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Characterization of a Highly Thermostable Alkaline Phosphatase from the Euryarchaeon *Pyrococcus abyssi*

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This work reports the first isolation and characterization of an alkaline phosphatase (AP) from a hyperthermophilic archaeon. An AP gene from *Pyrococcus abyssi*, a euryarchaeon isolated from a deep-sea hydrothermal vent, was cloned and the enzyme expressed in *Escherichia coli*. Analysis of the sequence showed conservation of the active site and structural elements of the *E. coli* AP. The recombinant AP was purified and characterized. Monomeric and homodimeric active forms were detected, with a monomer molecular mass of 54 kDa. Apparent optimum pH and temperature were estimated at 11.0 and 70°C, respectively. Thus far, *P. abyssi* AP has been demonstrated to be the most thermostable AP, with half-lives at 100 and 105°C of 18 and 5 h, respectively. Enzyme activity was found to be dependent on divalent cations: metal ion chelators inhibited activity, whereas the addition of exogenous Mg(II), Zn(II), and Co(II) increased activity. The enzyme was inhibited by inorganic phosphate, but not by molybdate and vanadate. Strong inhibitory effects were observed in the presence of thiol-reducing agents, although cysteine residues of the *P. abyssi* AP were not found to be incorporated within intra- or interchain disulfide bonds. In addition, *P. abyssi* AP was demonstrated to dephosphorylate linear DNA fragments with dephosphorylation efficiencies of 93.8 and 84.1% with regard to cohesive and blunt ends, respectively.

Alkaline phosphatases (APs) (orthophosphate monoester phosphohydrolases [alkaline optimum]; EC 3.1.3.1.) are classically described as homodimeric nonspecific metalloenzymes which catalyze phosphomonesterase reactions (45). The fact that they are widely found in nature, from bacteria to mammals indicates that APs are included in fundamental biochemical processes (38). Despite the fact that their physiological function is not clear, their induced production under inorganic phosphate starvation in many species (especially procaryotic organisms) indicates that they play a vital role in the phosphate metabolism. In mammals, they are linked with transport mechanisms (30).

Many APs have been characterized since the 1960s. The *Escherichia coli* AP has been widely studied in terms of biosynthesis (11, 26), structure, and catalytic properties (9). Numerous mammalian AP cDNAs have been cloned, and the corresponding enzymes have been characterized (5, 48, 49). Alignments of the deduced protein sequences have shown a strong conservation of the catalytic site, which involves a serine residue and three metal ions per monomer, two Zn(II) and one Mg(II) (27). However, mammalian APs differ from their *E. coli* counterpart in terms of Mg(II) secondary ligand (33), membrane anchoring (45), glycosylation (27), etc. APs represent a large research field as they are good models to study metal ion-dependent catalysis and are used in several application fields (molecular biology [3] and immunodetection [43]).

Although *Archaea* have been studied for a few decades, relatively few archaeal APs have been described. All of them are from halophilic species. APs have been isolated and characterized from three species of the genus *Halobacterium* (6, 15, 16). In 1990, Goldman et al. described an AP from the halophilic archaeon *Haloarcula marismortui* (17). So far, no APs from hyperthermophilic archaeons have been isolated and characterized. As studying thermostable enzymes appears interesting for the understanding of life at high temperatures, as well as for industrial processes, new APs exhibiting this property have been investigated. Thermostable APs from the following thermophilic bacteria have been described: *Thermotoga neapolitana* (12), *Thermus caldophilus* (36), *Thermus thermophilus* (37), and *Bacillus stearothermophilus* (32).

Pyrococcus abyssi is a heterotrophic hyperthermophilic euryarchaeon isolated from a deep-sea hydrothermal vent with an optimal growth temperature of 100°C (14). As its complete genome sequence is known and is publicly available (http://www.genoscope.fr), AP patterns were identified and allowed to isolate an archaeal AP gene. In this report, we describe the first characterization of a thermostable AP isolated from a hyperthermophilic archaeon.

MATERIALS AND METHODS

Organisms and growth conditions. *P. abyssi* (strain Orsay) was used in this study (14). The strain was grown in 2216S medium (4) at 95°C. *E. coli* HMS 174(DE3) harboring pLysS, purchased from Novagen, was used as the host strain for the recombinant plasmid of pARHS (10), and *E. coli* DH5α was used for the pBSK vector in dephosphorylation studies. *E. coli* strains were grown in 2×YT medium (Bacto Peptone, 16 g · liter^{-1}; yeast extract, 10 g · liter^{-1}; NaCl, 10 g · liter^{-1}) on a rotary shaker at 37°C for various times. Ampicillin was added to 2× YT to give a final concentration of 100 μg · ml^{-1}. Isopropyl-β-D-thiogalactopyranoside (IPTG) was added to give a final concentration of 0.5 mM to induce gene expression (3).

Cloning of the AP gene. Based on the *P. abyssi* genome sequence (http://www.genoscope.fr/Pab/, accession number 2366), the *phoA*$_{P.abyssi}$ gene was amplified by PCR on a DNA Thermal Cycler (Stratagene). Genomic DNA extrac-

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tion was performed on a *P. abyssi* culture as previously described (3), and the DNA was used as a template. The two primers, including *Nde*I and *Bam*HI restriction sites (in boldface type), were as follows: AP1 (5′-GTTTCCATTTGC **TCATATG**TCTCCAAGCGG-3′, sense) and AP2 (5′-CTCACCTCAA**GGATCC** TTAAGAAGAAGC-3′, antisense). By introducing a start codon at the beginning of the putative mature protein gene, AP1 was designed so that the putative signal peptide sequence of the *P. abyssi* AP would not be amplified. AP2 was targeted to the stop codon of the $phoA_{P.abyssi}$ gene, introducing the *Bam*HI restriction site next to the TAA codon. In addition to the template, the 50-μl reaction mixture contained 100 pmol of each primer, 10 nmol of an equimolar deoxynucleoside triphosphate mix (Eurogentec S.A.), *Taq* DNA polymerase buffer containing 5 mM $MgCl_2$ (Qbiogene), and 1.5 U of *Taq* DNA polymerase (Qbiogene). The mixture was subjected to eight cycles of amplification (30 s at 94°C, 30 s at 45°C, and 1 min 30 s at 72°C). A PCR product with the expected size was digested with *Nde*I and *Bam*HI and cloned in a pARHS vector, producing the recombinant plasmid pPabAP, and transformed into *E. coli* HMS 174(DE3) pLysS using standard procedures (3). Using this strategy, the putative mature enzyme was produced in the cytoplasm of *E. coli.*

Expression and purification of the recombinant AP. An overnight culture of *E. coli* HMS 174(DE3) pLysS, harboring pPabAP, was diluted in a 1:20 (vol/vol) ratio and grown until the optical density at 600 nm reached 0.8 (Milton Roy Spectronic 401, from Bioblock Scientific). The culture was induced by the addition of IPTG, to give a concentration of 0.5 mM, and incubated for an additional period of 4 h. Cells were harvested by centrifugation, washed in 50 mM sodium phosphate buffer (pH 7.5) containing 50 mM NaCl, and then resuspended in 50 mM sodium citrate buffer (pH 5.5). After sonication (375 W; 40% amplitude; Vibracell sonifier), cell debris was removed by centrifugation (10,000 × *g* for 10 min). The resulting supernatant was heated for 20 min at 80°C, and precipitated proteins were removed by another centrifugation as described above. As *E. coli* HMS 174(DE3) is not a $phoA_{E.coli}$ deletion strain, controls were used to evaluate the extent of *E. coli* AP contamination. The supernatant was subsequently dialyzed against sodium phosphate buffer (50 mM, pH 7.5) and loaded on an anion exchange column (ResourceQ; Pharmacia) equilibrated with the same buffer. Bound proteins were eluted by a linear gradient of NaCl (0 to 1 M in sodium phosphate buffer pH 7.5). Active fractions were eluted with 250 mM NaCl. The latter were pooled, loaded on a second anion exchange column (MonoQ; Pharmacia), and eluted by a linear gradient of NaCl (0 to 0.4 M in sodium phosphate buffer, pH 7.5). All chromatographic steps were performed at 4°C.

Protein samples were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using the method of Laemmli (29). Protein samples for SDS-PAGE were prepared by heating them for 10 min at 100°C in a 1:5 ratio volume of sample buffer (60 mM Tris-HCl buffer, pH 6.8; 2% SDS [wt/vol], 14.4 mM 2-mercaptoethanol [MCE], 25% glycerol [vol/vol], and 1% bromophenol blue [wt/vol]). Proteins were observed by staining them with Coomassie brilliant blue. Molecular mass standard was obtained from Pharmacia.

AP assay and protein determination. The standard assay for AP activity was carried out at 70°C, for 2.5 min, using 2.5 mM *p*-nitrophenylphosphate (pNPP) (Sigma Chemical Co.) as the substrate in 50 nM NaOH-glycine (pH 10.0) buffer, containing 0.5 mM $CoCl_2$, except as otherwise indicated. The release of *p*-nitrophenol in the reaction mixture (1 ml) was continuously measured spectrophotometrically at 410 nm using a Uvikon XL spectrophotometer (from BIOTEK Instruments), over the linear period. Enzyme and substrate blanks were also included. One unit of enzyme activity is defined as the amount of enzyme required to release 1 μmol of *p*-nitrophenol from pNPP in 1 min. Protein concentration was determined by the method of Bradford, using bovine serum albumin as the standard (7). For routine enzymatic characterization assays, 252 ng of purified AP was added to the 1-ml reaction mixture. Each value is the mean of at least three assays.

Quaternary structure analysis. Native gel electrophoresis was performed using 4 to 12% gradient polyacrylamide minigels (Bio-Rad). Samples were added in a 4:5 ratio (vol/vol) to native electrophoresis buffer (60 mM Tris-HCl buffer, pH 6.8; 25% glycerol; 1% bromophenol blue). Zymograms were performed by incubation of minigels in 50 mM NaOH-glycine (pH 10.0) buffer, containing 0.5 mM $CoCl_2$ and 2.5 mM 4-methylumbelliferylphosphate (MUFP) (Sigma Chemical Co.) as the substrate, for 20 min at 70°C. Active bands were observed under UV light, and proteins were visualized after staining with Coomassie brilliant blue. Molecular mass standard was obtained from Pharmacia.

Temperature and pH optima, thermal stability, and kinetic parameters. The apparent optimum temperature was determined by running the standard assay at temperatures ranging from 20 to 90°C in 3-(cyclohexylamino)-1-propanesulfonic acid (CAPS) buffer (0.2 M; pH 11.0). The apparent optimum pH of the enzyme was determined by running the standard assay, using Tris-HCl buffer (0.2 M) and CAPS buffer (0.2 M) for the pH ranges 6.5 to 10.5 and 9.0 to 12.5, respectively. Thermostability was determined using a diluted enzyme (0.63 mg of purified enzyme per ml was diluted 1:10 [vol/vol] in 50 mM Tris-HCl buffer, pH 8.0) and incubated at 90, 100, 105, and 115°C. Samples were taken at time intervals, and the residual activity was determined by the standard assay. pH was adjusted at room temperature for all the buffers used. Michaelis-Hill constants were determined from data obtained by determining the initial rate of pNPP hydrolysis under the assay conditions described above with 1 mM $CoCl_2$, using a range from 0.1 to 10 mM substrate.

Cation and inhibitor effect studies. The effects of monovalent and divalent cations were examined for their influence on enzyme activity. Increasing concentrations of NaCl and KCl were added to 50 mM NaOH-glycine (pH 10.0) containing 2.5 mM pNPP, and enzymatic activity was measured by standard procedures. Several divalent cations were also tested under similar conditions. All of them were used in their chloride form. The effect of inhibitors was determined by carrying out the same procedure, minus addition of $CoCl_2$.

DNA dephosphorylation procedure. pBSK was digested using *Eco*RI- or *Eco*RV-producing, respectively, cohesive and blunt-ended linearized vectors. Linearized vector (1.1 μg) was incubated in dephosphorylation buffer and 50 U of AP for 2 h. The recombinant AP from *P. abyssi* was used in 0.2 M Tris-HCl buffer (pH 10.0) at 70°C. Commercial calf intestinal AP (CIAP) (Roche) was used as a positive dephosphorylation control in its commercial dephosphorylation buffer at 37°C. The number of AP units was determined by carrying out the standard assay procedure under dephosphorylation conditions of buffer and temperature. Following this, 0.55 μg of AP-treated linear vector was ligated in the presence of 2 U of T4 DNA ligase along with its commercial buffer (Roche) to give a final volume of 20 μl, which was incubated overnight at 16°C. Finally, 50 μl of competent *E. coli* DH5α cells was transformed by 1 μl of the ligation mixture. Preparation of the competent cells and transformations were performed using the standard $CaCl_2$ transformation protocol (3). Transformed cells were selected by plating on 2×YT agar medium containing ampicillin (100 $\mu g \cdot ml^{-1}$). After linearization and dephosphorylation, DNA fragments were purified using a Spin Column (Qiagen) and resuspended in ultrapure water. Negative dephosphorylation control was performed by avoiding the dephosphorylation step. The percent dephosphorylation efficiency [*DE*(%)] was calculated from the following equation:

$$DE(\%) = \left(1 - \frac{\text{CFU/ml}_{AP}}{\text{CFU/ml}_{\text{control}}}\right) \times 100$$

where CFU/ml is the number of CFU per milliliter of transformed cells, CFU/ml_{AP} is CFU/ml after ligation performed on dephosphorylated vectors, and $\text{CFU/ml}_{\text{control}}$: is CFU/ml after ligation performed on nondephosphorylated vectors.

RESULTS

***P. abyssi* AP gene presents classical and unique features.** Among the 1,765 open reading frames deduced from the complete genome sequence of *P. abyssi* (available at http://www.genoscope.fr/Pab/), the $phoA_{P.abyssi}$ gene encoding an AP was identified as a 1,485-bp fragment located between positions +1001913 and +1003397 on the *P. abyssi* chromosome. Its putative genomic environment is composed of five genes encoding various phosphate transporting proteins, suggesting the existence of a pho regulon, as previously observed in other species (22, 44). The deduced product of $phoA_{P.abyssi}$ is a protein of 495 amino acids, with a theoretical molecular mass and isoelectric point of 54.5 kDa and 4.90, respectively. Hydropathy analysis of the N-terminal end of the protein revealed a domain with a high hydrophobicity (data not shown), suggesting that it is a signal peptide. In accordance with the "−3, −1 rule," the most probable cleavage site for the signal peptidase is between A25 and S26 (35, 46). Significant peptide sequence similarities were found with other APs already described, ranging from less than 30% for eucaryotic counterparts to approximately 35% for procaryotic APs, with the exception of *Pyrococcus furiosus* AP, which is 80% similar. In addition, alignment of peptide sequences from various APs

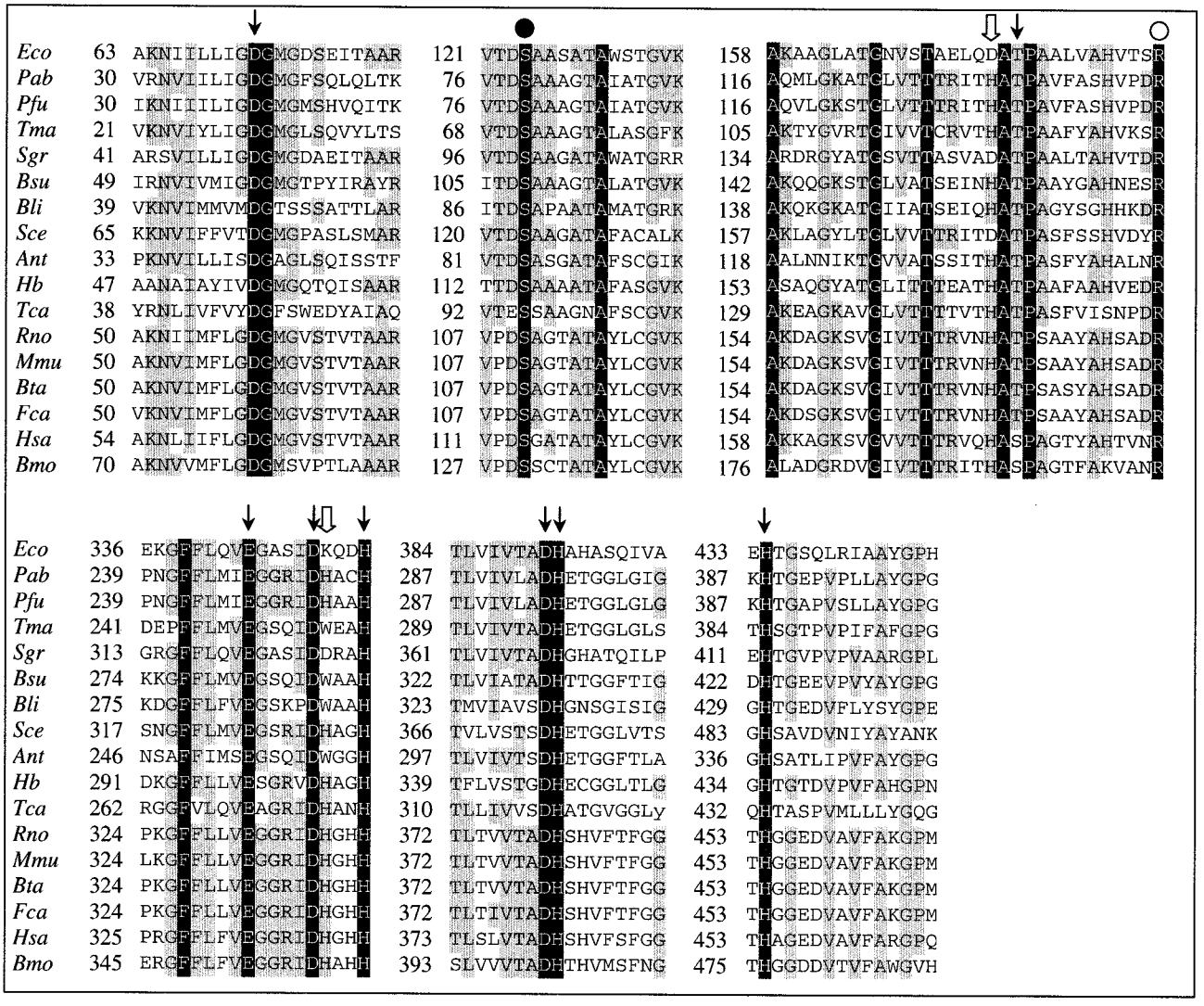

FIG. 1. Conservation of the active site. Shown is the peptide sequence alignment of *P. abyssi* AP with other members of the AP family. Abbreviations are as follows (sequences were obtained from the accession numbers indicated in parentheses): *Eco*, *E. coli* (pir, PAECA); *Pab*, *P. abyssi* (pir, E75081); *Pfu*, *P. furiosus* (Pf, 962748); *Tma*, *T. maritima* (pir, 72410); *Sgr*, *Streptococcus griseus* (pir, S17780); *Bsu*, *Bacillus subtilis* AP IV (pir, B69676); *Bli*, *Bacillus licheniformis* (genpept, AAG10093); *Sce*, *Saccharomyces cerevisiae* (pir, S69648); *Ant*, *Antarctic bacterium* (emb, CAB82508.1); *Hb*, *Halobacterium* sp. (genpept, AAG20936); *Tca*, *T. caldophilus* (genpept, AAF13361); *Rno*, *Rattus norvegicus* (pir, A28114); *Mmu*, *Mus musculus* (pir, A40172); *Bta*, *Bos taurus* (pir, A29600); *Fca*, *Felis catus* (pir, S66467); *Hsa*, *Homo sapiens* placental (pir, PAHUA); *Bmo*, *Bombyx mori* (pir, S19607). Strictly conserved residues and residues conserved more than 60% are highlighted in black and gray, respectively. Symbols above the sequences indicate the conserved residues which are involved in the active site, according to the following legend: ↓, metal ion coordination (Zn1, Zn2, Mg primary ligand); ⇓, metal ion coordination (Mg secondary ligand); ●, phosphoseryl intermediate formation; ○, phosphate coordination. Numbering of the sequences is given for precursors. Alignments were performed using the GAP program (Genetics Computer Group Wisconsin Package) in pairwise comparison.

with the mature product of the $phoA_{P.\ abyssi}$ gene (Fig. 1) shows conservation of well-defined regions which are conserved throughout the entire family. These regions contain the amino acid residue S102 (mature *E. coli* AP numbering), which has been shown to be incorporated within the phosphoseryl intermediate, in addition to the residues involved in the coordination of the two Zn(II) and the Mg(II) ions. However, the secondary ligands of the Mg(II), i.e., D153 and K328 (mature *E. coli* AP numbering), are replaced by H108 and H228 in the *P. abyssi* AP. These amino acid substitutions have been shown to be specific to mammalian APs (33). By comparison of AP sequences from *P. abyssi* and *E. coli*, it was concluded that the 10 strands of central β-sheet are conserved (data not shown). Thus, the topology of the *E. coli* AP central β-sheet is most likely conserved in the archaeal enzyme. Also, a putative Ca (II)-binding domain (Ef-hand) was deduced from the C-terminal sequence analysis and found to be specific to *P. abyssi* and *P. furiosus* AP sequences.

Expression and purification of *P. abyssi* AP. A 1,410-bp fragment was cloned in pARHS vector, encoding a 470-amino-acid protein which corresponds to the putative mature protein. The recombinant AP was expressed in *E. coli* HMS 174(DE3) pLysS cells harboring the pPabAP plasmid. The *E. coli* strain harboring the plasmid pPabAP was grown until mid-log phase

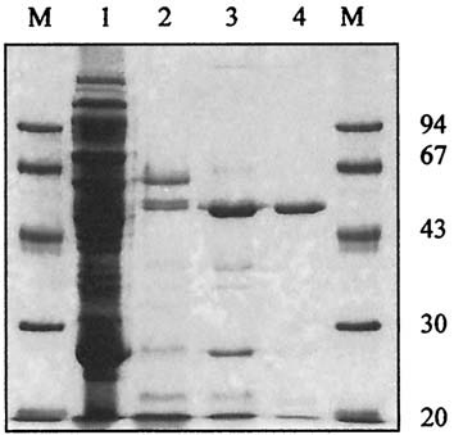

FIG. 2. Purification of the recombinant *P. abyssi* AP. Results of SDS–12% PAGE, showing the different steps of AP purification, are presented. Recombinant AP was purified from the soluble cellular fraction of *E. coli* HMS 174(DE3) pLysS, harboring pPabAP plasmid, induced cells. Lanes M, molecular mass standard; lane 1, soluble cellular crude extract; lane 2, crude extract after thermal treatment; lane 3, ResourceQ pooled active fractions; lane 4, purified fractions after MonoQ chromatography.

and induced by IPTG. SDS-PAGE analysis of cell-free heat-treated extract of induced cells revealed an additional band of 52 kDa, corresponding to the calculated molecular mass of the $phoA_{P.\ abyssi}$ product without its putative signal peptide (data not shown). The 52-kDa band was absent in extracts of *E. coli* HMS 174(DE3) pLysS (data not shown). A low expression rate was observed (approximately 5% of the soluble cellular protein fraction). Optimization of the expression was attempted. Neither IPTG concentration (0.1 to 2.0 mM) nor induction time (4 h to overnight) variations showed significant changes in the final recombinant enzyme concentration. Negligible AP activity was observed in the insoluble cellular fraction. Moreover, cytotoxicity of the recombinant enzyme was observed as cell lysis after induction of expression. Therefore, an induction time of 4 h was preferred to overnight induction. Nevertheless, recombinant AP could be easily purified to homogeneity by a three-step purification procedure consisting of heat incubation (80°C, 20 min, pH 5.5), which results in denaturation of the majority of *E. coli* proteins, followed by two anion exchange chromatography steps (data not shown). In particular, it appears that the heat incubation at pH 5.5 completely denatured *E. coli* AP as shown by zymogram and standard assay using MUFP and pNPP as substrates, respectively. Purification was estimated at approximately 450-fold, and the enzyme was determined to be at least 95% pure by SDS-PAGE (Fig. 2). Finally, purified AP solution (0.63 mg · ml^{-1}) was stored in phosphate buffer (pH 7.5) containing 50% glycerol at −20°C.

***P. abyssi* AP exists in mono- and homodimeric forms.** Electrophoresis of the purified recombinant AP using a 4 to 20% gradient of polyacrylamide gel under native conditions revealed the presence of two active forms (Fig. 3). Their respective molecular masses were estimated at 54 and 106 kDa. Since the 54-kDa value is consistent with the calculated molecular mass of the cloned gene product, the recombinant *P. abyssi* AP appears to exist in two forms, monomeric and homodimeric, both of which are active (Fig. 3). As one cysteine residue is present in the deduced amino acid sequence, the hypothesis of a dimeric form maintained with an interchain disulfide bond was clarified by treating the *P. abyssi* AP with SDS before running the electrophoresis experiment. Treatment of the AP with SDS yielded only the monomeric form (Fig. 3), and it was therefore concluded that the homodimeric form is maintained by noncovalent interactions, such as ion pairs and/or hydrophobic interactions, rather than an interchain disulfide bond.

***P. abyssi* AP presents high pH and temperature activation and high thermostability.** Apparent optimum pH of the AP was determined to be 11.0 in 0.2 M CAPS buffer. The enzyme exhibited at least 60% of its optimal activity over a rather narrow pH range, from 10.0 to 11.5 (Fig. 4A). Significant activity was observed over a pH range from 9.0 to 12.0. Furthermore, thermal activation studies enabled the determination of an apparent optimum temperature of 70°C in 0.2 M CAPS buffer (pH 11.0) (Fig. 4B). 25% of the maximum activity was found at 37°C. Moreover, the enzyme exhibited at least 80% of its optimal activity over a range of temperature from 60 to 80°C. Thermal stability was determined from enzyme incubation over a range of temperatures (90 to 115°C) (Fig. 4C). Half-lives of the *P. abyssi* AP at 100 and 105°C were estimated at 18 and 5 h, respectively. Eighty percent of the activity was recovered after incubation at 90°C for 36 h. Denaturation of the enzyme was observed to occur in less than 30 min at 115°C. Activation energy of the thermal denaturation was estimated at 262.1 kJ · mol^{-1}, according to the Arrhenius relationship.

***P. abyssi* AP is strongly dependent on divalent cations.** As APs are classically described as metalloenzymes (38), the effect of divalent cations was examined (Table 1). A strong inhibition in the presence of 1 mM EDTA in the reaction mixture was observed, whereas the enzyme exhibited a twofold activation in the presence of 1 mM Mg(II), Mn(II), Zn(II), or Co(II). Such results are a signature of strong dependency on divalent cations and confirm the metalloenzymatic nature of the *P. abyssi* AP. Increasing concentrations of Mg(II) in the reaction mixture provided a linear activation of the enzyme in the 0 to 2

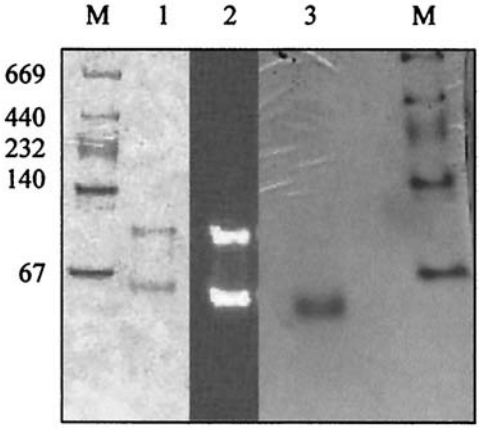

FIG. 3. Quaternary structure analysis. Results of 4 to 20% gradient PAGE, showing active forms and denaturation studies of the purified recombinant AP, are presented. Lanes M, molecular mass standard; lane 1, purified AP after Coomassie blue staining; lane 2, purified AP after incubation of the gel under enzymatic reaction conditions, using MUFP as substrate; lane 3, purified AP after 10% SDS treatment (10 min, 100°C) and Coomassie blue staining.

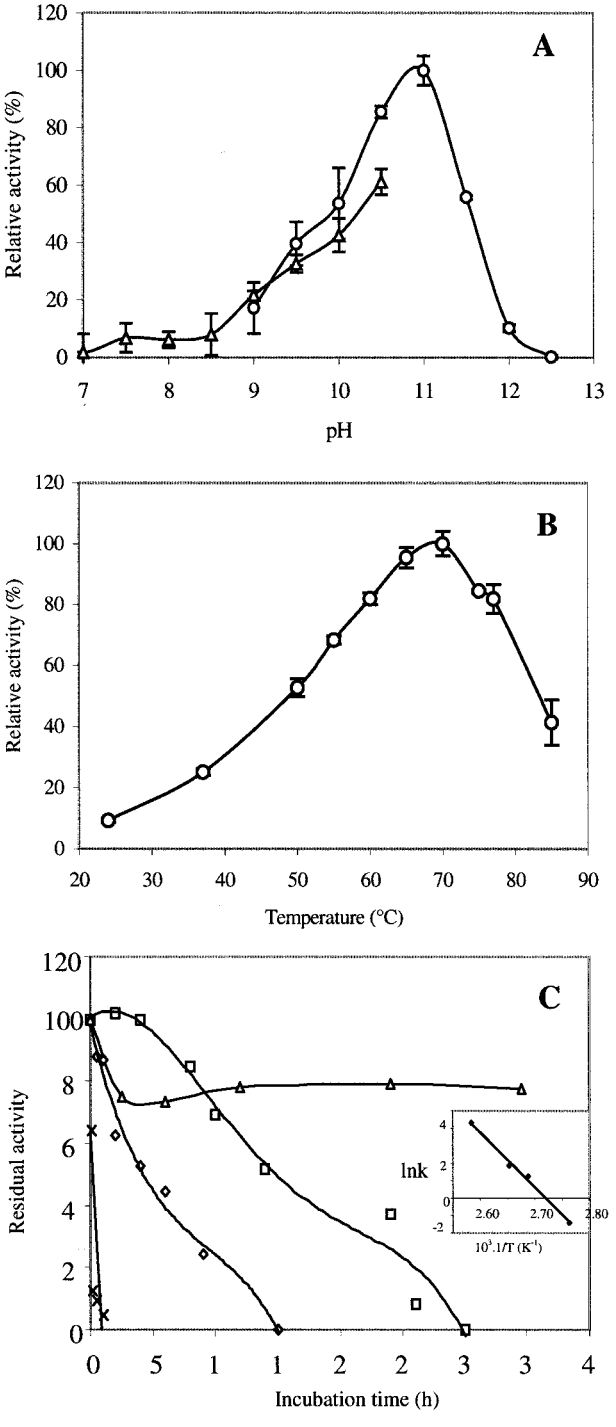

FIG. 4. (A) Optimum pH. Enzymatic activity was assayed at 70°C in the presence of 2.5 mM pNPP as a substrate, using 0.2 M Tris-HCl buffer, pH 7.0 to 10.5(△), and 0.2 M CAPS buffer, pH 9.0 to 12.5 (○). (B) Optimum temperature. Enzymatic activity was assayed at 22 to 85°C in the presence of 2.5 mM pNPP as a substrate, using 0.2 M CAPS buffer, pH 11.0. (C) Thermal stability. Residual activity versus incubation time at 90°C (△), 100°C (□), 105°C (◇), and 115°C (×) is shown. The insert graph shows lnk versus $10^{-3} \cdot 1/T$, where k is the rate constant from the Arrhenius relationship and T is the temperature of incubation (K).

TABLE 1. Effect of divalent cations

Effector (1 mM)	Relative activity (%)[a]
None	100.0 A
Mg(II)	223.1 A
Mn(II)	206.1 B
Zn(II)	204.0 B
Co(II)	186.6
Ca(II)	82.8 B
Cu(II)	61.9
Ni(II)	44.5 A
EDTA	10.9 A

[a] Standard deviations (SD) were <5% except for values with an A (5% ≤ SD < 10%) and those with a B (10% ≤ SD < 15%).

mM range, followed by a plateau in the 2 to 10 mM range (data not shown). This is consistent with the presence of a Mg(II)-binding site which has become saturated with the addition of exogenous Mg(II). Despite the elucidation of a Ca(II)-binding site in the deduced peptide sequence, this cation was found to have no significant effect on enzymatic activity. Cu(II) and Ni(II) showed inhibitory effects. The influence of ionic strength was tested. Increasing concentrations of NaCl and KCl had an inhibitory effect on AP activity. At 2 M of NaCl or KCl, 60 and 90% of the enzyme activity, respectively, was recovered (data not shown).

***P. abyssi* AP catalytic properties: effect of substrate concentration and inhibitors.** Michaelian kinetic parameters were estimated using the Lineweaver-Burk representation (Fig. 5). The curve is made up of two linear parts which allowed the calculation of two (V_{max}, K_m) doublets. Thus, from 0.1 to 0.7 mM, obtained values were as follows: $V_{max1} = 4.07$ μmol · min^{-1} and $K_{m1} = 166.33$ μM; from 0.8 to 10 mM, obtained values were as follows: $V_{max2} = 8.31$ μmol · min^{-1} and $K_{m2} = 1204.98$ μM. Moreover, inhibitors were tested (Table 2): classical phosphatase inhibitors (inorganic phosphate, molybdate, and vanadate), denaturing agents (SDS, urea), metal ion chelators (EDTA, EGTA), and thiol-reducing agents (dithiothreitol [DTT], MCE). Inorganic phosphate inhibited AP activity with only 22.2% of the control activity recovered in the presence of 10 mM Na_2HPO_4. This is in accordance with the general inhibitory effect of inorganic phosphate on APs. Molyb-

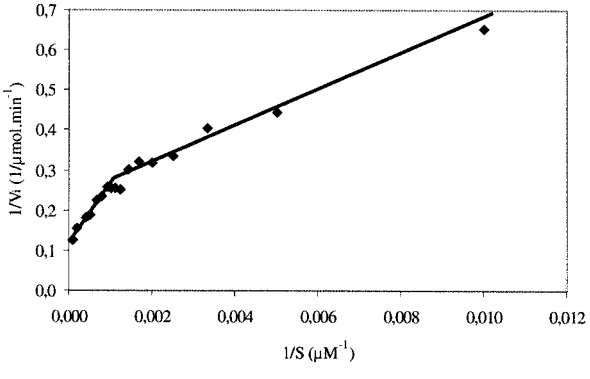

FIG. 5. Kinetic parameters: $1/V_i$ versus $1/S$. Enzymatic activity was assayed under standard condition with 1 mM $CoCl_2$ using a range of pNPP concentrations from 0.1 to 10 mM. Abbreviations: V_i, initial velocity; S, pNPP concentration.

TABLE 2. Spectrum of inhibitors

Inhibitor	Concn (mM)	Relative activity (%)[a]
None		100.0*
SDS	30 (1%)	16.4*
Urea	2,000	107.8
	4,000	106.5*
Molybdate	1	101.2*
	10	86.3
Vanadate	1	88.2*
	10	73.4*
Inorganic phosphate	1	74.8
	10	22.2
EDTA	1	10.7
	5	5.6*
EGTA	1	17.8
	5	4.6
DTT	0.2	79.7
	1	2.2
	2	1.6
MCE	2	58.5*
	5	32.1
	10	14.1

[a] Standard deviations were <5% except for values with an asterisk (5% ≤ standard deviation <10%).

date and vanadate were shown to have slight inhibitory effects, with 86.3 and 73.4% of the control activity retained, respectively, at an added concentration of 10 mM, respectively. SDS exhibited a strong inhibitory effect, whereas 2 and 4 M urea did not appear to have any effect. Metal ion chelators greatly inhibited enzymatic activity. Thiol-reducing agents (DTT and MCE) showed strong inhibitory effects. Upon addition of 2 mM DTT or MCE to the reaction mixture, residual AP activity was 1.6 and 58.5%, respectively.

***P. abyssi* recombinant AP is effective for DNA dephosphorylation.** APs are classically used in molecular biology to remove 5′-terminal phosphate from DNA fragments. As such, *P. abyssi* AP was tested against cohesive (*Eco*RI-linearized pBSK) and blunt (*Eco*RV-linearized pBSK) ends. The efficiency of dephosphorylation was estimated by comparing transformation rates after ligation of dephosphorylated and nondephosphorylated plasmids. Complete ligation of non-AP-treated fragments was observed when the experiment was carried out using cohesive ends as observed on agarose gel (Fig. 6A, lane 5). However, *P. abyssi* AP- and CIAP-treated fragments were generally found to be nonligated (Fig. 6, lanes 6 and 7, respectively). Dephosphorylation efficiency [*DE*(%)] was estimated at 93.8% for *P. abyssi* AP and 99.7% for CIAP. Similar results were observed on experiments using blunt ends, however, with partial ligation of non-AP-treated fragments (Fig. 6B). *DE*(%) was estimated at 84.1% for *P. abyssi* AP and 98.7% for CIAP. Obtained *DE*(%) values indicate the ability of *P. abyssi* AP to dephosphorylate linear DNA fragments, thus preventing self-ligation.

DISCUSSION

The *P. abyssi phoA* gene encoding an AP was cloned and the recombinant enzyme expressed in *E. coli*. The present work reports the first characterization of an AP from a hyperthermophilic archaeon. AP activity has already been found in *Pyrococcus horikoshii*, but the enzyme has not been isolated and characterized (18). In addition, comparison of the *P. abyssi* and *P. horikoshii* genomes was performed, and the *P. abyssi* AP sequence found no homologue in the *P. horikoshii* genome. Analysis of the deduced peptide primary structure revealed highly conserved patterns with other APs sequences. Comparison with the *E. coli* enzyme enabled identification of the strong conservation of both the catalytic site (metal ion coordination, phosphoseryl intermediate, and phosphate coordination) and secondary structure. This is consistent with previous studies on other APs (23, 27). This enzyme was expressed in *E. coli* HMS 174(DE3) harboring pLysS and recombinant pPabAP plasmids. Low expression levels were obtained, and experiments showed cytotoxicity of the recombinant AP. In addition, analysis of *P. abyssi* AP nucleotide sequences showed the presence of rare arginine-encoding codons AGG and AGA, occurring at 2.8 and 1.5%, respectively, whereas in *E. coli* they do so at only 0.14 and 0.21% (25). Thus, the level of expression might be improved by coexpressing the recombinant AP and $tRNA_{UCU}$ (28, 47).

Properties of the recombinant enzyme have been elucidated, and since the folding conditions and the posttranslational modifications are different between the native and the recombinant AP biosynthesis, properties reported here do not allow any conclusions about the native AP. An apparent optimum pH was found to be 11.0 in 0.2 M CAPS buffer. *P. abyssi* AP is among the most alkaline APs characterized. APs from thermophilic bacteria showed high pH optima, e.g., 9.9 for the *T. neapolitana* enzyme (12) and 10.0 and 11.5 for the *T. thermophilus* APs (37). Mammalian APs possess higher pH optima than the *E. coli* enzyme, and it has been proposed that substitutions of D153 and K328 residues into corresponding histidine for the mammalian enzymes are responsible for this increase in pH (20, 33). The high optimum pH observed may be explained by the similarity of *P. abyssi* AP to that found in mammalian APs. The apparent optimum temperature was found to be 70°C in the 0.2 M CAPS (pH 11.0) buffer. Because *P. abyssi*'s optimal growth temperature is near 100°C (14), the determined thermoactivation optimum value appeared lower than expected. Nevertheless, other recombinant proteins from *P. abyssi* present optimum temperature activity far below 100°C. Thermal stability was determined, and half-lives at 100 and 105°C were

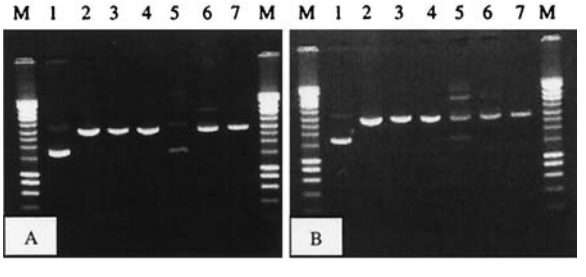

FIG. 6. DNA dephosphorylation. Shown are results of 0.8% agarose gel electrophoresis analysis of the DNA dephosphorylation procedure. pBSK vector was used as a substrate after being digested by *Eco*RI (A) or *Eco*RV (B). Lanes M, DNA ladder (Eurogentec S.A.); lanes 1, circular pBSK; lanes 2, linearized pBSK; lanes 3, *P. abyssi* AP-treated linear pBSK (2 h, 70°C); lanes 4, CIAP-treated linear pBSK (2 h, 37°C); lanes 5, non-AP-treated plasmid after ligation reaction; lanes 6, *P. abyssi* AP-treated plasmid after ligation reaction; lanes 7, CIAP-treated plasmid after ligation reaction.

estimated at 18 and 5 h, respectively. Approximately 80% of the activity is conserved after a 36-h incubation at 90°C. These results show that the *P. abyssi* AP is the most thermostable AP described so far, in comparison to the *T. neapolitana* AP, which exhibits a half-life of about 4 h at 90°C in the presence of $CoCl_2$ (12). Catalytic properties of the *P. abyssi* AP were determined. Phosphotransferase activity is commonly observed by using Tris-HCl or ethanolamine buffers (9, 31), which increases enzyme activity by promoting the phosphotransferase reaction. A fourfold increase in activity in Tris-HCl relative to NaOH-glycine buffer was observed with the *P. abyssi* AP in the same conditions of temperature, pH, and substrate. Michaelis constants were found to be $V_{max1} = 4.07\ \mu mol \cdot min^{-1}$ and $K_{m1} = 166.33\ \mu M$ and $V_{max2} = 8.31\ \mu mol \cdot min^{-1}$ and $K_{m2} = 1204.98\ \mu M$. *P. abyssi* AP presents K_m values which are consistent with those found for several APs (33). Lineweaver-Burke representation allowed the observation of two behaviors. V_{max2} is approximately twofold the V_{max1} value, and K_{m2} is about sevenfold the K_{m1} value. Since zymogram analysis of *P. abyssi* AP highlighted two active forms, the kinetic observations could correspond to the presence of both the monomer and the homodimer in the enzymatic reaction mixture. However, this hypothesis has to be confirmed by isolating the two enzyme forms and studying them separately. A spectrum of inhibitors was tested on the *P. abyssi* AP, using molybdate and vanadate as phosphotyrosylphosphatase inhibitors (1). More precisely, vanadate is defined as an inhibitor of phosphoryltransfer reactions, especially if they involve a stable-covalent phosphoseryl-enzyme intermediate (6). Vanadate appeared to be a weak inhibitor of *P. abyssi* AP. This is not consistent with previous studies on other APs (1, 6, 21) and may be a result of the experimental conditions (pH 10.0, 70°C) or of an original catalytic mechanism. In contrast, thiol-reducing agents exhibited a strong inhibitory effect on AP activity. Although *P. abyssi* AP contains no intra- or interchain disulfide bonds, cysteine residues (one per monomer) seem critically implied in the enzyme activity. Strong inhibition in the presence of 1% SDS and lack of inhibition in the presence of 2 and 4 M urea are consistent with properties of other APs (40).

APs are metalloenzymes (38), and they are all inhibited by metal ion chelators such as EDTA. They are classically considered to be Zn(II)- and Mg(II)- dependent enzymes, especially *E. coli* and mammalian APs (27). However, activation following Mn(II), Co(II), or other metal ion addition has already been observed among other APs (6, 8, 17, 31, 32, 37, 41, 50). The *P. abyssi* AP was found to be activated by Zn(II), Mg(II), Mn(II), and Co(II). Increasing the concentrations of Mg(II) results in an increase in enzymatic activity followed by a plateau (data not shown). Activation following the addition of Mg(II) has been observed with mammalian APs and largely characterized using *E. coli* AP mutants. Studies of *E. coli* AP concerning the secondary ligands of Mg(II) (D153 and K328 [mature *E. coli* AP sequence numbering]) have led to the conclusion that replacement of these residues with histidine results in the transformation of an Mg(II)-binding site into a Zn(II)-binding site. The affinity between Mg(II) and its binding site became lower for the (D153H, K328H) mutant AP, and optimal activity was recovered by the addition of Mg(II) (20, 24, 33, 34). The residues D153 and K328 in *E. coli* AP correspond to the residues H108 and H228 in *P. abyssi* AP. Both are similar to corresponding residues found in mammalian APs. This appears to be consistent with *P. abyssi* AP behavior towards Mg(II). Furthermore, the purified enzyme is active when no cations are added and inhibited by metal ion chelators. This may suggest that strong metal-enzyme interactions exist and that the purified AP is partially metalated. Therefore, *P. abyssi* AP may possess several metal binding sites. Such a hypothesis is consistent with the analysis of the sequence and other APs (17, 27, 42). Finally, reactivation of the *P. abyssi* AP apoenzyme was attempted by incubation of the latter in the presence of various divalent cations, at various temperatures. No reactivation was observed under these experimental conditions. Reactivation of AP apoenzymes has already been observed with varied efficiency (2, 12). However, drastic treatment of the purified *P. abyssi* AP using metal ion chelators appeared irreversible, suggesting important catalytic and structural roles of metal ions. Under present experimental conditions, *P. abyssi* AP presented two active forms: monomer and homodimer. APs are classically described as being homodimeric, which is the case with *E. coli* and mammalian APs. However, many monomeric (13, 19, 40) and oligomeric (17) forms have been described. Further studies may provide clarification of the parameters governing the equilibrium between the two forms.

APs are commonly used in molecular biology for the construction of recombinant plasmids. APs remove 5′-terminal phosphate from DNA fragments and, as such, AP-treated linearized plasmids are unable to exhibit self-ligation and are more likely to integrate an exogenous gene insert. *P. abyssi* AP was tested under plasmid dephosphorylation conditions using a linear cohesive or blunt-ended pBSK vector. Dephosphorylation efficiency with regard to cohesive and blunt ends was estimated at 93.8 and 84.1%, respectively. Bovine AP was found to be more effective than the *P. abyssi* AP; however; dephosphorylation conditions of the latter were not optimized. Although many APs have been isolated and characterized, only commercial *E. coli* AP (bacterial AP) and CIAP are routinely used in molecular biology. Such properties are rarely reported. DNA dephosphorylation has been performed with the Antarctic bacterium strain TAB5 AP (39). As *P. abyssi* AP presented great thermostability and good efficiency in DNA dephosphorylation, further studies may be required to elucidate its potential use in such applications.

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