Reconstitution of FhuA, an *Escherichia coli* Outer Membrane Protein, into Liposomes

BINDING OF PHAGE T5 TO FhuA TRIGGERS THE TRANSFER OF DNA INTO THE PROTEOLIPOSOMES*

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The *Escherichia coli* outer membrane protein FhuA catalyzes the transport of ferrichrome and is the receptor of bacteriophage T5. Purified FhuA was reconstituted into liposomes. The size of the proteoliposomes and the distribution of the proteins in the vesicles were determined by freeze fracture electron microscopy. Unilamellar vesicles with a diameter larger than 200 nm were observed frequently. FhuA was symmetrically oriented in the proteoliposomes. Reconstituted FhuA was functional as binding of phage T5 induced the release of phage DNA and its transfer inside the vesicles.

FhuA is a 78.9-kDa *Escherichia coli* outer membrane protein that catalyzes the high affinity transport of the ferric siderophores ferrichrome and albomycin across this membrane. It is also the receptor for bacteriophage T1, T5, and Ph80 and for colicin M (for review, see Ref. 1). Molecular modeling suggests that the protein contains about 30 transmembrane β-strands connected by loop regions. One of these external loops, extend-

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Functional Reconstitution of FhuA in Liposomes

Reconstitution of FhuA into Liposomes and Morphology of the Proteoliposomes—98% of the liposomes prepared by reverse phase evaporation and observed in FFEM were unilamellar. Their diameter varied between 100 and 200 nm (data not shown). FhuA was reconstituted into the liposomes following the procedures described in Ref. 20 and using OG since the protein was purified and functional in this detergent (6). The lipid:protein mol ratio (3800) was chosen so that individual proteins (particles) could easily be observed by FFEM. It should be kept in mind that because of steric hindrance no more than 20 phages are likely to bind on a proteoliposome having a diameter of 200 nm.

RESULTS

Reconstitution of FhuA into Liposomes and Morphology of the Proteoliposomes—In the first the purified protein solubilized in OG was added to a suspension containing preformed liposomes saturated with detergent coexisting with mixed lipid/detergent micelles. This stage (called stage II) is generally obtained for an OG concentration of 22 mM (i.e. just below the critical micellar concentration). In the second assay, the protein was added to a suspension of mixed detergent/lipid micelles. This stage (stage III) is observed when detergent is added to the liposomes at a concentration well above the critical micellar concentration (i.e. 45 mM). Upon lowering the detergent concentration by adsorption onto Bio-Beads the protein either reincorporates directly into the liposomes saturated in detergent (stage II) or associates with the lipids upon removal of the detergent to form proteoliposomes (stage III). These two procedures generally lead to different orientations of the protein (20). Proteoliposomes were then observed by FFEM. Figs. 1 and 2 represent, respectively, the FFEM and the size and particle distribution histograms of the different preparations. The histograms were obtained over a population of 100–200 vesicles. Calculations were done according to Heegaard et al. (21). Fig. 1A shows the result of a reconstitution performed at stage II. The bulk of the population comprised vesicles with smooth convex and concave fracture surfaces and corresponding to liposomes. Some multilamellar vesicles were also observed. Only a few liposomes contained proteins, which were visualized as globular intramembranous particles. The particles were distributed equally between the concave and convex fracture surfaces. Large and numerous aggregates of particles were observed; these probably correspond to protein aggregates. The histograms Fig. 2, A and B, illustrate these observations: 70% of the vesicles had a mean diameter of 50–100 nm and contained no more than 10–20 particles/proteoliposome. Stage III reconstitution led to two distinct populations of vesicles (Fig. 1B); small vesicles without particles and larger ones containing particles that were distributed equally between the convex and concave fracture surface. Neither protein aggregates nor multilamellar vesicles were observed. 50% of the proteoliposomes had an average diameter of 100–200 nm and contained 40 particles. The diameter of 40% of the proteoliposomes was larger than 200 nm, and these vesicles contained approximately 150 particles (Fig. 2, A and B).
Some of the vesicles had even a diameter varying between 500 and 800 nm (not shown on Fig. 1).

An aliquot of stage III preparation was treated with phage T5 so that the final phage:protein ratio was 1:100 (about one phage/proteoliposome). The suspension was incubated for 30 min at 30 °C and then treated with DNase (20 μg/ml) and MgSO4 (2.5 mM) to degrade ejected DNA. Fig. 1C shows that the vesicle morphology and size were not modified by the presence of phage. Phage structures were occasionally observed: they appeared as clusters of particles, the morphology of which was different from that of the protein aggregates shown in Fig. 1A. Their size corresponds to that expected for the phage capsid (90 nm in diameter).

Proteoliposomes reconstituted at stage III were subjected to centrifugation on a flotation sucrose gradient essentially as described in Ref. 17. Fractions were collected, and the proteins and lipids were estimated from the tryptophan fluorescence (FhuA contains 9 Trp) (4) and radioactivity, respectively. No fluorescence was recovered at the bottom of the gradient, indicating that all of the protein had been incorporated into the liposomes. Protein and lipids were found in a unique peak centered at 18% sucrose (w/v). Radioactivity was also found, albeit at a low level, throughout the gradient (data not shown). These results are in line with the ultrastructural data. Further functional analysis was limited to the proteoliposomes prepared at stage III.

**Functionality and Orientation of FhuA in the Proteoliposomes**—Previous experiments have shown that the addition of purified FhuA solubilized in OG to phage T5 resulted in the release of DNA from the phage capsid. Release of the DNA was measured from the increase of the fluorescence of YO-PRO-1, a dye that intercalates between the free DNA base pairs. The intensity of fluorescence of the dye is directly proportional to the number of DNA base pairs, and, provided that the fluorescence had been calibrated with DNA solutions of known number of base pairs, it is possible to design a quantitative assay of protein functionality (6).

To improve the effect of successive treatment of FhuA with detergent and Bio-Beads, the fluorescence assay was applied first to proteoliposomes solubilized by the addition of 25 mM OG (Fig. 3). The successive addition of proteoliposomes, phage T5, and OG to the cuvette containing YO-PRO-1 resulted in an increase of the fluorescence which started after a lag of about 30 s corresponding to the solubilization of the proteoliposomes and reached a plateau 5 min later. Quantification of the DNA ejected indicated that 90 ± 8% of the DNA was released at the plateau. Addition of DNase in the presence of Mg2+ to hydrolyze the released DNA caused an extinction of the fluorescence signal which returned to its initial level in the absence of DNA. The same fluorescence changes were observed with the FhuA preparation not submitted to the reconstitution procedure. This indicates that the reconstitution protocol has had no deleterious effects on the protein.

We showed previously that the rate of fluorescence increase does not correspond to the rate of DNA ejection, which occurs in few seconds, but to the rate of binding of the phage to FhuA: the higher the FhuA to phage ratio was, the shorter the time it took for the phage to bind. Typically, increasing the concentration of FhuA from 0.015 to 100 nM reduced the half-time of fluorescence increase from 30 min to 30 s (6). Therefore, measuring the initial rate of fluorescence increase should allow the number of active proteins to be determined. We took advantage of this observation to determine the orientation of FhuA in the proteoliposomes. The initial rate of fluorescence increase was first measured for variable concentrations of FhuA solubilized in OG ranging from 0.7 to 2.5 μg/ml. As expected, this rate increased linearly with protein concentration (Fig. 4). This rate was then determined for the same concentration of protein but reconstituted in the liposomes. The linear dependence was still observed, but the slope was half that measured for the solubilized protein. Solubilization of the proteoliposomes by the addition of OG before adding the phage resulted in the same linear dependence of the rate as that determined for the solubilized protein. These results confirm that all of the protein molecules have been inserted in the vesicles but that only about half of them are accessible to the phage. The protein is therefore symmetrically oriented in the vesicles.

**Binding of Phage T5 to Reconstituted FhuA Triggers the Transfer of DNA into Proteoliposomes**—A prerequisite to the determination of entrapped DNA with the fluorescent probe YO-PRO-1 is that the dye should be present both inside and outside the vesicles. Indeed, the channel that is unmasked in FhuA upon binding of T5 allows the diffusion of ferrichrome (7, 22). Since the molecular mass of YO-PRO-1 (629 Da) is close to that of ferrichrome (800 Da), it is likely that the dye diffuses through FhuA upon binding of the phage. Therefore proteoliposomes were prepared in the presence of 4 μM YO-PRO-1. Phage T5 was first added to a cuvette also containing 4 μM YO-PRO-1 (Fig. 5). The fluorescence increased slowly and reached a plateau of 450 a.u. in about 15 min. This increase was DNase-insensitive and corresponded to diffusion of some...
dye in the phage capsid. Proteoliposomes were then added. The fluorescence increased after a lag of 2–3 min and reached a new steady state corresponding to 800 a.u. 15 min later. Proteoliposomes were then solubilized by OG. Ferrichrome, which competes with phage for binding to solubilized FhuA (6), was first added to prevent phage attachment to any new receptor and especially to those whose binding site was oriented inside the vesicles and which had become accessible upon solubilization. Addition of DNase and Mg$^{2+}$ decreased the fluorescence to 150 a.u. immediately (data not shown). The difference between the plateau value (800 a.u.) and the final value (150 a.u.) therefore represents the total DNA whether released inside or outside the proteoliposomes. When DNase and Mg$^{2+}$ were added to the proteoliposomes, but in the absence of OG, the fluorescence was only partially decreased (450 a.u. at steady state). Since DNase cannot diffuse through the liposomes, this indicated that only part (54%) of the DNA was ejected in the liposomes and that the only proteoliposomes were found. It is likely that this DNA was entrapped in the liposomes prior to solubilization.

DISCUSSION

The data presented in this paper indicate that FhuA can be functionally reconstituted into liposomes. Two reconstitution protocols were chosen because they were likely to result in different orientations of the protein in the liposomes. Reconstitution of FhuA was less efficient when the protein was added to liposomes saturated with detergent coexisting with mixed lipid/detergent micelles (stage II); few proteins were inserted into the liposomes, and large protein aggregates that were not incorporated into the liposomes were found. It is likely that upon removal of the detergent, aggregation of the protein has preceded insertion in the liposomes and that the only proteoliposomes formed originate from mixed detergent/lipid micelles. In contrast, when reconstitution was performed at stage III, i.e. from totally solubilized liposomes, neither protein aggregates nor multilamellar vesicles were found, and large proteoliposomes (mean diameter ranging from 100 to 200 nm, some of them having a diameter of 500–800 nm) were observed which contained fairly large amounts of proteins. The fact that the proteoliposomes were larger than the initial liposome preparation suggests that the protein incorporates into the liposomes during their formation and that the open vesicles tend to fuse and form large vesicles. The difference in size of the vesicles formed upon reconstitution at stage II or III is probably due to the rate of removal of detergent, which is in turn dependent on the initial detergent/lipid ratio and on detergent concentration (23).

Functionality of reconstituted FhuA was assessed from a fluorescence assay of the DNA released upon binding of phage T5. Interaction of T5 with proteoliposomes prepared at stage II resulted in some release of DNA (data not shown). However, the preparation also contained protein aggregates, making it difficult to discriminate between phage bound to the aggregates or to proteoliposomes. For the different reasons given above proteoliposomes prepared at stage III were more suitable for functional assays. The protein was shown to be symmetrically oriented in these liposomes. DNA released from the phage was found both outside and inside the proteoliposomes. The DNA present outside is likely to originate from aberrant phage. These phages eject their DNA outside the bacterial envelope and represent 10–30% of the T5 stocks (24). DNA might also be mistracted as a consequence of inappropriate interactions between the phage and the receptor. This could occur in vesicles having a small curvature radius. We are rather confident that our measurements have allowed us to determine entrapped DNA and that released upon solubilization of the liposomes since binding to any newly accessible receptor was prevented by the addition of ferrichrome. Such a precaution was not taken by Tosi et al. (11), therefore rendering their data on T5 DNA transfer in liposomes difficult to interpret. Similarly, Roessner et al. (12) measured injection of phage λ DNA into small unilamellar vesicles containing the LamB receptor after disruption of the liposomes with chloroform or phospholipase followed by DNase treatment. We cannot exclude that this treatment has unmasked inside-oriented receptors to which phage λ would have bound. Our experiments strongly suggest that no bacterial component other than FhuA is required for DNA transport. They however indicate that only 10–20% of the total phage DNA is transferred into the liposomes. The simplest explanation is that transfer takes place only in the largest (500–800 nm in diameter) liposomes, which represent only a small part of the population. The state of compaction of T5 DNA in the liposomes is not known. We cannot exclude that polyamines present in the phage capsid would be transferred along with the DNA into the liposomes. Phage λ DNA (49 kilobase pairs) compacted with polyamines appears as a sphere of less than 100 nm in diameter in electron microscopy (25). If T5 DNA is also compacted by polyamines then it could fit into the proteoliposomes. The route used by DNA to cross the liposomes also remains unknown. DNA transfer is accompanied neither by significant perturbations of the vesicles morphology nor by changes in permeability to solutes of high molecular mass (i.e. cytochrome c) (26). The simplest model therefore still supposes that the DNA diffuses through the channel opened by T5 in FhuA.

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