Purification and biochemical characterization of a poly(ADP-ribose) polymerase-like enzyme from the thermophilic archaeon *Sulfolobus solfataricus*

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A poly(ADP-ribose) polymerase-like enzyme, detected in a crude homogenate from *Sulfolobus solfataricus* by means of activity and immunoblot analyses, was purified to electrophoretic homogeneity by a rapid procedure including two sequential affinity chromatographies, on NAD⁺-agarose and DNA-Sepharose. The latter column selected specifically the poly(ADP-ribosyl)ating enzyme with a 17% recovery of enzymic activity and a purification of more than 15000-fold. The molecular mass (54–55 kDa) assessed by SDS/PAGE and immunoblot was definitely lower than that determined for the corresponding eukaryotic protein. The enzyme was proved to be thermophilic, with a temperature optimum of approx. 80 °C, and thermostable, with a half-life of 204 min at 80 °C, in good agreement with the requirements of a

thermozyme. It displayed a K_m towards NAD⁺ of $154 \pm 50 \mu\text{M}$; in the pH range 6.5–10.0 the activity values were similar, not showing a real optimum pH. The enzyme was able to bind homologous DNA, as evidenced by the ethidium bromide displacement assay. The product of the ADP-ribosylating reaction co-migrated with the short oligomers of ADP-ribose (less than 6 residues) from a eukaryotic source. Reverse-phase HPLC analysis of the products, after digestion with phosphodiesterase I, gave an elution profile reproducing that obtained by the enzymic digestion of the rat testis poly(ADP-ribose). These results strongly suggest that the activities of the purified enzyme include the elongation step.

INTRODUCTION

Poly(ADP-ribose) polymerase (PARP; EC 2.4.2.30) is the enzyme responsible for hydrolysing the NAD⁺ molecule at the (1,*N*-nicotinamide) glycosidic bond, transferring the resulting ADP-ribose (ADPR) to acceptor proteins and elongating the first residue to synthesize linear or branched polymers of ADPR [1–3]. Its nuclear localization (it is strictly associated with the chromatin) makes this enzyme peculiar to eukaryotic organisms and able to contribute to the regulation of many nuclear events (DNA repair, transcription and gene expression) related to changes in chromatin structure [3,4].

PARP has so far not been detected in lower eukaryotes such as yeast or prokaryotic cells, which lack a nuclear compartment; until now no information has been available on its occurrence in the Archaea domain.

A recent definition describes Archaea as 'the closest living prokaryote relatives of the eukaryotes' [5]. The micro-organisms belonging to this domain, generally living in very extreme environmental conditions, are regarded as very peculiar prokaryotes [6]. They are morphologically similar to eubacteria, but they lack many strictly eubacterial features and show a number of molecular characteristics such as the transcriptional machinery [5,7], suggesting a special relationship with eukaryotes. The resemblance to eukaryotic organisms is strongest among the sulphur-dependent Archaea. The members of the genus *Sulfolobus* possess transcription [5,8] and translation factors [9] like those found in eukaryotes.

Sulfolobus solfataricus is a thermophilic sulphur-dependent archaeon isolated from an acidic hot spring in Agnano (Napoli, Italy), but present in all volcanic areas, where it lives optimally at 87 °C and pH 3.5 [10]. The morphology and the main

physiological features of this micro-organism have been widely studied [10,11] and enzymes from various metabolic pathways have been purified and characterized [12–15]. The wide interest in such enzymes arises from both their biotechnological potential and the increasing knowledge of the mechanisms that stabilize these molecules at high temperatures.

In our laboratory a thermophilic and thermostable ADP-ribosylating system was identified in this archaeon [16,17]. We demonstrated that most ADP-ribosylated proteins were modified in an enzymically catalysed reaction [16]. The enzyme was detected immunochemically in the *sulfolobal* homogenate by means of polyclonal anti-PARP antibodies [18], and the reaction product behaved as oligo(ADPR) on PAGE [17]. These results suggested that the ADP-ribosylating system from *S. solfataricus* might share common features with the eukaryotic nuclear mechanism.

Here we describe the purification and characterization of the ADP-ribosylating enzyme from *S. solfataricus*, here denoted PARPss; this is, to our knowledge, the first example of a PARP-like activity described from any archaeal species.

EXPERIMENTAL

Materials

PMSF, β -NAD⁺, ADPR, ethidium bromide (EtBr), agarose, BSA, proteinase K (EC 3.4.21.64), RNase A (EC 3.1.27.5) and phosphodiesterase I (PDE I; EC 3.1.4.1) were purchased from Sigma. Prestained molecular mass markers were from Bio-Rad, and the reagents for PAGE were from Serva. [U-adenine-¹⁴C]NAD⁺ (280 mCi/mmol), [adenylate-³²P]NAD⁺ (1000 Ci/mmol) and MP-Hyperfilm (RPN 6V-36) were products of the Radiochemical Centre, Amersham (Little Chalfont, Bucks.,

Abbreviations used: ADPR, ADP-ribose; ADPRT, mono(ADPR) transferase; EtBr, ethidium bromide; PARP, poly(ADP-ribose) polymerase; PARPss, *sulfolobal* PARP-like enzyme; PDE, phosphodiesterase; PR-AMP, phosphoribosyl-AMP; RP-HPLC, reverse-phase HPLC.

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U.K.). NAD⁺-agarose was purchased from PL-Biochemicals. DNA-Sepharose was prepared by the method of [19], by using CNBr-activated Sepharose from Pharmacia; the ligand was genomic DNA from *S. solfataricus*, purified as described below. Rat testis [³²P]-polyADPR and anti-PARP antibodies were gifts from Dr. M. Malanga (University of Naples, Napoli, Italy) and Dr. Felix Althaus (University of Zürich, Zürich, Switzerland) respectively.

Cell culture and homogenate preparation

S. solfataricus strain MT-4 (DSM No. 5833) isolated from an acidic hot spring in Agnano (Naples, Italy), was grown at 87 °C (pH 3.5) in a 90-litre fermenter in standard medium and collected during the stationary phase; the collected cells (70 g wet weight) were used to prepare the homogenate as described in [16]. All buffers used for enzymic preparations contained 0.1 mM PMSF. The crude homogenate was treated as described in [16], except that it was used for further purification without being heated at 85 °C.

Enzyme purification

The crude homogenate was centrifuged at 12000 *g* for 10 min and the pellet was resuspended in 50 mM Tris/HCl, pH 7.5, containing 0.5 M NaCl, 0.2 % (v/v) Triton X-100 and 0.1 mM PMSF by a rapid homogenization with Polytron. The suspension was stirred overnight at 4 °C and centrifuged at 39000 *g* for 60 min. Solid (NH₄)₂SO₄ was added to the supernatant (Triton extract) to 70 % satn. After overnight stirring at 4 °C, the precipitate was collected by centrifugation at 12000 *g* for 60 min, then dissolved in the minimal volume of 100 mM Tris/HCl, pH 7.5. After extensive dialysis against the same buffer, the dialysate (1.5 mg/ml) was loaded on an NAD⁺-agarose column (3 mg of protein/ml of resin; 4 cm × 55 cm), equilibrated in the same buffer, at a flow rate of 1 ml/min. The running flow rate was 6 ml/min. The column was washed with the equilibrium buffer containing 0.2 M NaCl, until *A*₂₈₀ reached zero. The elution was performed with 0.5 M NaCl in the same buffer. The active fractions were pooled, diluted with 100 mM Tris/HCl, pH 7.5, to 0.01 M salt concentration (1–1.5 mg/ml protein) and loaded on a DNA-Sepharose column (5 m-units/ml of resin), equilibrated in the same buffer. The washing and the elution were performed as for NAD⁺-agarose chromatography. The active fractions (300 µl of each), collected in Eppendorf tubes containing 30 µl of glycerol to stabilize enzyme activity, were pooled, except in some experiments (DNA-binding assay and kinetic analyses); for these measurements the most active fraction eluted from the column was used. Protein determination was performed as described in [16].

Enzymic activity assay

The standard incubation procedure and the thermostability controls have been described in detail [16]. The activity was routinely assayed in sealed vials at 80 °C and pH 8.0 (final volume 125 µl) in the presence of 0.64 mM [¹⁴C]NAD⁺ or [³²P]NAD⁺ (10000 c.p.m./nmol) and determined as radioactivity insoluble in 25 % (w/v) trichloroacetic acid [16]. In the reaction mixture, the reducing agent dithiothreitol was omitted because it inhibited the enzymic activity, as previously reported for the crude homogenate [16]. Specific enzyme activities were calculated from the initial rates and are expressed in m-units/mg of protein. One unit is defined as the amount of enzyme required to convert 1 µmol of NAD⁺/min under standard conditions.

Immunoblotting and activity blotting

SDS/PAGE [15 % (w/v) gel] was performed as in [16]. The Western blot procedure, followed by either immunochemical analysis with polyclonal anti-PARP antibodies or activity blotting, was as described previously [18]. Three aliquots (30–80 µg) of each sample (crude extract and/or purified enzymic fraction) were subjected to electrophoresis on the same gel; two of them were electroblotted. For the activity blot the lane with the homogenate was overloaded (100 µg). The third sample was stained with Coomassie Blue. The Western blotted nitrocellulose filter (Bio-Rad) was cut into single strips, each corresponding to one sample, and subjected to the specific analyses.

Kinetic analyses

The *K_m* of the enzyme purified on the DNA-Sepharose column was determined under standard conditions at increasing substrate concentrations and calculated from the Lineweaver–Burk plot (mean values from three different experiments in duplicate).

The dependence of enzyme activity on pH was measured for 10 min at 80 °C in 100 mM buffer as reported in the figure legend, within the pH range 4.0–10.0, in the presence of a saturating concentration of NAD⁺ (0.64 mM).

Purification of DNA from *S. solfataricus*

Buffer A [20 mM Tris/HCl (pH 7.8)/200 mM EDTA] was added to the crude homogenate (1:1, v/v). The mixture was diluted 1:4 (v/v) with buffer B [10 mM Tris/HCl (pH 7.8)/100 mM EDTA/0.5 % SDS] and the sample was digested with pancreatic RNase A (20 mg/ml) for 1 h at 37 °C, followed by incubation with proteinase K (100 mg/ml) for 2.5 h at 50 °C. Extraction with phenol and precipitation with ethanol were as described in [17]. The DNA concentration was determined by the diphenylamine method [20], with calf thymus DNA (Sigma) as standard; the size of the purified nucleic acid was checked by 1 % (w/v) agarose-gel electrophoresis [17].

EtBr displacement assay

The EtBr fluorescence assay was essentially the same as that described by Wellman [21]. The DNA purified from *S. solfataricus* was 0.16 µg/ml in 5 mM Tris/HCl, pH 8.0, containing 1.27 µM EtBr (0.25 µg/ml). Fluorescence was measured in a spectrofluorimeter with excitation at 525 nm (5 nm bandwidth) and emission at 600 nm (10 nm bandwidth). The data were corrected for fluorescence of free EtBr. In each experiment the fluorescence of a sample containing only the drug and DNA (the sample with maximal fluorescence) was measured and used to normalize other data. The purified enzyme was tested as ligand at increasing concentrations. In parallel, the experiment was performed with a known DNA-binding protein, p7ss, from the same archaeon [16]; the negative control was BSA. The binding constants were calculated by the following formula:

$$K_{\text{EtBr}}[\text{EtBr}] = K_{\text{appl}}[\text{ligand}]$$

where [ligand] is the ligand concentration at which the fluorescence is decreased by 50 % of maximal, and *K_{EtBr}* was $2.5 \times 10^6 \text{ M}^{-1}$.

Temperature effect and thermostability

The effect of temperature on the activity was determined by incubating an appropriate amount of purified enzyme (50 µ-units) under standard conditions, at temperatures in the range 25–100 °C. The reaction was stopped by ice-cooling the vials

before the addition of 25% (w/v) trichloroacetic acid (final concentration). In the Arrhenius plot, the activity was expressed as the logarithm of the ADPR concentration (nM/min per litre).

The thermostability of the purified enzyme was measured by incubating the active fraction from DNA-Sepharose in 100 mM Tris/HCl, pH 8.0, at 80 and 100 °C. At different intervals, aliquots (50 μ -units) were withdrawn to assay the enzymic activity under standard conditions on the addition of 0.64 mM [32 P]NAD⁺ (10 000 c.p.m./nmol).

Reaction products

The purified enzyme was incubated in the presence of 0.64 mM [32 P]NAD⁺ (40 000 c.p.m./nmol) under standard conditions (final volume 500 μ l). The ADP-ribosylated proteins were precipitated by 50% (v/v) trichloroacetic acid (25% final concentration). BSA (50 μ g) was added to enhance protein precipitation. The precipitate, collected by a 10 min centrifugation in a Microfuge (maximum speed), was suspended to the original volume with 10 mM Tris/HCl (pH 11)/8 mM NaOH/1.0 mM EDTA. Proteinase K (50 μ g/ml) was added and the mixture was incubated overnight at 37 °C to detach the product from the proteins, which, in parallel, were digested by the protease. After extraction with chloroform/3-methylbutan-1-ol (49:1, v/v), the aqueous phase was dried and loaded on a 20% (w/v) polyacrylamide gel, prepared and electrophoresed as described by Panzeter and Althaus [22]. The dried gel was exposed to an MP-Hyperfilm sheet for autoradiography (6 days).

In a parallel experiment, after incubation of the enzymic fraction with [32 P]NAD⁺ as described above, the reaction mixture was cooled over ice and loaded on a Sephadex G-25 column, assembled in a syringe and adapted to a centrifuge tube to perform the column centrifugation [16]; the elution was made in water.

The labelled V_0 eluate was re-chromatographed under the same conditions and again the 32 P-labelled proteins that were eluted at V_0 were collected. The dried fraction was incubated at pH 11 as described above, followed by extraction with organic solvent. An aliquot of the aqueous phase (800 c.p.m.) was analysed by reverse-phase HPLC (RP-HPLC) on a Beckman apparatus (model 342), equipped with a C_{18} column (0.5 cm $\times$ 25 cm; S5 ODS2 Spherisorb; 0.5 μ m). The sample and the column were equilibrated in 5 mM KH_2PO_4 , pH 5.6 (solvent A). The elution was started with solvent A; after 3 min the percentage of solvent B (100% methanol) was brought to 5% in 1 min. At 5 min the elution was continued with a linear gradient [5–30% (v/v) solvent B] in 20 min. Fractions containing 0.5 ml were collected at a flow rate of 0.5 ml/min. The radioactivity was measured as Čerenkov counts.

The remaining aqueous phase was digested overnight with PDE I (1 mg/ml) in 10 mM Tris/HCl (pH 8.8)/0.2 mM EDTA/2 mM $MgCl_2$ at 37 °C. After a 20 min incubation in the presence of proteinase K (50 μ g/ml) and organic extraction, the soluble products were analysed by RP-HPLC as described above. Under the same conditions authentic unlabelled NAD⁺, ADPR, AMP and poly(ADPR) were also chromatographed.

The labelled peaks that were eluted from the column were analysed by TLC by the method of [16] and the TLC plates were exposed for autoradiography for 3–10 days, depending on the radioactivity loaded.

In parallel, [32 P]NAD⁺ (3500 c.p.m.) and [32 P]poly(ADPR) (5000 c.p.m.) were incubated and analysed under the same conditions.

The radioactivity associated with peaks a (AMP) and b [phosphoribosyl-AMP (PR-AMP)] of Figure 6(C) was used

to calculate the average chain length of ADPR, also taking into account the contribution of the contaminating NAD⁺ (Figure 5A) to produce AMP in the presence of PDE. The already-described [1] ratio (AMP + PR-AMP + PR₂-AMP)/(AMP – PR₂-AMP), in which PR₂-AMP represents di(phosphoribosyl)-AMP, was used; PR₂-AMP was taken as zero.

RESULTS

Purification of the enzyme

Purification was performed as outlined in the Experimental section. Table 1 summarizes the procedure used to purify the ADP-ribosylating enzyme (0.8–0.9 m-unit/g of cells, wet weight) from *S. solfataricus*. In a typical experiment, the initial centrifugation of the crude homogenate, prepared from 70 g of bacteria, followed by the extraction in the presence of salt and detergent, led to the recovery in the Triton extract of approx. 50% of the starting activity. After precipitation of the Triton extract by 70%-satd. $(NH_4)_2SO_4$ and an extensive dialysis of the precipitate, an active fraction was obtained with a doubled specific activity.

The affinity chromatographies on NAD⁺-agarose and DNA-Sepharose were crucial steps to recover an enzyme purified nearly to homogeneity. The specific activity of the active peak from the NAD⁺-agarose column was 80-fold that of the previous step. The maximal increase (16 600-fold) was achieved by means of DNA-Sepharose column chromatography, with a good yield of total activity (17%). The specific activity of the purified enzyme, reported in Table 1, represents the value measured for the pooled active fractions. This fraction, analysed by non-reducing SDS/PAGE [15% (w/v) gel], gave a single protein band corresponding to an apparent molecular mass of 54–55 kDa (Figure 1A). The purified enzyme proved to be stable for at least 2 months when stored at –20 °C in the presence of 10% (v/v) glycerol.

The purified enzyme was identified by both immunochemical analysis and activity blotting (Figure 1B), after SDS/PAGE and Western blotting of the purified protein [18].

Kinetic parameters and catalytic properties

The enzymic activity was routinely assayed at 80 °C; the analysis of kinetic parameters by the Lineweaver–Burk plot indicated a K_m of $154 \pm 50 \mu$ M. The molecular mass of 54 kDa, reported above, was used to calculate k_{cat} ($4 \times 10^{-2} s^{-1}$), assuming first-

Table 1 Purification of the sulfolobal ADP-ribosylating enzyme

Results from one of the ten experiments performed; 70 g of bacteria were used. Enzyme assays were performed at 80 °C as described in the Experimental section.

Step	Protein (mg)	Activity (m-units)*	Specific activity (m-units/mg)	Recovery (%)	Purification (fold)
Homogenate	12 000	60	0.005	100	1.0
Triton extract	3 800	28	0.008	47	1.6
$(NH_4)_2SO_4$ precipitate	2 000	28	0.014	47	2.8
Dialysed precipitate	950	28	0.030	47	5.8
NAD-agarose	4.6	11	2.400	18	480
DNA-Sepharose	0.12	10	83.000	17	16 600

* 1 m-unit is defined as the amount of enzyme able to synthesize 1 nmol ADPR per min under standard assay conditions.

Figure 1 SDS/PAGE and Western blot of the ADP-ribosylating enzyme from *S. solfataricus*

(A) SDS/PAGE [15% (w/v) gel] of samples at different purification steps. The gel (5 cm × 7 cm) was loaded with Triton extract (80 µg) (lane 1), (NH₄)₂SO₄ precipitate after dialysis (50 µg) (lane 2), NAD⁺-agarose active fraction (20 µg) (lane 3), or enzyme from DNA-Sepharose (3 µg) (lane 4). Proteins were stained with 0.05% Coomassie Brilliant Blue. The numbers at the left refer to molecular masses (in kDa) of the standard proteins. (B) SDS/PAGE [15% (w/v) gel] of a crude homogenate (60 µg) (lane 1) and the purified enzyme (4 µg) (lane 2). Staining was with Coomassie Brilliant Blue. The gel corresponding to duplicate lanes 1 and 2 was subjected to Western blotting. The blotted nitrocellulose strips were analysed by activity blotting with [³²P]NAD⁺ (lanes 1' and 2') and by immunoblotting with chicken anti-PARP antibodies (lanes 1' and 2'), diluted 1:1000, by the method of [18].

order kinetics, where the only variable was the substrate NAD⁺, because PARPss is both enzyme and second substrate. The ratio k_{cat}/K_m was 259 s⁻¹·M⁻¹.

Enzymic thermostability and activity dependence on pH and temperature

The thermostability of the purified enzyme was determined at 80 and 100 °C. At 80 °C the half-life of the enzyme was 204 min, whereas with the same time of exposure at a temperature of 100 °C the activity decreased below 15%.

The PARPss exhibited high activity levels between pH 6.5 and 10.0, with no significant change within this range (Figure 2A). A noticeable, although still unexplainable, activity was measured at acidic pH, below pH 4.0 (results not shown).

The temperature dependence of the activity is shown in Figure 2(B). The temperature optimum was found to be 80 °C. The Arrhenius plot showed a break point in the range 50–60 °C. This transition was also demonstrated by CD analyses, showing an increase in negative ellipticity between 50 and 60 °C, and indicating that the protein gained a more structured conformation in this range of temperatures (results not shown). Above

Figure 2 pH and temperature dependence of the ADP-ribosylating enzyme from *S. solfataricus*

(A) pH dependence of the enzymic activity. The enzyme from DNA-Sepharose was incubated in 0.1 M buffer (sodium acetate, pH 4.0–5.6; potassium phosphate, pH 5.6–7.5; Tris/HCl, pH 7.5–10) and the activity was assayed under standard conditions. The results are representative of five experiments. In the pH range 6.5–10.0 the activity levels were similar. Abbreviation: mU, m-unit. (B) Arrhenius plot of PARPss activity. Activity was determined with the same enzyme preparations as in (A), under standard conditions, in the temperature range 30–100 °C. Values are means for three independent experiments. The transition point in the range 50–60 °C was also confirmed by CD (results not shown).

80 °C, the descending branch of the plot corresponded to the decrease of catalytic activity measured at 90 and 100 °C.

Binding of the enzyme to homologous DNA

The first evidence that PARPss has an affinity for DNA was obtained by the binding of the enzymic fraction to the DNA-Sepharose column. The DNA–protein interaction was further tested by the EtBr displacement assay. The fluorescence of the EtBr solution containing DNA alone was set at 100%; on addition of the different ligands, the decrease in fluorescence measured the ability of the analysed protein to displace the drug from DNA (Figure 3). The maximal decrease was observed for the archaeal DNA-binding protein, p7ss, at a very low concentration of the ligand compared with the curve obtained for PARPss. In the presence of the ADP-ribosylating enzyme the fluorescence was decreased to 50% at 0.3 µM ligand. No effect was observed with BSA. The apparent binding constants were calculated for the different ligands by using the formula reported in the Experimental section. At the ligand concentration decreasing the fluorescence to 50%, the constant determined for

Figure 3 DNA-binding properties of PARPss

The EtBr assay was performed at fixed concentrations of homologous DNA (0.16 $\mu\text{g}/\text{ml}$) and EtBr (1.27 μM). I is the fluorescence of sample with ligand, DNA and EtBr; I_B the fluorescence of sample with DNA and EtBr (the sample with maximal fluorescence) and I_0 the fluorescence of free EtBr. Symbols: $\square$, p7ss protein; $\circ$, BSA; $\triangle$, PARPss from DNA-Sepharose. The decrease in fluorescence depended on the ability of the ligand to displace the drug from DNA. The experiment was performed in duplicate.

Figure 4 Autoradiography of the electrophoresed alkali-digested ^{32}P -labelled products from the automodified enzyme

The ^{32}P -labelled products obtained after alkali digestion of the modified proteins were analysed by PAGE and autoradiography by the method of [22]. Lane 1, $^{32}\text{P}[\text{NAD}]^+$ (800 c.p.m.); lane 2, ^{32}P -labelled polymers (2000 c.p.m.) from a eukaryotic source, the rat testis; lane 3, the labelled digest from the DNA-Sepharose active eluate (1100 c.p.m.); lane 4, the labelled Triton extract (1500 c.p.m.). At the left, the migrations of $^{32}\text{P}[\text{NAD}]^+$ (NAD) and Bromophenol Blue (B) are indicated; at the right, the numbers refer to the length of the ADPR chains with the same mobilities as NAD^+ and Bromophenol Blue. The length of the ADPR chains was estimated with reference to NAD^+ , which migrates close to a four-unit oligomer, and Bromophenol Blue, which has a mobility corresponding to an eight-residue chain. The strong band running close to the front is AMP produced from ADPR monomer under the alkaline conditions applied.

PARPss was 1.4×10^7 , two orders of magnitude lower than the K_{app} for p7ss (7.0×10^9) and comparable to the K_{app} of a heterologous DNA-binding protein, rat testis H1 histone (2.5×10^6).

Figure 5 RP-HPLC of ^{32}P -labelled products after incubation at pH 11 and organic extraction

(A) $^{32}\text{P}[\text{NAD}]^+$ (800 c.p.m.). A limited conversion of $^{32}\text{P}[\text{NAD}]^+$ to ADPR is also evident (peak b). (B) ^{32}P -labelled products from the DNA-Sepharose active eluate (800 c.p.m.). The arrows indicate the elution volumes of the standards. The peaks from HPLC were analysed by TLC followed by autoradiography [16]. (A') Peaks b–d from (A); (B') peaks a–d from (B).

A decrease in fluorescence in the presence of PARPss, although to a smaller extent, was also measured when the archaeal DNA was replaced by the nucleic acid from a eukaryotic source (calf thymus) (results not shown).

Reaction products

The incubation of the ^{32}P -labelled material in the alkaline buffer (pH 11) detached the products from proteins, which were further digested by the proteinase K action. By means of PAGE and autoradiography, the water-soluble radioactivity recovered after organic extraction was resolved in a pattern resembling that of short oligomers (less than 6 units) of ADPR (Figure 4). The strongest band was at the level of one residue (mono-ADPR); the weakest label corresponded to a five-unit chain.

After incubation at pH 11 followed by RP-HPLC, the elution profile of the sulfolobal ^{32}P -labelled products showed that most radioactivity was co-eluted with authentic ADPR (peak b) and, to a smaller extent, with peak c (Figure 5B). For comparison, the elution profile of $^{32}\text{P}[\text{NAD}]^+$ treated under the same conditions as the sample is also shown (Figure 5A). The TLC analysis and autoradiography of peak b revealed the presence of ADPR and

Figure 6 RP-HPLC of ^{32}P -labelled products after digestion with PDE

(A) ^{32}P -NAD $^{+}$ (2500 c.p.m.); (B) rat testis ^{32}P -poly(ADPR) (3500 c.p.m.); (C) ^{32}P -labelled products from the DNA-Sepharose active eluate (1500 c.p.m.). The peaks from the three columns were further analysed by TLC and autoradiography. The TLC of peaks a from (A), (B) and (C) gave the results in the upper panel (lanes A', B' and C' respectively). In the lower panel (lanes B' and C'), the results for peaks b from (B) and (C) are shown. Abbreviation: PR $_2$ -AMP, di(phosphoribosyl)-AMP.

of a large amount of radioactivity with an R_f lower than that of the monomeric product (Figure 5B'). Peak c also corresponded to a product migrating more slowly than ADPR.

After digestion with PDE, the elution profile of the ^{32}P -labelled products was that shown in Figure 6(C), in comparison with PDE-digested ^{32}P -NAD $^{+}$ (Figure 6A) and ^{32}P -poly(ADPR) (Figure 6B).

Figure 6(A) shows only one large peak, which co-migrated with authentic AMP; this was the product expected after digestion of the pyridinic substrate with PDE.

In Figure 6(B), alongside a minor peak of radioactivity associated with peak a, high labelling was present at the elution volume of authentic ADPR (peak b). This is consistent with the elution time of PR-AMP, resulting from the digestion of rat testis ^{32}P -poly(ADPR) with PDE.

Figure 6(C) reproduces the elution profile obtained after the digestion of ^{32}P -poly(ADPR) with PDE. In addition to peak a, which was co-eluted with authentic AMP, the main labelling was associated with peak b.

As confirmed by TLC (Figure 6, lanes A', B' and C'), peak a from Figures 6A, 6B and 6C corresponded to compounds with the same R_f ; peak b from Figure 6C co-migrated with the authentic PR-AMP (see the profile of Figure 6B).

The equation in the Experimental section was applied to calculate the average chain length of the products from *S. solfataricus*; a length of 4.6 residues was determined.

DISCUSSION

In *S. solfataricus*, the endogenous levels of NAD $^{+}$, comparable to those in *Escherichia coli* (micromolar concentrations), are enough to satisfy both the oxidative needs of the archaeon and the involvement of the pyridinic compound in the synthesis of ADPR. In fact this reaction, *in vitro*, accounts for less than 2% of the total hydrolysis of NAD $^{+}$ [16].

The ADP-ribosylating enzyme was purified more than 15000-fold from *S. solfataricus* and showed some kinetic properties, such as the micromolar K_m , comparable to those of the eukaryotic protein.

The dependence of the activity on temperature was confirmed by its steady increase in the range 20–80 °C, with the optimum at 80 °C, in agreement with the thermophilic nature of the micro-organism. Like several other thermophilic enzymes, the archaeal PARPs showed a thermal conformational transition, as revealed by the break at approx. 60 °C in the Arrhenius plot and confirmed by CD analyses (results not shown). The activity of the enzyme from *S. solfataricus* also displayed a pH dependence; in the pH range 6.5–10 only 10–20% difference was measured in the activities, a property already demonstrated for other thermophilic enzymes [23].

The purified archaeal enzyme had a native molecular mass of approx. 54 kDa. This dimension is nearer that measured for prokaryotic and eukaryotic mono-ADPR transferases, although other features of the protein from the micro-organism strongly suggested a strict relationship with the eukaryotic-specific PARP (116 kDa) [24]. In fact the 54 kDa molecular mass of PARPs is not far from that of other PARP proteins identified in plant cells of *Arabidopsis thaliana* (72 kDa) and in the nematode *Caenorhabditis elegans* (54 kDa) [25,26]. These intermediate properties exhibited by the microbial enzyme probably reflect the position that the *Sulfolobales* occupy in the evolutionary scale, closer to Eukarya than to Bacteria [5]. In fact, the enzyme from *S. solfataricus* cross-reacted with antibodies raised against either mono(ADPR) transferase (ADPRT) or PARP [18]. However, the PARPs was found to undergo automodification (peculiar to mesophilic PARP) but at a higher temperature (80 °C), as revealed by the activity blot; like the nuclear enzyme it was able to elongate the ADPR chain up to five residues. Furthermore, in common with the nuclear counterpart, the enzyme was able to interact with the homologous DNA (a property used in its purification by affinity chromatography). The weak effect of DNA on the enzymic activity (results not shown) is still under study.

These observations suggest that, although *S. solfataricus* lacks the nuclear compartment, the 54 kDa PARP-like ADP-ribosylating enzyme might be involved in structural or functional events of the bacterial nucleoid. Previous results showed that in this micro-organism a 7 kDa DNA-binding protein, with a possible structural role, undergoes ADP-ribosylation *in vitro* by the endogenous enzyme [17].

It is evident that PARPs represents a very special ADP-ribosylating enzyme, able to operate under temperature conditions that would be lethal for the mesophilic enzyme. The

molecular basis of this high resistance and a correlation with the eukaryotic PARP will be clarified, at least in part, by the determination of the enzyme's structure.

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