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The role of citrate and phosphate anions in the mechanism of iron(III) sequestration by ferric binding protein: Kinetic studies of holoprotein formation of wild type and engineered mutants of FbpA†

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Abstract

Ferric binding protein A (FbpA) plays a central role in the iron acquisition processes of pathogenic *Neisseria gonorrhoeae*, *Neisseria meningitidis*, and *Haemophilus influenza*. FbpA functions as an iron shuttle within the periplasmic space of these Gram-negative human pathogens. Iron is picked up by FbpA at the periplasmic aspect of the outer membrane with concomitant acquisition of a synergistic anion. Here we report the kinetics and mechanisms involved with iron(III) loading into iron-free FbpA using iron(III) citrate as an iron source in the presence of excess citrate or phosphate (physiologically available anions) at pH 6.5. In the presence of excess phosphate, iron(III) citrate loads into apo-FbpA in three kinetically distinguishable steps, while in the presence of excess citrate only two steps are discernible. A stable intermediate containing iron(III) citrate bound FbpA is observed in each case. The observation of an additional kinetic step and moderate increase in apparent rate constants suggests an active role for phosphate in the iron insertion process. To further elucidate a mechanism for iron-loading, we report on the sequestration kinetics of iron(III) citrate in the presence of phosphate with binding site mutant apo-FbpAs, H9E, E57D, E57Q, Q58A, Y195F, and Y196H. Tyrosine mutations drastically alter the kinetics, and reduce iron sequestration ability. H9E, E57D, and E57Q have near native iron sequestration behavior; however iron binding rates are altered enabling assignment of sequential side chain interactions. Additionally, this investigation elaborates on the function of FbpA as a carrier for iron chelates as well as “naked” or free iron as originally proposed.

Iron is one of the most abundant elements in the earth's crust. Much of the biosphere has incorporated this element as an essential mineral; that is, most of life on this planet cannot exist without iron. Paradoxically, ferric iron (Fe³⁺) is almost insoluble under normal

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Supporting Information **Available:** This material is available free of charge via the Internet at <http://pubs.acs.org>. Available supplementary information includes the following: (1) additional kinetic plots, (2) *in silico* models of FbpA mutants, and (3) MALDI-TOF and CD characterization of point mutations.

physiological conditions, and is capable of catalyzing the formation of reactive oxygen species, thus requiring seclusion from the aerobic, aqueous, and neutral pH of the cellular environment. Plants, higher animals, and other life forms have developed many specialized mechanisms that compete directly for iron within the environment. One such competition is exemplified by the struggle for iron between a human host and a bacterial pathogen. The role of iron as an essential cofactor in the metabolic pathways of both microorganisms and their hosts has shaped the relationship between host and pathogen. The host's defense mechanism is to exclude free iron as a nutritional resource through complexation with proteins (1). Pathogens counter this host defensive mechanism through exploitation of the host proteins as a nutritional resource, and as a result use the host's control of iron to provide a soluble iron store.

Iron is a limiting growth factor for most microbes, and the efficient solubilization, transport, and storage of iron in pathogenic bacteria are indices of virulence (2). Gram-negative bacteria such as *Neisseria* and *Haemophilus* acquire iron from human iron transport proteins, lactoferrin and transferrin, and low molecular weight iron chelators such as citric acid (3). Using receptors on their outer membranes, these bacteria dock the human proteins or iron chelators and induce release of their iron load. Once iron has passed from the host protein through the bacterial external membrane, it is shuttled across the periplasm to the inner cell membrane. To prevent hydrolysis and redox cycling, iron is transported across the periplasm via a Fe^{3+} specific binding protein named ferric binding protein (FbpA). FbpA is a 34 kDa periplasmic binding protein which sequesters iron and serves as the auxiliary domain of the ABC (ATP-binding cassette) transporter FbpABC, which actively transports iron across the inner cellular membrane (4, 5). The binding event of iron to FbpA in the periplasm serves as a nodal point for iron entry into the cytoplasm since both iron salts (e.g., iron phosphate) and iron chelates (e.g., iron citrate) are transported by FbpA.

Neisseria are obligate human pathogens, and as such are restricted to growth in biological fluids. Low molecular weight ionic nutrients must pass through the periplasm, which lies between the porous outer membrane and the semi-permeable cytoplasmic membrane. The anion composition of the periplasm is not well characterized, but likely reflects the external host or cellular environment (6). *In vivo*, transferrin-bound-iron is the major nutrient iron source for *Neisseria*. When transferrin-bound-iron is not available, other sources of labile, chelated iron, if present in sufficient amounts, will sustain bacterial growth such as when the host defense system fails and iron concentration is raised artificially (7-9).

In humans, the labile iron pool, commonly referred to as "non transferrin bound iron, NTBI" is thought to consist of low-molecular-weight, low-affinity chelators such as citrate, phosphate, or adenosine triphosphate (10). These small ferric ion complexes can cross the outer membrane of Gram-negative bacteria through non-specific porins, and *Neisseria* have been reported to use citric acid as a heterologous siderophore (11-13). Phosphate and citrate concentrations in host extracellular fluids (1.0 and 0.1 mM respectively) are expected to be normal constituents of the low molecular weight component of the periplasm (14, 15). However, the role of citrate and phosphate in the iron acquisition process of *Neisseria* has not been definitively determined.

It has been shown that FbpA has a high binding affinity for Fe^{3+} ($K_a = 10^{18} \text{ M}^{-1}$) in the presence of phosphate, and that concomitant exogenous anion binding is requisite (16). Many anions complement tight iron binding, including phosphate, arsenate, oxalate, nitrilotriacetate (NTA), sulfate, and citrate, and anion exchange is facile (17-21). The kinetics of both iron loading and iron release are paramount to the function of FbpA in shuttling iron across the periplasm between two membrane bound receptor proteins. The geometry of the residues (2 Tyr, 1 Glu, and 1 His) (22-24) used as donor groups for iron

play a significant role in FbpA's iron binding strength, and in the mechanism for sequestration and release. Here the mechanism of the iron loading process is probed using mutagenesis and rational design to provide different donor groups that are capable of satisfying the octahedral geometry requirements of iron. This study complements the previous mechanistic inferences derived from static crystals (22-24) and is directed towards two objectives: to establish the ability of the physiological chelator citrate to deliver iron to FbpA in the absence and presence of phosphate anion, and to establish the kinetics and mechanism for the sequestration process at pH 6.5, the presumed pH of the periplasm. Although a role for phosphate was established previously (25), here we wish to explore whether this effect is also observed with the more biologically relevant citrate iron carrier, or if the mechanistic behavior of phosphate was dependent on NTA's presence as the iron carrier.

Experimental Procedures (Materials and Methods)

Materials

Citric acid monohydrate, NaOH, 2-(4-morpholino)-ethane sulfonic acid (MES), KCl, NaCl, and KOH were purchased from Fisher Scientific. Sodium phosphate dibasic (Na_2HPO_4), sodium perchlorate, tris(hydroxymethyl)aminomethane (Tris base) were purchased from Sigma-Aldrich. Chelex-100 was purchased from Bio-Rad. All solutions were prepared using ultrapure 18 M Ω -cm equivalent water (Hydropure) in acid-washed glassware.

Stock Solutions

Sodium Perchlorate—A stock solution of sodium perchlorate, NaClO_4 was prepared from sodium perchlorate hydrate, $\text{NaClO}_4 \cdot x\text{H}_2\text{O}$, and filter sterilized through a 0.22 μm CA sterile filter system (Corning). Standardization of the NaClO_4 solution was performed by passing an aliquot through a strongly acidic cation exchange column packed with DOWEX 50W-X8, H^+ , 20-50 mesh resin (J. T. Baker Chemical), and titrating the flow through with NaOH to a phenolphthalein end point.

Ferric Perchlorate—An acidic iron perchlorate $\text{Fe}(\text{ClO}_4)_3$ stock solution was prepared by dissolving $\text{Fe}(\text{ClO}_4)_3$ in 0.1 M HClO_4 . The determination of iron (0.104 M Fe^{3+} /0.1 M HClO_4) was performed by ultraviolet spectrophotometry (26).

Ferric Citrate—To avoid iron hydrolysis, working ferric citrate solutions were prepared by slow addition of an appropriate volume of acidic $\text{Fe}(\text{ClO}_4)_3$ stock solution (0.104 M Fe^{3+} /0.1 M HClO_4) to a vigorously stirred solution of MES, citrate, and NaClO_4 where the initial pH was adjusted to near neutral (approximately 6.7) by titration with 1 M NaOH before adding the acidic $\text{Fe}(\text{ClO}_4)_3$. In the neutral pH region with increasing citrate concentrations, iron complexation by citrate is favored over $\text{Fe}(\text{OH})_n$ polymer formation, and the solutions remain a clear, golden color with no reddish-brown precipitate (27).

The ferric citrate equilibrium speciation was modeled using Hyperquad Simulation and Speciation (HySS) software for all concentrations in this study using previously reported affinity constants, keeping in mind the observation that iron is bound by FbpA at a 1:1 molar ratio (28). The speciation of iron(III)citrate depends on the citrate/iron(III) ratio, the concentrations of iron and citrate, the ionic strength of the solution, and the solution pH. Ferric citrate speciation was calculated using several models. Considering citric acid to have three dissociable protons (H_3L), the model provided by Königsberger generates a fairly constant source of the 1:1 and the 1:2 species $[\text{Fe}(\text{Cit})\text{OH}]^{1-}$ and $[\text{Fe}(\text{Cit})_2\text{OH}]^{4-}$ at all concentrations used in this study (29, 30). Working with an alternate model proposed by Silva et al. using stability constants for citric acid with four dissociable protons (H_4L) at an

ionic strength of 0.1 M, the prevalent iron(III)citrate species are $[\text{Fe}(\text{Cit})_2]^{5-}$ and $[\text{Fe}(\text{Cit})_2\text{H}]^{4-}$ over the concentration range used in our study (29-31). These models only offer an approximation of our system as input parameters are not available at our exact conditions (e.g., ionic strength and concentration). Given that iron(III)citrate speciation is indeed complex, and the addition of apo-FbpA will perturb the speciation further, throughout this manuscript the terminology $\text{Fe}(\text{Cit})'$ will indicate that a mixed species distribution is present at the given iron(III) and citrate concentrations. The lack of variability of kinetic plots obtained over a range of $[\text{Fe}(\text{Cit})']$ and $[\text{Cit}]$ concentrations, and $[\text{Cit}]/[\text{Fe}^{3+}]$ ratios suggest that within the range of concentrations used in our studies, minor variations in iron(III)citrate speciation do not influence our results or interpretations. The relevant concentrations in the stopped-flow cell after mixing were: $[\text{Fe}^{3+}] = 0.05 - 1 \text{ mM}$ as $\text{Fe}(\text{Cit})'$, $[\text{citrate}] \approx 0.8 - 7.1 \text{ mM}$, $\text{MES} = 50 \text{ mM}$, $I = 0.15$ (NaClO_4), $\text{pH} = 6.5$.

For reactions in the presence of excess phosphate, solutions were made similarly, except phosphate was present in solution prior to the addition of $\text{Fe}(\text{ClO}_4)_3$. Under these conditions more than 99% of Fe^{3+} is present as the ternary complex $\text{Fe}(\text{Cit})(\text{H}_x\text{PO}_4)^{y-}$ (32). The relevant concentrations in the stopped-flow cell after mixing were: $[\text{Fe}^{3+}] = 0.1 - 1 \text{ mM}$ as $\text{Fe}(\text{Cit})(\text{H}_x\text{PO}_4)^{y-}$, $[\text{citrate}] \approx 0.1 - 1.1 \text{ mM}$, $[\text{phosphate}] = 1.0 - 10 \text{ mM}$, $\text{MES} = 50 \text{ mM}$, $I = 0.15$ (NaClO_4), $\text{pH} = 6.5$.

The $\text{Fe}(\text{Cit})'$ concentrations of the stock solutions (before mixing) ranged from: $0.1 - 2 \text{ mM}$. Phosphate concentrations ranged from $2 - 20 \text{ mM}$. All $\text{Fe}(\text{Cit})'$ solutions were allowed to stabilize overnight after preparation, and filter sterilized through a sterile SFCA $0.20 \mu\text{m}$ syringe filter (Corning) prior to use. Buffer pH adjustments were made using an Orion pH-meter model 230A.

Mutagenesis, Protein Expression, and Purification—The engineered iron binding mutants of FbpA were modeled on a computer using coordinates deposited into the Protein Data Bank. Substitutions for the iron binding side chains were added in standard geometries. A discussion of the replacements is included in the supplementary information together with kinemages representing the mutants selected for expression and kinetic analysis (Supplementary Figures S.9-S.14). Recombinant FbpA cloned from *Neisseria gonorrhoeae* strain F62 was expressed in an *E. coli* background with purification conducted as previously described (4). The Gene Editor System (Promega) was used to perform site-directed mutagenesis under the manufacturer's conditions using pSBGL as the template (28). Plasmid DNA was isolated from single putative mutants using standard miniprep techniques and was subjected to automated DNA sequencing using an ABI Prism 3100 sequencer operated by the Department of Microbiology and Molecular Genetics Shared Resources Facility, University of Pittsburgh. Oligonucleotide primers used for mutagenesis are listed in Table S. 1 in Supplementary Information. Purified FbpA was bound to a carboxymethyl sepharose column, and iron-loaded FbpA was converted to apo-FbpA on the column by addition of 10 volumes of 1 mM citrate in 0.1 M Tris base at $\text{pH} 8.0$. Apo-FbpA was eluted using a NaCl gradient in buffers rendered iron-free by exposure to Chelex-100. Fractions were collected in acid-washed glassware and extensively dialyzed against Chelex-100 treated 0.05 M MES with 0.2 M KCl , and concentrated using an Amicon centrifugal filter device (Millipore). Purified FbpA mutants were characterized using MALDI-TOF MS and circular dichroism (CD) analysis in order to monitor expected mass shifts and protein secondary structure (See Supplementary Information Figures S.15-S.18 for MALDI-TOF MS and Figures S.19-S.24 for CD spectra). Aliquots were frozen and kept at liquid nitrogen vapor temperature until used for kinetic analysis.

In preparation for kinetic analysis, all protein solutions were dialyzed into Chelex-100 treated 0.05 M MES, 0.1 M NaCl prior to dialysis exchange into Chelex-100 treated kinetics

buffer (0.05 M MES, 0.15 M NaClO₄, pH 6.5) to prevent precipitation between K⁺ and ClO₄⁻. Each dialysis treatment was allowed to proceed overnight in at least 1000:1 buffer to protein volume ratios.

Physical Measurements—Spectral studies were performed using a Varian Cary 100-Bio UV-VIS spectrophotometer and Applied Photophysics stopped-flow SX.18 MV equipped with a diode array spectrophotometer with an approximate range of 380-750 nm for rapid-scan measurements. Rapid-scan experiments provided evidence for the assignment of kinetic phases and conversion to final product. Kinetic studies were performed using an Applied Photophysics stopped-flow spectrophotometer SX.18 MV, and were monitored with both fluorescence and single wavelength absorbance detection, with equivalent values of k_{obs} obtained for both detection modes. All kinetic data illustrated in the figures were obtained in fluorescence mode with excitation at 280 nm under pseudo-first-order (generally 10-fold excess) conditions of excess Fe(Cit)['] in 50 mM MES, I = 0.15 (NaClO₄), pH 6.5 at 25 °C. Total fluorescence was monitored using a 305 nm cutoff filter. Each kinetic data point in the figures represents an average of 2-7 replicate runs. Estimated errors are less than the data point size unless otherwise indicated. The dead time of the instrument is 1.4 ms as determined using the test reaction between 2,6-dichlorophenolindophenol and L-ascorbic acid (33). In all kinetic experiments probing citrate or phosphate dependencies, citrate or phosphate were present in both FbpA and Fe(Cit)['] solutions prior to mixing.

Results

General Observations

Our objective in this work was to investigate the kinetics and mechanism of iron delivery from iron citrate to apo-FbpA in the absence and presence of phosphate. Time-resolved stopped-flow absorbance and fluorescence spectroscopy was used to detect the involvement of FbpA binding site side chains during sequestration of iron citrate. A generic form of the reaction in the absence and presence of phosphate is shown in eq. 1, where apo-FbpA represents the anion bound form of the protein (apo-FbpA-Cit

or apo-FbpA-PO₄), FeL represents the iron(III)-chelate used to deliver Fe³⁺ (iron(III)-citrate or iron(III)-citrate-phosphate), and the product is the ternary iron(III)-protein-anion complex (FeFbpA-L). Results presented in subsequent sections demonstrate that iron sequestration proceeds via a kinetically distinguishable two-step process in the absence of phosphate, and a three-step process in the presence of phosphate to produce FeFbpA-Cit as the kinetic product in both cases. Ultimately, in the presence of excess phosphate, anion exchange occurs, and FeFbpA-PO₄ is formed.

Kinetics of Fe(Cit)['] Insertion Into apo-FbpA

The reaction of Fe(Cit)['] (see Experimental Procedures) with apo-FbpA in the presence of variable excess citrate concentrations was investigated as illustrated in eq. 2.

Under our experimental conditions apo-FbpA exists as greater than 94% FbpA-Cit (19). The spectral change that occurs during the Fe(III) sequestration reaction shown in eq. 2 is illustrated in Figure 1. A quasi-equilibrium intermediate species is formed during the first process with $t_{1/2} = 580$ msec ($[\text{Fe(Cit)}'] = 0.1$ mM, $[\text{Citrate}] = 5$ mM, $[\text{FbpA}] = 70$ μM, pH

= 6.5), as indicated by the spectrum labeled as Ic in Figure 1. In a second slower process, the intermediate species converts with $t_{1/2} = 280$ sec to the spectrum labeled IIc in Figure 1. With $\lambda_{\max} = 474$ nm, this ligand-to-metal-charge-transfer (LMCT) band is consistent with the formation of FeFbpA-Cit (18). The broad $\lambda_{\max}$ for the reaction product FeFbpA-Cit was confirmed independently using the Cary spectrophotometer in kinetic scanning mode, the final scan of which was compared directly with a spectrum of FeFbpA-Cit prepared separately. At longer times in the presence of a large excess of citrate, a third step (not shown) eventually results in the loss of the LMCT absorbance band as FeFbpA-Cit is de-ferrated to form iron citrate with $t_{1/2} = 5$ h. As components of the total iron citrate speciation denoted as Fe(Cit)' in this manuscript, nonreactive ferric citrate complexes must establish an equilibrium yielding a reactive FeCit species before interacting with FbpA in a mechanism similar to that discussed by Bates for transferrin (34). Based on the disagreement of the complex speciation models, it is not apparent if the formation of a reactive 1:1 FeCit species like that reported by Bates is involved at these experimental conditions (34). However, the dependence on citrate:Fe ratio is similar and does suggest that the formation of a reactive iron citrate species is involved in iron sequestration by FbpA. The rate of transfer of iron to FbpA and transferrin at low concentration is dependent on ferric citrate, but at high excess of citrate to iron, the reverse reaction becomes significant (34).

The kinetics of the steps involved in reaction 2 were investigated by stopped-flow absorbance and fluorescence spectroscopy as a function of Fe(III) and citrate concentrations. For transition metals, recognition of the metal center is communicated by fluorescence quenching where the quenched state indicates metal bound to the fluorophore (35, 36). Although the 280 nm excitation wavelength generates both Trp and Tyr fluorescence, the use of both detection modes made it possible to determine whether the observed changes in fluorescence were due to (1) metal binding resulting in Tyr quenching or (2) fluorescence quenching due to changes in the environments of Trp residues. Both detection modes gave equivalent kinetic results where the fluorescence decline (Figure S.1) corresponds to the Fe³⁺ induced production of ionized tyrosine, the same species responsible for the increase in absorbance seen at $\lambda_{\max} = 474$ nm (Figure 1), i.e., the changes in fluorescence are comparable to those monitored in absorbance mode where only the tyrosines are reporting an increase in the LMCT band (25). The decrease in fluorescence (Figure S.1) is ascribed to complex formation with the phenolates of the tyrosine residues during iron uptake in a process similarly described for transferrins (37, 38).

The observed pseudo-first order rate constant for Step Ic ($k_{\text{obs}}^{\text{Ic}}$) is independent of citrate and Fe(Cit)' (Figure S.2) concentrations. The plots agree within experimental error and level at $k_{\text{obs}}^{\text{Ic}} \approx 1.3(2) \text{ s}^{-1}$. We assume that this process is associated with a change in the conformation of the protein possibly induced by iron interaction at the N-terminal domain accompanied by proton loss as observed for transferrins (37, 38). The second slower step (Step IIc) continues to the completion of the reaction to form FeFbpA-Cit. The observed pseudo-first order rate constant of the second step ($k_{\text{obs}}^{\text{IIc}}$) is not dependent on citrate concentration (Figure S.3) but shows saturation behavior with respect to Fe(Cit)' (Figure 2).

Our proposed working model for the overall process in reaction 2 is illustrated in Scheme 1. We propose that Step Ic in which 50% of the fluorescence is quenched is composed of three processes where FbpA and Fe³⁺_{aq} are in equilibrium with citrate (Reaction 3 and Reaction 4) as pre-equilibria to apo-FbpA-Cit reacting with Fe(Cit)' (Reaction 5). (See Experimental Section for a discussion of Fe(Cit) speciation as a function of Fe³⁺/citrate ratio.) In reaction 5, "*" denotes an intermediate state in which Fe(Cit)' may be fully or partially complexed in the secondary coordination sphere by the FbpA binding site. Step IIc in which the other 50% of the fluorescence quenching occurs may be interpreted to involve the reorganization of Fe(Cit)FbpA-Cit* to form a stable FeFbpA-Cit complex, where either the chelating citrate or

a citrate pre-equilibrated with apo-FbpA is released from the binding site upon protein closure (Reaction 6). This is consistent with the observed $\lambda_{\max}$ and absorptivity (Figure 1).

Step Ic

Step IIc

Scheme 1.

Step Ic corresponds to reactions 3-5, with reaction 5 as rate limiting, and reactions 3 and 4 as rapid pre-equilibria. Step IIc corresponds to reactions 3-6, where 3-5 are pre-equilibria and reaction 6 is rate limiting. At pseudo-first order conditions (where Fe(Cit)'_t concentration is much greater than FbpA concentration), the experimentally observed rate for the overall process including Steps Ic and IIc is defined in eq. 7.

$$\text{Rate}_{\text{obs}} = k_{\text{obs}}^{\text{IIc}} [\text{FbpA}]_{\text{total}} \quad (7)$$

From the overall rate expression in eq. 7, and considering reaction 5 as a pre-equilibrium step, $k_{\text{obs}}^{\text{IIc}}$ is defined as in eq. 8.

$$k_{\text{obs}}^{\text{IIc}} = \frac{k_6 K_5 [\text{Fe(Cit)'}]_t}{1 + K_5 [\text{Fe(Cit)'}]_t} \quad (8)$$

A fit of the data according to eq. 8 is shown in Figure 2 and the obtained parameters are: $K_5 = 1.0(2) \times 10^4 \text{ M}^{-1}$ and $k_6 = 4.8(2) \times 10^{-3} \text{ s}^{-1}$ (Table 1, column 3). Inclusion of a term for the back reaction did not result in convergence of the parameters. According to the data presented, we conclude that excess citrate has no influence on the insertion process of Fe(Cit)'_t into apo-FbpA.

Kinetics of Fe(Cit)'_t Insertion Into apo-FbpA in the Presence of Phosphate

The reaction of Fe(Cit)'_t with apo-FbpA in the presence of excess phosphate is illustrated in eq. 9.

Depending on conditions and in the absence of exogenous citrate, greater than 35% of apo-FbpA is complexed with phosphate at the anion binding site (FbpA-PO₄) prior to mixing with Fe(Cit)', which in the presence of phosphate yields Fe(Cit)(PO₄) (19). The spectral change that occurs during the Fe(III) exchange reaction in eq. 9 is shown in Figure 3. Contrary to reaction 2 in the absence of exogenous phosphate, two quasi-equilibrium intermediate species (labeled Ip and Iip Figure 3) are formed with $t_{1/2} = 110$ msec and 1.7 sec (when [Fe(Cit)'] = 0.1 mM, [Phosphate] = 5 mM, [FbpA] = 50 μM, pH = 6.5). In the second process, the intermediate species indicated by spectrum (Iip) is converted with $t_{1/2} = 50$ sec to the spectrum labeled IIIp in Figure 3 ($\lambda_{\text{max}} = 474$ nm). This is consistent with the formation of FeFbpA-Cit (18). Eventually ($t_{1/2} = 20$ h) the spectrum indicating FeFbpA-Cit formation shifts to $\lambda_{\text{max}} = 481$ nm (data not shown), suggesting that citrate anion exchange occurs to form FeFbpA-PO₄ (18). An active role for phosphate in the Fe(III) sequestration process may be inferred from the fact that the Fe(Cit)' sequestration reaction occurs more rapidly in the presence of phosphate and with the detection of an additional kinetic intermediate.

The relative amplitudes of the fluorescence mode kinetic traces for iron insertion into FbpA from Fe(Cit)' in the presence of phosphate are ca 10%:45%:45% for the first, second, and third step respectively. The working model for the overall process described in reaction 9 is illustrated in Scheme 2, where Step Ip corresponds to reactions 10-12, Step Iip to reactions 10-13 and Step IIIp to reaction 14.

Step Ip

Step Iip

Step IIIp

Scheme 2.

In Step Ip no dependence on phosphate (Figure S.4) or Fe(Cit)' (Figure S.5) concentrations were observed. This suggests that the rate determining step represented by $k_{\text{obs}}^p = 6 \text{ s}^{-1}$ is likely due to a change of the conformation of the protein active site.

We observe saturation dependence on phosphate concentration (Figure 4) and no dependence on Fe(Cit)' concentration (Figure S.6) for the pseudo first order rate constant associated with Step Iip. The rate determining process in Step Iip is illustrated in reaction 13. Reactions 10-12 from Step Ip may be considered as pre-equilibria for reaction 13. The phosphate concentration dependence of the rate in Figure 4 may be attributed to the relative

shift in equilibrium reaction 10 with increasing phosphate concentration. This assertion is supported by a plot of % total protein complexed as apo-FbpA-PO₄ as a function of [H_xPO₄^{y-}] (Figure 4, insert) (19).

In Step IIIp, no dependence on phosphate (Figure S.7) or Fe(Cit)' (Figure S.8) were observed, suggesting that the quasi-equilibrium in reaction 13 lies far to the right. We interpret this as an indication of another change in the conformation of the protein once the binding site is loaded with iron, also similar to the mechanism seen for lactoferrin (37). Leveling of $k_{\text{IIIp}}^{\text{app}}$ to form FeFbpA-Cit is at $1.5 \times 10^{-2} \text{ s}^{-1}$ (Figure S.7) which is about an order of magnitude faster than without phosphate (Figure S.3; see also Table 2, Row 3, Columns 2 and 4).

Comparison of Kinetics of Iron Transfer as a Function of Citrate and Phosphate Concentrations

For comparative analysis, in Table 2 we define a phenomenologically “apparent” first order rate constant ($k^{\text{app}}/\text{sec}^{-1}$) for each step in each system (with and without phosphate) at a fixed set of conditions for the Fe insertion reactions (2) and (9): [FbpA] = 7 μM ; [PO₄ or Cit] = 5 mM; [Fe(Cit)'] = 1.0 mM; pH 6.5; MES = 50 mM; I = 0.15 (NaClO₄). For the Fe³⁺ insertion process we observe that substituting PO₄³⁻ for excess citrate and Fe(Cit)(PO₄) for Fe(Cit)', results in a change from a 2-step to a 3-step resolvable system (Table 2).

Considering the relative % fluorescence quenching observed for each of the steps we tentatively conclude that there is an initial step in the presence of phosphate that is resolvable, and accounts for 10% of the observed fluorescence quenching (Step Ip), and is not resolvable in the absence of phosphate. The lack of this step in the absence of phosphate may have several origins, but it is likely that citrate may not pre-organize the Tyr's in the FbpA binding site to the same extent PO₄³⁻ does. Also, citrate as a synergistic anion in the cleft of apo-FbpA is more sterically demanding than phosphate. The interaction of Fe(Cit)' with a partially occupied apo-FbpA would place the approaching iron further away from the phenolic Tyr's during the initial step. This could cause less efficient quenching and result in the presence of iron not being reported. Also, there may be two initial steps in the citrate system, but both may be of the same magnitude and overlap enough that their half-life is not resolvable. As a result, what is defined as Step Ic for the citrate system may actually be a blending of two non-resolvable rate constants, while the presence of PO₄³⁻ enables resolution by increasing the rate of the early process.

The process represented by Step IIp (45% fluorescence quench) may be compared with Step Ic (50% fluorescence quench), and that represented by Step IIIp (45% fluorescence quench) with Step IIc (50% fluorescence quench) (see rows 2 and 3 in Table 2). $k_{\text{I}}^{\text{app}}$ for Step Ic and $k_{\text{II}}^{\text{app}}$ for Step IIp are comparable in value (Table 2, row 2, col 2 and 4) and we observe an increase of approximately an order of magnitude for the last step (Step IIc and IIIp) in the presence of PO₄³⁻ to produce FeFbpA-Cit in both cases (c.f. $k_{\text{II}}^{\text{app}}$ and $k_{\text{III}}^{\text{app}}$ in Table 2, row 3, col 2 and 4). These comparative data suggest that PO₄³⁻ plays a role in the stepwise Fe³⁺ insertion process.

Kinetics of Fe(Cit)' Insertion Into Engineered apo-FbpA Mutants in the Presence of Phosphate

Kinetics experiments with protein point mutations were carried out in order to elucidate the involvement of specific binding site side chains in the Fe³⁺ sequestration process. A description of *in silico* experiments designed to evaluate the rational design of iron-binding mutants of FbpA based on clashes and energetics is found in the Supplementary Information. Mutations were chosen that did not incorporate significant changes to the

conformation of neighboring side chains, and that would test the kinetic properties of FbpA relevant to iron sequestration. The mutations were chosen to minimize effects on the ability of the remaining residues to form coordinate covalent bonds to Fe^{3+} while providing alternate Fe^{3+} Lewis base ligands or removal thereof.

The point mutations utilized may be divided into two groups, those in the N-terminal domain (E57D, E57Q, H9E, and Q58A), and those in the C-terminal domain (Y195F and Y196H). Except for the tyrosine mutants, the overall amplitudes and shapes of the fluorescence mode kinetic traces for each of the mutants (E57D, E57Q, H9E, and Q58A) are similar to wild type, and suggest a similar iron binding motif (data not shown). The effects of protein point mutations on Fe^{3+} sequestration kinetics can be readily seen through a comparison of the ratio "R" of the rate constants for the corresponding steps ($R = k_{\text{mutant}} / k_{\text{wild type [wt]}}$), and fluorescence quenching amplitudes for the wild type (WT) protein. The reaction conditions used for comparison of the reaction of $\text{Fe}(\text{Cit})'$ with apo-FbpA in the presence of excess phosphate (eq. 9), and the ratios of first order rate constants for the three steps, $k^{\text{Ip}}_{\text{obs}}$, $k^{\text{IIp}}_{\text{obs}}$, and $k^{\text{IIIp}}_{\text{obs}}$, are shown in Figure 5.

The E57D mutant on the N-terminal domain offers the same hard oxygen donor group, but is not expected to be at an optimized distance relative to WT for coordination to the Fe^{3+} center. Figure 5 illustrates the effect of the E57D mutant on $k^{\text{Ip}}_{\text{obs}}$, $k^{\text{IIp}}_{\text{obs}}$, and $k^{\text{IIIp}}_{\text{obs}}$ data collected at both variable phosphate, and $\text{Fe}(\text{Cit})'$ concentrations in the presence of excess phosphate. The strongest influence is on $k^{\text{Ip}}_{\text{obs}}$, where in both cases R is approximately 0.5. This significant deviation from $R = 1$ observed for Step Ip suggests direct involvement of Glu57 early in the FeL binding process (Eq 9).

N-terminal domain effects were tested further by substitution of Glu57 with Gln, providing a polar, uncharged amide oxygen in a side-chain of equivalent length and volume. Here the results are less dramatic with no change in the variable iron concentration R values for E57Q (Figure 5), but R is less than 1 with respect to variable phosphate concentration data for Step Ip ($k^{\text{Ip}}_{\text{obs}}$) $R = 0.65$ (Figure 5). This is consistent with the results for the E57D mutant further confirming direct involvement of Glu57 early in the FeL sequestration process.

The third N-terminal domain mutation H9E, produces the most dramatic effect on Step Ip (R is approximately 0.35) for both $\text{Fe}(\text{Cit})'$ and phosphate variable concentration data (Figure 5), consistent with initial sequestration events occurring in the N-terminal domain sites E57 and H9.

Although not directly involved in Fe^{3+} sequestration, the Q58A mutation tested the effects of synergistic anion hydrogen-bonding on iron sequestration kinetics (23). Due to limited expression levels and purification yields for Q58A, only data at the highest iron concentration were collected (10 mM $\text{Fe}(\text{Cit})'$ after mixing). Although Q58 is not directly involved in Fe^{3+} coordination, early stages ($k^{\text{Ip}}_{\text{obs}}$ and $k^{\text{IIp}}_{\text{obs}}$) produced an R value of approximately 1.5 (Figure 5) suggesting more open access to the Fe^{3+} binding site with this mutation.

A limited number of mutants were expressed to explore a maximum number of effects, and so only two Tyr mutants were examined: (1) Y195F which results in loss of iron coordination from one residue; and (2) Y196H which provides an alternate Lewis base for coordination (hard (O) versus borderline (N) Lewis base). Very limited expression levels and yields were acquired for Y195F and Y196H, and as a result only data at the highest iron concentration were collected (10 mM $\text{Fe}(\text{Cit})'$ after mixing). The shapes of the time domain fluorescence kinetic traces for both Tyr mutants were drastically altered from wild type (data not shown). R values for Y195F and Y196H are significantly enhanced for Step Ip ($R > 3$; Figure 5) suggesting that changes in exogenous anion H-bonding capability may produce a

more openly accessible Fe^{3+} binding site in the early stages of sequestration. Step IIp for Y196H was also enhanced ($R \approx 1.75$) whereas Y195F ($R = 0.5$) was reduced compared to wild type (Figure 5). Step IIIp was not observed for either mutant (Figure 5). Further, the Y196H mutant was able to sequester iron as indicated by the presence of a weak LMCT band in the visible region of associated absorbance spectra, whereas Y195F did not develop a detectable LMCT band at the concentrations tested (data not shown).

Discussion

Periplasmic binding proteins, including FbpA, capture their ligand via a *pac man* or *Venus fly trap* model (39). X-ray crystal structures of the apo- and holo- forms of FbpA show that the N-terminal and C-terminal domains close over the active site upon iron sequestration (22-24). The domains exhibit little change in secondary structure as a 21 degree hinge rotation occurs in the beta sheet section stretching between the two domains, pulling the protein from the open to the closed conformation. It is proposed that the C-terminal domain is pre-organized for iron coordination through phosphate binding to the protein, and that Tyr195 and Tyr196 move little upon iron insertion. However, the rotamers of the N-terminal residues, His9 and Gly57, change conformation significantly upon binding iron.

Through comparison with previous results obtained for delivery of Fe^{3+} to apo-FbpA from Fe(NTA) in the absence and presence of phosphate (25), and with data presented here, we suggest that the Fe^{3+} sequestration process is initiated through coordination at the N-terminal domain side chains. Further, we advocate that FbpA may also be considered as a transporter of FeX (X = coordinating synergistic anion) in addition to “naked” or free Fe^{3+} as previously suggested (23, 40). A discussion of these assertions follows.

It is instructive to compare results for $\text{Fe}(\text{Cit})'$ insertion into apo-FbpA with the corresponding kinetics for Fe(NTA) insertion (25). In the absence of PO_4^{3-} , the insertion occurs in two distinguishable steps to produce FeFbpA-Cit and FeFbpA-NTA , respectively. An intermediate is formed ($(\text{FeX})\text{FbpA-X}^*$; X = Cit, NTA) with an equilibrium constant of 10^4 M^{-1} in each case (Table 1). Although the initial step is zero order in $\text{Fe}(\text{Cit})'$, but first order in Fe(NTA), at mM concentrations the rates are comparable with a $t_{1/2}$ of 0.6-1.0 sec for formation of the intermediate in each case at an identical set of arbitrary conditions (Table 1). The relaxation of the intermediate $(\text{FeX})\text{FbpA-X}^*$ to FeFbpA-X (X = Cit, NTA) proceeds approximately 4 $\times$ faster in the NTA case with a rate constant $4.8 \times 10^{-3} \text{ s}^{-1}$ for X = Cit and $2.2 \times 10^{-2} \text{ s}^{-1}$ for X = NTA (Table 1). Although the rates of relaxation to FeFbpA-X are different, both $\text{Fe}(\text{Cit})'$ and Fe(NTA) react via the same process through an intermediate assembly $(\text{FeX})\text{FbpA-X}^*$. The presence of phosphate in both $\text{Fe}(\text{Cit})'$ and Fe(NTA) insertion reactions increases the kinetically observable steps from 2 to 3 to produce FeFbpA-Cit and FeFbpA-NTA , respectively (25).

Scheme 3 summarizes our kinetic data obtained using wild type FbpA and inferences from our FbpA mutant studies for the stepwise process of iron insertion into FbpA in the absence of phosphate. There is no apparent iron or citrate involvement in the kinetics of Step Ic. Consistent with this observation is the comparison of parameters for $\text{Fe}(\text{Cit})'$ and Fe(NTA) in Table 1 and Table 2 (row 2, col 2 & 6), which shows analogous first step kinetics for these two complexes. Assuming protein side-chain involvement occurs in the same sequence in the absence as well as presence of phosphate, initial sequestration of Fe^{3+} occurs at the N-terminal domain as indicated by the mutant studies. A 50% relative fluorescence amplitude change characteristic of iron tyrosine fluorescence quenching suggests the coordination of at least one tyrosine residue in the first step of the Fe^{3+} insertion process. This is confirmed by absorbance measurements consistent with Tyr-Fe LMCT band formation.

In Step IIc of the $\text{Fe}(\text{Cit})'$ system, the second tyrosine enters into the iron coordination shell (Scheme 3) as indicated by a second large fluorescence quenching amplitude change (50%) equal to that of the first step. Comparing the rate constants of the second steps, iron transfer from citrate is $5\times$ slower than from NTA at similar experimental conditions (Table 1). This may reflect differences in the lability of the first coordination shell in $\text{Fe}(\text{Cit})'$ and $\text{Fe}(\text{NTA})$.

Scheme 4 represents an iron sequestration model for FbpA in the presence of phosphate, and is based on our results from wild type and mutant FbpA. Here FeX enters as a unit when introduced to apo-FbpA- PO_4 . The pre-equilibrium reaction involving phosphate and FbpA results in the pre-organization of the binding site tyrosines on the C-terminal domain (24, 41). In the first step of the Fe-citrate-phosphate mechanism (Scheme 4), the lower level of fluorescence quenching (approximately 10%) suggests that the fluorescent side chains may be less accessible and indicates that $\text{Fe}(\text{Cit})'$ is close but not bound as efficiently to the binding site tyrosines (Tyr195 and Tyr196). Interaction with the N-terminal residues is suggested by the N-terminal residue mutation results (Figure 5) in a mechanism similar to that observed in the first step of the Fe-NTA-phosphate system (25).

The second step of iron transfer is dependent upon phosphate concentration and likely reflects the equilibrium of eq. 10 (Figure 4, insert), while the role of the iron chelator (citrate) is less obvious. Fluorescence is quenched by another 45% in step IIp and suggests iron coordination by one of the binding site tyrosines (Scheme 4). The final change in fluorescence intensity (approximately 45% quenching) in step IIIp indicates coordination by the second binding site tyrosine (Scheme 4).

Comparison of the wild type kinetics to that of the binding mutants provides additional insight into the mechanism presented in Scheme 4. The loss of only one phenolate residue (Y195F), had a strong effect on iron sequestration, even though the *in silico* analysis of the replacement indicated molecular-goodness-of-fit leaving three properly aligned residues available for iron binding. Compared to the drastic effects of the single Tyr replacement to a nonbinding residue (Y195F), the Y196H mutant offered a binding site still outfitted with four functional groups capable of binding iron. Thus the mutation Y196H made it possible to test C-terminal domain effects without losing iron-coordination. Histidine is shorter than tyrosine, but this borderline hard/soft Lewis base is capable of coordinating Fe^{3+} , and may even incorporate a bridging water molecule if present in the binding pocket provided there is room.

For each of the Tyr mutants only one Tyr residue remained to report iron interactions. Although the lack of a strong LMCT band is generally indicative of an inability to sequester iron, here the reduced absorptivity is proposed to reflect the fact that only one of the two tyrosines is available to report the presence of iron. If the iron binding pocket of the Tyr mutants is more accessible due to lack of Tyr pre-organization, the initial interaction of an approaching iron cation with the remaining Tyr could presumably result in more efficient quenching during Step Ip. The reduced quenching amplitude of Step IIp of Y195F and the loss of the kinetics mode fluorescence quenching Step IIIp for both protein mutants demonstrate a disruption in the ability to sequester iron, and is a strong indication of C-terminal domain effects later in the mechanism of the iron sequestration process. This is interesting in that although Y196H exhibits a weak LMCT band indicating Fe^{3+} coordination, Y196F does not exhibit a detectable LMCT band. The apparent loss of efficient iron sequestration for Y195F may be a result of the loss of phosphate- Fe^{3+} -Tyr195 interactions which are predicted to stabilize the closed form of the protein.

A final step (Step IVp) occurs outside the time limits governing stopped-flow reactions reported here, but absorbance data clearly show anion exchange occurs in the final transition

to the phosphate form of the assembly FeFbpA-PO₄, which is stable with respect to iron loss over the period of several days. A similar observation is made for the Fe(NTA) system where FeFbpA-NTA is formed initially as the kinetic product, but eventually on a slower timescale, FeFbpA-PO₄ is formed as the thermodynamic product. A comparison of half lives for this final anion exchange is as follows: For FeFbpA-Cit/PO₄ $t_{1/2}$ = 20 hr ([Fe(Cit)]' = 0.1 mM, [H_xPO₄^{y-}] = 5.0 mM, [FbpA] = 50 μM, 50 mM MES, 150 mM NaClO₄, pH 6.5, 25 °C), and for FeFbpA-NTA/PO₄ $t_{1/2}$ = 2 hr ([Fe(NTA)] = 0.1 mM, [H_xPO₄^{y-}] = 1.0 mM, [FbpA] = 50 μM, 50 mM MES, 150 mM NaClO₄, pH 6.5, 25 °C) (25). In each case the kinetically controlled product in the presence of excess phosphate is FeFbpA-X (X = Cit, NTA) (Equation 15), which eventually exchanges to the thermodynamically controlled product FeFbpA-PO₄ (Equation 16). That the anion exchange requires a much longer time in the citrate system most likely reflects the fact that apo-FbpA has a 50× higher affinity for citrate over NTA (19).

These data illustrate that for the small molecule iron carriers citrate and NTA, FeX reacts with apo-FbpA-PO₄ to form FeFbpA-X as the kinetically controlled product (Equation 15), with eventual anion exchange in the presence of excess phosphate to FeFbpA-PO₄ (Equation 16). This further suggests that FbpA should be considered a FeX carrier when being loaded via a small molecule source, rather than just a naked Fe³⁺ carrier. This concept was first introduced by Shouldice, et al., in a structural paper showing FeEDTA bound to a mutant of FbpA (42). This shows that FbpA can accommodate an iron/anion-ligand complex by adopting an open conformation, in the case of Shouldice, et al. with Fe-EDTA bound to form a Fe-EDTA-FbpA assembly (42, 43). In this model FbpA may be more like a siderophore periplasmic binding protein (e.g., FepB) (44) which transports iron coordinated to a siderophore through the periplasm of *E. coli*; in the case of FbpA, the anion (e.g., citrate or NTA) takes the place of the siderophore carrier. That FbpA can incorporate different anions suggests that sequestration of FeX involves an adaptive protein-protein interface suited to binding alternative anions in order to achieve an iron-bound form of FbpA.

An overall objective of our work is to understand the *in vivo* mechanisms of iron citrate and iron phosphate uptake in *Neisseria* in order to understand the role and significance of these processes in pathogenesis. The ability of *Neisseria* to use ferric citrate as an iron source, and the ease of exchange of iron between citrate and FbpA may aggravate the outcome of infections and impose an increased risk in host cells with compromised iron withholding defenses (9, 12). Generalized iron overload associated with non-transferrin bound iron in dialysis patients and in conditions such as hemochromatosis and thalassaemia is known to result in an increased risk of infection by microorganisms which utilize low-molecular mass iron complexes including complexes with citrate (45). Whether or not sequestration of iron from ferric citrate by FbpA plays an important role in gonococcal and meningococcal infections remains to be further examined. However, the fact that citrate is available to *Neisseria* and can be used for iron exchange with FbpA clearly suggests such a role.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

FbpA	ferric binding protein
Fe(Cit)'	the distribution of iron(III) citrate complexes at the conditions given (see Experimental Section)
WT	wild-type
CD	circular dichroism
LMCT	ligand-to-metal-charge-transfer band

Figure 1.

Absorbance Spectra for apo-FbpA-Cit + FeCitrate

(A) Absorbance spectra ($\lambda_{\text{max}} = 474 \text{ nm}$) corresponding to quasi-equilibrium species I_c ($t_{1/2} = 0.58 \text{ s}$) and II_c ($t_{1/2} = 280 \text{ s}$) in Scheme 3. Conditions: $[\text{Fe}(\text{Cit})'] = 0.1 \text{ mM}$, $[\text{Citrate}] = 5 \text{ mM}$, $[\text{FbpA}] = 70 \text{ }\mu\text{M}$, $[\text{MES}] = 50 \text{ mM}$, $\text{pH} = 6.5$, $I = 0.15 \text{ (NaClO}_4\text{)}$, $25 \text{ }^\circ\text{C}$. (B) Time domain profile for species I_c (time domain data for species II_c determined similarly)

Figure 2.

k_{obs} vs $[Fe(Cit)']$ for Step IIc: FbpA-Cit + $Fe(Cit)'$

All solutions in 50 mM MES, $I = 0.15$ ($NaClO_4$), $pH = 6.5$, $25\text{ }^{\circ}C$. Fluorescence mode conditions (after mixing): [Citrate] = 5 mM, [FbpA] = $7\text{ }\mu M$. The solid line represents a fit of Equation 8 to the data. Estimated errors are less than the data point size.

Figure 3.

Absorbance Spectra for apo-FbpA-PO₄ + Fe(Cit)(PO₄)

(A) Absorbance spectra ($\lambda_{\text{max}} = 474$ nm) corresponding to quasi-equilibrium species Ip ($t_{1/2} = 0.11$ s), IIp ($t_{1/2} = 1.7$ s) and IIIP ($t_{1/2} = 50$ s) in Scheme 4. Conditions: [Fe(Cit)]' = 0.1 mM, [FbpA] = 50 μ M, [H_xPO₄^{y-}] = 5 mM, [MES] = 50 mM, pH = 6.5, I = 0.15 (NaClO₄), 25 °C. (B) Time domain profile for species Ip. (C) Time domain profile for species IIIP. Time domain data for species IIp determined similarly.

Figure 4.

k_{obs} vs $[\text{H}_x\text{PO}_4^{y-}]$ for Step IIp: $\text{FbpA-PO}_4 + \text{Fe}(\text{Cit})(\text{PO}_4)$.

All solutions in 50 mM MES, $I = 0.15$ (NaClO_4), $\text{pH} = 6.5$, 25°C . Fluorescence mode conditions (after mixing): $[\text{Fe}(\text{Cit})'] = 0.1$ mM, $[\text{FbpA}] = 7$ μM . Estimated errors are less than the data point size unless otherwise indicated. Insert: Plot of % formation of apo-FbpA- PO_4 as a function of phosphate concentration (Eq 10) (19).

Figure 5.

Average Rate Constant Ratios (R ; Mutant/Wild Type).

Rate constant ratios ($R = k_{\text{mutant}}/k_{\text{WT}}$) for pseudo-first order rate constants for Reaction 9 in the presence of excess phosphate or excess iron citrate. Conditions after mixing for a series of reactions where rate data were obtained over a range of phosphate concentrations (in the Figure legend $\text{PO}_4(k_n)$ ($n = 1, 2, 3$) represents k_{IP} , k_{IIP} , and k_{IIIP} respectively in Scheme 4): $[\text{Na}_2\text{HPO}_4] = 1, 2, 3, 5, 7, \text{ and } 10 \text{ mM}$, $[\text{apo-FbpA}] = 0.007 \text{ mM}$, $[\text{Fe}(\text{Cit})'] = 0.1 \text{ mM}$; $\text{mM} [\text{MES}] = 0.05 \text{ M}$; $[\text{NaClO}_4] = 0.15 \text{ M}$; $\text{pH} = 6.5$. Conditions after mixing for a series of reactions where rate data were obtained over a range of $\text{Fe}(\text{Cit})'$ concentrations (in the Figure legend $\text{Fe}(k_n)$ ($n = 1, 2, 3$) represents k_{IP} , k_{IIP} , and k_{IIIP} respectively in Scheme 4): $[\text{Fe}(\text{Cit})'] = 0.1, 0.2, 0.3, 0.5, 0.7, \text{ and } 1.0 \text{ mM}$, $[\text{apo-FbpA}] = 0.007 \text{ mM}$; $[\text{Na}_2\text{HPO}_4] = 5 \text{ mM}$; $[\text{MES}] = 0.05 \text{ M}$; $[\text{NaClO}_4] = 0.15 \text{ M}$, $\text{pH} = 6.5$.

Scheme 3.

Iron Sequestration Model for apo-FbpA + Fe(Cit)

(Mono-citrate iron species used in illustration for clarity, Step I and II exhibit equal fluorescence quenching amplitudes: step I – 50%; step II – 50%)

Scheme 4. Iron Sequestration Model for Apo-FbpA + Fe(Cit)(PO₄)

(Fluorescence quenching amplitudes: step I – 10%; step II – 45%; step III – 45%)

Table 1Comparative Kinetic Data for Fe(Cit)['] and Fe(NTA) Insertion into FbpA in the Absence of Phosphate

Reaction	Parameter	X = Citrate ^a	X = NTA ^b
FeX + apo-FbpA-X $\rightleftharpoons$ (FeX)FbpA-X*	$t_{1/2}$ ^c	0.58(9) sec	1.0(1) sec
	k_5	1.3(2) sec ⁻¹ ^d	2.6(1) $\times 10^3$ M ⁻¹ sec ⁻¹
	K_5	1.0(2) $\times 10^4$ M ⁻¹ ^e	1.1(1) $\times 10^4$ M ⁻¹
(FeX)FbpA-X* $\rightarrow$ FeFbpA-X	k_6	4.8(2) $\times 10^{-3}$ sec ⁻¹ ^e	2.2(1) $\times 10^{-2}$ sec ⁻¹

^aThis work. All solutions in 50 mM MES, I = 0.15 (NaClO₄), pH = 6.5, 25 °C. Error in the final digit is in parentheses.

^bData from (25). All solutions in 50 mM MES, I = 0.15 (NaClO₄), pH = 6.5, 25 °C. Error of the final digit is in parentheses.

^cFor Step I, the Fe(Cit)['] reaction does not exhibit a [Fe(Cit)[']] dependence, while the Fe(NTA) reaction is first order with respect to [Fe(NTA)]. For purposes of comparison, the half life ($t_{1/2}$) for the formation of the intermediate (FeX)FbpA-X* (X = NTA or Cit) was determined at identical conditions: [Fe(Cit)[']] = [Fe(NTA)] = 0.4 mM; [FbpA] = 7 – 8 μ M; 50 mM MES, I = 0.15 (NaClO₄), pH = 6.5, 25 °C.

^dError in the final digit is in parentheses and reports the reproducibility of the series of experiments whose results are given in Figure S.2. The value reported is an average of the rate constants from Figure S.2: citrate dependency, 1.1(1) and Fe(Cit)['] dependency, 1.5(1), which are similar and thus are both considered representative of the kinetic process defined as Step Ic.

^eValue obtained from a fit of Eq 8 to data in Figure 2.

Table 2
Calculated Apparent Rate Constants for Fe(Cit)' and Fe(NTA) Insertion into apo-FbpA in the Absence and Presence of Phosphate

FeX	Fe(Cit)'		Fe(Cit)(PO ₄)		Fe(NTA)		Fe(NTA)(PO ₄)	
	Parameter Value/sec ⁻¹ <i>a</i>	% <i>e</i>	Parameter Value/sec ⁻¹ <i>f</i>	% <i>e</i>	Parameter Value/sec ⁻¹ <i>j</i>	% <i>e</i>	Parameter Value/sec ⁻¹ <i>m</i>	% <i>e</i>
1	N/A <i>b</i>	N/A <i>b</i>	Step Ip k _I ^{app} = 6.0 <i>g</i>	10	N/A <i>b</i>	N/A <i>b</i>	Step I k _I ^{app} = 80 <i>n</i>	10
2	Step Ic k _I ^{app} = 1.3 <i>c</i>	50	Step IIp k _{II} ^{app} = 0.6 <i>h</i>	45	Step I k _I ^{app} = 2.8 <i>k</i>	50	Step II k _{II} ^{app} = 0.7 <i>o</i>	45
3	Step IIc k _{II} ^{app} = 0.0020 <i>d</i>	50	Step IIIP k _{III} ^{app} = 0.015 <i>i</i>	45	Step II k _{II} ^{app} = 0.022 <i>l</i>	50	Step III k _{III} ^{app} = 0.0093 <i>p</i>	45

^a Apparent first order rate constant for Step Ic (k_I^{app}), and Step IIc (k_{II}^{app}), calculated at the following conditions: 7 μM [FbpA], 0 mM [PO₄], 5 mM [Cit], 1.0 mM [Fe(Cit)'], 50 mM [MES], I = 0.15 (NaClO₄), pH = 6.5, 25 °C.

^b Not observed.

^c This work. See Figure S.2.

^d This work. See Figure 2.

^e Percent of total fluorescence quenching observed for this step.

^f Apparent first order rate constant for Step Ip (k_I^{app}), Step IIp (k_{II}^{app}), and Step IIIP (k_{III}^{app}) calculated at the following conditions: 7 μM [FbpA], 5 mM [PO₄], 0 mM [Cit], 1.0 mM [Fe(Cit)'], 50 mM [MES], I = 0.15 (NaClO₄), pH = 6.5, 25 °C.

^g This work. See Figure S.5.

^h This work. See Figure S.6.

ⁱ This work. See Figure S.8.

^j Apparent first order rate constant for Step I (k_I^{app}), and Step II (k_{II}^{app}) calculated at the following conditions: 8 μM [FbpA], 0 mM [PO₄], 1.0 mM [Fe(NTA)], 50 mM [MES], I = 0.15 (NaClO₄), pH = 6.5, 25 °C.

^k From (25). Value calculated by extrapolating data in Figure 6 to 1.0 mM Fe(NTA).

^l From (25), Figure 3.

^m Apparent first order rate constant for Step I (k_I^{app}), Step II (k_{II}^{app}), and Step III (k_{III}^{app}) calculated at the following conditions: 7 μM [FbpA], 5 mM [PO₄], 1.0 mM [Fe(NTA)], 50 mM [MES], I = 0.15 (NaClO₄), pH = 6.5, 25 °C.

n_{From} (25), Figure 3.

o_{From} (25); extrapolated from data in Figure 4A.

p_{From} (25); calculated from data in Figure 5 and eq (12).