Extremophiles (2006) 10:393–402
DOI 10.1007/s00792-006-0508-1

ORIGINAL PAPER

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Identification of the first archaeal oligopeptide-binding protein from the hyperthermophile *Aeropyrum pernix*

Received: 21 December 2005 / Accepted: 4 January 2006 / Published online: 25 April 2006

Abstract The archaeon *Aeropyrum pernix* grows optimally at 90°C and derives energy primarily from aerobic degradation of complex proteinaceous substrates. The ability of these nutrients to sustain growth is generally associated with the presence of oligopeptide transport systems, such as the well-known protein-dependent ATP-binding cassette (ABC) transporters. This study is concerned with the isolation and characterisation of the first archaeal oligopeptide-binding protein ($OppA_{Ap}$) from the extracellular medium of *A. pernix*. The protein shows a pI of 3.9 and a molecular mass of about 90 kDa under native conditions. By using a proteomic approach, the $OppA_{Ap}$-encoding gene was identified (APE1583) and about 55% of the protein amino-acid sequence was validated. The extracellular purified protein was able to efficiently bind oligopeptide substrates such as Xenopsin. The amount of a liganded peptide to $OppA_{Ap}$ was about 70% at 90°C using a 1/100 (w/w) $OppA_{Ap}$/substrate ratio. Sequence comparisons showed a weak but significant similarity of $OppA_{Ap}$ with bacterial oligopeptide binding proteins. Furthermore, APE1583 neighbouring genes encode for the cognate components of an ABC transport system, suggesting that these ORFs are organised in an operon-like structure, with $OppA_{Ap}$ as the extracellular component for the uptake of oligopeptides.

Communicated by G. Antranikan

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Keywords Archaea · ABC transport · Oligopeptide binding protein · *Aeropyrum pernix* · MALDI Mass Spectrometry

Introduction

Aeropyrum pernix is an obligate aerobic hyperthermophilic archaeon originated from deep-sea hydrothermal vents that grows heterotrophically at temperatures between 70 and 100°C, pH values 5.0–9.0, and salinity (NaCl) ranging from 1.5 to 8% (Sako et al. 1996). These geothermal marine fields contain volatile compounds such as CO_2, CH_4, H_2S, and proteinaceous materials of plant and animal origin that can act as potential carbon sources. Although *A. pernix* derives energy primarily from aerobic degradation of peptides and amino acids, the nutrient requirements of this microorganism are complex and the mechanisms of nutrient uptake are not understood.

In general, peptide uptake requires specific systems for the binding and transport of the substrate nutrients across the cellular membranes. In bacteria, a well-studied mechanism for oligopeptide uptake is mediated by protein-dependent ATP-binding cassette (ABC) transporters (Tam and Saier 1993; Monnet 2003). These systems, highly conserved in bacteria, are poorly described in *Archaea*.

ATP-binding cassette transporters form a class of proteins found in all domains of life and are involved in a variety of processes such as substrate uptake or export, osmosensing, osmoregulation, and chemotaxis (Detmers et al. 2001). In contrast to secondary transporters, ABC transporters are independent of the electrochemical gradient of protons or sodium ions across the cytoplasmic membrane, and use the hydrolysis of ATP to translocate and accumulate solutes at higher concentrations inside the cell. Bacterial ABC uptake systems are divided into two main classes: the carbohydrate (CUT) and the di/oligopeptide uptake transporters

classes. The translocator components of each class are composed of two integral membrane proteins (permeases), both having six transmembrane segments, two peripheral membrane proteins that bind and hydrolyse ATP (ATPase), and an extracellular substrate-binding protein (SBP) involved in binding and delivering of substrates to the permease domain. At a genetic level, genes encoding for these protein components are typically organized in an operon structure. These transport operons are strongly conserved despite the extensive shuffling of gene locations driving bacterial evolution.

The components of the transport systems that show the most pronounced difference in terms of sequence are the substrate binding proteins (SBPs) (Monnet 2003). In Gram-negative bacteria, SBPs reside in the periplasmic space (Goodell and Higgins 1987; Tynkkynen et al. 1993), while in Gram-positive bacteria they are anchored to the outer surface of the cell membrane *via* N-terminally linked lipids (Tynkkynen et al. 1993; Alloing et al. 1994; Jenkinson et al. 1996). The archaeal SBPs belonging to the two transporter classes, carbohydrates and di/oligopeptide, differ in size, domain organisation, and type of signal peptides (Albers et al. 2004). SBPs of the carbohydrates class exhibit an N-terminal signal peptide followed by a long stretch of hydroxylated amino acids, and it is assumed that these proteins can be anchored to the cytoplasmic membrane via N-terminal lipid modifications, or N-terminal transmembrane domains. On the other hand, SBPs of the di/oligopeptide class contain an N-terminal bacterial-like signal peptide and a hydrophobic domain at the C-terminal end, which is preceded by a stretch of hydroxylated amino acids. Therefore, the catalytic domain is believed to be membrane-anchored by a carboxy-terminal membrane-spanning domain.

Analysis of archaeal sequenced genomes revealed that most of the SBPs that have been identified belong to the carbohydrate transporter class, and studies on these binding proteins showed that they have a very high affinity for their carbohydrate substrates, enabling the microorganisms to scavenge all available nutrients in substrate-poor environments (Albers et al. 1999; Horlacher 1998). Interestingly, some of these sugar-binding proteins isolated from *Sulfolobus solfataricus* are found to display the same domain organisation of the di/oligopeptide SBPs (Helferink et al. 2001). On the other hand, several putative di/oligopeptide-binding proteins have been identified on the *Pyrococcus furiosus* and *S. solfataricus* genomes, but none of them has been demonstrated to exhibit the predicted uptake activity (Albers et al. 2004).

The present study describes the isolation and characterisation of the first archaeal ABC transporter oligopeptide-binding protein ($OppA_{Ap}$). The protein, purified to homogeneity from the conditioned media of *A. pernix* cultures, was examined by peptide mass finger printing analysis, and the corresponding open reading frame (ORF APE1583) was identified on the *A. pernix* genome. The deduced sequence shares weak but significant similarities to several bacterial peptide-binding proteins of ABC transport systems. The purified protein was characterised and it was shown to exhibit peptide-binding activity, confirming that it belongs to the oligopeptide-binding protein family.

Materials and methods

Enzymes and reagents

Trypsin, dithiothreitol, and alfa-cyano-4-hydroxycinnamic acid were purchased from Sigma. Trifluoroacetic acid (TFA) HPLC grade was from Carlo Erba. All other reagents and solvents of the highest purity were available from Baker.

Strain and growth conditions

Aeropyrum pernix K1 (JCM 9820) was grown at 90°C in a 10 l fermenter, as described by Sako (1), in Marine Broth (Difco) (37.4 g/l) and sodium tiosulphate (10 g/l). Cells were harvested in the stationary phase (48 h of growth), and the culture medium devoid from cells by continuous flow centrifugation was concentrated 50-fold by ultrafiltration (Minitan System; Millipore Corporations, Bedford, MA).

Protein isolation and purification

During the purification, the oligopeptide binding protein was monitored by SDS-PAGE analysis. The amount of the protein of interest was estimated by subjecting samples after each purification step to SDS-PAGE and by scanning individual lanes using a Biorad Instrument ChemiDOC Laser Scanning Densitometer.

Proteins from 10 L of filtered medium (110 mg of total proteins) were precipitated by the addition of $(NH_4)_2\ SO_4$ up to 90% saturation at 4°C and centrifuged at 12,000*g* for 30 min. The precipitate was resuspended in 50 mM Tris–HCl pH 8.0 and extensively dialysed against the same buffer. The proteic solution (90 mg of total proteins, 22.2% (w/w) of binding protein) was then loaded onto an anionic exchange chromatography column of DEAE-cellulose "DE52" (2×24 cm; Whatman, USA), equilibrated with 50 mM Tris–HCl buffer pH 8.0. Bound proteins were eluted by a 400-ml linear gradient from 0.0–0.1 M NaCl in the equilibration buffer at a flow rate of 1.2 ml/min. Fractions containing the protein of interest were pooled, dialyzed against 50 mM Tris–HCl buffer pH 8.0, and concentrated by polyethylen glycol (MW 12,000 Da, Fluka, USA). The protein sample (30 mg of total proteins, 46.6% (w/w) of binding protein) was then loaded onto an anionic exchange chromatography column UNO Q-12 (BioRad, USA), equilibrated with 50 mM Tris–HCl buffer pH 8.0, and connected to a FPLC sys-

tem. The protein was eluted with a linear gradient 0–1 M NaCl at a flow/rate of 2 ml/min. The fractions of interest (17 mg of total proteins, 58.8% (w/w) of binding protein) were dialyzed against 50 mM Tris–HCl buffer pH 8.0 containing 1.0 M ammonium sulphate and applied to a HiTrap Phenyl Sepharose HP column (Amersham, USA), pre-equilibrated with the same buffer. The column was washed at a flow rate of 1 ml/min and elution was performed with a linear gradient of ammonium sulphate from 1 M to 0 in 50 mM Tris–HCl buffer pH 8.0. The collected fractions containing the protein under investigation (12 mg of total proteins, 66.7% (w/w) of binding protein) were concentrated on an Amicon PM-10 membrane and loaded onto an anionic exchange chromatography column Mono Q (Pharmacia) in a SMART system (Pharmacia Biotech, USA), equilibrated with 50 mM Tris–HCl pH 8.0 buffer. The column was washed at a flow rate of 0.05 ml/min with 0.2 ml of the equilibration buffer, and a 0.0–1.0 M NaCl linear gradient in the equilibration buffer (1 ml) was applied. Fractions containing the protein of interest, designed $OppA_{Ap,}$ were pooled, desalted, and concentrated (6 mg of total proteins, 100% of binding protein) for further characterization.

Protein production

Aliquots (70 ml) of *A. pernix* culture broth collected at different growth times (18, 21, 44 h) were centrifuged at 9,000*g* and the supernatants were saturated with 90% ammonium sulphate. After centrifugation at 29,000*g* and 4°C for 20 min, the protein pellets were resuspended in 50 mM Tris–HCl pH 8.0, treated with PMSF (2 mM final concentration), and dialysed against the same buffer over night at 4°C. Hence, the samples concentrated to a final volume of 0.2 ml were analysed by SDS-PAGE, loading each lane with equal volumes (0.016 ml) or equal amounts of total proteins.

Electrophoresis and isoelectrofocusing

Polyacrylamide (12.5%) gel electrophoresis in 0.1% SDS was carried out according to Laemmli (Laemmli 1970). For molecular mass determination, the gel was calibrated with pre-stained protein markers (Cell Signalling Technology). Prior to electrophoresis, the sample was diluted in the denaturing sample buffer (BioRad, USA) and heated in a boiling water bath for 5 min. Alternatively, the sample was precipitated by TCA (20%) and the pellet was washed twice with ethylic ether, followed by washing with acetone. The precipitated protein was then resuspended in the denaturing sample buffer (BioRad) and heated in a boiling water bath for 5 min. Proteins were visualised by Coomassie brilliant blue R-250 staining. Analytical isoelectric focusing in the pH range of 3.0–10.0 was performed on a 5% acrylamide gel slab with a Model 111 MiniIEF Cell system (BioRad) following the manufacturer's instructions. Proteins were stained using Coomassie Brilliant Blue G250.

Determination of molecular mass

The molecular mass of the native $OppA_{Ap}$ was determined by gel filtration chromatography on a Superose^{TM} 12 HR 10/30 FPLC column. The column was eluted with 50 mM sodium phosphate buffer pH 7.0 containing 0.15 M NaCl (flow rate 1 ml/min). Calibration of the column was performed using the following standard gel filtration calibration kit (Amersham): Thyroglobulin (670 kDa), IgG (158 kDa), Ovalbumin (44 kDa), Myoglobin (17 kDa), Vitamin B12 (1.3 kDa).

In situ digestions

Protein bands stained with Coomassie brilliant blue were excised from a preparative SDS electrophoresis on a 12.5% polyacrylamide gel and subjected to in situ digestion as previously described (Medugno et al. 2003). Enzymatic digestions were carried out with trypsin (12.5 ng/μl) in 50 mM ammonium bicarbonate buffer pH 8.5.

Sequence analysis

Automated N-terminal degradation of the purified $OppA_{Ap}$ in solution was performed using a Perkin-Elmer Applied Biosystems 477A pulsed-liquid protein sequencer equipped with a model 120A phenylthiohydantoin analyser for the online identification and quantification of phenylthiohydantoin amino acids.

Glycosylation assay

$OppA_{Ap}$ and control standard glycoproteins (supplied with the Roche DIG Glycosylation Detection Kit) were directly spotted onto Immobilon membrane and detected immunologically after binding to 3-O-succinyl-ε-aminocaproic acid hydrazide conjugated with digoxigenin, following manufacturer's instructions (Roche, Switzerland).

Peptide binding assay

Binding of peptides to the purified $OppA_{Ap}$ was assayed using several peptides of length ranging from 8 to 13 amino-acid, all from Sigma. Purified protein (0.05 and 0.1 nmol) was added to 0.1 ml of 50 mM sodium phosphate buffer pH 7.0 containing 5 nmol of the peptide substrate for each assay. The mixtures were incu-

bated at 60 or 90°C for 30 min and successively kept in ice to stop the reaction. Each sample was filtered through a Microcon YM (Millipore) cutoff 10,000 Da at 4°C and 12,000g. Eluate recovered after each filtration (0.09 ml) was analysed by reverse-phase HPLC (DIONEX BioLC) on a μBondapak C_{18} column (3.9×300 mm, Waters), eluted with a linear gradient (0–60% acetonitrile in 0.1% TFA) at a flow rate of 1 ml/min. Control samples incubated in the absence of the purified protein or substrate peptides were run in parallel. The amount of bound peptide expressed as percentage was extrapolated by comparing the peak area of residual peptide after incubations in the presence or absence of $OppA_{Ap}$.

Sequence and structural analysis

The sequence database was searched using the BLAST-PSI program (Altschul et al. 1997) and a total of six homologous proteins from archaeal organisms were retrieved. These include polypeptides from *Pyrococcus abissy* (NP_127427.1), *Pyrococcus horikoshii* (NP_143789.1), *Pyrococcus furiosus* (NP_577919.1), *Pyrobaculum aerophilum* (NP_560480.1), *Sulfolobus solfataricus* (G90281), and *Termococcus kodakarensis* (YP_184217.1). Multiple sequence alignments were generated with the ClustalW program (Thompson et al. 1997). The sequence of $Oppa_{Ap}$ was submitted to the 3D-PSSM fold recognition server (Kelley et al. 2000).

Results

Protein purification and production

A novel protein involved in the small peptide transport and metabolism was purified and characterised from the culture medium of the hyperthermophilic archaeon A. pernix. SDS-PAGE analysis of conditioned media of cells grown up to stationary phase revealed the presence of a major band migrating at approximately 42 kDa (Fig. 1a).

The isolation of the protein was performed by selective ammonium sulphate precipitation of *A. pernix* stationary phase culture broth, followed by fractionation with two anionic exchange chromatography steps on DEAE and UNO Q-12 columns at pH 8.0. The protein of interest, monitored by SDS-PAGE analysis, was eluted with a saline gradient at approximately 0.5 M

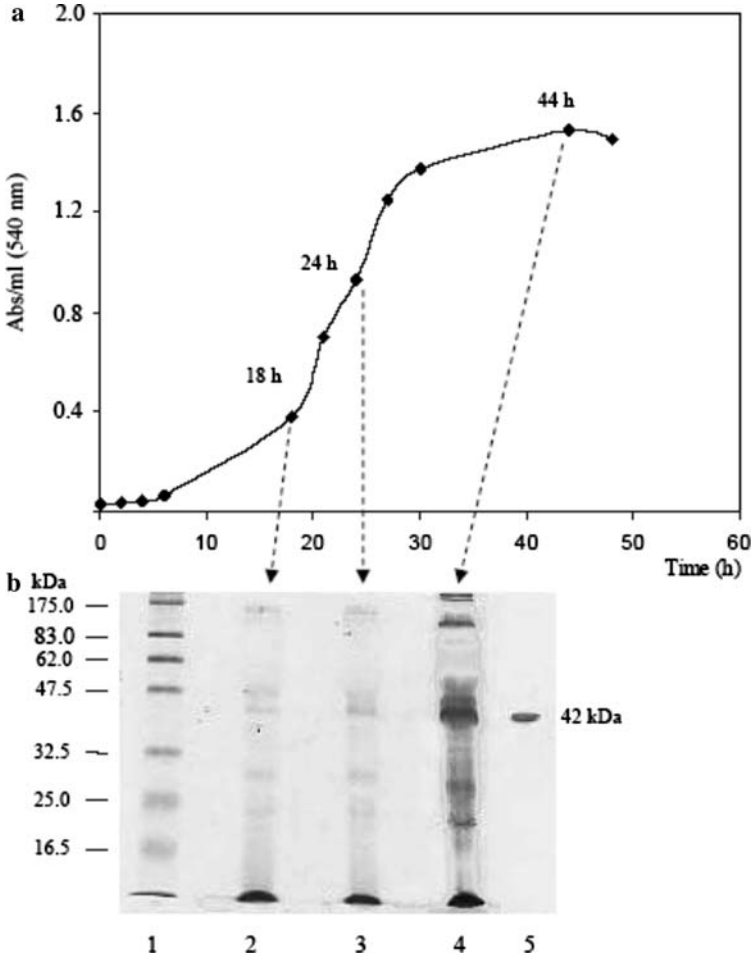

Fig. 1 (**a**) Kinetic growth of *A. pernix* at 90°C. Aliquots of the culture were recovered at regular intervals, and the optical density was determined at 540 nm. (**b**) SDS-PAGE analysis of the extracellular proteins at different growth times; each lane was loaded with 0.016 ml of sample. Lane 1, Molecular weight markers broad range; lane 2, culture broth sample collected after 18 h of growth; lane 3, culture broth sample collected after 21 h of growth; lane 4, culture broth sample collected after 44 h of growth; lane 5, purified protein

NaCl from DEAE and 0.4 M NaCl from UNO Q-12 column. Further purification was achieved by using hydrophobic interaction chromatography (Phenyl Sepharose) and anion exchange chromatography on Mono Q column at pH 8.0. The protein, eluted as a single sharp peak by a saline gradient at about 0.4 M NaCl, appeared to be homogeneous when analysed by SDS-PAGE (Fig. 1b), isoelectrofocusing, and gel filtration chromatography. The final yield of the protein purification was about 6 mg from 10 l of the microbial culture.

In order to study the production of the purified protein as a function of the growth time, equal volumes (0.016 ml) of culture broth samples collected at different time intervals were examined by SDS-PAGE. As shown in Fig. 1b, an increasing intensity of the 42 kDa band was observed reaching the maximum in the late stationary phase, where it appeared as the prominent component of the extracellular fraction. Similar results were obtained when equal amounts of total proteins were loaded for each lane (data not shown).

Protein identification by peptide mass finger printing analysis

The identification of the purified protein was obtained by excision of the 42 kDa band from the gel, in situ digestion with trypsin, and MALDI MS analysis. The peptide molecular masses, recorded either in reflector or linear mode, are listed in the Table 1. The signals selected from the reflector mass spectra were used to search the non-redundant NCBI database using the Mascot software package. Results (score 114) recognised the open reading frame (ORF) APE1583, on the *A. pernix* genome, as the gene encoding the purified protein, proving that 20 mass signals (ranging between m/z 786.4 and m/z 2607.6) exactly matched the masses of tryptic peptides from the predicted amino-acid sequence of the DNA database. Moreover, the negligible probability score (70 score) of the second best candidate provided by MASCOT confirmed the effectiveness of this identification. The remaining MALDI mass tryptic peptides were then successfully mapped along the identified amino-acid sequence, allowing assigning 72% of the overall mass signals detected after tryptic digestion. All these data concurred to validate about 55% of the deduced sequence from APE1583 (Fig. 2a). Notably, the mass peak detected at m/z 1301.7 was interpreted as the peptide Q47-K58 in which the glutamine has been converted to pyroglutamate, with this modification causing a difference of –17.0 Da with respect to the theoretical mass of the unmodified peptide (1318.4 Da). Indeed, the failure of the N-terminal sequence analysis of $OppA_{Ap}$, possibly because of N-terminal blocking, could confirm this hypothesis.

These findings clearly revealed that the purified protein coincided with the APE1583-encoded product (930 amino acids), which shares significant sequence similarity with different bacterial oligopeptide-binding proteins (Opp), and suggested that it could be considered the first archaeal member ($OppA_{Ap}$) of this family.

Table 1 Peptide released from $OppA_{Ap}$ following proteolysis experiments performed with trypsin

Peptides	Measured mass value	Expected mass value
47–58[b]	1301.7	1301.5
59–85	2908.5	2909.3
115–130	1751.8	1750.9
131–151	2249.4	2248.5
152–172	2363.1	2363.1
152–182	3511.5	3510.8
173–182	1164.6	1164.6
173–183	1292.7	1292.7
186–194	1055.6	1055.6
195–214	2152.0	2153.5
195–230	3948.7	3949.2
297–306	1175.6	1175.5
307–319	1665.3	1664.9
320–325	786.4	786.4
328–349	2315.3	2315.2
334–349	1690.9	1689.8
336–349	1428.8	1427.7
350–370	2607.6	2608.9
371–381	1361.7	1361.6
382–388	800.4	800.3
407–421	1828.9	1828.8
407–425	2342.2	2342.1
430–437	912.6	912.5
438–451	1710.9	1710.9
438–453	1982.8	1982.3
452–472	2365.2	2367.7
473–493	2170.4	2169.4
513–533[a]	2347.2	2347.1
534–560[a]	3047.4	3046.3
561–568	904.5	904.0
698–713	1736.8	1736.7
698–725	2979.3	2978.2
714–725	1257.8	1259.4
714–745	3391.8	3390.9
746–761	1797.2	1797.0
762–772	1338.7	1338.7
807–818	1369.8	1369.7
819–833	1580.6	1578.6
819–834	1680.6	1679.7

[a]Oxidized peptide
[b]Putative N-terminal pyroglutamate peptide

Molecular properties

The purified $OppA_{Ap}$ has a pI of 3.9 and resulted in being glycosylated at the unique putative N-glycosylation site in correspondence of N480 of the precursor amino-acid sequence (Fig. 2).

The molecular mass was 90 kDa under native conditions (gel filtration chromatography), which could be consistent with the amino-acid sequence of the protein in a processed form, starting from Q47 residue, as predicted by the results from MALDI-MS peptide mapping (theoretical mass value 98.5 kDa). Unfortunately, attempts to obtain either MALDI or ES mass spectra from the intact protein were unsuccessful. However, the

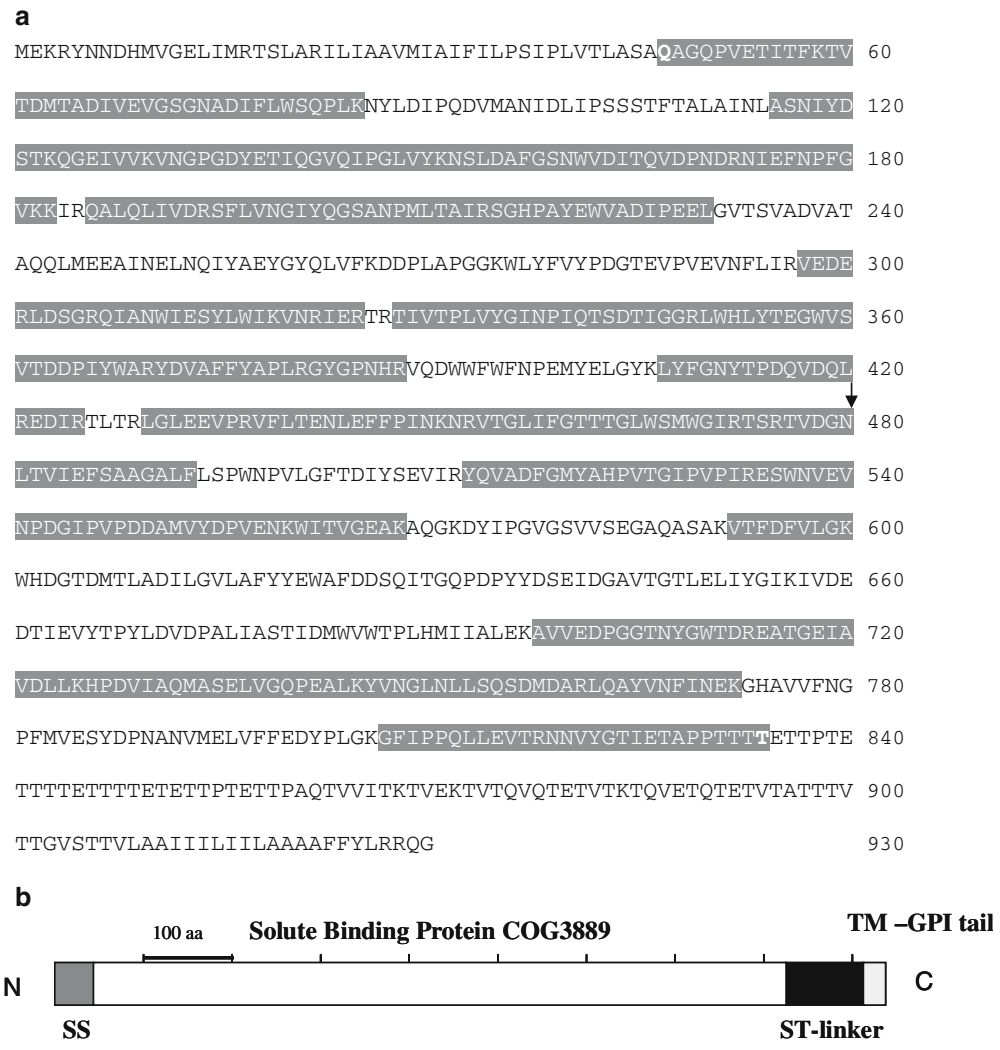

Fig. 2 (**a**) Amino acid sequence deduced from the ORF APE1583. The coverage sequence obtained from tryptic digestion is indicated with solid underlining. The arrow refers to N-glycosilation site. (**b**) Domain structure of $OppA_{Ap}$ precursor: SS, signal sequence (1–44 aa), SBP catalytic domain (45–823 aa), ST threonine stretch residues (824–907 aa), C-terminal GPI-signal sequence (899–930 aa) containing TM (transmembrane domain, 904–926 aa)

apparent molecular mass of the purified $OppA_{Ap}$ determined under denaturing conditions (SDS-PAGE) was 42 kDa (Fig. 1a, lane 5), accounting for about half value of the full-length amino-acid sequence. These results could be interpreted as an anomalous migration of the protein during SDS-PAGE analysis, possibly due to a high resistance of $OppA_{Ap}$ to the thermal and/or SDS denaturation, which have been well documented for several thermophilic proteins (Gentile et al. 2002; Tomschy et al. 1994). To clear up this point, extensively denaturating procedures of $OppA_{Ap}$ were adopted. Indeed when the protein was subjected to precipitation with TCA before the SDS-PAGE analysis, a different electrophoretic pattern was observed showing two additional bands, migrating approximately at 57 and 27 kDa, in concomitant with a decrease in intensity of the 42 kDa band (data not shown). This behaviour prompted the consideration that the extracellular-binding protein is assembled into a heterodimeric complex, high stable under the standard denaturing conditions. Further structural investigations are in progress to ascertain the oligomeric organization of $OppA_{Ap}$.

Bioinformatic analysis

The sequence database was searched using the BLAST-PSI program (Altschul et al. 1997) and a total of six homologous proteins from archaeal organisms were retrieved. These include polypeptides from *Pyrococcus abissy* (NP_127427.1), *Pyrococcus horikoshii* (NP_143789.1), *Pyrococcus furiosus* (NP_577919.1), *Pyrobaculum aerophilum* (NP_560480.1), *Sulfolobus solfataricus* (G90281), and *Termococcus kodakarensis* (YP_184217.1). The level of sequence homology is rather low (ranging from 9 to 17%) except for a subgroup including the proteins from *P. furiosus*, *P. horikoshii*, and *T. kodakarensis*. Five of these sequences are included in the COG (Clusters of Orthologous Groups of proteins) database (group COG3889).

A low but significant level of sequence homology can be detected between the archaeal proteins and a number of bacterial oligopeptide binding proteins. The 3D-PSSM fold recognition server, which threads the sequence through a library of known protein structures and selects the models with a significant score, was used

to compare the sequence of the archaeal protein with known folds. When the $OppA_{Ap}$ amino-acid sequence is submitted to 3D-PSSM, the two most confident matches were oligopeptide-binding proteins (PDB accession codes: 1DPE and 1JET), confirming the likelihood that the archaeal and bacterial periplasmic proteins share the same topology.

The $OppA_{Ap}$ is synthesized as a protein with a domain organization, schematically represented in Fig. 2b. Signal P algorithm (Bendtsen et al. 2004), adapted for Gram-positive bacteria, predicted the presence of a putative signal sequence and a cleavage site at residues A46-Q47, indicating Q47 as the N-terminal residue of the mature $OppA_{Ap.}$ These predictions are in agreement with the results obtained by mass spectrometry analysis. Following the catalytic domain, a stretch of threonine residues (ST-linker, residues 824–907) has been recognized adjacent to a C-terminal transmembrane helix, spanning from residues 908–926 (Fig. 2b). Further investigation identified a specific sequence motif required for GPI lipid attachment of protein to the cytoplasmic membrane, in the C-terminal region (899–930 aa), supporting the existence of a GPI modification machinery as previously proposed (Eiesenhaber et al. 2001).

The *A. pernix* genomic organization around APE1583 shows the presence of four downstream ORFs oriented in the same transcription direction (Fig. 3). Analysis of the deduced amino-acid sequences revealed APE1582 (accession no. A72537) and APE1581 (accession no. H72536) code for putative transport permease proteins, while APE1578 (accession no. E72536) and APE1576 (accession no. C72536) code for putative oligopeptide transport ATP-binding proteins. The distance in nucleotides between each ORF ranges from two to 30 nucleotides except for APE1583 that is located 289 bp upstream of APE1582. Therefore, this genomic region could be organized in an operon-like structure and the $OppA_{Ap}$ can be suggested as a subunit of an ABC transport system (Fig. 3). Interestingly, the archaeal orthologues of APE1583 display an identical chromosomal organization to that described for APE1583 with the exception of *P. aerophilum* (Fig. 3). All the other components of the putative archaeal operon share a significant level of sequence identity (30–60%).

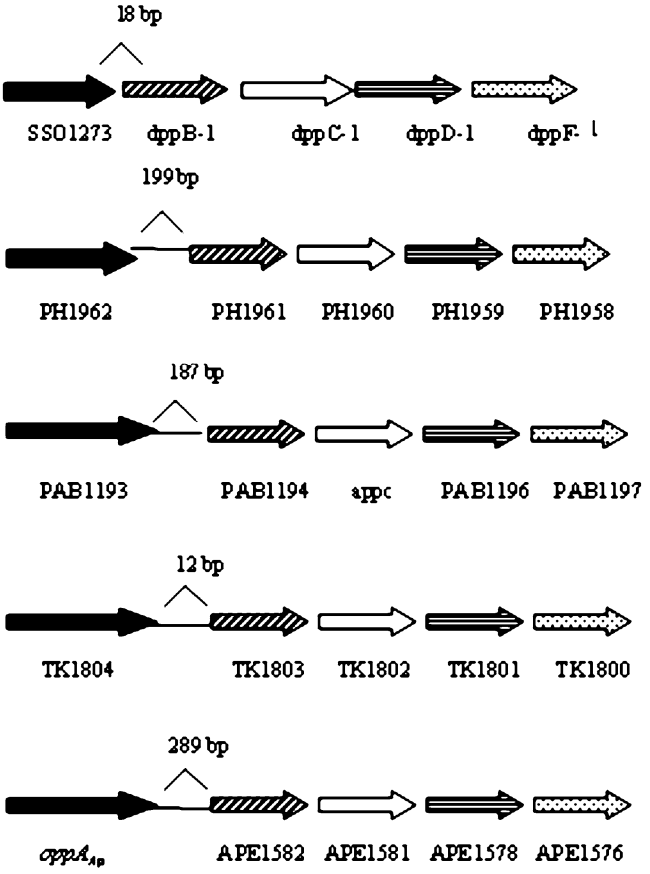

Fig. 3 Organization of the genomic region around $OppA_{Ap}$ and its homologous in *P. abyssi* (PAB1193), *P. horikoshii* (PH1962), *S. solfataricus* (SSO1273), and *T. kodakarensis* (TK1804). Homologous genes are indicated by the same shading pattern. The length of the arrow indicates the relative sizes of the proteins

Binding activity

Oligopeptide-binding activity of the purified protein was assayed using peptides differing in amino-acid length, ranging from eight to 13 amino-acids (data not shown). Among these, Xenopsin (EGKRPWIL) was revealed to be the most efficiently recognised by the protein and therefore chosen as a substrate for further investigations. Binding activity was investigated by reverse phase HPLC analysis, varying parameters such as $OppA_{Ap}$/substrate ratios (P/S), temperature, and incubation time (Fig. 4). Results indicated that the amount of bound Xenopsin (%), extrapolated from the unliganded peptide, improved from 12 to 52% over to the negative control (reaction mixture without protein) when the P/S ratio increased from 1:100 to 1:50 (w/w) at 60°C for 30 min, respectively. A further improvement of peptide binding (66%) was achieved by incubating the assay mixture at 90°C. A prolonged incubation time up to 60 min did not affect the binding activity, producing the same results obtained after 30 min of incubation (Fig. 4). These preliminary findings prove unambiguously that the isolated A. pernix protein exhibits peptide-binding activity and validates its annotation as an oligopeptide-binding protein.

Discussion

Binding-protein dependent ABC transporters are the best-studied groups of proteins in *Archaea* involved in carbon source uptake (Albers et al. 2004). All the archaeal binding proteins part of these ABC systems studied to date are equipped with a very high affinity for their substrates. This useful property allows the archaeal

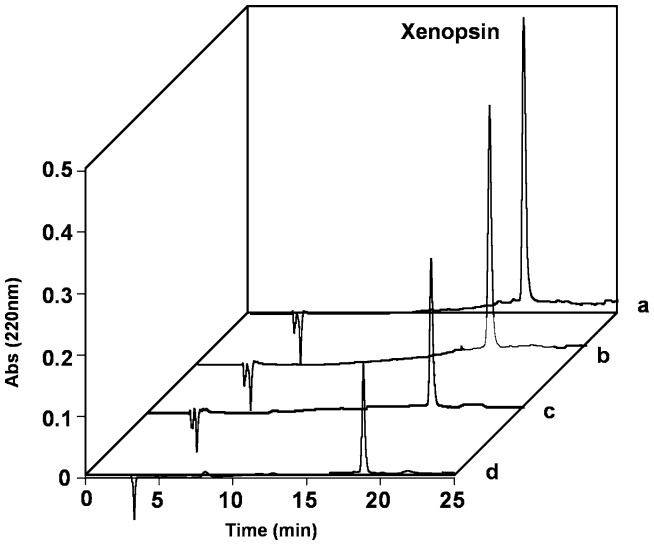

Fig. 4 Binding of Xenopsin peptide to the purified $OppA_{Ap}$. Reverse phase HPLC analysis of Xenopsin on a Waters C18 column in the absence and in the presence of $OppA_{Ap}$ under different conditions of incubation mixture. (**a**) Xenopsin incubated for 30 min without the protein (control). The incubation of the substrate at 60°C and 90°C produced the same result. (**b**) Xenopsin incubated at 60°C for 30 min with an $OppA_{Ap}$/substrate ratio 1:100 (w/w). (**c**) Xenopsin incubated at 60°C for 30 min with an $OppA_{Ap}$ /substrate ratio 1:50. (**d**) Xenopsin incubated at 90°C for 30 min with an $OppA_{Ap}$ /substrate ratio 1:50

cells to utilize carbon sources efficiently in substrate-poor environments such as hydrothermal vents in the deep sea or the hot sulphuric pools.

In the present study, we describe the isolation and characterisation of the first archaeal oligopeptide-binding protein, designed $OppA_{Ap}$, from the hyperthermophile *Aeropyrum pernix*. By mean of an integrated proteomic and genomic approach, we identified the $OppA_{Ap}$-encoding gene (APE1583) and partially mapped the amino-acid sequence of the isolated protein. Characterisation of $OppA_{Ap}$ binding activity suggested that this protein could be involved in nutrient uptake as part of an ABC transport system whose component genes are located downstream APE1583.

Preliminary studies on this binding protein by SDS-PAGE under different denaturing conditions suggested an oligomeric organization with two polipeptide chains assembled into a heterodimeric complex which showed high stability towards thermal and SDS denaturation. However, these hypotheses require further investigations to be tested.

Based on its functional role, $OppA_{Ap}$ was expected to be membrane anchored, analogous to Gram-positive bacterial OppA (Monnet 2003), where it can deliver the bound substrates to the other ABC membrane components. Conversely, our data showed that $OppA_{Ap}$ production in the extracellular medium was a function of growth-time, reaching a maximal concentration in the late stationary phase of growth. This phenomenon has been described for Gram-positive bacteria, such as *Bacillus cereus* (Gohar et al. 2002; Antelmann et al. 2001) and *Bacillus subtilis* (Perego et al. 1991), where the releasing of OppA, associated to the cytoplasmic membrane via N-terminal lipid moiety, occurred in the stationary phase of growth through a proteolytic cleavage occurring inside the N-terminal domain. This is believed to provide a strategy to regulate the level of OppA, even though the mechanism governing the release is poorly understood. However, unlike the bacterial counterparts, archaeal oligopeptide-binding proteins are assumed to be membrane anchored *via* a transmembrane (TM) domain present in the carboxyl-terminal region (Albers et al. 2004). The analysis of the APE1583-encoded sequence revealed the presence of a TM domain, together with a putative signal sequence for GPI lipid modification at the C-terminal end, suggesting that $OppA_{Ap}$ could be originally anchored to the membrane via a C-terminal domain before releasing into the external media with the major part of the protein located outside the cell. This prediction seems to be confirmed by the identification of a signal peptide (M1-A46) that could be involved in targeting the protein to the membrane, and by mass spectrometry and sequence analyses, which recognised Q47 in pGlu modified form as the putative N-terminal residue. Further structural investigations are needed to clarify the exact membrane topology of $OppA_{Ap}$.

Structural studies mainly performed on OppA from Gram-negative bacteria have revealed that the binding proteins comprise three domains, two of which (I, III) form the cleft of the binding site, connected by a flexible hinge (Tame et al. 1995; Nickitenko). The binding mechanism, referred as "Venus fly-trap", presumes a conformational change between the liganded and unliganded protein (Quiocho and Ledvina 1996). The manner in which the substrate is bound may be sequence dependent or independent (Tame et al. 1994; Lanfermeijer et al. 2000). Preliminary results clearly demonstrated that $OppA_{Ap}$ is able to bind oligopeptides of 8–13 amino-acid residues, showing a preference towards the octapeptide Xenopsin.

However, the in vivo functional activity of $OppA_{Ap}$ for oligopeptide uptake requires the presence of all the components forming the ABC transporter system. Analysis of *A. pernix* genome shows that the gene APE1583 encoding $OppA_{Ap}$ is located close to the ORFs coding for the subunits of a complete ABC transporter system. These ORFs seem to be organized in an operon-like structure, except for APE1583 that should be considered as a single gene.

This is not surprisingly since some bacteria, such as *Listeria monocytogenes* (Borezee et al. 2000), *Mycoplasma hominis* (Henrich et al. 1999), and *Streptococcus gordonii* (Jenkinson et al. 1996), have developed a similar strategy to render the expression of *OppA*-encoding genes more independent.

It is believed that this phenomenon represents one of the possible mechanisms to increase the expression of *OppA* ABC component and to produce these proteins at a higher level with respect to the other subunits of the

transport system. Based on these findings, it can be assumed that $OppA_{Ap}$ is a subunit of a complete ABC oligopeptide transport system present in *A. pernix*. So far, several archaeal transport operons, identified as putative oligopeptide transporters, were found surrounded by genes encoding sugar-degrading enzymes, and in some cases they were shown to play a functional role solely in sugar uptake (Elferink et al. 2001). The absence of genes involved in sugar metabolism in the close vicinity of the *A. pernix* ABC transport system strongly confirms the hypothesis that it is specifically implicated in peptide uptake. Remarkably, this transport system is found in five phylogenetically distant archaeal genomes, with both the sequences of the genes involved and their order on the genomes conserved, suggesting the occurrence of a new archaeal subfamily of oligopeptide transporters class.

Acknowledgments We thank Dr. Giuseppe Ruggiero for expert assistance with bacterial cultures, Dr. Raffaele Cannio for critical review of the manuscript, and Vitale Carratore for expert assistance with sequence analysis. This work is supported by grants from the Ministero dell'Università e della Ricerca Scientifica (Progetti di Rilevante Interesse Nazionale, PRIN 2002, Rome Italy); Centro Regionale di Competenza in Biotecnologie Industriali –BIOTEK-NET- Naples, Italy; MIUR-Decreto Direttoriale prot. no. 1105/2002

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