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Iron(III) hydroxamate transport across the cytoplasmic membrane of *Escherichia coli*

Wolfgang Köster

Mikrobiologie II, Universität Tübingen, Auf der Morgenstelle 28, W-7400 Tübingen, Federal Republic of Germany

Summary. Transport of iron(III) hydroxamates across the inner membrane into the cytoplasm of *Escherichia coli* is mediated by the FhuC, FhuD and FhuB proteins and displays characteristics typical of a periplasmic-binding-protein-dependent transport mechanism. In contrast to the highly specific receptor proteins in the outer membrane, at least six different siderophores of the hydroxamate type and the antibiotic albomycin are accepted as substrates. A *fhuB* mutant (deficient in transport of substrates across the inner membrane) which overproduced the periplasmic FhuD 30-kDa protein, bound [^{55}Fe] iron(III) ferrichrome. Resistance of FhuD to proteinase K in the presence of ferrichrome, aerobactin, and coprogen indicated binding of these substrates to FhuD. FhuD displays significant similarity to the periplasmic FecB, FepB, and BtuE proteins. The extremely hydrophobic FhuB 70-kDa protein is located in the cytoplasmic membrane and consists of two apparently duplicated halves. The N- and C-terminal halves [FhuB(N) and FhuB(C)] were expressed separately in *fhuB* mutants. Only combinations of FhuB(N) and FhuB(C) polypeptides restored sensitivity to albomycin and growth on iron hydroxamate as a sole iron source, indicating that both halves of FhuB were essential for substrate translocation and that they combined to form an active permease. In addition, a FhuB derivative with a large internal duplication of 271 amino acids was found to be transport-active, indicating that the extra portion did not disturb proper insertion of the active FhuB segments into the cytoplasmic membrane. A region of considerable similarity, present twice in FhuB, was identified near the C-terminus of 20 analyzed hydrophobic proteins of periplasmic-binding-protein-dependent systems. The FhuC 30 kDa protein, most likely involved in ATP binding, contains two domains representing consensus sequences among all peripheral cytoplasmic membrane proteins of these systems. Amino acid replacements in domain I (Lys→Glu and Gln) and domain II (Asp→Asn and Glu) resulted in a transport-deficient phenotype.

Offprint requests to: W. Köster

Key words: *E. coli* - Iron transport - *fhuCDB* - Ferric hydroxamate binding - ATP binding

Introduction

Uptake of ferric hydroxamates across the outer membrane of *Escherichia coli* requires highly specific receptor proteins. Their activity depends on the cytoplasmic-membrane-associated TonB, ExbB, and ExbD proteins (Braun et al. this issue and references therein). For the subsequent translocation across the inner membrane, a single system (composed of the FhuC,D,B proteins) accepts all ferric hydroxamates and exhibits properties typical of periplasmic-binding-protein-dependent transport systems (Fecker and Braun 1983; Braun et al. 1983; Köster and Braun 1986, 1989; Burkhardt and Braun 1987; Coulton et al. 1987). The *fhuACDB* genes are organized in an operon (Fig. 1).

Recent studies indicated that the hydrophilic FhuD protein, carrying a potential signal sequence, was synthesized as a precursor and exported through the cytoplasmic membrane. Since it was localized in the periplasm, the FhuD protein was a candidate for an iron(III)-hydroxamate-binding protein. Although soluble proteins in the periplasmic space were found to be involved in transport of siderophores of different types (Pierce and Earhart 1986; Ozenberger et al. 1987; Köster and Braun 1989; Staudenmaier et al. 1989; Elkins and Earhart 1989), no binding to any of these proteins has been shown. Therefore, [^{55}Fe]iron(III) ferrichrome uptake in a FhuD-overproducing strain was measured, and resistance of FhuD against proteinase K was studied in the absence and presence of different iron(III) hydroxamates. The transmembrane component mediating the translocation of iron(III) hydroxamates across the cytoplasmic membrane was identified as the FhuB protein. This extremely hydrophobic integral membrane protein has twice the size of most nonpolar components of binding-protein-dependent transport systems. The N- and C-terminal halves exhibit a striking

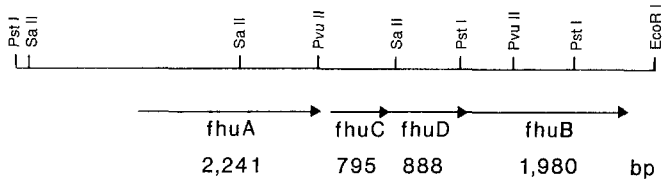

Fig. 1. Physical map of the *fhu* locus. The horizontal arrows indicate the location, size, and transcription polarity of the *fhuA,C,D, B* genes. The numbers of base pairs is given below. For *fhuC-fhuD* and *fhuD-fhuB* overlapping translation stop and start signals are found

similarity to each other (Köster and Braun 1986, 1989), to BtuC of vitamin B_{12} transport (Friedrich et al. 1986), to FecC and FecD of iron(III) dicitrate transport (Staudenmaier et al. 1989), and to FepD and FepG of iron(III) enterochelin transport (S. Chenault, C. Earhardt and M. McIntosh, personal communications). It was of interest to see whether both halves of the FhuB protein, seemingly derived from an ancient fusion of two identical or similar genes, were required for transport, or whether one half was sufficient. Moreover, the question was studied as to whether the proposed two halves of FhuB acted as separate entities (functional and structural domains), or whether they had to be linked in a single polypeptide.

The FhuC protein has been assigned an inner membrane location (Fecker and Braun 1983; Köster and Braun 1989). It is thought to be a peripheral cytoplasmic membrane protein facing the cytosol, and since its primary structure displays regions of strong similarity to those found in ATP-binding proteins of pro- and eucaryotes (Burkhardt and Braun 1987; Coulton et al. 1987), it is most likely involved in energy coupling. Typical for the hydrophilic inner-membrane-associated proteins periplasmic-binding-protein-dependent systems are two highly conserved regions, in the following designated domain I and II. ATP binding has been shown with HisP, which is involved in histidine uptake in *Salmonella typhimurium* (Hobson et al. 1984) and OppD of the oligopeptide transport system (Gallagher et al. 1889). Direct evidence for ATP hydrolysis concomitant with the transport process was obtained for histidine uptake into reconstituted proteoliposomes (Ames et al. 1989; Bishop et al. 1989), and maltose uptake into right-side-out vesicles (Dean et al. 1989) and into proteoliposome vesicles (Davidson and Nikaido 1990). We introduced point mutations into the two potential ATP-binding domains of FhuC to study the influence on iron(III) hydroxamate transport (Becker et al. 1990).

FhuD protein

Binding of iron(III) hydroxamates

The periplasmic FhuD protein was found to be absolutely necessary for uptake of ferric hydroxamates into the cell. This was concluded from the finding that strains KO293(*fhuD*) and KO295(*fhuD*) were resistant to albomycin and unable to grow on ferrichrome and coprogen as sole iron sources, but could be complemented by plasmid pWK480 carrying only the *fhuD* gene inserted into the pT7-5 vector (Tabor and Richardson 1985) downstream of the phage T7 gene 10 promoter. Complementation to albomycin sensitivity and to wild-type-like growth was achieved even in the absence of the T7 RNA polymerase.

Binding of [^{55}Fe]iron(III) ferrichrome to *E. coli* cells was determined in strains KO293 *fhuD*, KO280 *fhuB*, KO280 *fhuB* (pWK480), HE21 *fhuB fhuA* (pWK480) and strain HE22 *fhuB tonB* (pWK480). For this experiment the pWK480(*fhuD*$^+$) bearing strains also carried pGP1-2 (coding for the T7 RNA polymerase; Tabor and Richardson 1985) which led to FhuD overproduction under the conditions used. The *fhuB* strains were used to prevent uptake into the cytoplasm resulting in an accumulation of the radioactive labelled substrate in the periplasm. The experiment was carried out as a pulse/chase experiment, because reversible binding to FhuA and FhuD was expected. Cells of the logarithmic growth phase were incubated for 7 min with [^{55}Fe]iron(III) ferrichrome (1.5 M). After 8 min, 760 μM non radioactive ferrichrome was added and further incubated for 9 min. Samples were withdrawn, filtered and washed twice with 5 ml 10% LiCl as described previously (Hantke and Zimmermann 1981). Prior to labeling, cells were grown at 30° C in nutrient broth to a density of 4×10^8 cells/ml, harvested, suspended in medium, incubated for 30 min at 42° C and then for 60 min at 37° C. From the radioactivity the iron ions

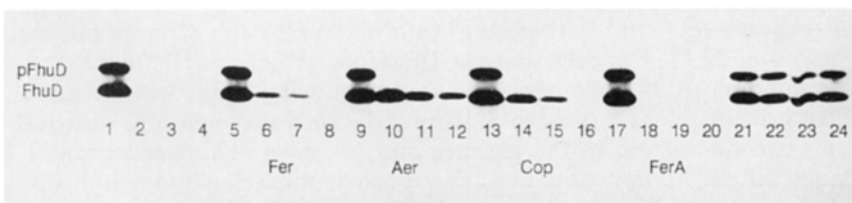

Fig. 2. Protection of the FhuD protein against proteinase K digestion by iron(III) hydroxamates. [^{35}S]Methionine-labeled FhuD protein in the periplasmic fraction of the FhuD-overproducing strain AB2847(pWK480 pGP1-2) was treated with proteinase K (30 μl/ml, 50° C) in the absence (lane 1–4) or in the presence of 30 μM ferrichrome (5–8), aerobactin (9–12), coprogen (13–16), and ferrichrome A (17–20). Lanes 21–24 show the untreated control. Samples were taken after 0, 30, 60, and 120 min. Only the relevant section of the fluorography after SDS/PAGE is shown

bound per cell were calculated. The values for KO293 (pulse: 5400/chase: 2000), KO280 (6900/2200), HE21 (pWK480) (5100/2500), and HE22 (pWK480) (6500/2800) were significantly lower than for KO280 (pWK480) (17400/2600). Release from the periplasmic FhuD was slower than from FhuA and the unspecific binding sites. These data, showing that the periplasmic FhuD is essential for ferric hydroxamate transport, fit perfectly with the model of a FhuA- and TonB-dependent uptake across the outer membrane and a periplasmic-binding-protein-dependent transport across the inner membrane (Köster and Braun 1990b).

Evidence for binding of ferric hydroxamates to the FhuD protein was also supported by the fact that ferrichrome, aerobactin, and coprogen were able to protect FhuD against proteinase K digestion (Fig. 2). No protection was obtained with ferrichrome A, ferric dicitrate, and Fe^{3+} ions or in the absence of any iron(III) hydroxamate. The effect was specific to FhuD, since pro-FhuD and bovine serum albumin were degraded at the same rate in the presence or absence of the relevant siderophores (Köster and Braun 1990b).

Similarity of FhuD to FecB, FepB, and BtuE

The uptake systems for iron(III) hydroxamates, iron(III) dicitrate, iron(III)-enterochelin, and vitamin B_{12} share many similar properties. Although the precise function of BtuE (DeVeaux et al. 1986) has not been determined, in all these systems the existence of a periplasmic component presumably involved in substrate binding was reported (Taylor et al. 1972; Bradbeer et al. 1978; Pierce and Earhart 1986; Elkins and Earhart 1989; Köster and Braun 1989; Staudenmaier et al. 1989). Therefore, it was of interest to see if similarities were also established in the polypeptide primary structures. An alignment of the FhuD, FecB, FepB, and BtuE amino acid sequences is presented in Fig. 3, demonstrating a striking similarity along the entire length. Especially noteworthy is the existence of several motifs, often including proline residues, which may be structurally important. Interestingly, some of these characteristic motifs were detected in BtuE which was found to be not essential for vitamin B_{12} uptake (Rioux and Kadner 1989). BtuE is only about 60% of the size of the other polypeptides and seems to be the least conserved protein in this group. Possibly this gene represents an incomplete copy of an ancestral gene.

These data, together with the evidence for iron(III) hydroxamate binding to FhuD, strongly support the idea that FhuD, FecB, FepB and possibly BtuE play an equivalent role in the corresponding uptake systems. The functional and structural relatedness suggests a common origin for the periplasmic binding proteins of the 'iron transport family'.

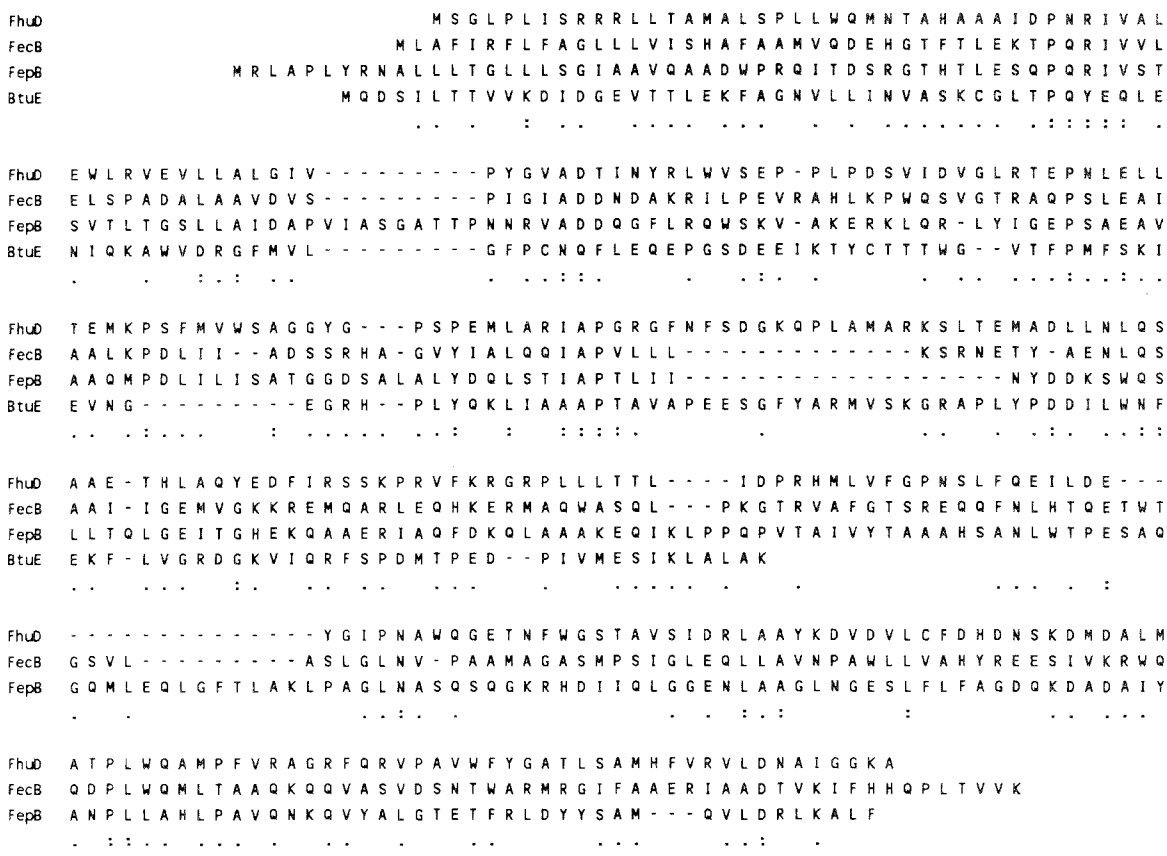

Fig. 3. Alignment of the FhuD, FecB, FepB and BtuE primary sequences. Identical amino acids in two proteins are indicated by dots, identical residues in at least three polypeptides are marked by colons below the sequences

FhuB protein

Model of FhuB membrane topology

The extremely hydrophobic FhuB protein exhibits all characteristics typical of an integral inner-membrane protein. Based on the amino acid sequence and the hydrophobicity profile (Köster and Braun 1986), a two-dimensional model of the topology of the FhuB protein in the cytoplasmic membrane is presented in Fig. 4. The model predicts 20 transmembrane segments termed 1–10 for FhuB(N) and 1a–10a for FhuB(C) to indicate homologous regions. The spacer region between the two homologous parts of FhuB (represented by the heavy line in the cytosolic loop connecting transmembrane segments 10 and 1a, Fig. 4) consists of only six amino acids, and is too short to function as a membrane-spanning segment. As a result the hydrophilic N- and C-termini of FhuB(N) and FhuB(C) are located on the same side of the membrane. Only a small loop lacking any charged amino acids exists between the apolar segments 7/8 and 7a/8a so that these regions may not span the membrane their entire length, but be embedded in or associated with the lipid bilayer. Charged amino acids are exclusively located at the end of hydrophobic spans or within the connecting loops except for a highly concerved Asp in the nonpolar stretches 9 and 9a. Remarkably, the basic amino acids Lys and Arg are three times less prevalent in the proposed periplasmic than in the cytosolic connecting loops. The net charge is -2 at the periplasmic and $+22$ at the cytoplasmic side of the membrane. From this point of view the model fits perfectly with the 'positive inside rule' for integral proteins of the bacterial cytoplasmic membrane and eukaryotic organelle membranes (von Heijne 1986). Arg and Lys residues are found 2–4 times more in the nontranslocated segments than in the translocated domains of complex membrane proteins (von Heijne and Gavel 1988). In further support of the model it has been shown for several integral membrane proteins that the positively charged N-terminus preceding a transmembrane region is located at the cytoplasmic side of the inner membrane (Eckert and Beck 1989; von Heijne 1989). The loops (Fig. 4) are potential candidates for substrate binding and interaction with the periplasmic FhuD and the cytoplasmic FhuC protein. The same criteria as used for FhuB resulted in models consisting of 10 transmembrane segments for the functionally equivalent and structurally similar FecC, FecD and BtuC proteins, which, however, are only half the size of FhuB.

Separate expression of FhuB(N) and FhuB(C)

E. coli KO280(*fhuB*) is deficient in the uptake of siderophores of the hydroxamate type (ferrichrome, aerobactin, coprogen) and the antibiotic albomycin. Under iron-limiting conditions no gowth on NBD (nutrient broth, 200 μM 2,2′-bipyridyl) plates is detectable since synthesis of enterochelin (enterobactin) is blocked due to an *aroB* mutation. Transport of iron(III) hydroxymates and sensitivity to albomycin was restored by pWK339 encoding wild-type FhuB (Köster and Braun 1986; Fig. 5).

A number *fhuB* derivatives were cloned into plasmids pUC19 (Yanish-Perron et al. 1985) and pSU9 (Martinez et al. 1988). To determine the activity of the FhuB derivatives, strain KO280 was transformed with the plasmids listed in Fig. 5. None of the truncated FhuB derivatives restored albomycin sensitivity to strain KO280. On NBD plates no growth zone was detectable around filter paper discs impregnated with ferrichrome, coprogen, shizokinen or rhodotorulic acid. Growth was supported by dihydroxybenzoic acid which is transported via the *fep* uptake system. To examine whether the separately cloned and independently expressed FhuB(N) and FhuB(C) proteins combined in one cell were able to restore transport activity of chromosomal *fhuB*$^-$ cells, various combinations of genetically compatible plasmids were tested. In agreement with former observations, it was found that both FhuB(N) and FhuB(C) are required for iron(III) hydroxamate transport across the inner membrane. Most striking was the finding that FhuB(N) and FhuB(C) did not have to form a single polypeptide chain. This is a very interesting case in which an integral membrane protein was cut into two halves which were individually

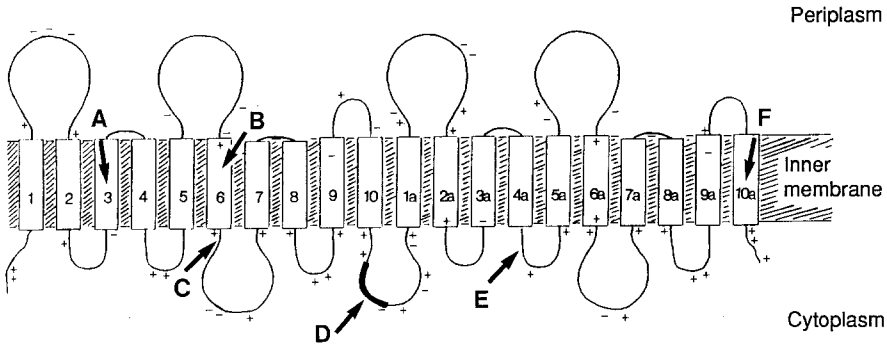

Fig. 4. Two-dimensional schematic model of the FhuB topology in the cytoplasmic membrane. A+ indicates Arg, Lys, and a −Asp, Glu residues. Transmembrane segments are represented by boxes numbered 1–10 and 1a–10a to demonstrate the internal homology. Arrows designated A–F point to positions at which the FhuB derivatives were altered. The heavy line in the cytosolic loop connecting segments 10 and 1a represents the 'spacer' of six amino acids which combines the homologous parts of FhuB

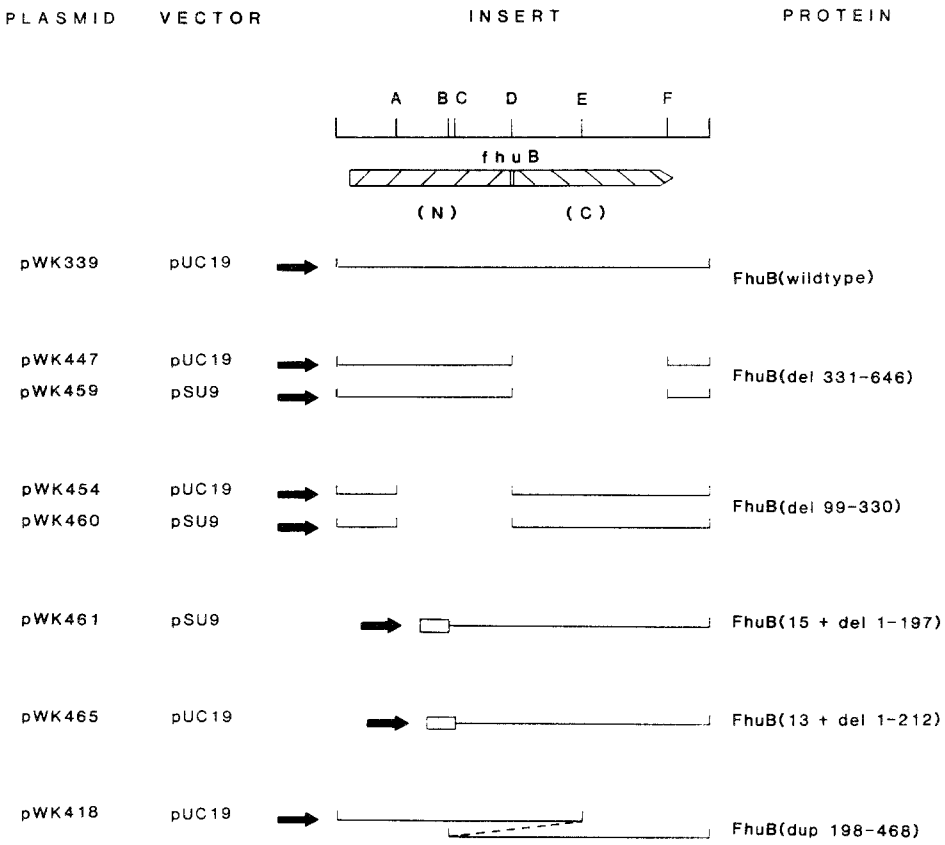

Fig. 5. Physical map of the *fhuB* region and list of plasmids carrying various portions of the gene. Vertical lines designated A–F correspond to the arrows A–F used in Fig. 4 and represent cleavage sites of relevant restriction enzyme (A, D, F = *Bss*HII, B = *Pvu*II, C = *Hpa*I, and E = *Nae*I). The hatched arrow symbolizes the orientation and extension of *fhuB* subdivided into *fhuB*(N) and *fhuB*(C) to demonstrate the internal homology. The *E. coli* UV5 promoter region is represented by filled arrowheads. The open bars stand for the 'in-frame' cloned polylinker-derived codons (see text)

inactive but functional when combined. Complementation of KO280 to albomycin sensitivity and growth on iron(III) hydroxymates was achieved by FhuB (del 331–646) in combination with three different FhuB derivatives carrying deletions in the N-terminal half (Figs. 4, 5). FhuB (del 331–646) had lost the region between arrows D and F (Fig. 4) which was almost the complete FhuB(C). The remainder of the nonpolar segment 10a was fused to the C-terminal of FhuB(N). The highest activity of this C-terminal-deleted FhuB protein was obtained in combination with FhuB (13+ del 1–212). In this construction, expressed under lacUV5 promoter control, the 212 N-terminal amino acids of FhuB were replaced by 13 residues encoded by the pUC polylinker. FhuB (13+ del 1–212) starts with a cytoplasmic loop (at arrow C, Fig. 4) fused to 13 mainly polar amino acids. In both constructs FhuB (del 331–646) and FhuB (13+ del 1–212) the insertion and proper orientation within the lipid bilayer of an intact FhuB(C) and FhuB(C) seems to be maintained. Since sensitivity to albomycin and growth with hydroxamates as sole iron sources was comparable to the wild-type FhuB, it is evident that the restored FhuB activity was sufficient to supply the cells with iron. The FhuB polypeptide was not precisely cut into two halves in the presumed linker region but rather contained additional sequences which most likely interfered with the correct insertion and the assembly of the two parts in the cytoplasmic membrane. It was also expected that only a small portion of the separately synthesized FhuB(N) and FhuB(C) polypeptides find each other in the cytoplasmic membrane and assemble correctly into a functional permease. From these points of view it was more surprising to gain transport activities with the constructions used. An unspecific import of the iron(III) hydroxamates into the cytoplasm as a result of a structurally disturbed inner membrane due to the improper insertion of the FhuB halves could be excluded. No transport activity was obtained with only one half of FhuB, and the wild-type *fhuB*$^+$ but *fhuC*$^-$ strain KO286 (pWK 459, pWK465) and the *fhuB*$^+$*fhuD*$^-$ strain KO293 (pWK459, pWK465).

The two other N-terminal deletions were less effective. Cells synthesizing FhuB (del 331–646) in combination with FhuB (del 99–330), or alternatively FhuB (15-del 1–197), were partially sensitive to albomycin and exhibited weak growth on NBD plates with the siderophores tested. FhuB (del 99–330) was missing a major part of FhuB(N) between arrows A and D (Fig. 4), FhuB (15+ del 1–197) consisted of 15 mainly hydrophilic residues followed by the rest of FhuB (starting at arrow B, Fig. 4). In both derivatives amino acids originally located within presumed membrane-imbedded segments were fused to hydrophilic domains. This may have affected insertion of these derivatives into the membrane. (Köster and Braun 1990a).

The plasmid pWK418 (Fig. 5) conferred albomycin sensitivity and growth on iron(III) hydroxamates to the same extent as pWK339. FhuB (dup 198–468) consists of the three major domains, FhuB(N) and FhuB(C) joined by the N-terminal part of FhuB(C) (up to arrow E, Fig. 4) and the C-terminal portion of FhuB(N) (start-

ing from arrow B, Fig. 4). The model predicts one fusion site to be located in a transmembrane segment and the other in a cytosolic loop. Surprisingly, the 271 additional amino acids between FhuB(N) and FhuB(C) did not abolish substrate binding and translocation across the cytoplasmic membrane. This indicates a high degree of plasticity of single FhuB subdomains imbedded in the membrane (Köster and Braun 1990a).

On SDS/polyacrylamide gels FhuB was only detectable as a diffuse band after radiolabelling; its electrophoretic mobility was dependent on the experimental conditions employed (Köster and Braun 1986, 1989). Upon heating in SDS, it formed aggregates which barely entered the separating gel. These uncommon properties, which FhuB shares with several other integral membrane proteins, could be explained by its very hydrophobic nature. The wild-type protein and the FhuB derivatives were expressed in the T7 system (Tabor and Richardson 1985). FhuB derivatives carrying the internal deletions and the duplication were made in amounts similar to the wild-type protein. After SDS/PAGE followed by fluorography, radioactive polypeptides were detected for all of the FhuB derivatives. In general, the FhuB derivatives exhibited the same unusual properties as wild-type FhuB (Köster and Braun 1990a).

Similarities between integral membrane proteins

No extensive similarities have been reported among integral membrane proteins of periplasmic-binding-protein-transport systems. Regions of significant similarity have only been observed in those nonpolar components which are involved in translocation of identical or similar substrates, such as siderophores and vitamin B_{12}, or certain sugars. The conserved region EAA---G---- ----I-LP found at a distance of about 90 amino acids from the C-terminus of the MalG, MalF, HisM, HisQ, PstA, PstC, and OppC proteins (Dassa and Hofnung 1985) is not present in FhuB (Köster and Braun 1986) or BtuC (Friedrich et al. 1986). C-terminal sequences of hydrophobic proteins of binding-protein-dependent uptake systems were analyzed. As shwon in Fig. 6, a region of significant similarity comprising 32 amino acids was found in each of the 20 proteins studied. This region includes the published sequence (Dassa and Hofnung 1985) and is present in FhuB(N) and FhuB(C). Although the sequence EAA (three residues upstream of the highly conserved G at position 17 in Fig. 6) was found in only 8 out of 20 proteins, and I-LP (position 27–31) in only 4, the percentage of identical amino acids in two randomly chosen proteins was high. Remarkable is a 47% identity between ProW from *E. coli* and SfuB from *Serratia marcescens,* since these proteins are involved in the uptake of completely different substrates (glyine betaine and iron, respectively) and originate from different organisms. The integral membrane proteins of the 'iron transport family' show a stronger similarity to each other and to RsbC, AraH, OppB, and OppC than to other proteins. The most conserved amino acids are localized between positions 10–20 (Fig. 6).

The integral membrane proteins of osmotic-shock-sensitive transport systems are thought to interact with the corresponding ATP-binding proteins which display a substantial similarity to each other (Ames 1986; Higgins et al. 1988). If structurally conserved regions of the

Protein	Conserved region	Distance	Reference
FhuB(N)	R P L T L M G L D D G V A R N L G L A L S L A R L A A L S L A I	417	Köster and Braun 1986
FhuB(C)	R W L T I L P L G G D T A R A V G M A L T P I R I A L L L L A A	86	Köster and Braun 1986
BtuC	R P M N M L A L G E I S A R Q L G L P L W F W R N V L V A A T G	86	Friedrich et al. 1986
FecC	N Q L N L L N L S D S T A H T L G V N L T R L R L V I N M L V L	84	Staudenmaier et al. 1989
FecD	R D L D L L A L A L G D A R A L G V S V P H I R F W A L L L A V	85	Staudenmaier et al. 1989
LivH	R A C R A C A E D L K M A S L L G I N T D R V I A L T F V I G A	95	Nazos et al.1986
RbsC	R Y I Y A V G G N E A A T R L S G I N V N K I K I I V Y S L C G	93	Bell et al.1986
AraH	R N T L A I G G N E E A A R L A G V P V V R I K I I I F V L S G	99	Scripture et al. 1987
OppB	M I E V L H S N F I R T A R A K G L P M R R I I F R H A L K P A	78	Hiles et al. 1987
OppC	T L S L K R K E F I E A A Q V G G V S T A S I V I R H I V P N V	84	Hiles et al. 1987
ProW	G I N Q V P A D L I E A S R S F G A S P R Q M L F K V Q L P L A	96	Gowrishankar 1989
SfuB	G I A Q A P V E L E N V A R S L G K S P A Q A L W S T T L R L A	77	Angerer et al. 1990
ChlJ	A L E G V D V K L E Q A A R T L G A G R W R V F F T I T L P L T	84	Johann and Hinton 1987
MalG	Y F E T I D S S L E E A A A L D G A T P W Q A F R L V L L P L S	85	Dassa and Hofnung 1985
MalF	L L K A I P D D L Y E A S A M D G A G P F Q N F F K T T L P L L	92	Froshauer and Beckwith 1984
UgpA	A L Q S I P R S L I E A A A I D G A G P I R R F F K I A L P L I	84	Overduin et al. 1988
UgpE	Q F F M L P D E L V E A A R I D G A S P M R F F C D I V F P L S	85	Overduin et al. 1988
HisM	A I R S V P H G E I E A A R A Y G F S S F K M Y R C I I L P S A	80	Higgins et al. 1982
HisQ	A F M A V P K G H I E A A T A F G F T H G Q I F R R I M F P A M	82	Higgins et al. 1982
PstC	V F E Q T P V M M K E S A Y G I G C T T W E V I W R I V L P F T	96	Surin et al.1985
PstA	M L K L V P Y S L R E A A Y A L G T P K W K M I S A I T L K A S	90	Surin et al. 1985

Fig. 6. Conserved region in the primary structure of hydrophobic components of binding-protein-dependent transport systems. Identical amino acids at equivalent positions in several proteins are underlined. Numbers following the sequences give the distance to the C-terminus

peripheral membrane proteins are involved in the interaction with the integral membrane proteins, similar sequences should also be present in the integral membrane proteins. The region EAA---G--------I-LP present in several nonpolar proteins was taken as an evidence for such an interaction (Dassa dand Hofnung 1985; Hiles et al. 1987). Although this motif was not found in the Fhu, Btu, Fec and several other proteins, the conserved region (Fig. 6), present twice in FhuB and once in all the otherwise different nonpolar proteins, may interact with the membrane-associated ATP-binding proteins. In the predicted model of FhuB (Fig. 4) the major part of this region forms a cytosolic loop connecting segments 6 and 7 (6a and 7a) so that an interaction with FhuC would be possible. In the model of MalF (Froshauer et al. 1988) the conserved region is likewise located on the cytoplasmic side of the inner membrane, forming a hydrophilic loop which connects two membrane-spanning regions. The predicted MalF topology differs from the FhuB model in that the conserved region is followed by two (and not four) transmembrane segments.

FhuC protein

Amino acid replacements in potential ATP-binding sites

For the transport of iron(III) hydroxamates, the hydrophilic FhuC protein was found in the cytoplasmic membrane fraction and exhibited strong similarities to the two conserved domains of ATP-binding proteins. By oligonucleotide-site-directed mutagenesis, based on the method of Nakamaye and Eckstein (1986), point mutations were introduced into the two domains of FhuC which form the presumed ATP-binding sites. Lysine residue 50 (domain I) was replaced with glutamine and glutamate, and aspartate residue 172 (domain II) with asparagine and glutamate. In addition, a deletion from residue 180 to residue 207 following domain II was introduced. As a control, asparagine 16 was substituted by aspartate. The mutagenized *fhuC* genes were cloned into the low-copy-number vector pHSG575 (Takeshita et al. 1987), the high-copy-number plasmid pUC18 (Yanish-Perron et al. 1985) and, since the FhuC protein can only be observed when overexpressed, into the expression vector pT7-5 (Tabor and Richardson 1985). Therefore, *fhuC* and its derivatives cloned downstream of the gene 10 promoter of phage T7 were transcribed by the T7 RNA polymerase in *E. coli* BL21(DE3) (Moffatt and Studier 1987). All subclones used for complementation assays produced FhuC proteins in amounts similar to wild-type FhuC. The FhuC proteins were of the expected molecular masses of 30 kDa, except for the protein carrying the deletion. None of the *fhuC* derivatives, whether on low-copy or high-copy vectors, were able to restore sensitivity to albomycin and growth on ferrichrome when transformed into the *fhuC* mutants LF947 (Fecker and Braun 1983) and KO281. The exception was *fhuC* (N16D) carrying a mutation outside the ATP-binding domains which conferred albomycin sensitivity and growth on ferrichrome to the same level as pWK380 carrying *fhuC* wild type.

Walker et al. (1982) detected two domains whose sequences were conserved in ATP-binding proteins. Detailed investigations on the adenylate kinase of eukaryotic cells (Fry et al. 1986), and the F_1-ATPase of *E. coli* (Duncan et al. 1986) revealed the consensus seqeunce GXXGXGKT in domain I (X are arbitrary amino acids), and ZZZZDEP in domain II (Z are hydrophobic amino acids). The membrane-associated transport proteins of the periplasmic-binding-protein-transport systems display the same motifs with the exception that nearly all contain S instead of T in domain I (Higgins et al. 1988; Staudenmaier et al. 1989; Angerer et al. 1990). Domain I seems to form a loop in which the lysine residue (number 50 in FhuC) is directly involved in nucleotide binding. The aspartate residue (number 172 of FhuC) of domain II is thought to bind Mg^{2+}-ATP directly. The only example of a prokaryotic transport protein where the substitutions G48I and K49Q in domain I abolished transport activity is the PstB protein for phosphate transport (Cox et al. 1989). No amino acid replacement was described for domain II. Lysine 50 and aspartate 172 were selected because both seem to play a direct role in ATP binding and hydrolysis. Surprisingly, substitution of aspartate by glutamate completely abolished FhuC activity. Domain II has been proposed to form a pocket from which water is excluded with the help of the four hydrophobic amino acid residues. For water exclusion the pocket has to fit the shape of the nucleotide tightly. Apparently, extension of aspartate by a CH_2 group to form glutamate suffices to disrupt nucleotide binding or hydrolysis. Replacement of aspartate by asparagine also abolished activity which can be explained if the negatively charged carboxyl group is required for Mg^{2+}-ATP binding. Substitution of lysine 50 by glutamine and glutamate altered the charge and the size of this particular amino acid and rendered FhuC inactive. The results are consistent with the hypothesis that the two domains represent active sites of the FhuC protein and are involved in ATP-coupled energization of iron(III) hydroxamate transport across the inner membrane. (Becker et al. 1990).

Model of ferric hydroxamate transport

Uptake of siderophores of the hydroxamate type and albomycin across the outer membrane is catalyzed by the FhuA, FhuE, and Iut receptors and the TonB, ExbB, ExbD proteins. Translocation of all the iron(III) hydroxamate compounds across the cytoplasmic membrane is mediated by the FhuB,C,D protein (Fig. 7).

FhuD in the periplasmic space is thought to bind the substrates and to pass them to FhuB. It has not been shown whether FhuD has to take the substrates directly from the receptor, which may include a physical interaction between the two proteins, or whether the substrates are released into the periplasm. From the similarity of the primary sequences, it can be concluded

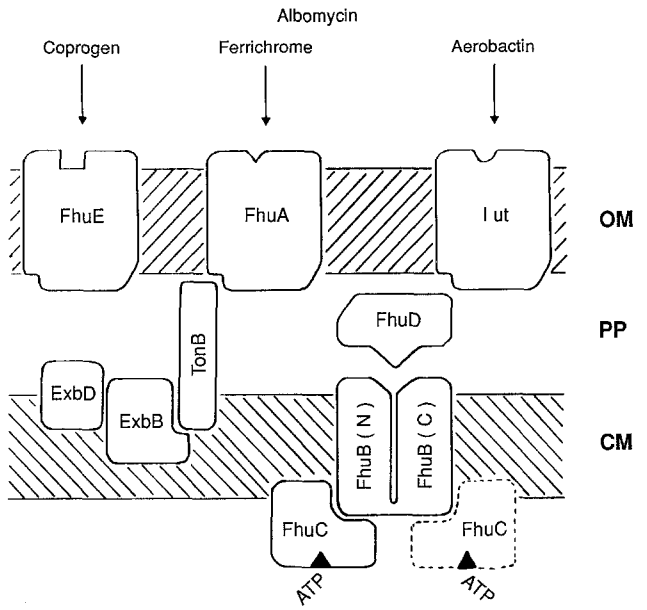

Fig. 7. Model of iron(III) hydroxamate transport (for details see text). OM, outer membrane; PP, periplasm; CM, cytoplasmic membrane

that FecB and FepB act in a similar manner in ferric dicitrate and ferrienterochelin (ferrienterobacin) transport. The FhuB protein is the central component of the uptake system. It mediates the actual substrate translocation across the lipid bilayer interacting somehow with FhuD on the periplasmic and FhuC on the cytostolic side of the inner membrane. In most of the comparable systems (*fec, fep, his, opp, mal, pst, ugp*) two nonpolar components were found in the cytoplasmic membrane which may act as heterodimers. Only one hydrophobic protein was observed for the *btu, sfu, ara, rbs* and *pro* uptake systems, probably acting as homodimers. In the iron(III) hydroxamate uptake also only one apolar component is involved. But this protein is unique in that it has twice the size of the other integral membrane proteins. Presumably originating from a duplication of an ancestral gene, it consists of two homologous parts and may function as a pseudo heterodimer.

The FhuC protein is most likely involved in energy coupling. Found to be associated with the cytoplasmic membrane, this hydrophilic protein is thought to interact with FhuB (and the phospholipids in the membrane?) facing the cytosol. Evidence for this location was supported by the finding that the otherwise protease-sensitive FhuC was not degraded in proteinase-K-treated spheroplasts (Köster and Braun, unpublished results).

The FhuB-FhuC stoichiometry has not been determined. It is possible that only one FhuC interacts with the FhuB(N)-(C) complex. However, we favour two FhuC proteins participating in transport, one being associated with FhuB(N), the other one with FhuB(C). This assumption is supported by the following data. Firstly, the ATP-binding proteins AraG and RbsA for arabinose and ribose transport, respectively, are double the size of the other ATP-binding proteins and exhibit an internal similarity (Scripture et al. 1987; Bell et al. 1986). Secondly, two ATP-binding proteins, OppF and OppD, were reported to be involved in oligopeptide uptake of *Salmonella typhimurium* (Hiles et al. 1987). Thirdly, eucaryotic proteins correlated with multiple drug resistance (Mdr) (Gros et al. 1986; Chen et al. 1986), cystic fibrosis in man (CFTR) (Riordan et al. 1989), and a-factor pheromone production in yeast (STE6) (McGrath and Varhavsky 1989) are thought to catalyze an energy-dependent export of substrates, and consist of a single polypeptide chain composed of two nonpolar membrane domains and two hydrophilic ATP-binding domains.

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