Molecular Cloning and Functional Expression of a Protein-Serine/Threonine Phosphatase from the Hyperthermophilic Archaeon Pyrodictium abyssi TAG11

BIANCA MAI,1 GERHARD FREY,1* RONALD V. SWANSON,2 ERIC J. MATHUR,2 AND K. O. STETTER1

Lehrstuhl für Mikrobiologie, Universität Regensburg, 93053 Regensburg, Germany,1 and Diversa Corp., San Diego, California 921212

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An open reading frame coding for a putative protein-serine/threonine phosphatase was identified in the hyperthermophilic archaeon Pyrodictium abyssi TAG11 and named Py-PP1. Py-PP1 was expressed in Escherichia coli, purified from inclusion bodies, and biochemically characterized. The phosphatase gene is part of an operon which may provide, for the first time, insight into a physiological role for archaeal protein phosphatases in vivo.

Reversible phosphorylation of proteins plays an important role in a wide variety of cellular processes, including regulation of metabolic pathways, cell differentiation, and signal transduction (4, 6). Serine, threonine, and tyrosine residues are the main targets for reversible phosphorylation in members of the domains Eucarya and Bacteria (7). The eukaryal protein-serine/threonine phosphatases can be classified in four major groups, PP1, PP2A, PP2B, and PP2C, according to their substrate specificity, metal ion dependence, and sensitivity to inhibitors (5). Sequence comparison of the primary structures of PP1, PP2A, and PP2B all have a common catalytic core of about 280 amino acids (1).

In Archaea, the third domain of life forms (28), little is known about protein phosphorylation and the proteins involved therein. Recently, serine/threonine-specific protein phosphatase activities have been identified in the thermoadapted euryarchaeota Sulfolobus solfataricus (PP1-arch1 [11, 16]) and in two euryarchaeota, Methanosarcina thermophila (PP1-arch2 [18]) and Haloflexx volcanic (17). The enzymatic activity of each of these proteins is dependent on the presence of divalent metal ions. PP1-arch2 from M. thermophila is sensitive to inhibitors of eukaryal protein-serine/threonine phosphatases, whereas the other archaeal phosphatases are not. The deduced amino acid sequences of PP1-arch1 and PP1-arch2 show similarity (27 to 31%) to sequences of the eukaryal PP1/PP2A/PP2B superfamily of protein-serine/threonine phosphatases (17). Nothing is currently known about the physiological role of these protein phosphatases in vivo. Members of the genus Pyrodictium (24, 26) are among the most thermophilic organisms known to date. Growth occurs from 80 up to 110°C, with an optimum between 100 and 105°C, depending on the species (25). Strains of Pyrodictium are often chemolithoautotrophs, gaining energy by the formation of H2S from elemental sulfur and molecular hydrogen. Pyrodictium species are further characterized by the synthesis of a unique extracellular sulfur (24). This network consists of hollow tubules (cannulae), which are composed of at least three protein subunits (14, 21). Based on their N-terminal sequences, we have isolated the corresponding genes, canA, canB, and canC (17a). Downstream of canB, an additional open reading frame, ppp1, encoding a polypeptide with homology to the archaeal and eukaryal protein-serine/threonine phosphatases was identified. In this paper we report the expression, purification, and functional characterization of the corresponding polypeptide, Py-PP1 (Pyrodictium PP1). The demonstration that canB and ppp1 are cotranscribed suggests a possible role for Py-PP1 in regulation or modification of the extracellular network.

MATERIALS AND METHODS

Expression in Escherichia coli. The gene for Py-PP1 was amplified by PCR (3 min at 94°C; 34 cycles of 1 min 10 s at 94°C, 1 min 20 s at 55°C, and 1 min 35 s at 72°C; 10 min at 72°C) from 5 ng of p34/1-9 plasmid DNA, using Taq Extender Polymerase (Stratagene, Heidelberg, Germany). The primers were designed to introduce an Ndel site at the initiation codon (5'-TGGAAGAAGGCTG-3') and a Ndel site downstream of the stop codon (5'-GG CGGAGCCCGCCTCTATGCTGCTCAG-3'). The resulting PCR product was cloned into the NdeI/NorI sites of expression vector pET17B (AGS, Heidelberg, Germany) to form plasmid pETpp1.

Purification of recombinant Py-PP1. The cell pellet obtained from 600 ml of culture was washed with 160 ml of buffer containing 20 mM Tris-HCl (pH 7.5), pelleted again, and resuspended in 30 ml of the same buffer. Cells were lysed by two passages through a French pressure cell at 20,000 lb/in2. The crude extract was centrifuged for 10 min at 10,000 rpm in a Sorvall SS34 rotor. The pellet was resuspended in 25 ml of buffer containing 50 mM Tris-HCl (pH 8.0), 20 mM EDTA, 200 μg of lysisyme per ml, 5 M urea, and 2% Triton X-100 and incubated for 20 min at room temperature. Insoluble proteins were precipitated (10 min, 11,000 rpm, JA20 rotor). Denatured Py-PP1 was renatured by dilution (1:100) into renaturing solution (50 mM Tris-HCl [pH 8.0], 20 mM EDTA, 20 mM dithiothreitol, 5 M guanidinium-Cl), and incubated for 2 h at room temperature. Particles were removed by centrifugation (10 min, 10,000 rpm, JA20 rotor). Denatured Py-PP1 was renatured by dilution (1:100) into renaturing solution (50 mM Tris-HCl [pH 8.0], 20 mM EDTA, 5 mM dithiothreitol, 0.5 M l-arginine; precool to 4°C) and incubated at 4°C overnight. Finally, the protein solution was concentrated about fourfold (Amicon ultrafiltration cell; 10 K Polysulfon membrane [Sartorius, Göttingen, Germany]) and dialyzed extensively against 50 mM Tris-HCl (pH 8.0)−25 mM NaCl. Aliquots were stored frozen at −80°C or refrigerated at 4°C.

Enzyme activity assay. The protein phosphatase activity of Py-PP1 was tested with a serine/threonine phosphatase assay system from Promega (Heidelberg, Germany). The standard assay mixture contained 50 mM Tris-HCl (pH 6.0 at 90°C), 25 mM NaCl, 2.5 mM MnCl2, 5.5 μg of serine/threonine phosphopeptide (RRARpTYY), and 100 ng of enzyme in a total volume of 50 μl. Phosphatase activity was monitored for 10 min at 90°C by subtracting the tubes completely in a water or glycerol bath. Reactions were stopped by transferring the tubes to ice water. After cooling to 0°C, released Pi was quantitated with malachite green as described elsewhere (16). All data presented are corrected for spontaneous Pi release, which was monitored under the conditions described. Assays were linear with respect to time and amount of enzyme added.
Standard procedures. Protein concentrations were determined by the method of Heil and Zillig (9). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described by Laemmli (15).

Sequence analysis. The Wisconsin Package software (Genetics Computer Group, Madison, Wis.) was used for sequence analysis.

Nucleotide sequence accession number. The nucleotide sequence for the Py-PP1-encoding gene reported in this paper has been submitted to the EMBL data bank and assigned accession no. Y12396.

RESULTS

Cloning and sequence analysis. The gene for the B subunit of the extracellular network of *P. abyssi* TAG11 was isolated from a λgt11 clone on a 4.5-kbp EcoRI fragment (canB [ORF1]) and black (pypl) boxes. Subclone p34EX, containing a 1.3-kbp EcoRI/XhoI fragment, was sequenced on both strands. The pypl gene sequence was completed by sequencing flanking regions in p34/1-9. The orientations of primers used for RT-PCR experiments (P1F, P1R, P2F, and P2R [Fig. 4]) are indicated by half-arrows. The scheme is not drawn to scale. (B) Sequence alignment of Py-PP1 with PP1-arch1 from *S. solfataricus* (accession no. U35278), PP1-arch2 from *M. thermophila* (U96772), and several eukaryal protein-serine/threonine phosphatases. The eukaryal sequences chosen for the alignment have high similarity scores by pairwise comparison with Py-PP1. Amino acids conserved in at least six of the seven sequences shown are shaded. B.oI., *Brassica oleracea* (P48487); A.th., *Arabidopsis thaliana* (P30366); S.ce., *Saccharomyces cerevisiae* (P32598); H.sa., *Homo sapiens* (P37140).
unambiguously over their entire lengths; conserved residues are scattered over a wide range. The archaeal proteins are shortened at their N termini relative to the eukaryal proteins. These complexes were purified by washing in a buffer containing 5 M urea and 2% Triton X-100 (Fig. 2B, lane 5). The archaeal proteins are scattered over a wide range. The archaeal proteins are shorter than their eukaryal counterparts. Additional residues which introduce gaps in the alignment (Fig. 1B, at positions 140, 165, 200, 230, and 250).

Expression of Py-PP1 in vivo. To investigate the enzymatic properties of Py-PP1, we have established a highly efficient expression system in E. coli. The putative phosphatase gene was cloned into the expression vector pET17B by a PCR-based approach, yielding the construct pETpp1. Crude extracts of the recombinant cells were analyzed by SDS-PAGE before and after induction with IPTG, a polypeptide of the expected size (33 kDa) was synthesized in cells containing pETpp1. Purification steps were analyzed by SDS-PAGE. Lane 1, size standard; lanes 2 and 3, crude extract before and after induction with IPTG; lane 4, pellet after passage through a French press; lane 5, washed pellet; lane 6, renatured protein. See Materials and Methods for details.

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form but rather was trapped within inclusion bodies. Soluble Py-PP1 was obtained by denaturation in guanidinium chloride and subsequent renaturation. A phosphorylated peptide was used as the substrate for the activity assays, thereby permitting the experiments to be performed at physiological temperatures without denaturing the substrate. Metal ion (Mn$^{2+}$, Ni$^{2+}$, and Co$^{2+}$) dependence of Py-PP1 as well as the effects of specific inhibitors were found to be similar to those described for PP1-arch from *S. solfataricus* and the phosphatase activities detected in two euryarchaeota, *H. volcanii* and *M. thermophila* (18, 19). No homologous sequences were detected in the genomes of *Methanococcus jannaschii* (2), *Methanobacterium thermoautotrophicum* (22), and *Archaeoglobus fulgidus* (13), demonstrating that the protein is not universally conserved among the *Archaea*.

In *Eucarya*, protein phosphatases play an important role in the regulation of metabolic pathways and the transfer of extracellular stimuli to the nucleus. Substrate specificity and phosphatase activity are modulated by different regulatory subunits which bind to the catalytic polypeptides (6, 10). Nothing...
is yet known about the roles of protein phosphatases or their regulation in Archaea. Active PP1-arch1 has been isolated as monomer from *S. solfataricus* and is presumedly not stably complexed with other (regulatory) polypeptides in vivo (17). The gene organization in *P. abyssi* TAG11 provides the first evidence for a possible function of Py-PP1 phosphatase. The gene for Py-PP1 is located downstream of an open reading frame, *canB*, which codes for a component of the extracellular network of *P. abyssi* (17a). Sequence comparison with promoter regions of other archaeal genes revealed that the phosphatase gene is not preceded by a TATA box, the main component of archaeal promoters (8). We could demonstrate by RT-PCR that the phosphatase gene and *canB* are cotranscribed on one mRNA. The experiments also revealed that *canB* alone is transcribed much more efficiently. The data indicate that the cotranscript is produced by a read-through at the terminator of *canB* (possible termination sites [27] are found immediately downstream of *canB*). The cotranscription of these two genes suggests a possible regulatory role of the phosphatase activity on the expression or synthesis of the proteinaceous extracellular network of *Pyrodictium*. Investigations on the involvement of Py-PP1 in the synthesis of this unique structure are in progress.

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**REFERENCES**