The Structure of the Iron-binding Protein, FutA1, from Synechocystis 6803*

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Cyanobacteria account for a significant percentage of aquatic primary productivity even in areas where the concentrations of essential micronutrients are extremely low. To better understand the mechanism of iron selectivity and transport, the structure of the solute binding domain of an ATP binding cassette iron transporter, FutA1, was determined in the presence and absence of iron. The iron ion is bound within the “C-clamp” structure via four tyrosine and one histidine residues. There are extensive interactions between these ligating residues and the rest of the protein such that the conformations of the side chains remain relatively unchanged as the iron is released by the opening of the metal binding cleft. This is in stark contrast to the zinc-binding protein, ZnuA, where the domains of the metal-binding protein remain relatively fixed, whereas the ligating residues rotate out of the binding pocket upon metal release. The rotation of the domains in FutA1 is facilitated by two flexible β-strands running along the back of the protein that act like a hinge during domain motion. This motion may require relatively little energy since total contact area between the domains is the same whether the protein is in the open or closed conformation. Consistent with the pH dependence of iron binding, the main trigger for iron release is likely the histidine in the iron-binding site. Finally, neither FutA1 nor FutA2 binds iron as a siderophore complex or in the presence of anions, and both preferentially bind ferrous over ferric ions.

Bioavailable iron is a limiting nutrient for primary production in large areas of the oceans. This concentration of free iron in aquatic environments is dynamic and varies greatly depending upon the local environment. Fe3+ is notoriously insoluble in water at neutral pH values, whereas Fe2+ is very soluble but highly susceptible to oxidation by atmospheric oxygen. Microbes play a large role in the cycling of iron between the ferric and ferrous forms and generally reduce ferric iron under anaerobic conditions by using it as a final electron acceptor. Conversely, microbes can oxidize ferrous iron under aerobic conditions when other compounds such as nitrate are the final electron acceptors. Organisms can import either form of iron. A number of bacteria, algae, and Cyanobacteria increase the bioavailability of ferric iron through the secretion of organic molecules, such as siderophores, into the extracellular environment. These compounds have exceptionally high binding affinities for iron (association constants of ~10¹⁹ M⁻¹) and essentially scavenge ferric iron from the extracellular environment before the organism imports the entire complex into the cell (e.g. Ref. 1). In some cases these siderophores may actually facilitate a photochemical reduction of the bound ferric ion (2). Alternatively, ferric iron can be locally reduced to the more soluble ferrous form and imported directly. This latter can be accomplished by the organisms itself as is the case with some algal species that use ferric chelate reductase to reduce the iron before import or via a symbiotic organism such as bacteria associated with the roots of freshwater and marine macrophytes that reduce ferric iron in the rhizosphere (3).

Both Gram-negative and Gram-positive bacteria have evolved ATP binding cassette-type transport systems for the high affinity uptake of transition metal ions such as iron, manganese, and zinc, particularly at low extracellular levels of these metals (4–6). The ATP binding cassette-type binding proteins from a number of bacteria have been grouped into clusters on the basis of their sequence homologies and the metal ligand identities (7). In Synechocystis 6803, the futA1 (str1295) and futA2 (str0513) genes encode periplasmic-binding proteins that have been proposed to be part of a high affinity iron transport system. Before in vitro studies with purified FutA1 expressed as a GST-fusion protein in Escherichia coli suggested that the protein binds ferric iron with an association constant of about 1 × 10¹⁹ M⁻¹ (8). Similarly, in Synechocystis 6803 mutants in which futA1 and futA2 were deleted, iron uptake was reduced to 37 and 84%, respectively, compared with wild type (8). When both genes were deleted, the resulting iron uptake activity was less than 5% that of wild type. Because high concentrations of citrate inhibited iron uptake, it was concluded that the iron was

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The atomic coordinates and structure factors (code 2PT2 and 2PT1) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

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2 The abbreviations used are: GST, glutathione S-transferase; TEV, tobacco etch virus; HEPPS, 4-(2-hydroxyethyl)-1-piperazinepropanesulfonic acid; MES, 4-morpholineethanesulfonic acid.
not being transported as a ferric citrate complex. These results suggested that there was at least some redundancy in the functions of FutA1 and FutA2. Recently, it was shown that FutA2 binds iron with approximately the same relative affinity as FutA1 (9). However, when futA2 is deleted, the mutants accumulate normal levels of iron-containing ferredoxin in the periplasm but fail to accumulate copper in plastocyanin. This result combined with the fact that Synechocystis 6803 expresses FutA2 to high levels led to the conclusion that it might not be directly involved in iron transport but, instead, sequesters iron in the periplasm and acts as a metallochaperone. As so, they suggested that it might assist in iron import as well as prevent these ions from competing with other metal binding periplasmic proteins such as those which bind copper.

Here we report the high resolution structure of FutA1 in the presence and absence of bound iron. FutA1 has a C-clamp structure that is very similar to that of Campylobacter jejuni (10) and binds iron via four tyrosine and one histidine residues in the absence of anions or siderophores. Upon release of the bound iron, the two domains of the protein rotate about two β-strands running along the back of the protein. There are extensive interactions between the chelating residues and the rest of the protein such that these residues remain in a relatively fixed conformation upon the release of iron and the opening of the iron-binding site via domain movement. From analysis of the changes in the contact surface area, this rocking motion may require relatively small changes in the total energy of the protein and, therefore, may have minimal impact on the release or binding of iron. Although a previous iron binding analysis of a FutA1-GST fusion protein suggested that FutA1 binds ferric iron, binding analysis presented here clearly demonstrates that the specificity of FutA1 is for the ferrous form instead, with an association constant of about 1 $\times 10^{19}$ M$^{-1}$. This observation is consistent with the homology of FutA1 to the C. jejuni iron-binding protein that is also specific for ferrous iron. An amino acid sequence alignment between FutA1 and FutA2 indicates that the iron-binding site of FutA2 is also composed of four tyrosines and one histidine. Therefore, it is not surprising that we found that purified FutA2 binds ferrous rather than ferric iron. Consistent with this finding is the fact that the iron binding configuration of these proteins is conserved among bacterial strains that favor low oxygen environments where ferrous iron is more prominent.

**MATERIALS AND METHODS**

Cloning of FutA1 (slr1295) and FutA2 (slr0513) from Synechocystis PCC 6803—The solute binding domain of FutA1 (residues 30–360) from Synechocystis PCC 6803 was PCR-amplified from genomic DNA and ligated into the expression vector pET-28a (Novagen). The pET-28a (Novagen) vector was transformed with the ligation mixtures and then plated onto LB media supplemented with 30 μg/ml kanamycin. After ~16 h of growth at 37 °C, the colonies were harvested from the plates and used for the inoculation of 2 × 4-liter baffled flasks containing tryptose broth (TB) medium supplemented with 30 μg/ml kanamycin and 30 μg/ml chloramphenicol. The cells were grown at 37 °C with shaking (230 rpm) to an $A_{600}$ of ~0.4, at which time the temperature was lowered to 22.5 °C for the remainder of the incubation. Thirty minutes after lowering the temperature, protein expression was induced by the addition of 0.5 mM isopropyl 1-thio-β-d-galactopyranoside. The cells were allowed to grow for an additional 16 h before harvesting by centrifugation. The cell paste was frozen in liquid nitrogen and stored at −80 °C.

FutA1 Purification—Approximately 10 g of frozen cells were thawed in 50 ml of cold buffer A (25 mM NaH$_2$PO$_4$, 300 mM NaCl, 10 mM imidazole, pH 8.0) with one “Complete EDTA-free protease inhibitor” tablet (Roche Applied Science). Cells were lysed on ice by four 45-s cycles of sonication separated by 3 min of cooling. Cellular debris was removed by centrifugation at 4 °C for 30 min at 30,000 × g. The clarified lysate was loaded onto a 5-mL HiTrap Chelating HP cartridge (GE Healthcare) charged with Ni$^{2+}$ and pre-equilibrated with buffer A. After loading, the column was washed with about 40 ml of 90% buffer A, 10% buffer B (25 mM NaH$_2$PO$_4$, 300 mM NaCl, 300 mM imidazole at pH 8.0) followed by an elution via a 10–100% linear gradient of buffer B. FutA1-containing fractions were pooled based on SDS-PAGE analysis and dialyzed against 20 mM HEPES, pH 7.5, with 200 mM NaCl overnight at 4 °C. For removal of the His tag, recombinant TEV (rTEV) protease was added to dialyzed protein at a molar ratio of ~1 rTEV:100 FutA1, and the mixture was incubated for 4 h at ~23 °C, then 16 h at 4 °C. The recombinant TEV (rTEV) protease contained an N-terminal His$_6$ tag that allowed the separation of cleaved FutA1 from both His-tagged FutA1 and rTEV via Ni$^{2+}$ affinity chromatography, as described above. Native FutA1 was dialyzed overnight at 4 °C against 20 mM HEPES, pH 7.5, with 100 mM NaCl. For long-term storage and crystallization, FutA1 was concentrated to ~47 mg/ml based on an extinction coefficient of ~1.16 ml/(mg-cm) as calculated by the program Protean (DNAnstar, Inc., Madison, WI).

Crystallization of Apo and Iron-loaded FutA1—Initial crystallization conditions were obtained using the Crystal Screen by Hampton Research. Before crystallization, a final concentration of 1 mM ammonium ferric sulfate (Sigma-Aldrich) and 1 mM citrate were added to FutA1, which enhanced the deep red-brown color of the purified protein. Ferric iron was used in these early experiments because literature had suggested that FutA1 was a ferric-binding protein (8). It should be noted, however, that only high concentrations of freshly prepared iron solutions enhanced the color of the protein. As discussed under “Results,” this is more than likely due to the fact that FutA1...
binds ferrous iron and not ferric iron. Colorless apoFutA1 crystals were obtained at room temperature via the hanging drop method of vapor diffusion against 2.2–2.8 M ammonium sulfate, 100 mM succinate, pH 5.0, and 5% isopropanol. These crystals were of the orthorhombic space group P2₁2₁2₁ with cell dimensions of a = 59.1 Å, b = 60.7 Å, and c = 91.7 Å. Red-colored crystals of Fut1 complexed with iron were obtained in a similar manner as the apoprotein structure, with a substitution of 100 mM HEPPS, pH 8.0, and the omission of isopropanol. The iron-containing FutA1 crystals were of the monoclinic space group C2 with cell dimensions of a = 98.3 Å, b = 65.7 Å, c = 50.3 Å, and β = 102.4°.

**High Resolution X-ray Data Collection—**X-ray data from apoFutA1 crystals were collected using a Bruker AXS, Inc. and a Proteum R Smart 6000 CCD detector connected to a Bruker-Nonius FR591 rotating anode generator. The data were processed with SAINT (Bruker AXS, Inc.) and scaled with SCALEPACK (13). X-ray data processing statistics are presented in Table 1.

**Structure Determination and Refinement—**The structure of apoFutA1 was solved via molecular replacement with the program AMoRe using the structure of the Bordetella ferric-binding protein (PDB code 1Y9U) as a search model (10, 14). The apoFutA1 model was refined using a combination of manual model-building in O and maximum-likelihood refinement with CNS (15, 16). The iron-loaded structure of FutA1 was determined via molecular replacement with the program AMoRe using the refined apoFutA1 as a search model, then refined in a similar manner as the apoprotein structure. All relevant refinement statistics are presented in Table 1. The apoFutA1 structure contained no ions in the metal binding pocket but showed weak electron density for 3 sulfate anions which were modeled with an occupancy of 0.5. The iron-loaded FutA1 contained a single iron atom in the ligand binding pocket, four sulfate anions (one modeled at half-occupancy), and a single molecule of ethylene glycol.

**FutA2 Purification—**FutA2 was purified and refolded from inclusion bodies as previously described (9). Approximately 2 g of cells were thawed in 35 ml of inclusion body buffer (50 mM HEPES, pH 7.5, 500 mM NaCl, 5 mM MgCl₂, 1% Triton X-100) with 10 mM β-mercaptoethanol, DNase I, and lysozyme. Cells were stirred for 1 h at room temperature followed by sonication for 20 s, then centrifuged at 30,000 × g for 30 min. The supernatant was discarded, and the pellet was resuspended in 30 ml of inclusion body buffer with 10 mM β-mercaptoethanol then centrifuged a second time. The pellet was then resuspended in inclusion body buffer with 2 M urea and centrifuged again. The remaining pellet contained nearly pure FutA2 inclusion bodies. This pellet was washed twice with ultrapure water, then resuspended in 30 ml of 6 M urea in 50 mM Tris, pH 7.5, and incubated on ice for 1 h. Insoluble material was removed by centrifugation, and the remaining supernatant containing FutA2 was refolded by diluting 17-fold into cold 50 mM Tris, pH 7.5, with 0.1 mM ferrous ammonium sulfate over 48 h. This mixture was then centrifuged to remove insoluble debris, diluted with an equal amount of 50 mM Tris, pH 7.5, and loaded onto a 5-ml HiTrap Q HP cartridge (GE Healthcare). FutA2 was eluted with 50 mM Tris, pH 7.5, 500 mM NaCl. The red-colored fractions were harvested and dialyzed against 20 mM Hepes, pH 7.0, and further purified on a 5-ml HiTrap Q HP cartridge. Purified FutA2 had an absorbance peak at ~434 nm, indicating the presence of bound iron.

**Iron Removal from FutA1 and FutA2—**Purified FutA1 and FutA2 were stripped of iron before binding experiments by dialysis against 50 mM MES, pH 6.0, 10 mM 2,2′-dipyridyl, 100 mM dithionite. Chelating and reducing agents were then removed by dialyzing twice against 20 mM Tris, pH 8.0, 200 mM NaCl.

**Iron Analysis—**The actual concentration of ferric and ferrous iron in the stock solutions was measured spectrophotometrically at 396 and 515 nm as previously described (17). The E₃₉₆ measurement represents the sum of the ferric and ferrous iron using an empirically determined extinction coefficient of 3.6 × 10³ M⁻¹ cm⁻¹. The E₅₁₅ measurement measured the ferrous concentration alone with an extinction coefficient of 1.12 × 10⁴ M⁻¹ cm⁻¹. From these two values, the actual concentrations for the various iron species were calculated.

### Table 1

| Data collection and refinement statistics | 2PT2 | 2PT1 |
|-----------------------------------------|------|------|
| PDB accession code                      | Iron-bound | Apo |
| Space group                             | C2    | P2₁,2₁,2₁ |
| Cell parameters                         | a, b, c (Å) | 98.3, 65.7, 50.3 |
|                                         | 90.0, 102.4, 90.0 |
| Resolution (Å)                          | 20.1-1.95 (20.2-2.19) |
| Rₚ/₀                                    | 50-2.0 (2.00-2.02) |
| Completeness (%)                        | 92.8 (86.8) |
| Redundancy                              | 4.8 (3.4) |
| Commissional refinement                 | Resolution (Å) |
|                                         | 50-2.0 (2.00-2.02) |
| No. reflections                         | 20,599 |
| Redundancy                              | 21,638 |
| Completeness (%)                        | 92.8 (86.8) |
| Redundancy                              | 4.8 (3.4) |
| Root mean square deviations             | Bond lengths (Å) |
|                                         | 0.0049 |
| Bond angles (°)                         | 1.2 |
| Ramachandran analysis                   | Most favored (%) |
|                                         | 95.3 |
| Additional allowed (%)                  | 4.3 |
| Generously allowed (%)                  | 0.4 |
| Disallowed (%)                          | 0.0 |

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**Unpublished Material**
Iron Binding Experiments—Iron binding to FutA1 and FutA2 was performed as has been previously described (8). Briefly, a 70 μM solution of FutA1 was prepared in 20 mM Tris, pH 8.0, that contained 200 mM NaCl. A 0.5-ml sample was added to a cuvette, and 8-μl aliquots of 1.0 mM stock solutions of iron were sequentially added. The actual concentration of ferric and ferrous iron in the stock solutions of ammonium ferrous sulfate hexahydrate \((\text{NH}_4)_2\text{Fe(SO}_4\text{)}_2\cdot6\text{H}_2\text{O}\), ammonium iron (III) sulfate \((\text{NH}_4\text{Fe(SO}_4\text{)}_2\cdot12\text{H}_2\text{O})\), iron (II) chloride \((\text{FeCl}_2)\), and iron III chloride \((\text{FeCl}_3)\) was determined using the spectrophotometric analysis described above immediately before use. After each addition of iron, the absorbance spectrum was measured between 300 and 560 nm using a Beckman Coulter DU 800 spectrophotometer and the associated software analysis package. Similar binding experiments were performed with FutA2 with a 44 μM solution of FutA2. The binding affinity of FutA1 for ferrous iron was performed using the citrate competition experiment as previously described (8, 18). A 70 μM solution of FutA1 was saturated with a solution of ferrous iron and dialyzed overnight to remove the excess iron. The sample was then placed in a cuvette, and a spectrum was collected. Aliquots of sodium citrate were then added, mixed, and allowed to incubate for ~10 min to allow the protein and citrate to establish an equilibrium. After each incubation, a new spectrum was collected. The optical density at 411 nm was plotted versus the final concentration of citrate, and the resulting curve was fitted to Equation 1 to determine the binding constant of ferrous iron to FutA1 relative to citrate.

\[
A = \frac{A_{\text{max}} \times [\text{citrate}]}{K_d + [\text{citrate}]} \quad \text{(Eq. 1)}
\]

where \(A_{\text{max}}\) is the initial absorbance of the FutA1/Fe\(^{2+}\) complex at that wavelength, and \(K_d\) is the apparent dissociation constant relative to citrate.

**Analysis of Domain Contacts**—The buried surfaces between the two domains of FutA1 were determined by using the programs MSPDB, MS, MSSEP, and MSAV (19) and ATMSRF (20) and a solvent probe radius of 1.7 Å. The protein was divided into the N-terminal (residues 45–144 and 277–325) and C-terminal (residues 145–276 and 326–360) domains in both the apo and iron-bound structures. The contact areas between these two domains were calculated for both conformations (summarized in Table 2) on an atom-by-atom basis. The differences between these contacts were then mapped onto the surface of the N-terminal domain for display purposes.

**RESULTS**

The structure of iron-bound FutA1 is highly similar to that of CFbpA from *C. jejuni* (10), perhaps more than the homology of the proteins might have suggested (41% identical and 64% similar to each other). Using all of the residues in the two proteins for alignment, the root mean square deviation between Cα backbones is ~1 Å (Fig. 1). Sequence homology studies on CFbpA from *C. jejuni* put both FutA1 and FutA2 into the same class of anion-independent iron-binding proteins (10). Although residues 30–360 of FutA1 were included in the expression system, only residues 45–360 were clearly defined in the electron density.

As is typical of the solute-binding proteins from the ATP binding cassette transporters, FutA1 has two domains with the binding site lying in the cleft between the two domains. One domain is composed mainly of the N-terminal portion of the

**TABLE 2**

| Residue type          | Apo Main chain | Apo Side chain | Iron-bound Main chain | Iron-bound Side chain |
|-----------------------|----------------|----------------|-----------------------|-----------------------|
| Acid: Asp, Glu        | 25             | 97             | 21                    | 70                    |
| Base: His, Lys, Arg   | 63             | 85             | 55                    | 165                   |
| Polar: Asn, Glu, Ser, Thr | 32          | 49             | 32                    | 68                    |
| Small: Ala, Gly       | 20             | 22             | 28                    | 47                    |
| Hydrophobic: Cys, Ile, Leu, Met, Pro, Tyr | 47         | 190            | 58                    | 128                   |
| Aromatic polar: Trp, Tyr | 49           | 348            | 31                    | 272                   |
| Total surface area    | 1028           |                | 977                   |                       |
restricts domain movement (e.g. Ref. 21), FutA1 has two β-strands connecting to two domains of the protein. It is likely that this change accounts for the domain movement between the apo versus iron-bound forms of the protein.

**Iron Binding Environment in FutA1**—From the electron density, it was readily apparent that iron was bound in the colored FutA1 crystals (Fig. 2).\(^3\) As was found in the cFbpA structure (10), iron is chelated by four tyrosine and one histidine residues. These tyrosine residues are apparently held firmly in place by a strong secondary shell of interacting residues. Arg-145 forms hydrogen bonds with both Tyr-55 and Tyr-241, and Asn-239 also interacts with Tyr-241. These interactions also likely improve iron-tyrosine interactions by increasing the electronegativity of the tyrosine hydroxyls. Unlike the periplasmic iron-binding proteins from Neisseria gonorrhoeae (nFbpA) (22) and Haemophilus influenzae (HiFbpA) (23), there are no apparent anion interactions with the bound iron. The only non-protein atom in the immediate vicinity of the iron is an apparent water molecule that interacts with the Tyr-242 hydroxyl and a sulfate molecule that interacts with that water molecule and the imidazole ring of His-54. In a nearly identical configuration found in the Neisseria FbpA, the iron is bound to FutA1 in a distorted trigonal bipyramidal configuration. Tyrosine residues 55, 185, and 242 form the trigonal plane with angles of 101, 119, and 138°. His-54 and Tyr-241 lie perpendicular to the plane formed by the three tyrosines and form the remaining two coordination sites.

**Changes in FutA1 Conformation upon Iron Binding and Release**—As shown in Fig. 3, when iron binds to FutA1, the two domains pivot about the two β-strands that run along the back of the protein and clamp down upon the bound iron. During refinement of the apo structure, Asp-103 remained an outlier in the Ramachandran plot analy-

\(^3\) MolView (34) was used for the creation of Figs. 2–5.
The density for this residue is unambiguous, and it is more than likely that this strained backbone configuration is real. Interestingly, this residue is at the flex point of β-strand during domain motion. The iron-binding site is essentially two half-sites that come together as the two domains rotate toward each other. On the N-terminal domain lies His-54 and Tyr-55, and on the C-terminal domain are the remaining three tyrosine residues; Tyr-185, Tyr-241, and Tyr-242. The extensive interactions with the secondary shell of residues in the iron-binding site keep the residues in relatively fixed orientations as the binding cleft opens and closes (Fig. 4). The only residue that rotates into and out of the iron-binding site is His-54. Because iron readily dissociates from FutA1 at pH values less than six (data not shown), it seems likely that His-54 is a major trigger for iron binding and release.

**Changes in Domain Contact during Iron Binding and Release**—In the absence of iron FutA1 adopts an open conformation. This suggests that even though Asp-103 is in a distorted conformation in the apo form, this domain orientation is still energetically favored over a closed conformation without iron bound. To better understand the basis for this preference, the contact area between the two domains was analyzed in the presence and absence of bound iron. The changes in these contact regions were then calculated and mapped onto the surface of the N-terminal domain (Fig. 5). The bottom of this figure shows the rendered surface of the N-terminal domain of the apo form of FutA1 with a ribbon representation on top. The portions of the surface that remain unchanged upon opening and closing of the iron binding cleft are colored white. Unique areas of contact that are formed upon closing and opening of the iron binding cleft are colored blue and red, respectively. The area and nature of these contacts are summarized in Table 2. It is very apparent in Fig. 5 that the two domains rock back and forth by flexing at the two β-strands running along the back of the protein. In particular, it is interesting to note how the helix in the C-domain denoted by the arrow fits well in a groove formed by the N-domain in the apo form, and this interaction is eliminated as iron binds and the
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cleft is closed. As shown in Table 2, the apo form of FutA1 has an additional \(50-\text{Å}^2\) buried surface area between the two domains than does the iron-bound form. Although this is a rather modest change compared with the \(1000\text{ Å}^2\) involved in the domain-domain contact, the nature of the interactions changed more significantly. Compared with the iron-bound form, the apo form has an additional \(140-\text{Å}^2\) hydrophobic and aromatic buried surface. It is possible that without iron bound, these differences favor the open conformation of FutA1. However, together with the distorted conformation of Asp-103 in the apo form, it seems likely that the energy differences between these two conformations are relatively small such that the energy cost of the conformational changes that occur upon iron binding do not have a large impact on affinity.

Iron Binding Experiments—Although previous studies using a FutA1-GST fusion protein had suggested that FutA1 binds the ferric form of iron, the binding specificity of FutA1 alone was determined in the context of the atomic structure. Because of the inherent instability of ferrous iron, the ferric and ferrous iron content of the various stock solutions were measured as described under “Materials and Methods.” The ammonium ferrous sulfate hexahydrate solution and ferrous chloride solutions both contained \(1.0\text{ mM Fe}^{2+}\) and \(0.6\text{ mM Fe}^{3+}\), whereas the ammonium iron (III) sulfate and ferric chloride solutions were mainly \(1.0\text{ mM Fe}^{3+}\) with a small amount of \(0.01\text{ mM Fe}^{2+}\). Although there were initial concerns that the mixed nature of the iron solutions might confound the binding analysis, this turned out not to be the case because of the ion selectivity detailed below.

From spectral analysis it is clear that FutA1 binds ferrous but not ferric iron (Fig. 6). When either iron III chloride or ammonium iron (III) sulfate is added to FutA1, the only change in the spectrum was a slight overall increase in absorbance across the whole spectrum measured and may be due to an increase in light scattering. In stark contrast, the addition of ferrous iron causes a marked absorbance with its maximum at \(411\text{ nm}\). The increase in optical density is linear with respect to the \(\text{Fe}^{2+}/\text{FutA1}\) ratio and abruptly levels off when the ferrous iron is at an equimolar ratio to FutA1. This is in contrast to what was found with the FutA1-GST fusion protein. In that study, whereas the binding of ferrous iron was not examined, the addition of ferric ions caused an increase in absorbance at \(453\text{ nm}\). The major difference between these results is the fact that the previous analysis was performed on a FutA1-GST fusion and may be responsible for the differing results.

The binding of ferrous iron to FutA1 was estimated by using sodium citrate, with a known association constant, to compete with FutA1 for iron binding as previously described (8, 18). As shown in Fig. 7, millimolar concentrations of sodium citrate are necessary to compete with the ferrous iron bound to FutA1. When the \(A_{411}\) is plotted versus the concentration of citrate, the apparent \(K_d\) is \(20\text{ mM}\). Because the concentration of FutA1 was \(70\text{ µM}\), this suggests that FutA1 binds ferrous iron \(280\) times better than does citrate. That citrate binds iron with an association constant on the order of \(10^{19}\text{ M}^{-1}\) suggests that FutA1 binds \(\text{Fe}^{2+}\) with an association constant on the order of \(1\times 10^{19}\text{ M}^{-1}\) or better.

FutA1 and FutA2 are highly homologous iron-binding proteins, 50% identical and 67% similar in sequence. Therefore, similar iron binding experiments were performed on expressed FutA2 protein (Fig. 8). As shown here, FutA2 is also specific for ferrous iron. Interestingly, the maximum absorption peak for the FutA2-ferrous complex is at \(434\text{ nm}\) as opposed to the
A ~411-nm peak seen in the FutA1-ferrous complex. This is likely due to some subtle difference in the iron binding environment since sequence alignments strongly suggest that all of the residues contacting the bound iron are identical in both proteins.

**DISCUSSION**

The conformational changes associated with iron binding to FutA1 greatly contrast with what has been observed with the cluster 9 solute-binding proteins of which we have studied the structure of the zinc-binding protein, ZnuA. Unlike FutA1, ZnuA from *Synechocystis* has a long helix extending along the back of the protein and binds zinc with high affinity via three histidine residues in the solute binding cleft. Recent studies on the apo and metal-bound forms of ZnuA have shown that metal binding cleft remains relatively unchanged upon binding of metal (24). Similarly small domain motion was observed in the structure of the metal-binding protein, TroA (25), although they observed an apparent “collapse” of the metal binding upon the release of metal ion that was not observed in the case of ZnuA. However, as the zinc disassociates from the binding site, two of the three histidine residues rotate out of the metal binding cleft. This is nearly the opposite to what is observed in the case of FutA1. In this case the ligating residues remain relatively fixed in each domain, and the metal-binding site is created as the two domains close down upon the metal ion. Similarly large domain motion upon metal binding was also observed in the anion-dependent ferric iron-binding proteins, MhFbpA (26) and hFbp (27), although there appears to be more rearrangement of the metal ligating residues in these structures than observed here with FutA1. These differences lead to a number of interesting questions. Why does one class of metal-binding proteins undergo such large conformational changes, whereas another does not? It could be argued that perhaps the relatively few coordination interactions in ZnuA is not amenable to having ligating residues split between the two domains. However, some of the manganese transporters (28) have the same number of chelating residues as does FutA1 and yet belong to the more rigid cluster 9 solute binding class of proteins.

It seems possible that the split metal-binding site in FutA1, and therefore the need for large domain changes, is necessary because of the nature of the metal recognition site. The secondary shell of residues around the bound iron is apparently important to facilitate iron interactions with the cluster of tyrosine hydroxy groups. Perhaps this rigidifies the metal-binding site such that release has to be mediated by cleaving the metal-binding site in two via these observed conformational changes. It is also not clear what the various “triggering” mechanisms are among these various metal-binding proteins. In the case of FutA1 it is easy to envision how cytosolic ATPase activity might transmit conformational changes through the metal transport pore to metal-binding protein to stimulate the release of metal. This could be accomplished via opening the metal binding cleft or by affecting the one residue in the metal-binding site that undergoes significant conformational changes, His-54. However, it is not as apparent how the more rigid cluster 9
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solute-binding proteins are triggered by their cytosolic ATPases to release the bound metal.

The specificity of FutA1 for ferrous over ferric iron was not expected based on previous studies (8). In those studies the addition of FeCl₃ to a solution of FutA1-GST fusion protein caused an increase in absorption with a maximum at 453 nm. By monitoring the change in absorption and using sodium citrate to compete with FutA1 for iron binding, the association constant was estimated to be \( \sim 1 \times 10^{19} \text{M}^{-1} \). It should be noted, however, that possible binding of Fe³⁺ was not tested. When FutA1 and FutA2 were individually deleted from the organism, the uptake of \(^{59}\text{Fe}³⁺\) was inhibited by 63 and 16%, respectively, and when both were simultaneously deleted, iron uptake was nearly eliminated. The addition of 0.1 mM sodium citrate also entirely blocked iron uptake. Unexpectedly, with FutA1 expressed alone, we observed an increase in absorption with a maximum at 411 nm upon iron binding. However, in this case two different sources of Fe³⁺ did not bind to FutA1, whereas Fe²⁺ bound with affinity similar to what was observed with Fe³⁺ in the previous studies. What is the cause for the apparent change in iron selectivity and apparent shift in absorption maximum? Clearly the major difference between these two studies is that FutA1 was expressed as a GST fusion in the previous analysis. The change in absorption maximum could be either due to iron binding to a different site on these two proteins or that the GST affects the local environment of the bound iron in the fusion protein. Alternatively, it could also be that GST or perhaps contaminating glutathione related to the GST might have reduced at least some of the ferric to ferrous iron (29), or it may be that the ferric solutions contained significant amounts of ferrous ions. It seems unlikely that the GST fusion itself would change specificity of FutA1 from ferrous to ferric binding.

The binding of ferrous iron by FutA2 in this investigation contrasts the results of the work done by Waldron et al., which concluded that FutA2 binds ferric iron \textit{in vitro} (9). The data presented here represent the first test of both ferrous and ferric iron binding to FutA1 or FutA2. Based on the close amino acid sequence similarity of FutA1 and FutA2, including the conservation of the residues lining the iron-binding cleft, it is not surprising that both proteins favor the same ionic state of iron.

The protein-ligand environments of the various iron-binding proteins are fairly diverse. Among those that bind anions concomitantly with the metal, ferric-binding proteins such as Tf (30) and HiFbpA (31) bind the iron via two tyrosine hydroxyls, solvent atoms, and acidic groups with an octahedral coordination geometry. In contrast, the iron-binding protein from \textit{Mannheimia haemolytica} (MhFbpA), binds iron via three tyrosine residues with two additional interactions coming from an associated carbonate ion (26). Here, two tyrosines and one oxygen from the carbonate form a distorted trigonal plane with the second carbonate oxygen and the third tyrosine residues lying above and below this plane. Unlike the anion-dependent binding of ferric ions to the iron-transport protein from \textit{N. gonorrhoeae} (22), MhFbpA can still bind iron in the absence of anion. However the solute binding cleft is open in the absence of anion in the case of MhFbpA. In a nearly identical configuration found in nFbpA, the iron is bound to FutA1 in a distorted trigonal bipyramidal configuration. Tyrosine residues 55, 185, and 242 form the trigonal plane and His-54 and Tyr-241 are above and below and essentially perpendicular to the plane formed by the three tyrosines.

The structures of FutA1 and the \textit{C. jejuni} FbpA (cFbpA) clearly show that the iron can be bound directly to these transport proteins without the assistance of siderophores and anions. From the binding analysis, it is also clear that this configuration apparently favors the binding of ferrous over ferric iron. In contrast, the Fe³⁺ appears to bind the iron transport protein from \textit{N. gonorrhoeae} as an anion-metal complex (22). Previous structural and genomic analysis clustered FutA1 and cFbpA with a group of iron-binding proteins called Class III that all share this four tyrosine, one histidine iron binding configuration (10). Results from these studies suggest that Class III iron-binding proteins favor ferrous iron, whereas the siderophore and anion-mediated iron-binding proteins favor ferric iron. Although it is not clear why one ligand configuration favors ferric and the other ferrous, the biological reason for this selectivity may be clearer. Interestingly, Class III iron-binding proteins are found in Cyanobacteria or bacteria that grow in low oxygen or anoxic conditions. When Cyanobacteria appeared \( \sim 2.7 \) billion years ago, there was an abundance of carbon dioxide but little to no oxygen (32). It, therefore, makes sense that these ancient bacteria might have created a mechanism for ferrous iron import. Similarly, the other members of this class of iron-binding proteins live in low oxygen environments that favor the ferrous form of iron. Therefore, it appears that the binding of \textquoteleft\textquoteleft naked\textquoteright\textquoteright iron favors ferrous iron binding and evolved at a time of a low oxygen environment and is now also being used by bacteria that live in relatively hypoxic environments. In the case of the Cyanobacteria, it is entirely possible that they have since adapted to changing levels of oxygen by creating a ferric reductase in a manner akin to how it enveloped its RuBisCO with the carboxysome; that is, rather than change the specificity of FutA1/FutA2 to bind ferric iron, create a cellular mechanism to circumvent the problem. Most recently, DrgA of \textit{Synechocystis} 6803 has been shown to have significant ferric reductase activity, although it is unclear as to where in the cell this reduction is carried out (33).

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