Metal-Dependent Conformers of the Periplasmic Ferric Ion Binding Protein†

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ABSTRACT: One of the better understood structural correlates of Fe^{3+} binding by the transferrins is the conformational shift demonstrated by both lobes. FbpA, a prokaryotic protein involved in periplasmic iron transport, has previously been shown to be structurally and functionally homologous to the transferrins. Similar to each individual lobe of the transferrins, it is hypothesized that FbpA exists in two distinct conformations depending on whether metal is bound. Evidence for these changes is provided by the differential susceptibility of FbpA to trypsin digestion. Binding of Fe^{3+} by FbpA significantly decreases the ability of trypsin to digest wild-type protein. Construction of a null binding mutant, Tyr195Ile, confirms that protein "locked" in the apo-conformation is similarly susceptible to trypsin. This mutant also marks the initial characterization of an FbpA molecule unable to bind iron, suggesting that the Tyr195 residue is directly involved in iron binding. Other FbpA mutants which do bind iron show moderate resistance to digestion which suggests that they remain in the holo-protein conformation when binding Fe^{3+}. The conformational states of FbpA may have important implications in protein−protein recognition during transport of Fe^{3+} between membranes, and may explain how these proteins function in the context of periplasm-to-cytosol Fe^{3+} transport.

Fbp[1] (Ferric ion binding protein) is a periplasmic binding protein expressed by *Neisseria gonorrhoeae* and *Neisseria meningitidis* (collectively referred to as the pathogenic Neisseria) and is critical for the transport of growth-essential Fe^{3+} from the periplasm into the bacterial cytosol (*1, 2*). Fbp is characteristic of the well-described class of periplasmic binding proteins, but is unique in that it functions in the transport of free iron (Fe^{3+}) (*1*). Fbp-mediated, high-affinity Fe^{3+} transport is a specific and efficient mechanism of iron acquisition which, in combination with other factors, allows pathogenic Neisseria to aggressively multiply within the human host (*3*). The vigorous host response to the resulting bacterial load causes the pathology associated with these organisms; thus, Fbp plays a critical role in gonococcal and meningococcal disease.

fbpA is the first gene in an operon (*fbpABC*) which encodes three open reading frames (*4*). As such, the protein encoded by this gene is referred to throughout this paper as FbpA. FbpA functions as a periplasmic binding protein, shuttling Fe^{3+} across the periplasmic space and cytoplasmic membrane (*5*). Based upon relatively low-resolution chemical modification studies, the binding of Fe^{3+} by FbpA appears to occur in a manner highly analogous to the transferrins (*6*). Preliminary crystallographic analysis supports this observation (T.A.M.). The second gene of this operon, *fbpB*, is predicted to encode a cytoplasmic permease that removes Fe^{3+} from FbpA and transports this cation across the inner membrane (*4*). The third genetic unit, *fbpC*, encodes a putative nucleotide binding protein that supplies the energy for transport of Fe^{3+} to the cytoplasmic permease using energy derived from the hydrolysis of a phosphate bond (*4*). The proposed functions of FbpB and FbpC have not been explored biochemically, but rather are inferred based on homology with, and the similar operon organization of, well-defined examples of periplasm-to-cytosol transport of other small ligands (*e.g.*, maltose and histidine) in *E. coli* and related organisms (*7, 8*). Homologs of the pathogenic Neisseria *fbpABC* operon have been described for members of the Pasteurelleaceae (*hitABC*) (*9, 10*) and the Enterobacteriaceae (*sfuABC*) (*11*−*13*). For all of the known periplasm-to-cytosol transporters, the participation of a periplasmic binding protein, a cytoplasmic permease, and a nucleotide binding protein is critical for successful transport of the ligand. By inference, this transport process must involve protein−protein interactions among these three protein components. These interactions may be dependent upon conformational changes in any of the proteins involved in the transport process (*14, 15*).

An early indication that human transferrin assumes distinct conformational states dependent on Fe^{3+} cations bound was the differential susceptibility to trypsin digestion of holo-hTf versus apo-hTf (*16*). It was recognized that binding of iron to either lobe of hTf correlates with a decrease in the sensitivity of the protein to trypsinization. In our previous work comparing the structure and function of the transferrins and FbpA, we have demonstrated significant evidence of gross structural and functional homology between the two protein families (*17*). However, data on the specific conformations of FbpA, and their linkage to the coordination of Fe^{3+}, have not yet been described.

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[1] Abbreviations: Fbp, ferric ion binding protein; Fe^{3+}, free ferric iron; NTA, nitrilotriacetic acid; PCR, polymerase chain reaction; ELISA, enzyme-linked immunosorbant assay; hTf, human transferrin; Tyr195Ile, Tyr to Ile change at position 195 of wild-type FbpA; Tyr264Phe, Tyr to Phe change at position 264 of wild-type FbpA.

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This study focuses on FbpA and the different conformations that this protein may attain. The ability to exist in two distinct conformations is a characteristic of another family of Fe^{3+} binding proteins, the eukaryotic transferrins (*18*). The transferrins are bilobed molecules that bind a single Fe^{3+} cation per lobe. During the process of delivering Fe^{3+} to eukaryotic cells, hTf undergoes a conformational change depending on whether Fe^{3+} is bound; this conformational shift is critical for receptor recognition and subsequent Fe^{3+} uptake (*19*). FbpA is roughly the size of a single transferrin lobe and stoichiometrically coordinates a single molecule of Fe^{3+}, using an identical set of amino acid side chain and anion ligands (*5, 17, 20*). It may thus be considered a structural homolog of a mono-lobed transferrin. Like the transferrins, the role of FbpA is to transport Fe^{3+} between membranes, though the site of action is different. In the case of FbpA, transport of Fe^{3+} occurs between the outer and inner membranes (the periplasmic space). In the case of the transferrins, transport of Fe^{3+} occurs in the extracellular space between plasma membranes. Thus, FbpA and the transferrins possess a functional homology that correlates with their structural homology.

The structural and functional similarities between each lobe of the transferrins and FbpA homologs suggest that these proteins may undergo similar conformational changes during iron transport. Whether FbpA can exist in two distinct conformations, as a holoprotein (metal-bound) form or an apoprotein (metal-free) form, has not been previously demonstrated. As for the transferrins, a structural shift may be important to the high-affinity transport of Fe^{3+}, and play a crucial role in the efficient interaction of FbpA with other members of the periplasm-to-cytosol transport system (*4*).

This study provides the first physical evidence that FbpA exists in two conformational states. This is demonstrated by evaluating the trypsin sensitivity of the apo- and Fe^{3+}-bound forms of FbpA. Compelling evidence that trypsin sensitivity is a useful technique to study metal-dependent conformers of FbpA is provided by analysis of wild-type protein, as well as site-directed mutants of FbpA that either do or do not bind metal. These proteins also demonstrate that site-directed mutants of FbpA can be constructed which do not bind Fe^{3+} or which are attenuated in their ability to bind Fe^{3+}. We correlate the metal ion binding activity of these mutants with their spectroscopic profiles and trypsin sensitivity. These data support the hypothesis that FbpA assumes two structurally distinct conformations, dependent upon whether a metal ion is bound to this molecule. Ultimately, these studies shed light on the function of FbpA in the dynamic process of Fe^{3+} transport from the periplasm to cytosol.

MATERIALS AND METHODS

Strains and FbpA Wild-Type and Site-Directed Plasmid Constructs. Native FbpA was obtained from the construct pSBGL in an *Escherichia coli* strain DH5α background, as previously described (*17, 21*). Site-directed mutagenesis was performed using two different techniques. For production of the Tyr264Phe mutant, the Altered Sites system (Promega, Madison, WI) was utilized as instructed by the manufacturer. For these studies, the *Hin*dIII/*Eco*RI fragment encoding FbpA in pSBGL was cloned into the identical site in the pSELECT background. The oligonucleotide 5′-CGGATTCAAAGGATATTCGGCACG-3′ was used to convert the tyrosine to a phenylalanine at position 264 of the mature protein.

Alternatively, primer overlap extension was used to create the Tyr195Ile mutant (*22, 23*). For this mutant, complementary oligonucleotide primers were synthesized incorporating a two-base change that encodes the Tyr195Ile amino acid change using the oligonucleotide 5′-GCGAAATAGATGCCGCCGTCATCAACAACTACATCTGGCACGC-3′ and the complement of this primer. These primers were paired with the M13 forward and reverse primers (Promega) and amplified using pSBGL as the *fbpA* template to produce two overlapping half-fragments. A second round of amplification was performed using these combined products as template and M13 forward and reverse primers to produce full-length, mutated FbpA. The M13 primers in this round also contained engineered *Nco*I and *Bam*HI sites, which were used to digest the product and ligate it into the multiple cloning site of pET-11D. Successful ligations were selected upon transformation of *E. coli* BL21(DE3); protein expression was induced via addition of IPTG to log phase cultures per instructions (Stratagene, La Jolla, CA).

Purification of FbpA and Mutants. FbpA and mutant proteins were purified from *E. coli* using a modification of the method previously described (5, 17). Briefly, 2 L bacterial cultures were prepared under conditions in which FbpA or mutant protein was maximally expressed. These organisms were washed once in cold phosphate-buffered saline and suspended in 25 mL of 1 M Tris, pH 8.0. The cells were lysed by the addition of 25 mL of a 4% (w/v) cetyltrimethylammonium bromide solution followed by shaking for 1 h at 37 °C. Particulates were removed by centrifugation (7000*g* for 15 min at 4 °C), and the solubilized material was diluted to a final volume of 1 L by the addition of Milli-Q H_2O. Diluted lysate was clarified by filtration and applied to a CM-Sepharose CL-6B (Sigma Chemical Co., St. Louis, MO) column (6 cm diameter, 15 cm length), equilibrated as previously described. The detergent-solubilized extract was eluted from the column using a NaCl gradient. Peak fractions were identified by their absorbance at 280 nm and confirmed as purified FbpA by SDS−PAGE. Native FbpA was quantitated using a previously established extinction coefficient at 280 nm (*17*). FbpA mutants used for this study were quantitated using a modified Lowry procedure as previously described (*20*).

Deferration and Metal Saturation of FbpA and FbpA Mutants. For these procedures, all glassware was prepared by acid washing, and buffers were rendered metal free by treatment with Chelex 100 (BioRad, Freemont, CA). For deferration, a solution of purified FbpA, diluted to 1 mg/mL in 10 mM Tris, pH 8.0, was loaded onto a 10 mL CM-Sepharose column. The bound protein was then exposed to 10 volumes of 5 mM citrate in 10 mM Tris, pH 8.0, followed by washing with 10 mM Tris, pH 8.0. If apo-FbpA was desired, the column was eluted with 0.5 M NaCl in 10 mM Tris, pH 8.0. Deferration was confirmed by an absence of visible absorbance at 481 nm in the protein solution. Column-bound apo-protein could also be loaded with other metals by saturation with appropriate complexes. For Tb^{3+} saturation, the column-bound apo-FbpA was exposed to 250 mL of 5 mM Tb^{3+}•citrate in 10 mM Tris, pH 8.0, followed by elution as described above; Tb^{3+} saturation was confirmed through luminescence spectroscopy (see below).

Mass Spectrometry. The masses of the expressed proteins were determined by pneumatically-assisted electrospray mass spectrometry with a Perkin Elmer/Sciex API I mass spectrometer equipped with an atmospheric pressure ionization source and an articulated IonSpray interface (Perkin Elmer, Norwalk, CT). Proteins were dissolved at a concentration of 10 mg/mL in 99:1 $H_2O-CH_3CO_2H$, 25 μL of which was introduced into the ionization source in a flowing stream (40 μL/min) of 99:99:2 $H_2O-CH_3OH-CH_3CO_2H$ delivered through glass capillary tubing by a Hewlett Packard 1090 Series II liquid chromatograph equipped with a Hewlett Packard 1040 diode array UV detector (Hewlett Packard). Effluent was monitored by a primary diode array detector with a wavelength of 214 nm and by mass spectrometry. The ionspray interface was maintained at 5 kV. High-purity air was used as the nebulizing gas and was maintained at an operating pressure of 40 psi. The orifice of the mass spectrometer was operated at 70 eV. High-purity N_2 heated to 55 °C was used as the curtain gas, flowing at 0.6 L/min. The quadrupole was scanned in the appropriate *m/z* range in 8−11 s/scan at a resolution of *m/z* 0.1. Protein masses were reconstructed from the average of >10 multiply-charged envelopes of quasi-molecular ions by the Fenn algorithm as implemented by the mass spectrometer manufacturer (*24*), resulting in a resolution of ±2 atomic mass units in the reconstructed mass ranges studied.

Trypsin Sensitivity of Apo- and Holo-FbpA and Mutants. For these experiments, 50 μg of deferrated FbpA (or site-directed mutant) was equilibrated for 30 min at room temperature in the presence or absence of 1.1 molar equiv of Fe^{3+}•NTA, in a total volume of 150 μL of 40 mM Tris, pH 8.0, and 65 mM NaCl. To this was added a 1/100 molar equiv of trypsin (Sigma) and incubated at 25 °C. Samples (30 μL) were removed at various time points, added to an equal volume of final sample SDS buffer [20% (w/v) sodium dodecyl sulfate, 20% (v/v) glycerol, 200 mM Tris, pH 6.8, and 5% (v/v) 2-mercaptoethanol], and immediately boiled for 5 min. The extent of trypsinization was assessed after analysis of the sample by SDS−PAGE (12% gel) and visualization by Coomassie staining. FbpA trypsin susceptibility was quantitated by densitometric analysis of scanned gels using 1-DIM software (Biomed Instruments, Fullerton, CA).

Spectroscopy of FbpA and Site-Directed Mutants. The visible absorbance of Fe^{3+}•FbpA and mutants was measured using 2 mg/mL (≈60 μM) solutions of protein in 200 mM NaCl and 10 mM Tris, pH 8.0. Absorbance spectra were acquired as previously described using a Hitachi UV/Vis spectrophotometer (Hitachi Corp., Tokyo, Japan). Tb^{3+}•FbpA and Tb^{3+}•mutants were studied by lanthanide luminescence spectroscopy as previously described (*17*). For these experiments, 10 μM solutions of each purified protein were prepared as above and dialyzed in 10 mM Tris, 25 mM $NaHCO_3$, and 200 mM NaCl, pH 8.0. Luminescence spectra were recorded on a Perkin Elmer LS-50B spectrofluorometer equipped with a pulsed xenon lamp with computer correction. Excitation spectra from 200 to 350 nm using a fixed emission wavelength (λ_{em}) of 545 nm were recorded as a measure of the energy transfer from donor residues in the local metal ion binding environment for each variant protein to the bound Tb^{3+} cation. Data were recorded as ASCII files and graphically manipulated with the Excel software program (Microsoft Corp., Renton, WA).

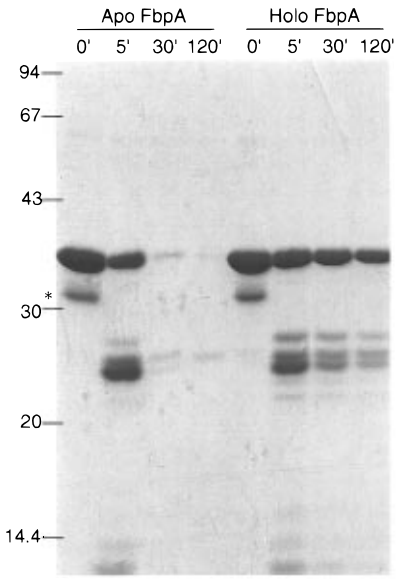

FIGURE 1: Trypsin sensitivity of apo- and holo-FbpA. The sensitivity of apo-FbpA to trypsin digestion was compared with apo-FbpA incubated in the presence of stoichiometric concentrations of Fe^{3+}•NTA. For each of these preparations, trypsin sensitivity was monitored as a function of time by SDS−PAGE. The results demonstrate that Fe^{3+}−FbpA is much more resistant to proteolysis than apo-FbpA alone. The asterisk indicates an impurity present in the FbpA preparation.

RESULTS

Trypsin Susceptibility of Holo- and Apo-FbpA. The susceptibility of apo- and holo-FbpA to trypsin digestion was followed over time with SDS−PAGE. This analysis (Figure 1) demonstrates that holo-FbpA is more resistant to trypsinization than apo-FbpA. Under the conditions used, little (about 2%) apo-FbpA remained undigested at 30 min, whereas approximately 35% of the holo-FbpA was protected from trypsin digestion. After even 120 min of digestion, some holo-FbpA remained refractory to trypsinization, whereas apo-FbpA was completely proteolyzed (Figure 1). This differential sensitivity to trypsin is consistent with FbpA existing in two conformational states which are dependent upon the binding of Fe^{3+}. These data mirror the studies performed with the transferrins, which show that conformational shifts in each lobe occur coincident with Fe^{3+} binding (*16*, *18*).

Site-Directed Mutants That Test the Metal Ion Dependent, Two-Conformer FbpA Hypothesis. Further evidence that the differential trypsin susceptibility of apo- and holo-FbpA truly represents two distinct conformations was tested by the generation and evaluation of site-directed mutants specifically altered in their Fe^{3+} binding properties. For these studies, Tyr residues were targeted based on previously described chemical modification data, which suggested that at least two of the Tyr residues in the FbpA sequence must play a critical role in Fe^{3+} binding by FbpA (*17*). Of the 11 Tyr that are found within FbpA, only 5 are conserved among FbpA, HitA, and SfuA. These conserved residues are located at amino acid positions 5, 107, 194, 195, and 264 of the mature FbpA. Mutations involving two of these residues, Tyr195Ile and Tyr264Phe, were informative for further investigation of the issue of FbpA metal-dependent conformational changes.

Successful construction and expression of FbpA mutants was confirmed by electrospray mass spectrometry by correlating the change in molecular mass between each mutant

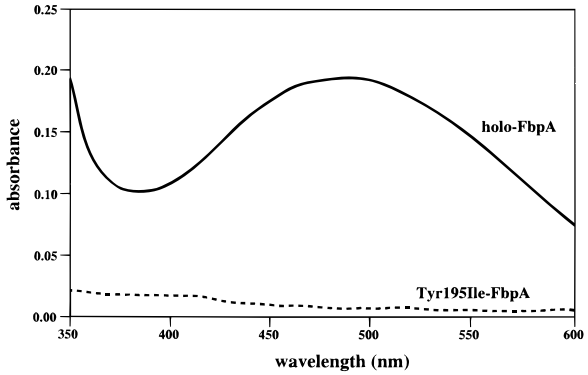

FIGURE 2: Absorbance spectra of holo-FbpA and Fe^{3+}-saturated Tyr195Ile. The UV/Vis spectra of equimolar concentrations of the holo-FbpA and Fe^{3+}-saturated Tyr195Ile were recorded as described under Materials and Methods. The spectra demonstrate that the absorbance at 481 nm, characteristic of both the Fbps and the transferrins, does not exist for the Tyr195Ile mutation, suggesting it is unable to bind Fe^{3+}.

from that of native FbpA. Wild-type FbpA yielded an apparent molecular mass of 33 606 Da, in agreement with DNA sequence data. The Tyr195Ile mutant possessed a calculated molecular mass of 32 572 Da, and the Tyr264Phe mutant demonstrated a calculated molecular mass of 32 590 Da. Each of these values is within two atomic mass units of their predicted mass, which is within the level of resolution of the instrument for a protein of this mass range. Furthermore, the mass spectroscopy profiles indicated that each protein preparation was of high analytical purity.

Visible Spectral Comparison of FbpA, Tyr195Ile, and Tyr264Phe. One of the signature characteristics that FbpA shares with the transferrins is a strong visible absorption in the 480 nm range, which is due to the presence of Fe^{3+} bound by a characteristic ligand field. This Fe^{3+} binding motif is shared by both FbpA and the transferrins. Figure 2 demonstrates that the substitution of an Ile for Tyr at position 195 completely abrogated any Fe^{3+}•FbpA visible absorbance, suggesting that this Tyr is critical to the coordination of Fe^{3+} by FbpA. By contrast, Tyr264Phe possessed a visible spectrum that is similar to wild-type Fe^{3+}−FbpA (data not shown). These results predict that the Tyr195Ile FbpA mutant exists in an apo-conformation, and that the Tyr264Phe FbpA mutant can bind metal, albeit with less efficiency than the wild-type protein.

An alternative explanation of the Tyr195Ile FbpA mutant data is that this change may result in an FbpA that is conformationally distinct from apo-FbpA. Refuting this argument are studies which demonstrated that a panel of monoclonal antibodies against FbpA react equally well to this mutant in a standard solid phase ELISA (*25*)(data not shown) (*26*). Furthermore, the chromatographic behavior of the Tyr195Ile FbpA mutant is indistinguishable from apo-FbpA in terms of elution from a cation exchange column (data not shown). These data provide supportive evidence that the Tyr195Ile FbpA mutant is "locked" in the apo-FbpA conformation. It also suggests that Tyr195 of FbpA is a ligand directly involved in the coordination of Fe^{3+}.

Tb^{3+} Luminescence Spectra of FbpA and Mutants. The use of Tb^{3+} as a probe for metal−protein interactions provides a tool for examining the aromatic amino acid side chain involvement in the metal binding environment of appropriate proteins, such as FbpA. We have previously

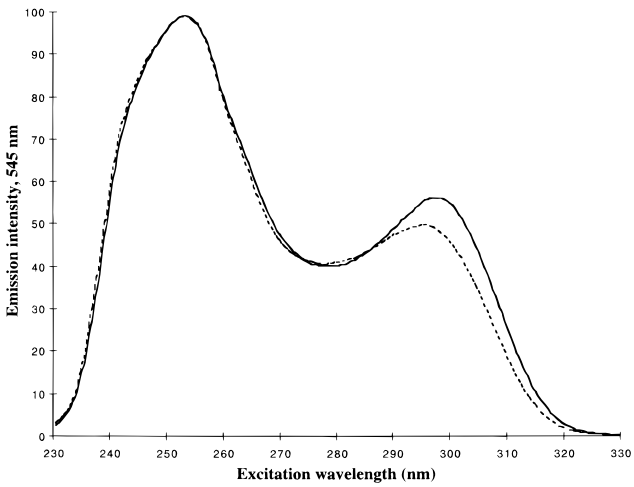

FIGURE 3: Comparison of FbpA and site-directed mutant Tb^{3+} luminescence profiles. The apo•forms of FbpA (solid line) and Tyr264Phe (dashed line) were incubated with Tb^{3+} as described under Materials and Methods. The spectra shown are plots of Tb^{3+} luminescence at the characteristic 545 nm emission as a function of excitation wavelength.

reported that FbpA binds Tb^{3+}, and that the aromatic amino acid sensitized luminescence spectra of Tb^{3+}•FbpA and Tb^{3+}•hTf are similar (*17*). For the present experiments, metal-free FbpA and mutants were incubated with Tb^{3+}•NTA as previously described, and their excitation spectra were recorded in the UV range where aromatic amino acids absorb, using an emission wavelength of 545 nm (Figure 3). While wild-type FbpA demonstrated a characteristic excitation spectrum from 200 to 350 nm, the Tb^{3+}−Tyr195Ile complex demonstrated no detectable Tb^{3+}-dependent emission upon excitation (data not shown). These data suggest that Tb^{3+} does not interact with the Tyr195Ile FbpA binding site. Tb^{3+}−Tyr264Phe complexes demonstrated a Tb^{3+} excitation spectrum that was nearly identical to native FbpA (Figure 3). Because the fluorescence intensity of Tb^{3+} is distance-dependent (r^{-6}), a significant change in a ligating residue should be reflected in an altered excitation spectrum. Because Tyr264Phe did not show such a change, it is unlikely that Tyr264 directly participates in metal binding. Preliminary metal binding studies suggest, however, that this mutant has a decreased affinity for metal by approximately 2 orders of magnitude (T.A.M., unpublished data). An interpretation consistent with these results is that Tyr264 does not directly coordinate metal cations, yet is involved in other structural interactions which act to mildly destabilize the holo-conformation which this protein assumes upon binding of Tb^{3+} or Fe^{3+}. The Tb^{3+}−FbpA interaction represents a probe for functional metal binding that can be applied to FbpA variants in order to assess the environment of the metal binding site.

Trypsin Susceptibilities of the Tyr195Ile and the Tyr264Phe Mutants. The FbpA mutants described above were tested for their iron-dependent sensitivity to trypsin. Both the Tyr195Ile and Tyr264Phe mutants were equally sensitive to trypsin exposure in the absence of Fe^{3+}•NTA (Figure 4) and demonstrated proteolytic kinetics similar to the wild-type apo-FbpA. However, in the presence of Fe^{3+}•NTA, Tyr264Phe demonstrated protection from trypsin digestion, whereas Tyr195Ile demonstrated no metal-dependent resistance to trypsin digestion (Figure 4A). This result is consistent both with the suggestion that Tyr264Phe assumes

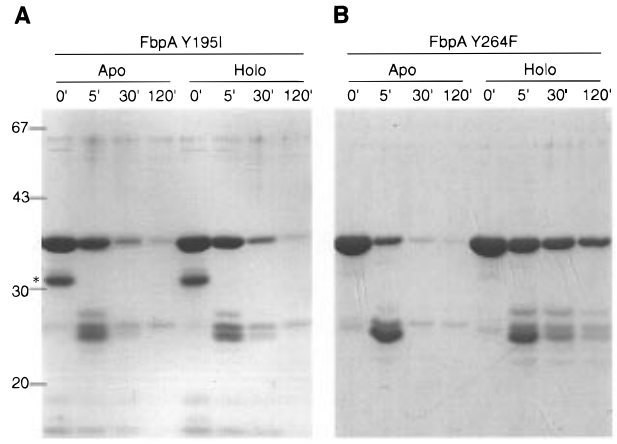

FIGURE 4: Protease sensitivity of site-directed FbpA mutants. The Fe^{3+}-dependent sensitivity of Tyr195Ile (panel A) and Tyr264Phe (panel B) was examined by SDS−PAGE in a time-course experiment from 1 to 120 min. Both proteins were exposed to trypsin over time in the presence and absence of Fe^{3+}. While Tyr195Ile is susceptible to trypsin despite the presence of Fe^{3+}, the decrease in Tyr264Phe sensitivity to digestion in the presence of Fe^{3+} mirrors the results from wild-type FbpA. The asterisk indicates an impurity present in the FbpA preparation.

the wild-type holo-protein conformation upon coordination of Fe^{3+}, as well as with the assumption that Tyr195Ile is "locked" in the apo-protein configuration. These data confirm and extend the results of the spectroscopic studies, providing further evidence for the existence of distinct conformational states in FbpA.

DISCUSSION

This study provides evidence that two conformers of FbpA exist, depending upon whether metal is bound. This supports the hypothesis based on previous studies which suggests that Fe^{3+} binding by FbpA and the transferrins is accomplished through analogous mechanisms (*17*). Similarly, it has been proposed that FbpA functions as a classic periplasmic binding protein (*4, 5*). The three-dimensional structures of the transferrins and many periplasmic binding proteins have been resolved, and common structural properties have been demonstrated (*27*). One such property is a significant shift in conformation between ligand-free and ligand-bound states. Thus, given its similarities to both the transferrins and the periplasmic binding proteins, FbpA would be expected to undergo structural changes depending upon coordination of metal. However, prior to this study, formal proof for this phenomenon in FbpA had not been reported.

The data described here address the issue of ligand-induced conformation by providing evidence that FbpA can exist as two independent conformers. This structural change may be of particular importance for the transport of Fe^{3+} by bacterial pathogens that express a Fe^{3+} transport system homologous to FbpABC (*1*). Proof of this change in conformation is derived from two biochemical approaches: sensitivity to tryptic digestion and metal binding properties of both the wild-type and site-directed mutants of FbpA. Holo-FbpA is relatively resistant to trypsin digestion, as is a site-directed mutant that is proficient in iron binding (Tyr264Phe). By contrast, trypsin sensitivity is demonstrated for apo-FbpA, as well as for the null binding mutant, Tyr195Ile. These differences are similar to the trypsin susceptibility patterns of apo- and holo-transferrin, and are consistent with other studies of both the transferrins and the periplasmic binding proteins that demonstrate significant structural differences between apo- and holo-proteins (*12, 16*). However, direct evidence that the conformational transitions of FbpA are similar to these well-characterized protein families must await more detailed structural studies.

Another significant finding reported in this study is the identification of Tyr195 as a key iron binding residue. Mutation of this residue, which renders the protein unable to efficiently bind Fe^{3+}, appears to represent an FbpA variant that is "locked in" the apo-protein conformation. One reasonable conclusion from studies of this mutant is that Tyr195 is directly involved in Fe^{3+} binding by FbpA. Although the possibility that the radical substitution of an Ile for a Tyr leads to a dramatic alteration in FbpA conformation cannot be formally excluded, the similarity of trypsin sensitivity between apo-FbpA and Tyr195Ile strongly suggests that the mutant is structurally identical to the metal-free state of the wild-type protein. While attempts at preparing the more subtle Tyr to Phe mutation at position 195 were not successful (data not shown), previous studies have demonstrated that, like the transferrins, Tyr plays a critical role in metal coordination. The observation that this residue is conserved among the FbpA homologs HitA and SfuA is further evidence that this residue is directly involved in Fe^{3+} coordination (*1*).

Like Tyr195, Tyr264 is conserved among the FbpA homologs. Mutating this residue to Phe results in a protein that demonstrates a nearly identical visible absorbance spectrum as FbpA, yet has a 100-fold lower affinity for Tb^{3+} (data not shown). A similar decrease in the affinity of this mutant relative to the wild-type protein can be demonstrated for Fe^{3+} interactions using citrate competition (T.A.M., unpublished data). The trypsin sensitivity pattern of this protein is similar to wild-type FbpA. Thus, this mutation appears to result in a protein that is able to exist in either an apo- or a holo-conformer, but which is attenuated in its metal binding properties. One interpretation of this observation is that Tyr264 is not directly involved in the liganding of metal but may be important in other ways, through interdomain hydrogen bonds or other tertiary structure interactions. Further structural studies of FbpA will be informative in addressing the role that this conserved Tyr plays in metal binding.

The capacity of periplasmic binding proteins to exist in either a ligand-bound or a ligand-free conformation has been assumed to be a critical property for the transport of ligand through the periplasm and across the cytoplasmic membrane. This model purports that ligand-bound periplasmic binding protein will have a different affinity for the respective cytoplasmic permease than the ligand-free form of the periplasmic binding protein. This assumption has recently been challenged by an appreciation for the restrictions on protein diffusion within the Gram-negative periplasm, and by the report that the periplasmic histidine binding protein (HisJ) has an affinity for its cytoplasmic permease component which is independent of whether ligand is bound to HisJ (*29*). Similar results have been shown for MalE, the maltose periplasmic binding protein (*30*). Accompanying these experimental data have been theoretical arguments for the lack of importance of conformational discrimination in periplasm-to-cytosol transport of maltose (*31*). These reports tie ligand transport efficiency to the concentration of the

periplasmic binding component and affinity of the periplasmic binding protein for its specific ligand, throwing some previous assumptions about the role of periplasmic binding protein conformations into doubt.

Though it shares many basic functional and structural characteristics with proteins such as HisJ and MalE, FbpA differs from these well-established models of periplasm-to-cytosol ligand transport in two significant ways. The first involves the nature of the ligand which is transported. The chemistry of Fe^{3+} is significantly different from amino acids or sugars, owing to the extremes of toxicity and solubility demonstrated by this element. Free Fe^{3+} is both highly insoluble (K_{sp} 10^{-38} M at physiological pH) and able to catalyze the production of $OH^{\cdot}$ and O_2^- through Fenton chemistry (*32*). Thus, simple diffusion of this element in its free form across the periplasm to a bound receptor/transporter complex is chemically discouraged. However, this does not exclude the possibility that a "periplasmic mobilizer" of Fe^{3+} may exist, which facilitates the diffusion of this metal through the periplasm while limiting its potential toxicity. The second difference derives from FbpA's high affinity for the ligand it transports. We have previously estimated that FbpA binds Fe^{3+} with a K_d on the order of 10^{-20} M (*5*); this can be compared to HisJ, which binds histidine with a K_d of 3×10^{-8} M (*33*), or MalE, which binds maltose in the micromolar range (*34*). This difference in ligand affinity may manifest itself in a biologic scenario where, similar to the transport of Fe^{3+} by transferrin, discrimination between the conformational states of FbpA by the cytoplasmic permease FbpB may be critical. One other interaction which may be dependent on FbpA conformation is the transfer of iron from transferrin to FbpA. In pathogenic Neisseria, the outer surface receptor complex of TbpA/B binds human transferrin, which is followed by mobilization of transferrin-bound Fe^{3+} into the periplasm (*3*). It is possible that the interaction at the inner leaflet of the outer membrane between FbpA and the TbpA/B complex is also dependent upon the conformational state of the periplasmic binding protein. Preliminary evidence for this interaction can be found in the observation by Schryvers and colleagues that a 37 kDa protein copurified with TbpA (*35*). Whether differential recognition of FbpA conformers governs interactions with either TbpA or FbpB will be the focus of future studies in the periplasm-to-cytosol transport of Fe^{3+} by pathogenic Neisseria.

REFERENCES

1. Mietzner, T. A., Tencza, S. B., Vaughan, K. G., Adhikari, P. A., and Nowalk, A. J. (1996) *Curr. Top. Microbiol. Immunol. 225*, 113.
2. Mietzner, T. A., and Morse, S. A. (1994) *Annu. Rev. Nutr. 14*, 471.
3. Mietzner, T. A., and Cohen, M. (1996) in *The molecular approach to new and improved vaccines* (Levine, M. M., Kaper, J., et al., Eds.) p 817, Marcel Dekker, Inc., New York.
4. Adhikari, P., Berish, S., Nowalk, A., Veraldi, K., Morse, S., and Mietzner, T. (1996) *J. Bacteriol. 178*, 2145.
5. Chen, C. Y., Berish, S. A., Morse, S. A., and Mietzner, T. A. (1993) *Mol. Microbiol. 10*, 311.
6. Nowalk, A. J., Tencza, S. B., and Mietzner, T. A. (1994) *Biochemistry 33*, 12769.
7. Ames, G. F. (1986) *Annu. Rev. Biochem. 55*, 397.
8. Tam, R., and Saier, M. H., Jr. (1993) *Microbiol. Rev. 57*, 320.
9. Sanders, J. D., Cope, L. D., and Hansen, E. J. (1994) *Infect. Immun. 6*, 4515.
10. Adhikari, P., Kirby, S. D., Nowalk, A. J., Veraldi, K. L., Schryvers, A. B., and Mietzner, T. A. (1995) *J. Biol. Chem. 270*, 25142.
11. Angerer, A., Gaisser, S., and Braun, V. (1990) *J. Bacteriol. 172*, 572.
12. Angerer, A., Klupp, B., and Braun, V. (1992) *J. Bacteriol. 174*, 1378.
13. Zimmerman, L., Angerer, A., and Braun, V. (1989) *J. Bacteriol. 171*, 238.
14. Schuman, H. A. (1992) *J. Biol. Chem. 257*, 5455.
15. Wolf, A., Lee, K. C., Kirsch, J. F., and Ames, G. F. (1996) *J. Biol. Chem. 271*, 21243.
16. Brock, J. H., Arzabe, F., Lampreave, F., and Pineiro, A. (1976) *Biochim. Biophys. Acta 446*, 214.
17. Nowalk, A. J., Tencza, S. B., and Mietzner, T. A. (1994) *Biochemistry 33*, 12769.
18. Welch, S. (1992) in *Transferrin: The iron carrier* (Welch, S., Ed.) p 61, CRC Press, Boca Raton, FL.
19. Welch, S. (1992) in *Transferrin: The iron carrier* (Welch, S., Ed.) p 25, CRC Press, Boca Raton, FL.
20. Mietzner, T. A., Bolan, G., Schoolnik, G. K., and Morse, S. A. (1987) *J. Exp. Med. 165*, 1041.
21. Berish, S. A., Chen, C. Y., Mietzner, T. A., and Morse, S. A. (1992) *Mol. Microbiol. 6*, 2607.
22. Horton, R. M., Ho, S. N., Pullen, J. K., Hunt, H. D., Cai, Z., and Pease, L. R. (1993) *Methods Enzymol. 217*, 270.
23. Morrison, H. G., and Desrosiers, R. C. (1993) *Biotechniques 14*, 454.
24. Covey, T. R., Bonner, R. F., Shushan, B. I., and Henion, J. (1988) *Rapid Commun. Mass Spectrom. 2*, 249.
25. Burroughs, S. E., Horrocks, W. D., Jr., Ren, H., and Klee, C. B. (1994) *Biochemistry 33*, 10428.
26. Goldstein, R. F., and Leung, E. (1990) *Ann. Biochem. 190*, 220.
27. Mietzner, T. A., Barnes, R. C., Jeanlouis, Y. A., Shafer, W. M., and Morse, S. A. (1986) *Infect. Immun. 51*, 60.
28. Anderson, B. F., Baker, H. M., Norris, G. E., Rice, D. W., and Baker, E. N. (1989) *J. Mol. Biol. 209*, 711.
29. Quiocho, F. A. (1990) *Philos. Trans. R. Soc. London, B 326*, 341.
30. Ames, G. F. L., Liu, C. E., Joshi, A. K., and Nikaido, K. (1996) *J. Biol. Chem. 271*, 14264.
31. Merino, G., Boos, W., Shuman, H. A., and Bohl, E. (1995) *J. Theor. Biol. 177*, 171.
32. Bohl, E., Shuman, H. A., and Boos, W. (1995) *J. Theor. Biol. 172*, 83.
33. Welch, S. (1992) in *Transferrin: The iron carrier* (Welch, S., Ed.) p 1, CRC Press, Boca Raton, FL.
34. Wolf, A., Shaw, E. W., Oh, B., De Bondt, H., Joshi, A. K., and Ames, G. F. (1995) *J. Biol. Chem. 270*, 16097.
35. Hor, L. I., and Shuman, H. A. (1993) *J. Mol. Biol. 233*, 659.
36. Schryvers, A. B., and Morris, L. J. (1988) *Mol. Microbiol. 2*, 281.

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