IMPROVED METHOD FOR THE ISOLATION OF THE A AND A* PROTEINS OF BACTERIOPHAGE ϕX174

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1. Introduction

The A protein (mol. wt 55 000) encoded by the gene A of bacteriophage ϕX174 is a multifunctional enzyme that is active in the replication of the viral DNA. This protein initiates a rolling circle type of DNA replication by introducing a break in the viral strand of RFI DNA at the origin of replication [1,2]. It is the only phage-coded protein necessary for this type of DNA replication and related phages (e.g., G4) use a similar protein.

Many aspects of the functioning of this protein are being studied in vitro, e.g., DNA binding properties, nuclease activity, activity during DNA replication. This made desirable the development of a faster isolation procedure that yields a protein which is sufficiently pure. The one we describe here is based on affinity chromatography and makes use of a single-stranded DNA-cellulose column [3] and a heparin–agarose column [4]. An important advantage of this method, apart from the relatively short purification time required, is that in the same purification procedure not only pure A* protein but also pure and enzymatically active A* protein is obtained [5]. The A* protein (mol. wt 37 000) is also encoded by gene A of ϕX174. The synthesis of the A* protein starts at an internal initiation site within gene A and the same reading frame is used for both proteins. Therefore the A* protein is identical to the carboxyl part of the A protein.

Consequently it can be expected that both proteins have certain properties in common. It was indeed found that both proteins co-purify to a large extent. A complete separation of the A and A* proteins from each other was achieved by heparin–agarose chromatography and a remarkable change in the substrate specificity of the A* protein under altered conditions was detected.

2. Materials and methods

2.1. *Conditions*

Escherichia coli strain HF4704 (*sup*$^-$, *thy*, *uvr*A) was grown in 10 l synthetic TPG medium [6] to 5×10^8 cells/ml at 37°C. Cells were infected with the lysis-defective ϕX174 mutant *am3* with a multiplicity of infection of 5 and incubated for 30 min at 37°C. The culture was centrifuged (10 000 × *g*) for 15 min at 4°C and the pellet was washed with 1.2 l 150 mM Tris–HCl at pH 7.5, 50 mM NaCl and 5 mM EDTA. The final cell pellet was resuspended in 300 ml 150 mM Tris–HCl at pH 7.5, 50 mM NaCl, 10% (w/v) sucrose, 4 mM dithiothreitol and 1 mM EDTA. The cell-suspension was rapidly frozen in liquid nitrogen and stored at –20°C. ϕX174 superhelical covalently closed (RFI) DNA was prepared in [7]. Polyethyleneimine (PEI) was from Serva. A 2% solution in distilled water was neutralized to pH 7.5 with HCl.

2.2. *Preparation of affinity columns*

Heat-denatured calf thymus DNA (Worthington) was covalently coupled to Whatman CF11 cellulose powder by irradiation with ultraviolet light [8]. Non-covalently bound DNA was removed from the cellulose by rinsing packed columns with 5 bed vol. of a solution containing 10 mM NaOH and 0.25 mM EDTA. The amount of covalently bound DNA was determined by eluting a 1 ml single-stranded (ss) DNA-cellulose column with DNase I in the presence

of Mg^{2+} at 20°C and measuring the extinction of the eluate at 260 nm. The ssDNA–cellulose preparations used contained ⩾800 μg DNA/ml bed volume. Heparin (Sigma) was coupled to CNBr-activated agarose (Sepharose 6B, Pharmacia) as in [4].

2.3. *Gel electrophoresis*

Agarose (Serva) horizontal slab gels (1.4%) were run in 40 mM Tris–acetate, 20 mM Na-acetate and 2 mM EDTA (pH 7.7) in the presence of ethidium bromide (5 μg/ml) and photographed under ultraviolet light (366 nm). SDS–polyacrylamide gel electrophoresis was on 12.5% vertical slab gels prepared as in [9].

2.4. *Assay for nuclease activity*

Reaction mixtures with 25 μl total vol. contained: 0.5 μg φX174 RFI DNA, 50 mM Tris–HCl at pH 7.5, 5 mM $MgCl_2$ or 5 mM $MnCl_2$, 150 mM NaCl, 5 mM dithiothreitol and 5 μl column fractions. After 30 min incubation at 37°C EDTA (25 mM) and 10 μg proteinase K (Merck) preincubated for 30 min at 37°C were added and the incubation was continued for 30 min at 37°C. The reaction mixtures were subsequently run on an agarose gel for 3 h at 60 mA/200 V.

2.5. *Determination of protein*

Protein concentrations were determined as in [10] using bovine serum albumin as a standard.

3. Results and discussion

The stored suspension of infected cells (300 ml) was thawed and incubated with lysozyme (150 μg/ml) in a 50°C waterbath under continuous shaking until the suspension reached 37°C. Solid NaCl was added to 1.0 M. The viscous suspension was then chilled in ice. Cell debris and most of the chromosomal DNA were removed by centrifugation at 100 000 × *g* for 30 min, 0°C. The remaining nucleic acids were precipitated by adding an equal volume of 2% (v/v) PEI to the 100 000 × *g* supernatant. In this step, NaCl is lowered to 0.5 M, resulting in the quantitative precipitation of nucleic acids whereas the A and A* proteins stay in solution. The white fluffy precipitate was removed by centrifugation at 30 000 × *g* for 30 min, 0°C. Protein was precipitated by adding powdered ammonium sulphate (0.25 mg/ml) to the supernatant over 15 min. The precipitate was collected by centrifugation (30 000 × *g*, 15 min, 0°C), resuspended in 80 ml 60% saturated ammonium sulphate solution in distilled water (pH 8.0) in order to remove trace amounts of PEI, and spun down again. The pellet (120 mg protein) was dissolved in 80 ml 100 mM Tris–HCl at pH 7.5, 350 mM NaCl, 20% (v/v) glycerol, 10 mM β-mercaptoethanol and 1 mM EDTA (buffer A + 350 mM NaCl). This protein solution was applied at 5 ml/h to an ssDNA–cellulose column (0.9 × 10 cm) pre-equilibrated in buffer A + 350 mM NaCl. The column was washed with 5 bed vol. buffer A + 350 mM NaCl and subsequently developed with a 40 ml linear gradient of 0.35–2.0 M NaCl in buffer A. From the ssDNA–cellulose column 2 mg protein was eluted with a peak at 0.65 M NaCl. Analysis of the column fractions on SDS–polyacrylamide gels showed that the A and A* proteins are eluted between 0.5–1.2 M NaCl and are poorly separated from each other (fig.1a).

Fractions containing A and A* protein (fractions 6–25) were pooled and dialysed against buffer A + 350 mM NaCl. A heparin–agarose column (0.9 × 13 cm) was equilibrated in buffer A + 350 mM NaCl and the protein solution was applied at 5 ml/h. The column was washed with 5 bed vol. buffer A + 350 mM NaCl and the A and A* proteins were eluted with a 80 ml linear gradient of 0.35–2.0 M NaCl in buffer A. Fig.1b shows the protein elution pattern of the heparin–agarose column as determined on SDS–polyacrylamide gels. The A protein is eluted with a peak at 0.65 M NaCl and the A* protein with a peak at 0.8 M NaCl. The fractions containing mainly A protein and mainly A* protein were pooled separately and subjected to a second purification step on heparin–agarose. This resulted in fractions of pure A protein and pure A* protein (fig.1c), with final yields of 50 μg and 500 μg, respectively.

The A protein is known to have a sequence specific endonuclease activity [2,5]. It nicks superhelical φX174 RFI DNA once, at the site of the origin of replication, whereas, e.g., RFI DNA from the phages PM2 and M13, and the plasmid pACYC177 are not cleaved. Therefore the A protein nuclease activity can easily be distinguished from other nucleases by using a mixture of φX174 and pACYC177 RFI DNA as a substrate. It was found that already after the ssDNA–cellulose column the A protein-containing fractions had only nuclease activity for φX174 RFI DNA and not for pACYC177 RFI DNA (fig.2, lanes 1, 2). The A* protein has no nuclease activity on

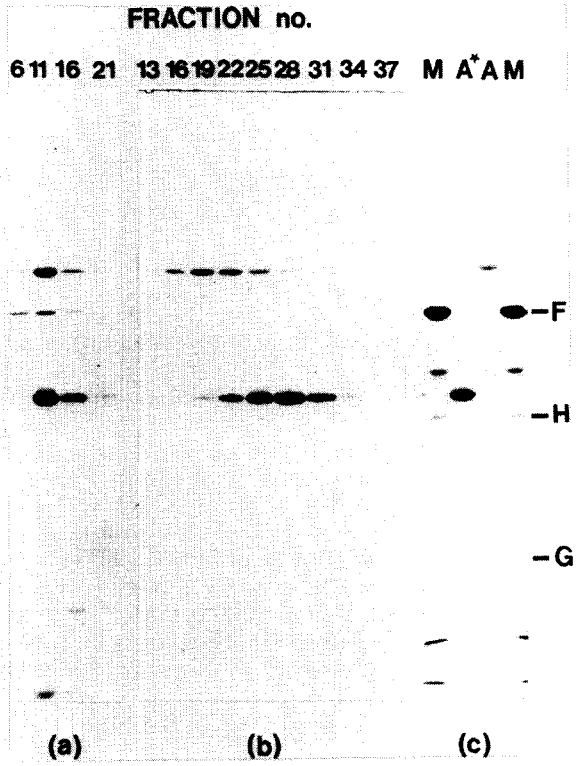

Fig.1. SDS-polyacrylamide gel electrophoresis of: (a) ssDNA–cellulose column fractions; 50 fractions of 0.8 ml were collected during elution from 0.35–2.0 M NaCl with the start of the gradient at fraction 1; (b) heparin–agarose column fractions; after the start of the gradient at fraction 10, 66 fractions of 1.2 ml were collected during elution from 0.35–2.0 M NaCl, fractions 15–22 (A protein) and fractions 23–34 (A* protein) were pooled and separately subjected to a second heparin–agarose purification step; (c) pure A and A* protein after the final heparin–agarose column, fractions containing pure A protein were found <0.65 M NaCl, fractions containing pure A* protein at >0.8 M NaCl. The bacteriophage coat proteins (lanes M), F, H and G with mol. wt 48 000 36 000 and 22 000, respectively, are used as markers [14].

ϕX174 RFI DNA under the conditions used. This means that no further purification is necessary if only the site specific nuclease activity is required. Both proteins are endonucleolytically active on viral (+) ssDNA, but in different ways. The A protein nicks only once, at the site of the origin of replication, whereas the A* protein has the ability to nick the viral DNA at numerous places, with a preference for the origin site, as in [5].

Nuclease activity of the A and A* proteins on double-stranded DNA was also studied in the presence of Mn^{2+}, a metal ion which is known to alter the specificity of several enzymes that interact with DNA [11–13]. Incubation of ϕX RFI DNA with A or A* protein in the presence of Mn^{2+} resulted in both cases in the formation of RFII DNA. Besides, a small amount of relaxed RF DNA (RFIV) moving in front of RFI DNA agarose gels containing ethidium bromide, was found (fig.2, lanes 3–5). In a control experiment, in which Mg^{2+} was added instead of Mn^{2+}, no nuclease activity of the A* protein and no formation of RFIV DNA by the A protein were found (fig.2, lanes 6–8). The specificity of the nick in the presence of Mn^{2+} was indirectly tested by

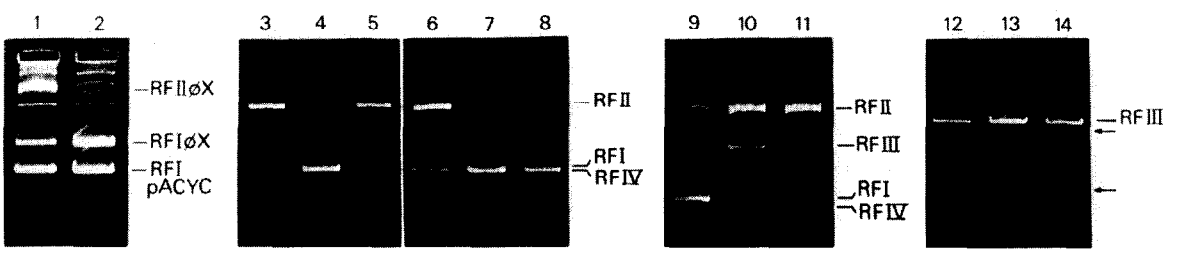

Fig.2. Agarose gel electrophoresis: Lane 1, a mixture of ϕX174 and pACYC177 RFI DNA incubated with ssDNA–cellulose column fraction 16 with Mg^{2+} as a cofactor; lane 2, control without protein; lanes 3–5, ϕX RFI DNA incubated with 0.075 μg A protein (lane 3), without protein (lane 4) and with 0.085 μg A* protein (lane 5) with Mn^{2+} as a cofactor; lanes 6–8, ϕX RFI DNA incubated as in lanes 3–5 but with Mg^{2+} as a cofactor; lanes 9–11, ϕX RFI DNA incubated with 0.38 μg A protein (lane 11), with 0.43 μg A* protein (lane 10) and without protein (lane 9) with Mn^{2+} as a cofactor; lanes 12–14, ϕX RFI DNA incubated as in lanes 9–11 and subsequently digested with PstI restriction enzyme. Arrows mark the positions of the fragments with 80% and 20% genome length.

incubation of RFI DNA with excess amounts of A or A* protein; under these conditions RFII, RFIV and linear RFIII DNA are formed. A subsequent digestion with *PstI* restriction enzyme converted RFII and RFIV DNA into RFIII DNA; RFIII DNA itself was cleaved into two fragments, representing 80% and 20% of the genome length, respectively (fig.2, lanes 9–14).

From the appearance of specific fragments that correspond exactly with the position of the *PstI* restriction site relative to the origin site, it may be concluded that the A and A* proteins both nick specifically at the origin site and are able to nick also the complementary strand under these conditions. The small amount of RFIV DNA which is formed is apparently due to nicking-closing activity of the A and A* proteins in the presence of Mn^{2+}.

The characteristics of the nuclease activities of the A and A* proteins under various conditions and with different substrates as described above, allow a convenient discrimination between both phage proteins.

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