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IN THE THERMOPHILIC ARCHAEON *Sulfolobus solfataricus* A DNA-BINDING PROTEIN IS *IN VITRO* (ADPRIBOSYL)ATED

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SUMMARY-A small protein with high affinity for homologous DNA was isolated from *Sulfolobus solfataricus* homogenate by mineral acid extraction. It was purified using a two-step procedure including CM-cellulose and RP-HPL chromatographies. The protein was electrophoretically homogeneous, had a molecular weight of 7.147 kDa and an amino acid composition with a high content of lysine and glutamic acid residues. The protein was able to protect DNA against thermal denaturation and DNAase I digestion in a dose-dependent manner. After incubation of the sulfolobal homogenate in the presence of ^{32}P-NAD, followed by the purification steps, the protein was modified by ADPribose, as revealed by reaction product analysis, SDS-PAGE and autoradiography.

The packaging of DNA into chromatin in Eukaryotes requires several levels of organization, from the nucleosome structure to the 30nm fiber (1). On the other hand, in Bacteria, the assembly of DNA in the nucleoid conformation is simpler, with free supercoils and compaction with histone-like proteins (2, 3 and references therein).

In Archaea, which morphologically resemble Bacteria (4), but share many biochemical and genetic properties with Eukaryotes (4-6), the presence of nucleosome-like structures has been suggested (3).

The Archaea domain includes extreme thermophiles like the sulphur-dependent genus *Sulfolobus*, whose chromosome structure has been widely investigated in order to answer the question of mainteinance of structural integrity at very high temperatures (3 and references therein).

A chromosome-like organization of DNA in these microrganisms implies that specific proteins must be involved both in nucleosomal assembly and in its conformational changes related to archaeal DNA metabolism.

Among archaeal proteins structurally related to histones (2, 7-10), the HMf protein from *Methanothermus fervidus* more strictly resembles eukaryotic histones (10)

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and it has been suggested it is involved in the formation of nucleosome-like structures in Archaea (3).

A family of highly basic proteins of estimated molecular masses 7-8-10kDa was found in *S.acidocaldarius* (8, 9). Among these, the microheterogeneous 7kDa protein has a characteristic N-terminal sequence of lysines in the order X-K-X-K-X-K-X-K (9).

The interest in archaebacterial chromosomal proteins was stimulated by research aimed at the study of the post-translational modification of proteins by (ADPribosyl)ation in the archaeon *S.solfataricus* (11, 12), and particularly focused on the identification of specific proteins as possible acceptors of ADPribose in these microrganisms. Modification of proteins by single or polymeric ADPribose is a wide phenomenon occurring in almost all organisms (reviewed in 13). ADPribose (ADPR) is synthesized from NAD by specific enzymes, mono(ADPR)transferases and poly(ADPR)polymerase (13). The former enzymes are ubiquitous in Prokaryotes and Eukaryotes, the last one is exclusive of eukaryotic cells, being localised in the nucleus, closely associated to chromatin.

In Eukaryotes nuclear proteins with a high affinity for DNA (histones, HMGs and others) were indicated as the best substrates in this reaction (13). Therefore, the presence of chromosomal-like proteins and their modification by ADPR in the thermophilic *Sulfolobus solfataricus*, isolated from volcanic hot springs (14), was investigated.

This paper deals with the properties of a small basic protein, purified from this archaeon, which exhibits a high affinity for homologous DNA, is able to protect the nucleic acid against thermal denaturation and nuclease digestion, and undergoes *in vitro* (ADPribosyl)ation by endogenous enzymes.

MATERIALS AND METHODS

Protein purification - *S.solfataricus* cells (strain MT-4, DSM 5833) were grown according to (14) and the homogenate was prepared as described in (12). The homogenate was made 5% perchloric acid by addition of 10% acid (v/v), re-homogenized with Polytron (2 minutes at max speed) and stirred for two hours at 4^{0}C. After centrifugation for 30 minutes at 12,000 rpm, the supernatant (PCA extract) was collected and dyalysed against 7.5mM sodium borate buffer, pH 8.8 overnight. The dialysed sample (138 mg proteins) was loaded onto a CM-cellulose 32 column (cm 1.5x22) equilibrated in the same buffer and connected to a chromatographic system equipped with a UV monitor (Bio-Rad). The protein was eluted step-wise with the same buffer containing 1M NaCl. 1ml Fractions were collected at a flow rate of 1ml/min.

1M NaCl fraction was dyalysed against H_2O and analysed by HPLC on a C_4 column (Mackeray-Nagel, cm 0.5 x 24, 0.5μ particles). Solvent A was 0.1% trifluoroacetic acid; solvent B was 95% CH_3CN in solvent A. The protein was eluted with a 20%-55% B linear gradient in 70 minutes.

Protein analyses - Homogeneity of the protein was checked by SDS-PAGE (18%) (12), electrospray and aminoacid sequence analyses. Protein concentration was measured as in (12). Aminoacid composition of the protein was determined after 24 hours hydrolysis in 6N HCl at 110°C, on a Beckman 119 aminoacid analyzer; sequence data were obtained by automatic Edman degradation on a Beckman Protein A 473 sequencer.

DNA purification - 10mM Tris-HCl buffer, containing 10mM EDTA was added v/v to the homogenate. The solution was diluted 4 times by addition of the same buffer with 0.1M

EDTA/0.5% SDS and incubated in the presence of RNAase A (SIGMA, type XIV; 10μg/ml) for 1h at 37°C. Thereafter proteinase K (SIGMA; 50μg/ml) was added and the incubation prolonged for 2.5 hours at 50°C. The incubation mixture was extracted with phenol (saturated with 10mM Tris-HCl, pH 8.0)/chloroform/isoamylic alcohol (49:49:2; v/v/v). The aqueous phase was dyalysed overnight against 10mM Tris-HCl, pH 7.5 (2 changes x 5 liters). DNA concentration was determined by A_{260} (1.0 A_{260} unit = 50 μg nucleic acid). DNA recovery was about 2.4mg/g bacteria.

Thermal denaturation - DNA (20μg/ml) was analysed either in 1mM Na-phosphate pH 7.8 or in 0.25mM EDTA pH 8.0, the latter being described as non-aggregating conditions (15). In the reconstitution experiments, the DNA/protein ratio ranged between 1:0.4-1:6 (w/w). A_{260} was recorded with a CARY 1 spectrophotometer (Varian) equipped with a Peltier system and an Epson computer. Temperature was increased of 5°C per minute from 20°C to 110°C. % Hyperchromicity was calculated as $A_{°C}-A_{20°}/A_{110°}-A_{20°}$ x100.

Nuclease digestion - DNA was digested by incubating 28μg DNA in the presence of pancreatic DNAase I (0.43 Units; SIGMA) in 10mM Tris-HCl buffer, pH 7.5, 10mM NaCl, 10mM $MgCl_2$ (final volume 50μl). After incubation at 6°C for 5 minutes, the reaction was stopped by addition of 10mM EDTA. In the experiment in the presence of p7ss, the protein was added to DNA in various amounts and left 10 minutes at room temperature before adding the nuclease. After phenol-chloroform extraction as above, the aqueous phase was analysed by agarose (1.0%) gel electrophoresis in the presence of 0.1%SDS (16).

(ADPribosyl)ation of sulfolobal protein - (ADPribosyl)ating activity was assayed according to (12) by using ^{32}P-NAD (Amersham, 60,000 cpm/nmol) as substrate. To purify the ADPribosylated protein, 1-2mg crude homogenate was incubated with ^{32}P-NAD under stantard conditions. Reaction was stopped by addition of trichloroacetic acid (20% final concentration) to the mixture. The pellet collected after centrifugation (10 minutes at 6,000 rpm) was washed twice with 20% acid and suspended in 5% perchloric acid (300μl), followed by purification steps as described above. The purified fraction was analysed by SDS-PAGE and autoradiography by exposure of the gel to Hyper-film (Amersham) for 2-3 days.

Reaction product analysis - This analysis was performed after detachment of mono- or poly-ADPR from the protein according to (17). The ^{32}P-labelled protein, obtained as described in the previous section, was incubated in alkaline solution (pH 11) in the presence of Proteinase K (50μg/ml) at 60°C. After phenol/chloroform extraction, the aqueous phase, was subjected to column centrifugation on Sephadex G-25 (12) in order to separate ADPribose of different lenght (mono- from oligo-ADPR) and was analysed by polyacrylamide gel electrophoresis and autoradiography (17).

RESULTS AND DISCUSSION

A rapid and simple procedure was followed to purify a small protein, indicated as p7ss, from *Sulfolobus solfataricus*, providing a good yield (1.3 mg protein/g bacteria, wet weight). The perchloric acid extract contained large amounts of the protein (Fig.1). Acid extraction and CM-cellulose chromatography reproduced the steps described for the purification of H1 histone and HMGs (18), except that protein elution from the column was achieved step-wise with 1M NaCl.

The three peaks from the HPLC column (Fig. 2, fractions 13-20) corresponded to the protein, which was homogeneous in SDS-PAGE, and migrated with a molecular mass lower than 10kDa (Fig.1). The exact molecular weight was calculated as 7.147 kDa by electrospray analysis, which produced also evidence of mono-, di- and tri-methylated isoforms (data not shown), thus explaining the HPLC profile.

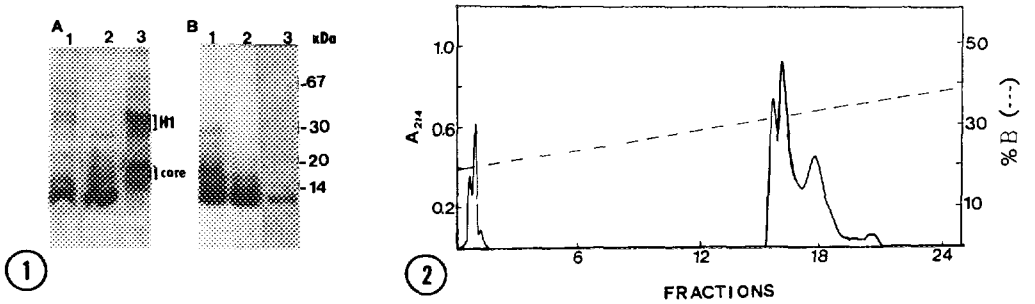


Fig.1. SDS polyacrylamide gel (18%) electrophoresis of p7ss from various purification steps. A.(1) nucleoprotein fraction, (2) perchloric acid extract, (3) calf thymus H1 and core histones, B. (1) perchloric acid extract, (2) 1M NaCl fraction from CM-cellulose, (3) fractions13-20 from HPLC. 10μg-40μg proteins were loaded. The gel (cm 7x5) was stained in 0.5% Coomassie blue.

Fig. 2. RP-HPLC of 1M NaCl fraction from CM-cellulose. Several runs of 0.8 mg protein each were performed. 1ml Fractions were collected at a flow-rate of 1ml/min. Fractions 13-20 were pooled, lyophilised and used for further analyses.

Alternatively to the acid extraction of the whole homogenate, we also recovered in good yield this protein from a nucleoprotein fraction prepared as described by Searcy (15) (data not shown). This fraction contained both DNA and p7ss (Fig.1) in a 1: 6 (w/w) ratio. The presence of p7ss in the nucleoprotein fraction suggested its possible interaction with DNA.

Aminoacid composition (Table I) indicated a high content of both basic (24%) and acidic (20%) residues in a ratio fairly resembling that found in both eukaryotic HMGs (18) and DNA binding proteins from bacterial sources (8, 9, 15).

The partial aminoacid sequence (Fig.3) indicated that the protein could belong to the 7kDa protein family studied by Reinhardt in *S.acidocaldarius* (8, 9). From the N-terminus the sequence almost overlapped those described and exhibited the same order of lysines (X-K-X-K-X-K-X-K). However some differences were observed with regards to the N-terminal residue for 7a and 7b (9) and the substitutions, not all conservative, of some aminoacids (Fig.3).

p7ss exhibited a high affinity for homologous DNA and was able to stabilize it against thermal denaturation in a dose-dependent manner (Fig.4). The results shown in the figure were obtained by incubating *S.solfataricus* DNA with p7ss in 1mM Na-phosphate, but a similar effect was observed also in non-aggregating medium (EDTA). In the presence of p7ss, the melting point was shifted towards higher temperatures. Maximum shift was recorded at a DNA/protein ratio of 1:6 (w/w), where denaturation occurred above 100^{o} C. This melting profile exactly overlapped the one recorded for the nucleoprotein fraction, containing DNA and p7ss in the same ratio (data not shown).

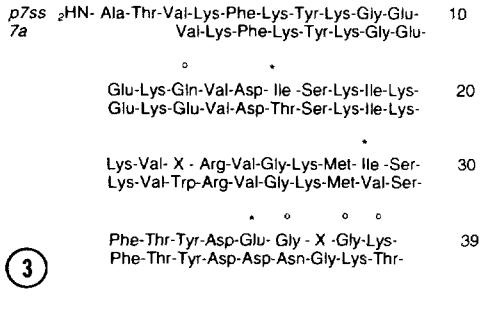
The sensitivity of DNA to nucleases was lowered in the presence of the protein. The electrophoretic pattern of DNA digested with DNAase I, in the presence of p7ss, was comparable to that of undigested DNA, whereas naked DNA was

Table I - Amino acid composition (mol%) of *S. solfataricus* p7ss compared to other DNA- binding proteins

Amino acid	p7ss[a]	7a[b]	HTa[c]	H1[d]	HMG-E[e]
Asx	5.4	10.7	6.0	2.5	5.3
Glx	14.0	11.6	13.8	3.7	16.0
Ser	5.4	4.8	7.7	5.6	8.2
Thr	4.7	5.0	5.7	5.9	5.2
Ala	5.6	7.4	8.0	24.3	9.9
Val	7.1	11.0	2.4	5.4	4.2
Ile	4.3	1.5	7.7	1.5	traces
Leu	5.4	5.0	3.7	4.5	2.8
Met	2.5	3.3	0.3	-	-
Phe	3.2	3.0	5.0	0.9	traces
Tyr	3.1	3.5	1.0	0.9	traces
Trp	ND[f]	ND[f]	-	-	-
Pro	traces	traces	2.6	9.2	9.1
Gly	13.4	6.5	6.6	7.2	11.8
His	traces	-	0.2	-	traces
Lys	20.4	18.5	15.8	26.8	19.2
Arg	3.2	5.9	6.7	1.8	8.0
% Basic res.	23.6	24.4	22.7	28.6	27.2
%Acidic res.	19.6	22.3	19.8	6.2	21.3
Lys/Arg	6.3	3.1	2.4	14.9	2.4
Mol. wt.(kDa)	7.1	7.0	10.0	21.0	ND[f]

a: Purified from either perchloric acid extract or nucleoprotein fraction of *S.solfataricus*.
b: From *S.acidocaldarius* (9).
c: From *Thermoplasma acidophilum* (15).
d: From calf thymus (18).
e: From mouse (18).
f: ND= not determined.

completely degraded (Fig.5). Bovine serum albumin, which does not exert any DNA binding property, had no protecting effect (Fig.5, lane 5), thus excluding any unspecific DNA/protein aggregation.



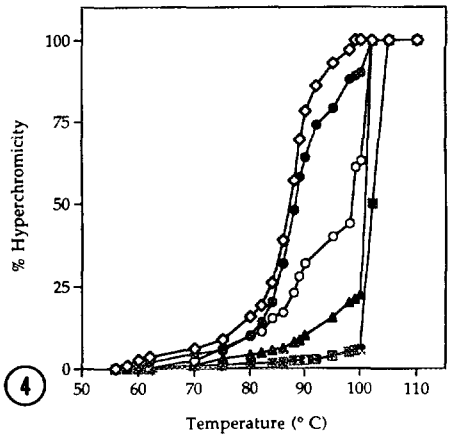


Fig. 3. N-terminal sequence of p7ss. For comparison, the amino acid sequence of one of the 7kDa proteins from *S.acidocaldarius* (9) was shown. (*) conservative and (°) non conservative substitutions were highlighted. X indicates not identified residues.

Fig. 4. Melting profiles of *S.solfataricus* DNA in the absence (◊) and in the presence of p7ss in 1:0.4 (✱), 1:1 (o), 1:3 (▲) and 1:6 (▣) DNA/protein ratios (w/w).

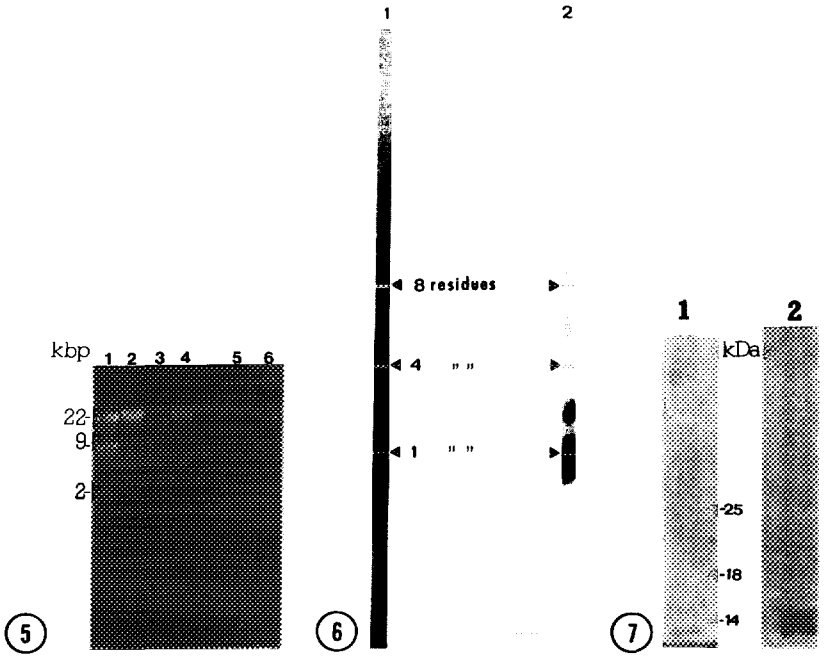


Fig. 5. Agarose (1%) gel electrophoresis of *S.solfataricus* DNA. (1) EcoRI/HIND III digested phage DNA; *S.solfataricus* DNA before (2) and after (3) DNAase I digestion; DNA digested with nuclease in the presence of p7ss in 1:1 (4) and 1:0.5 (6) (w/w) ratio; (5) nuclease digested DNA in the presence of bovine serum albumine in 1:1 ratio.

Fig. 6. ADPribose residues (2,500 cpm) detached from p7ss at pH 11, after column centrifugation on Sephadex G-25. (1) Fraction eluted at Vo. Radioactivity corresponds to 2-5 residues. (2) Fraction retained on Sephadex. Mono-ADPribose is prevalent. Part of this labelling was identified by TLC (12) as 5'-AMP produced during incubation at pH 11.

Fig. 7. SDS-PAGE (1) and autoradiography (2) of purified ^{32}P-labelled p7ss. 1.4µg protein (900 cpm) was loaded.

The ^{32}P-labelled p7ss was obtained by incubating the sulfolobal homogenate in the presence of ^{32}P-NAD and thereafter purified as above. The specific radioactivity of the protein was 4,500 cpm/nmol protein.

An amount of the labelled protein (5,000 cpm) was used to analyse reaction products. The products were detached from p7ss, extracted and an aliquot (1,000 cpm) analysed by TLC followed by autoradiography. A clear spot corresponding to authentic ADPribose was revealed, whereas no contaminating NAD was observed (data not shown). The remaining product (2,500 cpm) were subjected to column centrifugation on Sephadex G-25 and the eluates analysed by SDS-PAGE, followed by autoradiography (Fig. 6). The eluate corresponding to Vo contained mainly products migrating as short oligomers (up to 5-6 residues of ADPribose) (Fig. 6, lane 1), whereas the fraction retained on the column was enriched in monomers and dimers (Fig. 6, lane 2).

The modified protein was revealed by SDS-PAGE and autoradiography (Fig. 7). A shadowed band was observed, suggesting a slight increase of molecular weight

by the presence of the modifying residues. Whether the DNA-binding properties of p7ss are modulated by (ADPribosyl)ation is under study.

Modification of p7ss by ADPribose, if confirmed *in vivo*, might represent a fine regulatory mechanism of protein/DNA interactions.

This finding is particularly interesting in the light of the results described above, which clearly indicate that the purified protein belongs to the class of those with high affinity for DNA.This property was also supported by the high similarity of p7ss with the proteins described by Reinhardt for a different strain of *Sulfolobus* (8, 9). It is worth noting that, although the two *Sulfolobus* strains (*solfataricus* and *acidocaldarius*) are different in specific features such as ribosomal proteins and RNA polymerase patterns (9), they share homologous DNA-binding proteins, suggesting a common structural motif to interact with DNA. Therefore, studying the nature of DNA-binding proteins can help to reveal the conserved regions which may be important to interact with DNA and contribute to understand the way the circular genome is stabilized in the archaeal cell, expecially in the light of its possible chromosomal-like structure.

The DNA-binding properties of p7ss and probably their modulation through chemical modification of the protein (perhaps by ADPribose), must be regarded as important for general knowledge on the structural and functional mechanisms involved in the regulation of archaeal DNA assembly.

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