Modeling Ligand-gated Receptor Activity

FhuA-MEDIATED FERRICHROME EFFLUX FROM LIPID VESICLES TRIGGERED BY PHAGE T5*

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An in vitro assay of iron-ferrichrome translocation across the FhuA protein of outer membranes from Escherichia coli has been devised. Upon reconstitution into large lipid vesicles, bacteriophage T5 binds to this polyvalent receptor, triggering a conformational change that resulted in channel opening. This facilitates the translocation of an iron(III)-siderophore, without the complexities involved in the in vivo process. Efflux of $^{55}$Fe(III)-ferrichrome across FhuA channels was determined quantitatively by monitoring the release of trapped radioactivity. The assay is rapid, reliable, and specific, because other bacteriophages, such as φ80, fail to trigger channel opening of the FhuA receptor.

Understanding the function of ligand-gated channel proteins on the basis of their conformational changes has attracts much interest in recent years (1). Although the number of sequences of proteins belonging to this family has increased rapidly (2), structures are available for a mere few of them and at limited resolution (3). This is caused on the one hand by the difficulties in overexpressing these proteins, particularly those from eucaryotic sources (4), and on the other by the problems besetting the preparation of three-dimensional crystals of membrane proteins, which of course are prerequisite for the determination of high resolution structures by x-ray analysis. We have therefore chosen the iron(III)-ferrichrome translocating FhuA protein from Escherichia coli outer membranes (5–9) as a model of ligand-gated proteins. Proteins from this source have proven very useful in the determination of structures at atomic resolution (10–13). The molecular genetics of E. coli, moreover, are well suited for the investigation of the structural basis of their functional properties (14–16).

At limited availability of iron in the medium, its transport into the cytoplasm requires a complex cascade of events (17). First, ferri ions are chelated by one of several siderophores (18). Second, the iron-siderophore complex binds to its specific surface-exposed receptor. Binding is a necessary but not sufficient condition to trigger channel opening in a third step. This process requires energy, which is transduced from the plasma membrane to the channel protein by a protein complex consisting of at least three components, the products of the genes tonB, exbB, and exbD (19, 20). The TonB protein appears to play a key role in this energy transduction by an as yet poorly understood allosteric transition, which results in the interaction of the TonB protein with a specific motif, the so-called TonB box, which is found in several channel proteins (21–23). Fourth, the substrate is scavenged by a ligand-specific binding protein in the periplasmic space (24). In a fifth step, the complex is delivered to a specific, active transport in the plasma membrane. In the final, sixth step, the oxidized form of iron is reduced in the cytoplasm to the ferrous state (25, 26). The rate-limiting step of this cascade appears to be the energy requirement of the translocation process across the outer membrane. The function of the TonB complex is needed also for infection of the cell with the several viruses and bacterial toxins that use FhuA as a receptor (phages T1, φ80, and colicin M), with the only exception being bacteriophage T5. This virus has been shown to trigger channel opening and ion flux through the FhuA protein in the absence of energy transduction (27). It thus circumvents this requirement, whereas the other ligands may serve as controls.

We are presently crystalizing the FhuA protein. This requires not only that the protein be available in large quantities but also that its native state can be assayed by a routine procedure throughout the lengthy process of crystallization. We here report an assay in which the FhuA protein and $^{55}$Fe(III)-ferrichrome were co-incorporated into large lipid vesicles: the former into the membrane and the latter trapped in the internal compartment. Interaction of T5 phage with the receptor protein, monitored by a fluorescent dye that interacts with the ejected phage DNA, caused the release of radiolabeled Fe(III)-ferrichrome through the FhuA channel. Using radioactive iron, efflux from vesicles could readily be quantitated.

EXPERIMENTAL PROCEDURES

Protein Purification—FhuA protein was overexpressed in an E. coli BL21(DE3) strain not expressing OmpF, OmpC, PhoE, LamB, and OmpA proteins (gift of Dr. A. Prilipov), using the plasmid pHK763 carrying the fhuA gene (kindly provided by Dr. H. Killmann). The protein was solubilized from outer membranes using octyl-POE1 (Alexis, Laülfingen, Switzerland) as the detergent and purified in a procedure analogous to that used for porins from E. coli (28, 29). After a final size exclusion chromatography step, SDS-polyacrylamide gel electrophoresis of the heat-treated protein revealed a single band corresponding to a polypeptide with a mass of 80 kDa.

Ferrichrome Labeling—Ferrichrome, kindly provided by Dr. J. B. Neillands, was labeled with $^{55}$Fe(II) according to Weaver and Konisky (30). The final solution was 2.03×$^{10}^{-7}$ M, as determined by its absorbance at 426 nm. The specific activity was 55 Ci/mol1.

Bacteriophages—Phage T5 (the gift of Dr. V. Braun) and φ80 (BIOzentrum) were isolated and stored as described (27), using E. coli B8 and DH5α (Biozentrum) for production of bacteriophages T5 and φ80, respectively. Using the same strains, phage titers of T5 and φ80 were determined to be 5×$^{10}^{12}$ pfu/ml and 7×$^{10}^{12}$ pfu/ml, respectively.

Reconstitution and Ferrichrome Entrapping—For the preparation of lipid vesicles, 2 μCi of $^{[14]}$Clipidimipolyphosphatidylycholine (DuPont NEN) was added to 40 mg of lipids (4:1 phosphatidylcholine:phosphatidylglycerol, Avanti Polar Lipids Inc.) in chloroform. Liposomes were obtained by using the reverse phase evaporation technique in HEPES buffer (20 mM HEPES, 0.15 M NaCl, pH 7.2) as described by Szoka and Papahadjopoulos (31). The final lipid concentration was 10.9 mM FhuA.

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1 The abbreviations used are: octyl-POE, octyl-polyoxyethylene; OG, n-octyl-β-D-glucoside; pfu, plaque-forming units.

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Fig. 1. Translocation studies of ferrichrome across FhuA channels. Gel filtration chromatography (Sephacryl S-200 in HEPES buffer) separated free (fractions 20–30) from entrapped $^{55}$Fe-ferrichrome, which eluted with $^{14}$C-labeled vesicles (fractions 9–14). A, the second of two consecutive vesicle purification steps is shown. Fraction 13 was used for subsequent analyses. B, elution pattern following incubation of the purified vesicles with T5 in the presence of DNase I and Mg$^{2+}$ and Ca$^{2+}$ ions (1 mM each). C shows a similar experiment in which phage $\Phi 80$ was used instead of T5. D shows the elution pattern after the addition of octylglucoside to the proteoliposomes (in the absence of phases). Incubations prior to sample applications and elution buffer were as described under “Experimental Procedures.” The left ordinate refers to $^{14}$C-labeled vesicles (open squares), whereas the right ordinate refers to $^{55}$Fe label (closed circles), whereas the right ordinate refers to $^{55}$Fe-labeled vesicles (open squares).

Fig. 2. Phage T5 DNA ejection following interaction with reconstituted FhuA protein. The time course of the fluorescent signal arising from the dye YO-PRO 1 interacting with double-stranded DNA is shown. The addition of phage T5 at $t = 90$ s (arrow from below) to a suspension of proteoliposomes containing reconstituted FhuA protein (curve A) caused an instantaneous rise in fluorescence, reflecting a small amount of naked DNA present in the phage stock. At $t = 1400$ s, the addition of proteoliposomes did not cause any further change in signal intensity (arrow 1). Treatment with DNase I (arrow 2) hydrolysed the fraction of DNA ejected into the solution (~20%). Subsequent solubilization of proteoliposomes by detergent (octyl-POE, arrow 3) resulted in further signal decrease. If vesicles were solubilized before treatment with DNase I (dotted line), the fluorescence intensity rapidly increased and reached a value corresponding to that obtained in a control experiment (curve C), in which DNA was released from the phage T5 upon interaction with detergent-solubilized FhuA protein (no lipids present). A further control experiment showed liposomes that did not contain FhuA protein (curve B). No signal arose upon partitioning of dye molecules into the vesicle bilayer (not shown), because the baseline during the first 100 s of the experiment remains at the same level without further addition.

Results

Proteoliposomes reconstituted with FhuA protein were freed from extraneous $^{55}$Fe-ferrichrome by gel filtration chromatography in two steps, of which the second reveals a negligible peak of free ferrichrome (Fig. 1A). When stored at 4°C, the vesicles remained stable over a period of 24 h. Upon the addition of phage T5 (1 per 30 proteoliposomes), ejection of DNA was revealed by the increase of the fluorescent intensity of the dye YO-PRO 1 (see Fig. 2), which intercalated into double-stranded DNA (36). An instantaneous rise, seen in all curves, could be attributed to the interaction of the dye with free DNA originating from the small fraction of lysed T5 phages present in the added phage stock solution. The subsequent gradual rise (Fig. 2, curve A) was attributed to two components. Phage DNA ejected in the medium interacts with the fluorescent dye; the contribution of phage DNA injected into the vesicle interior is more difficult to quantitate. When the curve reached a plateau, all T5 phages had bound to a receptor protein molecule, as...
supported by the observation that the addition of proteoliposomes after 1400 s had no further effect on the fluorescence signal (Fig. 2, arrow 1). Treatment of the solution with DNase I (Fig. 2, arrow 2) caused a rapid decrease of the fluorescence intensity of curve A by about 30%. This showed that the fraction of phage DNA existing in solution was immediately accessible to enzymatic hydrolysis. The liposome-entrapped DNA could also be degraded but only upon solubilization of the vesicles with a detergent (Fig. 2, arrow 3). This result represented strong evidence that phage DNA did enter the liposomes. Solubilization of the vesicles performed before addition of DNase I (Fig. 2, dotted line) caused a rapid increase in fluorescence intensity. The value reached corresponded to the level seen if detergent-solubilized FhuA protein was present without liposomes (control experiment; Fig. 2, curve C). This result appeared to account for the quenching of the DNA-dye complex in the lipid bilayer and its release upon the addition of a detergent. The residual fluorescence, seen in curves A and C, was due to oligonucleotides resulting from DNase I activity (37).

Translocation of Fe(III)-ferrichrome was shown in Fig. 1 (B–D). Phage T5 triggered channel opening of the FhuA protein reconstituted in lipid vesicles, facilitating the efflux of approximately 85% of the labeled ferrichrome (Fig. 1B). The control experiments showed that without phage, less than 15% of the 55Fe label was released, an amount also observed if no phage was added under otherwise identical conditions. The addition of octylglucoside, causing the solubilization of the proteoliposomes, released >95% of the Fe(III)-ferrichrome into the medium (Fig. 1D). The remaining 55Fe label was eluted in the excluded volume adsorbed to lipid/protein/detergent aggregates.

DISCUSSION

We have designed an in vitro assay system that allows the quantitative determination of translocation of an iron(III)-siderophore complex through FhuA channels. The energy requirement of the in vivo translocation process has been circumvented by exploiting the properties of phage T5, which upon binding to the membrane-incorporated FhuA protein, causes DNA ejection. This process is specific, because it was not observed with other ligands such as phage φ80. A significant difference in the time courses of T5 phage DNA ejection with FhuA protein, either reconstituted or solubilized in liposomes, was observed, for which three explanations may apply: (a) The effective concentration of binding sites exposed to the solution is half that of the total protein if the inserted membrane protein is oriented randomly. (b) Accessibility of surface-exposed binding sites may be considerably reduced, due to a taut conformation of the protein in the lipid bilayer. (c) When phage T5 DNA is injected into vesicles, the fluorescence probe YO-PRO 1 may give rise to a reduced signal. This may be due to limited accessibility of dye to the vesicles, quenching of the signal, or both. The observation that the fluorescence intensity rose significantly upon solubilization of the vesicles by detergent revealed that phage DNA was indeed trapped in the liposomes. The results from these experiments provide strong evidence that the FhuA protein was reconstituted in the vesicles in an active form and that it allowed DNA to cross the lipid bilayer. This should now give the opportunity to address the question of the specificity of transported solutes and of the mechanism by which macromolecules cross biological membranes.

Our choice of gel filtration chromatography to quantitate ferrichrome efflux is based on the accuracy and reproducibility with which potential adsorption of ferrichrome to vesicles and leakage can be distinguished from actual efflux. Thus, we could determine that ferrichrome efflux following incubation with phage φ80 amounts to about 15% of the total release of 55Fe label from the vesicles. This tallied with the value of another control experiment in which no phage was added at all and thus represented the background leakage from liposomes under the reaction conditions used. The value of 85% of ferrichrome released during incubation with phage T5 can be assigned to leakage on the one hand and to a fraction of about 80% of liposomes that encountered a phage particle on the other. The ratio of phage-to-liposome constant, efflux could be maximized by adjusting the ratio of incorporated FhuA protein per vesicle to a value between 5 and 10. Now that these values have been established, it will be possible to use filter assays for the routine determination of efflux with large sets of samples.

In conclusion, iron(III)-ferrichrome transport across the outer membrane of E. coli, the physiologically relevant in vivo function of the FhuA protein, could be assessed in an in vitro system using bacteriophage T5 as the triggering ligand. This strategy avoids the problems involved in the energy transduction from the plasma membrane to the channel protein. The assay allows the specificity of the gating ligand (phage T5 versus φ80) to be assessed and should now provide a tool to determine the vectoriality and specificity of the transported solutes, as well as the question of DNA transfer. As to our structural studies of the receptor protein (29), its functionality can now be monitored at any stage during purification and crystallization. In addition, it opens the possibility for kinetic studies of the transport rate through the FhuA protein and sets the stage for reconstitution of the entire translocation cascade.

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