 mM NaCl and 5 mM imidazole). After extensive washing, the protein was eluted with a linear gradient of 5–200 mM imidazole in binding buffer. Fractions containing PrpZHIS were pooled, dialysed against final buffer (20 mM Tris/HCl, pH 8-0, 10% v/v glycerol) and stored at −70 °C. Under these conditions, approximately 1 mg pure protein was obtained per litre of bacterial culture. Protein concentrations were measured with the Bio-Rad Protein Assay Kit, using BSA as the standard. The PrpZHIS protein was visualized on SDS-PAGE gels stained with Coomassie blue. For Western blotting, the recombinant protein was detected using an anti-His-Tag monoclonal antibody (Novagen) and the His-Tag AP LumiBlot system (Novagen) according to the
Phosphatase assays using PNPP as a substrate. Tablets of PNPP (Sigma) were dissolved immediately before use. Unless specified, reactions were performed in 200 μl assay buffer containing 50 mM CAPS/NaOH (pH 9–5), 0–0.02 % (v/v) β-mercaptoethanol (BME), 10 mM PNPP and 5 mM MnCl₂ or MgCl₂. Reactions were initiated by adding purified PrpZH₃ and incubated for 30 min at 37 °C. The release of p-nitrophenol (PNP) was followed by measuring the A₄05 using a PowerWave microplate spectrophotometer (Bio-Tek Instruments). The molecular absorbance coefficient of PNP under these assay conditions was 17 800 cm⁻¹ M⁻¹ (Li, 1984). One unit of activity was defined as the release of 1 nmol PNP min⁻¹ at 37 °C. Specific activity was defined as the number of enzyme units (μg protein)⁻¹. Under these conditions, release of PNP was linear for about 60 min. The optimal pH for PNPP hydrolysis was determined with 50 mM concentrations of the following buffers: MES/NaOH (pH 6–5), HEPES/NaOH (pH 7–0–7–5), Tris/HCl (pH 8–8–5) and CAPS/NaOH (pH 9–11). The optimal concentrations of Mn²⁺ or Mg²⁺ for enzyme activity were determined in 50 mM CAPS/NaOH (pH 9–5) by varying the concentration of MnCl₂ or MgCl₂ in the assay buffer. The effect of various inhibitors on PrpZH₃ activity was assayed in the presence of 5 mM MnCl₂ in 50 mM Tris/HCl (pH 8–5) to prevent precipitation of some inhibitors at higher pH. Kinetic parameters of PrpZH₃ with PNPP as the substrate were determined in 50 mM CAPS/NaOH (pH 9–5) in the presence of 5 mM MnCl₂ using substrate concentrations ranging from 0.1 to 25 mM. Data were fitted to the Michaelis–Menten equation by non-linear regression analysis.

Phosphatase assays using phosphopeptides. Phosphatase activity of PrpZH₃ was measured by using various synthetic phosphopeptides as substrates. Phosphorylated peptides were purchased from Promega [RRA(pT)VA] and Upstate Biotechnology [RRA(pS)VA and RRLIEDAE(pY)AARG]. The release of inorganic phosphate (Pₐ) was monitored by measuring the absorbance of the molybdate–malachite green–phosphate complex using the Serum/Threonine Phosphatase assay system (Promega). Reactions were performed in 50 μl assay buffer containing 50 mM CAPS/NaOH (pH 9–5), 0–0.02 % (v/v) BME, 1 mM phosphate peptide and 5 mM MnCl₂. Reactions were initiated by adding 40 ng purified PrpZH₃ incubated for 30 min at 37 °C and stopped by adding 50 μl of a molybdate dye/additive mixture. After 15 min incubation at room temperature to allow colour development, the A₄₀₀ was measured. The amount of Pᵢ generated was determined by using a standard curve. Optimum pH for enzyme activity against the phosphothreonine peptide was determined as described above.

Phosphatase assays using phosphorylated myelin basic protein (MBP). MBP was radiolabelled at Ser/Thr residues using the cAMP-dependent protein kinase (PKA) (New England Biolabs). Reactions were performed in the presence of [γ-³²P]ATP as specified by the manufacturer. Phosphorylated MBPs were purified by TCA precipitation and extensive dialysis against buffer containing 25 mM Tris/HCl (pH 7–5), 0–1 mM EDTA, 2 mM DTT and 0–0.1 % (v/v) Brij 35. Assays were performed by incubating purified PrpZH₃ with 1–8 μM [³²P]MBP in 50 mM Tris/HCl (pH 8–0), 0–0.02 % (v/v) BME and 5 mM MnCl₂. At various time points, reactions (15 μl) were stopped by the addition of 4 x Laemmli SDS sample buffer (250 mM Tris/HCl, pH 6–8, 8 % SDS, 40 % glycerol, 0–0.2 % bromphenol blue, 4 % BME). Reaction products were applied to 15 % SDS-PAGE gels. Gels were dried under vacuum and exposed to a phosphor screen (Kodak). Radiolabelled products were visualized using an FX scanner (Bio-Rad) and quantified by image analysis using the Quantity One software (Bio-Rad). Optimal pH for PrpZH₃ activity against [³²P]MBP was determined as described above. For the determination of kinetic parameters, initial velocities were measured at substrate concentrations ranging from 0.5 to 50 μM. Reactions were stopped by the addition of 20 % TCA. Following centrifugation, the release of radiolabelled Pᵢ was determined by scintillation counting.

RESULTS

PrpZ is a member of the PPM family

The prpZ gene (ORF sty4824) was identified from the S. enterica serovar Typhi CT18 genome sequence (Parkhill et al., 2001). It encodes the PrpZ protein, which consists of 387 amino acid residues with an estimated molecular mass of 42.7 kDa and an isoelectric point of 5.6. Analysis of the PrpZ amino acid sequence revealed the presence of two domains. The N-terminal domain, which consists of approximately 260 amino acid residues, shows homology to PP2Cs (Fig. 1a). The C-terminal domain, consisting of 127 amino acid residues, shares no homology with any known protein (Fig. 1a). A BLASTP sequence similarity
search using the PrpZ N-terminal domain (residues 1–260) identified more than 100 bacterial homologues from both Gram-negative and Gram-positive species. The amino acid sequence of the PrpZ phosphatase domain is 23–28% identical to the various bacterial homologues and 18% identical to the human PP2Cα. Multiple sequence alignments reveal that PrpZ contains the motifs that are conserved among PP2Cs (data not shown) (Bork et al., 1996). Importantly, these conserved motifs contain the invariant residues involved in the binding of both the two metal ions (Asp 42, Asp 66, Asp 202 and Asp 240) and the phosphate group of substrates (Arg 37) (Das et al., 1996). Thus, sequence similarity and conservation of the essential catalytic residues between serovar Typhi PrpZ and human PP2Cα suggest that PrpZ is a PP2C of the PPM family.

Cloning, expression and purification of PrpZHis

To determine whether the prpZ gene encoded a functional protein phosphatase, the ORF corresponding to the PrpZ protein was PCR-amplified and cloned into expression vector pET20b(+) to generate plasmid pET-PrpZHis. The recombinant PrpZHis protein consists of the complete coding sequence of PrpZ fused to a C-terminal tag of six histidine residues. E. coli BL21(DE3) cells transformed with plasmid pET-PrpZHis were analysed for production of PrpZHis by SDS-PAGE. Upon IPTG induction, a protein that migrated with an apparent molecular mass of 51 kDa was detected in the soluble fraction of cell lysates (Fig. 1b, lane 2). To confirm that the overproduced protein corresponded to PrpZHis, a Western-blot analysis was performed using a monoclonal antibody directed against the C-terminal His tag. As shown in Fig. 1(c), the PrpZHis protein was detected by the antibody and migrated with an apparent molecular mass of 51 kDa. The calculated molecular mass of PrpZHis (43-8 kDa) is less than the apparent molecular mass deduced from SDS-PAGE (51 kDa). Following purification to apparent homogeneity by Ni2+-NTA chromatography (Fig. 1b, lane 3), the PrpZHis protein was subjected to MS analysis. The mass determined by MS (43 827 Da) was identical to the theoretical mass of PrpZHis (43-8 kDa), indicating that PrpZHis runs aberrantly on SDS-PAGE.

Phosphatase activity of PrpZHis using PNPP or a phosphothreonine peptide as substrates

PP2Cs have been shown to require divalent cations such as Mg2+ or Mn2+ for activity (Cohen, 1989). First, we determined the optimal pH for PrpZHis activity in the presence of 5 mM MnCl2 or MgCl2 using PNPP as a substrate. In the presence of MnCl2, the pH profile showed a maximum at pH 9-5 (Fig. 2a). In comparison, when MgCl2 was substituted for MnCl2, PrpZHis activity at pH 9-5 was reduced 40-fold (Fig. 2a). In the presence of 5 mM MgCl2, PrpZHis was essentially inactive at neutral pH and the optimal pH was 10-5 (Fig. 2a). To determine which divalent cations are effective in activating PrpZHis phosphatase activity, assays were performed in the presence of increasing concentrations of MgCl2, MnCl2, CaCl2 and ZnCl2. The release of PNP was greatly induced by the addition of Mn2+, with 2 mM MnCl2 stimulating half-maximal activity of PrpZHis (Fig. 2b). In contrast, Mg2+ barely stimulated PrpZHis activity (Fig. 2b). No activity was detected with Ca2+ or Zn2+ (data not shown). Thus, with PNPP as the substrate, PrpZHis shows a strong preference for Mn2+ over Mg2+ and its activity is optimal at alkaline pH.
To examine the phosphatase activity of PrpZHis further, we used a synthetic peptide substrate containing a phosphothreonine residue [RRA(pT)VA]. Enzymatic activity was determined by measuring the release of Pi as described in Methods. In the presence of 5 mM MnCl₂, maximum PrpZHis activity towards the phosphothreonine peptide was observed at pH 9.0 (Fig. 3a). Strikingly, we found that PrpZHis activity was stimulated to similar levels when 5 mM MgCl₂ was substituted for MnCl₂ (Fig. 3a). In the presence of 5 mM MgCl₂, the optimal pH for PrpZHis activity was 9.0–9.5. Maximal PrpZHis activity was achieved at 5 mM MnCl₂ or MgCl₂ (data not shown). These data indicate that Mn²⁺ and Mg²⁺ have a similar effect on the dephosphorylation of the phosphothreonine peptide. Thus, the metal-ion requirement of PrpZHis depends on the substrate used.

To confirm that PrpZHis is a PP2C, we examined the effect of various inhibitors of protein phosphatases, acid phosphatases and alkaline phosphatases on PrpZHis activity using PNPP or the phosphothreonine peptide as substrates (Table 1). Okadaic acid, a potent inhibitor of protein phosphatases 1 and 2A, did not inhibit PrpZHis activity. The same results were obtained for trifluoperazine, an inhibitor of protein phosphatases 2B, and ammonium molybdate, a general inhibitor of PPPs. Levamisole and sodium tartrate, which respectively inhibit alkaline phosphatases and acid phosphatases, had no effect on PrpZHis activity. As shown in Table 1, inhibition of PrpZHis was detected only with non-specific protein phosphatase inhibitors like sodium fluoride, sodium pyrophosphate and sodium phosphate when present at millimolar concentrations. As expected from a metal-dependent protein phosphatase, inhibition was also observed with EDTA. Altogether, these data confirm that PrpZHis has all the features of a PP2C.

**Fig. 3.** Dephosphorylation of synthetic phosphopeptides by purified PrpZHis. (a) Influence of pH on PrpZHis activity. The phosphatase activity of PrpZHis was measured under standard conditions using the peptide RRA(pT)VA (1 mM) as a substrate. PrpZHis (18 nM) was incubated in the presence of 5 mM MnCl₂ (●) or 5 mM MgCl₂ (■). Buffers are described in Methods. (b) Michaelis–Menten plot of phosphopeptide dephosphorylation against substrate concentration. The activity of PrpZHis was measured in 50 mM CAPS/NaOH (pH 9.5) in the presence of 5 mM MnCl₂. PrpZHis (18 nM) was incubated with RRA(pT)VA (●), RRA(pS)VA (○) or RRUJEDAE(pY)AARG (▲). Specific activity is defined as pmol P_i released min⁻¹ (μg protein)⁻¹.

**Table 1.** Effect of various inhibitors on the catalytic activity of PrpZHis

Assays were performed in 50 mM Tris/HCl (pH 8.5) in the presence of 5 mM MnCl₂ using 10 mM PNPP or 1 mM RRA(pT)VA as substrate.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Concentration</th>
<th>Relative activity (%) with:</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>PNPP</td>
<td>RRA(pT)VA</td>
</tr>
<tr>
<td>None (control)</td>
<td>—</td>
<td>100</td>
</tr>
<tr>
<td>Okadaic acid</td>
<td>0.1 μM</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>1 μM</td>
<td>128</td>
</tr>
<tr>
<td>Trifluoperazine</td>
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<tr>
<td>Ammonium molybdate</td>
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<td>125</td>
</tr>
<tr>
<td>Levamisole</td>
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</tr>
<tr>
<td>Sodium tartrate</td>
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<td>84</td>
</tr>
<tr>
<td></td>
<td>10 mM</td>
<td>89</td>
</tr>
<tr>
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<td>66</td>
</tr>
<tr>
<td></td>
<td>100 mM</td>
<td>37</td>
</tr>
<tr>
<td>EDTA</td>
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<td>66</td>
</tr>
<tr>
<td></td>
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<td>0</td>
</tr>
<tr>
<td>Sodium pyrophosphate</td>
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</tr>
<tr>
<td></td>
<td>5 mM</td>
<td>ND</td>
</tr>
<tr>
<td>Sodium phosphate</td>
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<td>21</td>
</tr>
<tr>
<td></td>
<td>25 mM</td>
<td>ND</td>
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</table>

ND, Not done (as the inhibitors would interfere with the molybdate–malachite green complex).
Table 2. Kinetic parameters of PrpZ<sub>His</sub> with different substrates

For PNPP and phosphopeptides, assays were performed in 50 mM CAPS/NaOH (pH 9.5) in the presence of 5 mM MnCl<sub>2</sub>. For MBP, assays were performed in 50 mM Tris/HCl (pH 8.0) in the presence of 5 mM MnCl<sub>2</sub>.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>K&lt;sub&gt;m&lt;/sub&gt; (mM)</th>
<th>k&lt;sub&gt;cat&lt;/sub&gt; (s&lt;sup&gt;-1&lt;/sup&gt;)</th>
<th>k&lt;sub&gt;cat&lt;/sub&gt;/K&lt;sub&gt;m&lt;/sub&gt; (s&lt;sup&gt;-1&lt;/sup&gt; mM&lt;sup&gt;-1&lt;/sup&gt;)</th>
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<tbody>
<tr>
<td>PNPP</td>
<td>5.7</td>
<td>7.4</td>
<td>1.3</td>
</tr>
<tr>
<td>RRA(pT)VA</td>
<td>0.13</td>
<td>2.5</td>
<td>19.2</td>
</tr>
<tr>
<td>RRA(pS)VA</td>
<td>0.24</td>
<td>0.9</td>
<td>3.7</td>
</tr>
<tr>
<td>RRLIEDAE(pY)AARG</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>(pS/T)MBP</td>
<td>0.019</td>
<td>1.1</td>
<td>5.73</td>
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<tr>
<td>(pY)MBP</td>
<td>0.0026</td>
<td>0.1</td>
<td>4.16</td>
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*K<sub>m</sub> was too high for accurate determination.

Substrate specificity of PrpZ<sub>His</sub>

To examine the substrate specificity of PrpZ<sub>His</sub>, we used synthetic peptide substrates containing a phosphothreonine [RRA(pT)VA], a phosphoserine [RRA(pS)VA] or a phosphotyrosine [RRLIEDAE(pY)AARG] residue. Kinetic parameters of PrpZ<sub>His</sub> for these synthetic peptides were determined (Table 2). The PrpZ<sub>His</sub> phosphatase showed a fivefold reduction in catalytic efficiency (k<sub>cat</sub>/K<sub>m</sub>) for the serine phosphopeptide, compared with the threonine phosphopeptide. This decreased catalytic efficiency for the phosphoserine peptide was due to both an increase in K<sub>m</sub> and a decrease in k<sub>cat</sub> (Table 2). These data indicate that PrpZ<sub>His</sub> displays a preference for the phosphothreonine peptide. As shown in Fig. 3(b), PrpZ<sub>His</sub> was able to release P<sub>i</sub> from the phosphothreonine peptide, which is not a usual substrate for PP2Cs. Because the phosphotyrosine peptide substrate was not present at saturating levels (i.e. K<sub>m</sub> > 1 mM), the kinetic parameters could not be determined with good accuracy. Compared with PNPP, we found that the phosphothreonine and phosphoserine peptides are better substrates for PrpZ<sub>His</sub> with k<sub>cat</sub>/K<sub>m</sub> values approximately one order of magnitude higher (Table 2). Overall, these data show that PrpZ<sub>His</sub> displays a preference for phosphothreonine residues and is also able to dephosphorylate phosphothreonine residues.

PrpZ<sub>His</sub> dephosphorylates phospho-Ser/Thr and phospho-Tyr MBP

To examine the ability of PrpZ<sub>His</sub> to dephosphorylate protein substrates, we performed phosphatase assays using [32P]MBP phosphorylated at either Ser/Thr residues or Tyr residues. First, we determined the optimal pH of the reaction by incubating [32P]MBP phosphorylated at Ser/Thr residues with purified PrpZ<sub>His</sub> in the presence of 5 mM MnCl<sub>2</sub> or MgCl<sub>2</sub> and measuring the release of [32P]P<sub>i</sub>. In the presence of MnCl<sub>2</sub>, optimal PrpZ<sub>His</sub> activity occurred at pH 8.0, since higher pHs induced precipitation of [32P]MBP (Fig. 4a). In the presence of MgCl<sub>2</sub>, optimal activity was observed at pH 9.5 (Fig. 4a). Interestingly, substantial amounts of [32P]MBP were dephosphorylated at neutral pH, regardless of the divalent cation present in the reaction (Fig. 4a). Similar pH optima were obtained for [32P]MBP phosphorylated at Tyr residues (data not shown). Substrate specificity of PrpZ<sub>His</sub> was further determined by measuring the dephosphorylation of [32P]MBP (Fig. 4b). **Fig. 4.** Dephosphorylation of [32P]MBP by PrpZ<sub>His</sub>. (a) Influence of pH on PrpZ<sub>His</sub> activity against phospho-Ser/Thr MBP. PrpZ<sub>His</sub> (15-6 nM) was incubated with 1-8 μM [32P]MBP in the presence of 5 mM MnCl<sub>2</sub> (●) or 5 mM MgCl<sub>2</sub> (▲). Buffers are described in Methods. After 10 min of incubation, reactions were stopped by the addition of 4 × Laemmli SDS sample buffer. Reaction products were analysed on 15% SDS-PAGE. Amounts of radiolabelled P<sub>i</sub> released were quantified with a PhosphorImager. (b) Kinetics of dephosphorylation of MBP phosphorylated either at Ser/Thr residues (●) or at Tyr residues (▲). PrpZ<sub>His</sub> (15-6 nM) was incubated with 1-8 μM [32P]MBP in 50 mM Tris/HCl (pH 8-0) in the presence of 5 mM MnCl<sub>2</sub>. At indicated time points, reactions were stopped by the addition of 4 × Laemmli SDS sample buffer and analysed as indicated in (a).
phosphorylated at Ser/Thr residues or Tyr residues over time (Fig. 4b). Assays were performed at pH 8 in the presence of 5 mM MnCl₂. In the absence of PrpZHis [³²P]MBP was stable for at least 20 min under these experimental conditions (data not shown). Addition of purified PrpZHis stimulated the dephosphorylation of phospho-Ser/Thr MBP as well as phospho-Tyr MBP (Fig. 4b). The initial rate of dephosphorylation of [³²P]MBP phosphorylated at Tyr residues was slightly slower than that of [³²P]MBP phosphorylated at Ser/Thr residues (Fig. 4b). Similar results were obtained when reactions were performed in the presence of MgCl₂ (data not shown). Dephosphorylation of [³²P]MBP was strictly dependent on the presence of Mn²⁺ or Mg²⁺, since the presence of 10 mM EDTA inhibited the reaction (data not shown). Kinetic parameters were also determined for [³²P]MBP phosphorylated at either Ser/Thr residues or Tyr residues. As shown in Table 2, the $k_{cat}/K_m$ value for phospho-Tyr MBP was slightly lower than that for phospho-Ser/Thr MBP. These data confirmed the ability of PrpZHis to dephosphorylate protein substrates phosphorylated at Tyr residues. Taken together, these data show that PrpZHis is a PP2C with dual substrate specificity.

**DISCUSSION**

In this study, we characterized the enzymic properties of PrpZ, a novel eukaryotic-type Ser/Thr protein phosphatase identified by the sequencing of the *S. enterica* serovar Typhi CT18 and Ty2 genomes (Deng et al., 2003; Parkhill et al., 2001). We showed that the purified recombinant PrpZHis protein is a functional protein phosphatase. PrpZHis displays the hallmarks of PP2Cs, as indicated by its amino acid sequence similarity to other PP2Cs, its Mn²⁺- or Mg²⁺-dependent enzymic activity and its insensitivity to okadaic acid. In addition, we found that PrpZHis possesses the ability to dephosphorylate phosphotyrosine residues, in vitro, which is unusual for a PP2C. PrpZ is the first PP2C to be identified in enterobacteria.

Comparison of PrpZHis reactivity towards different substrates (PNPP, phosphopeptides and phosphorylated MBP) revealed important differences with respect to pH range and metal-ion requirement. Using PNPP as a substrate, the highest specific activity of PrpZHis was observed at pH 9-5 in the presence of MnCl₂ (Fig. 2a). With the same substrate, alkaline pH optima were also observed for the *B. subtilis* PrpC and the *Synechocystis* PCC 6803 PpH A PP2Cs (Obuchowski et al., 2000; Ruppert et al., 2002). Although PrpZHis was barely active towards PNPP and the phosphothreonine peptide at neutral pH (Figs 2a and 3a), we found that it readily dephosphorylates [³²P]MBP at pH 7-5 (Fig. 4a). In good agreement, both the *B. subtilis* PrpC and the *Synechocystis* PCC 6803 PpH A PP2Cs were shown to dephosphorylate protein substrates at a pH close to 7-5 (Obuchowski et al., 2000; Ruppert et al., 2002). Thus, our data show that PrpZHis is active at physiological pH (pH 7-5) towards a protein substrate like [³²P]MBP.

Like other PP2Cs, PrpZHis activity is strictly dependent on Mn²⁺ or Mg²⁺ in vitro. Using PNPP as the substrate, PrpZHis showed a strong preference for Mn²⁺ (Fig. 2b). This preference for Mn²⁺ over Mg²⁺, with PNPP as a substrate, is similar to that of other PP2Cs like the Stp1 phosphatases of *Streptococcus agalactiae* and *Pseudomonas aeruginosa* (Mukhopadhyay et al., 1999; Rajagopal et al., 2003), the *B. subtilis* PrpC phosphatase (Obuchowski et al., 2000), the *Myxococcus xanthus* PpH1 phosphatase (Treuner-Lange et al., 2001), the *Synechocystis* PCC 6803 PpH A phosphatase (Ruppert et al., 2002) and the PstP (MstP) phosphatase of *Mycobacterium tuberculosis* (Chopra et al., 2003). Using the phosphothreonine peptide as a substrate, we found that Mg²⁺ is as effective as Mn²⁺ in catalysing substrate dephosphorylation (Fig. 3a). Similar differences in metal requirement were also observed for other bacterial PP2Cs. For example, Mg²⁺ was only twofold less effective than Mn²⁺ in stimulating the *Myxococcus xanthus* PpH1 activity towards phosphopeptides, whereas hydrolysis of PNPP was strictly Mn²⁺-dependent (Treuner-Lange et al., 2001). Altogether, these data show that information obtained with the substrate PNPP may not reflect the enzymic properties obtained with other substrates like phosphopeptides or phosphorylated proteins.

An important question is to identify the metal ion used in *vivo* by PrpZ. The intracellular concentration of Mg²⁺ (1-10 mM) is at least one order of magnitude higher than that of Mn²⁺ (10-100 µM) (Finney & O’Halloran, 2003). Based on our finding that both Mg²⁺ and Mn²⁺ can stimulate the dephosphorylation of [³²P]MBP at neutral pH (Fig. 4a), it appears most likely that Mg²⁺ is the main physiologically relevant metal ion for PrpZ activity *in vivo*. In enterobacteria, levels of intracellular Mn²⁺ have been reported to vary over two orders of magnitude, reaching the millimolar range under appropriate environmental conditions (Kehres & Maguire, 2003). Thus, it cannot be ruled out that increasing concentrations of intracellular Mn²⁺ may further stimulate PrpZ activity. Variations in the intracellular concentration of Mn²⁺ might be one of the mechanisms used *in vivo* to regulate the activity of PrpZ.

Using synthetic phosphopeptides, we showed that PrpZHis displays a preference for phosphothreonine over phosphoserine (Table 2). A similar preference for phosphothreonine residues has also been reported for the *Synechocystis* PCC 6803 PpH A and the *Myxococcus xanthus* PpH1 PP2Cs (Ruppert et al., 2002; Treuner-Lange et al., 2001). Unexpectedly, we found that PrpZHis also dephosphorylates the phosphothreonine peptide (Table 2). The ability of PrpZHis to dephosphorylate phosphothreonine was confirmed with MBP phosphorylated at Tyr residues (Fig. 4b). These data indicate that PrpZHis has dual specificity *in vitro*. In contrast, the *Mycobacterium tuberculosis* PstP (MstP) PP2C showed little or no activity with phosphothreonine protein substrates (Boitel et al., 2003; Chopra et al., 2003). To date, the PpH A protein of *Synechocystis* PCC 6803 is the only PP2C that has been reported to dephosphorylate...
phosphotyrosine residues in vitro (Ruppert et al., 2002). This ability to dephosphorylate phosphotyrosine residues has also been observed for other Ser/Thr phosphatases of the PPP family. For example, PrpE of B. subtilis and the protein Ser/Thr phosphatase encoded by bacteriophage λ showed activity towards phosphotyrosine (Barik, 1993; Iwanicki et al., 2002). Thus, until the physiological substrate(s) of PrpZ is identified and characterized, we cannot rule out the possibility that PrpZ might hydrolyse phosphotyrosine residues in vivo.

The physiological function of PrpZ is unknown. Like the S. enterica SptP and Yersinia YopH tyrosine phosphatases, PrpZ might be a type III effector protein injected into host cells through one of the two S. enterica type III secretion systems. This possibility is unlikely, because PrpZ does not contain the N-terminal domain of type III effector proteins that is essential for both targeting to the secretion apparatus and chaperone recognition (Smith et al., 2001). Alternatively, PrpZ might be a cytosolic protein that modulates the phosphorylation level of a still unknown protein substrate. This substrate might be a protein whose function is regulated through reversible phosphorylation by PrpZ and an opposing protein kinase. Many of the genes encoding bacterial PP2Cs are genetically linked with Ser/Thr protein kinase genes. Accordingly, two ORFs encoding proteins with homology to Ser/Thr protein kinases (sty4822 and sty4823) were found in the same genomic region as the prpZ gene. Thus, it is most likely that PrpZ and these two protein kinases are part of the same signalling pathway. Recently, a microarray study showed that transcription of the prpZ gene decreased by more than threefold upon cell exposure to hydrogen peroxide, suggesting that PrpZ might be involved in a signalling pathway controlling oxidative stress (Porwollik et al., 2003). Since prpZ and the neighbouring protein kinase genes are present in S. enterica serovar Typhi but absent from the closely related S. enterica serovar Typhimurium, understanding the roles of PrpZ and the opposing protein kinases is likely to provide important insights into differences in lifestyle and/or pathogenesis between serovar Typhi and serovar Typhimurium.

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