MAJOR OUTER MEMBRANE PROTEIN *d* OF *ESCHERICHIA COLI* K12

Purification and in vitro activity of bacteriophage k3 and f-pilus mediated conjugation

Loek VAN ALPHEN, Louis HAVEKES and Ben LUGTENBERG

Department of Molecular Cell Biology, Section Microbiology, and Institute for Molecular Biology, State University, Transitorium 3, Padualaan 8, Utrecht, The Netherlands

Received 4 January 1977

1. Introduction

The outer membrane of Gram-negative bacteria consists of LPS (lipopolysaccharide), phospholipids and proteins [1]. One of the major outer membrane proteins is heat-modifiable [2] in that its apparent molecular weight on SDS (sodium dodecyl sulphate)–polyacrylamide gels is dependent on the temperature used during preparation of the sample. The heat-modifiable protein is referred to as protein 3a [3], II* [4], B [2] or *d* [5]. Mutants which lack protein *d* can be isolated by selection for bacteriophage K3 resistance. They are poor recipients in F-pilus mediated conjugation [6–8]. This suggests that protein *d* is the receptor for bacteriophage K3 and is directly involved in the F-pilus mediated conjugation.

In this paper we describe the isolation of protein *d* of *Escherichia coli* K12 and its in vitro activity in inactivating bacteriophage K3 and inhibiting the F-pilus mediated conjugation.

2. Experimental

2.1. *Strains and growth conditions*

The following *E. coli* K12 strains were used. Late exponential phase cells of strain CE 1036 [9,10] grown in brain/heart medium were used for the isolation of protein *d*. Under these conditions outer membrane protein *b* is hardly, and *c* is not at all, present in this strain, whereas protein *d* is present in increased amounts (fig.1). Stationary-phase cells of strain PC 0205 [9], grown in yeast broth, were used for the isolation of LPS. Strains AB 1157 (*lacY, strA*) [11] and CSH 36 (F'-*proA, proB, lac*$^+$) [12] were used as recipient and donor strain respectively in F-pilus mediated conjugation. *E. coli* B was used for phospholipid isolation, as an indicator strain for phage titrations and for multiplication of phage K3. The composition of the media has been described elsewhere [9].

Bacteriophage K3 was purified on a linear 30–60% (w/v) sucrose-gradient containing 10 mM potassium phosphate buffer, pH 7.8, by centrifugation for 2 h in a Beckman SW 27^I rotor at 23 500 rev./min.

2.2. *Isolation of phospholipids, LPS and protein* d

Phospholipids were extracted from whole cells according to Bligh and Dyer [13].

LPS was isolated according to Galanos et al. [14] and possible traces of phospholipids were removed [13].

Protein *d* was purified as follows:

2.2.1. Step I

Cells were harvested and washed with 0.9% NaCl. Cell envelopes (32 g wet wt) were isolated as described before [5] from 170 g cell-paste.

2.2.2. Step II

The cell-envelopes were resuspended in 120 ml 2 mM Tris–HCl, pH 7.8/10 mM $MgCl_2$/2% Triton X-100 and incubated for 10 min at room temperature. After centrifugation for 60 min at 225 000 × *g* the pellet was washed with 2 mM Tris–HCl, pH 7.8.

2.2.3. Step III

The pellet was resuspended in 100 ml

10 mM Tris–HCl, pH 7.5, containing 2% SDS, 0.05% β-mercaptoethanol and 5 mM EDTA. The suspension was stirred for 30 min at 30°C and centrifuged for 1 h at 48 000 × *g*. The extraction was repeated once and the supernatants were combined.

2.2.4. Step IV

The protein was precipitated by adding cold acetone to a final concentration of 90%. Precipitated material was centrifuged at 4°C and washed twice with 90% acetone. The precipitate was then lyophilized and extracted twice with 85 ml chloroform/methanol (2:1) and lyophilized again.

2.2.5. Step V

The dry preparation (3 g) was extracted with 300 ml 2 mM Tris–HCl, pH 7.8/2 mM EDTA/2% Triton X-100/8 M urea during 30 min at 60°C. The suspension was centrifuged for 30 min at 20 000 × *g*. The protein was recovered from the supernatant by acetone precipitation as described in Step IV and lyophilized.

2.2.6. Step VI

The lyophilized material (260 mg protein) was dissolved in 10 ml 10 mM Tris–HCl, pH 7.5/1.5% SDS/5 mM EDTA and dialyzed overnight at 30°C against the same buffer. The dialyzed solution was chromatographed into two portions on a 4.3 × 92 cm Bio-Gel P150 column at 30°C [4], using the same Tris–SDS–EDTA buffer as the eluent. Protein fractions were combined and rechromatographed. Protein was recovered from the pooled peak fractions by acetone precipitation, washed five times with 90% acetone and twice with water and lyophilized.

Protein purity was checked by SDS–polyacrylamide gel electrophoresis of samples incubated either for 2 h at 37°C or for 5 min at 100°C. Possible contamination with LPS was measured by determination of 3-deoxy-D-manno-octulosonic acid as described before [10]. For amino acid analysis aliquots of the purified protein were hydrolyzed with 6 N HCl in vacuo at 110°C for 24 h and 72 h. Analyses were carried out on a Technicon TSM and a Beckman Unichrom amino acid analyser.

2.3. *Reconstitution of biologically active protein* **d**

The procedure basically consists of the one described by Nakae [15] for the incorporation of membrane proteins into LPS–phospholipid vesicles. Phospholipid (1 μmol in 20 μl chloroform) was evaporated in a 7 ml conical centrifuge tube under a vigorous stream of nitrogen. LPS (0.15 μmol) and protein *d* (100 μg), suspended in distilled water by sonication at 45°C, were added. After brief sonication (30 s) the mixture was evaporated at 45°C as described and resuspended by sonication (30 s) in 200 μl 100 mM NaCl/10 mM $MgCl_2$/1 mM Hepes buffer, pH 7.4. After incubation for 30 min at 45°C the suspension was cooled slowly [15]. In controls one or two of the constituents were omitted.

2.4. *Inactivation of phage K3*

Mixtures of protein *d*, phospholipid and LPS were diluted 20- and 50-fold in yeast broth supplemented with KCN and chloramphenicol in final concentrations of 2.5 mM and 100 μg/ml respectively. After preincubation for 10 min at 25°C 2×10^7 plaque-forming-units of phage K3 were added/ml. Samples were taken after various intervals, diluted immediately 10 000-fold in cold medium and tested for plaque-forming-units. The inactivation constant K_i was calculated using the formula $\log P_t/P_0 = K_i.N.t$, in which P_0 and P_t represent the number of plaque-forming-units at times 0 and t respectively and N represents the concentration of protein *d* (μg/ml). The curve obtained resulted in a straight-line over the incubation period (30 min).

2.5. *Inhibition of F-pilus mediated conjugation*

An 100 μl mixture of protein *d*, LPS and phospholipids, prepared as described in section 2.3, was added to 20 μl of a 100-fold diluted culture of logarithmically growing donor cells (corresponding to $4–6 \times 10^4$ cells). After preincubation for 25 min at 37°C 100 μl of a 100-fold diluted culture of logarithmically growing recipient cells was added. After incubation for 30 min at 37°C, mating aggregates were disrupted by violent agitation. The mixture was diluted appropriately and spread on selective minimal medium agar plates, containing lactose as the carbon source and streptomycine to prevent growth of donor cells. The plates were incubated for 24 h at 37°C and lac^+ colonies were counted.

3. Results and discussion

3.1. *Purification and characterization of protein* d

The procedure for the purification of protein *d* from *E. coli* B described by Garten et al. [4] resulted in a large loss of protein *d* when *E. coli* K12 was used as the protein source. Therefore the method was modified as follows. The SDS–Mg^{2+}-extraction (step 2 of ref. [4]) was omitted and replaced by Step II (section 2.2.2) in order to remove cytoplasmic membrane proteins, phospholipids and part of the LPS from the cell-envelopes [16]. The Triton–EDTA extraction (step 4b in ref [4]) was replaced by a Triton–EDTA–urea extraction (Step IV) resulting in loss of merely non-proteineous material. Gel patterns of the materials obtained after various purification steps are shown in fig.1 (A–E). The purified protein was still susceptable to a heat induced increase in its apparent molecular weight on SDS–polyacrylamide gel electrophoresis (fig.1F, 1G). The elution profile of the Bio-Gel P150 column is shown in fig.2. Starting from 170 g cell-paste, 120 mg pure protein was obtained, which corresponds with a yield of 30% of protein *d*. Another 20 mg was obtained from the side fractions of the column peak with a purity of more than 90%. The LPS content of the purified protein was less than 1% (w/w). The amino acid composition is shown in table 1. Comparison with the data of the same protein of *E. coli* strains B [4] and NRC 482 [2] showed that the amino acid composition is similar except for small differences in the amounts of methionine and tryptophan.

3.2. *Inactivation of phage K3*

The activity of purified protein *d* in inactivating bacteriophage K3 is summarized in table 2. The purified protein, either suspended in buffer by sonication as described in section 2.3. or solubilized in buffer containing Triton X-100, has little effect on the phage. Treatment of the protein according to a

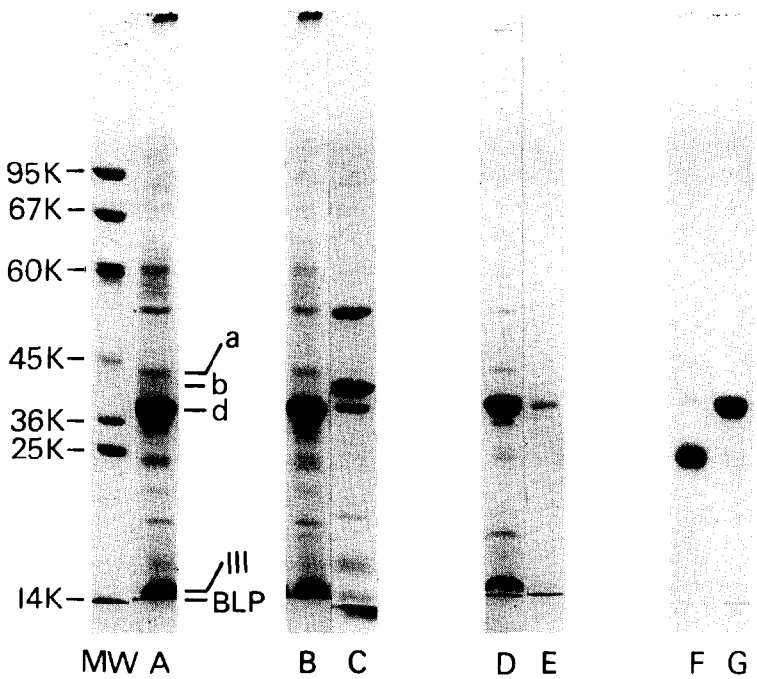

Fig.1. Protein composition of the products of the various purification steps. (A) Cell envelopes (Step I). (B and C) Supernatant and pellet respectively of the SDS–EDTA extraction (Step III). (D and E) Supernatant and pellet respectively of the Triton–EDTA–urea extraction (Step V). (F) Purified protein *d*. (G) Purified protein *d* in its heat-modified form (d(mod)). *Abbreviations:* MW molecular weight standard mixture, BLP Braun's lipoprotein. See references 3 and 5 for nomenclature of the proteins. All samples were boiled for 5 min except in the case of F.

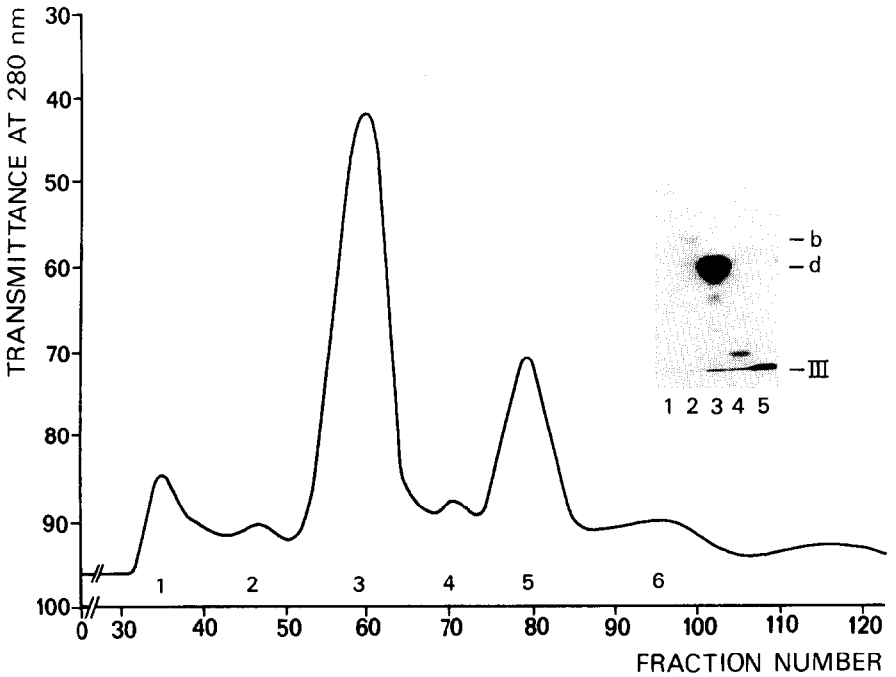

Fig.2. Elution profile of the first chromatography step on Bio-Gel P150. Electrophoretic profiles of boiled samples of the different peak fractions are shown in the insert. The symbols are the same as those of fig.1. Fractions contained 10 ml.

Table 1
Amino acid composition of protein *d*

Amino acid	Residues (mol/100 mol)[a]
Asp	12.6
Thr	6.3
Ser	4.3
Glu	8.9
Pro	5.9
Gly	10.7
Ala	9.5
Met	1.2
Ile	4.2
Leu	7.1
Tyr	5.1
Phe	3.0
Val	7.6
Lys	5.6
His	1.8
Arg	4.4
Trp	1.5
'Cys	0.4

[a] Corrected for loss due to hydrolysis

method, which presumably causes incorporation of the protein into LPS–phospholipid vesicles [15], resulted in a mixture that strongly inactivated the phage. Omission of phospholipids from the mixture resulted in an equally active preparation although supplementation of *d* with phospholipids alone increased its activity significantly. LPS or phospholipids alone were not active. Purified outer membrane protein *c* (manuscript on purification in preparation) was unable to replace protein *d*. Pretreatment of the *d*–LPS mixture at 100°C (without SDS) strongly decreased the inhibitor activity. The inactivation of bacteriophage K3 by the *d*–LPS complex resembles the adsorption of K3 to intact cells in that it is irreversible.

The results of table 2 strongly suggest that LPS is specifically required to restore the biological activity of the protein. An interaction between *d* and LPS in vivo therefore is very likely.

3.3. *Inhibition of F-pilus mediated conjugation*

The influence of mixtures of protein *d*, LPS and

Table 2
Inactivation of bacteriophage K3[a]

Inactivator[b]	Rate constant K_i (ml/min/μg)
None	$< 0.02 \times 10^{-4}$
d	0.33×10^{-4}
d (in Triton)	0.18×10^{-4}
d + LPS + phospholipids	50×10^{-4}
d + LPS	50×10^{-4}
d + Phospholipids	0.57×10^{-4}
LPS[c]	$< 0.02 \times 10^{-4}$
Phospholipids[c]	$< 0.02 \times 10^{-4}$
c + LPS + phospholipids[c]	$< 0.02 \times 10^{-4}$
d + LPS (after 5 min 100°C)	3.2×10^{-4}

[a] Experiments were performed as described in section 2.4
[b] The inactivating mixture was prepared as described in section 2.3, except when *d* was solubilized in Triton X-100. In that case a solution in 1% Triton was subsequently diluted 20-fold
[c] An apparent K_i was calculated using a value for N as if protein *d* was present

phospholipids on F-pilus mediated conjugation is shown in table 3. Protein *d*–LPS complexes exerted a very strong inhibition whereas the additional presence of phospholipids had no influence. Protein *d* alone had no effect whereas LPS alone had a strong effect. This effect has been found before and it was then concluded to be aspecific [11]. Decreasing the LPS concentration in protein *d*–LPS mixtures resulted in inactive samples (not shown). Mixtures of protein *d* and phospholipid alone were hardly inhibitory (not shown). When the mixture of donor cells and LPS–protein *d* complexes was diluted 10-fold prior to the addition of recipient cells, the inhibition of exconjugant formation was decreased about 6-fold, indicating that the inhibition is reversible.

Table 3
Inhibition of F-pilus mediated conjugation[a]

Inhibitor	Exconjugants (%)
None	100
d	95–105
d + LPS + Phospholipids	1–2
d + LPS	1–2
LPS	10–15

[a] Conjugation was performed as described in section 2.5. The inhibitor was prepared as described in section 2.3

The present results show that isolated protein *d*, complexed with LPS, can function as an inhibitor of F-pilus mediated conjugation. However the results do not discriminate between two possible in vivo properties of the protein *d*–LPS complex, namely (i) the primary receptor for F-pili or (ii) a structure on the recipient's cell-surface which is required for stabilization of mating aggregates. The latter step occurs after the primary contact formation and before DNA-transfer (M. Achtman, personal communication). Comparison of the inhibition of F-pilus mediated conjugation and of inactivation of bacteriophage K3 by protein *d*–LPS complexes demonstrates that the inhibition of F-pilus mediated conjugation is relatively inefficient, which is probably caused by the reversibility of the inhibition. An explanation for this reversibility is that it is an intrinsic property of protein *d*–LPS complexes. Irreversible binding of donor and recipient cells may be a multi-step process which also requires other components of the recipient cell. Alternatively the reversibility may be caused by an imperfect reconstitution of the inhibitor of the

F-pilus mediated conjugation although the complex is an almost perfect inactivator for bacteriophage K3. Further experiments are necessary for the elucidation of the exact role of protein *d*–LPS complexes in F-pilus mediated conjugation.

Acknowledgements

The excellent technical assistance of Marieke Paulssen was gratefully acknowledged. We thank Dr H. M. Verheij and W. C. Puijk for performing the amino acid analyses and Nelke van Selm for the isolation of LPS.

References

[1] Osborn, M. J., Gander, J. E., Parisi, E. and Carson, J. (1972) J. Biol. Chem. 247, 3962–3972.
[2] Reithmeier, R. A. F. and Bragg, P. D. (1974) FEBS Lett. 41, 195–199.
[3] Schnaitman, C. A. (1974) J. Bacteriol. 118, 442–453.
[4] Garten, W., Hindennach, I. and Henning, U. (1975) Eur. J. Biochem. 59, 215–221.
[5] Lugtenberg, B., Meijers, J., Peters, R., Van der Hoek, P. and Van Alphen, L. (1975) FEBS Lett. 58, 254–259.
[6] Skurray, R. A., Hancock, R. E. W. and Reeves, P. (1974) J. Bacteriol. 119, 726–735.
[7] Havekes, L. M., Lugtenberg, B. J. J. and Hoekstra, W. P. M. (1976) Molec. Gen. Genet. 146, 43–50.
[8] Manning, P. A. and Reeves, P. (1976) J. Bacteriol. 127, 1070–1079.
[9] Lugtenberg, B., Peters, R., Bernheimer, H. and Berendsen, W. (1976) Molec. Gen. Genet. 147, 251–262.
[10] Van Alphen, W., Lugtenberg, B. and Berendsen, W. (1976) Molec. Gen. Genet. 147, 263–269.
[11] Havekes, L., Tommassen, J., Hoekstra, W. and Lugtenberg, B. (1977) J. Bacteriol. in press.
[12] Miller, J. H. (1972) in: Experiments in molecular genetics, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.
[13] Bligh, E. G. and Dyer, W. J. (1959) Can. J. Biochem. Physiol. 37, 911–917.
[14] Galanos, C., Lüderitz, O. and Westphal, O. (1969) Eur. J. Biochem. 9, 245–249.
[15] Nakae, T. (1975) Biochem. Biophys. Res. Commun. 64, 1224–1231.
[16] Schnaitman, C. A. (1971) J. Bacteriol. 108, 545–552.