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Preparation and biophysical characterization of recombinant *Pseudomonas aeruginosa* phosphorylcholine phosphatase

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ABSTRACT

Pseudomonas aeruginosa infections constitute a widespread health problem with high economical and social impact, and the phosphorylcholine phosphatase (PchP) of this bacterium is a potential target for antimicrobial treatment. However, drug design requires high-resolution structural information and detailed biophysical knowledge not available for PchP. An obstacle in the study of PchP is that current methods for its expression and purification are suboptimal and allowed only a preliminary kinetic characterization of the enzyme. Herein, we describe a new procedure for the efficient preparation of recombinant PchP overexpressed in *Escherichia coli*. The enzyme is purified from urea solubilized inclusion bodies and refolded by dialysis. The product of PchP refolding is a mixture of native PchP and a kinetically-trapped, alternatively-folded aggregate that is very slowly converted into the native state. The properly folded and fully active enzyme is isolated from the refolding mixture by size-exclusion chromatography. PchP prepared by the new procedure was subjected to chemical and biophysical characterization, and its basic optical, hydrodynamic, metal-binding, and catalytic properties are reported. The unfolding of the enzyme was also investigated, and its thermal stability was determined. The obtained information should help to compare PchP with other phosphatases and to obtain a better understanding of its catalytic mechanism. In addition, preliminary trials showed that PchP prepared by the new protocol is suitable for crystallization, opening the way for high-resolution studies of the enzyme structure.

Introduction

Pseudomonas aeruginosa is an ubiquitous and opportunistic Gram-negative bacterium and the cause of many urinary-tract, respiratory, corneal, and surgical wound infections [1–3]. For instance, it is the primary cause of chronic lung infections and mortality in patients with cystic fibrosis [4–6].

This bacterium has high metabolic versatility and the ability to exploit very different environments. It grows in iso- or hyper-osmotic media, with choline, betaine, dimethylglycine, or carnitine as nutrients, and at high or low inorganic phosphate concentrations. Phosphorylcholine phosphatase (PchP)[1] [7] and hemolytic phospholipase C (PlcH) [7–10] are two of the many pathogenic factors produced by *P. aeruginosa*. In hyperosmolarity, as in infected human tissues [11], or in isoosmolarity, PlcH and PchP are involved in the sequential and coordinated hydrolysis of phosphatidylcholine or sphingomyelin to produce choline and phosphate as sources of carbon, nitrogen, and phosphorus [7,12]. Several years ago, we purified PchP from the periplasmic extracts of *P. aeruginosa* and characterized some of its biochemical properties [13]. More recently, we established that PA5292 (PAO1 genome (http://www.Pseudomonas.com) encodes the 349-residue PchP precursor with a 22-residue N-terminal signal peptide, and that the 327-residue mature protein is exported to the periplasmic space [14].

PchP exhibits very low sequence similarity to other proteins. Nonetheless, we identified in PchP three conserved motifs that allowed its classification as a member of the bacterial haloacid dehalogenases (HAD) hydrolase superfamily: I, ^{31}DMDNT^{35}; II, ^{166}SAA^{168}; and III, ^{261}GDTPDSD^{267} (residue numbering is for the mature protein) [15]. By site-directed mutagenesis and kinetic experiments we established that the three motifs are involved in

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[1] *Abbreviations used:* PchP, *Pseudomonas aeruginosa phosphorylcholine* phosphatase; CD, circular dichroism; *p*-NPP, *p*-nitrophenylphosphate; PAGE, polyacrylamide gel electrophoresis; SEC, size-exclusion chromatography.

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the catalysis of *p*-nitrophenylphosphate (*p*-NPP) and demonstrated that the enzyme activity was dependent on the presence of Mg^{2+}, Zn^{2+}, or Cu^{2+} [15,16].

From these studies it was also evident that the study of its transcriptional regulation and the basic physicochemical properties of PchP were mandatory for further advances in the characterization of its biological, biochemical, and medical relevance. To that end, it was also evident the need of a reliable protocol for producing large amounts of pure native protein. The latter two issues are the main focus of this work.

Materials and methods

General details

p-NPP was from Sigma (St. Louis, Missouri). Protein purity was assessed by SDS–PAGE. Least square fit was done using the Solver module of Microsoft Excel 2000. Mass spectroscopy was performed on a VG Quatro II (VG Biotech, Altrinchan, UK) triple quadrupole instrument equipped with an electrospray ionization source. Thiol determination with 5,5́-dithiobis (2-nitrobenzoic acid) (DTNB) was performed as described [17,18]. The molar extinction coefficient at 280 nm (see Results) was used to measure the concentration of PchP in pure preparations. The enzymic activity of PchP was measured as previously described [13]. Briefly, the assay was performed at 37 °C in 100 mM sodium acetate, pH 5.0, 2 mM $MgCl_2$, and 0-12 mM *p*-NPP. The reaction was stopped after 15 min by dilution 1:6 in 0.1 M sodium hydroxide, and the production of *p*-nitrophenol was measured at 410 nm. The pH dependence of K_M and k_{cat} was analyzed using the equation for two active site ionizing groups described in Ref. [19]. Controlled proteolysis with trypsin was carried out at 37 °C in 50 mM Tris–HCl, pH 8.0. Crystallization trials were performed using the hanging drop method.

Molecular biology

The DNA encoding PchP was excised from pTYB12-$PchP_{349}$ or from pTYB12-$PchP_{327}$ [15] and subcloned into the NdeI and BamHI sites of pET9b (Invitrogen, Corp.) Cys → Ala variants of PchP were prepared as described in Ref. [15].

Protein expression and purification

The resulting constructs, pET9b-$PchP_{349}$ or pET9b-$PchP_{327}$, encoding pro- or mature PchP, respectively (UniProtKB ID Q9HTR2_PSEAE), were used to electro transform BL21(DE3) *Escherichia coli* cells. Single colonies of transformed cells grown on kanamycin containing plates were used to prepare a 100-mL starter culture in Luria Bertani (LB) broth containing 50 mg/l kanamycin (12 h at 30 °C in an orbital shaker at 200 rpm). The starter culture (50 mL) was transferred to a 2-L flask containing 1 L of fresh medium and growth was continued up to $OD_{620\ nm}$ = 1.0 (∼3 h). Then, the expression of recombinant protein was induced by adding 1% lactose. After a 3-h induction, cells were harvested by centrifugation (15 min, 6000 rpm, 15 °C) and frozen at −20 °C.

PchP purification was as follows: five grams of cells were thawed with 5 mL of 50 mM Tris–HCl,100 mM NaCl, 1 mM EDTA, pH 8.0 (Buffer A) at 4 °C and immediately disrupted by pressure (1000 psi; French Pressure Cell Press; Thermo IEC, Needham Heights, MA, USA). The homogenate was centrifuged (10 min, 4 °C, 12,000*g*) and the pellet of crude inclusion bodies and cellular debris saved for further purification. Inclusion bodies were freed of several contaminants by several cycles of suspension in 5 mL of Buffer A with the additives listed below, viscosity reduction by repeated extrusion through a polyethylene Pasteur pipette, incubation (30 min at 37 °C), and centrifugation (15,000 g, 10 min at 4 °C). The additives for each cycle were: (a) 10 mM $MgCl_2$, 20 μg mL^{-1} DNase I; (b) 0.2 mg mL^{-1} lysozyme; (c,d) 0.5 % v/v Triton X-100 and 10 mM EDTA. Finally, distilled water replaced Buffer A in cycles (e–g). The purified inclusion bodies were incubated 2 h a room temperature with gentle rolling in 5 mL of 25 mM Tris–HCl, 6.5 M urea, 1 mM DTT, 0.5 mM EDTA, 5.0 mM glycine, pH 8.5 (Buffer B). After centrifugation (12,000 g, 10 min at 4 °C), the supernatant was loaded into a Q-Sepharose (Amersham Biosciences) column (1.5 × 6.5 cm) equilibrated with Buffer B. Protein elution was accomplished using a gradient of NaCl (0.0–0.5 M; 200 mL) in Buffer B. Fractions containing 95-98% pure PchP judging by SDS–PAGE were pooled and dialyzed. Dialysis buffer was first 10 mM Tris–HCl, 100 mM NaCl, 0.1 mM DTT, 0.1 mM EDTA, pH 8.5, second 10 mM Tris–HCl, 100 mM NaCl, 3 mM $MgCl_2$, pH 8.5, and third 10 mM Tris–HCl, 100 mM NaCl, 5 mM $MgCl_2$, pH 8.5 (Buffer C). After dialysis, protein aggregates were removed by centrifugation (12,000*g*, 10 min at 4 °C) and the clear supernatant of pure PchP was subjected to a preparative size exclusion chromatography on a Sephadex G75 column (2 × 40 cm) equilibrated with Buffer C.

Optical and hydrodynamic studies

CD instrument and measurement setting were described previously [20]. Samples were dissolved in Buffer C. Near-UV measurements were carried out at 20 °C with a 1.0-cm cell containing 15-μM protein. For far UV measurements, cell and protein concentration were 0.1 cm and 1.5 μM, respectively. The final spectra were smoothed using a 40-point moving window and a 4th order polynomial [21]. Results are given in molar ellipticity units based in the concentration of peptide bonds (far UV) or protein (near UV).

Fluorescence measurements were performed with a K2 ISS spectrofluorometer (ISS, Champaign, IL). Protein solutions were prepared in Buffer C. Excitation was at 295 nm (8 nm bandwidth), and data were acquired at 1-nm intervals between 250 and 450 nm. Quantum yield was calculated as described previously [22].

Analytical size-exclusion chromatography (SEC) [23] was carried out at 22 °C with a Superose 12 10/300 column (GE healthcare) equilibrated with Buffer C. Calibration of the column was done with a set of standard globular protein: thyroglobulin (670 kDa), bovine IgG (158 kDa), chicken ovalbumin (44 kDa), and equine myoglobin (17 kDa). The total solute-accessible volume of the column was measured adding cyanocobalamin (1.3 kDa) to the protein standards. Light scattering measurements were performed with a miniDAWN instrument (Wyatt Technology Corp, Santa Barbara, CA). Samples of PchP were subjected to SEC on a Pharmacia Superdex 200 (10/30) column and eluted peaks were monitored at 690 nm with a light scattering detector and at 280 nm with a UV detector (Rainin Dynamax, NY). Molar mass and hydrodynamic radius (R_h) were calculated using the software Astra provided with the instrument.

Thermal unfolding

Unfolding transitions as a function of temperature were monitored by CD at 220 nm. Protein concentration was 1.5 μM, and a 0.1 cm cell was used. Temperature was varied 2 °C min^{-1}, from 0 to 85 °C, and the melting curve was sampled at 0.2-min intervals. Data were fitted to the following equation [19],

$$\Delta G_{NU} - RT\ln(f_u/f_n) = \Delta H_{Tm} + \Delta C_P(T - T_m) - T\{(\Delta H_{Tm}/T_m) + \Delta C_p \ln(T/T_m)\} \quad (1)$$

$$S = f_n(S_{0,N} + l_N T) + f_u(S_{0,U} + l_U T), \quad (2)$$

where f_U and f_N are the unfolded and folded fractions at equilibrium and $f_U + f_N = 1$, T_m is the temperature at which $f_U = f_N$, S is the observed CD signal, $S_{0,N}$ and $S_{0,U}$ are the intrinsic CD signals for the native and unfolded state, respectively, l_N and l_U are the slopes for the assumed linear dependence of $S_{0,N}$ and $S_{0,U}$ with the temperature. Samples were in Buffer C, with or without a 5 M excess of EDTA.

Metal binding

The affinity of the enzyme for Mg^{2+} was measured by micro dialysis using a 10-well Microdialyzer Spectra/Por (Spectrum Laboratories, Inc.). Dialysis buffer was 10 mM Tris–HCl, 100 mM NaCl, pH 8.5 treated with Chelex 100 (Sigma) to remove potential metal contaminants. After Chelex treatment, Mg^{2+} was added to the dialysis buffer to the desired final concentration (25 and 60 μM). In each assay, a constant free metal concentration was equilibrated with increasing protein concentrations (25–120 μM). Samples were equilibrated with the circulating buffer for 36 h at 4 °C, and the concentration of Mg^{2+} in the buffer and in the wells was measured using the dye Calmagite (Sigma) [24]. The following equations describe a binding to two sites with different affinity:

$$M \times L/ML = K_{D1} \quad (3)$$

$$ML \times L/MLL = K_{D2} \quad (4)$$

$$M_t = M + ML + MLL \quad (5)$$

$$M_t = K_{D1} \times ML/L + ML + ML \times L/K_{D2} \quad (6)$$

$$L_b = ML + 2MLL = M_t[1/(K_{D1}/L + 1 + L/K_{D2}) + 2L/(K_{D1} \times K_{D2}/L + K_{D2} + L)], \quad (7)$$

where M_t, ML, MLL, L, L_b, K_{D1}, and K_{D2} are molar concentrations of PchP, PchPMg, PchP$(Mg)_2$, free Mg^{2+}, bound Mg^{2+}, and the two dissociation constants, respectively. Eq. (7) was fit to the data. Note that in the dialysis experiment L is held constant and therefore the equation represents a straight line with ordinate = 0. Also, if $K_{D2} >> K_{D1}$, the equation reduces to:

$$L_b = ML = M_t/(K_D/L + 1), \quad (8)$$

which is the equation for binding to a single site.

Results and discussion

Expression and purification of PchP

Escherichia coli cells transformed with either pET9b-$PchP_{349}$ or pET9b-$PchP_{327}$ express large amounts of PchP. Using the protocol described in Materials and methods, 50 mg of tag-free, 95-98% pure PchP per liter of culture were regularly obtained, which constitutes a significant improvement over previous expression and purification procedures using fusion constructs [16] and provides a reliable source material for the structural study of this enzyme.

PET9b-$PchP_{349}$ and pET9b-$PchP_{327}$ encode pro-PchP and mature PchP, respectively. SDS-PAGE (not shown) revealed that in the lysates of cells transformed with pET9b-$PchP_{349}$ only a very small fraction of pro-PchP is present and the main product is mature PchP, identical in electrophoretic mobility to the single product obtained with cells transformed with pET9b-$PchP_{327}$. Mass and N-terminal analysis confirmed that, in both cases, the main product is processed PchP starting at residue Thr 23 (experimental mass = 37089 ± 4 Da; calculated mass = 37089.5 Da; Uniprot entry Q9HTR2_PSEAE). This result is in agreement with the signal peptide sequence and signal peptidase cleavage site predicted by SignalP (SignalP v.3.0's neural network and hidden Markov model implementations [25]) and confirms previous findings of Massimelli et al. [14]. Pro-PchP possess a typical signal peptide [26,27] and type I signal peptidase motif (AXA) [28]. The results imply that *P. aeruginosa* pro-PchP is efficiently processed in *E. coli*, and that processing precedes the formation of insoluble inclusion bodies. One interesting possibility is that the formation of inclusion bodies of mature PchP might occur in the periplasm of the bacterium, as reported before for a variant of TEM β-lactamase [29]. Since the expression of PchP from pET9b-$PchP_{349}$ afforded higher protein yields, all further experiments were carried out with the protein prepared using this construction.

Most of the expressed PchP is sequestered in inclusion bodies. Soluble PchP was obtained by treatment of the inclusion bodies with 6.5 M urea solutions. After purification by ionic exchange chromatography in concentrated urea, the refolding of the enzyme was accomplished removing the denaturant by dialysis. During dialysis, about 30% of the protein precipitates. The yield of soluble protein is highly dependent on the pH of the dialysis buffer and drops dramatically below pH 8.5. The final product of the refolding reaction is a mixture of different aggregation states that can be resolved chromatographically (see below), and native PchP was isolated by preparative gel filtration on a Sephadex G75 column.

General properties of refolded PchP

The aggregation state of native PchP (Fig. 1) was established unambiguously by multiangle light scattering, yielding a R_h value of 36.8 ± 0.4 Å and a particle mass of 74.6 ± 3.1 kDa. These results

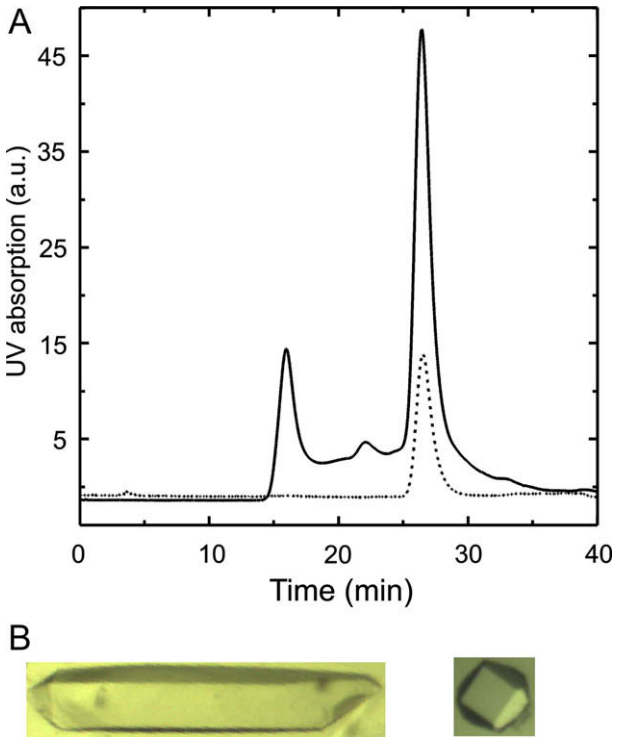

Fig. 1. (A) An analytical SEC chromatogram of the refolding product of PchP is shown (solid line). The peak eluting at 16.1 min corresponds to a catalytically-inactive aggregate of PchP (af-PchP). The fraction eluting at 26.5 min is catalytically-active, native PchP. Af-PchP isolated by preparative SEC (see Methods) is converted into native PchP after a ~10 day incubation at 4 °C, given rise to a peak with chromatographic behavior identical to that of native PchP (dotted line). On the other hand, af-PchP behaves as a chromatographic particle of 700 kDa or larger. A minor component with an elution volume corresponding to that of a PchP tetramer is also present. (B) Representative crystals of PchP obtained by the preparative procedure described in Materials and methods. Crystallization conditions were 20 °C, 0.1 M sodium acetate, 4% PEG 8000, pH 4.8 (left) and 0.1 M sodium phosphate, 0.1 M potassium phosphate, 0.1 M MES, 2 M sodium chloride pH 6.5 (right). Protein concentration was 8 mg mL^{-1}. The major axis of the crystal shown on the left is ~0.2 mm.

suggest that the elementary hydrodynamic particle for native PchP is a dimer.

The second major fraction populated after refolding elutes at the total accessible volume of the analytical SEC column implying a R_s > 80 Å and compatible with an aggregate of PchP of 700 kDa or larger (Fig. 1). This high-order aggregate possesses only residual activity but contains structured protein (see below), and thereafter it will be referred to as *alternatively folded* PchP (af-PchP).

The relative proportion of native and af-PchP after refolding is highly dependent on Mg^{2+} concentration. At 5 mM or higher Mg^{2+} concentration, native PchP is predominant, but irreversible aggregation and precipitation are also increased. At less than 5 mM Mg^{2+}, the yield of soluble protein increases, but this condition favors af-PchP at the expenses of native PchP. Thus, the chosen refolding conditions (5 mM Mg^{2+}) maximizes the recovery of native PchP.

Interestingly, af-PchP is a kinetically trapped, non native intermediate state that is transformed very slowly into native PchP. After 7–10 days a 4 °C, isolated af-PchP generates with high yield PchP with full catalytic activity and native R_s (see Fig. 1).

The catalytic parameters of the dimeric native PchP for the hydrolysis of *p*-NPP were determined (Table 1) and found to be in excellent agreement with the values reported previously for PchP expressed in *E. coli* as an N-terminal fusion and purified in the folded state [16].

PchP contains two cysteine residues, Cys 87 and Cys 84. To establish if they form a disulfide bond, the thiol content of native PchP was determined by the Ellman reaction [17,18]. In the presence of 6 M urea, the measurements indicated no free thiol groups. The same result was obtained with af-PchP. After treatment with DTT, full reactivity toward DTNB was observed (two thiol groups per molecule). Thus, it is concluded that PchP contains a disulfide bridge. This disulfide is intramolecular, since SDS-PAGE showed no differences in mobility for reduced and no reduced samples (not shown). Since native PchP treated with DTT retains full enzymic activity, the disulfide is not essential for keeping the protein in the native state. This finding was confirmed preparing by site specific mutagenesis the C87A and C94A PchP mutants, which have k_{cat}/K_m values of 4.4×10^4 and 2.4×10^4 M^{-1} s^{-1}, respectively, values compatible with a fully active conformation.

Additional evidence of a well-folded conformation and high purity for native PchP was obtained from preliminary crystallization screens that yielded crystals in several experimental conditions (Fig. 1). PchP crystals were subjected to X-ray diffraction but unfortunately the achieved resolution (4.0 Å) was not sufficient for further analysis. Refinement of the crystallization process is in progress.

CD and fluorescence studies

PchP possesses 21 tyrosine, 7 tryptophan, and 7 phenylalanine residues. If PchP aromatic residues were fully solvated, its $E_{M,\ 280\ nm}$ would be 62900 M^{-1} cm^{-1} [30]. The experimental determination using a previously described procedure [20] yielded an $E_{M,\ 280\ nm}$ of 70839 ± 151 M^{-1} cm^{-1} (mean ± SD; n = 3), which indicates that the aromatic groups in the native state are in a hydrophobic environment.

CD spectra of native PchP and af-PchP are shown in Fig. 2. The far-UV CD spectrum of native PchP corresponds to a protein with a significant content of secondary structure. Analysis of the spectrum using the standard methods implemented in Dicroprot [31] yielded 25–32% α-helix and 15–20% β-strand content. Thus, the result suggests that PchP is a α/β or α + β class protein and is consistent with the structural model proposed earlier by Beassoni et al. [16]. The near-UV CD spectrum of native PchP has strong signals, which reflects the asymmetric environment of aromatic residues in the folded protein. In the 250–270 nm range, positive bands from the vibronic transitions of phenylalanine residues are prominent. Tyrosine (270–285 nm) and tryptophan (285–290 nm) positive bands are also discernible. The CD spectra provide a basic reference for quality control and conformational integrity assessment of PchP preparations.

The far UV CD spectrum of af-PchP shows a strong reduction in the α-helical content in favor of beta or coil conformations. Be-

Table 1
Catalytical properties of native PchP[a].

Parameter	Value	Units
K_m	$2.90 \pm 0.3 \times 10^{-3}$ ($3.2 \pm 0.5 \times 10^{-3}$)[b]	M
k_{cat}	$8.3 \pm 0.6 \times 10^{1}$ ($8.1 \pm 1.6 \times 10^{1}$)	s^{-1}
k_{cat}/K_m	$2.9 \pm 0.7 \times 10^{4}$ (2.5×10^{4})	M^{-1} s^{-1}

[a] Means ± SE of five separate experiments.
[b] In parentheses are values reported by Beassoni et al. [16]; of these, k_{cat} had been miscalculated and was recalculated for this work from the specific activity data reported by the same authors.

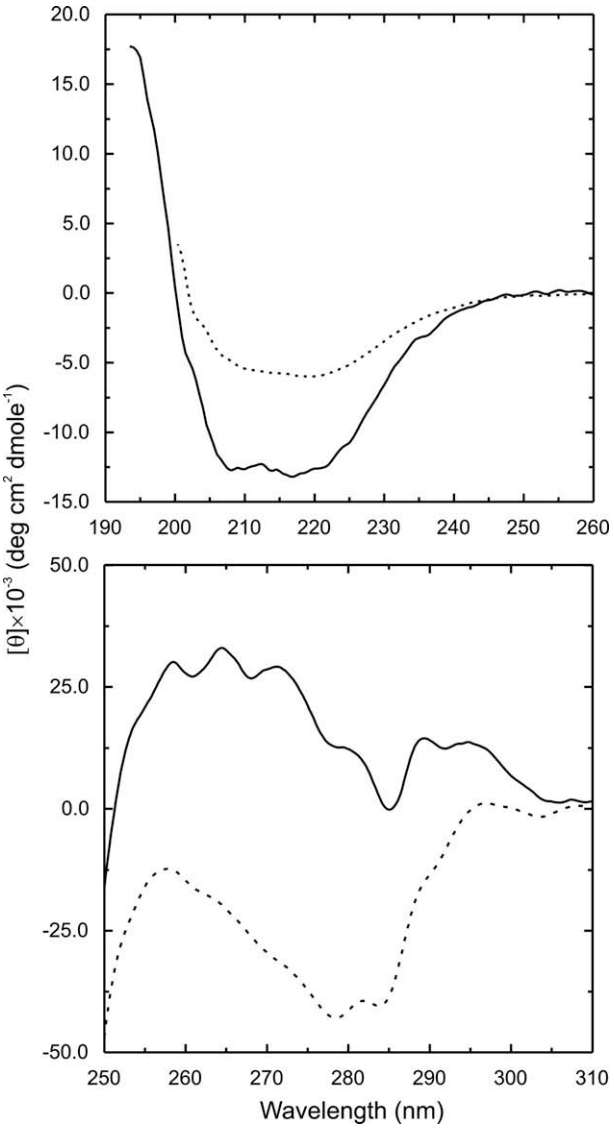

Fig. 2. Optical activity of PchP. Far and near UV CD regions of the spectra are shown in the upper and lower panels, respectively. The solid and dotted lines correspond to native PchP and af-PchP, respectively. As recommended in Ref. [35], CD intensity is expressed per dmole of peptide bond (far UV) and per dmole of protein monomer (near UV).

cause of increased light scattering, no reliable data in the 190–200 nm range were obtained in this case, and this precluded the use of Dicroprot for estimating the content of each secondary structure type. Strikingly, the near-UV CD spectrum of af-PchP is as strong as that of the native form but dominated by negative contributions of tyrosine and tryptophan residues. This strongly suggests that the aromatic residues are rigidly packed, although the tertiary structure must differ from the native fold.

Upon excitation at 295 nm the fluorescence emission of native PchP exhibits its maximum at 330 nm (see Fig. 1 in Supplementary Material). In comparison, free tryptophan emits at λ_{max} = 356 nm. This 26-nm blue shift suggests that most PchP tryptophan residues are solvent shielded by the native structure. The calculation of the quantum yield for PchP gives a value of 0.10 ± 0.07, which suggests that most PchP tryptophans residues experience above average quenching from nearby protein groups.

The fluorescence properties of af-PchP are also compatible with the shielding of its tryptophan residues from the solvent. Particularly, shape and λ_{max} of the af-PchP emission spectrum are indistinguishable from those from the native PchP spectrum. Interestingly, the fluorescence of af-PchP is even more quenched than in the case of native PchP, yielding a quantum yield value of 0.07 ± 0.05. This suggests that in both cases specific tertiary contacts between tryptophan residues and side-chains with the capacity to act as quenchers are preserved.

Mg^{2+} binding

Controlled proteolysis evidenced that PchP refolded without Mg^{2+} is conformationally unstable (see Fig. 2 in Supplementary Material). It is also known that divalent cations are required for PchP catalysis. Based on kinetic analysis it was proposed a compulsory ordered mechanism in which the metal binds to the active site prior to the substrate *p*-NPP [16,32]. However, a direct proof of the capability of the free enzyme to bind the metal in the absence of substrate was lacking. We addressed the issue measuring binding of Mg^{2+} to the free enzyme by dialysis equilibrium at pH 8.5 and 4 °C with different protein:metal molar ratios (Fig. 3). The best fit to the data was found with two binding sites (Eq. (7)) and affinity values of 210 and 700 μM for K_{D1} and K_{D2}, respectively. An equation assuming a single binding site (Eq. (8)) also fits reasonably well the data, and in this case the single K_D value is 177 μM is obtained. These results are consistent with the determination of the Mg^{2+} effect on V_{max} for *p*-NPP hydrolysis by PchP produced as an N-terminal intein fusion, which gave a K_D value of 600 μM [16]. From a mechanistic point of view, the dialysis binding data confirm that the presence of substrate is not obligatory for Mg^{2+} binding.

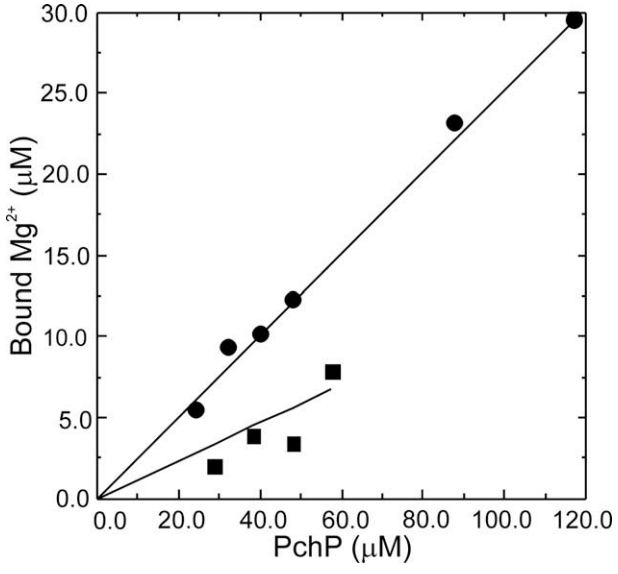

Fig. 3. Equilibrium binding of Mg^{2+} to PchP. Microdialysis experiments were performed at 4 °C against a large excess of 10 mM Tris–HCl, 100 mM NaCl, pH 8.5 containing 25 and 60 μM Mg^{2+}(squares and circles, respectively). Protein and metal contents of the compartments were measured after a 36 h equilibration. Straight lines are the result of the global fit of Eq. (3) to the data. Values in the abscissa are total (bond plus unbound) PchP.

Dependence of the enzymic activity on pH

It was reported that the highest specific activity of PchP toward *p*-NPP was at pH 5 [13]. To get a better understanding of the pH dependence of the catalyzed reaction K_M and k_{cat} were determined as a function of pH in the 4.5–5.7 range. The results are shown in Fig. 4. Below and above these pH values, K_M increases sharply and the kinetic plateau could not be reached, impeding reliable fit of the data. The parabolic curves for K_M and k_{cat} have opposite orientations and are centered at ~pH 5.2, where the maxima of k_{cat} and the minima for K_M are observed. A simple model for two ionizing groups at the active site [19] satisfactorily fits to the data and yields apparent pK_a values of 4.2 and 6.1. This result can be rationalized considering the theoretical model of PchP active site proposed recently by our group—which is based on the conservation of sequence motif among members of the HAD family and mutagenesis experiments [16]—and the mechanism of catalysis proposed for members of the same family for which detailed structural and biochemical information is available [33,34]. The basic mechanism of PchP phosphatase activity is a rate limiting nucleophilic attack of the Asp 31 carboxylate to the phosphorous of the bound $ROPO_3H$-Mg^{2+} complex. This first step results in the phosphorylated enzyme intermediate. Subsequently, a water molecule activated by proton abstraction acts as the nucleophile that releases the phosphate group and regenerates the free enzyme. The proposed general base that activates the water molecule is Asp 33. The apparent pK_a value of 4.25 may reflect the ionization of Asp 31. The second pK_a value is more difficult to assign. However, it may represent the ionization of the phosphate group, which may need to be protonated to allow the attack of ionized water.

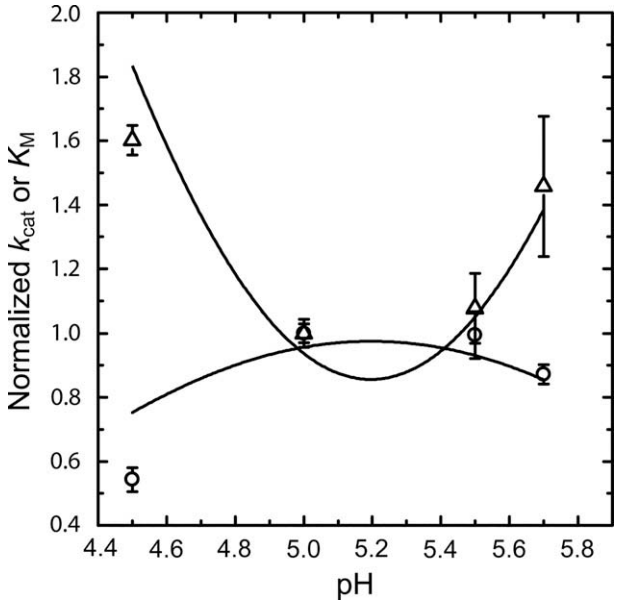

Fig. 4. Catalytic parameters of PchP as a function of pH. k_{cat} (circles) and K_M (triangles) were normalized as the ratio to the respective values at pH 5.0. Bars represent the error of the mean of 6–8 independent experiments.

Unfolding

The thermal stability of PchP and the effect of Mg^{2+} on it were assessed by far UV CD measurements. The signal was recorded as the protein samples were heated (Fig. 5) or cooled (not shown) at uniform rate in the 4–85 °C range. PchP was dissolved in Buffer C containing 5 mM Mg^{2+}, or 1 mM Mg^{2+} and 5 mM EDTA. In both cases, the conformational changes that ensued from heating were irreversible, which precludes derivation of formal energy parameters. Notwithstanding, by fitting the data to Eqs. (1) and (2), apparent T_m, ΔC_P, and ΔH were obtained, which serve for comparative purposes. In the presence of 5 mM Mg^{2+}, the enzyme is exceedingly stable with a T_m of 65.8 °C, ΔC_P = 2.18 kcal mol^{-1} K^{-1}, and ΔH = 76.8 kcal mol^{-1} for the major phase of ellipticity change. This sigmoid change in the signal is preceded by a much smaller change between 30 and 50 °C that in this particular case was modeled as if it were part of the baseline change. In the presence of EDTA, the enzyme becomes unstable and looses a fraction of the ellipticity at room temperature. Heating the Mg^{2+}-free protein elicits a conformational change that is much less cooperative and of less amplitude than the corresponding transition of the Mg^{2+} stabilized protein. The derived apparent T_m, ΔC_P, and ΔH in this case were 34.3 °C, 1.54 kcal mol^{-1} K^{-1}, and 31.3 kcal mol^{-1}, respectively, which evidences a huge reduction in thermal stability.

Conclusions

A reliable procedure for the preparation of large amounts of properly folded PchP was devised, and its basic chemical and biophysical properties have been determined. The obtained product is of high quality and purity, crystallized in preliminary screening, and thus shows promise as a starting point for X-ray studies and structure determination. A complex folding behavior of PchP has also been unveiled, involving a kinetically trapped alternatively folded state. A procedure for the separation of this nonnative state from native PchP has been devised, which is of interest for basic biophysical studies and for improving the quality of PchP preparations that will be subjected to structural characterization. The catalytic properties of the enzyme have been characterized and the high affinity of the enzyme for Mg^{2+} in the absence of substrate demonstrated. In addition, spectroscopic features for this protein have been identified that would allow the efficient quality control of the enzyme preparations and serve as monitors for more advanced folding studies. The degree of thermal resistance of the enzyme was established, and it was found that in the absence of Mg^{2+} the protein looses its structure, providing a lead for potential inhibitory agents. In summary this work sets standards and provides clues that may be of great help in future basic and applied studies of this enzyme.

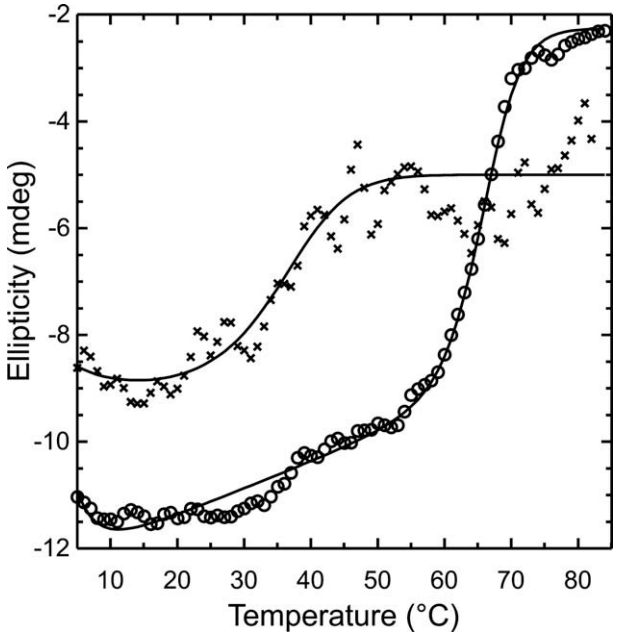

Fig. 5. Thermal unfolding of PchP. The CD signal was monitored at 220 nm while the cell was heated at a uniform rate of 2 °C per min. Circles correspond to native PchP in Buffer C (i.e., in the presence of 5 mM Mg^{2+}). Crosses correspond to the protein in the same buffer depleted of Mg^{2+} by the addition of EDTA. The lines represent the fit of the data to Eqs. (1) and (2). For clarity, only one every four experimental data are shown. The corresponding T_m for each transition is given in Results.

Conflict of interest statement

The authors declare that they have no competing financial interests.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.pep.2010.01.006.

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