Purification and Structural and Functional Characterization of FhuA, a Transporter of the *Escherichia coli* Outer Membrane[†]

Pascale Boulanger,[‡] Marc le Maire,[§] Mélanie Bonhivers,[‡,||] Sandrine Dubois,[‡] Michel Desmadril,[⊥] and Lucienne Letellier*[,‡]

Laboratoire des Biomembranes, URA CNRS 1116, Université Paris-Sud, Bât. 432, F-91405 Orsay Cedex, France, Section de Biophysique des Protéines et des Membranes, DBCM, CEA et CNRS URA 2096, CEA-Saclay, F-91191 Gif-sur-Yvette Cedex, France, and Laboratoire d'Enzymologie Physicochimique et Moléculaire, URA CNRS 1131, Université Paris-Sud, Bât. 432, F-91405 Orsay Cedex, France

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ABSTRACT: The *Escherichia coli* outer membrane ferrichrome transporter FhuA was purified chromatographically in a neutral detergent (octyl glucoside or dodecyl maltoside). The amount of dodecyl maltoside bound to the protein (1.2 ± 0.15 g/g of FhuA) and the Stokes radius of the FhuA−dodecyl maltoside complex (R_S = 4.2 nm) were determined using size exclusion chromatography. Sedimentation equilibrium and velocity experiments indicated that the FhuA preparation was monodisperse and that the protein was monomeric. The value found for the frictional coefficient of the protein−detergent complex (1.18) suggested a globular shape for the complex. Sedimentation experiments gave values for the molecular mass of the FhuA−dodecyl maltoside complex (180 kDa) and for the Stokes radius in complete agreement with those calculated from size exclusion chromatography. The circular dichroism spectrum indicated a 51% β-sheet content. Functionality of the purified protein was assessed from fluorescence measurements using the DNA probe YO-PRO-1. Interaction of nM concentrations of FhuA with bacteriophage T5 resulted in the release of 90 ± 8% of the phage DNA. The limiting step in DNA ejection was binding of the phage to its receptor. Release of DNA took place in a few seconds. Ferrichrome (0.8 μM) competed with the phage for binding to FhuA and prevented DNA ejection.

FhuA is a 78.9 kDa protein (Coulton et al., 1986) which belongs to a family of *Escherichia coli* outer membrane transporters. This minor protein (10^3 copies per bacterium) catalyzes the high-affinity transport of the ferric siderophores ferrichrome and albomycin across the outer membrane [for a recent review see Braun (1995)]. Transport of ferrichrome across the outer membrane of *E. coli* also requires the participation of TonB, a protein anchored in the cytoplasmic membrane which extends in the periplasm and interacts with FhuA and of ExbB and ExbD, two inner membrane proteins that form a complex with TonB [reviewed in Postle (1993) and Braun (1995)]. FhuA is also the receptor for the bacteriophage T1, T5, and Φ80 and for colicin M (Hantke & Braun, 1978). Modeling suggests that FhuA contains transmembrane β-strands and loops regions connecting these transmembrane regions. In particular, one of the external loops extending from residue 316 to 356 is involved in binding of the ligands (Carmel & Coulton, 1991; Killmann & Braun, 1992; Koebnik & Braun, 1993; Moeck et al., 1995). Hoffman et al. (1986) have designed a protocol to extract the protein in octyl glucoside from envelopes. This procedure yielded a protein which retained receptor activity for phages and siderophores but the purity of which rendered it unsuitable for electrophysiological purpose. Therefore the protein was further purified using a combination of anion exchange chromatography, chromatofocusing, and size exclusion chromatography. The purified protein, which did not by itself form channels in planar lipid bilayer, was converted into an ion channel upon binding of phage T5 (Bonhivers et al., 1996). The electrophysiological characteristics of the channel showed similarities with those of the channel formed by a FhuA derivative (FhuA Δ322−355) from which an external loop which constitutes the binding site for phage T5 and ferrichrome had been removed (Killmann et al., 1993). These observations suggested that binding of phage T5 to loop 322−355 of FhuA unmasked an inner channel in FhuA (Bonhivers et al., 1996). This raised the question of the relevance of this channel opening to the physiological process of phage DNA transfer through the outer membrane. Although several observations suggested that the linear double-stranded DNA of 121 kbp is channeled through the envelope and that phage proteins participate in the formation of these channels (Letellier & Boulanger, 1989; Boulanger & Letellier, 1992; Guihard *et al.*, 1992), little is understood about this mechanism at the molecular level. The fact that the purified protein showed electrophysiological activity upon interaction with T5 rendered the protein well suited for *in vitro* studies of phage−receptor interactions. Early experiments have shown that after 10 min of incubation of phage T5 with isolated membrane extracts containing FhuA, 70% of the DNA

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* Corresponding author. Phone: +33 1 69 15 64 29. FAX: +33 1 69 85 37 15. E-mail: letellie@psisun.u-psud.fr.

[‡] URA CNRS 1116.

[§] CEA-Saclay.

[||] Present address: John Hopkins University, Department of Biological Chemistry and Medicine, 420 Physiology Building, 725 North Wolfe Street, Baltimore, MD 21205.

[⊥] URA CNRS 1131.

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became DNase-sensitive (Zarybnicky & Zarybnicka, 1973). Tosi et al. (1984) also showed that interaction of phage T5 with partially purified FhuA caused the release of 48−68% of the DNA in 2 h. *In vitro* interaction of phage λ with a crude extract from *E. coli* containing LamB also caused a partial and slow release of DNA (50% in 30 min) (Roa & Scandella, 1976). These investigations, although indicating that DNA ejection can take place *in vitro* are far from reflecting the efficiency and rapidity of the process *in vivo*. Furthermore DNA ejection could be only indirectly assayed either by a DNase sensitivity assay which required separation of the released DNA from the phage particle by sedimentation or by a plaque inhibition assay, conditions which might obscure the details of DNA release kinetics. New fluorescent nucleic acid probes have recently become available. These dyes, such as YO-PRO-1[1] (Haughland, 1994), belong to a family of membrane impermeant benzoxazolium−4 quinolinium dyes with high binding constants for double-stranded DNA (estimated to $10^{10}-10^{12}$ M^{-1}), high extinction coefficient ($\epsilon = 52\,000$ cm^{-1} M^{-1}), and quantum yield (0.44) (Glaser & Rye, 1992; Rye et al., 1992, 1993; Selvin, 1992). It has been proposed that they would also bind to DNA by intercalation (Carlsson et al., 1994). They thus appeared well suited for direct *in vitro* quantitative assays of phage DNA ejection.

In this paper we first present some structural properties of FhuA. The purity of the protein, its state of oligomerization, and the MM of the protein−detergent complex were assessed from a combination of size exclusion chromatography and sedimentation experiments. We then present a fluorescence quantitative analysis of the interaction of FhuA with phage T5. This study reveals that almost all the DNA of the phage was released and that this ejection took place in a few seconds.

MATERIALS AND METHODS

Chemicals. The following detergents were used: OG from Bachem (Basel, Switzerland), DM and Triton X-100 from Sigma. [1-^{14}C]DM (specific activity: 2GBq/mmol) was synthesized by the Centre d'Etudes de Saclay, France. Standard proteins for SEC were from Pharmacia.

Purification of the FhuA Protein

E. coli K12 AB2847 (pHK232), a strain overproducing FhuA, was used for purification. The first steps of purification of FhuA involving solubilization of the envelopes and treatment with OG-EDTA were essentially as described by Hoffmann et al. (1986). The OG extract was suspended in 50 mM Tris, pH 7.8, 1 mM EDTA, 33 mM OG, and further purified chromatographically using an FPLC system and columns from Pharmacia (Uppsala, Sweden). All purification steps were performed at 20 °C and all buffers, except otherwise indicated, contained 33 mM OG and 5 mM NaN_3. Buffers were filtered and degassed, and samples were centrifuged before use.

[1] Abbreviations: CD, circular dichroism; CMC, critical micellar concentration; C10DAO, d_{10}-decyl-*N*,*N*′-dimethylamine oxide; DM, *n*-dodecyl β-D-maltoside (dodecyl maltoside); OG, octyl β-D-glucopyranoside (octyl glucoside); PBS, phosphate-buffered saline; PFU, plaque-forming unit; R_S, Stokes radius; SEC, size exclusion chromatography; YO-PRO-1, quinolinium, 4-[(3-methyl-2(3*H*)-benzoxazolylidene)methyl]-1-[3-(trimethylammonio)propyl]-, diiodide.

Anion Exchange Chromatography. The OG extract (0.8 mg/mL, 12 mL) was first loaded onto a Hi TrapQ column previously equilibrated with buffer A (25 mM Tris, pH 7.8, 20 mM EDTA). Proteins were eluted by applying a 0−100% gradient of buffer B (25 mM Tris, pH 7.8, 300 mM NaCl). Fractions containing FhuA were dialyzed against buffer A and chromatographed on a MonoQ HR5/5 columnn using similar elution conditions as above. Fractions containing FhuA were collected, dialyzed overnight at 4 °C against buffer C (25 mM Bis-Tris, pH 6.3).

Chromatofocusing. Fractions issued from the anion exchange chromatography were applied onto a MonoP HR 5/5 column equilibrated with buffer C. The pH gradient was generated with polybuffer 74 (1/10) pH 4. Fractions containing FhuA were collected and concentrated by ultrafiltration on Centricon 30 (Amicon).

Size Exclusion Chromatography. FhuA was finally purified on a Superose 12 HR 10/10 column equilibrated with buffer D. For most studies buffer D was 25 mM Tris, pH 7.2, 150 mM NaCl, 33 mM OG. For circular dichroism experiments NaCl was only 20 mM. For analytical ultracentrifugation experiments and for determination of the Stokes radius buffer D was 25 mM Tris, pH 7.2, 150 mM NaCl, 2 mM DM. Fractions containing FhuA were collected and either used immediately or stored at −20 °C.

Calibration of the Superose 12 Column and RS Determination. The following globular proteins of known Stokes radius were used for calibration: thyroglobulin (8.6 nm), ferritin (6.3 nm), catalase (5.2 nm), aldolase (4.6 nm), bovin serum albumin (3.5 nm), ovalbumin (2.8 nm), chymotrypsinogen (2.1 nm), and ribonuclease A (1.75 nm) (le Maire et al., 1986, 1996). Harlan et al. (1995) verified the usefulness of these proteins (except for the two smaller ones) as calibration standards in buffers containing various detergents, including DM used here. Dextran Blue 2000 was used as marker of void volume (V_o). The total column volume (V_t) was measured using $NaNO_3$ (80 mM). Proteins and markers were solubilized in buffer D containing 2 mM DM.

Measurement of Detergent Binding to FhuA. Determination of FhuA Concentration

Detergent binding to FhuA was determined using SEC and [1-^{14}C]DM. The first two steps of purification of FhuA (anion exchange chromatography and chromatofocusing) were performed in OG. The fractions containing FhuA were concentrated by ultrafiltration on Centricon 30 in the presence of 2 mM DM and loaded onto the Superose 12 HR 10/10 column equilibrated with buffer D containing the radiolabeled detergent (0.6 MBq/mmol). The fractions containing FhuA were pooled and rechromatographed on the same column to ensure complete exchange of OG for DM. Flow rate was 0.5 mL/min. The fractions collected were counted for detergent radioactivity, and the protein content was determined as described by le Maire et al. (1991).

Accurate determination of the protein concentration is required for estimation of the detergent/protein ratio. For this purpose the amino acid composition of the sample was determined as follows: the protein was precipitated in 80% (vol/vol) acetone at −5 °C for 24 h. The pellet that represented 80% of the initial protein content was solubilized in formic acid/H_2O/acetonitrile (10/50/40) and lyophilized. The residue was hydrolyzed in 6 N HCl at 105 °C for 24 h.

Analyses were performed with a Biotronik LC 2000 analyzer equipped with a Dionex DC6A resin column (Durrum Chemical Corporation) and a Spectra-Glo fluorimeter (Gilson). The amount of protein was calculated from the observed amounts of the six more abundant amino acids and from the amino acid composition of FhuA. The value found, which was 20% lower than that obtained using the Lowry method with BSA as standard, was used throughout the calculations.

Analytical Ultracentrifugation Experiments. Sedimentation equilibrium and sedimentation velocity measurements were performed in the Beckman OPTIMA XL-A analytical ultracentrifuge (AN 60-Ti rotor) (Laboratoire d'Enzymologie, CNRS, Gif-sur-Yvette) using the DM-solubilized FhuA protein.

Determination of the Sedimentation Coefficient, Frictional Ratio, and R_S from Analytical Ultracentrifugation. We have followed the formalism of Tanford et al. (1974) in which the contribution of the bound detergent δ_D in g/g of protein can be allowed for by the relation

$$M^*(1 - \bar{v}^*\rho) = M_p[(1 - \bar{v}_p\rho) + \delta_D(1 - \bar{v}_D\rho)]$$

where M^* is the MM of the anhydrous protein−detergent complex, $\bar{v}^*$ its partial specific volume, and ρ the solvent density, M_p is the MM of the anhydrous protein component of the sedimenting particle, and $\bar{v}_p$ and $\bar{v}_D$ are the partial specific volumes of the protein and of the bound detergent, respectively.

Circular Dichroism Spectrum of Purified FhuA. The CD spectrum of FhuA was measured at 20 °C in a Jobin Yvon Mark V dichrograph using a 0.2 mm wide cuvette. FhuA (0.24 mg/mL, final concentration) was solubilized in 10 mM Tris, pH 7.2, 10 mM NaCl, 16.5 mM OG. Deconvolution of the spectra was done using the algorithm from Antrade et al. (1993).

Preparation and Purification of Phage T5 and Φ80. T5st*am*N5 (T5) phage was produced on *E. coli* Fsuβ^+, a permissive host (Zweig & Cummings, 1973). Φ80 was produced on *E. coli K12* AB 2847 (Hoffmann et al., 1986). Phage stocks were prepared and purified as described in Bonhivers et al. (1996). They were resuspended in 10 mM Tris-HCl, pH 7.2, 150 mM NaCl. The final titers were 1×10^{13}/mL and 7×10^{12}/mL for T5 and Φ80, respectively.

Extraction of DNA from T5 Phage. DNA was released from T5 by submitting the phage to an hyperosmotic shock in 5 M LiCl at 46 °C. The preparation was further incubated at 46 °C for 10 min (Konopa & Taylor, 1975). An aliquot of the preparation was layered on the top of a CsCl-step gradient. After centrifugation, only one band was found which migrated at a density (1.3 g/cm^3) corresponding to that of ghosts (i.e., phage depleted of DNA) (not shown). This suggested that nearly all phage had released their DNA. The amount of DNA released was deduced from the known phage titer (1×10^{13}/mL) and the number of T5 DNA base pairs (121 kbp/phage) (Zweig & Cummings, 1973).

FhuA Receptor Activity As Determined from the Plaque Inhibition Assay. Receptor activity was determined by measuring the capacity of FhuA to bind (inactivate) phage T5. The phage suspension was incubated at 37 °C in the presence of FhuA as described under "Fluorescence Experiments" with final FhuA to T5 ratio (mol/mol) of either 180 or 4500. At given times, aliquots were rapidly diluted to prevent further binding of the phage to FhuA, mixed with the indicator strain *E. coli* Fsuβ^+ and plated. Infectious centers were determined and compared to a control treated identically but not containing FhuA.

Fluorescence Experiments. Fluorescence experiments were performed with an SLM 8000 spectrofluorimeter in a 1×0.4 cm cuvette thermostated at 37 °C. Excitation and emission wavelengths were set at 491 and 509 nm, respectively, and slits were 4 nm for excitation and emission. Unless specified otherwise, YO-PRO-1 (Molecular Probes, 1 mM in dimethyl sulfoxide) was 500-fold diluted in 20 mM Hepes, pH 7.2, 150 mM NaCl, 25 mM OG (Hepes buffer). FhuA and the phage were successively added, and the fluorescence was monitored continuously as a function of time. The final volume of the suspension was 1 mL. Recording of the fluorescence signal was delayed by about 4 s (i.e., the time needed for mixing of the suspension). Stirring of the suspension was avoided to prevent the appearance of bubbles due to the detergent. The absorbance at the excitation wavelength (491 nm) was always kept below 0.1 to avoid inner filter effects.

RESULTS

Purification and Structural Properties of FhuA

Purification of FhuA and Determination of the Stokes Radius of the Detergent−FhuA Complex. FhuA was extracted from *E. coli* envelopes following the protocol described by Hoffmann et al. (1986) and further purified in three steps as described in Materials and Methods. After passage on the Superose 12 gel filtration column a single peak was obtained which corresponded to a unique polypeptide migrating at an apparent MM of 80 kDa (Figure 1A, inset). The same elution profile was obtained whether the protein was solubilized in OG (not shown) or DM (Figure 1A).

Size exclusion chromatography is a method currently used for determination of the size and MM of proteins. For water-soluble globular proteins as for most membrane proteins, the Stokes radius is an appropriate size parameter governing elution position for different kinds of gel columns (le Maire et al., 1996). The Superose 12 column was calibrated with proteins of known Stokes radius solubilized in DM. The Stokes radius of the FhuA−detergent complex was estimated using the calibration curves R_S versus K_d with $K_d = (V_e - V_o)/(V_t - V_o)$ and where V_e represents the retention volume of the protein. Blue Dextran and $NaNO_3$ served as markers of the void (V_o) and total (V_t) volumes. The value obtained was about 4.2 nm (Figure 2 and Table 1).

Measurement of Detergent Binding to FhuA. Determination of the MM of the protein in the protein−detergent complex requires the measurement of the amount of bound detergent. Bound detergent has to be measured above the CMC to maintain the protein in an homogeneous state (Møller et al., 1986). DM was used rather than OG because of its low CMC (0.18 mM compared to 20 mM for OG). This allows a better accuracy on the detergent binding ratio. OG was exchanged for DM on the gel filtration column and as described by le Maire et al. (1976). Two successive columns in DM were performed to ensure complete exchange of OG for DM. The optical density profile after the second chromatography is shown in Figure 1A, while Figure 1B

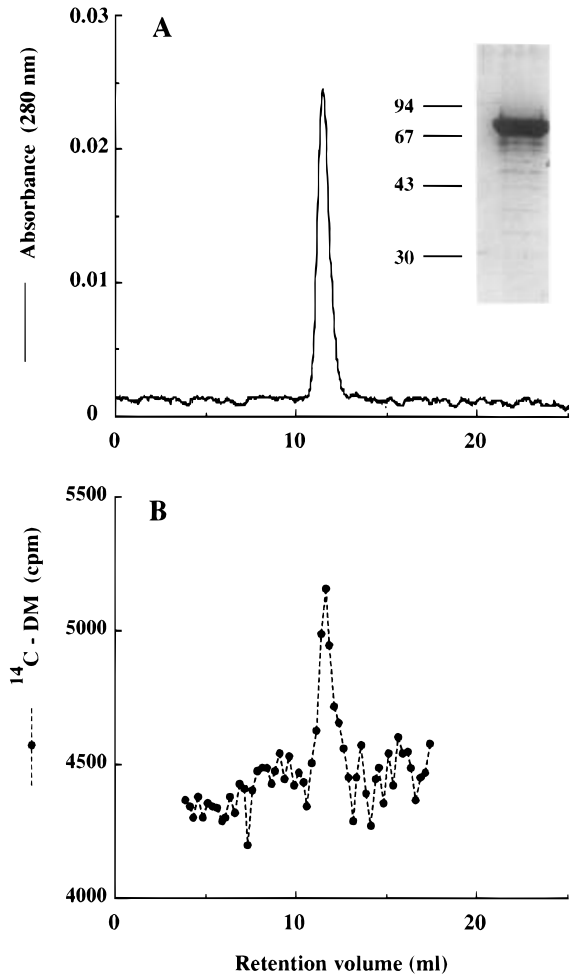

FIGURE 1: Size exclusion chromatography on Superose 12 and [^{14}C]-DM binding level. (A) Elution profile of FhuA after the second passage on the Superose 12. Experimental conditions are described in Materials and Methods. An absorbance of 0.02 corresponds to 0.1 mg of protein/mL. Inset: SDS−PAGE (Coomassie Blue staining) of the peak fraction. (B) [1-^{14}C]DM content of the fractions obtained. Aliquots of 50 μL were counted for radioactivity.

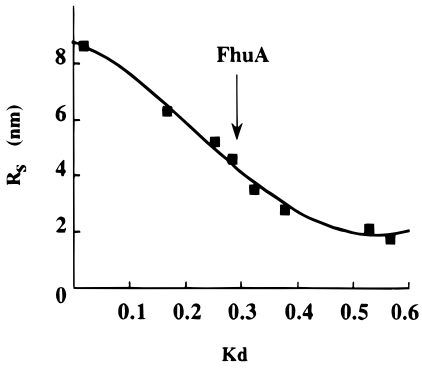

FIGURE 2: Calibration curve of water-soluble proteins on the Superose 12 column. Proteins (1−5 mg/mL) of known Stokes radius (R_S) (see Materials and Methods) were solubilized in 25 mM Tris, pH 7.2, 150 mM NaCl, 2 mM DM and applied onto the Superose 12 column equilibrated with the same buffer. Elution was performed at a flow rate of 0.5 mL/min. K_d values were calculated from the retention volumes V_e. The curve is the fit obtained with a polynomial of degree 4 (correlation coefficient = 0.992). The arrow indicates the K_d value of the FhuA−DM complex.

represents the detergent elution profile. The base line corresponded to free DM micelles and monomers. The protein peak (see Figure 1A) was associated with a rise in detergent concentration above the base line. Binding levels were calculated from this increase in detergent concentration. The value calculated for δ_D was 1.2 ± 0.15 g/g of FhuA. It corresponds to about 185 DM molecules per polypeptide chain (mean of 3 experiments).

Table 1: FhuA Structural Parameters As Deduced from SEC and Analytical Centrifugation

column data (1)	
R_S (nm)	4.2
DM binding (g/g of FhuA)	1.20 ± 0.15
sedimentation equilibrium data (2)	
$M^*(1 - \bar{v}^*\rho)$	40 800
M^*	185 000
M_p[a]	84 000 ± 5 000
sedimentation velocity data (3)	
$s_{20,w}$ (10^{-13} s)	7.8
combined data (1 and 3)	
M^*	172 000
M_p[a]	78 000 ± 4 000
combined data (2 and 3)	
R_S (nm)	4.5
f/f_{min}	1.18
protein sequence	
M_p[b]	78 804
$\bar{v}_p$ (cm^3/g)[c]	0.720
$\bar{v}^*$ (cm^3/g)[d]	0.776

[a] The standard deviation reflects only the error in the binding measurements and not on $M^*(1 - \bar{v}^*\rho)$. [b] Deduced from the cDNA sequence. [B] based on the amino acid composition using the method of Cohn and Edsall (1943). [d] Based on the values of υ_p and $\bar{v}_D = 0.824$ (cm^3/g) (at 20 °C) (Møller & le Maire, 1993).

Determination of the Molecular Mass of the Protein. The state of aggregation and the MM of FhuA was assessed from sedimentation equilibrium and velocity experiments. The plot ln C versus r^2 was linear throughout the centrifugation cell (Figure 3A), indicating that the protein solution was homogenous and that there was no change in average MM with concentration and therefore no protein self-aggregation. This can also be seen from the sedimentation velocity profiles shown in Figure 3B. The recordings show sedimentation boundaries after centrifugation at 4 min intervals. No rapidly sedimenting component was observed during acceleration of the rotor to the operating speed (60 000 rpm). The shape and width of the concentration profile between the depleted and protein-containing parts of the fluid column indicated sample homogeneity: the variation of the concentration of the protein as a function of the radial distance (dA/dr) approached a Gaussian distribution (not shown). The values of $M^*(1 - \bar{v}^*\rho)$ and the sedimentation coefficient (7.8 S) calculated from the sedimentation equilibrium and velocity experiments are given in Table 1.

M_p, the MM of the anydrous protein component of the sedimenting particle, can be calculated independently either from the sedimentation equilibrium experiments or from a combination of the sedimentation coefficient and the R_S determined by size exclusion chromatography. The values obtained are respectively 84 000 ± 5 000 and 78 000 ± 4 000 (Table 1), unambiguously indicating that the protein is monomeric. The frictional ratio was calculated according to Tanford et al. (1974). The value found (1.18) (see Table 1) indicates that the protein−detergent complex has an overall globular shape with an usual hydratation.

Circular Dichroism of the Purified FhuA Protein. Measurement of the CD spectrum of the protein in the far UV requires the salt concentration of the protein to be reduced. This was accomplished during SEC. Figure 4 represents the

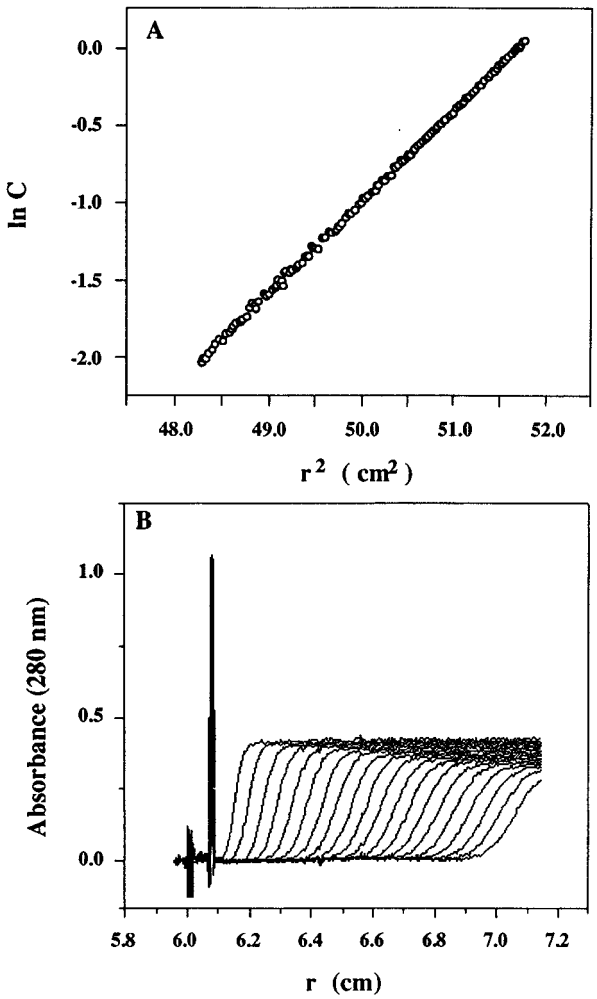

FIGURE 3: Sedimentation equilibrium (A) and velocity (B) of the FhuA−DM complex. FhuA (0.32 mg/mL corresponding to an A_{280} = 0.36) eluted from a single chromatograph on Superose 12 equilibrated in 25 mM Tris, pH 7.2, 150 mM NaCl, and 2 mM DM was used. (A) Sedimentation equilibrium was performed for 20 h at 8000 rpm, 20 °C, and the absorbance (*C*) was measured at 280 nm. (B) Sedimentation velocity was performed at 60 000 rpm 20 °C; scans were taken at 4 min intervals; r represents the radial distance.

CD spectrum of the protein in the presence of 16.5 mM OG (conditions which do not impair functionality; see below). The CD spectrum is typical of an all-β-protein displaying a large positive peak at 195 nm. Deconvolution of the spectrum using the Algorithm of Antrade et al. (1993) indicated the following content: 51% β-sheet, 2% α-helix, and 47% random. Similar values of the β-sheet content were obtained by comparing the spectrum observed to that of a known all-β-protein (not shown).

Functional properties of FhuA

In Vitro Interaction of FhuA with T5, but Not with Φ80, Induced a Quasi-Total Release of Phage DNA. The fluorescence signal of YO-PRO-1 (2 μM) was calibrated with phage T5 DNA solutions prepared as described in Materials and Methods and of known concentrations. The fluorescence emission intensity increased linearly with the amount of DNA up to 0.7×10^{15} bp (0.8 μg of DNA) and saturated above this value. Similar results were obtained whether or not OG was added (data not shown). Saturation was observed for a DNA base pairs to YO-PRO-1 mole ratio above 1, in agreement with the intercalation of one YO-PRO-1 molecule in between two base pairs (Carlsson et al., 1994). Saturation therefore reflects only the limitation in available dye. Higher concentrations of YO-PRO-1 resulted in concentrational quenching of the probe and could therefore not be used.

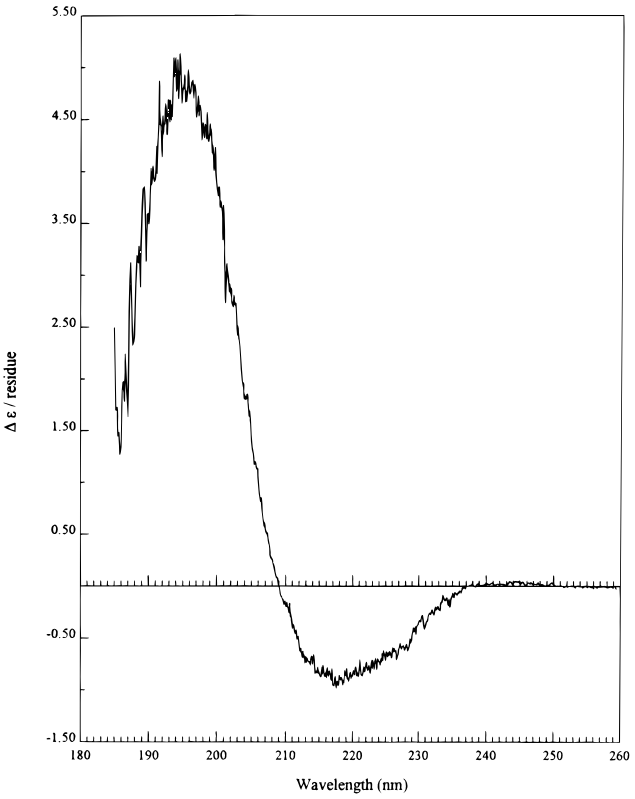

FIGURE 4: Circular dichroism spectrum of FhuA purified in OG. The purified FhuA protein (0.24 mg/mL) was solubilized in 10 mM Tris, pH 7.2, 10 mM NaCl, and 16.5 mM OG. Measurements were carried out at 20 °C over an optical pathway of 0.2 mm.

A preliminary requisite to the analysis of the DNA ejection process was that the dye only reported the DNA ejected and not that encapsulated in the phage head. Figure 5 shows that this is indeed the case. Phage T5 (1.5×10^8/mL, 20 ng of DNA) was first added to a cuvette containing YO-PRO-1 and Hepes buffer not containing OG. It immediately induced a small increase in fluorescence which did not vary with time. This increase, which represented about 10−15% of the total fluorescence measured in the presence of FhuA is likely to correspond to naked DNA that is often present in phage preparations (Tosi et al., 1984). Addition of variable concentrations of FhuA (30−220 nM) to phage T5 resulted in a large increase in fluorescence which started after a lag of about 30 s and reached the same steady state regardless of the concentration of FhuA added. The time required to reach this plateau increased from 15 to 30 min when the FhuA concentration was decreased from 220 to 30 nM, respectively. Quantification of the DNA ejected using known amount of free T5 DNA indicated that 90 ± 8% of the total DNA was released at the plateau. The same figure shows that addition of DNase (10 μg/mL, final concentration) to hydrolyze the released DNA decreased the fluorescence to its initial level in the absence of phage DNA. YO-PRO-1 therefore fluoresces only in the presence of intact and free DNA and not in the presence of individual nucleotides.

FhuA also serves as a receptor for phage Φ80. However, in contrast to T5, infection by Φ80 also requires the

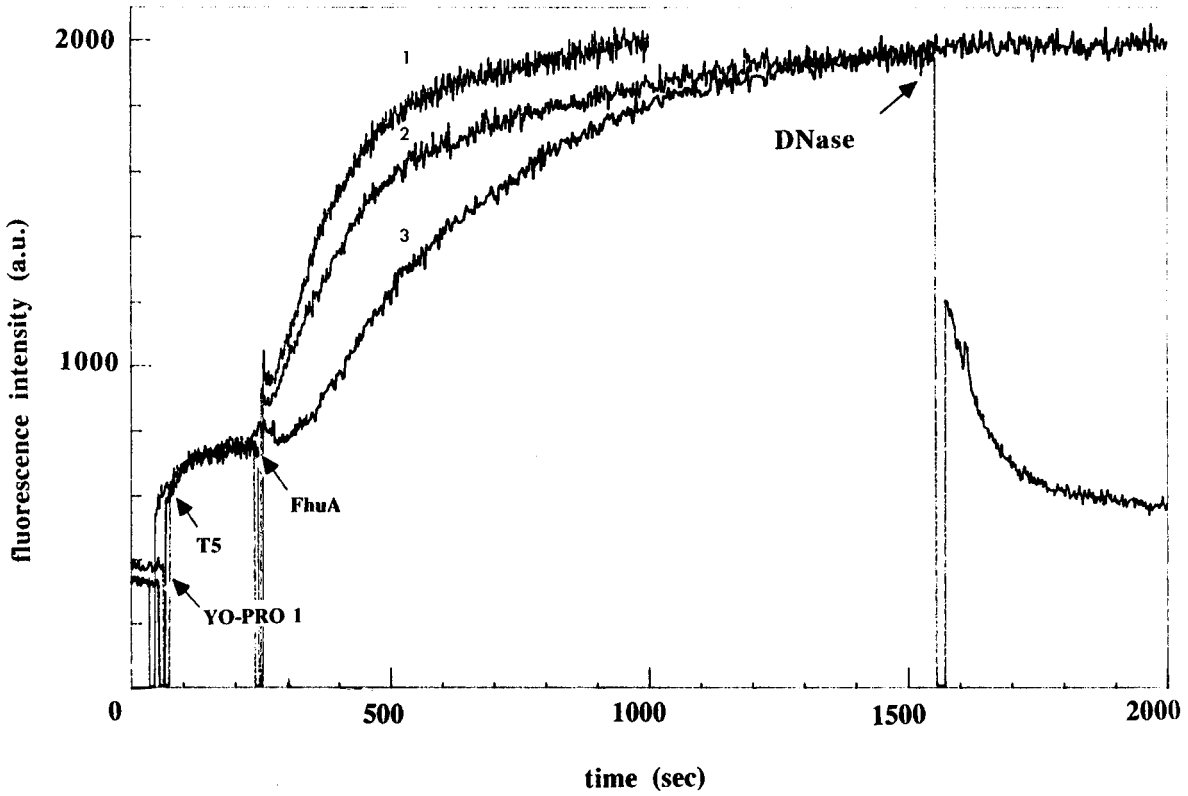

FIGURE 5: Phage T5 DNA ejection as measured with YO-PRO-1. YO-PRO-1 (2 μM) was incubated in 20 mM Hepes, pH 7.2, 150 mM NaCl, and at 37 °C. T5 (1.5×10^8 phage/mL) and FhuA were successively added so that the final concentration of OG never exceeded 1 mM. Emission wavelength was set at 509 nm ($\lambda_{ext} = 491$ nm). DNase (10 μg/mL, final concentration) and $MgSO_4$ (1 mM) were added where indicated. FhuA concentrations: 220 nM (curve 1), 110 nM (curve 2), and 30 nM (curve 3).

cytoplasmic membrane-anchored protein TonB. Experiments similar to those described in Figure 5 were performed with Φ80: no ejection of phage DNA was observed even with concentrations of FhuA up to 100 nM (data not shown) and even if the protein was solubilized in the presence of 33 mM OG (see below).

Effect of OG and FhuA Concentration on the Kinetics of Phage T5 DNA Ejection. Aliquots of FhuA, initially solubilized in 33 mM OG, were diluted in Hepes buffer containing variable concentrations of the detergent and phage T5 was then added. The extent of DNA release remained constant (90%, data not shown), whatever the OG concentration added. However the time required to attain half of the steady state value of the fluorescence ($t_{1/2}$) increased from 30 to 340 s when the OG concentration was decreased from 33 to 0.3 mM (Figure 6). It is interesting to note that $t_{1/2}$ remained constant for OG concentrations above its CMC (i.e., 18 mM).

The effect of variable concentrations of FhuA on $t_{1/2}$ was then determined in Hepes buffer containing 25 mM OG. Figure 7 shows that an increase of the FhuA concentration from 0.015 to 100 nM resulted in a decrease of the $t_{1/2}$ from 30 min to 30 s.

Correlation between Phage DNA Ejection and Number of Phage Bound to FhuA. For phage DNA to be ejected, collision between the phage particle and the receptor must first take place. The observation that the kinetics of fluorescence increase depended on the concentration of FhuA suggested that collision between the two particles might be a limiting step in DNA ejection. Aliquots of the phage−receptor suspensions prepared at different phage/receptor ratio and used for the fluorescence experiments were

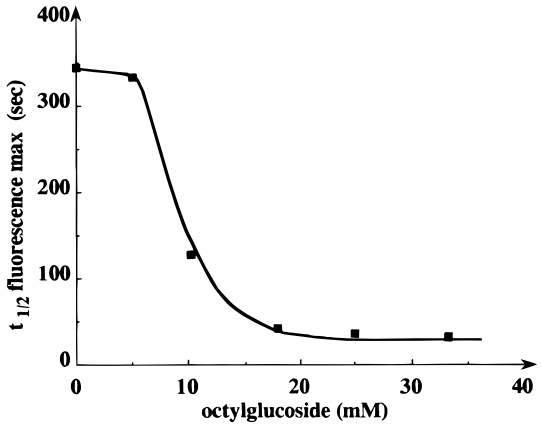

FIGURE 6: Effect of variable concentrations of octyl glucoside on T5-induced fluorescence increase. YO-PRO-1 (2 μM) was incubated in 20 mM Hepes, pH 7.2, 150 mM NaCl, containing the concentrations of OG indicated. FhuA (27 nM) and T5 (1.5×10^8 phage/mL) were then added and the fluorescence increase followed as a function time as shown in Figure 5. The $t_{1/2}$ of fluorescence maximum is defined as the time required for fluorescence to attain 50% of the plateau value. The plateau value corresponded in all cases to 90 ± 8% DNA released.

analyzed for PFU. The number of phage bound to FhuA was deduced from the phage titer and the number of infectious centers found. Figure 8A and 8B show that the curves representing the % of phage bound to FhuA and the % of fluorescence increase versus time were superimposable, indicating that the kinetics of fluorescence increase reflects the kinetics of binding of the phage to its receptor.

Competition between Ferrichrome and Phage T5 for Binding to FhuA. FhuA catalyzes the high-affinity transport of the siderophore ferrichrome through the *E. coli* outer

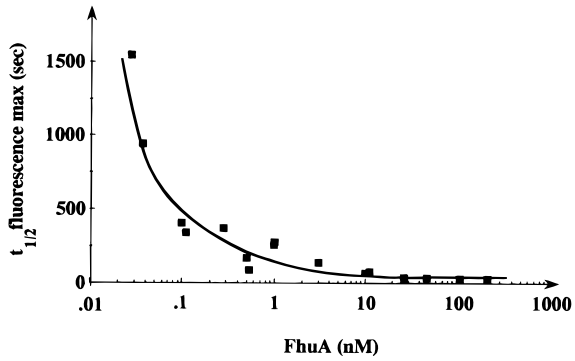

FIGURE 7: Effect of FhuA concentration on the $t_{1/2}$ of phage T5 DNA release. Conditions were as described in legend to Figure 6 except that the OG concentration was 25 mM.

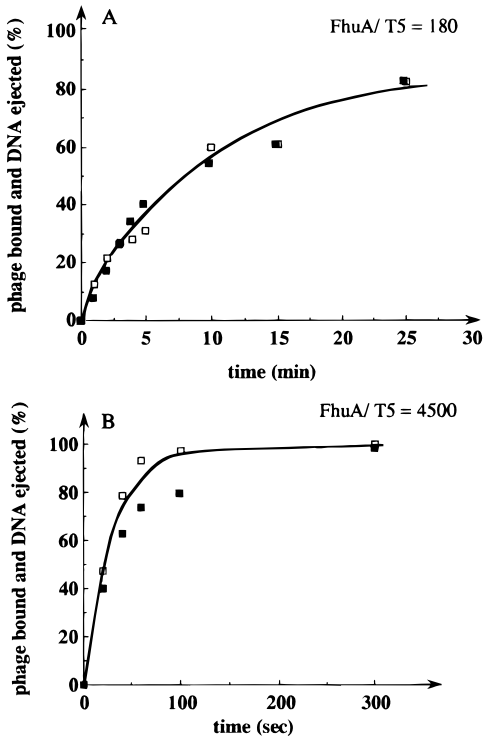

FIGURE 8: Relationship between YO-PRO-1 fluorescence increase induced by T5 and the number of phage bound to FhuA. The intensity of fluorescence of YO-PRO-1 was measured as a function of time for different FhuA/T5 ratios (T5: 1.5×10^8 phage/mL) and in the presence of 25 mM OG as described in legend to Figure 5. At the times indicated aliquots of the suspension were taken for measurement of YO-PRO fluorescence (●) and PFU (□). 100% of fluorescence corresponds to 100% of DNA released. The % of phage bound was deduced from the number of PFU.

membrane. Furthermore, addition of μM concentrations of ferrichrome before phage T5 totally prevents infection by the phage (Hantke & Braun, 1978). The ferric complex of ferrichrome was allowed to bind to FhuA prior to phage T5 addition and the T5-induced fluorescence of YO-PRO-1 was measured as a function of time. The fluorescence intensity at steady state varied inversely with the concentration of ferric-ferrichrome. Figure 9 represents the % of inhibition of phage DNA ejection calculated from the steady state values versus the concentration of ferrichrome. Phage DNA ejection was totally prevented for ferrichrome concentrations >0.8 μM. The desferri complex of ferrichrome was less efficient in inhibiting DNA ejection since 0.1 μM of the siderophore inhibited DNA ejection only by 40% compared to 70% for the ferric complex (data not shown). In a second

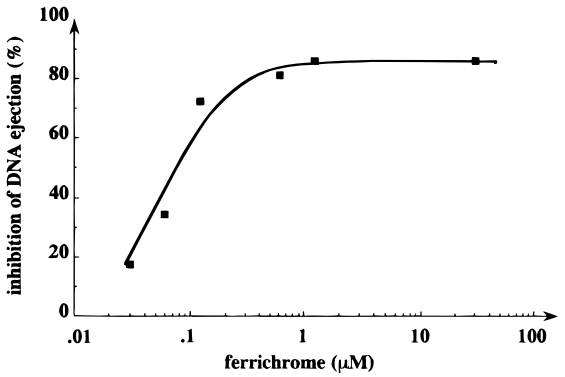

FIGURE 9: Effect of ferrichrome concentration on the amount of phage T5 DNA released. Variable concentrations of ferrichrome were added to a cuvette containing 20 mM Hepes, pH 7.2, 150 mM NaCl, 25 mM OG and YO-PRO-1 (2 μM). FhuA (25 nM) and phage T5 (1.5×10^8 phage/mL) were then added. The fluorescence increase of YO-PRO-1 induced by ejection of the DNA was measured at 37 °C as a function of time. The % of inhibition of DNA release was calculated when the fluorescence of YO-PRO-1 attained the steady state. 0% of inhibition is defined as the steady state value of the fluorescence in the absence of ferrichrome.

series of experiments where ferrichrome (1 μM) was added after the onset of fluorescence increase induced by T5, the fluorescence increase was immediately arrested (not shown). Given the results described in Figure 8, it is likely that this arrest reflects binding of ferrichrome to receptors unoccupied by phage. Further addition of phage T5 after ferrichrome did not lead to a new increase of the fluorescence, indicating that the affinity of ferrichrome for FhuA is such that T5 cannot displace it from its binding site.

CONCLUSIONS

We have purified the *E. coli* outer membrane transporter FhuA by applying successively anion exchange chromatography, chromatofocusing, and SEC. The purity of the protein was ascertained by the presence of a unique peak after SEC associated with a unique polypeptide band on SDS−PAGE. Further proof that the purified protein was devoid from porin contamination was given by planar lipid bilayer experiments where concentrations of FhuA as high as 10 ng/mL did not result in any appearance of channels (Bonhivers et al. 1996). It is likely that the purified protein−detergent complex is devoid of lipid and lipopolysaccharide contamination since anion exchange chromatography has proven to remove efficiently the lipolysaccharide (Garavito & Rosenbusch, 1986) and lipids (Møller & le Maire, 1993). Furthermore Jalal and der Helm (1989) in purifying FepA, another *E. coli* outer membrane transporter, reported that chromatofocusing, under conditions similar to ours, totally removed phospholipids.

Monodispersity of the purified FhuA−detergent complex protein was ascertained by sedimentation equilibrium/velocity measurements. Recordings showed that the FhuA preparation neither contained aggregates nor aggregated during centrifugation. The combined results of SEC and sedimentation studies indicated a unique species of MM ranging from 172 to 185 kDa. Given the number of bound detergent molecules determined by gel filtration (185/FhuA molecule) and the likely negligable contribution of lipids and lipopolysaccharides, this corresponds to a MM of the protein of 80 kDa. This value undoubtly indicates that the purified protein is monomeric. This monomeric state is likely to

correspond to the functional state *in vivo* since the purified protein retained functionality. This is also in line with the results of Schultz et al. (1989), who showed that expression of partially inactive FhuA in the presence of active FhuA did not diminish its activity, and therefore that heteromultimers were not formed. This is in contrast with the results obtained on FepA, another *E. coli* siderophore receptor, which shares both sequence and function homologies with FhuA (Lundrigan & Kadner, 1986; Rutz et al., 1992), that appears to exist in a dimeric/trimeric state (Skare et al., 1993; Liu et al., 1993).

Calibration of the gel filtration column with soluble proteins of known Stokes radius provided R_S value for the FhuA−DM complex close to that obtained directly from a combination of sedimentation equilibrium and velocity experiments: $R_S = 4.2$ nm versus 4.5 nm (see Table 1). These data confirm that water-soluble proteins can be used for determination of R_S values of membrane proteins solubilized in DM. Calibration in DM appears therefore better suited than with polyoxyethylene-type detergents which show an anomalous behavior probably due to the polyoxyethylene part of the detergent and lead to an overestimate of R_S (le Maire et al., 1989).

The CD spectrum of FhuA indicates that the protein is primarily organized in a β-sheet structure. The value found (51%) is in very good agreement with the value (50.4%) predicted by Koebnick and Braun (1993) and based on the primary sequence of the protein. Therefore FhuA behaves like the majority of proteins of the outer membrane of Gram negative bacteria for which the secondary and even tertiary structure is known (OmpF, PhoE, LamB) and which are essentially organized in β-strands (Cowan et al., 1992; Kreusch et al., 1994; Schirmer et al., 1995).

Determination of the detergent binding ratio can provide some information on the surface area of the hydrophobic domain of the protein (Møller & le Maire, 1993). The molar ratio found (185 DM/FhuA) is intermediate between the value found for the photosynthetic reaction center from *Rhodobacter spheroides* (148) and that obtained for cytochrome oxidase (215) (Møller & le Maire, 1993). This suggests that a membrane protein built on a scaffold of β-barrel type binds a comparable number of detergent molecules per protomer as α-helical type membrane proteins [see also Zulauf (1985)]. If we assume, as this seems to be the case (Pebay-Peyroula et al., 1995), a comparable organization of the detergent bound on α-helical and β-barrel proteins, then FhuA might be rather large: on the basis of model calculations (Møller & le Maire, 1993), the perimeter of FhuA would be in the range of 12−18 nm. It is of interest to make a comparison with what is known for other β-barrel proteins such as porins. To our knowledge, there are no estimates of the amount of DM bound to trimers of porins in solution. However, Pebay-Peyroula et al. (1995) performed a neutron-scattering study of tetragonal crystals of OmpF porin in two detergents, C10DAO and OG. On the basis of the volume of the detergents' belts, the numbers of these detergent molecules per porin trimer were estimated to be 410 and 350, respectively, for a perimeter of the porin trimer of about 24 nm. These estimates of bound detergents are consonant with the range of perimeter values estimated above for FhuA.

The frictional ratio f/f_{min} ($= R_S/R_{min}$) of the FhuA−DM complex is low (1.18) and similar to that generally obtained for globular water-soluble proteins (Tanford, 1961) or some detergent-solubilized membrane proteins like *R. spheroides* reaction center (Møller et al., 1986). This suggests that the FhuA−detergent complex is also globular. The 3D structures of several bacterial porins have been determined (Weiss & Schulz, 1992; Cowan et al., 1992, 1995; Kreusch et al., 1994; Schirmer et al., 1995). Each monomer forms a 16- or 18-stranded antiparallel β-barrel containing a channel. A long loop forms a constriction inside the channel. This loop which is located on the external face of the outer membrane is involved in ion or ligand selectivity of the channel. FhuA as well as FepA belong to a family of transporters which also carry an external loop involved in binding of ligands. Deletion of the loop converts FepA (Rutz et al., 1992) and FhuA into diffusion channels (Killmann et al., 1993; Braun et al., 1994). We have also recently shown that binding of phage T5 to the external loop of wild type FhuA is sufficient to convert the transporter into a channel (Bonhivers et al., 1996). If, as predicted by Koebnick and Braun (1993), FhuA contains 32 transmembrane β-strands with the external loop connecting β-strands 15 and 16, then the question of the organization of the protein can be raised: is it formed of a single barrel of 32 β−strands or, by analogy with the porins, by two β−barrels connected by the loop? Resolving the 3D structure of the protein will therefore be a major future challenge.

It has been long known that the interaction of phage λ and T5 with their partially purified receptors trigger the release of the phage DNA. In all cases DNA ejection required 10−30 min and was only partial and thus was significantly slower than that of DNA transfer through the bacterial envelope [reviewed in Boulanger and Letellier (1989)]. Three non-exclusive hypotheses may explain these discrepancies. First DNA ejection *in vivo* requires bacterial components which are missing in the isolated receptor preparations; second, the conformation of the receptor *in vitro* does not match that *in vivo*; third, the experimental conditions for measurement of DNA ejection were not appropriate. The experiments described here show that the third hypothesis explains part of these discrepancies.

We have used YO-PRO-1, a fluorescent DNA probe which binds to double-stranded polynucleotides by intercalating between the base pairs, to analyze the kinetics of DNA ejection induced upon interaction of phage T5 with FhuA. Our results show that phage bound to FhuA ejected virtually all their DNA but that the limiting step in ejection was binding of the phage to FhuA: the higher the FhuA/phage ratio was, the shorter time it took for the phage to bind. Given the known amount of phage bound at any time (see Figure 8) we can therefore conclude that total release of the DNA is achieved in a few seconds. T5 DNA release *in vitro* clearly occurs in one step, whereas DNA transfer *in vivo* occurs in two steps (Lanni, 1960; Guihard et al., 1992; Bonhivers & Letellier, 1995). Thus, there must be additional host factors that contribute to this unusual transfer of DNA in two steps *in vivo*.

FhuA caused the release of phage T5 DNA even when the protein was kept in a buffer not containing detergent. The fact that this outer membrane protein retained functionality in the absence of detergent is in agreement with the observation that several *E. coli* outer membrane proteins (porins) also retain electrophysiological activity even when diluted in the absence of detergent [reviewed in Letellier and

Bonhivers (1996)]. Nevertheless the $t_{1/2}$ of fluorescence increase was ten times slower in the absence than in the presence of OG. The most likely interpretation is that the FhuA proteins form aggregates in the absence of detergent and that these aggregates are inactive. Binding of T5 would then be slowed down because fewer active receptors would be available. Alternatively, the kinetics of binding of T5 could be slowed down if removal of the detergent changed the conformation of FhuA. Ferrichrome competed with phage T5 for binding to FhuA at concentrations similar to that found *in vivo* (Hantke & Braun, 1978; Hoffmann et al., 1986). This indicates that the conformation of FhuA solubilized in detergent is very similar to that *in vivo*.

The availability of an active purified membrane protein and sensitive techniques such as fluorescence and planar lipid bilayer (Bonhivers et al., 1996) will allow further analysis of the function of FhuA and of its interaction with phage at the molecular level.

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REFERENCES

Antrade, M. A., Chacon, P., Merelo, J. J., & Moran, F. (1993) *Prot. Eng. 6*, 383−390.
Bonhivers, M., & Letellier, L. (1995) *FEBS Lett. 374*, 169−173.
Bonhivers, M., Ghazi, A., Boulanger, P., & Letellier, L. (1996) *EMBO J. 15*, 1850−1856.
Boulanger, P., & Letellier, L. (1992) *J. Biol. Chem. 267*, 3168−3172.
Braun, V. (1995) *FEMS Microbiol. Rev. 16*, 295−307.
Braun, V., Killmann, H., & Benz, R. (1994) *FEBS Lett. 346*, 59−64.
Carlsson, C., Larsson, A., Jonsson, M., Albisson, B., & Nordén, B. (1994) *J. Phys. Chem. 98*, 10313−10321.
Carmel, G., & Coulton, J. W. (1991) *J. Bacteriol. 173*, 4394−4403.
Cohn, E. J., & Edsall, J. T. (1943) *Proteins, Amino Acids and Peptides*, p 372, Reinhold, New York.
Coulton, J. W., Mason, P., Cameron, D. R., Carmel, G., Jean, R., & Rode H. N. (1986) *J. Bacteriol. 165*, 181−192.
Cowan, S. W., Schirmer, T., Rummel, G., Steiert, M., Ghosh, R., Pauptit, R. A., Jansonius, J. N., & Rosenbusch, J. P. (1992) *Nature 358*, 727−733.
Cowan, S. W., Garavito, R. M., Jansonius, J. N., Jenkins, J. A., Karlsson, R., Konig, N., Pai, E. F., Pauptit, R. A., Rizkallah, P. J., Rosenbusch, J. P., Rummel, G., Schirmer, T. (1995) *Structure 3*, 1041−1050.
Garavito, R., & Rosenbusch, J. P. (1986) *Methods Enzymol. 125*, 311−328.
Glaser, A. N., & Rye, H. S. (1992) *Nature 359*, 859−861.
Guihard, G., Boulanger, P., & Letellier, L. (1992) *J. Biol. Chem. 267*, 3173−3178.
Hantke, K., & Braun, V. (1978) *J. Bacteriol. 135*, 190−197.
Harlan, J. E., Picot, D., Loll, P. J., & Garavito, R. M. (1995) *Anal. Biochem. 224*, 557−563.
Haughland, R. P. (1992−1994) *Handbook of Fluorescent Probes and Research Chemicals* (Larison, K. D., Ed.) pp 224−229, Molecular Probes Inc., Eugene, OR.
Hoffmann, H., Fischer, E., Kraut, H., & Braun, V. (1986) *J. Bacteriol. 166*, 404−411.
Jalal, M. A. F., & van der Helm, D. (1989) *FEBS Lett. 243*, 366−370.
Killmann, H., & Braun, V. (1992) *J. Bacteriol. 174*, 3479−3486.
Killmann, H., Benz, R., & Braun, V. (1993) *EMBO J. 12*, 3007−3016.
Koebnik, R., & Braun, V. (1993) *J. Bacteriol. 175*, 826−839.
Konopa, G., & Taylor, K. (1975) *Biochem. Biophys. Acta 399*, 460−467.
Kreusch, A., Neubuser, A., Schittz, E., Weckesser, J., & Schulz, G. E. (1994) *Protein Sci. 3*, 58−63.
Lanni, Y. T. (1960) *Virology 10*, 514−529.
le Maire, M., Møller, J. V., & Tanford, C. (1976) *Biochemistry 15*, 2336−2342.
le Maire, M., Aggerbeck, L. P., Monteilhet, C., Andersen, J. P., & Møller, J. P. (1986) *Anal. Biochem. 154*, 525−535.
le Maire, M., Viel, A., & Møller, J. (1989) *Anal. Biochem. 177*, 50−56.
le Maire, M., Chabaud, R., & Hervé, G. (1991) *Laboratory Guide to Biochemistry, Enzymology, and Protein Physical Chemistry*, p 38, Plenum Press, New York.
le Maire, M., Ghazi, A., & Møller, J. V. (1996) in *Cross Evaluation of Stategies in Size-Exclusion Chromatography* (Potschka, M., Dubin, P., Eds.) ACS Symposium Series 635, pp 36−51, American Chemical Society, Washington, DC.
Letellier, L., & Boulanger, P. (1989) *Biochimie 71*, 167−174.
Letellier, L., & Bonhivers, M. (1996) in *Handbook of Biological Physics* (Kaback, R. H., Konings, W. N., & Lolkema, J. S., Eds.) pp 615−637, Elsevier, New York.
Liu, J., Rutz, J. M., Feix, J. B., & Klebba, P. E. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 10653−10657.
Ludrigan, M. D., & Kadner, R. J. (1986) *J. Biol. Chem. 261*, 10797−10801.
Moeck, G. S., Ratcliffe, M. J., & Coulton, J. W. (1995) *J. Bacteriol. 177*, 6118−6125.
Møller, J., & le Maire, M. (1993) *J. Biol. Chem. 268*, 18659−18672.
Møller, J. V., le Maire M., & Andersen J. P. (1986) *Progress in Protein−Lipid Interactions*, Vol. 2, pp 147−197, Elsevier Science Publishers B.V., Amsterdam.
Pebay-Peyroula, E., Garavito, R. M., Rosenbusch, J. P., Zulauf, M., & Timmins, P. A. (1995) *Structure 3*, 1051−1059.
Postle, K. (1993) *J. Bioenerg. Biomembr. 25*, 591−601.
Roa, M., & Scandella, D. (1976) *Virology 72*, 182−194.
Rutz, J. M., Liu, J., Lyons, J. A., Goranson, J., Armstrong, S. K., McIntosh, M. A., Feix, J. B., & Klebba, P. E. (1992) *Science 258*, 471−475.
Rye, H. S., Yue, S., Wemmer, D. E., Quesada, M. A., Haughland, R. P., Mathies, R. A., & Glaser, A. N. (1992) *Nucleic Acids Res. 20*, 2803−2812.
Rye, H. S., Dabora, J. M., Quesada, M. A.,Mathies, R. A. & Glazer, A. N (1993) *Anal. Biochem. 208*, 144−150.
Schirmer, T., Keller, T. A., Wang, Y.-F., & Rosenbusch, J. P. (1995) *Science 267*, 512−514.
Schultz, G., Ulrich, F., Heller, K., & Braun, V. (1989) *Mol. Gen. Genet. 216*, 230−238.
Selvin, P. (1992) *Science 257*, 885−886.
Skare, J. T., Ahmer, B. M., Seachord, C. L., Darveau, R. P., & Postle, K. (1993) *J. Biol. Chem. 268*, 16302−16308.
Tanford, C. (1961) *Physical Chemistry of Macromolecules*, p 391, John Wiley & Sons, New York.
Tanford, C., Nozaki, Y., Reynolds, J. A., & Makino, S. (1974) *Biochemistry 13*, 2369−2373.
Tosi, F., Labedan, B., & Legault-Demare, J. (1984) *J. Virol. 50*, 213−219.
Weiss, M. S., & Schulz, G. E. (1992) *J. Mol. Biol. 227*, 493−509.
Zarybnicky, V., & Zarybnicka, A. (1973) *Virology 54*, 318−329.
Zulauf, M. (1985) in *Physics of Amphiphiles: Micelles, Vesicles and Microemulsions*, Proceedings of the International School of Physics "Enrico Fermi". (Degiorgio, V., & Corti, M., Eds.) pp 663−673, North Holland Press, Amsterdam.
Zweig, M., & Cummings, D. J. (1973) *Virology 51*, 443−453.

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