The role of the synergistic phosphate anion in iron transport by the periplasmic iron-binding protein from *Haemophilus influenzae*

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The acquisition of iron from transferrin by Gram-negative bacterial pathogens is dependent on a periplasmic ferric-ion-binding protein, FbpA. FbpA shuttles iron from the outer membrane to an inner membrane transport complex. A bound phosphate anion completes the iron co-ordination shell of FbpA and kinetic studies demonstrate that the anion plays a critical role in iron binding and release *in vitro*. The present study was initiated to directly address the hypothesis that the synergistic anion is required for transport of iron in intact cells. A series of site-directed mutants in the anion-binding amino acids of the *Haemophilus influenzae* FbpA (Gln-58, Asn-175 and Asn-193) were prepared to provide proteins defective in binding of the phosphate anion. Crystal structures of various mutants have revealed that alteration of the

C-terminal domain ligands (Asn-175 or Asn-193) but not the N-terminal domain ligand (Gln-58) abrogated binding of the phosphate anion. The mutant proteins were introduced into *H. influenzae* to evaluate their ability to mediate iron transport. All of the single site-directed mutants (Q58L, N175L and N193L) were capable of mediating iron acquisition from transferrin and from limiting concentrations of ferric citrate. The results suggest that the transport of iron by FbpA is not dependent on binding of phosphate in the synergistic anion-binding site.

Key words: ferric-ion-binding protein A (FbpA), glycoprotein, *Haemophilus influenzae*, iron transport, periplasmic iron-binding protein, transferrin.

INTRODUCTION

Due to its redox capabilities, iron serves as a cofactor for many enzymes and proteins that mediate important biological functions. Most organisms have an absolute requirement for iron and have developed specialized systems for acquiring, transporting and storing this essential element [1]. In vertebrates, iron is transported through the body by the serum glycoprotein, transferrin, and in mammals a related glycoprotein, lactoferrin, is produced to sequester iron on mucosal surfaces and at sites of infection. The presence of transferrin or lactoferrin in most ecological niches within the vertebrate or mammalian host restricts the availability of iron for bacterial pathogens. As a consequence, high-affinity transport mechanisms for iron are required for growth and survival within the host [2].

The host-restricted pathogens *Haemophilus influenzae*, *Neisseria meningitidis* and *Neisseria gonorrhoeae* have adapted to the environment in the host by directly using the host glycoproteins as a source of iron for growth through the action of specific receptors at the cell surface [3,4]. Studies in a human gonococcal infection model demonstrate that the survival of *N. gonorrhoeae* is dependent on the presence of either the surface transferrin receptor or the surface lactoferrin receptor, preferably both [5,6]. This demonstration that the receptor-mediated uptake pathways are critical for survival almost certainly applies to other host-restricted pathogens in the Neisseriaceae and Pasteurellaceae, such as *H. influenzae* and *N. meningitidis*, and has been experimentally demonstrated for the pig pathogen *Actinobacillus pleuropneumoniae* [7].

The surface transferrin receptor, which consists of two outer membrane proteins, transferrin-binding proteins A and B (TbpA and TbpB), specifically binds to transferrin from the host or from very closely related species [8]. The receptor removes iron

from transferrin and transports it across the outer membrane by mechanisms that have not been delineated. TbpB, a bi-lobed, surface-exposed lipoprotein, is required for iron acquisition *in vivo* [7] but growth can occur in its absence under laboratory conditions with transferrin as a sole iron source [7,9–12]. TbpA, a homologue of the well-characterized TonB-dependent receptors [13,14], is predicted to consist of a C-terminal β -barrel and an N-terminal plug region [15]. TbpA is absolutely required for acquiring iron from transferrin, consistent with its proposed role as a channel for transport of iron across the outer membrane. This process is dependent on interaction with TonB [16,17] that provides energy derived from an inner membrane complex of TonB and the associated proteins ExbB and ExbD.

Iron acquisition from transferrin is also dependent on the presence of a periplasmic ferric-ion binding protein, FbpA [18,19]. FbpA, along with an inner-membrane permease complex consisting of transmembrane and ATPase components (FbpB and FbpC respectively), is responsible for the transport of iron from the periplasm to the cytoplasm [20,21]. FbpA from *H. influenzae* (and from *N. gonorrhoeae*) has been called a bacterial transferrin due to the similarity in its overall structure to a single lobe of transferrin and to the fact that it co-ordinates iron with a similar constellation of amino acids (histidine, glutamic acid, tyrosine and tyrosine residues) [22]. FbpA differs from transferrin in that in lieu of using a carbonate anion to complete the octahedral iron co-ordination complex, it uses a phosphate anion.

The presence of bound phosphate in the crystal structure may not definitively demonstrate that it is the anion used under physiological conditions but biochemical and kinetics studies appear to support that role. Phosphate binds to the iron-loaded form of FbpA with the highest affinity, and cannot be displaced by carbonate anion under conditions emulating the periplasm [23]. A series of biochemical and kinetic studies suggest that the anion plays a key

Abbreviations used: FbpA, ferric-ion-binding protein A; hLf, holo human lactoferrin; HRP, horseradish peroxidase; hTf, holo human transferrin; IPTG, isopropyl β -D-thiogalactoside; LA, Luria agar; PEG, poly(ethylene glycol); Tbp, transferrin-binding protein.

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The refined co-ordinates of *Haemophilus influenzae* N193L FbpA have been deposited in the PDB (PDB ID code 2O69).

role in iron binding and release by FbpA [24,25]. Analysis of the kinetics and mechanism of iron loading of FbpA suggests that the anion plays a critical role in inserting Fe^{3+} into the metal-binding site [25]. Similarly iron release studies suggest that the synergistic anion controls the release of iron, as a proton-assisted release of the phosphate anion is the preliminary step in iron release [24]. The key role that the phosphate anion plays in iron binding and release in *in vitro* studies implicates the synergistic anion as a potentially critical component in the mechanism of iron acquisition and prompted us to initiate studies to directly address this proposal. In the present study a series of site-directed mutants of the FbpA from *H. influenzae* altered in the anion-binding residues were prepared and tested for their ability to mediate iron uptake in reconstituted pathways.

EXPERIMENTAL

Strains and media

H. influenzae and *Escherichia coli* strains were stored in 30% (v/v) glycerol at -70°C . *H. influenzae* isolates were inoculated on to chocolate agar plates and incubated overnight at 37°C in a 5% CO_2 atmosphere. Liquid cultures of *H. influenzae* were routinely prepared in BNH broth [BHI broth base (Difco), 3.32 $\mu\text{g}/\text{ml}$ NAD (Sigma) and 10 $\mu\text{g}/\text{ml}$ haemin (Sigma)]. *E. coli* isolates were inoculated on to LA (Luria agar) plates (Gibco BRL) supplemented with 100 $\mu\text{g}/\text{ml}$ ampicillin (Sigma). Liquid *E. coli* cultures were prepared in LA broth (Luria broth base and 100 $\mu\text{g}/\text{ml}$ ampicillin).

Anaerobic disc diffusion assays

H. influenzae strains were tested for their ability to use hTf (holo human transferrin), hLf (holo human lactoferrin), haemin and ferric citrate (impregnated on concentration discs) for growth on iron-limited anaerobic agar media, buffered with 50 mM Hepes (Sigma; pH 7.4), as previously described [19]. The strains were subcultured in media lacking haemin in order to deplete iron stores prior to plating on the iron-limited growth media [19].

Construction of site-directed FbpA mutants

The various site-directed mutants of the *H. influenzae* *fbpA* gene were prepared by the Stratagene QuikChange® Site-directed Mutagenesis kit using the pT7-7 plasmid encoding the wild-type gene as the starting template. Sequence analysis confirmed that the desired mutations were the only changes in the coding sequence. A gene replacement vector for *H. influenzae* was prepared by cloning 1.26 kb of the region immediately upstream of the *fbpA* gene adjacent to a 1.36 kb DNA fragment containing the *fbpAB* intergenic region and a portion of the coding sequence for the *fbpB* gene. Restriction sites engineered between these two regions were used to clone in a chloramphenicol-resistance cassette derived from the pTnMax4 plasmid [26] or the kanamycin-resistance determinant derived from the pUC4K plasmid. The plasmid containing the chloramphenicol-resistance cassette was linearized by digestion with *SstI* and used directly to transform *H. influenzae* strain H36 using M-IV competence-inducing media [27]. The resulting chloramphenicol-resistant strain, H306, was used as a parent for all subsequent transformations. The wild-type or mutant *H. influenzae* *fbpA* genes were subcloned from the pT7-7 vector into the gene replacement vector between the upstream region and kanamycin-resistance determinant. The resulting plasmids were linearized by *SstI* digestion and used to directly transform the chloramphenicol-resistant parent strain. The strain transformed with the wild-type *fbpA* gene, H317, was used as the control wild-

Figure 1 Expression of mutant FbpAs

(A) Osmotic shock fractions isolated from *E. coli* strain BI21DE3/pLysS containing recombinant pT7-7 expression plasmid with the wild-type or mutant *fbpA* gene from *H. influenzae* strain H36. The osmotic shock fractions were subjected to SDS/PAGE analysis and protein was detected by staining with Coomassie Blue. WT, wild-type gene; Q58L, plasmid with Q58L mutant gene; N193L, plasmid with N193L mutant gene; N175L, plasmid with N175L mutant gene. (B) Immunoblot analysis of whole-cell wild-type and recombinant *H. influenzae* strains. WT, kanamycin-resistant wild-type *fbpA* strain H317; Q58L, Q58L *fbpA* mutant strain H369; N193L, N193L *fbpA* mutant strain H370; N175L, N175L *fbpA* mutant strain H371. The blot was developed with polyclonal anti-*H. influenzae* FbpA polyclonal rabbit antisera (1:2000) and goat anti-rabbit HRP (horseradish peroxidase) conjugate (1:3000). The blot was developed with polyclonal anti-*H. influenzae* FbpA polyclonal rabbit antisera (1:2000) and goat anti-rabbit HRP conjugate (1:3000). Numbers in parentheses underneath depict quantity of protein expressed by each mutant [$\text{pg} \cdot \mu\text{L}^{-1} \cdot (10^9 \text{ bacteria})^{-1}$] by densitometry analysis of FbpA protein expression for whole-cell wild-type and recombinant *H. influenzae* strains as compared with quantified recombinant wild-type FbpA from *E. coli*.

type strain in all subsequent growth studies to ensure that any observed growth differences could not be attributed to the presence of the kanamycin cassette.

Expression and purification of FbpA protein

The different FbpAs were expressed from a recombinant pT7-7 plasmid after induction with IPTG (isopropyl β -D-thiogalactoside) in the BL21 (DE3)/pLysS host strain. A modified osmotic shock procedure provided relatively pure preparations of protein (Figure 1A). Samples were extensively dialysed against 10 mM Tris (pH 8.0) at 4°C , and subjected to cation-exchange chromatography on a BioCad HPLC system as a final purification step. The column was washed with 10 vol. of 20 mM Tris (pH 7.5) to remove unbound proteins and eluted with a gradient of 0–1.5 M NaCl. After concentration, the samples were dialysed extensively against 10 mM Tris (pH 8.0) at 4°C . The resulting preparations were deemed pure based on SDS/PAGE analysis. Following dialysis the samples were concentrated using Microsep™ 10K microconcentrators to 10 mg/ml. In order to remove any bound iron, a 4000-fold molar excess of citrate was added along with 1 mM EDTA at pH 8.0 to each protein sample. The iron-removal buffer was then removed through exchanges by several successive treatments with the microconcentrator using 10 mM Tris buffer (pH 8.0). The samples were exposed to a buffer containing 5 mM phosphate and then subjected to several exchange treatments against 10 mM Tris buffer (pH 8.0). A solution of freshly prepared 16 mg/ml ferric chloride in 100 mM sodium citrate and 100 mM sodium carbonate (pH 8.0) was added to the samples to provide a 5-fold molar excess of iron, and any excess metal was removed

by extensive dialysis against 10 mM Tris buffer (pH 8.0). These protein preparations were used in spectral studies.

Spectral studies

Scans of UV–visible spectra (310–600 nm) were performed on the iron-loaded wild-type FbpA and FbpA mutant proteins using the Wavescan software package provided with the Ultrospec 2000 from Amersham Biosciences. All experiments were performed in 1.5 ml quartz cuvettes kept at 20 °C. Calculations were conducted using the visible absorbance maxima for each respective protein.

Relative iron affinity was measured and calculated using a citrate competition assay [21]. Iron-loaded samples of FbpA at 50 μ M were exposed to increasing concentrations of citrate (0–384 mM final concentration) in a buffer (10 mM Tris, pH 8.0). After incubation for 20 min at 20 °C, the absorbance was recorded at each citrate concentration. The relative affinity constant for the protein was then calculated based on the reported association constant of citrate for ferric iron at a similar pH [21].

Purification and crystallization of N193L FbpA

The purified protein preparation was concentrated using Microsep™ 10K microconcentrators to greater than 30 mg/ml. Crystallization conditions were as previously described [28] with the following exceptions. Crystals of N193L were grown at 4 °C by the hanging drop technique from 4 ml drops containing 15 mg/ml of the N193L FbpA, 18 % (v/v) PEG [poly(ethylene glycol)] 550 monomethyl ether and 0.05 M Tris (pH 8.5). The drops were equilibrated against a 1 ml reservoir containing 36 % PEG 550 monomethyl ether and 0.1 M Tris (pH 8.5). Diffraction-quality pink crystals typically grew after 4 days. For cryocrystallography, the crystals were placed in a cryoprotectant solution identical with the reservoir solution with a final concentration of 20 % (v/v) ethylene glycol for a short time before being placed in the nitrogen gas stream to be cooled to 100 K.

Data measurement and structure solution

Initial X-ray analysis revealed that all crystals belong to the orthorhombic space group $P2_12_12$ with unit cell dimensions of $a = 105.61$ Å (1 Å = 0.1 nm), $b = 75.43$ Å, $c = 33.44$ Å, $a = b = \gamma = 90^\circ$ for N193L. Crystallographic data were recorded in the same manner as the data for the H9A mutant previously described [29]. The steps taken and programs used in determining the high-resolution N193L structure were identical with those utilized in solving the previous H9A crystal structure [29]. Ramachandran plots of the mutant structure revealed that greater than 90 % of the residues are in the most favoured regions with all other residues falling in additional allowed regions indicating that the structures are of high quality. The refined co-ordinates of *H. influenzae* N193L FbpA have been deposited in the PDB (PDB ID code 2O69).

RESULTS

Production and characterization of site-directed FbpA mutants

The three amino acids whose side chains are involved in binding to the synergistic phosphate anion, Gln-58, Asn-175 and Asn-193, were targeted for site-directed mutagenesis. For simplicity and consistency we decided to convert each of the three anion-co-ordinating amino acids into leucine. Site-directed mutagenesis was performed by the QuikChange® mutagenesis system (Stratagene) using an expression plasmid containing the wild-type *H. influenzae* *fbpA* gene as starting template.

Initial expression experiments were performed with a pT7-7 expression plasmid with the wild-type or mutant *fbpA* gene. All

Figure 2 Electron density maps (green for the $2F_o - F_c$ map contoured at 1σ) showing the anion-binding site

(A) The Q58L mutant protein; (B) the N193L mutant protein. This Figure was generated using Raster 3D (Photorealistic Molecular Graphics). For the spheres, iron atoms are orange and the water molecules are light blue.

of the mutant genes were confirmed by DNA sequence analysis prior to performing the functional and structural studies. The recombinant plasmids containing the wild-type and mutant proteins were introduced into the BL21DE3/pLysS host strain and the production of recombinant protein after induction with IPTG was assessed by SDS/PAGE analysis.

The FbpAs were clearly the major protein in the samples from a modified osmotic shock procedure (Figure 1). The faster migration of several of the anion mutants, particularly the Q58L protein, on SDS/PAGE was unexpected (Figure 1A). The altered migration was not attributed to a substantive change in the size of the polypeptide chain from proteolysis or aberrant expression, since the intact proteins were observed by protein crystallography (Figure 2). The mutant proteins were purified from the osmotic shock fluid by cation-exchange chromatography, concentrated, converted into the apo form and then iron-loaded prior to performing spectral analysis. Spectral analysis revealed absorbance changes characteristic of members of the transferrin superfamily upon addition of iron to the mutant FbpAs (Table 1). Several of

Table 1 Spectral properties of the wild-type and mutant FbpAs

Spectra were obtained from 310 to 600 nm for the fully iron-loaded forms of the indicated proteins. The molar absorption coefficient (ϵ) was calculated with the ProtoParam bioinformatics tool at <http://ca.expasy.org>. The log K_{S1} was calculated as described by Chen et al. [21]. WT, wild-type.

FbpA	$\lambda_{\max}$ (nm)	$\lambda_{\min}$ (nm)	Molar absorption coefficient at 280 nm ($M^{-1} \cdot cm^{-1}$)	Citrate competition assay log K_{S1}
WT	481	378	39 400	20.2
Q58L	469	380	39 400	19.4
N175L	466	375	39 400	19.5
N193L	490	396	39 400	19.5

Table 2 X-ray data collection and refinement statistics for the N193L FbpA mutant

Data collection	
Wavelength (Å)	1.5418
Resolution (Å)	43–2.0
Completeness (%) [*]	99.7 (100.0)
$I/\sigma(I)$	7.0 (1.7)
R_{sym} †	0.067 (0.390)
Redundancy	3.8
Refinement statistics	
Resolution (Å)	20–2.0
Completeness for range (%)	99.6
Number of reflections‡	17 742
Working set	16 777
Free set	965
R -factor§, R -free	0.204, 0.259
No. of protein atoms	4549
No. of water molecules	127
Fe atoms	1
Mean B -factors (Å ²)	40.85
rmsd (root mean square deviation) from ideality	
Bond lengths (Å)	0.019
Bond angles (°)	1.592

^{*} Number in parentheses is the statistic for the highest resolution shell.
[†] $R_{\text{sym}} = \sum_h (\sum_j |I_{j,h} - \langle I_h \rangle|) / \sum_h I_{j,h}$, where h = set of Miller indices and j = set of observations of reflection h .
[‡] $F > 2.0\sigma$.
[§] R -factor = $\sum_{hkl} |F_o - F_c| / \sum_{hkl} |F_o|$.

the mutant proteins had a shift in the $\lambda_{\max}$ relative to the wild-type FbpA, suggesting an alteration of the Tyr(O)–Fe interaction. The ability of the mutant proteins to bind iron was assessed by a citrate competition assay (Table 1) demonstrating that the reduction in iron binding relative to wild-type protein was modest.

Phosphate (5 mM final concentration) was added to the protein preparations to ensure that sufficient phosphate was present to occupy the anion-binding site. The preparations were concentrated to greater than 30 mg/ml protein and used to grow crystals by vapour diffusion using the hanging drop technique. Diffraction-quality crystals were obtained after 4 days growth. In the present study we report on the structure of N193L. The N175L [29] and Q58L [30] structures were described previously. Crystals of the iron-loaded N193L mutant protein were placed in a cryoprotectant solution, frozen and subjected to X-ray diffraction analysis. A model for the N193L protein was derived from the processed data by molecular replacement using the structure of the wild-type apo protein (1D9V) as the initial phasing model. The crystallographic data from this analysis are summarized in Table 2.

The N193L protein crystallized in an open conformation, with substantial separation between the N-terminal and C-terminal

domains, analogous to the apo form of the wild-type protein [31], similar to what was observed with the other FbpAs with mutated iron or anion ligating residues [29,30,32]. In contrast with the structure of the Q58L protein (Figure 2A), no electron density was visible around the iron in the N193L structure except for density outside of the anion-binding pocket describing three water molecules (Figure 2B). The anion-binding pocket was also devoid of electron density representing the phosphate anion in the N175L structure [29] indicating that alteration of either of these C-terminal residues abrogated binding of the synergistic anion. The iron atom was associated with the C-terminal domain in proximity to the tyrosine hydroxyls from Tyr-196 and Tyr-195 for Q85L and N193L (Figures 2A and 2B respectively).

Function of the mutant FbpAs in iron acquisition

The mutant genes encoding Q58L, N175L and N193L were introduced into *H. influenzae* to replace the wild-type *fbpA* gene so that the ability of the mutant FbpAs to function in iron acquisition could be evaluated [19]. The parent strain used for transformation (H306) contained a chloramphenicol-resistance cassette in place of the *fbpA* gene. The control wild-type strain used in the growth experiments (H317) was generated by transformation of H306 with the wild-type *fbpA* gene so that it had the identical gene arrangement as the mutant strains. Comparison of H317 with the original parent wild-type strain (H36) in growth studies and detection of FbpA by immunoblot analyses indicated that the control strain H317 was essentially identical with respect to expression of FbpA and growth on various iron sources (results not shown). To assess the expression and stability of the mutant FbpAs in *H. influenzae*, Western blot analysis was performed on the set of *H. influenzae* mutant strains grown under iron-restricted conditions. This analysis was performed on the intact cells used for the plate disc diffusion growth experiments described below and thus truly reflects the levels of FbpA available for supporting growth on exogenous iron sources. As illustrated in Figure 1(B) the level of protein in the mutant strains was comparable with that present in the strain containing the wild-type gene. Densitometry analysis was performed on repeat Western blots with varying concentrations of recombinant FbpA isolated from *E. coli* in order to quantify the level of protein (Figure 1B, numbers). The numbers indicate that the differences in protein concentration were less than 2-fold. The proteins in several of the strains migrated faster than the wild-type protein (Figure 1B), analogous to what was observed with the recombinant proteins (Figure 1A).

To evaluate function of the FbpAs in the intact iron uptake pathway, the set of strains were tested for growth on various sources of iron in an anaerobic disc diffusion plate assay as described previously [19]. In this assay, growth of an iron-limited culture of cells around discs impregnated with different sources of iron is assessed. The growth around discs containing haem and the lack of growth around discs containing human lactoferrin for all of the strains confirm that the growth is dependent on ability to use the exogenous iron source (Table 3). In this assay, strains expressing any of the three individual site-directed mutants (Q58L, N175L or N193L) were clearly able to use transferrin as a source of iron for growth. As observed previously [19], growth on levels of ferric citrate less than 40 nmol required a functioning FbpABC pathway. Since all of the strains expressing the mutant proteins grew on lower levels of ferric citrate (Table 3), the results suggest that all of the mutant proteins are capable of donating iron to the inner membrane transport complex comprising FbpB and FbpC. Mutant strains expressing N175L or N193L required 20 nmol of ferric citrate to support growth, whereas only 10 nmol

Table 3 Growth of *H. influenzae* strains expressing wild-type and mutant FbpAs

Growth on the indicated iron sources was determined by the anaerobic plate assay and assessed by the distance of detectable growth beyond the concentration disc as follows: (–) no detectable growth; (+) 0.5 mm; (++) 0.5–2 mm; (+++) greater than 2 mm. The amounts applied to the discs were 2.5 nmol of haem, 2.5 nmol of lactoferrin (hLf), 2.5 nmol of transferrin (hTf) and from 10 to 40 nmol of ferric citrate (FC 40 to FC 10). Ferric citrate solutions were prepared by diluting the stock solution (consisting of 27.03 mg/ml ferric chloride dissolved in citrate/bicarbonate buffer) into citrate/bicarbonate buffer (100 mM sodium citrate and 100 mM sodium bicarbonate buffer, pH 7).

FbpA expressed	Strain number	Growth on indicated iron source						
		Haem	hLf	hTf	FC 40	FC 30	FC 20	FC 10
Wild-type	H317	+++	–	+++	++	++	++	+
No FbpA	H374	+++	–	–	+	–	–	–
Q58L	H369	+++	–	+++	++	++	++	+
N175L	H371	+++	–	+++	++	++	+	–
N193L	H370	+++	–	+++	++	++	+	–

of ferric citrate was necessary to support the growth of strains expressing the wild-type or Q58L FbpAs.

DISCUSSION

Host-adapted pathogens from the families Pasteurellaceae and Neisseriaceae rely on an iron acquisition pathway for growth and survival in the host that involves direct binding of host transferrin by a receptor complex at the cell surface [5,7]. Although genetic studies have established the identity and confirmed the role of various components in the iron acquisition pathway [4,33], there has been relatively little advance in our understanding of the specific mechanisms involved in the iron acquisition process.

Significant advances have been made in our understanding of iron–siderophore and vitamin B₁₂ uptake systems due to an impressive amount of structural information that has become available for the pathway components [34]. These include the mechanism of iron–siderophore binding to the outer membrane receptor, the transmembrane signalling to the periplasmic regions for recruitment of TonB interactions and the gating of the channel by movement of external loops. Although there certainly are common features and many parallels between acquisition of iron bound to siderophores and to transferrin, there are also substantial differences that could involve mechanistic differences in the transport processes. In contrast with import of iron–siderophore complexes, which are soluble and transported intact, the ferric ion is removed from transferrin at the cell surface and, due to its relative insolubility in neutral aqueous solutions, may require direct transfer to the periplasmic binding protein or association with anions to retain solubility. Furthermore, binding of iron–siderophore to the periplasmic binding protein is not associated with a substantial conformational change [35], in contrast with the periplasmic ferric binding protein [31], suggesting that the removal of iron or iron–siderophore at the inner membrane may involve quite distinct mechanisms.

FbpA proteins present in *H. influenzae*, *N. meningitidis* and *N. gonorrhoeae* are relatively close homologues [36]. Recent extensive studies probing the kinetics of iron binding and release by FbpA have provided considerable insights and have led to hypotheses regarding the iron uptake process *in vivo* [24,25,37,38]. In regard to the transfer of iron from the outer membrane receptor complex to FbpA, the authors have proposed two hypotheses, one involving reduction and reoxidation of the iron atom [25]. In both hypotheses the synergistic anion associated with the binding site

of FbpA is required for iron binding and is proposed to play important structural and thermodynamic roles in the process. However, the present study demonstrates that the site-directed mutants in the anion-binding ligands, N175L and N193L, that are defective in binding the synergistic anion (Figure 2) [29], were capable of supporting growth of iron-limited cells with exogenous transferrin (Table 3). This suggests that a bound phosphate anion is not essential for transfer of iron from the outer membrane transferrin receptor complex to FbpA.

Previous kinetic studies indicated that dissociation of the synergistic anion was important for the release of ferric iron, suggesting that it would expedite the process of iron donation to the bacterial permease, FbpB [24,37]. In this model, the dissolution of the thermodynamically stable complex of FbpA with ferric iron and phosphate would begin with protonation of the synergistic anion [24]. A competing ferric-ion-binding site, possibly provided by the FbpB *in vivo*, would provide the driving force for release of Fe³⁺ from FbpA [37]. However, the results in the present study (Table 3) suggest that strains expressing mutant proteins that were defective in binding of the phosphate anion (Figure 2) were capable of donating iron to the inner membrane complex. Clearly, alternative models for iron removal from FbpA at the inner membrane interface need to be considered.

The lack of electron density in the anion-binding pocket of FbpA as demonstrated by crystallography of N193L (Figure 2B) and N175L [29] provides convincing evidence that no phosphate anion is present. Although this cannot completely exclude the possibility that anion-binding pocket is capable of binding an anion under physiological conditions in the periplasm, it strongly suggests that mechanisms of iron transport that do not involve a synergistic anion need to be seriously considered. Although many of the present studies have been focused on the FbpA from *H. influenzae*, *N. meningitidis* and *N. gonorrhoeae*, which share the same distorted octahedral complex for iron, and collectively have an iron-co-ordination scheme analogous to transferrin [23,36], it is important to recognize that functional homologues exist with alternative iron co-ordination geometries. Thus the periplasmic iron-binding protein from *M. haemolytica* is also involved in mediating iron transport from transferrin, but has a drastically different iron co-ordination scheme [28,39]. Assuming that the mechanisms of iron transport are similar, then mechanisms of binding and release that require the involvement of specific anions or residues may be unlikely. An assortment of anion-independent FbpA structures from other pathogens have been reported and include *Yersinia enterocolitica* YfuA [39], *Serratia marcescens* SfuA [39] and *Campylobacter jejuni* FbpA [40].

The one common property of all of the structurally characterized FbpAs is their ability to undergo facile opening and closing of the ligand-binding cleft via hinge-like rotations of the N- and C-terminal domains. Additionally, it has been demonstrated in the present study, and others [39], that FbpAs from multiple classes are able to co-ordinate and transport ferric ion in an open and anion-free conformation. These observations are consistent with a mechanism of iron transport at the outer membrane where FbpA docks to the outer membrane complex in an open form, with a sterically accessible iron-binding cleft. The affinity of the open form for ferric ion, although weaker than the closed form, may be sufficient for iron acquisition from the transferrin receptor complex in the outer membrane. Anions likely increase the efficiency of this process, by inducing domain closure and dissociation of FbpA from the outer membrane proteins. At the inner membrane, the affinity difference for ferric ion between the opened and closed forms is likely the feature that is exploited for the final transport of iron into the cytoplasm, so that the role of the inner membrane complex may be to capture and stabilize an

open form of FbpA. Clearly more direct experimental evidence is required to demonstrate whether the mechanism of transport is not dependent on the mode of iron co-ordination.

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