Coordination of Iron by the Ferric Iron-Binding Protein of Pathogenic *Neisseria* Is Homologous to the Transferrins†

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ABSTRACT: The ferric iron-binding protein (Fbp) functions as a periplasmic-binding protein in the high-affinity active transport of growth-essential iron by pathogenic *Neisseria*. Fbp reversibly binds a single ferric ion per molecule of protein with high affinity. Similarly, the transferrins are a highly conserved family of bilobed vertebrate proteins that reversibly bind a single molecule of iron on each of the N- and C-terminal lobes. While evolutionarily divergent, iron binding by all described transferrin lobes is accomplished by a remarkably similar repertoire of residues, including two Tyr, one His, and one Asp, as well as a synergestic bicarbonate anion. With a molecular mass of *ca.* 34 kDa, Fbp approximates the size of a transferrin lobe. Given the similarities in iron-binding properties, it was investigated whether Fbp bound iron by a similar molecular strategy as the transferrins. The studies reported here demonstrate that the spectral properties of purified Fbp and human transferrin are similar in the visible range. Chemical modification of purified Fbp in the presence and absence of iron using the Tyr-specific modifier tetranitromethane demonstrates that between two and three Tyr residues are implicated in iron binding. A similar experiment using the His-specific reagent diethyl pyrocarbonate indicates that one of the six Fbp-encoded His residues is protected by iron. In addition, like the transferrins, a bicarbonate anion is required for the efficient coordination of iron by Fbp. The range of metals bound by Fbp and human transferrin, including the luminescent lanthanide terbium, is identical. Finally, terbium derivatives of Fbp and human transferrin yield virtually identical luminescence excitation spectra, implying a highly similar binding site environment. These studies suggest that the prokaryotic Fbp is a mono-sited analog for iron binding by the eukaryotic transferrins.

Pathogenic *Neisseria*, *N. gonorrhoeae* and *N. meningitidis*, are the etiologic agents of gonorrhea and meningitis, respectively, in humans. Both of these pathogens express a highly conserved iron-derepressible protein that binds a single ferric ion per molecule of protein. This protein is termed Fbp[1] for ferric iron-binding protein and is localized to the periplasmic space of pathogenic *Neisseria* (Berish et al., 1990; Mietzner et al., 1984, 1986, 1987; Mietzner & Morse, 1985). It has been proposed that Fbp functions as the periplasmic-binding component of a high-affinity active transport system for the assimilation of growth-essential iron from a related family of host-derived iron-binding proteins, the transferrins. This family includes human serum transferrin, lactoferrin, and melanotransferrin (Welch, 1992). Fbp has been purified to homogeneity; it is a basic protein (pI >9.35) comprised of 308 residues (Berish et al., 1990, 1991; Mietzner et al., 1987). It binds Fe^{3+} with an affinity approaching that of the transferrins (Chen et al., 1993; Crichton, 1990; Mietzner et al., 1987). Recombinant Fbp (rFbp) has been cloned and overexpressed in *Escherichia coli*, allowing for its efficient purification at near-gram quantities (Berish et al., 1990, 1991). This has facilitated the biochemical analysis of iron binding by Fbp and contributed to understanding the participation of this protein in iron acquisition by pathogenic *Neisseria*.

Fbp demonstrates functional and structural characteristics of a well-characterized protein family, the prokaryotic periplasmic-binding proteins (PBPs). The PBPs participate in the active transport of growth-essential nutrients across the periplasmic space of Gram-negative bacteria (Adams & Oxender, 1989; Ames, 1986; Anderson et al., 1987; Quiocho, 1990). Structurally and functionally well-characterized examples of this family include the His-, arabinose-, and sulfate-binding proteins, among others (Ames, 1986). What has not been structurally and functionally characterized with respect to this family is a PBP that binds a growth-essential metal such as iron.

It has been recognized that the transport of iron in Gram-negative bacteria proceeds by an active transport process requiring a PBP component. Several PBPs involved in iron transport have been genetically described for *Escherichia coli*. FepB, for ferric enterochelin-binding protein, functions in the periplasmic transport of iron associated with this chatechol-type siderophore (Elkins & Earhart, 1989); FhuD, for ferric hydroxamate-binding protein, functions in the transport of iron bound to the general class of hydroxamate siderophores (Crosa, 1989); and FecB, for ferric citrate-binding protein (Staudenmaier et al., 1989), functions in the transport of iron bound to citrate. Each of these PBPs participates in iron acquisition by different iron chelates; however, the structural constraints and biochemical properties of these iron-binding PBPs have not been investigated. It is inferred that each of these PBPs binds iron in the context of their specific metal chelate–iron complex.

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[1] Abbreviations: Fbp, ferric iron-binding protein; rFbp, recombinant Fbp; PBP, periplasmic-binding protein; Fe-PBP, iron PBP; DEPC, diethyl pyrocarbonate; TNM, tetranitromethane; Tf, transferrin; hTf, human transferrin; Lf, lactoferrin; hLf, human lactoferrin; NTA, nitrilotriacetate; TSB, tryptic soy broth; Amp, ampicillin.

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For pathogenic *Neisseria*, Fbp functions in the periplasmic transport of iron from external sources. It can be classified as a member of the PBP family based upon several observations: (i) that levels of the protein are inversely regulated by the concentration of the specific nutrient (free iron) in the media; (ii) that it is similar in size (*ca.* 30 kDa) to other PBPs; (iii) that it reversibly binds the free nutrient (Fe^{3+}) for the purpose of transport; and (iv) that it is compartmentalized to the periplasmic space (Ames, 1986; Chen et al., 1993; Mietzner et al., 1984, 1987). Thus, Fbp represents an example of a PBP that binds free iron. There have been homologs of Fbp reported to be expressed by *Serratia marcescens* (Angerer et al., 1990), *Escherichia coli* (Yura et al., 1992), and *Haemophilus influenzae* (Harkness et al., 1992). All these organisms are potential disease-producing pathogens. It has been argued that expression of a functional Fbp by pathogenic *Neisseria* is a necessary but by itself insufficient property for pathogenicity (Mietzner et al., 1986).

In spite of showing little primary sequence homology, the PBPs of enteric organisms possess similar structural characteristics (Adams & Oxender, 1989; Ames, 1986; Quiocho, 1990). All demonstrate a characteristic three-dimensional folding motif composed of five or six strands of noncontiguous β-sheets, covered on each side by connecting α-helices (Adams & Oxender, 1989; Quiocho, 1990). For example, inspection of the enterobacterial arabinose-binding and sulfate-binding protein crystal structures demonstrates α-helix/β-sheet contents of 45/25% and 42/32%, respectively (Pflugrath & Quiocho, 1988; Quiocho et al., 1977). Similarly, circular dichroism analysis of purified Fbp indicates an α-helix/β-sheet content of 32/27% (Chen et al., 1993). Thus, although pathogenic *Neisseria* are are only distantly related to Enterobacteriaceae, Fbp demonstrates structural and functional characteristics of the PBP family.

Inspection of the crystal structures of human lactoferrin and rabbit transferrin (Anderson et al., 1987; Bailey et al., 1988) reveals a structural correlation with the bacterial PBPs (Adams & Oxender, 1989; Quiocho, 1990). Each lobe of rabbit transferrin exhibits a folding motif nearly identical to that of the PBPs (Adams & Oxender, 1989). Like Fbp, the transferrins also bind a ferric ion with high affinity. The mechanism by which iron is bound to the transferrins has been well-studied and is highly conserved (Brock, 1985, 1989; Welch, 1992). Previously, a great deal of information regarding the iron-binding sites has been obtained by studies of purified transferrins using spectrophotometric, chemical modification, and fluorometric approaches (Brock, 1985; Luk, 1971; O'Hara et al., 1981; Rogers et al., 1977; Williams, 1982). These studies have been corroborated by the crystallographic analysis of human lactoferrin (Anderson et al., 1987) and rabbit transferrin (Bailey et al., 1988). The coordination of each Fe^{3+} ion involves four protein ligands, including a carboxylate oxygen of an Asp residue, the phenolate oxygens of two Tyr residues, and an imidazole nitrogen of a His residue (Anderson et al., 1989). In addition, a bidentate oxygen ligand is contributed by a bicarbonate anion bridged to an Arg residue and the dipole moment of a well-conserved α-helix (Anderson et al., 1989). In spite of the evolutionary divergence between the transferrins, these binding site residues are remarkably conserved (Welch, 1992).

Experimental results presented here provide evidence that Fbp binds iron with structural and functional homology to the transferrins. These include spectrophotometric, chemical modification, and fluorometric proofs that validate this homology. As such, Fbp represents a model system for studying the transient iron-binding mechanisms of both the transferrins and the PBPs involved in iron acquisition.

MATERIALS AND METHODS

Materials. The host iron-binding protein, hTf (97% Fe^{3+}-saturated and apoprotein), ferric citrate, citric acid, gallium sulfate, cupric sulfate, aluminum choride, zinc chloride, NTA, ferric chloride, terbium chloride hexahydrate, TNM, DEPC, cetyltrimethylammonium bromide (CTAB), Amp, TSB, CM-Sepharose CL-6B, and Trizma-(Tris) base were purchased from Sigma Chemical Co. (St. Louis, MO). rFbp isolations were performed using a Bio-Rad Econo system (Bio-Rad, Hercules, CA); desalting of deferrated rFbp employed Bio-Rad Econo-10DG disposable columns. When appropriate, glassware and plastics used with deferrated rFbp or metal–rFbp salts were acid-washed with Chromerge solution (Fisher Scientific, Pittsburgh, PA) and rinsed copiously with distilled–deionized H_2O to remove trace metal contamination. In addition, all aqueous solutions were exposed to Chelex-100 resin (Bio-Rad) to remove metal contamination.

Purification of rFbp. rFbp was purified from *E. coli* strain JM109 containing the plasmid pSBGL by a modification of the method previously described (Berish et al., 1991). Briefly, a single colony from an overnight growth of bacteria on LB agar containing 100 μg/mL Amp was used to inoculate 25 mL of TSB containing 100 μg/mL Amp in a sterile 250 mL flask. This culture was incubated for 8 h at 37 °C and used to inoculate 2 L of TSB with 200 μg/mL Amp. After the flask was shaken overnight at 37 °C, organisms were removed by centrifugation (5000*g* for 15 min at 4 °C). Cells were washed once in cold phosphate-buffered saline and resuspended 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) CTAB 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 dH_2O. Diluted lysate was clarified by filtration through Whatman no. 4 filter paper (Fisher Scientific). The clarified lysate was applied to a CM-Sepharose CL-6B column (6 cm diameter, 15 cm length) equilibrated by washing with 5 volumes of 1 M NaOH, followed by 5 volumes of 10 mM Tris base, pH 8.0, containing 1 M NaCl (high-salt buffer) and 5 volumes of 10 mM Tris base, pH 8.0 (low-salt buffer). The soluble CTAB extract was applied at a flow rate of 2.0 mL/min and visually banded as a red ring at the top of the column. rFbp was eluted from the column using a gradient of low- to high-salt buffer in a final volume of 500 mL. Fractions of 5 mL were collected and analyzed for absorbance at 280 nm followed by SDS–PAGE as previously described (Mietzner et al., 1984), and the rFbp-containing fractions was pooled.

Deferration/Referration of rFbp and hTf. Solutions of rFbp and hTf (10 mg/mL) were acidified by the addition of 0.1 volume of 0.1% acetic acid, and iron was chelated by the addition of a 2000-fold molar excess of sodium citrate, pH 8.0, over rFbp. Excess citrate and iron–citrate were removed by desalting over an Econo-10DG column and fractions collected in acid-washed tubes. Referration was accomplished through addition of stoichiometric amounts of Fe^{3+}–NTA to apo-rFbp or apo-hTf solutions. The absorbance of either apo- and Fe^{3+}–rFbp or apo- and Fe^{3+}–hTf proteins was obtained at 481 nm (Fbp) or 470 nm (hTf) as described under Results.

Sequence Analysis of the Transferrins and Fbp. Sequences of bLf, hLf, hTf, and Fbp were analyzed for their percent similarity and identity using the BESTFIT program from University of Wisconsin Genetics Group. BESTFIT analyses

were performed using standard default parameters of gap weight 3.000 and length weight 0.1. Sequences for bovine and human lactoferrin and for hTf were deduced from the mRNA sequences entered in GENBANK.

Formation of Non-Iron Metal–rFbp Complexes. Deferrated solutions of rFbp were dialyzed overnight versus 50 molar excess metal–citrate/10 mM Tris, pH 8.0, where the metal ion was either Cu^{2+}, Ga^{3+}, Al^{3+}, Zn^{2+}, Cr^{3+}, or Ni^{2+}. Excess metal–citrate chelates were removed by dialysis against 10 mM Tris, pH 8.0.

Formation of Tb^{3+}–rFbp Complexes. Tb^{3+}–rFbp complexes were isolated by a novel, previously unreported, chromatographic procedure. This was accomplished by binding purified rFbp on a CM-Sepharose CL-6B column matrix as described above. The bound protein was deferrated by washing overnight with 20 volumes of a buffer containing 5 mM citrate and 10 mM Tris, pH 8.0. Tb^{3+}–rFbp complexes were formed by exposing the column to 10 volumes of a buffer containing 1 mM Tb^{3+}–citrate and 10 mM Tris, pH 8.0. Tb^{3+}–rFbp complexes were then eluted using a NaCl gradient as described above.

Chemical Modification and Metal Protection. DEPC modification of rFbp or hTf His residues was performed similarly to that previously described (Rogers et al., 1977) for a 2.0 mg/mL solution of both the ferric iron and apo forms of either rFbp (54 μM) or hTf (25 μM). These were buffered with 1% acetic acid, pH 6.0, and DEPC was added in 10 molar excess. The absorbance at 240 nm was followed spectrophotometrically for 30 min. Using a value of ϵ_{240nm} = 3200 $M^{-1}cm^{-1}$ for *N*-ethoxyformylhistidine (Rogers et al., 1977), the moles of His modified per mole of rFbp were determined at several points during the reaction versus time.

TNM modification of Tyr residues was performed similarly to that previously described (Williams, 1982) for solutions of 2 mg/mL iron-saturated rFbp (54 μM), apo-rFbp (54 μM), iron-saturated hTf (25 μM), or apo-hTf (25 μM), in 200 mM NaCl/10 mM Tris, pH 8.0. A 250-fold molar excess of TNM over rFbp or hTf Tyr residues was added to each tube, and 1.5 mL aliquots were removed at times of 0, 1, 2, 3, and 20 h. The modification reaction was stopped by dialysis in 200 mM NaCl/10 mM Tris, pH 8.0, for 4 h. The extent of Tyr nitration was determined either by the A_{405} as previously reported (Teuwissen et al., 1973; Tsao et al., 1974; Williams, 1982) or by the decrease in Tyr content determined by amino acid analysis (Teuwissen et al., 1973; Williams, 1982). Modified Tyr was expressed as the number of Tyr residues per total rFbp-encoded Tyr. In addition, TNM protection of Tyr residues was assessed as a measure of metal binding for rFbp complexes with Cu^{2+}, Ga^{3+}, Al^{3+}, Zn^{2+}, Cr^{3+}, or Ni^{2+}. For these studies, the reaction time was held to 45 min, and Tyr modified was determined by the A_{405} described above.

Terbium Luminescence of rFbp and hTf. Solutions of Tb^{3+}–hTf and Tb^{3+}–rFbp (10 μM) were prepared in 10 mM Tris, 25 mM $NaHCO_3$, and 200 mM NaCl, pH 8.0, similar to previous reports (Luk, 1971; O'Hara et al., 1981). Tb^{3+}–EDTA (10 μM) was used as a positive control of trivalent terbium luminescence. Fluorescence spectra were recorded on an SLM spectrofluorometer (SLM Instruments, Inc., Champaign, IL) equipped with a 200 W xenon lamp. Data were recorded as ASCII files for data manipulation. Tb^{3+}–EDTA, Tb^{3+}–hTf, and Tb^{3+}–rFbp exhibited a characteristically strong Tb^{3+} emission at 545 nm. Excitation spectra, in which a fixed emission wavelength (λ_{em}) of 545 nm was used, were recorded to implicate energy transfer donor residues in the local binding environment of Tb^{3+} in each protein.

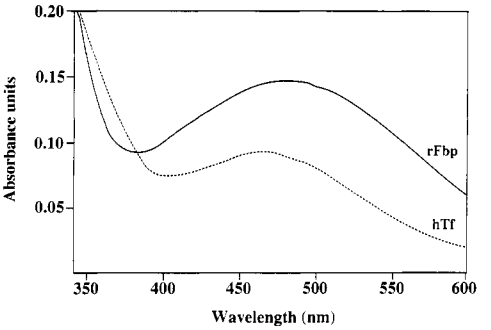

FIGURE 1: Absorbance spectra of Fe^{3+}–hTf and Fe^{3+}–rFbp. Both Fe^{3+}–hTf and Fe^{3+}–rFbp exhibit a broad absorbance spectrum in the visible range with a peak absorbance for Fbp at 481 nm and for hTf at 470 nm. Spectra were determined using samples containing 2 mg/mL protein (54 μM rFbp, 25 μM hTf) in 10 mM Tris/200 mM NaCl, pH 8.0.

Bicarbonate-Dependent Iron Binding by rFbp. Deferrated solutions of rFbp (250 μM) and hTf (100 μM) were prepared in 10 mM Tris (pH 8.0)/200 mM NaCl. Volatile CO_2 was removed from solutions by an overnight steady stream of N_2 with stirring. Samples were added to 1 mL volume, 1 cm path length cuvettes, and at time zero, an equimolar aliquot of degassed $Fe(NO_3)_3$ was added. At 10 min, a 500 molar excess of $NaHCO_3$, pH 8.0, over protein was added. Samples were monitored for 30 min at 470 nm (hTf) or 481 nm (rFbp). The increase in visible absorbance was used as a measure of iron binding. Data were expressed as a percentage of the maximum absorbance attained at 30 min.

RESULTS

Comparison of rFbp and hTf Visible Spectra, Iron-Binding Properties, and Molecular Size. As originally reported by Mietzner et al. (1987), purified concentrated Fbp exhibits a red color. This property is shared by the transferrins (Brock, 1985). The absorbance spectrum of a 2.0 mg/mL solution of iron-saturated rFbp and hTf in 10 mM Tris (pH 8.0) containing 200 mM NaCl is demonstrated in Figure 1. From this spectrum, it can be determined that rFbp has a maximum visible absorbance of 481 nm with $E_{481}^{1\%}$ = 0.72 with an ϵ = 2430 M^{-1} cm^{-1}. In our laboratory, hTf has a maximum visible absorbance of between 465 and 470 nm with $E_{470}^{1\%}$ = 0.43 and an ϵ = 3470 M^{-1} cm^{-1}, similar to that previously reported (Brock, 1985). It is well-recognized that the biochemical basis for the visible absorbance of the transferrins is the coordinated Fe^{3+} atom. On the basis of the spectral characteristics of purified rFbp, it is inferred that the iron associated with Fbp (Mietzner et al., 1987) is responsible for its absorbance in the visible range.

The transferrins exist as bilobed molecules, binding 2 moles of ferric iron per mole of protein and having molecular masses of between 70 and 80 kDa (*e.g.*, nonglycosylated apo-hTf has a molecular mass of 75 095 daltons) (Brock, 1985). Fbp has a molecular mass roughly half that size at 33 642 daltons (Berish et al., 1990) and runs at ≈37 kDa by SDS–PAGE (Mietzner, 1984). A comparison of the absorbance values, molecular masses, and iron-binding capacities of Fbp, hTf, and hLf is summarized in Table 1. From the similarities apparent here, a logical hypothesis can be formed that Fbp represents a mono-sited, half-transferrin. One characteristic property of the transferrins is the reversible binding of iron. It has been suggested that Fbp also shares this property (Chen et al., 1993). A standard condition for the removal of iron from hTf is the excess addition of the iron chelator citrate,

Table 1: Comparison of Biologic Properties of hTf, hLf, and Fbp

characteristic	transferrin (Welch, 1992)	lactoferrin (Welch, 1992)	Fbp (Mietzner et al., 1985)
molecular mass (daltons)	74 000–80 000	80 000–92 000	33 642
state of iron bound	ferric	ferric	ferric
Fe^{3+}-binding sites	two, one per lobe	two, one per lobe	one
vis spectra, Fe^{3+}-bound	A_{max} = 470 nm	A_{max} = 460 nm	A_{max} = 481 nm

at a pH of 4.5 (Brock, 1985). When iron-saturated hTf or rFbp is exposed to these conditions followed by separation from competing citrate and readjustment of the pH to 8.0, the absorbance in the visible range is nearly eliminated (data not shown). This decrease in the characteristic Fe^{3+} absorbance suggests that, like hTf, iron can be removed from rFbp by citrate at lowered pH. This is in contrast to lactoferrin, which retains iron unless subjected to pH 2.0 and excess citrate (Sawatzki, 1987). Upon the addition of increasing concentrations of iron and monitoring at the absorbance maxima of rFbp (481 nm) or hTf (470 nm), it can be demonstrated that the absorbance due to the Fe^{3+}–rFbp complex maximizes at an Fe^{3+}:Fbp molar ratio of 1:1 whereas the Fe^{3+}–hTf absorbance reaches its maximum at a molar ratio of 2:1 (data not shown). These values are as expected based upon earlier studies on the stoichiometries of Fe^{3+}–Fbp (Mietzner et al., 1987) and Fe^{3+}–hTf (Welch, 1992).

The Subset of Amino Acid Side Chains Participating in Iron Binding by Fbp Is Homologous to the Transferrins. As a group, the transferrins are slightly divergent with regard to amino acid sequence. For example, BESTFIT analysis of bovine Lf and hLf demonstrates a sequence similarity of 82% and an identity of 70%. Likewise, comparison of hLf and hTf indicates 76% similarity and 62% identity. In spite of the sequence differences among the transferrins, each iron-binding site of a transferrin half-molecule demonstrates a conserved iron-binding motif involving two Tyr residues, one His residue, one Asp residue, and a bicarbonate anion (Welch, 1992). The 309-residue sequence of Fbp shares little similarity with either the N- or the C-terminal hTf half-molecules (similarity of 39 and 38%, identity of 15 and 18%, respectively). No obvious regions of homology are apparent, nor can any key residues involved in iron binding be aligned. However, Fbp may still represent a functional homolog to a transferrin half-molecule if a similar repertoire of residues participates in iron binding. This has been historically demonstrated for the transferrins using protection from chemical modification (Feeney et al., 1983). This technique was employed in this study to quantitate the susceptibility of iron-saturated rFbp (Fe^{3+}–rFbp) and apo-rFbp to two side-chain-specific chemical modifiers—DEPC, specific for His, and TNM, specific for Tyr. By deduction, side chains protected from chemical modification represent iron-ligating residues.

The participation of any of the six His residues encoded by Fbp (Berish et al., 1990, Mietzner et al., 1987) in the iron–ligand interaction was investigated using DEPC modification. DEPC reacts stoichiometrically and specifically with the imidazole group of His residues to form an *N*-ethoxyformylimidazole derivative. This derivative exhibits an absorbance at 240 nm which can be used to quantitate modification of His in a protein. Quantitation of the His residues modified in Fe^{3+}–rFbp vs apo-rFbp suggests that between one and two His residues are protected by iron in this analysis (Figure 2). Previously published reports indicate

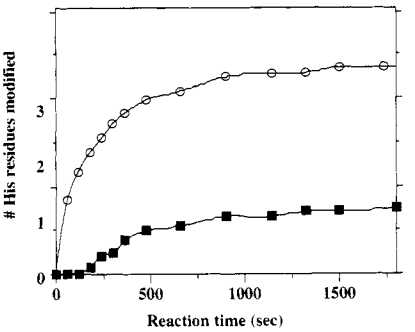

FIGURE 2: DEPC modification of rFbp His residues. Solutions of apo-rFbp (open circles) and Fe^{3+}–rFbp (closed squares) (54 μM) in 10 mM Tris and 200 mM NaCl were prepared. Samples were buffered to pH 6.0 with 1% acetic acid, and a 10 molar excess of DEPC was added. Moles of histidines modified per mole of protein was determined as described under Materials and Methods. Fe^{3+}–rFbp demonstrated protection of between one and two His residue per molecule of protein when compared to apo-rFbp.

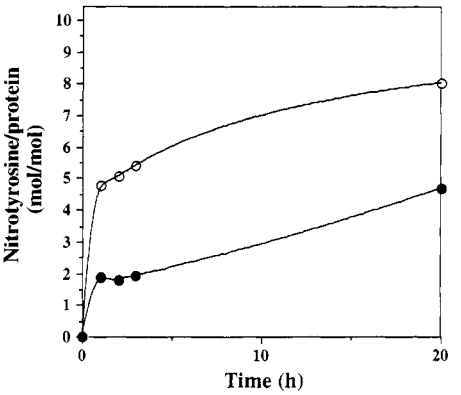

FIGURE 3: Fe^{3+} protection of Tyr from TNM modification. A 250 molar excess of TNM was added to solutions of apo-rFFbp (open circles) and Fe^{3+}–rFbp (closed circles) (54 μM) in 10 mM Tris, pH 8.0, and 200 mM NaCl, and the number of Tyr modified was determined at various time points over a total of 20 h as described under Materials and Methods. A difference of 2.5 nitrotyrosines between apo-rFbp and Fe^{3+}–rFbp is seen after 1 h.

that Fe^{3+} protects four His residues per molecule of hTf under slightly different experimental conditions, two per iron-binding lobe (Rogers et al., 1977); this is therefore similar to the Fe^{3+}–rFbp complex.

Based on DNA sequence data (Berish et al., 1990), a total of 11 Tyr residues are present in Fbp. TNM targets the phenol moiety of Tyr residues and forms an *o*-nitrophenolate derivative in a reaction that can be followed spectrophotometrically. Fe^{3+}–rFbp and apo-rFbp were reacted with TNM as described under Materials and Methods. Figure 3 demonstrates two rates of modification: an initial, rapid reaction occurring within the first hour that is much faster for apo-rFbp when compared to Fe^{3+}–rFbp, and a second rate that is much lower over the subsequent 20 h. At 1 h, 4.7 Tyr/rFbp were modified in the apo-rFbp preparation compared with 1.9 Tyr/rFbp in the Fe^{3+}–rFbp sample. After 1 h, the rates of apo-rFbp and Fe^{3+}–rFbp are roughly parallel and probably indicate the equal susceptibility of Tyr to TNM in these two forms over time. This difference of 2–3 Tyr residues protected by iron is very similar to that reported for each half-transferrin iron-binding lobe (Feeney et al., 1983).

To demonstrate that an analogous result can be obtained with hTf, a separate experiment was performed in which Fe^{3+}–rFbp and apo-rFbp were modified with TNM in parallel with iron-saturated hTf and apo-hTf (data not shown). The time of modification was held to 45 min, and 7.5 Tyr were modified

Table 2: Protection of rFbp Tyr Residues by Non-Iron Metals

metal	no. of Tyr modified per rFbp: with metal	without metal	ΔTyr protected per rFbp
Fe	5.2	2.8	2.4
Ga	5.0	3.1	1.9
Al	5.5	3.0	2.5
Cu	5.0	2.1	2.9
Zn	4.5	1.74	2.76
Cr	4.5	1.6	2.9

in iron-saturated transferrin versus 11.2 Tyr modified for the apo-hTf preparation. This difference of 3.7 Tyr (1.8 per iron-binding site) is consistent with previous chemical modification and confirmatory crystallographic evidence (Anderson et al., 1989; Feeney et al., 1983) indicating that 2–3 Tyr are involved in the ligand pocket. In an identical experiment using rFbp, 3.5 Tyr per molecule of Fe^{3+}–rFbp were modified vs 5.2 in apo-rFbp (a difference of 1.7 Tyr per iron-binding site). These results suggest that a similar number of Tyr residues are protected per binding site from TNM modification in both rFbp and hTf. The evidence that at least two Tyr and one His are involved in iron binding by rFbp is consistent with the hypothesis that it binds iron in a manner identical to the transferrins.

Promiscuity of Metal Binding by Fbp. The transferrins efficiently bind metals other than iron, including Al^{3+}, Ga^{3+}, Cu^{2+}, Zn^{2+}, Cr^{3+}, and Tb^{3+}, among a total of 31 elements (Welch, 1992). We have investigated the range of Fbp metal binding using TNM protection as a probe. In these experiments, purified rFbp was deferrated as described under Materials and Methods and introduced to a solution of citrate–metal (Fe^{3+}, Cu^{2+}, Zn^{2+}, Ga^{3+}, Cr^{3+}, Al^{3+}) at a 50-fold molar excess of metal over apo-rFbp. Color formation was noted only for Fe^{3+}–rFbp and Cu^{2+}–rFbp mixtures, the former demonstrating the typical red color and the latter resulting in a yellow color. Metal–rFbp complexes were subjected to chemical modification with TNM and compared with the susceptibility of apo-rFbp to modification. As demonstrated in Table 2, the protection of 2–3 Tyr residues was observed for each of these metals, suggesting that the same range of metal binding can occur within Fbp that is characteristic of the transferrins (Welch, 1992).

Terbium Luminescence of Tb^{3+}–hTf and Tb^{3+}–Fbp. Lanthanide luminescence has been used to study metal-binding proteins in a variety of systems in order to obtain information about the environment of the metal-binding site (Brittain et al., 1976; Horrocks, 1993). The nature of the metal-binding sites of transferrin and their physical separation has been studied utilizing trivalent lanthanide cations as luminescent probes (Luk, 1971; O'Hara et al., 1981). Tb^{3+} is a particularly useful probe as it is an excellent acceptor of energy from intrinsic protein fluorophores; when specifically bound to a protein such as hTf, it acts as the acceptor of fluorescent energy transferred from nearby Tyr or Trp residues and produces an excitation spectrum reflective of the residues in the local environment. Tb^{3+} was used in this study to probe residues proximal to the binding site of Fbp that transfer energy to bound Tb^{3+}. Figure 4 demonstrates the fluorescence excitation spectra of Tb^{3+}–hTf and Tb^{3+}–rFbp with λ_{em} = 545 nm. The virtually overlapping spectra, with maxima at 245 and 295 nm, suggest that the Tb^{3+} environment in these proteins is similar. Specifically, both spectra show characteristic excitation wavelengths of tyrosinate and Trp residues (295 nm) in the local environment of Tb^{3+} as well as a shoulder at 280 nm, indicative of nonionized Tyr luminescence in the vicinity of the binding site (Lakowicz, 1983). The similarity of the excitation spectra of Tb^{3+} in these proteins is further evidence that Fbp and the transferrins bind iron in a structurally similar fashion. Additionally, UV difference spectra of Tb^{3+}–hTf and Tb^{3+}–rFbp confirm the presence of tyrosinate residues in the local environment of the bound Tb^{3+} (data not shown).

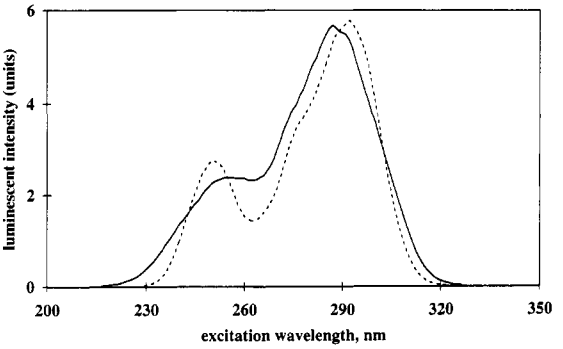

FIGURE 4: Excitation spectra of Tb^{3+}–hTf (dashed line) and Tb^{3+}–rFbp (solid line) with emission monitored at 545 nm. The rFbp spectrum was multiplied by 1.7 to normalize peak heights. The absorbance shoulder at 273 nm is the result of unfiltered monochromator doubling and is not sample-dependent. The similarities of the spectra indicate that both hTf and Fbp bind the Tb^{3+} ligand in a similar environment.

Dependence on a Bicarbonate Anion for Iron Binding. The participation of a bicarbonate anion in binding iron is a well-established characteristic of the transferrins (Brock, 1985). Because of the similarities in iron-binding properties among the transferrins, bicarbonate dependence for Fbp–iron interactions was investigated. Fe^{3+} bound to rFbp or hTf was estimated by an increase in absorbance in the visible range at 481 or 470 nm, respectively, analyzed over a time period of 10 min. Figure 5 expresses these data as a function of the percent iron-binding capacity of both rFbp and hTf. The data indicate an increase in absorbance up to 30% of the maximal which probably represents contamination of the system with atmospheric or undisplaced CO_2. After 10 min, a 500-fold excess of $NaHCO_3$ was added to the system, resulting in an increase of the iron-binding capacity for both rFbp and hTf to 100%; this suggests that like the transferrins, Fbp requires the participation of an anion such as bicarbonate in order to form a stable Fe^{3+}–protein complex.

DISCUSSION

This study focuses on the high-affinity iron-binding properties of Fbp. In spite of sharing little sequence similarity or identity with hTf, both proteins reversibly bind iron with high affinity (Anderson et al., 1987; Chen et al., 1993; Mietzner et al., 1987; Welch, 1992). The structural similarities among each half-lobe of the transferrins and the PBPs have been reviewed (Anderson et al., 1987, 1989; Baker et al., 1987). Previous studies, however, have not described the existence of a functional analog to the transferrins among the PBPs. The data described in this study address this deficit by demonstrating that the fundamental mechanism for binding iron by the prokaryotic Fbp (Mietzner et al., 1984, 1987; Mietzner & Morse, 1985) and the eukaryotic hTf (Brock, 1989) is maintained at the molecular level. Specifically, it is demonstrated that Fbp shares spectral and metal coordination properties with hTf.

The ability to isolate proteolytically derived half-transferrins that still retain substantial affinity for iron has been clearly established (Harris & Aisen, 1989), suggesting that the two iron-binding sites can function independently with regard to

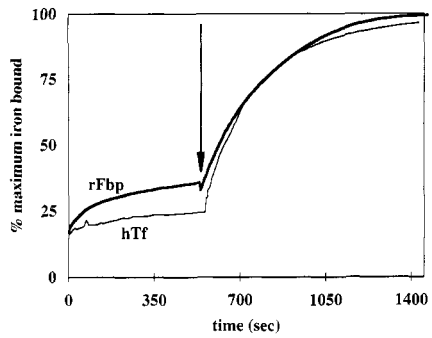

FIGURE 5: Dependence of iron binding by rFbp and hTf on $NaHCO_3$. Aliquots of $Fe(NO_3)_3$ were added to iron-free 250 μM solutions of rFbp (thick line) or hTf (thin line), and iron binding was determined as described under Materials and Methods. The arrow indicates the time point at which a 500-molar excess aliquot of $NaHCO_3$, pH 8.0, was added to the reaction. The data demonstrate that prior to the addition of bicarbonate, 20–30% saturation was achieved for both hTf and rFbp. After addition of excess bicarbonate, both proteins reached maximal absorbance.

iron binding. It has been proposed that the bilobed iron-binding motif of the transferrins arose from gene duplication (Greene & Feeney, 1968). As a result, there has been interest in identifying an evolutionarily related, single-domain half-transferrin (Welch, 1992). One report (Palmour & Sutton, 1971) suggested that the hagfish *Eptatretus stoutii* possessed a transferrin with a molecular mass of 44 kDa, approximately half that of the transferrins. This was later refuted (Williams et al., 1982) when the correct mass of the protein was demonstrated to be 75–80 kDa, in line with that of the transferrins. Thus, the existence of a single-domain half-transferrin remains elusive. Fbp has many properties in common with a predicted half-transferrin. At 34 kDa, it is roughly half the mass of hTf and binds only a single molecule of ferric iron using analogous protein ligands. Fbp and hTf share spectroscopic characteristics such as absorbance maxima in the 460–480 nm range and similar molar absorptivity per binding site. Fbp and hTf are both promiscuous in their range of metal binding, being identically capable of taking up a wide variety of transition and even lanthanide cations. The complement of residues implicated in metal ion binding by the transferrins is two Tyr, one His, and a bicarbonate anion per binding site. We have shown evidence here that a similar set of residues is implicated in iron binding by Fbp. Lastly, probes of the metal-binding sites of both proteins with the luminescent Tb^{3+} probe yield extremely similar excitation spectra, suggesting that the local structure of the binding site in these molecules is highly homologous. Though the sequences of the transferrins and Fbp show little identity with one another, these data suggest that a much higher degree of functional homology exists at the level of tertiary structure for iron binding by transferrins and Fbp.

Functional similarities between hTf and Fbp clearly exist. It is well recognized that the role of hTf in iron metabolism is to efficiently transport extracellular iron (Brock, 1989; Crichton, 1990; Crichton & Ward, 1992; Welch, 1992). Analogously, in the prokaryotic anatomy Fbp functions by transporting iron across the "extracytoplasmic" periplasmic space of pathogenic *Neisseria*, a region that separates the absorption of transferrin-bound iron from the cellular delivery of this iron. We have proposed that this process is implemented by a three-protein system that includes a membrane-bound permease, a nucleotide-binding protein, and a periplasmic nutrient-binding protein (Chen et al., 1993). Based on the capacity of Fbp to bind iron and its periplasmic localization (Chen et al., 1993; Mietzner et al., 1987), it is likely that this molecule is the periplasmic-binding component of a high-affinity iron-acquisition system to obtain iron from hTf.

The structure, function, and evolutionary relationships among the vast array of PBPs have recently been reviewed (Tam & Saier, 1993). This family can be divided into eight clusters depending on the molecular mass and nature of the bound solute. Fbp is classified as a member of cluster 1, with proteins that bind maltooligosaccharides, multiple sugars, and α-glycerol phosphates. The highly homologous protein SfuA, the PBP component of an iron-acquisition system in *Serratia marcescens* (Angerer et al., 1990; Berish et al., 1992), is also in this group. It is interesting that other PBPs participating in the transport of iron, *e.g.*, FecB, FepB, or FhuD, are grouped in cluster 8. The latter subset of proteins binds iron in the form of an iron–chelate complex, whereas Fbp binds iron directly, a property that is shared by the transferrins. This suggests that Fbp analogs may have branched at a point distinct from the iron–chelate-binding proteins in the evolution of the PBPs.

The partial homology (58.5% similarity, 36.8% identity) between Fbp and SfuA is useful in the prediction of residues important in coordination of iron by Fbp (Berish et al., 1992). Presumably, those residues important in function would be preserved between these proteins since it is proposed that they perform an analogous function in iron transport (Angerer et al., 1990; Chen et al., 1993). This comparison is not apparent between Fbp and any half-transferrin because they are so distantly related. A BESTFIT sequence alignment of Fbp and SfuA (Chen et al., 1993) suggests that of the 11 Tyr encoded by Fbp, only 5 (at Fbp sequence positions 5, 107, 194, 195, and 265) are conserved between these sequences. Likewise, of the six His residues encoded by Fbp, only two are conserved between SfuA and Fbp (at Fbp sequence positions 9 and 218). The involvement of the conserved Tyr and His residues in iron binding by Fbp is currently being investigated with site-directed mutagenesis. A recent series of papers has begun to utilize a systematic molecular biology approach toward understanding the structure/function relationships of the transferrins (Funk et al., 1990; Woodworth et al., 1991). Given the ease of manipulation of rFbp, a complementary series of experiments are underway. It is hoped that these experiments will contribute to additional insight into the functional properties of iron binding by both Fbp and the transferrins.

REFERENCES

Adams, M. D., & Oxender, D. L. (1989) *J. Biol. Chem. 264*, 15739–15742.

Ames, G. F.-L. (1986) *Annu. Rev. Biochem. 55*, 397–425.

Anderson, B. F., Baker, H. M., Dodson, E. J., Norris, G. E., Rumball, S. V., Waters, J. M., & Baker, E. N. (1987) *Proc. Natl . Acad . Sci. U.S.A. 84*, 1769–1773.

Anderson, B. F., Baker, H. M., Norris, G. E., Rice, D. W., & Baker, E. N. (1989) *J. Mol. Biol. 209*, 711–734.

Angerer, A., Gaisser, S., & Braun, V. (1990) *J. Bacteriol. 172*, 572–578.

Bailey, S., Evans, R. W., Garratt, R. C., Gorinsky, B., Hasnain, S., Horsburgh, C., Jhoti, H., Lindley, P. F., Mydin, A., Sarra, R., & Watson, J. L. (1988) *Biochemistry 27*, 5804–5812.

Baker, E. N., Rumball, S. V., & Anderson, B. F. (1987) *Trends Biochem. Sci. 12*, 350–353.

Berish, S. A., Mietzner, T. A., Mayer, L. W., Genco, C. W., Holloway, B. P., & Morse, S. A. (1990) *J. Exp. Med. 171*, 1535–1546.

Berish, S. A., Chen, C.-Y., Mietzner, T. A., & Morse, S. A. (1991) *Mol. Microbiol. 6*, 2607–2615.

Brittain, H. G., Richardson, F. S., & Martin, R. B. (1976) *J. Am. Chem. Soc. 98*, 8255–8260.
Brock, J. H. (1985) in *Metalloproteins* (Harrison, P. M., Ed.) Part 2, pp 183–262, MacMillan, London.
Brock, J. H. (1989) in *Iron in Immunity, Cancer and Inflammation* (Sousa, M., & Brock, J. H., Eds.) pp 35–54, Wiley, New York.
Chen, C.-Y., Berish, S. A., Morse, S. A., & Mietzner, T. A. (1993) *Mol. Microbiol. 10*, 311–318.
Crichton, R. R. (1990) *Adv. Protein Chem. 40*, 281–355.
Crichton, R. R., & Ward, R. J. (1992) *Biochemistry 31*, 11256–11264.
Crosa, J. (1989) *Microbiol. Rev. 53*, 517–530.
Elkins, M. F., & Earhart, C. F. (1989) *J. Bacteriol. 171*, 5443–5451.
Feeney, R. E., Osuga, D. T., Meares, C. F., Babin, D. R., & Penner, M. H. (1983) in *Structure and function of iron storage and transport proteins* (Urushizaki, I., et al., Eds.) pp 231–240, Elsevier Science Publishers, Amsterdam.
Funk, W., MacGillivray, R., Mason, A., Brown, S., & Woodworth, R. (1990) *Biochemistry 29*, 1654–1660.
Greene, F. C., & Feeney, R. E. (1968) *Biochemistry 7*, 1366–1371.
Harkness, R. E., Chong, P., & Klein, M. H. (1992) *J. Bacteriol. 174*, 2425–2430.
Harris, D. C., & Aisen, P. (1989) in *Iron Carriers and Iron Proteins* (Loehr, T. M., Ed.) pp 239–351, VCH Publishers, New York.
Horrocks, W. D. (1993) *Methods Enzymol. 226*, 495–538.
Lakowicz, J. (1983) *Principles of Fluorescence Spectroscopy*, Plenum, New York.
Luk, C. K. (1971) *Biochemistry 10*, 2838–2843.
Mietzner, T. A., Luginbuhl, G. H., Sandström, E. C., & Morse, S. A. (1984) *Infect. Immun. 45*, 410–416.
Mietzner, T. A., & Morse, S. A. (1985) in *The Pathogenic Neisseriae* (Schoolnik, G. K., Brooks, G. F., Falkow, S., Knapp, J. S., McCutchan, A., & Morse, S. A., Eds.) pp 406–415, American Society for Microbiology, Washington, D.C.
Mietzner, T. A., Barnes, R. C., Jeanlouis, Y. A., Shafer, W. M., & Morse, S. A. (1986) *Infect. Immun. 51*, 60–68.
Mietzner, T. A., Bolan, G., Schoolnik, G. K., & Morse, S. A. (1987) *J. Exp. Med. 165*, 1041–1057.
O'Hara, P., Yeh, S. M., Meares, C. F., & Bersohn, R. (1981) *Biochemistry 20*, 4704–4708.
Palmour, R. M., & Sutton, H. E. (1971) *Biochemistry 10*, 4026–4032.
Pflugrath, J. W., & Quiocho, F. A. (1988) *J. Mol. Biol. 200*, 163–180.
Quiocho, F. A. (1990) *Philos. Trans. R. Soc. London B 326*, 341–351.
Quiocho, F. A., Gilliland, G. L., & Phillips, G. N. (1977) *J. Biol. Chem. 252*, 5142–5149.
Rogers, T. B., Gold, R. A., & Feeney, R. E. (1977) *Biochemistry 16*, 2299–2305.
Sawatzki, G. (1987) in *Iron Transport in Plants, Animals and Man* (Winkelman, D. V. d. H. G., & Neilands, J. B., Eds.) pp 477–489, VCH Publishers, Weinheim, Germany.
Staudenmaier, H., Van Hove, B., Yaraghi, Z., & Braun, V. (1989) *J. Bacteriol. 171*, 2626–2633.
Tam, R., & Saier, M. H. (1993) *Microbiol. Rev. 5*, 320–346.
Teuwissen, B., Masson, P. L., Osinski, P., & Heremans, J. F. (1973) *Eur. J. Biochem. 35*, 366–371.
Tsao, D., Azari, P., & Phillips, J. L. (1974) *Biochemistry 13*, 408–413.
Welch, S. (1992) in *Transferrin: The iron carrier* (Welch, S., Ed.) pp 1–127, CRC Press, Boca Raton, FL.
Williams, J. (1982) *Biochem. J. 201*, 647–651.
Williams, J., Grace, S. A., & Williams, J. M. (1982) *Biochem. J. 201*, 417–419.
Woodworth, R., Mason, A., Funk, W., & MacGillivray, R. (1991) *Biochemistry 30*, 10824–10829.
Yura, T., Mori, H., Nagai, H., Nagata, T., Ishihama, A., Fujita, N., Isono, K., Mizobuchi, K., & Nakata, A. (1992) *Nucleic Acids Res. 20*, 3305–3308.