DOMAIN STRUCTURE OF BACTERIOPHAGE fd ADSORPTION PROTEIN

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1. Introduction

Bacteriophage fd is one of a group of closely-related filamentous male-specific coliphages (others are M13 and f1). The virion consists of a closed single-stranded loop of DNA, 6408 nucleotides in length [1], within a tubular array of 2700 subunits of coat protein [2]. At one end of the viral filament are ~5 copies of a second protein, the adsorption protein or A-protein [3]; there are also a few copies of 2 or 3 other, smaller proteins [4,5]. Much is known about the life cycle of the virus (review [6]).

The A-protein is required for adsorption of the phage to the host receptor, which is probably the tip of the F-pilus [7]. Treatment of the phage with the proteinase subtilisin results in digestion of the A-protein but not the coat protein, leaving a particle which is stable but not infectious [7,8]. Electron microscopy reveals that such a particle has lost several small knob-like structures located at one end of the native virion [9]. The amino acid sequence of the A-protein has been deduced by alignment of the N-terminal residues of the protein [3] with the translated DNA sequence of the phage [1,10]. Further analysis of the structure of the A-protein, and its role in adsorption to the host cell, has been hindered by its low abundance (~1% of the virus (w/w)) and its extreme insolubility; isolation of the protein requires complete denaturation of the virus with detergent [3,4].

Here, we show that mild digestion of phage fd with subtilisin releases a large, soluble N-terminal fragment of the A-protein. The fragment appears to compete with intact phage for attachment sites on the host cell. These results suggest models for the structure of the A-protein and its assembly into the virion at the host cell membrane.

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2. Materials and methods

2.1. *Materials*

Subtilisin was from Sigma Chemical Co; [*methyl*-^{3}H]acetimidate was synthesized as in [11].

2.2. *Growth, purification and radiolabelling of phage*

A sample of phage fd was kindly provided by Dr I. Molineux (Imperial Cancer Research Fund, London). The phage was grown on *Escherichia coli* 5274 (from the collection of J. Lederberg and provided by Dr J. Hewitt, University of Cambridge) and purified by polyethylene glycol precipitation and CsCl density centrifugation [12].

Purified phage was radiolabelled by treatment with 0.1 M [*methyl*-^{3}H]acetimidate (13 Ci/mol) in 0.1 M sodium borate (pH 10.0) for 15 min at 0°C [11]. Under these conditions, only Lys_8 of the major coat protein is amidinated (J. A., R. N. P., unpublished), although we know that minor coat proteins are modified too [11]. The modified phage were indistinguishable from native phage by the criteria of adsorption spectroscopy, SDS–polyacrylamide gel electrophoresis [13] and infectivity (plaque assay) and were stored at 4°C in 50 mM Tris–HCl buffer (pH 7.5) containing 1 mM EDTA.

2.3. *Preparation and purification of the A-protein fragment*

Phage fd (80 mg in 16 ml), in 5 g/l NH_4HCO_3, was treated with 80 μg subtilisin (1 mg/ml in 1 mM HCl) for 30 min at 37°C. The proteinase was inhibited by adding 40 μl phenylmethylsulphonyl fluoride (PMSF)

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(26 mg/ml in ethanol), and most of the phage particles were removed by centrifugation (45 000 rev./min Beckman SW50-1 rotor, 3 h). The supernatant was lyophilised and resuspended in 1 ml 50 mM Tris–HCl, 1 mM EDTA, 52 mg/l PMSF (pH 7.5) then applied to a column of Sephadex G-50 superfine (50 cm × 0.3 cm^2) equilibrated with the same buffer. Fractions (1 ml) were analysed for absorbance at 280 nm and by SDS–polyacrylamide gel electrophoresis. Fractions containing the A-protein fragment were pooled, lyophilised and dissolved in 0.1 ml glass-distilled water.

2.4. *Analysis of the A-protein fragment*

The protein (~0.07 mg) was dissolved in 0.1 M sodium hydrogen carbonate containing 10% propan-1-ol and treated with 25 mg diisothiocyanato-aminopropyl porous glass beads pore size 75 Å (DITC-glass) for 16 h at room temperature. The immobilised protein was degraded by the Edman procedure in a computer-controlled solid phase microsequencer of design and construction to be described elsewhere (J. E. W., in preparation). Phenylthiohydantoins were identified by reverse phase chromatography on Zorbax ODS [14].

2.5. *Biological activity of the A-protein fragment*

The adsorption activity of the fragment was investigated by diluting the stock solution with distilled water (1:19, by vol.) and testing its ability to compete with radiolabelled intact phage for binding sites on the host bacterium. Tubes containing 5 ml nutrient broth (5 g/l yeast extract, 10 g/l bactotryptone, 5 g/l NaCl) were inoculated with 50 μl of an overnight culture of *E. coli* 5274 and incubated for 6 h at 37°C with shaking, to an A_{595} of 0.8. The following were then added to pairs of tubes:

(1) [^{3}H]Amidinated bacteriophage fd (3.6 μg in 10 μl) representing ~40 phage particles/bacterial cell;
(2) [^{3}H]Amidinated bacteriophage fd (3.6 μg), 2 μl diluted A-protein fragment;
(3) [^{3}H]Amidinated bacteriophage fd (3.6 μg), 20 μl diluted A-protein fragment;
(4) [^{3}H]Amidinated bacteriophage fd (3.6 μg), predigested with subtilisin as for unlabelled phage.

The tubes were incubated for 20 min at 37°C, and the bacteria were then collected by centrifugation. The pellets were dissolved in 3 ml 1% SDS and the tubes heated to 100°C for 5 min. Six portions of 0.3 ml were taken from each tube for scintillation counting [11].

As a control, 4 mg phage fd was treated with subtilisin to which PMSF had already been added. The sample was centrifuged, the supernatant was lyophilized as before and then dissolved in 100 μl distilled water. The phage-binding experiment was repeated, but with 1/10th (10 μl) of the control lyophilisate replacing the A-protein fragment. The validity of the assay as a measurement of competition was checked by investigating the effect of 5-fold and 100-fold excesses of unlabelled phage on binding of [^{3}H]-amidinated phage.

3. Results

3.1. *Purification and app.* M_r *of the A-protein fragment*

Subtilisin digestion of phage fd caused the disappearance of A-protein and the corresponding appearance of a more mobile band on SDS–polyacrylamide

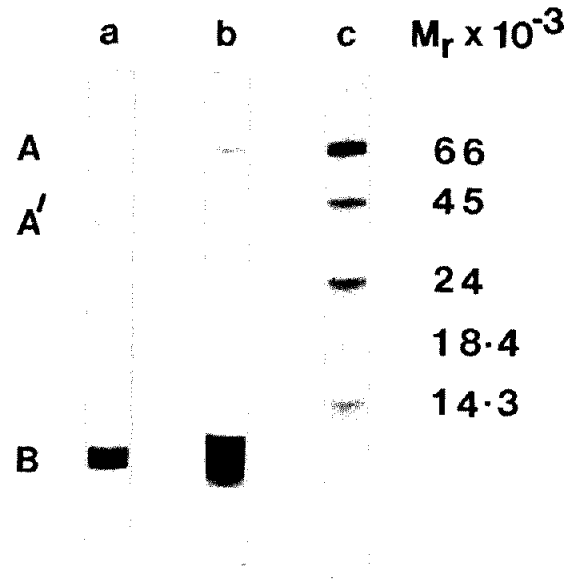

Fig.1. Characterization of the A-protein fragment by SDS–polyacrylamide gel electrophoresis. (a) Phage fd (0.25 mg) was treated with subtilisin (0.25 μg) for 30 min at 37°C and the phage particles were then removed by centrifugation. The supernatant was freeze-dried and analysed by SDS gel electrophoresis [13]. (b) A control sample (80 μg) of untreated phage fd. (c) A mixture of marker proteins, with assumed M_r-values in brackets, as follows: (1) bovine serum albumin (66 000); (2) egg albumin (45 000); (3) trypsinogen, PMSF-treated (24 000); (4) lactoglobulin (18 400); (5) lysozyme (14 300); (A) A-protein; (A') A-protein fragment; (B) major coat protein.

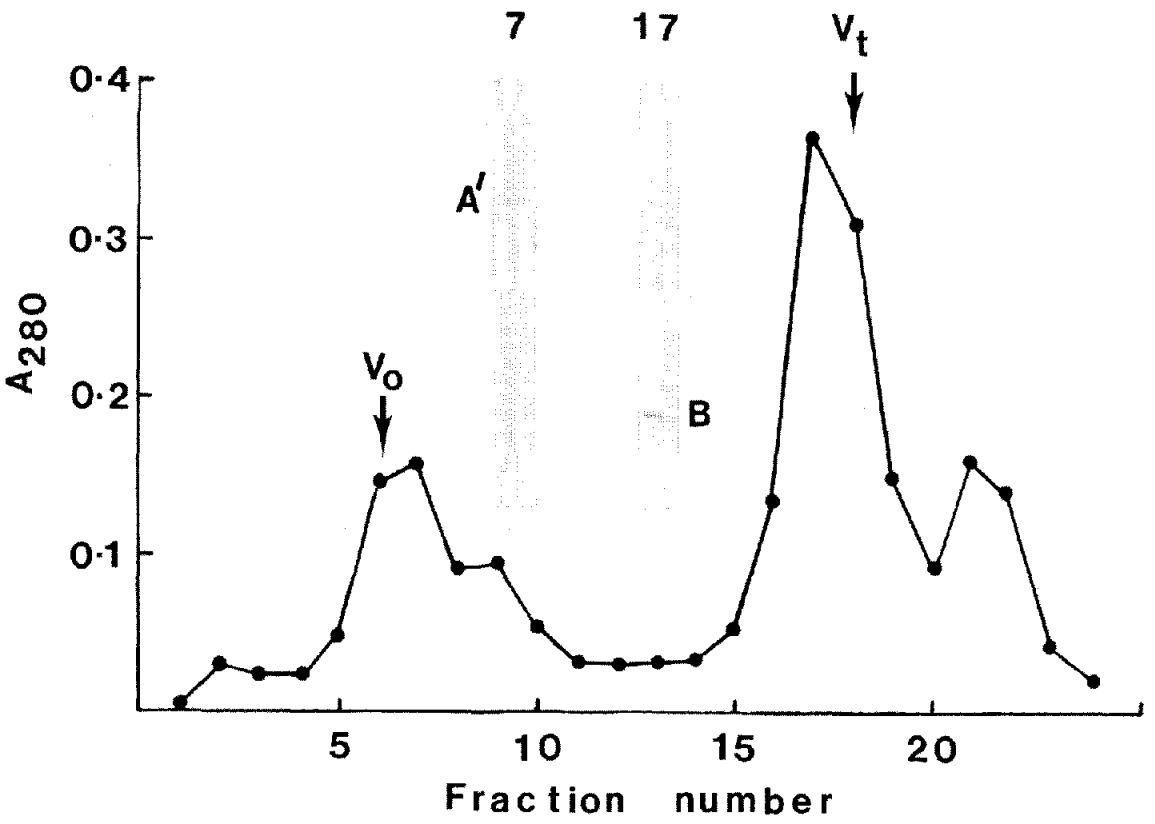

Fig.2. Purification of A-protein fragment by gel filtration. Phage fd (80 mg) was treated with subtilisin (80 μg) for 30 min at 37°C. Phage particles were removed by centrifugation and the supernatant was applied to a column of Sephadex G-50 superfine. Fractions (1 ml) were collected and tubes 5–7 were pooled and freeze-dried: (•) A_{280}; V_o, V_t, excluded and total volumes of the column, respectively. SDS polyacrylamide gel electrophoresis patterns of fractions 7 and 17 are shown inset.

gel electrophoresis. This component, a putative A-protein fragment, remained in the supernatant after the bulk of the phage particles had been pelleted by ultracentrifugation, and migrated with app. M_r 37 600 (fig.1). It eluted at the excluded volume during gel filtration on Sephadex G-50, whereas residual coat protein was retarded by the column and eluted at the total volume (fig.2). The fragment was recovered in ~15% yield as estimated by amino acid analysis, assuming M_r 37 600.

3.2. *N-Terminal sequence analysis of the A-protein fragment*

Edman degradation of the purified fragment revealed the sequence Ala–Glu–Thr–Val–Glu–Ser–X–Leu. This is the N-terminal sequence of the intact A-protein as determined by dansyl-Edman degradation [3] and translation of the phage DNA sequence [10], the latter identifying X as cysteine. Thus the fragment represents an N-terminal portion of the A-protein.

3.3. *Biological activity of the fragment*

The effect of the fragment on the attachment of [^{3}H]amidinated phage fd to host cells is summarised in table 1. Addition of the fragment led to a reduction in radiolabel bound by the cells; under these conditions, portions of 2 μl and 20 μl of the diluted fragment solution caused a decrease in phage binding to 84% and 27%, respectively, of the binding observed with [^{3}H]-labelled phage alone. The value of 27% is similar to that found with subtilisin-digested ^{3}H-labelled phage (29%, table 1), suggesting that it represents non-specific trapping of phage in the bacterial pellet.

In contrast, the control lyophilisate, prepared after

Table 1
Binding of A-protein fragment and ^{3}H-labelled phage fd to *Escherichia coli*

Expt.	Additions to 5 ml bacterial culture	Radioactivity (dpm) bound to bacterial cells after centrifugation (duplicate experiments)	Percentage of value found for ^{3}H-labelled phage alone
A. 1	[^{3}H]Phage (3.6 μg)	3180 3620	100
2	[^{3}H]Phage (3.6 μg) A-protein fragment (2 μl)	2690 3010	84
3	[^{3}H]Phage (3.6 μg) A-protein fragment (20 μl)	1090 750	27
4	Subtilisin-treated [^{3}H]phage (3.6 μg)	1140 860	29
B. 1	[^{3}H]Phage (3.6 μg)	2720 3360	100
2	[^{3}H]Phage (3.6 μg) Control lyophilysate (10 μl)	2300 3340	93
3	[^{3}H]Phage (3.6 μg) Unmodified phage (18 μg)	1380 1380	45
4	[^{3}H]Phage (3.6 μg) Unmodified phage (360 μg)	780 980	29

treating phage with subtilisin that had already been inhibited by PMSF, had no significant effect on phage adsorption (table 1). Thus the observed inhibition of phage adsorption by the preparation of A-protein fragment is probably caused by the fragment itself, rather than by PMSF, subtilisin or any other possible contaminants in the preparation.

The effects of 5- and 100-fold excesses of unlabelled phage on phage binding (table 1) confirm that the assay measured competition for binding sites on the bacteria. These amounts constitute ~200 and 4000 unlabelled phage particles/cell, respectively. As each bacterium has only a few F-pili [22], the adsorption sites should therefore be saturated under these conditions. Again, ~29% of the total radioactivity incorporated appears to represent non-specific association of phage protein with the bacterial pellet. Thus a 5-fold excess of unlabelled phage should reduce the incorporation of labelled phage to 41% of the original value, in reasonable agreement with the observed value of 45%.

4. Discussion

From these experiments, we conclude that subtilisin digestion of phage fd releases a large, soluble, N-terminal domain of the A-protein which appears to contain, at least in part, the site for attachment of the phage to the host cell.

This susceptibility of the A-protein to proteolysis suggests that the protein is folded into an N-terminal domain which is attached to the end of the phage filament by an exposed portion of peptide chain. In [9], knobbed structures at the end of the phage were observed by electron microscopy which were removed by subtilisin treatment; a soluble fragment of polypeptide chain was recovered, presumably derived from the A-protein, with app. M_r similar to that described here. Thus the knobbed structure is likely to be the N-terminal domain of the A-protein, leaving the C-terminal region responsible for attachment of the protein to the end of the viral filament. This region contains the only long sequence of hydro-

phobic amino acids in the protein, a run of 23 uncharged residues terminating 5 residues from the C-terminus [1,10]. It therefore might be suitable for binding to the viral coat protein, which is predominantly hydrophobic [15,16]. The fragment, prepared as in [9], has also been found to inhibit infection of cells by phage (W. Folkhard, personal communication). The specific binding activity, probably to the tip of the F-pilus, is therefore likely to be the first step in the process of viral infection.

There are thought to be ~5 copies of the A-protein at one end of the viral filament [3], but it is not known whether this implies a need for multiple binding of the A-protein to the pilus during infection. From the results in table 1, it appears that the A-protein fragment retains ~20% of the binding activity of intact A-protein in the native phage. This estimate could be influenced by damage to the fragment during isolation, and its interpretation would depend on the number of fragments required to block phage adsorption and their mechanism of interaction with the pilus. Retraction of the F-pilus is thought to be the mechanism by which the phage reaches the bacterial membrane [7,17]. Thus the isolated A-protein fragment should provide a convenient probe of the processes of phage attachment and entry into the host cell and for studies of A-protein interaction with purified pili in vitro

By comparison of the N-terminal amino acid sequence of the A-protein [3] with its gene sequence [1,10], it appears that the protein is initially synthesized with a 'leader' peptide similar to that found with the phage coat protein [18]. The coat protein is known to enter and span the bacterial inner membrane, with its N-terminus on the outside of the cell, before assembly into the phage filament [19]. Thus it is likely that the A-protein also enters the membrane and becomes oriented with its N-terminal domain on the outside, anchored in the lipid bilayer by the C-terminal region. In addition to its role in adsorbing the infecting virus to the cell [6–8], the A-protein has been found to play a part in the late steps of viral morphogenesis [20], perhaps in helping to terminate the assembly correctly when the viral DNA has been packaged [21]. The end of the filament carrying the A-protein, which would logically be the first part of the virus to enter the bacterial cell, is therefore likely to be the last part of the newly synthesized virus particle to emerge from the infected cell. A model that accommodates all these results is shown schematically in fig.3.

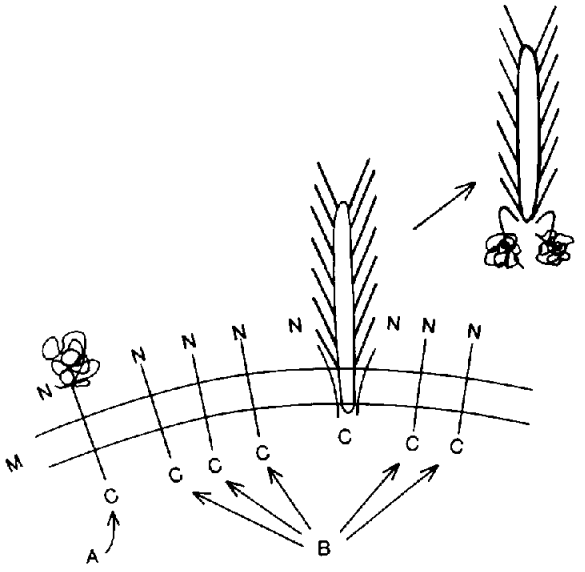

Fig.3. A schematic model for completion of assembly of bacteriophage fd. It is proposed that the A-protein is added to the last end of the phage particle to emerge through the bacterial cell membranes. Possible participation of another minor coat protein at this end of the filament [5] is not shown. The drawing is not to scale: (A) A-protein; (B) major coat protein; (M) *E. coli* inner cell membrane; (N) N-terminus; (C) C-terminus.

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