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The molecular class C acid phosphatase of *Chryseobacterium meningosepticum* (OlpA) is a broad-spectrum nucleotidase with preferential activity on 5′-nucleotides

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Abstract

The *olpA* gene of *Chryseobacterium meningosepticum*, encoding a molecular class C phosphatase, was cloned and expressed in *Escherichia coli*. The gene encodes a 29-kDa polypeptide containing an amino-terminal signal peptide typical of bacterial membrane lipoproteins. Expression in *E. coli* results in a functional product that mostly partitions in the outer membrane. A secreted soluble OlpA derivative (sOlpA) lacking the N-terminal cysteine residue for lipid anchoring was produced in *E. coli* and purified by means of two steps of ion exchange chromatography. Analysis of the kinetic parameters of sOlpA with several organic phosphoesters revealed that the enzyme was able to efficiently hydrolyze nucleotide monophosphates, with a strong preference for 5′-nucleotides and for 3′-AMP. The enzyme was also able to hydrolyze sugar phosphates and β-glycerol phosphate, although with a lower efficiency, whereas it was apparently inactive against nucleotide di- and triphosphates, diesters, and phytate. OlpA, therefore, can be considered a broad-spectrum nucleotidase with preference for 5′-nucleotides. Its functional behaviour exhibits differences from that of the *Haemophilus influenzae* OMP P4 lipoprotein, revealing functional heterogeneity among phosphatases of molecular class C.

Keywords: Acid phosphatase; *Chryseobacterium meningosepticum*; Nucleotidase

1. Introduction

Enzymes that dephosphorylate organic compounds (phosphatases) are ubiquitous among bacteria. Bacterial phosphatases can exert activity either in the cytoplasmic compartment or externally, after secretion across the cytoplasmic membrane. Secreted phosphatases can be found as soluble periplasmic proteins or membrane-bound lipoproteins, and are mostly involved in dephosphorylation of substrates encountered in the environment [1–3]. Some of them exhibit a broad substrate specificity (e. g. the *Escherichia coli* PhoA alkaline phosphatase [4]) while others are specialized for certain substrates (e. g. the *E. coli* AppA phytase [5] and CpdB 2′:3′-cyclic phosphodiesterase [6]). Different repertoires of secreted phosphatases are found in different bacterial species [3,7–9], reflecting the different evolutionary histories of microbial taxa and their need for adaptation to different ecological niches.

Among secreted bacterial phosphatases, the molecular class C enzymes are a recently recognized family of proteins that belong in the DDDD superfamily of phosphohydrolases [10]. Of members of this family, only the class C enzyme from *Haemophilus influenzae*, corresponding to the outer membrane lipoprotein OMP P4 (previously *e*(P4)) [11], has been purified and characterized in some detail [12–14]. This enzyme exhibits optimal activity at pH 5, is inhibited by EDTA, vanadate, and molybdate, but not by tartrate, fluoride, or inorganic phosphate, and shows a quite narrow substrate specificity with an apparent preference for aryl-phosphates [12]. In vivo, it was recently shown to be involved in nicotinamide mononucleotide (NMN) utilization by acting as an NMN 5′-nucleotidase [15]. A secreted

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soluble derivative of the OMP P4 lipoprotein lacking the lipid anchor, produced in *E. coli*, was found to retain identical functional activity while being easier to purify [13].

In this paper we report on the characterization of the *Chryseobacterium meningosepticum* OlpA enzyme, the first recognized phosphatase of molecular class C [10]. (Cloning and sequencing of the *olpA* gene was first reported at the 1997 Annual Meeting of the American Society for Microbiology, Miami, FL).

2. Materials and methods

2.1. Recombinant DNA methodology and bioinformatics

Basic recombinant DNA methodology was performed essentially as described by Sambrook et al. [31]. Construction of the *C. meningosepticum* CCUG4310 genomic library in the *E. coli* plasmid vector pACYC184 has been described previously [16]. The TPMG expression-cloning procedure [17] was used to isolate the phosphatase determinant from the library. The pBluescript SK plasmid (Stratagene, La Jolla, CA, USA) was used for subcloning procedures. *E. coli* DH5α (Life Technologies, Milan, Italy) was used as the host for genetic vectors and recombinant plasmids. DNA sequences were determined on both strands by the dideoxy-chain termination method and custom sequencing primers (MWG-Biotech, München, Germany). Multiple sequence alignment was performed using the CLUSTALW program at the server of the Pôle Bio-Informatique Lyonnais (http://pbil.univ-lyon1.fr/).

2.2. Construction of the expression plasmid pLL-OlpA

The expression plasmid pLL-OlpA, for production of soluble recombinant OlpA (sOlpA) in *E. coli*, was constructed as follows. The *olpA* gene was amplified from the first codon downstream that encoding the Cys^{17} residue (putatively involved in the attachment to the lipid moiety) to the stop codon by high-fidelity polymerase chain reaction (PCR) using primers CMEC1 (5′-tccatggccTCTGCTCAAAAAGCAGATCAC) and CMEC2 (5′-gtctagaacaTTATTGATTAATATTTTGAG) (the capitalized sequences correspond to those annealing to the *olpA* template), which also added an *Nco*I restriction site (underlined) at the 5′ end, and an *Xba*I restriction site (underlined) after the *olpA* stop codon. PCR amplification was performed using *Pfu* DNA polymerase (Stratagene) under the conditions recommended by the manufacturer. The resulting 0.75-kb amplimer was blunt-end cloned in the *Sma*I site of plasmid pUC18, subjected to confirmatory sequencing, and subcloned as an *Nco*I–*Xba*I fragment into plasmid pCombIII [18] digested with the same enzymes, to obtain recombinant plasmid pLL-OlpA. In this plasmid the amplified *olpA* sequence is fused in frame, at the 5′ end, with that encoding the PelB leader peptide targeting protein secretion in the periplasmic space, and the *pelB–olpA* gene fusion is expressed under the control of the P_{lac} promoter. *E. coli* MG×NAP, an MG1655 derivative in which the *aphA* gene coding for the class B acid phosphatase was disrupted by an interposon conferring kanamycin resistance [M.C. Thaller, unpublished], was used as the host for production of the recombinant soluble OlpA (sOlpA) protein.

2.3. Protein electrophoretic techniques

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described by Laemmli [19] using an acrylamide concentration of 12% in the resolving gel. Zymogram detection of the OlpA enzyme following SDS-PAGE and in-gel renaturation of proteins was performed using the phenolphthalein–methyl green detection system, as described previously [3,9]. Renaturation was carried out in 50 mM Tris–HCl (pH 7.0); equilibration and development for zymogram detection of the OlpA activity was carried out in 100 mM sodium acetate buffer (pH 6) containing 1 mM $MgCl_2$.

2.4. Cell fractionation procedures

To isolate the *E. coli* outer membrane fraction, bacterial cells, suspended in normal saline to an $A_{600} \cong 20$, were disrupted by sonication. The sample was then clarified by centrifugation (7000×*g* for 15 min at 4 °C) and the supernatant was centrifuged at 60,000×*g* for 30 min at 4 °C. The membrane-murein pellet was washed once with normal saline, resuspended in normal saline containing 1% (v/v) Triton X-100 and $MgCl_2$ 10 mM, kept for 30 min at 25 °C, and then centrifuged again (60,000×*g* for 30 min at 4 °C). The detergent-insoluble fraction, washed once again with normal saline and suspended in Tris–HCl 50 mM (pH 8.0), represented the outer membrane preparation. Contamination by cytoplasmic membranes, analysed by testing for NADPH dehydrogenase activity [20], was found to be lower than 10%. Periplasmic proteins were extracted by a spheroplasting procedure using lysozyme and EDTA, as described previously [21].

2.5. Purification of the sOlpA protein

The sOlpA protein was purified from *E. coli* MG×NAP(pLL-OlpA) grown aerobically in 3 l of Super Broth [22] containing 50 mM sodium phosphate buffer (pH 7.2) and ampicillin (250 μg/ml, we normally use this higher ampicillin concentration when growing *E. coli* strains with high-copy number vectors in very rich media such as Super Broth) at 30 °C. Isopropyl β-D-thiogalactopyranoside (Sigma Chemical, St. Louis, MO, USA) (0.5 mM final concentration) was added to the culture when it reached an OD_{600} of 1.5 (delayed addition of IPTG, compared to standard conditions, was found to be beneficial to sOlpA

expression in preliminary experiments). Three hours after induction, cells were harvested by centrifugation, and periplasmic proteins were extracted by a spheroplasting procedure using lysozyme and EDTA [21]. The sOlpA protein was precipitated from the periplasmic extract with 30% (w/v) polyethylene glycol (PEG) 6000 at 4 °C for 18 h. The precipitate was collected by centrifugation (45,000×*g* for 2 h at 4 °C), resuspended in 30 ml of 20 mM Tris–HCl (pH 7.5), and loaded (at a flow rate of 1 ml/min) onto a DEAE-Sepharose FF column (7 by 2.6 cm, Amersham-Pharmacia Biotech, Milan, Italy) previously equilibrated with the same buffer. Under these conditions the sOlpA protein eluted in the flow-through. The phosphatase-containing fractions were pooled, dialysed for 12 h at 4 °C against 50 mM sodium acetate buffer (pH 5.2) containing 0.1% PEG 6000, and loaded (at a flow rate of 1 ml/min) onto a CM-Sepharose FF column (13.5×1.6 cm, Amersham-Pharmacia) previously equilibrated with the same buffer. After washing the column, the bound proteins were eluted by a linear NaCl gradient (0 to 0.5 M) in the same buffer. The fractions containing phosphatase activity were pooled, dialysed for 12 h at 4 °C against 20 mM Tris–HCl (pH 7.5) containing 0.1% PEG 6000 and 1 mM $MgCl_2$, and stored at −20 °C until use. Protein concentration in solution was determined by the method of Bradford using a commercial kit (Bio-Rad Protein Assay, Bio-Rad) and bovine serum albumin as a standard.

2.6. Size-exclusion chromatography

Size-exclusion chromatography for determination of the molecular mass of native sOlpA was carried out with a Biosys 2000 system (Beckman Instruments, Fullerton, CA, USA) using a Ultraspherogel SEC 3000 column (30×0.75 cm, Beckman), protected by a Ultraspherogel SEC Guard column (4×0.75 cm, Beckman), equilibrated, and eluted with 100 mM sodium acetate buffer (pH 6.0) containing 50 mM NaCl. The column was calibrated with protein standards (Sigma) in the range 150,000–12,400 dissolved in the elution buffer. The peak fraction of each protein was determined at A_{280}. A linear plot of the partition coefficient versus the log of the molecular masses of the protein standards was used to estimate the molecular mass of the phosphatase.

2.7. Enzyme assays

Acid phosphatase activity was assayed using 2.5 mM *p*NPP as substrate in 100 mM sodium acetate buffer (pH 6) containing 1 mM $MgCl_2$. Incubation was at 37 °C for 10 min. The reaction volume was 0.3 ml. The *p*NP concentration was read at 414.5 nm after addition of 0.7 ml of 2 M NaOH (ε=+18,000 M^{-1} cm^{-1}). The pH optimum for activity of the purified enzyme was determined by measuring the phosphatase activity as described above, using 100 mM buffers of pH values ranging from 4 to 8, containing 1 mM $MgCl_2$, and an enzyme concentration of 2.2 nM. Sodium acetate was used in the range 4–6, while 2-(*N*-morpholino)ethansuflonic acid (MOPS, pK_a 7.31)/NaOH was used in the range 6–8. The effect of EDTA or other substances was assayed by measuring the phosphatase activity, as described above, after incubation of the purified enzyme (2.2 nM) with each substance, at the desired concentration, for 20 min at 25 °C in 100 mM sodium acetate buffer (pH 6.0). A control assay without any substance was always carried out in parallel. To investigate the role of divalent cations on enzyme activity, the purified enzyme was extensively dialysed (overnight at 4 °C) against 20 mM Tris–HCl (pH 7.5) containing 0.1% PEG 6000 and 1 mM EDTA; EDTA was then removed by dialysis against 20 mM Tris–HCl (pH 7.5) containing 0.1% PEG 6000. Restoration of enzymatic activity was investigated by adding increasing concentrations of $MgSO_4$, $CuSO_4$, and $ZnSO_4$.

The steady-state kinetic parameters (K_m and k_{cat}) of purified sOlpA with different substrates were determined under initial-rate conditions using the Hanes–Woolf linearization of the Michaelis–Menten equation [23]. In these experiments the phosphatase activity was assayed by measuring the released inorganic phosphate (Pi) by the acidified ammonium molybdate method [24] at 820 nm (ε=+14,000 M^{-1} cm^{-1}). When the K_m values were lower than 80 μM,

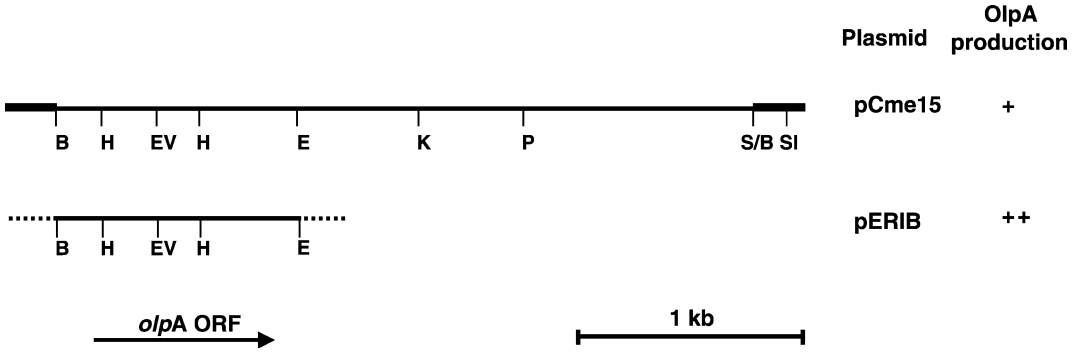

Fig. 1. Physical map of the insert of plasmid pCme-15, and subcloning strategy. Thin lines represent cloned DNA, while thick or dotted lines represent vector sequences from pACYC184 or pBluescript SK, respectively. Production of OlpA was assayed by zymograms after renaturing SDS-PAGE, as described in Materials and methods, on crude extracts prepared from late-exponential phase cultures. The location of the *olpA* ORF is indicated. B, *Bam*HI; E, *Eco*RI; EV, *Eco*RV; H, *Hin*dIII; K, *Kpn*I; P, *Pst*I; Sl, *Sal*I; S/B, *Sau*3AI/*Bam*HI junction.

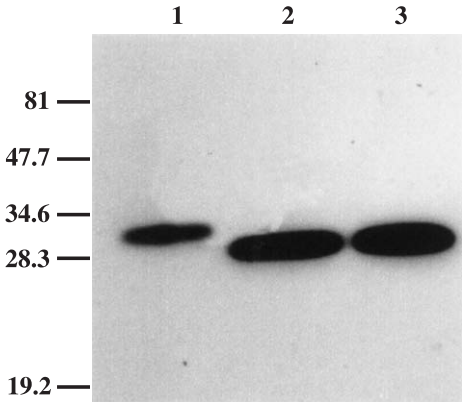

Fig. 2. Zymogram detection of the OlpA acid phosphatase following renaturing SDS-PAGE in different strains and cell fractions (see Materials and methods for experimental details). Lanes: 1, *E. coli* DH5α(pERIB) outer membrane fraction (the acid phosphatase activity present in this fraction constituted >90% of the total activity present in a crude extract of *E. coli* DH5α(pERIB)); 2, *E. coli* MG×NAP(pLL-OlpA) periplasmic extract (the acid phosphatase activity present in this fraction constituted >90% of the total activity present in a crude extract of *E. coli* MG×NAP(pLL-OlpA)); 3, purified sOlpA. Protein size standards are shown in kDa on the left.

they were measured as inhibition constants (K_is) in a competitive model, using 2.5 mM *p*NPP as the reporter substrate and reading the released *p*NP as described above. The K_i value was determined by the plot of V_o/V_i versus I, yielding a line whose slope is $K_m^S/(K_m^S+S)\times K_i$, where V_o and V_i are the initial rates of *p*NPP hydrolysis in the absence and presence of the inhibitor, respectively, I is the inhibitor concentration, S is the reporter substrate concentration, and K_m^S the Michaelis constant of the enzyme for the reporter substrate. The enzyme assays for measurement of kinetic parameters were carried out in a volume of 0.3 ml (the final volume after blocking was always 1 ml) at 37 °C, using an enzyme concentration of 2.2–4.4 nM.

3. Results

3.1. Cloning and expression of the C. meningosepticum olpA gene in E. coli

The *olpA* gene (EMBL/GenBank accession number Y12759) was isolated, by means of an expression-cloning procedure, from a genomic library of *C. meningosepticum* CCUG4310 constructed in the *E. coli* plasmid vector pACYC184. The recombinant plasmid pACme15, carrying the cloned phosphatase determinant, contained a 3.5-kb DNA insert. The phosphatase determinant was mapped within a 1.1 *Bam*HI–*Eco*RI fragment by subcloning analysis (Fig. 1). The origin of the cloned determinant from a single region of the *C. meningosepticum* genome was confirmed by hybridization of the 1.1-kb *Bam*HI–*Eco*RI DNA insert of plasmid pERIB (Fig. 1) with the genomic DNA of CCUG4310 in a Southern blot experiment (data not shown).

A sequence alignment of the OlpA protein with the four other known bacterial class C phosphatases, the OMP P4 lipoprotein of *H. influenzae* [11], the LppC lipoprotein of *Streptococcus equisimilis* [25,26], the HppA protein of *Helicobacter pylori* [27], and the SapS protein of *Staphylococcus aureus* [28], revealed that OlpA is quite divergent from any of them, sharing 35.7%, 33.4%, 31.9, and 31.3% amino acid identity with LppC, SapS, HppA, and OMP P4, respectively.

The deduced amino acid sequence of the *olpA* product contains an amino-terminal motif typical of bacterial membrane lipoproteins [29], with the cysteine residue at position 17 being the putative cleavage ligand for the lipid moiety. A lipoprotein nature of OlpA was supported by the fact that in *E. coli* DH5α(pERIB) the enzyme mostly partitioned in the outer membrane-containing fraction (Fig. 2), from which it could be extracted by 1% (w/v) zwittergent 3–14 (data not shown).

3.2. Production of a soluble OlpA derivative in E. coli, and protein purification

To facilitate protein purification, the *olpA* sequence encoding the lipoprotein leader peptide was replaced by a sequence encoding a bacterial leader peptide targeting protein secretion into the periplasmic space (that of PelB pectate lyase of *Erwinia carotovora* [30]). The engineered *pelB–olpA* gene fusion was placed under the transcriptional control of the P_{lac} promoter, and the resulting plasmid (named pLL-OlpA) was introduced into *E. coli* MG×NAP, an MG1655 derivative defective for production of the AphA acid phosphatase, that could have interfered in the purification process. MG×NAP(pLL-OlpA) produced an enzymatically active OlpA enzyme which, in cell fractionation experiments, mostly partitioned in the periplasmic fraction (Fig. 2).

Table 1
Summary of the purification steps of the sOlpA enzyme produced by *E. coli* MG×NAP(pLL-OlpA)

Purification step[a]	Total protein (mg)	Specific activity (units/mg protein)	Total activity (units)	Yield (%)	Purification (fold)
Periplasmic extract	64	469	30,000	100	1
PEG precipitate	12.6	1706	21,500	72	3.6
DEAE-Sepharose eluate	0.25	63,556	15,889	53	136
CM-Sepharose eluate	0.034	287,765	9784	33	614

One unit of activity is defined as the amount of enzyme hydrolyzing 1 nmol *p*NPP/min under the assay conditions described in Materials and methods.
[a] The activity present in the periplasmic extract was >90% of the total cell-associated activity.

The soluble OlpA derivative (sOlpA) produced by *E. coli* MG×NAP(pLL-OlpA) was purified from the periplasmic fraction prepared from this strain by an initial precipitation step with PEG 6000, followed by two sequential ion-exchange chromatography steps (Table 1). SDS-PAGE analysis of the purified sOlpA preparation revealed a single band of 30 kDa, and the preparation was estimated to be over 95% pure (Fig. 3). The yield of purified sOlpA was low (approximately 11 μg per liter of culture). Since the efficiency of the purification procedure was around 30% (Table 1), the low yield of purified protein mostly reflected a low level production of sOlpA by the *E. coli* host.

3.3. Enzyme characterization

The M_r of the purified sOlpA, estimated by size-exclusion chromatography, was approximately 30,000, suggesting that the native protein is found as a monomer.

The pH optimum of sOlpA with *p*NPP as substrate was found to be around 6. More than 50% of activity was retained in the pH range 5–7; a relatively sharp decrease of activity was observed at values lower than 4.5 or higher than 7 (Fig. 4).

The activity of sOlpA was inhibited by EDTA (IC_{50}, 0.5 mM), molybdate (IC_{50}, 0.25 mM), fluoride (IC_{50}, 1.5 mM), tartrate (IC_{50}, 7 mM), *o*-vanadate (IC_{50}, 1 mM), Pi (IC_{50}, 170 mM), adenosine (IC_{50}, 0.16 mM), NAD (IC_{50}, 0.3 mM), and NADP (IC_{50}, 0.35 mM).

Concerning divalent cations, the sOlpA activity was moderately inhibited by $ZnSO_4$, while it was stimulated by $MgSO_4$ and by $CuSO_4$. However, unlike $MgSO_4$, $CuSO_4$ appeared to be more stimulatory at the lower concentrations (Table 2). The sOlpA activity was almost completely abolished following extensive dialysis against EDTA, but it could be restored following readdition of divalent cations (Table 2). Mg^{2+} was apparently the most effective in restoring the activity, while Zn^{2+} was the least effective. Interestingly, with the presence of Cu cations EDTA-treated sOlpA recovered, at most, 142% of the activity, a value that was notably lower than that achievable by the native enzyme upon addition of Cu^{2+} (Table 2).

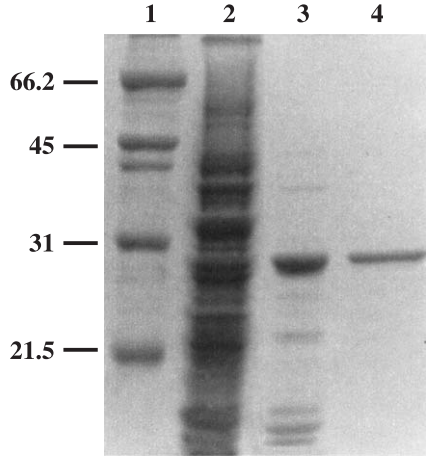

Fig. 3. SDS-PAGE analysis of the purification process of the sOlpA protein. Lanes: 1, protein size standards (in kDa); 2, *E. coli* MG×NAP(pLL-OlpA) periplasmic extract; 3, flow-through of DEAE-Sepharose FF; 4, purified sOlpA.

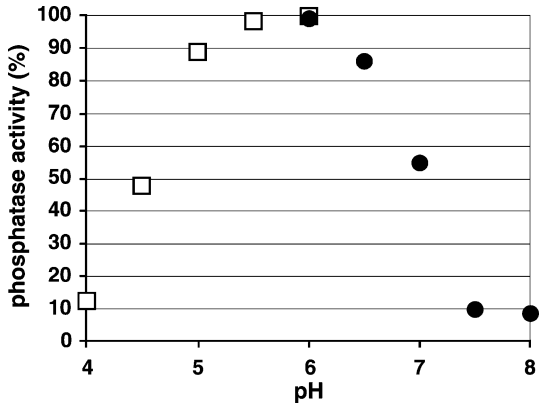

Fig. 4. Phosphatase activity of the purified sOlpA protein at different pH. Assays were performed using 2.5 mM *p*NPP as substrate in 100 mM buffers of pH values ranging from 4 to 8, containing 1 mM $MgCl_2$, and an enzyme concentration of 2.2 nM. Sodium acetate was used in the range 4–6, while MOPS/NaOH was used in the range 6–8. (□) activity measured in sodium acetate buffer; (●) activity measured in MOPS/NAOH buffer. No significant buffer-related modification of the enzyme activity was observed at the overlapping pH value.

Analysis of the kinetic parameters of sOlpA revealed that the enzyme efficiently hydrolyzed 5′- and 3′-nucleotide monophosphates (k_{cat}/K_M ratios, in the range 4×10^5–2×10^7 M^{-1} s^{-1}) with a notable preference for 5′-nucleotides and 3′-AMP (Table 3). Interestingly, the variability of the k_{cat}/K_M ratios observed with different nucleotide monophosphates (50-fold at maximum) was mostly due to a variation in the enzyme affinity for the various substrates, while the turnover rates only varied within a factor of 2 (Table 3). The enzyme was also able to hydrolyze *p*NPP (with an efficiency slightly superior to that exhibited with the less preferred 3′-nucleotides) and, somewhat less efficiently, ribose-5P. Hydrolysis of glucose-6-phosphate and β-glycerol phosphate was also detected, but the affinity for these substrates was exceedingly low, preventing the determination of individual kinetic parameters (Table 3). No

Table 2
Percent activity of sOlpA in the presence of divalent ions added either under native conditions or after metal removal by dialysis against EDTA

Sample	Native sOlpA	EDTA-treated sOlpA
Buffer	100 ± 4	7 ± 1
Mg 0.25 mM	142 ± 5	153 ± 5
Mg 0.5 mM	144 ± 5	160 ± 5
Mg 1 mM	154 ± 5	169 ± 4
Cu 0.25 mM	215 ± 7	142 ± 5
Cu 0.5 mM	174 ± 5	137 ± 4
Cu 1 mM	150 ± 5	124 ± 4
Zn 0.25 mM	85 ± 3	53 ± 2
Zn 0.5 mM	85 ± 3	70 ± 2
Zn 1 mM	87 ± 3	75 ± 3

Table 3
Kinetic parameters measured with the purified sOlpA protein

Substrate	K_M (μM)[a]	k_{cat} (s^{-1})	k_{cat}/K_M (M^{-1}/s^{-1})
5′-AMP	16 ± 1	173 ± 3	1.1×10^7
5′-GMP	11 ± 0.4	205 ± 4	1.9×10^7
5′-UMP	13 ± 0.8	267 ± 4	2.1×10^7
5′-CMP	13 ± 0.5	303 ± 9	2.3×10^7
3′-AMP	16 ± 0.7	160 ± 4	1.0×10^7
3′-GMP	164 ± 7	162 ± 3	9.9×10^5
3′-UMP	335 ± 9	138 ± 3	4.1×10^5
3′-CMP	530 ± 14	188 ± 6	3.6×10^5
β-glycerol-P	≫1000	N.D.[b]	2.0×10^3
*p*NPP	180 ± 6	129 ± 4	7.2×10^5
Ribose-5-P	534 ± 20	65 ± 1	1.2×10^5
Glucose-6-P	≫1000	N.D.	1.5×10^3

Data are mean values of three measurements ± S.D.
[a] Determined as K_i when in the direct assay K_M was lower than 80 μM.
[b] ND, not determined; in these cases only the k_{cat}/K_M ratio could be determined due to the very low affinity of the enzyme for the substrate. The S.D. was lower than 5%.

hydrolytic activity was detected against 2′:3′-cAMP, ADP, ATP, NADPH, NADP, and phytic acid, using a substrate concentration of 1000 μM and an enzyme concentration of 4.4 nM, for 15 min at 37 °C.

4. Discussion

Molecular class C is a recently discovered family of bacterial acid phosphatases that belongs in the DDDD superfamily of phosphohydrolases, and the *C. meningosepticum* OlpA protein was the first recognized enzyme of that class [10].

The *olpA* gene was shotgun cloned and expressed in *E. coli*, yielding a functional enzyme that mostly partitioned in the outer membrane-containing fraction. This fact, together with solubility in the detergent Zwittergent 3–14 and the presence of an amino-terminal leader peptide typical of bacterial membrane lipoproteins, strongly suggests that OlpA is found as a membrane lipoprotein, although the presence of a lipid anchor was not specifically investigated.

OlpA purification was facilitated by engineering the gene to produce a soluble periplasmic derivative (sOlpA), as previously done with OMP P4 [13], and biochemical characterization was carried out with sOlpA. The enzyme was able to efficiently dephosphorylate all types of nucleotide monophosphates, although with notable differences in the kinetic parameters for different compounds. The enzyme exhibited a remarkably high efficiency for 5′-nucleotides and for 3′-AMP, while being 10- to 50-fold less efficient toward the other 3′-nucleotides, whereby these differences were mostly influenced by variations of affinities rather than of turnover rates. Apart from *p*NPP and ribose-5-P, against which the enzyme also exhibited a good to moderate efficiency, other compounds appeared to be very poor substrates or were not hydrolyzed at all. In view of the above results, OlpA appears to behave as a broad-spectrum nucleotidase with an overall preference for 5′-nucleotides (or a 5′-nucleotidase with extended specificity for 3′-nucleotides) rather than a true nonspecific phosphatase. Since removal of the lipid moiety did not modify the OMP P4 structure and functions [13], and sOlpA produced in *E. coli* displayed predicted functions, data determined with sOlpA are expected to be representative of the native enzyme. Provided that this assumption is true, the physiological role of OlpA could be that of scavenging different types of mononucleotides to provide the cell with Pi and nucleosides. Additional experiments, including characterization of the native OlpA purified from its original host and generation of *olpA* mutants, will be necessary to provide further insight on the physiological role of this protein.

OlpA is a metal-dependent enzyme, as indicated by EDTA inhibition and restoration of activity by divalent cations. However, the preliminary results carried out with metals did not allow to draw definite conclusions on the nature of the metal(s) present in the native enzyme. In particular, data concerning restoration of protein activity by divalent cations might suggest that, for optimal activity, the OlpA protein needs two different cofactors. This point would deserve further investigation once larger amounts of the enzyme are available.

Comparison of sOlpA with OMP P4 of *H. influenzae*, which is the only class C enzyme that has been subjected to biochemical characterization in some detail [12–15], is not straightforward since the latter enzyme was not subjected to a detailed kinetic characterization with nucleotides or other substrates, and the assay conditions used in that work were somewhat different from those used here. However, some notable differences between the two enzymes are apparent. While OlpA is highly active on 5′-nucleotides and 3′-AMP, OMP P4 appears to be weakly active on 5′-nucleotides compared to aryl-phosphates [12,13]. Moreover, OlpA appears to be more susceptible to inhibition by fluoride and tartrate, compared to OMP P4 [12,13]. Finally, the OlpA behaviour with divalent cations after dialysis against EDTA was different from that observed with OMP P4 [12]. Compared to HppA, OlpA appears to be more susceptible to EDTA [27]. Overall, these data suggest that functional heterogeneity can be found among class C bacterial phosphatases from different species. It will be interesting to investigate the biochemical properties of other class C enzymes to ascertain whether and how the structural heterogeneity observed among members of this class is relevant to their functional behaviour.

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