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Overproduction, purification, and biochemical characterization of the dual specificity H1 protein phosphatase encoded by variola major virus

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Abstract

Smallpox, a highly contagious infectious disease caused by the variola major virus, has an overall mortality rate of about 30%. Because there currently is no specific treatment for smallpox, and the only prevention is vaccination, there is an urgent need for the development of effective antiviral drugs. The dual specificity protein phosphatase encoded by the smallpox virus (H1) is essential for the production of infectious viral particles, making it a promising molecular target for antiviral therapeutics. Here, we report the molecular cloning, overproduction, purification, and initial biochemical characterization of H1 phosphatase, thereby paving the way for the discovery of small molecule inhibitors.
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Keywords: Smallpox; Variola major; Dual specificity phosphatase; H1 phosphatase; H1L

Smallpox was officially eradicated more than 20 years ago [1], but there remains a serious concern that undeclared stocks of the variola major virus may still exist and could be used as a bioterrorist weapon [2–4]. Although there is a vaccine, it is not without risks and serious side effects [5,6]. Consequently, there is an urgent need for effective antiviral drugs. In addition to providing potential therapy for infected people, the availability of antiviral drugs could decrease the risks associated with the smallpox vaccine by providing a treatment for vaccine-associated complications.

Essential viral enzymes have frequently proven to be good targets for antiviral drugs [7]. The dual specificity protein phosphatase (H1) encoded by the smallpox virus is essential for the formation of mature, infectious viral particles [8] and is one of a small number of variola proteins that have been identified as promising molecular targets for therapeutic intervention [7]. Dual specificity phosphatases (DSPs)[1] are

operationally defined as members of the protein tyrosine phosphatase (PTPase) superfamily that have the capability to dephosphorylate both phosphotyrosine and phosphoserine/threonine residues *in vitro* [9]. Seeking a source of material for inhibitor screening and/or structure-based drug development, we have constructed a synthetic gene encoding the H1 phosphatase, overproduced the enzyme in *Escherichia coli*, purified it to homogeneity, and characterized its enzymatic activity *in vitro*.

Results

Expression and purification of variola H1 phosphatase

The amino acid sequence of the 171 residue variola major H1 phosphatase differs at only five positions from its counterpart in vaccinia virus. However, one of these amino acid substitutions is immediately adjacent to the active site cysteine in H1 (Cys110). For this reason, we felt that the vaccinia enzyme would not be an acceptable substitute for the variola phosphatase from the standpoint of drug development. Rather than modifying vaccinia DNA to encode the variola phosphatase, we instead elected to assemble an ORF from synthetic

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[1] *Abbreviations used:* DSPs, dual specificity phosphatases; PTPase, protein tyrosine phosphatase; TEV, tobacco etch virus; IMAC, immobilized metal affinity chromatography; PEG, polyethylene glycol; pNPP, *para*-nitrophenyl phosphate; ORF, open reading frame; PCR, polymerase chain reaction; MBP, maltose-binding protein; IPTG, isopropyl-β-D-thiogalactopyranoside.

oligodeoxyribonucleotides, which also gave us the opportunity to optimize the codon usage for expression in *E. coli* (Fig. 1). The synthetic variola phosphatase ORF was fused in frame to the C-terminus of an ORF encoding a dual His_6-MBP affinity tag [10,11]. This construct was designed so that the fusion protein could be cleaved by tobacco etch virus (TEV) protease to yield a recombinant H1 phosphatase with a single, non-native serine residue appended to its N-terminus.

The His_6-MBP-H1 fusion protein was expressed at a high level and in a soluble form in *E. coli* (Fig. 2), possibly due to the chaperone-like properties of the MBP [12]. After the fusion protein was partially purified by immobilized metal affinity chromatography (IMAC) and digested by

TEV protease, a significant amount of the released H1 phosphatase precipitated. Reducing agents, glycerol, polyethylene glycol (PEG), varying the salt concentration (0–2 M), changing the buffer composition (acetate, Tris, Bis–tris, Hepes, and phosphate), shifts in pH (5–9) or altering the TEV protease cleavage temperature had little effect on precipitate formation. The precipitate had low enzymatic activity and probably represents denatured or partially denatured enzyme. The remaining soluble fraction of the H1 phosphatase was purified to apparent homogeneity (Fig. 2) and was very stable with high enzymatic activity (see below). The overall yield of final product was only 5–10 mg from 1 L of culture or 10 g of wet cell paste (Table 1). The relatively low yield of pure material was due mainly to

Fig. 1. Nucleotide sequence of the synthetic open reading frame encoding variola major H1 phosphatase and amino acid sequence (single letter code) of the corresponding polypeptide. The engineered TEV protease cleavage site is indicated by an arrow. The catalytic cysteine residue is enclosed by a circle. Amino acids that differ between variola and vaccinia virus phosphatases are enclosed by squares.
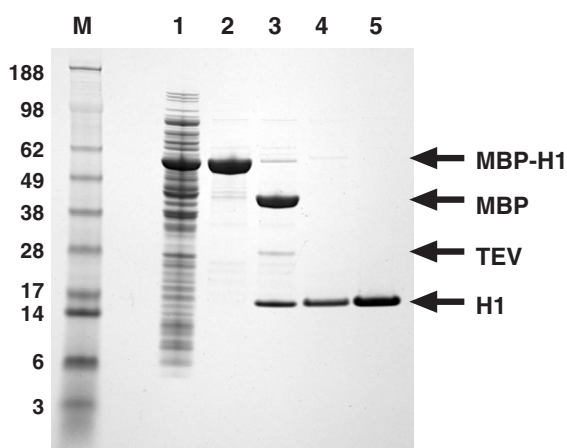
Fig. 2. Purification of variola H1 phosphatase as monitored by SDS–polyacrylamide gel electrophoresis. Lanes: M, molecular weight markers (kDa); 1, soluble intracellular protein (crude extract); 2, eluate from 1st Ni–NTA column; 3, products of TEV protease digest; 4, flow-through fraction from 2nd Ni–NTA column; 5, final product after gel filtration.

the tendency of H1 phosphatase to precipitate during purification.

Its retention time on a gel filtration column, in relation to a set of protein molecular weight standards, suggested that H1 phosphatase is predominantly monomeric with a small fraction of dimer. On the other hand, dynamic light scattering and analytical ultracentrifugation yielded results that were more consistent with a dimer (data not shown). Although the mammalian PRL-1 DSP has also been shown to form oligomers [13], the majority of DSPs characterized to date are monomeric in solution (e.g., cdc25, VHR, and PRL-3) [14–16]. The oligomeric state of vaccinia H1 phosphatase has not been investigated.

Enzyme kinetics

The chromogenic substrate *para*-nitrophenyl phosphate (pNPP), an analog of phosphotyrosine, was used to monitor the enzymatic activity of the H1 phosphatase. The optimum conditions for enzymatic activity were observed to occur in the pH range between 6.5 and 7, at a temperature of 30 °C, and in the absence of monovalent salt. Under these conditions, the reaction velocity was linear with respect to enzyme concentration up to at least 25 μg (6.3 μM). The enzyme exhibited classical saturation kinetics with respect to pNPP concentration (Fig. 3), with an apparent K_M of ca. $228 \pm 8\,\mu M$ and k_{cat} of $0.30 \pm 0.02\,s^{-1}$ at room temperature, and $293 \pm 11\,\mu M$ and $0.56 \pm 0.03\,s^{-1}$ at 30 °C, respectively. The k_{cat} and K_M of pNPP for vaccinia H1 phosphatase have not been reported, but the turnover number and Michaelis constant for variola H1 phosphatase are 7- and 27-fold lower, respectively, than the corresponding values for the human vaccinia H1-related phosphatase (VHR) [15]. The specificity constant (k_{cat}/K_M) of variola H1 phosphatase, which is a measure of catalytic efficiency, is 4-fold greater than that of VHR.

The activity of variola H1 phosphatase was inhibited by increasing ionic strength but stimulated by organic solvents such as DMSO, PEG, and alcohol (Fig. 4). The initial reaction velocity reached a maximum at 20% 2-propanol and 40% DMSO or PEG 400. Even modest concentrations of NaCl (5 mM) caused some precipitation of the H1 phosphatase, and enzymatic activity was completely obliterated at concentrations exceeding 10 mM. The acute salt-sensitivity of H1 phosphatase *in vitro* may indicate that other factors are needed to maintain its stability at higher physiological salt concentration *in vivo*.

Discussion

Until recently, it was a common misconception that protein phosphatases are highly promiscuous enzymes with
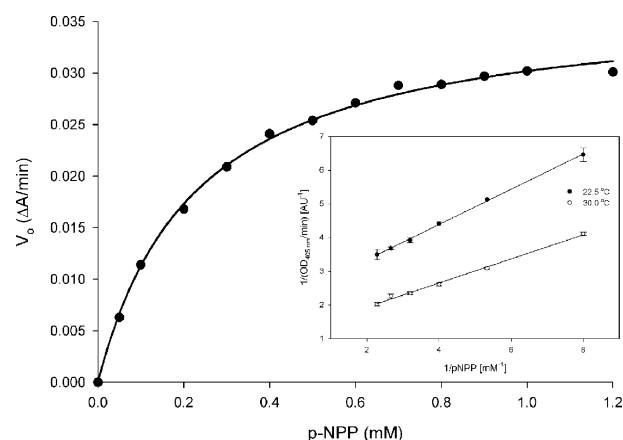

Fig. 3. Saturation curve for the hydrolysis of pNPP by variola H1 phosphatase plotted as initial velocity vs. substrate concentration. The reactions were initiated by adding 5.4 μg of enzyme (final concentration 1.4 μM) and monitored at 405 nm. The insert shows the Lineweaver–Burke plots for the reaction at room temperature and 30 °C, from which the K_M and v_{max} values were derived.

Table 1
Purification of H1 phosphatase

Fraction	Volume (ml)	Protein (mg/ml)	Phosphatase activity (U/ml)	Phosphatase total activity (U)[a]	Specific activity (U/mg)	Yield (%)	Fold purification
Soluble cellular extract	155	8.51	2.22	344.1	0.26	100	1.0
IMAC Run 1 bound	123	1.89	2.08	255.8	1.10	74	4.2
IMAC Run 2 effluent	360	0.09	0.18	64.8	2.00	19	7.7
Final product	1.30	5.00	16.55	21.5	3.31	6.3	12.7

[a] One unit equals 1 μmol *p*-nitrophenol produced per minute under standard assay conditions.
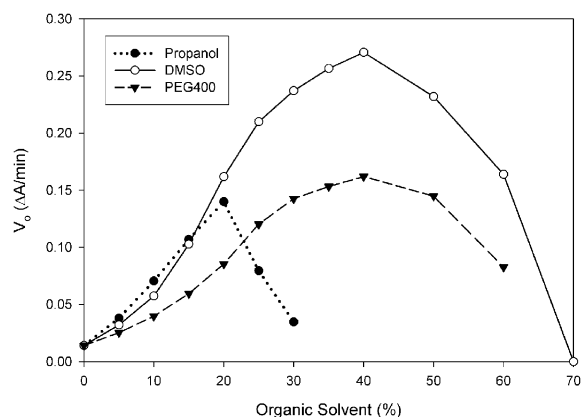
Fig. 4. The effect of organic solvents on the enzymatic activity of variola H1 phosphatase. Solid circles, open circles, and triangles represent 2-propanol, DMSO, and PEG 400, respectively. The enzyme evidently was denatured by propanol concentrations in excess of 20% (v/v), whereas the reaction velocity was reduced by DMSO and PEG 400 concentrations greater than 40% due to the high viscosity of the solution.

broad substrate specificity that merely play "housekeeping" roles. However, recent structural, biochemical and genetic studies have demonstrated that many of these enzymes exhibit a high degree of substrate specificity *in vivo* and regulate specific signal transduction events and physiological processes [17–20]. Accordingly, they may yet prove to be important molecular targets for therapeutic intervention in various human diseases.

Dual specificity phosphatases, such as variola H1, differ from the "classical" PTPases in that they are capable of dephosphorylating not only phosphotyrosine residues, but also phosphoserine and phosphothreonine. The total number of DSPs encoded by the human genome is predicted to be only 38; far less than the 428 predicted tyrosine and serine/threonine kinases [18], implying some degree of functional redundancy and/or substrate promiscuity. Nevertheless, the strict requirement of H1 phosphatase activity for the maturation of infectious viral particles [8] indicates that its role can not be assumed by any of its mammalian counterparts and suggests, therefore, that it may be possible to develop specific inhibitors of this enzyme.

Thus far, only two viral proteins have been identified as substrates of H1 phosphatase *in vivo*. The enzyme dephosphorylates the C-terminal tyrosine residue of A17 [21] and Ser85 of A14 [22], two virion membrane-associated proteins. Oligopeptides encompassing either of these sites may be useful starting points for the development of H1 phosphatase inhibitors. It is also possible that additional leads will emerge from the discovery of other proteins that are targeted by H1 phosphatase *in vivo*. Although relatively little is known about the substrate specificity of this enzyme at the present time, the availability of pure recombinant H1 phosphatase promises to facilitate an in-depth analysis of its specificity, yielding crucial information for the design of potent and specific inhibitors that could be used to combat smallpox.

Materials and methods

Assembly and molecular cloning of H1 phosphatase gene

A synthetic open reading frame (ORF) encoding the variola major H1 phosphatase was assembled by the method of Hoover and Lubkowski [23], using default parameters. In the process, a canonical recognition site for tobacco etch virus (TEV) protease (ENLYFQ/S) was added to the N-terminus of the ORF. AttB1 and attB2 recombination sites [24] were subsequently added to the N- and C-termini, respectively, of the synthetic gene by polymerase chain reaction (PCR) after which the PCR amplicon was inserted into pDONR201 (Invitrogen, Carlsbad, CA, USA) by recombinational cloning [24]. The nucleotide sequence of the synthetic ORF was confirmed experimentally. Next, the synthetic ORF was moved by recombinational cloning into the destination vector pDEST-HisMBP [10,11] to create pVPPase2. The latter plasmid produces H1 phosphatase in the form of a fusion with His-tagged *E. coli* maltose-binding protein (MBP) that can be cleaved by TEV protease to yield a phosphatase with a single non-native serine residue attached to its N-terminus. The MBP moiety includes an N-terminal hexahistidine tag to facilitate the purification of the fusion protein by IMAC.

Overproduction of H1 phosphatase in E. coli

The His_6-MBP-H1 fusion protein was overproduced in *E. coli* BL21(DE3) cells (Novagen, Madison, WI, USA). Single antibiotic-resistant colonies of cells transformed with pVPPase2 were used to inoculate 100 ml of Luria Broth supplemented with $100\,\mu g\,ml^{-1}$ ampicillin (American Bioanalytical, Natick, MA, USA). These cultures were grown by shaking ($250\,rev\,min^{-1}$) to saturation overnight at 37 °C and then diluted 50-fold into several liters of Luria Broth supplemented with $100\,\mu g\,ml^{-1}$ ampicillin and 0.2% (D+)-glucose monohydrate (Sigma–Aldrich, St. Louis, MO, USA). When the cells reached early log phase ($OD_{600nm} = 0.3–0.5$), the temperature was reduced to 30 °C and isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 1 mM. Four hours later, the cells were recovered by centrifugation at 5000*g* for 10 min and stored at −80 °C.

Purification of H1 phosphatase

All chromatography steps were carried out at 4 °C. Ten grams of *E. coli* cell paste was suspended in ice-cold 50 mM MES, pH 6.5, 200 mM NaCl, 25 mM imidazole (buffer A) containing Complete EDTA-free protease-inhibitor cocktail (Roche Molecular Biochemicals, Indianapolis, IN, USA), and 1 mM benzamidine HCl (Sigma–Aldrich). The cells were lysed with an APV-1000 homogenizer (Invensys, Roholmsvej, Germany) at 69 MPa and centrifuged at 30,000*g* for 30 min at 4 °C. The supernatant was filtered through a 0.22 μm polyethersulfone membrane and then

loaded onto a 12 ml Ni–NTA superflow column (Qiagen, Valencia, CA, USA) equilibrated in buffer A. The column was washed to baseline with buffer A and then eluted with a linear gradient to 250 mM imidazole. Fractions containing recombinant His$_6$MBP-H1 were pooled, concentrated using an Amicon YM30 membrane (Millipore Corporation, Bedford, MA, USA), diluted with 50 mM MES, pH 6.5, 200 mM NaCl buffer to reduce the imidazole concentration to about 25 mM, and digested overnight at 4 °C with His$_6$-tagged TEV protease [25]. The soluble fraction of the digest was applied to a 30 ml Ni–NTA superflow column equilibrated with buffer A and the H1 phosphatase emerged in the column effluent. The effluent was treated with dithiothreitol (5 mM) and ethylenediaminetetraacetic acid (1 mM) overnight, concentrated using an Amicon YM-10 membrane and applied to a 26/60 HiPrep Sephacryl S-100 prep-grade column (Amersham Biosciences, Piscataway, NJ, USA) equilibrated with 50 mM MES, pH 6 buffer. The peak fractions containing H1 phosphatase were pooled and concentrated to 5–7 mg ml^{-1} (determined spectrophotometrically using a molar extinction coefficient of 15360 M^{-1}cm^{-1}). Aliquots were flash-frozen with liquid nitrogen and stored at −80 °C. The final product was judged to be >95% pure by SDS–PAGE (see Fig. 2). The molecular weight of the H1 phosphatase was confirmed by electrospray mass spectrometry.

Enzyme kinetics

The enzymatic activity of variola H1 phosphatase was measured by using pNPP as the substrate in 200 μl reactions containing 50 mM Bis–Tris, pH 6.8, 1 mM ethylenediaminetetraacetic acid, 1 mM dithiothreitol, and 10% dimethylsulfoxide, both at room temperature (22.5 °C) and 30 °C. The reactions were initiated by adding 5.4 μg of purified enzyme to the reaction mixture (final concentration 1.4 μM) at various pNPP concentrations, incubating them at the appropriate temperature for 10 min, and then quenching the reactions by the addition of 800 μl of 1 N NaOH. The developed color was measured at 405 nm using methacrylate disposable cuvettes (Fisher Scientific, Pittsburgh, PA, USA) on a Shimadzu spectrophotometer. Otherwise identical reactions containing no enzyme were used to account for absorption due to non-enzymatic hydrolysis. The kinetic constants K_M and k_{cat} were determined by plotting the reciprocals of activity against varying pNPP and *para*-nitrophenolate (molar extinction coefficient of 18,000 M^{-1}cm^{-1}) concentrations. The effects of salts and organic compounds were determined by incubating with the enzyme for 10 min and the reactions were initiated by adding the substrate at a concentration equivalent to the K_M value.

Acknowledgments

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