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Inactivation in vitro of the *Escherichia coli* outer membrane protein FhuA by a phage T5-encoded lipoprotein

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Abstract

Bacteriophage T5-encoded lipoprotein, synthesized by infected *Escherichia coli* cells, prevents superinfection of the host cell by this virus. The molecular basis of its ability to inactivate the receptor of phage T5, the FhuA protein, was investigated in vitro. Fully competent T5 lipoprotein, with a His tag attached to the C-terminus, was purified in detergent solution. Co-reconstitution with homogeneous FhuA protein into liposomes revealed that the lipoprotein inhibited the irreversible inactivation of phage T5 by FhuA protein. This phenomenon correlated with the inhibition of phage DNA ejection determined by fluorescence monitoring. Addition of detergent abolished the interaction between T5 lipoprotein and FhuA protein. When the signal sequence and N-terminal cysteinyl residue of the lipoprotein were removed by genetic truncation, the soluble polypeptide could be refolded and purified from inclusion bodies. The truncated lipoprotein interfered with infection of *E. coli* by phage T5, but only at very high concentrations. Circular dichroism spectra of both forms of T5 lipoprotein exhibited predominantly β-structure. T5 lipoprotein is sufficient for inactivation of the FhuA protein, presumably by inserting the N-terminal acyl chains into the membrane, thus increasing its local concentration. An in vitro stoichiometry of 10:1 has been calculated for the phage-encoded T5 lipoprotein to FhuA protein complex.

Keywords: FhuA protein; Phage T5; Lytic conversion; Lipoprotein; Liposome

1. Introduction

In the early stage of infection of *Escherichia coli* by bacteriophage T5, a phage-encoded lipoprotein is expressed which not only prevents superinfection but also protects progeny phages from being inactivated by the receptor present in envelope fragments of lysed host cells [1,2]. The T5 lipoprotein has been proposed to exert its function by binding to the phage T5 receptor, the FhuA protein, on the surface of *E. coli* [3]. The interaction with, and the inhibition of, the FhuA protein has been dubbed lytic conversion [2]. The FhuA protein [4–6], which is multifunctional, requires the energy-transducing TonB system for all functions except the binding of phage T5 [7–12]. This interaction causes the TonB-independent opening of a channel through the FhuA protein [13]

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Abbreviations: SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; HEPES, 4-(2-hydroxyethyl)-piperazine-1-ethane-sulfonic acid; IPTG, isopropyl-β-D-thiogalactopyranoside; CD, circular dichroism

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and triggers T5 DNA ejection [14], invariably resulting in the irreversible inactivation of the virus. This has allowed us to establish an in vitro assay for the FhuA-mediated translocation of both ferrichrome and phage DNA across *E. coli* outer membranes [15,16]. Our results provided the basis for the study of the mechanism of action by which phage T5-encoded lipoprotein inhibits the FhuA protein.

We have now overexpressed the T5 lipoprotein in *E. coli* and purified it to homogeneity in detergent solution. We have also constructed a truncated lipoprotein lacking the N-terminal cysteine and consequently the covalently attached fatty acyl moiety. The polypeptide formed inclusion bodies and could be purified in aqueous solution. The in vitro assay system presented allows the function of the lipoprotein to be examined and lytic conversion to be understood at a molecular level.

2. Materials and methods

2.1. Expression and purification of acylated, C-terminally His-tagged lipoprotein

Phage T5 was prepared as described [13]. The DNA encoding T5 lipoprotein (*llp* gene) was amplified by PCR (Pwo DNA Polymerase, Boehringer Mannheim) using T5 DNA as template and the following primers: llpXbaIF, 5′-GTATCTAGAATTAAATAGGGGAGAATGTAA-3′; llpXhoIR, 5′-TTTCTCGAGGAAAACTCCCTCGCATGTATT-3′. The fragment obtained was cloned into expression vector pET32a(+) (Novagen) by means of *Xba*I and *Xho*I sites. This resulted in plasmid pIP-1, with the *llp* sequence under the control of a T7 promoter [17] and fused to a His_6 coding sequence at the C-terminus of the protein. Sequences were confirmed using the T7 sequencing kit (Pharmacia Biotech). For expression, *E. coli* strain B834(DE3) [18] was transformed with pIP-1. Cells were grown at 30°C in 2YT medium, containing 100 μg ml^{-1} ampicillin, until late stationary phase. For purification, 30 g of cells were broken in the French pressure cell, and membranes were prepared as described [19]. T5 lipoprotein was extracted from the membranes using 60 ml of 20 mM Tris, 50 mM imidazole, 0.1 M NaCl, 3% octyl-polyoxyethylene (octyl-POE, Alexis, Läuflingen, Switzerland), final pH 8.0, and stirring at 4°C overnight. The protein was applied to an affinity column (3 ml chelating Sepharose, Pharmacia Biotech, saturated with Ni^{2+}) preequilibrated with buffer A (20 mM Tris, 50 mM imidazole, 0.1 M NaCl, 1% octyl-POE, final pH 8.0). After washing, the lipoprotein was eluted by using the same buffer but containing 0.25 M imidazole. The protein was precipitated at −20°C with 10 vol. of ethanol (−70°C) for 20 min, collected by centrifugation and resolubilized at 4°C in buffer A overnight. Affinity chromatography was repeated once. The protein was dialyzed overnight against buffer A without imidazole at 4°C. The final protein concentrations were estimated [20] and gave a yield of 1.8 mg.

2.2. Expression in E. coli, refolding from inclusion bodies, and purification of lipoprotein truncated at the N-terminus

A lipoprotein mutant lacking the N-terminal cysteine was constructed as described above but using as primers llpNdeIF, 5′-TCGCATATGTCTACTTTTGGACCTAAAGAT-3′ and llpBamHIR, 5′-GATGGATCCTAAGGTGGTTTTTACTTAGAA-3′ with pET32a(+) as expression vector. The protein was overexpressed as above but at 37°C and 0.1 mM IPTG was added when the cultures reached an OD_{600} of 0.6. Inclusion bodies were isolated as described [21] and dissolved in buffer B (20 mM Tris, 0.1 M NaCl, pH 7.6) containing 6 M urea. Refolding was performed by slow (18 h) dilution at room temperature in buffer B (final urea concentration of 0.6 M), followed by 10-fold concentration and subsequent dialysis overnight against buffer B for complete removal of urea. After removing aggregates by centrifugation, gel filtration chromatography using a Sephadex G-50 column in buffer B followed. The protein was soluble at concentrations $\leq$1 mg ml^{-1}.

2.3. Physical characterizations of lipoprotein

SDS-PAGE was performed at 4°C as described [22]. Gels were stained with Coomassie brilliant blue. Matrix-assisted laser desorption/ionization mass spectroscopy (Maldi-MS) was performed as described [23]. CD spectra of the protein were recorded on a Jasco J-720 spectropolarimeter in a range from

250 to 195 nm, with dynode voltages < 0.6 kV. Prior to the measurement, lipoprotein was treated with 0.1 M EDTA for 3 h at room temperature, and EDTA was subsequently removed by gel filtration (Sephadex G50) in 20 mM NaP_i, 1% octyl-POE, pH 7.6.

2.4. Reconstitution of FhuA and lipoprotein

Homogeneous FhuA protein [19] and T5 lipoprotein were co-reconstituted ^{14}C-labeled liposomes as described [15]. Before use, labeled proteoliposomes were washed by gel filtration chromatography (9 ml Sephacryl S-200 HR, Pharmacia Biotech) in 'Hepes buffer' (20 mM HEPES, 0. 15 M NaCl, pH 7.2), and the fractions counted for ^{14}C in a Packard scintillation counter.

2.5. Phage inactivation assay

The procedure was adapted from Braun et al. [3]. FhuA protein (0.4 μg) was mixed with lipoprotein in 'Hepes buffer' containing 1 mM of both $MgSO_4$ and $CaCl_2$ to a final volume of 400 μl. Different ratios were used, and mixtures were either in detergent solution with octyl-POE at different concentrations, or reconstituted. Phage T5 (10 μl/5×10^4 pfu ml^{-1}) was added, and the mixture incubated at 37°C for 15 min. Ice cold 0.15 M NaCl (200 μl) was added, and 300 μl of the solution combined with 100 μl of freshly grown *E. coli* B^E (tester strain) to $OD_{600} \approx 0.5$ in 2YT medium, mixed with 3 ml of molten soft agar containing 1 mM both of $MgSO_4$ and $CaCl_2$ and plated onto nutrient agar plates. Plates were incubated at 37°C for 12 h and single plaques counted.

2.6. Phage T5 DNA ejection

Ejection of phage T5 DNA into the medium, and injection into proteoliposomes, were measured using YO-PRO 1 (Molecular Probes) in 1.5 ml 'Hepes buffer' (above) as described [14–16]. Measurements were performed at 37°C in a SLM 8000C fluorimeter (SLM-Aminco, Urbana, IL), using excitation and emission wavelengths of 491 nm and 509 nm, respectively. Data points were integrated over 0.9 s. At $t = 30$ s, co-reconstituted FhuA (7.5 pmol) and lipoprotein (in variable amounts) were added to the cuvette. At $t = 70$ s, 5×10^6 phage T5 particles were added. Where indicated, octyl-POE was added to a final concentration of 1% to solubilize liposomes, and 20 U DNase I was added to degrade DNA.

3. Results

T5 lipoprotein, expressed in *E. coli* strain B834(DE3) with a His tag at its C-terminus, rendered the cells as resistant to phage T5 as does the native protein, demonstrating that the construct produced active lipoprotein. When cells were grown at 30°C without induction, a new band appeared on SDS-PAGE, with a mobility corresponding to a mass of 9 kDa (Fig. 1). Attempts to prepare large quantities of the protein by induction with IPTG or by incubating cultures at 37°C resulted in cell lysis, indicating a toxic effect of overexpression. Purifica-

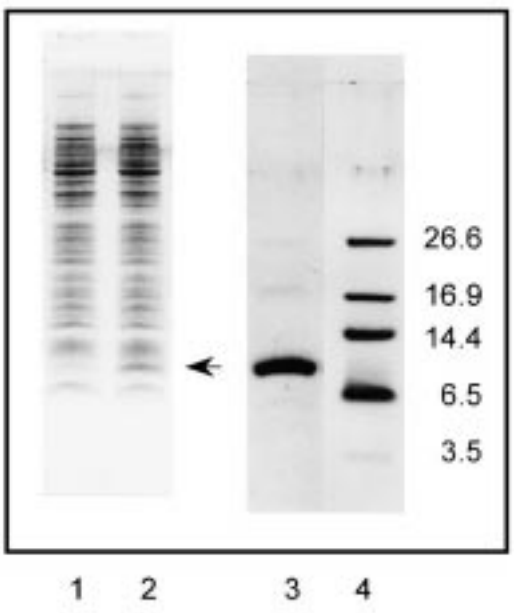

Fig. 1. Purity of overexpressed and isolated T5 lipoprotein with a His tag. Discontinuous SDS-PAGE was performed, and gels were stained with Coomassie brilliant blue. Lanes 1 and 2, total cells of the expression strain *E. coli* B834(DE3), with and without transformation. The band corresponding to T5 lipoprotein is indicated by the arrow. Lane 3, homogeneous lipoprotein, with its acyl chains presumably inserted into the SDS micelles. The gel pattern was similar to that of truncated lipoprotein. Lane 4, marker proteins, with molecular masses indicated in kDa on the right.

Table 1
Inhibition of FhuA protein by native and truncated lipoprotein

	Vesicles	FhuA protein	T5 lipoprotein	Truncated lipoprotein	Detergent	pfu
A	−	–	–	–	–	256 ± 23 (n = 4)
B	−	–	–	–	4	234 ± 16 (n = 2)
C	+	–	–	–	1	239 ± 44 (n = 6)
D	−	5 pmol	–	–	4	0 ± 0 (n = 3)
E	**+**	**5 pmol**	**–**	**–**	**1**	**2 ± 2** (n = 10)
F	+	5 pmol	–	–	4	1 ± 1 (n = 5)
G	−	5 pmol	50 pmol	–	4	0 ± 0 (n = 4)
H	**+**	**5 pmol**	**50 pmol**	**–**	**1**	**245 ± 49** (n = 7)
I	+	5 pmol	50 pmol	–	4	1 ± 1 (n = 2)
K	−	–	–	0.75 nM	–	220 ± 4 (n = 2)
L	−	–	–	7.5 nM	–	165 ± 5 (n = 2)
M	−	–	–	75 nM	–	0 ± 0 (n = 2)
N	−	–	–	75 pM	0.3	109 ± 9 (n = 2)
O	−	–	–	0.75 nM	0.3	30 ± 8 (n = 2)
P	−	–	–	7.5 nM	0.3	2 ± 1 (n = 2)

The results of the phage T5 inactivation study are given with the deviations and the number of experiments (n) performed. Plaque forming units (pfu) of uniform aliquots of bacteriophage T5 were determined after incubation with mixtures of FhuA protein and T5 lipoprotein. Incubations were carried out for 15 min, and aliquots of the mixtures were added to the tester strain (*E. coli* B^E), plated on agar plates, and incubated at 37°C. The proteins were used either in detergent-solubilized form, or reconstituted into vesicles. Detergent concentrations are expressed as multiples of the critical micelle concentration (CMC, for octyl-POE 6.7 mM or 0.24%). At 1×CMC, the effective concentration of detergent monomers is less than the CMC because of detergent binding to both protein and lipids, thus keeping the T5 lipoprotein in solution without solubilizing the liposomes. At concentrations of 4×CMC, vesicles were solubilized completely. Rows D–I: concentrations of proteins refer to the total volume of the incubation mixtures (400 μl). The critical values are given in bold. Rows K–P: truncated lipoprotein was incubated with phage T5 in the absence of purified FhuA protein. Concentrations of protein and detergent refer to the total volume of top agar (3 ml) and reaction mixture added (400 μl).

tion from 30 g of intact cells afforded 1.8 mg pure lipoprotein, which migrated as a single band in gel electrophoresis (Fig. 1). Mass spectroscopy [23] yielded a single peak with a mass of 8988 ± 14 Da, consistent with acylation of the N-terminus by three fatty acids, analogous to the posttranscriptional modification of cellular lipoprotein of *E. coli* [24]. When the signal peptide and the first residue (Cys) of the mature T5 lipoprotein were removed by truncation, a fatty acyl-free, water-soluble polypeptide was expressed and purified from inclusion bodies. CD spectra of both the native and the truncated T5 lipoprotein revealed a high content of β-structure (Fig. 2).

Inactivation of phage T5 by purified FhuA protein (Table 1) was found to be inhibited in vitro by isolated T5 lipoprotein. When proteoliposomes containing both FhuA and lipoprotein were incubated with phage T5, the integrity of the virions remained unaffected. For quantitative inactivation of the FhuA protein, and hence full phage recovery, a molar ratio of lipoprotein to FhuA protein of ≥10:1 is necessary. Addition of detergent (octyl-POE) solubilized the vesicles and restored the activity of FhuA protein, resulting in the inactivation of all virus particles. The truncated, soluble lipoprotein inhibited neither reconstituted nor detergent-solubilized FhuA protein (not shown). When added to tester strain, the soluble polypeptide inhibited infection of the cells by phage T5, with the inactivation of FhuA protein of the tester strain by one order of magnitude higher in the presence of detergent. In order to exclude an unspecific interaction of the polypeptide, we performed control experiments using albumin and horse myoglobin at similar concentrations, with the result that infection of the cells was not inhibited.

The results of the fluorescence studies (Fig. 3) are in excellent agreement with the inactivation studies described. Virus DNA ejection, which occurs upon interaction of phage T5 with FhuA protein, was inhibited in the presence of T5 lipoprotein. A rise in

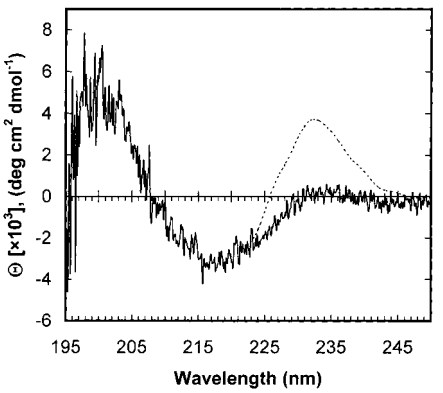

Fig. 2. Circular dichroism spectrum of lipoprotein. CD spectra of isolated T5 lipoprotein were recorded at 20°C in NaP_i buffer containing 1% octyl-POE. The protein concentration was 0.8 mg/ml, the path length of the cuvette 1 mm. When treatment of the protein with 100 mM EDTA was omitted (see Section 2), a peak with a maximum at 235 nm was observed (dotted line), possibly reflecting binding of a divalent cation to the polypeptide [25]. Activity in vitro was not influenced with or without EDTA treatment. The spectrum obtained with the truncated, non-acylated mutant of the T5 lipoprotein in the absence of octyl-POE is indistinguishable from that shown. The spectra obtained are indicative of β-sheet as the predominant secondary structure element.

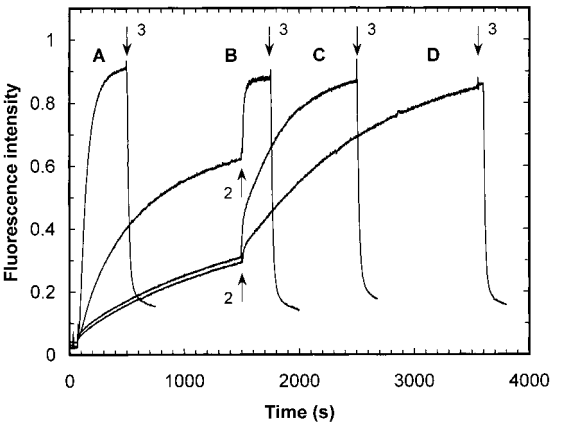

Fig. 3. Recovery of FhuA protein activity following solubilization of liposomes containing T5 lipoprotein and FhuA protein. The activity of FhuA protein (0.6 μg in all cases) with respect to triggering phage T5 DNA ejection was monitored by recording the time course of the fluorescence signal of YO-PRO 1 (1 μM), a dye interacting with double stranded DNA. At $t = 70$ s, phage T5 was added to a suspension of co-reconstituted T5 lipoprotein and FhuA protein (curves C and D). A gradual rise of the fluorescence was attributed to penetration of the dye into the phage capsid [19]. Addition of octyl-POE (1% final concentration, arrow 2) caused an increase of the fluorescence signal, indicative of phage DNA ejected in the medium. The higher the molar ratio of T5 lipoprotein to FhuA protein, the slower the recovery of FhuA protein activity. A ratio of 10:1 is shown in curve C, one of 100:1 in curve D. Free DNA was degraded by DNase I (20 U, arrow 3), causing a sharp decrease of the fluorescence signal. As control experiments, phage T5 was added to detergent-solubilized FhuA protein (curve A) as well as to FhuA protein reconstituted into lipid vesicles (curve B). In curve A, all the DNA is ejected into solution, whereas in curve B, a quenched signal is indicative of a significant amount of viral DNA injected into the liposomes [15]. Liposomes devoid of protein or containing only lipoprotein did not cause any phage DNA ejection.

fluorescence showed that addition of detergent and the resulting solubilization of the proteoliposomes restored the activity of FhuA protein. The kinetics of recovery of activity varied with the molar ratio of T5 lipoprotein to FhuA protein used.

4. Discussion

The irreversible inactivation of bacteriophage T5 by purified FhuA protein is inhibited in vitro by a phage-encoded lipoprotein. The receptor and the viral lipoprotein, co-reconstituted into liposomes, appear to form a complex which is not recognized by the virus. For complete inhibition, a molar excess of 10:1 of T5 lipoprotein over FhuA protein is necessary. An independent confirmation of the stoichiometry was obtained by fluorescence studies which also revealed that the molar ratio of T5 lipoprotein to FhuA protein affects the kinetics of recovery of FhuA protein activity upon detergent-mediated solubilization. The value of 10 mol lipoprotein per mol FhuA protein refers, of course, to the total concentrations supplied and thus depends on whether receptor and inhibitor are distributed evenly among liposomes, as has been observed with FhuA protein [16]. Moreover, it is significant to know whether T5 lipoprotein distributes randomly between inner and outer leaflets of the liposomes, and on what side of the membrane the interaction with FhuA protein occurs.

Aiming to resolve these topological questions, we have constructed and purified a mutant T5 lipoprotein lacking the N-terminally acylated cysteine. The resulting polypeptide is water-soluble and exhibits a

CD spectrum indistinguishable from that obtained with wild-type T5 lipoprotein. When incubated with cells of the *E. coli* tester strain at a ratio of soluble lipoprotein to surface-exposed FhuA protein of 10^6:1, the bacteria became completely resistant to the phage. This result reflects the dilution of the soluble protein in the bulk solvent, and suggests the role of N-terminal acylation of wild-type T5 lipoprotein to be that of increasing the effective local concentration in two dimensions. Indeed, truncation affects lipoprotein function in a way comparable to the addition of detergent to full-size lipoprotein: in either case, its interaction with the FhuA protein is weakened, with the result that the receptor function of the FhuA protein is much less inhibited. With respect to topology, the result obtained suggests that soluble T5 lipoprotein may prevent infection by binding to the extracellular face of the FhuA protein located at the surface of the tester strain. This would represent an alternative mechanism to that proposed previously, which suggested the inhibitory action of T5 lipoprotein to occur by insertion on the periplasmic side [3].

In conclusion, we have demonstrated that the presence of T5 lipoprotein is necessary and sufficient for the inactivation of the FhuA protein in vitro, and that the interactions between the two proteins are specific. Our results suggest that several lipoprotein molecules cluster around the FhuA protein in the lipid bilayer, thereby shielding, directly or indirectly, the receptor protein from recognition by the phage. The critical question to be addressed now is the interaction between receptor and lipoprotein at the molecular level. We are currently investigating the complex formation by studying the interaction of the integral FhuA protein and the phage T5 lipoprotein by structural methods.

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