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Structure–function relationships in the bifunctional ferrisiderophore FpvA receptor from *Pseudomonas aeruginosa*

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Abstract FpvA is the primary outer membrane transporter required for iron acquisition via the siderophore pyoverdine (Pvd) in *Pseudomonas aeruginosa*. FpvA, like other ferrisiderophore transporters, consists of a membrane-spanning β-barrel occluded by a plug domain. The β-strands of the barrel are connected by large extracellular loops and periplasmic turns. Like some other TonB-dependent transporters, FpvA has a periplasmic domain involved in a signalling cascade that regulates expression of genes required for ferrisiderophore transport. Here, the structures of FpvA in different loading states are analysed in light of mutagenesis data. This analysis highlights the roles of different protein domains in Pvd-Fe uptake and the signalling cascade and reveals a strong correlation between Pvd-Fe transport and activation of the signalling cascade. It is likely that conclusions drawn for FpvA will be relevant to other TonB-dependent ferrisiderophore transport and signalling proteins.

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Introduction

In order to circumvent the low bioavailability of iron(III) (Fe^{3+}) (Neilands 1995), many Gram-negative bacteria secrete small molecules called siderophores that have a high affinity for iron(III) (Boukhalfa and Crumbliss 2002). Once formed in the extracellular environment, ferric-siderophore complexes are transported into the periplasm by specific outer membrane transporters (Ferguson and Deisenhofer 2004). This process requires the energy-transducing protein TonB anchored in the inner membrane (Postle and Larsen 2007). Fluorescent *Pseudomonads* secrete siderophores called pyoverdines that are composed of a chromophore related to 2,3-diamino-6,7-dihydroxyquinoline linked to a dicarboxylic acid and a strain-specific peptide of variable length and amino acid sequence. Once loaded with iron, pyoverdine (Pvd) from the opportunistic human pathogen *Pseudomonas aeruginosa* PAO1 (Fig. 1a) is transported back into the bacteria by the outer membrane transporter FpvA (Poole et al. 1993). In addition to transport of Pvd-Fe, FpvA plays a role in a signalling cascade regulating the transcription of its own gene and genes encoding proteins involved in

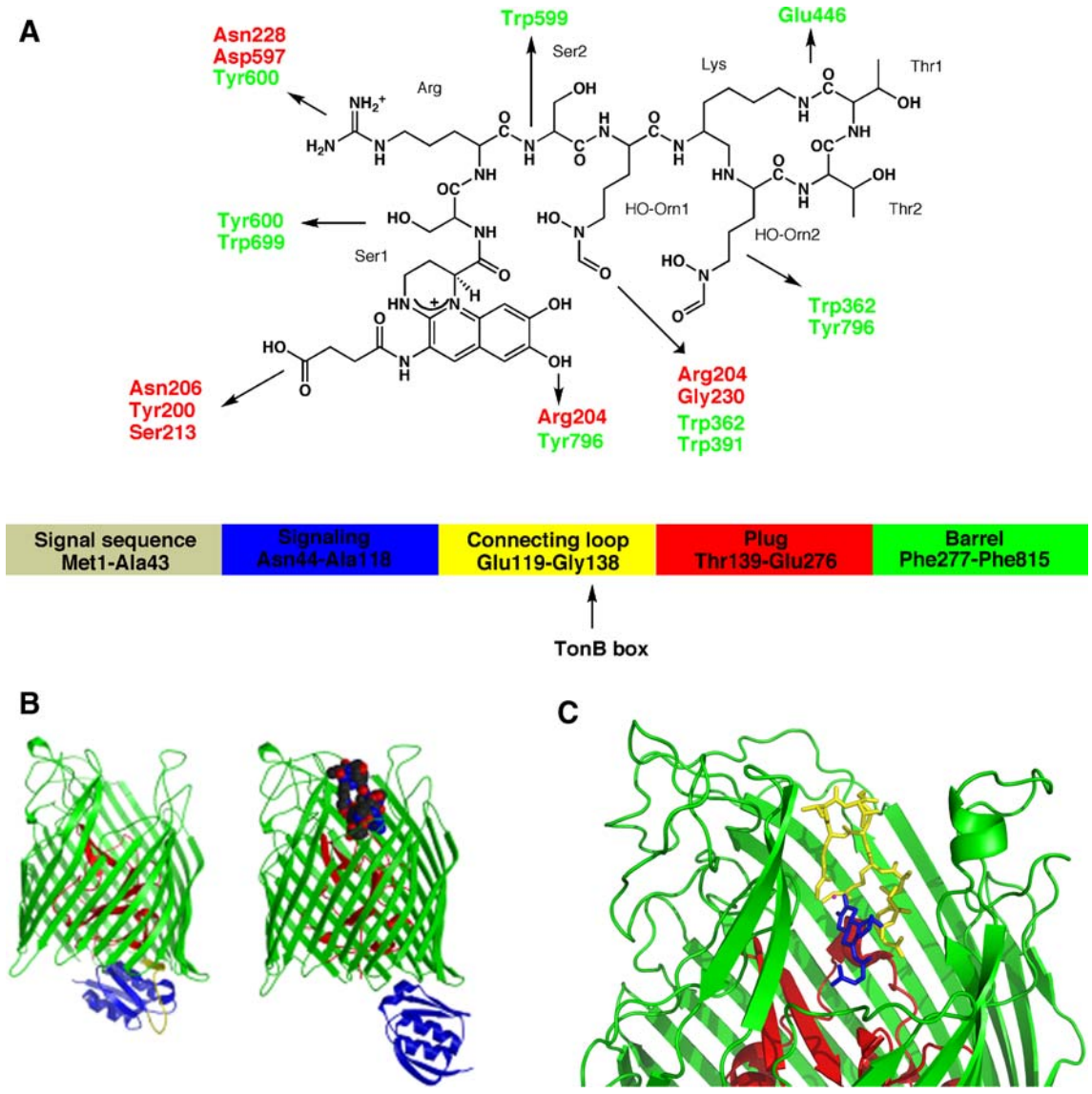

Fig. 1 **a** Interactions of Pvd with the residues of its binding site on FpvA. The structure of Pvd and the interacting residues of FpvA are shown. Residue labels are coloured based on their domain location: plug, *red*; barrel, *green*. **b** Crystal structures of FpvA and FpvA-Pvd-Fe. The β-barrel and plug domains of FpvA are shown in *green* and *red*, respectively, the signalling domain is shown in *blue*, the TonB box in *yellow* and the siderophore Pvd in *black–blue–red*. The TonB box was disordered in the FpvA-Pvd-Fe crystal structure and so is not shown. **c** Structure of Pvd-Fe in its binding site on FpvA. The chromophore is shown in *blue*, the peptide moiety in *yellow* and iron in *magenta*. The plug and barrel are *red*- and *green-coloured*, respectively

siderophore biosynthesis (Beare et al. 2003; Lamont et al. 2002; Visca 2004). FpvA, like all other siderophore outer membrane transporters for which structures have been determined (http://blanco.biomol.uci.edu/Membrane_Proteins_xtal.html), consists of a 22-stranded transmembrane β-barrel filled by an N-terminal domain called the plug domain (Fig. 1b). The β-strands of the barrel are connected by long extracellular loops and periplasmic turns. The subclass of TonB dependent transporters (including FpvA) that are part of signalling cascades have an additional N-terminal domain of about 90 residues located in the periplasm (Braun and Braun 2002; Hantke 2005; Visca et al. 2002). In FpvA, this domain

is required for regulation of expression of *fpvA* and of genes involved in the biosynthesis of Pvd. The signalling domain interacts with an antisigma factor FpvR that extends from the periplasm through the inner membrane into the cytoplasm and controls the activities of two sigma factor proteins PvdS and FpvI (Beare et al. 2003; Lamont and Martin 2003; Redly and Poole 2005).

The structures of FpvA in different loading status have been solved (Cobessi et al. 2005; Wirth et al. 2007; Brillet et al. 2007), and a large number of mutants of FpvA have been characterised by different research groups (James et al. 2005; Nader et al. 2007; Shen et al. 2005), but their phenotypes have not been discussed in the light of the FpvA structures. Here, we correlate the effects of different mutations with the biochemical and structural data available on FpvA and then discuss the mechanisms of Pvd-Fe transport and the initiation of the signalling cascade.

Ferripyoverdine binding

The binding sites for siderophores are located at the extracellular faces of the transporters and are composed of residues from both plug and barrel domains (Fig. 1). The structure and residue composition of siderophore binding pockets are specific to each transporter and enable specific high affinity binding of the cognate siderophore. The Pvd-Fe binding site of FpvA is mostly composed of aromatic residues from the plug and β-barrel domains (Fig. 1a, c; Wirth et al. 2007). It is only able to bind the Pvd shown in Fig. 1a and a similar Pvd produced by a strain of *P. fluorescens* (Schons et al. 2005). In FpvA-Pvd-Fe, Pvd-Fe is attached to the plug domain via the chromophore (Fig. 1c). The Ser1 and Arg residues of the peptide moiety of Pvd fold around the chromophore and the rest of the peptide folds over the chromophore. The peptide moiety, which determines the receptor specificity of the Pvd, interacts mainly with the β-barrel domain and the extracellular loops, rather than with the plug domain (Wirth et al. 2007). Binding studies with different Pvd analogues have shown that Ser_1 and the succinyl moiety linked to the chromophore of Pvd can be sterically hindered with no effect on binding or the iron uptake properties of Pvd-Fe (Schons et al. 2005). However, the second position of the peptide moiety in Pvd (Arg) cannot be subject to steric hindrance without a major decrease in affinity of the ferric-siderophore (Schons et al. 2005). In FpvA-Fe-Pvd, the Arg of Pvd points toward the plug domain and is covered by part of loop L7 and interacts with D597 of this loop. This interaction seems to be important for the correct positioning of the siderophore in its binding site. Introduction of short (4 or 8 residues) peptides in different positions of the FpvA binding site invariably affected both Pvd-Fe uptake and Pvd production (James et al. 2005). Site directed mutations had less effect with only 3 of 7 mutations (W362A, W391A and F795A) affecting Pvd-Fe uptake (Shen et al. 2005). Two of these mutations (W362A and W391A) are at residues that interact with Pvd-Fe (Fig. 1a). The mutations may also induce conformational changes in the siderophore binding site that disrupt its topology and hence recognition and binding of Pvd-Fe and/or the first step of Pvd-Fe uptake across the outer membrane. W362 is part of a loop (L3) that lines one side of the siderophore-binding pocket. This segment interacts with the hydroxyl ornithines of Pvd-Fe (Fig. 1a) and also with a small loop of the plug domain that lines the bottom of the binding pocket. Comparisons between FpvA and FpvA-Pvd-Fe show that both of these loops move to allow enough space for Pvd-Fe binding.

Pvd-Fe uptake

Ferrisiderophore uptake through transporters involves a sequential cascade of conformational changes of the different protein domains, with a "two gating" mechanism involving two major conformational changes having been proposed for FecA (Ferguson et al. 2002) and, by implication, other ferrisiderophore transporters. The first gate involves the large extracellular loops of the β-barrel and the second the plug domain.

Analyses of three FecA structures (FecA, FecA-Cit and FecA-Cit-Fe; Ferguson et al. 2002; Yue et al. 2003) have suggested a closing of the extracellular loops L7 and L8 over the binding site after the binding of siderophore-Fe, resulting in sequestration of the ligand in the binding site. In work with other receptors, ferrichrome-induced fluorescence quenching of fluorescein-labelled FhuA indicated movement of one or more extracellular loops of FhuA associated

with binding of ferrisiderophore (Bos et al. 1998) and spectroscopic and cross-linking studies of FepA showed movements of loops during uptake of enterobactin-Fe (Cao et al. 2003; Scott et al. 2002). These data are consistent with a mechanism in which ferrisiderophores are trapped in their binding sites on outer membrane transporters, ensuring that they have no possibility other than to be transported into the periplasm. Time-resolved fluorescence spectroscopy studies, based on the fluorescent properties of Pvd, Pvd-Ga and Pvd-Al, clearly indicate that apo Pvd and Pvd-metal are in different protein environments when bound to FpvA (Folschweiller et al. 2002). When loaded with metal, the siderophore in its binding site is less flexible and solvent-accessible and its environment is not as polar as in the FpvA-Pvd complex, suggesting that the extracellular loops form a lid that traps Pvd-metal in the binding site, consistent with findings with other receptors. However, the structures of FpvA and FpvA-Pvd-Fe do not show a change in the conformation of extracellular loops following ferrisiderophore binding (Brillet et al. 2007; Wirth et al. 2007). Changes of conformation may be not seen in the FpvA-Pvd-Fe structure because these extracellular loops are very flexible, and their conformation may therefore be imposed by the crystallization conditions.

Ten residues of the extracellular loops of FpvA have been mutated. Pvd-Fe uptake was affected by mutations in loops L4, L5, L7, L9 and L11 (James et al. 2005; Nader et al. 2007). In all cases decreases were observed in the Pvd-Fe uptake rates except for mutation S602C (loop L7) which caused a 1.7 fold increase in the amount of iron transported (Nader et al. 2007), indicating a important role for this residue and loop L7 in the Pvd-Fe uptake process. The effects of mutations in the extracellular loops suggest that one or more of these extracellular loops are important for the correct positioning of the ferrisiderophore in its binding site and for Pvd-Fe uptake.

The second gate in the two-gated mechanism for siderophore-Fe uptake is provided by the plug domain that fills the lumen of the β-barrel (Fig. 1b), blocking access to the periplasm. On the two-gating model, this gate only opens once siderophore-Fe is trapped in its binding site after closure of the first gate. The opening of the second gate is energy dependent. The energy is delivered by the energy transducing complex TonB-ExbB-ExbD anchored in the inner membrane, through interactions between the periplasmic C-terminal part of TonB and a part of the outer membrane transporter called the "TonB box" (Pawelek et al. 2006; Postle and Larsen 2007; Shultis et al. 2006). In FhuA the binding of ferrichrome induced the unwinding of an β helix adjacent to the TonB box (Ferguson et al. 1998; Locher et al. 1998). The structure of the TonB box in FpvA was only determined in the absence of Pvd-Fe, with this region being disordered in the FpvA-Pvd-Fe structure.

How does the second gate open? The exact mechanism is not known, but it has been proposed that a channel is formed by either a major change of conformation of the plug domain or by movement of this domain out of the β-barrel, or a combination of both (Eisenhauer et al. 2005; Endriss et al. 2003; Ma et al. 2007). Recent single-molecule unfolding experiments in the context of outer membrane transporters suggest that TonB could induce either large conformational changes or unfolding of the plug domain by exerting a very modest mechanical force upon the transporter (Gumbart et al. 2007). However, existing data are not sufficient to distinguish between these possibilities. The structures of FpvA and FpvA-Pvd-Fe show no differences in the folding of the plug domain and give no indications of the mechanism involved in the opening of the second gate (Brillet et al. 2007; Cobessi et al. 2005; Wirth et al. 2007). Only four mutations have been obtained in the FpvA plug domain, all by insertion of short peptides, and all of them caused a decrease in Pvd-Fe uptake (James et al. 2005). Such a phenotype is not surprising, since insertions in the plug domain are likely to have major effects on the conformation of this domain and its interaction with the barrel. As might be expected, mutations in the FpvA β-barrel also resulted in reduced uptake of Fe-Pvd.

Signalling cascade regulating gene expression

The N-terminal domain of FpvA, called the signalling domain, is located in the periplasm. It is not required for uptake of Pvd-Fe across the outer membrane but is required for the signalling cascade that controls expression of genes encoding Pvd synthesis enzymes, FpvA itself, an exotoxin and a protease (James et al. 2005; Shen et al. 2002; Visca 2004). This domain is composed of 2 α-helices sandwiched by 2 β-sheets

(Brillet et al. 2007; Wirth et al. 2007). The same fold is present in the signalling domains of PupA and FecA (Ferguson et al. 2007; Garcia-Herrero and Vogel 2005) and is probably conserved among this subfamily of TonB-dependent receptor proteins. Five different mutations in the FpvA signalling domain all affected Pvd production but had no effect on transport of Pvd-Fe (James et al. 2005) and deletion of this domain had the same effect (Shen et al. 2002). This reemphasises that this domain is required only for signalling and not for uptake of Pvd-Fe. Similarly, the signalling domain of FecA can be removed without reducing the transport rate (Enz et al. 2000).

Molecular genetic approaches strongly imply that the signalling domain of FpvA interacts with the periplasmic part of FpvR (Beare et al. 2003; Lamont et al. 2002; Shen et al. 2002). The FpvA and FpvA-Pvd-Fe structures (Fig. 1b) show that the positioning of this domain in the periplasm depends on the loading status of FpvA but its fold is not modified. In unloaded FpvA the 3-stranded β-sheet of the signalling domain interacts with residues of the TonB box of FpvA (Fig. 2). During the signalling cascade this domain must interact with the FpvR protein, which controls the activities of the sigma factors FpvI and PvdS. The interaction observed in FpvA between the TonB box and the signalling domain is very similar to the interactions between the TonB boxes of BtuB and FhuA with TonB (Pawelek et al. 2006; Shultis et al. 2006). Assuming that the interaction between FpvA and TonB is similar to BtuB-TonB and FhuA-TonB interactions, this suggests that TonB and the signalling domain are in competition for interaction with the TonB box. Unloaded FpvA is able to interact with TonB in vitro (Adams et al. 2006) so that at least under these conditions TonB is apparently able to displace the signalling domain and interact with the TonB box of FpvA. This suggests that TonB and the FpvA signalling domain have similar affinities for the TonB box.

How do mutations in other parts of FpvA affect signalling? At least three mutations in the siderophore binding pocket resulted in reduced signalling (James et al. 2005), most likely because binding of Pvd-Fe to FpvA is required for initiation of the signalling cascade. In the extracellular loops, all mutations affecting Pvd-Fe uptake also affected Pvd production (James et al. 2005; Nader et al. 2007), suggesting that closing of gate one is important for signalling as well as transport of Pvd-Fe. Twelve of 15 mutations in the FpvA β-barrel and all of the mutations in the plug domain affected signalling (James et al. 2005), showing that these parts of FpvA are important in the signalling cascade as well as for

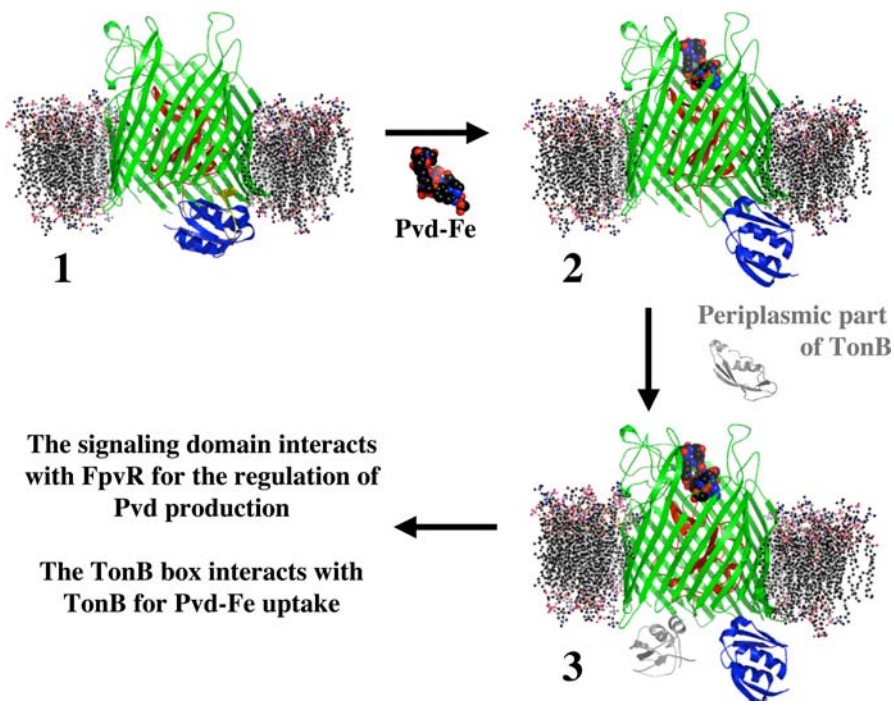

Fig. 2 Sequential mechanism of Pvd-Fe binding and transport leading to the signalling cascade. *1* When the Pvd-Fe binding site of FpvA is vacant the signalling domain interacts with the TonB box. *2* When FpvA is Pvd-Fe loaded the signalling domain is displaced. *3* The TonB box interacts with TonB. Modelling of TonB-FpvA-Pvd-Fe is based on the superposition of FpvA-Pvd-Fe onto FhuA-TonB (Brillet et al. 2007)

transport of Pvd-Fe. However, three mutations in the barrel (D553, Y554 and E637) affected Pvd production without affecting uptake of Pvd-Fe (James et al. 2005).

What is the relationship between Pvd-Fe transport and signalling?

Mutagenesis studies with FecA showed a parallel between ferric-dicitrate uptake and the signalling cascade (Sauter and Braun 2004). Excluding the signalling domain, most of the mutations in FpvA affect both functions suggesting that they are also linked in this receptor. Furthermore, a TonB protein is required for FpvA signalling in *P. aeruginosa* (M. Shirley and I. Lamont, unpublished data). These observations, coupled with structural studies of FpvA and the mechanism of interactions of TonB with ferrisiderophore transporters, suggest a sequential mechanism linking transport and signalling (Fig. 2). In this mechanism the starting point for both iron uptake and Pvd production (signalling cascade) is the binding of Pvd-Fe to FpvA which induces a change of conformation of the extracellular loops, trapping Pvd-Fe in its binding site. Once Pvd-Fe is trapped in its binding site on FpvA, a conformational change is transmitted from the binding site to the periplasmic N-terminal region reducing the affinity of the signalling domain for the TonB box. TonB is then able to interact with the TonB box with the signalling domain being displaced. The FpvA–TonB interaction allows transduction of the energy necessary for movement of the plug domain (formation of a channel) and translocation of Pvd-Fe into the periplasm. Displacement of the signalling domain from the TonB box allows it to interact with FpvR leading to gene expression. On this model, mutations that affect the first steps of Pvd-Fe transport (closing of the extracellular loops, interactions between TonB and the TonB box) also affect the signalling process. This proposed sequential mechanism is in agreement with the FpvA and FpvA-Pvd-Fe structures. The signalling domain is not involved in the transport mechanism, but interacts with FpvR only when FpvA is Pvd-Fe loaded and its displacement to interact with FpvR follows the beginning of the Pvd-Fe transport. Pvd-Fe and TonB are both required for displacement of the signalling domain and consequent signalling in vivo whereas in vitro, the presence of either Pvd-Fe or TonB (Adams et al. 2006) is sufficient to displace the signalling domain. On this model, the three mutations in the barrel that affect signalling while having no effect on transport of Pvd-Fe must affect the conformational switch associated with displacement of the signalling domain, such that TonB can interact with the TonB box and transport can occur but the displacement of the signalling domain is not appropriate for it to interact effectively with FpvR.

Conclusion

Crystal structures provide a precise but static view of a protein in the form in which it crystallises. Postulations derived from crystal structures as to how a protein might work have to be tested by functional studies, incorporating information from appropriate mutants. Here we combined biochemical and structural studies of FpvA in different loading states with functional data from mutant studies to better understand the changes of conformation undergone by FpvA during Pvd-Fe uptake and during the signalling cascade. The results of these analyzes are summarised in Fig. 2, which provides a plausible scheme emphasising the importance of interactions between the TonB box and the signalling domain or the TonB protein. On this scheme binding of Pvd-Fe to FpvA leads to a conformational change that allows TonB to interact with the TonB box and the signalling domain to interact with FpvR. This mechanism could apply to all siderophore transporters that have a signalling domain and are involved in regulation of gene expression as well as ferric siderophore uptake.

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