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Minireview

Energy-coupled transport through the outer membrane of *Escherichia coli* small deletions in the gating loop convert the FhuA transport protein into a diffusion channel

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Abstract

Active transport of Fe^{3+} as ferrichrome complex through the outer membrane of *Escherichia coli* is mediated by the FhuA outer membrane protein and the TonB–ExbB–ExbD protein complex in the cytoplasmic membrane. The required energy is provided by the electrochemical potential of the cytoplasmic membrane which is assumed to induce a conformation of the TonB protein that causes a conformational change in FhuA so that bound ferrichrome is released into the periplasmic space located between the outer and the cytoplasmic membrane. Excision of segments as small as 12 amino acids in the largest surface loop of FhuA converted FhuA into an open channel through which ferrichrome and antibiotics diffused independent of TonB–ExbB–ExbD. It is proposed that FhuA forms a closed channel which is opened by movement of the gating loop through a kind of allosteric interaction with TonB. The gating loop is also involved in binding of all FhuA ligands which in addition to ferrichrome are the phages T1, T5, ϕ80, colicin M and the antibiotic albomycin.

Key words: Gated FhuA channel; *E. coli*; Outer membrane

1. Introduction

Bacteria have developed highly intricate transport systems for substrates because they are faced with very low substrate concentrations in a frequently changing surrounding. Active transport through the cytoplasmic membrane may generate a steep concentration gradient inside versus outside the cells. In Gram-negative bacteria in contrast to Gram-positive bacteria the substrates have to move not only through the cytoplasmic membrane but also through the outer membrane. This is usually achieved by diffusion or facilitated diffusion. Diffusion through pore-forming proteins (porins) suffices to meet the growth requirements when the substrates are hydrophilic and not larger than 600 Da [1–4]. Facilitated diffusion with stereospecific binding to the channel proteins was found for maltose and maltooligosaccharides [5], nucleosides and deoxynucleosides [6]. In contrast to the diffusion processes, energy-consuming active transport through the outer membrane poses an unresolved problem since no energy source is known to exist outside the cytoplasmic membrane. However, active transport was proposed for the uptake of Fe^{3+} as siderophore complexes in Gram-negative bacteria and for vitamin B_{12} since these substrates remained bound to their receptors at the surface of unenergized cells, or of mutants which were devoid of the TonB, ExbB or ExbD activities [7,8]. The latter three proteins were implicated in energy-transfer from the cytoplasmic into the outer membrane [7–10]. Very recently decisive progress in the understanding of outer membrane transport was made by the demonstration that receptor proteins form channels [11–13] which may be opened for passing Fe^{3+} siderophores through the outer membrane. Once they have entered the periplasm the Fe^{3+} siderophores are actively transported through the cytoplasmic membrane by a binding-protein-dependent transport mechanism that consumes ATP.

2. The FhuA receptor transports ferrichrome through the outer membrane

The extreme insolubility of ferric iron (10^{-18} M at pH 7) requires solubilization by complexation which microorganisms achieve by siderophores, and higher organisms by binding iron to transferrin, lactoferrin and ferritin. Ferrichrome is one of the siderophores of the hydroxamate type [14]. It contains one Fe^{3+} molecule bound as a trihydroxamate complex in a cyclic hexapeptide consisting of 3 residues δ-*N*-acetyl-δ-*N*-hydroxy-L-ornithine and 3 residues glycine. Ferrichrome is too large to diffuse through the porins (M_r 740) and the concentration would be too low to support growth by simple diffusion through the outer membrane. For this reason ferrichrome binds to the FhuA receptor at the cell surface [15] where it is concentrated to be further translocated

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through the outer membrane. It binds to unenergized cells and to *tonB, exbB* and *exbD* mutants but remains at the cell surface receptor and is not taken up [16]. From the requirement of TonB, ExbB and ExbD for transport it was inferred that these proteins were involved in transport through the outer membrane [7]. Experimental conditions which bypassed the outer membrane bypassed also the need for TonB, ExbB and ExbD supporting the notion that these 3 proteins were only required for transport through the outer membrane and not for transport through the cytoplasmic membrane [7].

3. The basic structure of FhuA is a channel

The transmembrane arrangement of FhuA was derived from three sets of data, (i) the amino acid sequence derived from the nucleotide sequence [17], (ii) a single spontaneous Asp deletion (residue 348) which largely abolished FhuA activity [18] and (iii) the genetic insertion of tetra- to hexadecapeptides at 34 sites along the entire polypeptide [19]. The latter procedure rendered FhuA accessible to proteases, which cleaved either at the cell surface or in the periplasm depending on the location of the inserted peptide [19]. The proposed model (Fig. 1) contained a prominent surface loop (residues 316–356, henceforth termed gating loop) in which the Asp^{348} deletion was located, suggesting the gating loop to be important for FhuA activity. We therefore excised by genetic means portions of the loop. The smallest deletion comprised 12 amino acid residues and extended from residue 322 to 333 (FhuA Δ322–333).

The *fhuA* Δ322–333 gene was overexpressed, the resulting protein heated to 50°C to avoid irreversible denaturation, separated from the outer membrane proteins by SDS-PAGE and then eluted from the gel in buffer. Exactly the same procedure was applied to additional FhuA deletion derivatives, to FhuA wild-type and to an outer membrane totally devoid of FhuA. The FhuA samples were added to the KCl solution of one of two compartments which were separated by a black lipid membrane. Channel formation through spontaneous insertion of FhuA into the black lipid membrane was monitored by measuring the ion current through the FhuA channels in an applied electric field. Conductance was not increased by FhuA wild-type or FhuA ΔAsp^{348} (Fig. 2). However, excision of 12 amino acid residues, which in fact were only 8 residues because the genetic procedure used inserted Pro-Asp-Leu-Ala, resulted in a FhuA deletion derivative (FhuA Δ322–333) which formed stable channels in black lipid membranes (similar to those shown in Fig. 2 for FhuA Δ322–355). Additional derivatives were lacking 22 residues of FhuA plus 3 foreign residues (FhuA Δ334–355) and 34 (+3 residues, FhuA Δ322–355) which all formed stable channels of defined sizes in artificial membranes. Conductance increased the larger the segment was which had been excised within the gating loop (Table 1). Removal of regions extending be-

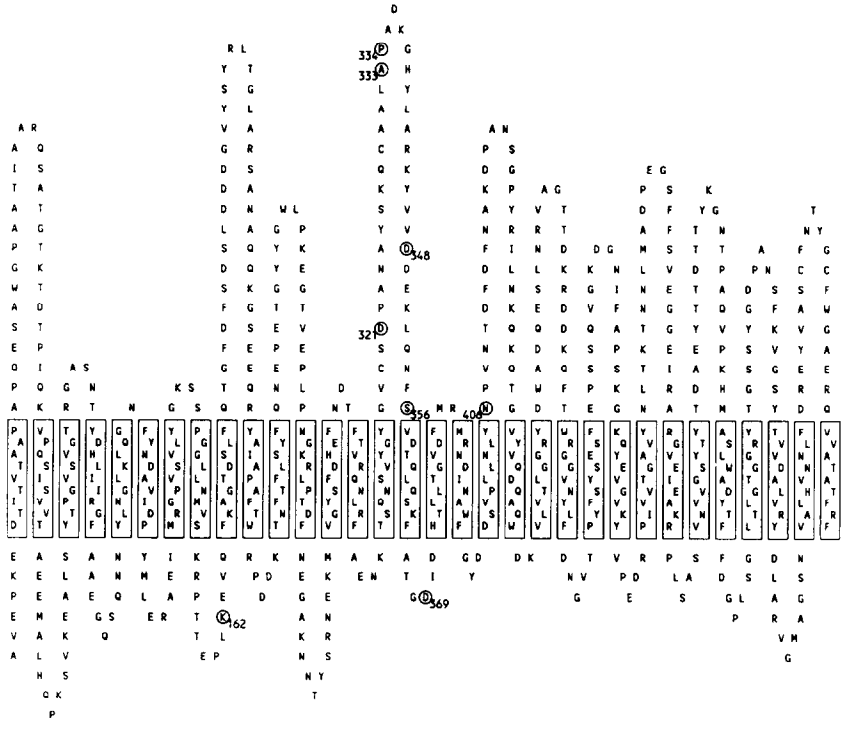

Fig. 1. Predicted transmembrane arrangement of the FhuA protein. Those residues were marked which flanked the deletions in the proposed gating loop.

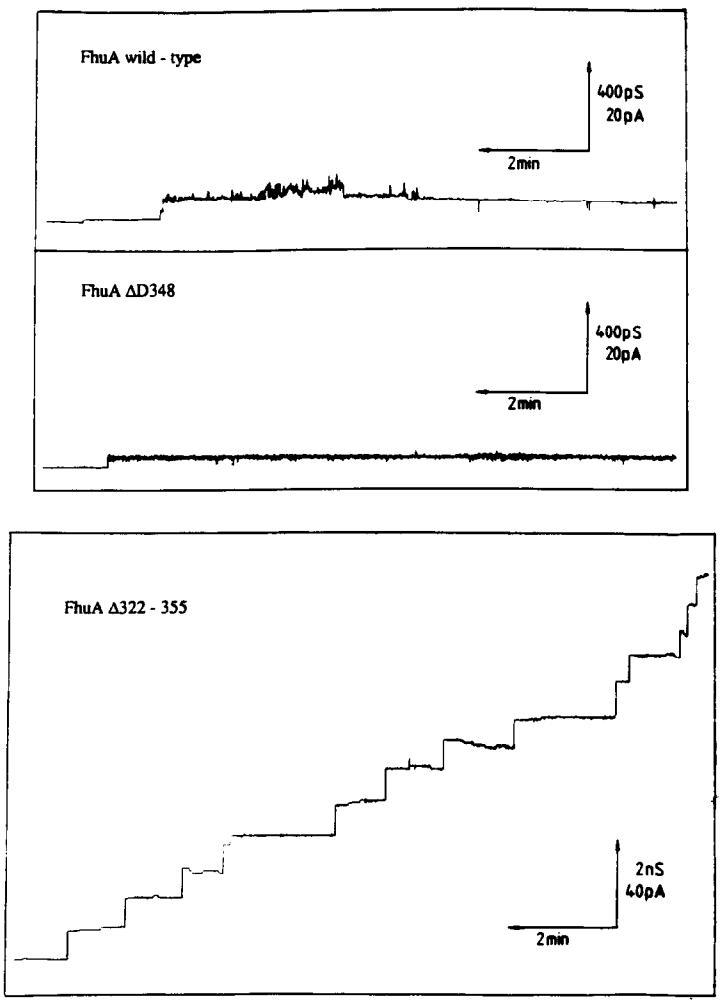

Fig. 2. Single channel recording of diphytanoyl phosphatidyl-choline membranes in the presence of 10 ng/ml each of FhuA wild-type (upper panel), FhuA ΔAsp^{348} (middle panel), and 5 ng/ml of FhuA Δ322–355. The aqueous phase contained 1 M KCl and the applied membrane potential was 50 mV in the experiments shown in the two upper panels, and 0.1 M KCl and 20 mV in the experiment shown in the lower panel. The experiments were performed at 20°C.

yond the gating loop (FhuA Δ322–405, FhuA Δ163–368) resulted in unstable channels which were somewhat smaller than the largest channel within the gating loop (FhuA Δ322–355). Either the channel diameter changed due the instability of FhuA resulting in a smaller average channel conductance, or removal of a larger portion of FhuA made the channel smaller. This would be conceivable if FhuA forms a β-barrel like the porins.

The results obtained with the artificial membranes were supported by growth promotion and transport experiments with viable cells. Cells lacking TonB activity and expressing FhuA wild-type did not grow indicating that the channel of FhuA wild-type was closed. TonB$^-$ cells expressing no FhuA (FhuA Δ1–714) or FhuA ΔAsp^{348} also showed no growth. In contrast, growth of TonB$^-$ cells which expressed FhuA Δ322–333 was weakly supported by ferrichrome as sole iron source (Table 1). Growth of TonB$^-$ cells improved the larger the deletion in the gating loop was (Table 1). The larger FhuA deletion derivatives extending beyond the gating loop allowed permeation of ferrichrome but were slighty less permeable than the largest deletion within the gating loop.

The larger FhuA deletion derivatives supported permeation of SDS and the antibiotic bacitracin which were excluded from passage through outer membranes containing FhuA wild-type or no FhuA. Diffusion of these structurally very different compounds and the low preference of FhuA Δ322–355 in artificial membranes for cations [12] indicated that FhuA Δ322–355 forms large ion-permeable channels.

Transformants of *tonB*$^+$ cells carrying FhuA wild-type showed the largest and the most dense growth zone. The growth zones of the *tonB*$^+$ cells expressing the FhuA deletion derivatives were the same as those of the *tonB*$^-$ cells which indicated that ferrichrome entered the periplasm through the open FhuA channels without the help of TonB. Only the smallest FhuA deletion derivatives,

FhuA ΔAsp^{348} and FhuA Δ322–333, showed better growth in *tonB*$^+$ than in *tonB*$^-$ cells, suggesting a residual response of these FhuA derivatives to TonB.

4. Opening of the channel

Evidence from the older literature that TonB is involved in energy transduction from the cytoplasmic membrane to the receptors in the outer membrane [20] gained support by localization studies of TonB. They demonstrated that TonB was anchored with the N-terminal end in the cytoplasmic membrane while the remainder was localized in the periplasm [21,22]. Site 160 of TonB was implicated in interaction with receptors since mutations in a homologous region among the receptors close to the N-terminal end (TonB box) were suppressed by mutations at site 160 [8,23]. In addition, cellular proteolysis of overexpressed TonB was prevented by overexpressed FhuA which no longer occurred in FhuA mutants with an impaired interaction with TonB [24].

Site-directed mutagenesis demonstrated that the histidine residue in the cytoplasmic membrane was important for TonB activity [25]. This histidine residue is conserved in all TonB proteins hitherto sequenced, included TonB of *Pseudomonas putida* which otherwise shows a much lower homology to the enterobacterial TonB sequences than the enterobacterial TonB proteins among themselves [26]. If TonB somehow changes its conformation in response to the energized state of the cytoplasmic membrane the histidine residue very likely plays an essential function.

TonB-dependent processes were strongly reduced in mutants altered in the *exb* locus. Sequencing of this locus revealed two genes, *exbB* and *exbD*, responsible for the Exb phenotype [27]. Because *exbBD* mutants displayed a residual activity in TonB-dependent reactions, ExbBD functions were considered to be accessory but not essential for TonB activity. This opinion changed when the residual activity of TonB in *exbBD* mutants was traced back to the TolQR activities which could partially replace the ExbBD activities [28,29]. The Tol proteins TolA, TolB, TolQ and TolR form another system by which macromolecules are translocated through the outer membrane [30]. Functional similarity was also demonstrated between TolA and TonB since replacement of the N-terminal region of TonB by the N-terminal region of TolA resulted in a partially active TonB molecule [31]. Both proteins display very different sequences but show homology in the N-terminal membrane portion [31,32].

The first indication of a physical interaction between TonB and ExbB came from studies which showed that plasmid-encoded ExbB prevents proteolysis of plasmid-encoded TonB [33]. ExbB also stabilized ExbD from which it was inferred that the 3 proteins form a complex in which ExbB binds TonB and ExbD. Stabilization of TonB by ExbB was also shown for the chromosomally encoded proteins [9], which could be cross-linked [34]. The preferential interaction of ExbBD with TonB and of TolQR with TolA [28,29,31] was confined to the N-terminal membrane portion which was the only difference between the TolA-TonB fusion protein and TonB [31].

ExbB is mainly located in the cytoplasmic membrane through which it is folded thrice, with the N-terminal end in the periplasm and the C-terminal end in the cytoplasm [31,35]. ExbD has a similar arrangement as TonB with the N-terminal end anchored to the cytoplasmic membrane and the remainder located in the periplasm [36]. ExbB interacts within or close to the cytoplasmic membrane with TonB [25]. ExbB and TolQ, and ExbD and TolR display the highest sequence homologies in the predicted transmembrane segments indicating that these regions are functionally very important [37]. In fact, two point mutants in transmembrane segments of TolQ were inactive [30,37].

Table 1
Properties of FhuA deletion derivatives

	Conductance (nS)	Growth zone (mm)		Transport rate[a]
		TonB$^+$	TonB$^-$	TonB$^-$
FhuA wild-type	0	42(+)	0	0%
FhuA ΔAsp^{348}	0	30(+)	0	ND
FhuA Δ322–333	1.5	26(+)	11(±)	51%
FhuA Δ334–355	1.75	18(+)	19(±)	89%
FhuA Δ322–355	3	26(+)	26(+)	209%
FhuA Δ322–405	2	24(+)	24(+)	129%
FhuA Δ163–368	2	24(+)	24(+)	190%

[a] The transport rates give the numbers of $Fe^{3+} \times 1{,}000$ per cell after 30 min incubation of *E. coli* HK99 *fhuA*$^-$ *tonB*$^-$ transformed with the various *fhuA* derivatives with 20 μM [$^{55}Fe^{3+}$]ferrichrome. The values of the transport rates are related to the transport rate of a FhuA wild-type *tonB*$^+$ cells. Single channel conductance was measured in 1 M KCl by using diphytanoyl phosphatidylcholine/n-decane membranes. (+) means dense; (±) weak growth zone. 0, no conductance, no growth. ND, not determined.

5. Conformational change in FhuA induced by TonB

FhuA not only serves as receptor for ferrichrome but also for the bacteriophages T1, T5, φ80 and colicin M, a protein toxin that kills *E. coli*. These ligands provide ample opportunities to study FhuA activities. Phages T1 and φ80 only bind to energized *ton*B$^+$ *exbB*$^+$ *exbD*$^+$ cells from which it was inferred that FhuA assumes an 'energized conformation' which differs from the 'unenergized conformation', and that TonB serves as coupling device between the energized cytoplasmic membrane and FhuA in the outer membrane [38]. Ferrichrome prevented binding of T5 to unenergized cells or to *tonB* mutants which demonstrated the remaining of ferrichrome at FhuA in

contrast to energized *tonB*$^+$ cells [16]. Colicin M remained bound to FhuA at the cell surface of unenergized or *tonB*$^-$ cells where it was degradable by added proteases in contrast to energized *tonB*$^+$ cells where it was taken up into the cells within a few minutes [39]. Although binding and infection by phage T5 does not need energized *tonB*$^+$ cells certain FhuA mutant proteins support T5 infection much better when combined with certain TonB mutant proteins [40] which suggests a change of the less active FhuA conformations into conformations that more adequately meet the structural requirements for T5 infection.

6. Conclusions

Isolated FhuA formed a closed channel which was converted into a TonB-independent permanently open channel by deletion of segments in the largest surface loop which we consider to be the principal gating loop that controls FhuA permeability. The highest conductance was obtained with the largest deletion of 34 residues within the gating loop (FhuA Δ322–355). The width of the channel derived from the conductance was about three times larger than the channels formed by the porins and showed little specificity for the solutes that passed through [12]. These data suggest that FhuA also forms closed channels in unenergized and in *tonB*$^-$, *exbB*$^-$ and *exbD*$^-$ cells. The closed form is the thermodynamicaly stable state. Opening of the channel consumes energy which is provided by the electrochemical potential of the cytoplasmic membrane through the action of the TonB–ExbB–ExbD protein complex. Energy transfer from the cytoplasmic membrane to FhuA in the outer membrane is thought to be achieved by TonB which is anchored in the cytoplasmic membrane and physically interacts with FhuA. The way TonB opens the FhuA channel may resemble the mechanism how a regulatory subunit of an enzyme allosterically controls the activity of the catalytic subunit. TonB adopts an energized conformation that opens FhuA. The open state of FhuA must be short since it does not increase the permeability of the outer membrane for solutes which have no FhuA binding specificity. In a growing cell FhuA fluctuates between the open and the closed state. Each time energy is provided by TonB FhuA is opened. On average only 0.1 molecule of ferrichrome pass through a FhuA channel per second in a rapidly growing cell which is many orders of magnitude below the rate potassium flows through gated channels of nerve cell membranes (10^7/s). The low ferrichrome transport rate may come from the strong binding of ferrichrome to FhuA and / or from the low frequency at which the FhuA channels are opened.

The conformational change of FhuA induced by TonB not only opens the channel but also dissociates ferrichrome from FhuA. Of the other FhuA ligands phage T1 and φ80 bind to the open state, phage T5, colicin M and the antibiotic albomycin bind to the closed state, if there exist no intermediary states between closed and open. Conceptually the major unresolved question is how TonB in the energetic ground state is converted to the energized conformation by the electrochemical potential of the cytoplasmic membrane, and what role ExbB and ExbD play in this reaction.

FepA is another receptor of which 135 and 139 residues were excised resulting in an open channel through which ferric enterobactin, ferrichrome, saccharides, SDS and antibiotics diffused without the help of TonB [11,13]. It is thus conceivable that all the ferric siderophore receptors and the vitamin B_{12} receptor form closed channels which are openend in an energy-coupled process through the action of TonB, ExbB and ExbD.

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