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ADPribosylation Reaction by Free ADPribose in *Sulfolobus solfataricus,* a Thermophilic Archaeon

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Abstract In the archaeon *Sulfolobus solfataricus,* protein ADPribosylation by free ADPribose was demonstrated by testing both [*adenine*-^{14}C(U)]ADPR and [*adenine*- ^{14}C(U)]NAD as substrates. The occurrence of this process was shown by using specific experimental conditions. Increasing the incubation time and lowering the pH of the reaction mixture enhanced the protein glycation by free ADPribose. At pH 7.5 and 10 min incubation, the incorporation of free ADPribose into proteins was highly reduced. Under these conditions, the autoradiographic pattern showed that, among the targets of ADPribose electrophoresed after incubation with ^{32}P-NAD, the proteins modified by free ^{32}P-ADPribose mostly corresponded to high molecular mass components. Among the compounds known to inhibit the eukaryotic poly-ADPribose polymerase, only $ZnCl_2$ highly reduced the ADPribose incorporation from NAD into the ammonium sulphate precipitate. A 20% inhibition was measured in the presence of nicotinamide or 3-aminobenzamide. No inhibition was observed replacing NAD with ADPR as substrate. J. Cell. Biochem. 66: 37–42, 1997.

Key words: archaeon; ADPribose; glycation; ADPribose transferase

Protein glycation by free ADPribose (ADPR), produced by NAD glycohydrolases (EC 3.2.2.5), ADPribosyl cyclase, or the rapid turnover of poly(ADPribose), is a well-documented phenomenon [Frei and Richter, 1988; Hilz et al., 1984; Cervantes-Laurean et al., 1993]. This so-called nonenzymatic mechanism flanks the enzymatic ADPribosylation, the NAD-dependent reaction, catalysed by specific enzymes: mono-ADPribose transferases, ubiquitous in all known organisms [Masmoudi and Mandel, 1987; Edmonds et al., 1989; Moss et al., 1983; Althaus and Richter, 1987], and poly-ADPribose polymerase (EC 2.4.2.30), only found in cells with the nuclear compartment (Althaus and Richter, 1987; Lautier et al., 1993].

The nonenzymatic process leads to the modification of acceptor proteins through arginine covalent linkage, or via Schiff base formation, with single units of free ADPribose [Frei and Richter, 1988; Hilz et al., 1984; Cervantes-Laurean et al., 1993]. This ADPR-protein linkage is the most resistant to treatment with 3 M NH_2OH [Frei and Richter, 1988]. A biological meaning of the protein glycation in vivo was also proposed by the same authors.

We demonstrated that a highly thermostable ADPribosylating activity is present in *Sulfolobus solfataricus*, a thermoacidophilic archaeon [Faraone-Mennella et al., 1995]. This sulphur-oxidizing microrganism, isolated from volcanic hot springs [De Rosa et al., 1975], belongs to the *Sulfolobus* species of the Crenarchaeota subdomain [Woese et al., 1990]. In the sulfolobal ADPribosylating system, mono-ADPribose was the main product of the reaction. Evidence was obtained that part of the ADPribose-protein complexes was stable to the treatment with 3 M NH_2OH at 37°C for up to 17 h, as in the nonenzymatic mechanism [Faraone-Mennella et al., 1995]. Since in this microrganism the enzymatic ADPribosylation was demonstrated identifying the enzyme by means of anti-PARP and anti-ADPRT antibodies [Faraone-Mennella et al., 1996], the question of the nonenzymatic reaction, previously proposed, was not fully addressed.

Abbreviations used: ADPR, ADPribose; ADPRT, ADPR transferase; PARP, poly(ADPR)polymerase.

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In this paper we give further experimental evidence supporting the previous results that in *S. solfataricus* the protein glycation by free ADPribose is actively operating under specific assay conditions and can be clearly distinguished from the NAD-dependent process.

MATERIALS AND METHODS

Materials

All reagents were of the highest purity available. Enzymes, coenzymes, nucleotides, and substrates were obtained from Sigma (St. Louis, MO). [*adenine*-^{14}C(U)]NAD (280 mCi/mmol) was acquired from the Radiochemical Center, Amersham (Arlington Heights, IL); [*adenylate*-^{32}P]NAD (10–50 Ci/mmol) was purchased by Dupont-NEN (Boston, MA). Molecular weight markers were acquired from Pharmacia (Gaithersburg, MD).

Cell Culture and Sample Preparation

Sulfolobus solfataricus (strain MT-4, DSM 5833) was isolated from a volcanic hot spring in Agnano (Naples). Cell culture, all procedures to obtain the homogenate and ammonium sulphate precipitate, and protein concentration were as described in Faraone-Mennella et al. [1995].

Synthesis of ADPR

Labelled ADPR was prepared as described by Payne et al. [1985] by alkaline incubation of a mixture of cold and [^{14}C]- or [^{32}P]-labelled NAD with specific radioactivity of 10^4 cpm/nmol and 4.5×10^5 cpm/nmol, respectively. The product was purified by RP-HPLC on a C_{18} column [Faraone-Mennella et al., 1995].

ADPribosylation of Sulfolobal Proteins

Forty percent ammonium sulphate precipitate (200 µg protein) was incubated under standard conditions (80°C, pH 7.5), already described [Faraone-Mennella et al., 1995], in the presence of 0.64 mM [*adenine*-^{14}C(U)]NAD (10,000 cpm/nmol) or 0.64 mM [*adenine*-^{14}C(U)]ADPR (10,000 cpm/nmol). The reaction was stopped by adding ice-cold 50% trichloroacetic acid (v/v). [^{14}C] incorporation into the 25% CCI_3 COOH-insoluble fraction was measured by liquid scintillation after treatment of the pellet as in Faraone-Mennella et al. [1995]. The blank for the enzymatic assay was obtained by incubating the *S. solfataricus* 40% ammonium sulfate precipitate, previously heated at 110°C for 24 h, with [*adenine*-^{14}C(U)]NAD or [*adenine*-^{14}C(U)]ADPR under the incubation conditions described for each experiment. Changes of the assay conditions or of the reaction mixture composition are reported in the figure legends. The thermal stability (80°C) of all compounds used was tested over the indicated times.

SDS-15% PAGE of the ^{32}P-Labelled Proteins

Sulfolobal 40% ammonium sulphate precipitate (200 µg), incubated with 0.64 mM [*adenylate*-^{32}P]NAD (4.5×10^5 cpm/nmol) or 0.64 mM [*adenylate*-^{32}P]ADPR (4.5×10^5 cpm/nmol) under standard conditions, was analysed by SDS-PAGE, followed by autoradiography [Faraone-Mennella et al., 1995].

RESULTS

ADPribosylation of the Sulfolobal Proteins

The time dependence of the protein glycation was first evidenced by measuring the radioactivity incorporation from increasing concentrations of [^{14}C]ADPR at two different incubation times (10 min and 40 min (Fig. 1). Appreciable levels of labelling were observed above 30 µM [^{14}C]ADPR for both times, but the maximal radioactivity was measured at 0.64 mM substrate after 40 min.

The uptake of [^{14}C] in the $(NH_4)_2SO_4$ precipitate incubated either with [^{14}C]ADPR or with [^{14}C]NAD, under standard conditions (80°C, pH

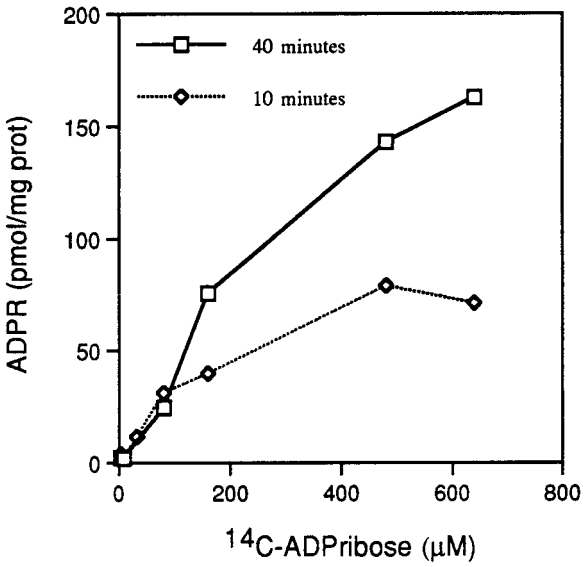

Fig. 1. ADPribose incorporation in the sulfolobal ammonium sulphate precipitate. The sample (200 µg protein) was incubated under standard conditions in the presence of increasing concentrations of [^{14}C]ADPR for 10 or 40 min. Mean values of two different experiments are shown.

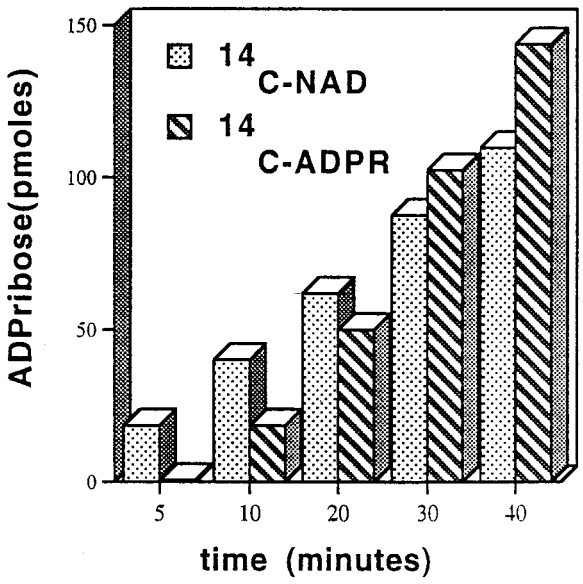

Fig. 2. Time courses of ADPribose uptake from NAD and ADPR in the sulfolobal ammonium sulphate precipitate. The same sample as in Fig. 1 was incubated under standard conditions in the presence of 0.64 mM [^{14}C]NAD or 0.64 mM [^{14}C]ADPR to measure, respectively, the NAD- and ADPR-dependent ADPribosylation (six determinations in duplicate on two different sulfolobal preparations; % error = 3–8%). Analysis of the variance, calculated for the points at 10 min and 40 min, indicated that the difference between the values for NAD and ADPR was significant with $P = 0.01$ (10 min) and $P = 0.05$ (40 min). Unspecific binding of radioactivity was taken into account by subtracting the value of the corresponding blank (see Materials and Methods).

7.5), is shown in Figure 2. The incorporation of radioactivity in the presence of the substrate [^{14}C]ADPR was lower than with [^{14}C]NAD up to 20 min incubation. When the incubation was carried over this time, an increase of the labelling from free ADPribose was evidenced.

The same experiment then was performed by adding 0.64 mM unlabelled ADPR to the reaction mixture and incubating the sample for a few minutes at 80°C before completing the mixture with the radioactive substrate (Fig. 3). While the labelling measured with [^{14}C]ADPR was always lowered to 50%, indicating an isotopic dilution with the unlabelled compound (Fig. 3A), the decrease of radioactivity incorporated from [^{14}C]NAD was evidenced only after 20 min (Fig. 3B). This result suggested that the initial rate of the NAD-dependent reaction is unaffected by the unlabelled ADPR (or affected at a not detectable extent) and that the protein glycation by free ADPribose takes place slowly, its contribution being better measurable at long incubation times (>20 min).

pH Dependence of the ADPribosylation Reaction

The pH of the incubation mixture was found to highly influence the incorporation of radioactivity into the $(NH_4)_2SO_4$ precipitate. The effect of the pH was measured following the incubation of the sample with either [^{14}C]NAD or [^{14}C]ADPR over the pH range 2.4–9.5 under conditions less favourable to protein glycation (10 min at 80°C) (Fig. 4). The incubation of the precipitate with 0.64 mM [^{14}C]ADPR showed the maximum percentage of [^{14}C] uptake at pH 3.6, whereas at basic pH the values were reduced to less than 20%; the [^{14}C] incorporation from [^{14}C]NAD was optimal in the pH range 7.5–9.4. Therefore, free ADPribose was preferentially bound to proteins, even at short incubation times, under acidic conditions. This observation is in line with previous reports that low pHs favour the nonenzymatic ADPribosylation mechanism in eukaryotic systems [Frei and Richter, 1988; Hilz et al., 1984] and that the ketoamines derived from an Amadori rearrangement between ADPribose and aminoacid residues, mainly lysine, are highly unstable at alkaline pH [Cervantes-Laurean et al., 1993].

Effect of Some Chemical Compounds on the [^{14}C] Labelling of 40% Ammonium Sulfate Precipitate

Table I shows the radioactivity incorporation into the sulfolobal precipitate in the presence of several chemicals, which are known to affect the poly-ADPR polymerase activity [Althaus and Richter, 1987]. The analysed compounds were chosen among purine analogs (theophylline) and molecules sharing structural similarities with the piridynic ring of nicotinamide (3-aminobenzamide).

3-aminobenzamide and nicotinamide, known to inhibit both PARP and ADPRT, gave a comparable low inhibition (about 20%–25%). No effect was obtained in the presence of either 4 mM teophylline, which causes 92% inhibition of the eukaryotic enzyme, or 2 mM NADP. On the other hand, DTT, a component of the reaction mixture to assay PARP, induced a dose-dependent decrease in radioactivity. $ZnCl_2$ highly inhibited the reaction, even at very low concentrations (<250 μM), as reported for PARP.

It is noteworthy that, when [^{14}C]ADPR was used as substrate, neither $ZnCl_2$ nor 3-aminobenzamide or nicotinamide showed any inhibitory effect (Table I). This result might be explained by considering that the binding of ADPR to proteins, taking place through a nonenzy-

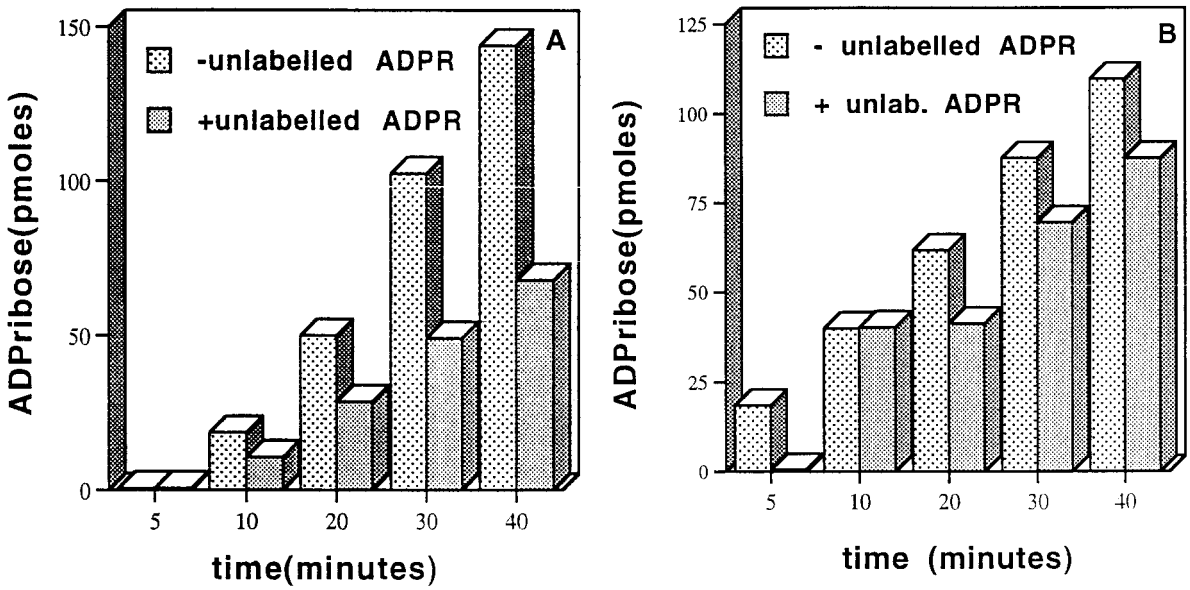

Fig. 3. [^{14}C] uptake in the $(NH_4)_2SO_4$ precipitate in the presence and absence of cold ADPR. Samples were incubated for 5 min at 80°C in the absence (control) or presence of 0.64 mM unlabelled ADPR for 5 min, followed by a standard assay with equimolar [^{14}C]ADPR **(A)** or [^{14}C]NAD **(B).** Mean of four determinations; standard error in the range as in Fig. 1.

matic reaction, was not affected by these compounds.

SDS-PAGE of the ADPribosylated Sulfolobal Proteins

With either NAD or ADPR as substrate, different patterns of modified proteins were obtained. *S. solfataricus* proteins, labelled in the presence of [^{32}P]NAD or [^{32}P]ADPR at pH 7.5 for 10 min, were analysed by SDS-PAGE followed by autoradiography (Fig. 5). The protein targets of free ADPribose (Fig. 5, lane 2) represented only part of the whole autoradiographic pattern of the sample incubated in the presence of [^{32}P]NAD (Fig. 5, lane 1). This result indicates that the proteins modified in the ADPribose-dependent reaction might be specific to the glycation mechanism, which preferentially involves lysine or arginine residues. A right comparison of the autoradiographic patterns from the NAD and ADPR reactions at acidic pH was not possible as the incorporation from [^{32}P]NAD under these conditions was not enough to give clear results.

DISCUSSION

The results reported here indicate that glycation by free ADPribose can be an active process in *S. solfataricus.*

In addition to the preliminary evidence indicating both the uptake of radioactivity from [^{14}C]ADPR and a high resistance of some sulfolobal ADPR/protein adducts to hydrohylamine [Faraone-Mennella et al., 1995], in this paper we demonstrate that some features of the nonenzymatic reaction make it distinguishable from NAD-dependent ADPribosylation. Protein glycation, which increases with the concentration of ADPribose, preferentially takes place with incubation for longer than 20 min. Furthermore, the reaction is highly enhanced under acidic conditions. This is in line with a previous report [Hilz et al., 1984], indicating the acidic pH as the preferential condition for the nonenzymatic reaction in rat liver mitochondria.

Cold ADPR does not affect incorporation of radioactivity from [^{14}C]NAD at short times, whereas it induces a isotopic dilution when incubated with [^{14}C]ADPR. On the other hand, chemicals like nicotinamide, 3-aminobenzamide, and $ZnCl_2$, which inhibit the enzymatic reaction to different extents, do not influence the protein glycation.

These observations further support the finding discussed above that in this microrganism a part of the ADPribose-protein adducts was highly stable after treatment with 3 M NH_2OH but very easily released by incubation in either 0.33 M NaOH or CHES buffer, pH 9.0 [Faraone-Mennella et al., 1995]. In this regard the reaction detected in *S. solfataricus* seems to follow the experimental conditions (pH and time depen-

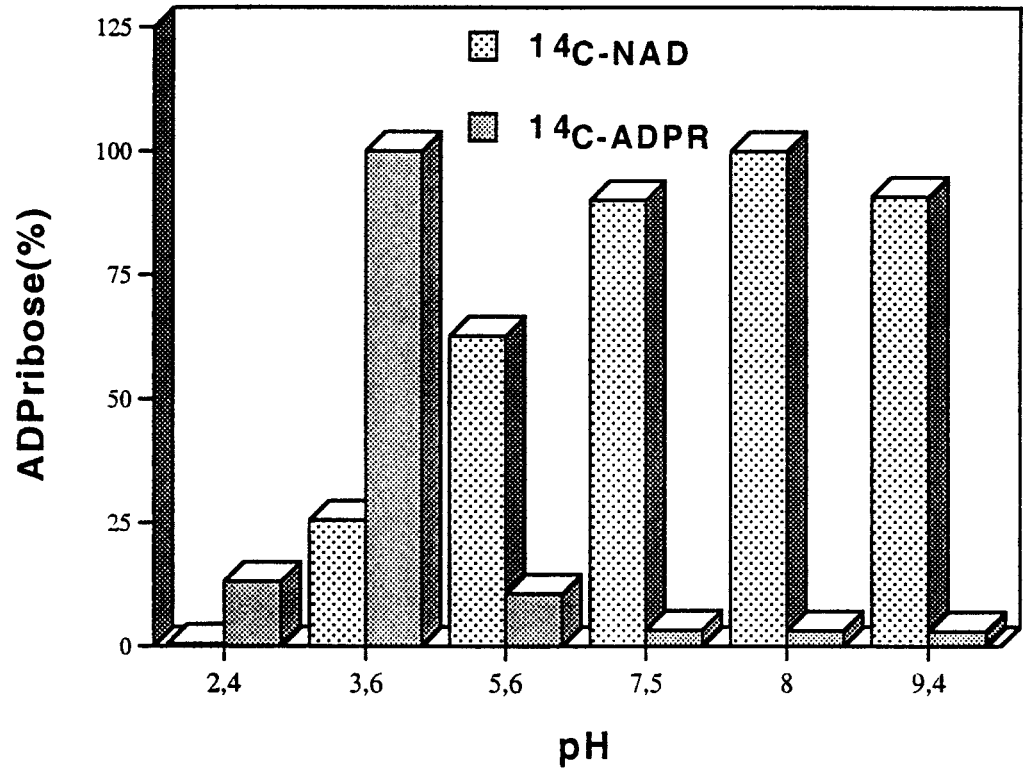

Fig. 4. Effect of pH on [^{14}C] uptake in the sulfolobal precipitate. Proteins (200 µg) were assayed under standard conditions at different pH values in the presence of 0.64 mM [^{14}C]NAD or 0.64 mM [^{14}C]ADPR. Buffers (0.1 M) were glycine-HCl (pH 2.4), sodium acetate (pH 3.6–5.6), potassium phosphate (pH 5.6–7.5), and Tris-HCl (pH 7.5–9.4). The maximum value measured over the pH range was taken as 100% (220 pmoles of incorporated ADPR for the experiment with ADPR as substrate at pH 3.6; 60 pmoles for NAD at pH 8.0). Mean values of two determinations, except for pH 3.6 (three assays in duplicate; error % 7.5 for ADPR, 2.0 for NAD) and pH 8.0 (four assays in duplicate; error % 2.0 for both substrates).

TABLE I. Effect of Some Chemicals on [^{14}C] Uptake in *S. solfataricus* Proteins*

Compound	mM	% radioactivity	
		[^{14}C]NAD	[^{14}C]ADPR
None	—	100	100
3-aminobenzamide	10.0	80	100
Nicotinamide	10.0	75	100
Dithiothreitol	2.0	63	ND
NADP	2.0	100	ND
Teophylline	4.0	100	ND
$ZnCl_2$	0.2	<10	100

*The ammonium sulphate precipitate (200 µg) was incubated under standard conditions in the presence or absence of the reported compounds at concentrations giving the maximal effect on the eukaryotic systems. ND, not determined.

dence, chemical stability) described for eukaryotic systems, except for the incubation temperature.

The ADPribose molecule has the potential reactivity for protein glycation through Schiff base formation with lysine residues, leading to ketoamine products which result from an Amadori rearrangement [Cervantes-Laurean et al., 1993]. The occurrence in vitro of histone or polylysine glycation by ADPribose was reported [Kun et al., 1976], and specific mitochondrial proteins were also shown as acceptors [Frei and Richter, 1988; Hilz et al., 1984]. Previous studies indicated that the mitochondrial nonenzymatic ADPribosylation involves specific acceptor proteins, regulating the import/export of Ca^{2+} [Frei and Richter, 1988; Hilz et al., 1984]. It is known that in the mitochondrial system the efflux of Ca^{2+}, as induced by organic peroxides, uses the activation of NAD glycohydrolase and nonenzymic ADPribosylation of a 30 kDa polypeptide to promote the release or transport of the bivalent cation. It was proposed that the potential for the glycation process is related to the formation and catabolism rates of free ADPribose [Cervantes-Laurean, 1993]. However, in vivo protein glycation, although postulated, was not clearly demonstrated [Kreymeyer et al., 1984].

In view of the proposed biological functions of this process in eukaryotes, the evidence that free ADPR modifies archaeal proteins might

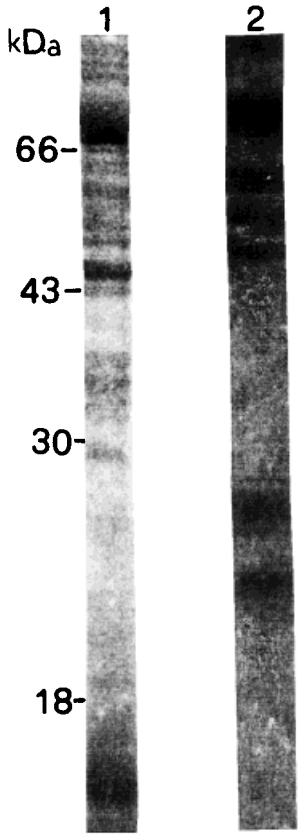

Fig. 5. Autoradiography of electrophoresed archaeal proteins. The [^{32}P]-labelled precipitate (200 µg) was electrophoresed on SDS-PAGE (12%) and exposed for autoradiography. **Lane 1:** 40% ammonium sulphate precipitate after 10 min incubation in the presence of 0.64 mM [^{32}P]NAD (4 days exposure). **Lane 2:** The same sample after 10 min incubation with 0.64 mM [^{32}P]ADPR (8 days exposure).

represent an interesting result, although no information is available today about the presence of an archaeal NAD glycohydrolase activity. This enzyme was demonstrated in most eukaryotic species, including yeast and moulds, but not in prokaryotes, where the NAD catabolic pathway involves a pyrophosphatase rather than a glicohydrolase activity (Olivera and Ferro, 1982).

Therefore, in *S. solfataricus*, study of the occurrence of such an enzyme and how both the enzymatic [Faraone-Mennella et al., 1996] and nonenzymatic ADPribosylation mechanisms influence the turnover and the endocellular levels of NAD is needed to identify the specific biological functions of the ADPribosylation process in this microorganism.

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