Immunochemical detection of ADP-ribosylating enzymes in the archaeon *Sulfolobus solfataricus*

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Received 25 October 1995; revised version received 29 November 1995

Abstract Polyclonal antibodies raised against eukaryotic mono-(ADPribose)transferase and poly(ADPribose)polymerase were used to test the presence of antigenic determinants in a crude extract of *Sulfolobus solfataricus*, a thermophilic archaeon. Samples from eukaryotic (bull testis) and bacterial (*E. coli*) sources were analysed for comparison. All tested antibodies reacted with the sulfolobal sample with a specificity comparable to that of the eukaryotic preparation, as revealed by ELISA test, activity assays in the presence of antibodies and immunoblot experiments. After electrophoresis and western blot of sulfolobal proteins, a band at a mass around 50 kDa was detected by immunostaining.

Key words: Archaea; *Sulfolobus*; Thermophiles; ADP-ribosylation; Immunoblotting; Antibody

1. Introduction

Sulfolobus solfataricus is a thermophilic sulphur-oxidizing archaeon, belonging to *Sulfolobales*, which share several biochemical and genetic features with eukaryotes [1,2]. In *S. solfataricus* we have evidenced the presence of an ADP-ribosylating system which is active at a temperature as high as 80°C [3].

Mono-ADP-ribosylation is common to both prokaryotes and eukaryotes, whereas poly(ADP)-ribosylation is typical of the eukaryotic cells, due its nuclear localization [4,5]. ADPRT and PARP (EC 2.4.2.30) split ADP-ribose from NAD and transfer it to acceptor proteins; in addition, the nuclear enzyme is able to elongate the ADPR chain and to synthesize linear or branched polymers [6]. The finding that the sulfolobal ADP-ribosylating system also exhibits the elongation step [3,7], seems unique for a prokaryotic organism.

The relationship between sulfolobal and eukaryotic ADP-ribosylation was evaluated by studies with antibodies raised against eukaryotic ADPRT, PARP and the catalytic site of PARP. The specific cross-reactivity of all tested antibodies with the sulfolobal sample suggests the presence of epitopes, in the archaeal proteins, structurally related to the eukaryotic enzymes.

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Abbreviations: ADPR, ADP-ribose; ADPRT, mono(ADPribose)transferase; BSA, bovine serum albumin; PARP, poly(ADPribose)polymerase; PBS, phosphate-buffered saline; PMSF, phenylmethylsulphonyl fluoride; TBS, Tris-buffered saline.

2. Experimental

2.1. Crude extract preparations

E. coli cells (Y9010; 320×10^6/ml) were lysed with lysozime (1 mg/ml) in 10 mM Tris-HCl, pH 8.0/10 mM EDTA (1 h, 25°C) and centrifuged to use the supernatant (12 mg protein/ml). Bull testis (2 g) was homogenized with Polytron (max speed) in 50 mM Tris-HCl (pH 7.3)/17% glycerol/12 mM 2-mercaptoethanol/0.5 mM EDTA/10 mM KH_2PO_4/0.5 M KCl/1 mM PMSF (1:4, w/v), and centrifuged (10,000 rpm, 20 min; Beckman centrifuge J2-21, JA20 rotor). The supernatant (extract), filtered through glass fiber (GF/F Whatman), was 11 mg protein/ml. Sulfolobal proteins were pelleted from the homogenate (0.33 g cells/ml; strain MT-4, DSM 5833) [3], suspended in 50 mM Tris-HCl (pH 7.5)/0.5 M NaCl/0.2% Triton X-100/0.1 mM PMSF (1:0.5, w/v), and stirred overnight at 4°C. After centrifugation (14,000 rpm, 20 min), the supernatant proteins were 12 mg/ml, as determined by the BCA method (Pierce, Rockford, USA) with BSA as the standard.

2.2. Polyclonal antibodies

Rabbit antiserum to the truncated form (aa 24–303) of rabbit ADPRT (anti-ADPRT), chicken anti-calf thymus PARP Igγ (anti-PARP), rabbit antiserum to the catalytic site of human PARP (anti-cat) were obtained by the courtesy of Dr. J. Moss (NIH, Bethesda, MD, USA), Dr. F. Althaus (University of Zurich, Switzerland) and Dr. G. de Murcia (CNRS, Strasbourg, France), respectively. Rabbit and chicken pre-immune controls were prepared according to Harlow and Lane [8].

2.3. ELISA test

The test was performed according to [8]. 5–20 μg protein of testis, *E. coli* and sulfolobal extracts in PBS, pH 8.0, were incubated per well in 96-well microtitre plates (3 h, 20°C) and washed twice with PBS/0.05% (v/v) Tween 20. The plastic was incubated with both polyclonal antibodies and pre-immune sera (1:500, v/v, 12 h × 4°C). Horseradish peroxidase-coniugated rabbit anti-chicken IgG (PIERCE) were used as secondary antibodies to detect anti-PARPs, and alkaline phosphatase-coniugated mouse anti-rabbit IgG to evidence anti-ADPRT and anti- cats [8], measuring the A_{405} by an EIA Reader (2550, Bio-Rad).

2.4. Enzymatic assays

Bull testis and *E. coli* extracts were assayed for PARP and ADPRT according to [9], sulfolobal fraction according to [3] (pH 7.5, 80°C), with 600 μM [^{14}C]NAD (CFA 197, Amersham; 10,000 cpm/nmol) or [^{3}H]adenine-NAD (TRK 931, Amersham; 12,000 cpm/nmol). 6–40 μU of each sample were incubated (12 h, 4°C) in PBS/0.1 mM PMSF, with the various antibodies or the pre-immune sera (1:500, v/v; final volume 50 μl), centrifuged (10,000 rpm × 10 min), and the supernatants (15 μl) assayed for enzymatic activity.

2.5. Western blot

The various extracts were analysed by SDS-12% PAGE (Mini Protean, Bio-Rad) [9]. Western blot of the proteins (60 μg) was according to [10]. Activity blot with [^{32}P]NAD (1000 Ci/mmol; 1 μCi/ml) and immunoblots with anti-PARPs (1:500, v/v) and anti-Cats (1:1,000, v/v) were as in [11] and [12] respectively; that with anti-ADPRTs (1:5,000, v/v) was according to [13]. The second antibodies were as in section 2.3.

SSDI 0014-5793(95)01455-1

3. Results and discussion

Table 1 shows the results of the ELISA test. Anti-ADPRT IgG reacted preferentially with *E. coli* extract. For bull testis, incubated with both anti-PARP and anti-cat antibodies, the measured values were higher, although comparable to those of the sulfolobal extract.

Table 1
ELISA test of crude extracts from different sources with antibodies raised against eukaryotic ADP ribosylating enzymes

Antibodies**	*E. coli**	Bull Testis*	*Sulfolobus solfatar**
anti-ADPRT	163 ± 10	46 ± 4	68 ± 3
anti-PARP	21 ± 8	138 ± 8	73 ± 5
anti-Cat	24 ± 5	132 ± 6	99 ± 5

The reported absorbance values (± standard error) have been subtracted of the corresponding blank.
*8 μg protein.
**1:500 dilution.

The effect of all tested IgG on sulfolobal enzymatic activity is shown in Fig. 1. An activity lower than in the control (60–70%) was measured after incubation of the sulfolobal extract with the three antibodies, with a similar behaviour in the presence of anti-PARP Igγ and anti-cat serum, which, in contrast, both unaffected the *E. coli* enzymatic activity.

The specificity of interaction between the different antibodies and the antigenic determinants in the analysed extracts were examined by immunoblots (Fig. 2). Immunostainings with anti-ADPRT serum (Fig. 2A, lane 1) revealed a single band slightly above 50 kDa. In the presence of anti-PARPs (Fig. 2A, lane 2), two bands were evident with a mobility around 50 kDa. Weaker stainings, not reproducibly detectable, were observed at 80 kDa and 26 kDa, likely non-specific staining, but possibly the 26 kDa and the 50 kDa fragments being proteolytic products of the 80 kDa band. As expected, with anti-PARPs, the testis fraction gave a band at 116 kDa (Fig. 2A, lane 3), whereas no staining was evidenced for *E. coli* sample (Fig. 2A, lane 4). As

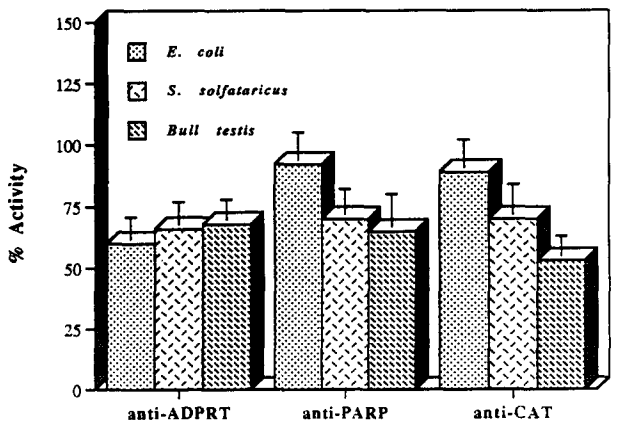

Fig. 1. Enzymatic assays in the presence of antibodies. Bull testis (0.5–1.0 mU/mg), *E. coli* (0.02 mU/mg) and *S. solfataricus* (0.12 mU/mg) enzymatic activity was assayed as described in section 2. The activities measured in the samples incubated with pre-immune sera (controls) were taken as 100%. The reported values (mean of 4 experiments in duplicate) were calculated as percent of the controls. The bars represent the standard errors.

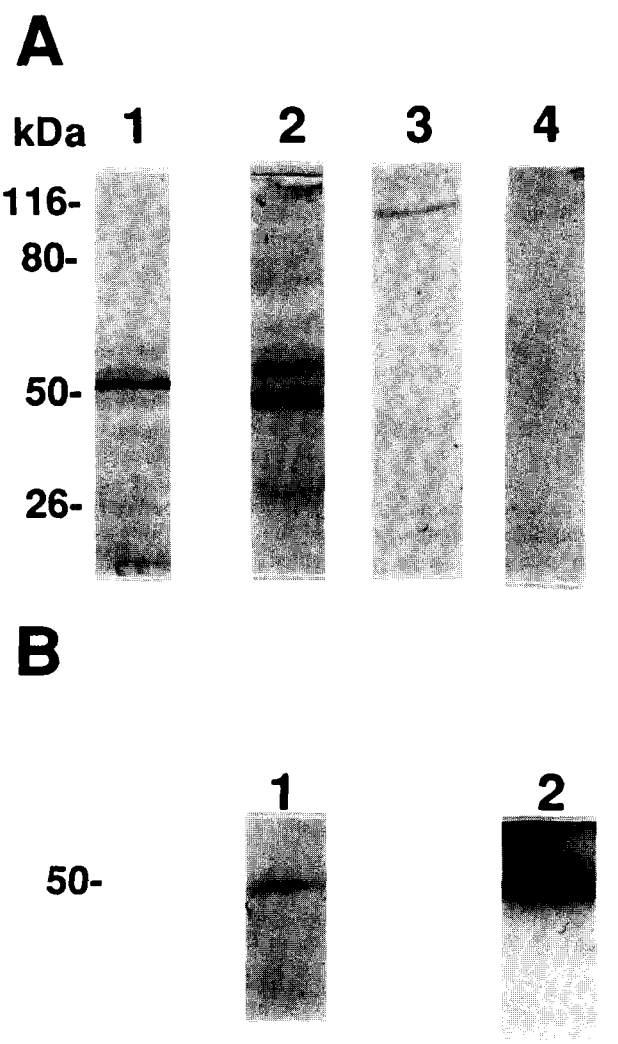

Fig. 2. SDS-10% PAGE and Western blot of the sulfolobal, testis and bacterial crude extracts. (A) From the Western blotted filter, a strip, corresponding to 40μg proteins of the sulfolobal extract, was cut and incubated in the presence of anti-ADPRT antibodies (1). An analogous strip with the same fraction (40 μg, lane 2), was incubated with anti-PARP Igγ; for comparison, bull testis (10 μg, lane 3) and *E. coli* (80 μg, lane 4) samples are shown. (B) The Western blotted sulfolobal proteins (40 μg), were first analysed for enzymatic activity by incubation of the filter in the presence of [^{32}P]NAD (80°C, 1 h) and exposed to autoradiography for three weeks (2). Thereafter the membrane was immunoblotted with anti-cats (1).

for the ELISA test, both controls reacted with anti-ADPRT (data not shown). In the sulfolobal extract with anti-cats (Fig. 2B, lane 1), again a band near 50 kDa was observed. The specificity of the reaction was confirmed, before immunostaining, by the activity blot of the same filter. This reaction is successful only with the polymerizing enzyme, which is itself acceptor of poly(ADPR). In the sulfolobal sample, the autoradiography evidenced a labelling corresponding to the immunostained band (Fig. 2B, lane 2). The negative result for both testis and *E. coli* was due to the high incubation temperature (80°C). However, under the conditions described for the eukaryotic enzyme, in the testis, but not in *E. coli*, was confirmed the presence of PARP activity (data not shown).

Although ADPRT and PARP catalyze the same initiation and transfer reactions, no structural relationship of the two classes of enzymes was actually demonstrated [14]. The structural complexity of PARP reflects the more specialized functions of this enzyme as the elongation and branching steps of the reaction and the automodification [6].

The evolutionary relationship of thermophilic Archaea with Eukarya [1,2] makes *S. solfataricus* a good candidate to investigate whether archaeal ADPribosylation reaction exhibits common features with the eukaryotic system. As evidenced by anti-ADPRT, this is the first report of a highly thermophilic (80°C) and thermostable archaeal ADPRT with possible structural similarity with the mesophilic counterparts. Still more relevant is the presence of a poly(ADPR)polymerase-like activity, which is exclusive of the eukaryotic cell and has never been described in any prokaryote. The philogenetic position of thermophiles as *Sulfolobus*, makes likely that the ADP-ribosylation system in this microrganism is evolutionarily intermediate between those of bacteria and eukaryotes. It is worth noting that anti-PARP IgG reacted with all the samples, except with the *E. coli* fraction. It is known that the structural features of PARP are different from those of all ADPRTs and very far from those of the bacterial enzymes. It is also meaningful that the sulfolobal sample (and not the *E. coli* one) gave a positive result with the activity blot analysis, specific for the eukaryotic enzyme.

The immuno-cross-reactivity toward the sulfolobal proteins indicates that all tested antibodies recognize structural regions common to both the eukaryotic enzymes and strongly suggests a relationship between the archaeal and the eukaryotic ADPribosylating systems.

Acknowledgements: We thank the Servizio Fermentazioni directed by Dr. A. Gambacorta, who prepared the sulfolobal homogenate, and the people cited in section 2, who generously provided antibodies. Work partly supported by CNR funds, Project of Biotechn. and Mol. Biol. (Extremophiles), 1995.

References

[1] Woese, C.R., Kandler, O. and Wheelis, M.L. (1990) Proc. Natl. Acad. Sci. USA 87, 4576–4579.
[2] Klenk, H.P. and Doolittle, F. (1994) Curr. Biol. 4, 920–922.
[3] Faraone-Mennella, M.R., De Lucia, F., De Maio, A., Gambacorta, A., Quesada, P., De Rosa, M., Nicolaus, B. and Farina, B. (1995) Biochim. Biophys. Acta 1246, 151–159.
[4] Poirer, G.G. and Moreau, P. (eds.) (1992) ADP-ribosylation Reactions, Springer-Verlag, Berlin.
[5] Moss, J. and Vaughan, M. (eds.) (1990) ADP-ribosylating Toxins and G Proteins, Am. Soc. Microbiol., Washington DC.
[6] Lautier, D., Lagueux, J., Thibodeau, J., Menard, L. and Poirer, G.G. (1993) Mol. Cell. Biochem. 122, 171–193.
[7] Faraone-Mennella, M.R. and Farina, B. (1995) Biochem. Biophys. Res. Commun. 208, 55–62.
[8] Harlow, E. and Lane, D. (1988) Antibodies, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
[9] Doi, S., Tanigawa, Y., Tsuchiya, M. and Shimoyama, M. (1993) Biochim. Biophys. Acta 1162, 115–120.
[10] Towbin, H., Staehelin, T. and Gordon, J. (1978) Proc. Natl. Acad. Sci. USA 76, 4350–4354.
[11] Simonin, F., Briand, J.P., Muller, S. and de Murcia, G. (1991) Anal. Biochem. 195, 226–231.
[12] Lamarre, D., Talbot, B., de Murcia, G., Laplante, C., Leduc, Y., Mazen, A. and Poirer, G.G. (1988) Biochim. Biophys. Acta 950, 147–160.
[13] Okazaki, I.J., Zolkievska, A., Nightingale, M.S. and Moss, J. (1994) Biochemistry 33, 12828–12836.
[14] Domenighini, M., Montecucco, C., Ripka, W.C. and Rappuoli, R. (1991) Mol. Microbiol. 5, 23–31.