Ex Vivo Analysis of Synergistic Anion Binding to FbpA in Gram-Negative Bacteria†

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ABSTRACT: Ferric binding protein, FbpA, is a member of the transferrin superfamily whose function is to move an essential nutrient, iron, across the periplasm and into the cytosol through formation of a ternary complex containing Fe^{3+} and a synergistic anion, X. Here we utilize SUPREX (*s*tability of *u*npurified *p*roteins from *r*ates of H/D *ex*change) to determine the identification and distribution of the synergistic anion in FeFbpA-X species in periplasmic preparations from Gram-negative bacteria. SUPREX is a mass spectrometry-based technique uniquely suited for thermodynamic analyses of protein–ligand complexes in complex biological mixtures such as periplasmic preparations. Model binary mixtures of FeFbpA-Cit and FeFbpA-PO_4 were initially characterized by SUPREX due to the likely presence of citrate and phosphate ions in the periplasm. *Ex vivo* SUPREX analyses were performed on FeFbpA-X species overexpressed in an *Escherichia coli* cell line and on endogenous FeFbpA-X species in *Neisseria gonorrheae*. Detected in the *E. coli* periplasmic extract were two distinct populations of FbpA, including one in which the protein was unliganded (i.e., apoFbpA) and one in which the protein was bound to iron and the synergistic anion, phosphate (i.e., FeFbpA-PO_4). FeFbpA-PO_4 was the only population of FbpA molecules detected in the *N. gonorrheae* periplasmic extract. This work provides the first determination of the identity of the *in vivo* anion bound to FeFbpA-X in the periplasm and substantiates the hypothesis that the synergistic anion plays a structural and functional role in FbpA-mediated transport of iron across the periplasm and into the cytosol.

FbpA is the single-lobed, monomeric, 34 kDa Fe^{3+} periplasmic binding protein of the Gram-negative pathogen *Neisseria gonorrheae* (*1–3*). As a member of the transferrin protein superfamily, FbpA binds iron with the assistance of a synergistic anion to form an extremely stable ternary complex with a log K'_{eff} for Fe^{3+} in the range of 16−18, depending on the identity of the synergistic anion (*4–6*). The crystal structure of holo-(iron-loaded) FbpA shows the synergistic anion as phosphate directly bound to the Fe^{3+} and protein anion binding site, similar to that found for the holo form of the homologous protein from *Haemophilus influenzae*, HitA (*3, 7*). In recent investigations, we and others have established that high-affinity Fe^{3+} sequestration can be accomplished, *in vitro*, with a variety of anionic, oxygen donor iron ligands including arsenate, citrate, nitrilotriacetate, oxalate, phosphate, pyrophosphate, and sulfate (*4–6, 8–10*). Our *in vitro* findings also revealed that anion exchange modulates the affinity of FbpA for Fe^{3+} as well as the Fe^{3+}/Fe^{2+} redox potential for sequestered iron and that this may play a role in a reductive mechanism for iron release from FbpA at the inner cell membrane.

The existence and identity of the synergistic anion in FeFbpA-X species *in vivo* are an important question. While there are crystallographic data that show phosphate as the synergistic anion (*3, 7*), the biological relevance of the crystallographic results requires further investigation. The authors of the crystallographic study noted that the presence of phosphate may have been an artifact of the purification or crystallization process. Moreover, in light of our recent thermodynamic results showing that phosphate does indeed impart the greatest thermodynamic stability to holo-FbpA (*4, 6, 9*), it is even more possible that the crystallographic studies showing phosphate in the anion site could be artifactual due to the presence of even trace amounts of phosphate at the purification/crystallization conditions. For example, given the enhanced stability of holo-FbpA complexes in the presence of phosphate, it is possible that even small amounts of phosphate present in the crystallization/purification process could significantly favor the phosphate−FbpA complex during crystallization, as the ease of obtaining protein crystals is often tied to protein stability (e.g., folded proteins with more conformationally restricted structures are often easier to crystallize than proteins with less conformationally restricted structures). The necessity for a synergistic anion to the *in vivo* function of ferric binding protein has also been questioned, based on a recent crystallographic study

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(*11*). Furthermore, the anion population of the periplasm is complex, and it has been noted to change with the host environment (*6, 12*). As we have shown that the anion in the ternary assembly FeFbpA-X is labile and can modulate a number of biologically relevant physicochemical properties of FbpA *in vitro* (*4–6, 9, 10, 13*), it is particularly important to determine the identity and distribution of the synergistic anion, X, in FeFbpA-X species found in unpurified bacterial periplasmic preparations.

Biophysical measurements on individual proteins and protein–ligand complexes in multicomponent biological mixtures such as periplasmic preparations are difficult to perform using traditional spectroscopy-based techniques. Optical spectroscopies such as circular dichroism and fluorescence measure the bulk properties of a sample, and they are not amenable to the analysis of unpurified protein samples that contain multiple proteins, DNA, and/or small molecules. In contrast to spectroscopy-based biophysical methods, mass spectrometry-based methods are particularly well-suited for the analysis of proteins and protein–ligand complexes in multicomponent biological mixtures. Here we utilize a mass spectrometry- and H/D exchange-based technique, termed SUPREX[1] (stability of unpurified proteins from rates of H/D exchange and MALDI mass spectrometry) to investigate the ligand binding properties of FbpA in unpurified bacterial periplasmic preparations.

We have previously reported on the application of SUPREX to the thermodynamic analysis of a wide variety of different protein and protein–ligand complexes (*9, 14–19*). This work included the application of SUPREX to synergistic anion binding in purified samples of a ferric binding protein, FbpA (*9*). Here, we extend these studies to the analysis of unpurified FbpA samples from periplasmic preparations. The ability to study proteins, like FbpA, in complex biological mixtures can be critically important for understanding what is truly occurring in complex biologic samples such as bacterial cell fractions. For example, the presence of other proteins, DNA, or small molecules *in vivo* may alter a protein's structure and/or function. Additionally, purification may influence the protein's apparent physical properties. This is an issue for synergistic anion binding studies involving ferric binding protein, which is the subject of this *ex vivo* study.

EXPERIMENTAL PROCEDURES

Reagents. Deuterium oxide (D_2O; 99.9% atom D), deuterium chloride (20 wt % in D_2O, 99.5% atom D), and sodium deuteroxide (40 wt % in D_2O, 99.9% atom D) were purchased from Sigma Aldrich. Guanidinium chloride (GdmCl) was purchased from Mallinckrodt (ACS grade). Deuterated guanidinium hydrochloride was prepared by repeated dissolution and lyophilization of fully protonated guanidine hydrochloride in D_2O until the calculated deuterium content was greater than 99%. Sinapinic acid (SA) was purchased from Sigma Aldrich. Trifluoroacetic acid (TFA), methanol (MeOH), acetonitrile (MeCN), 2-(4-morpholino)-ethanesulfonic acid (MES), and potassium chloride were purchased from Fisher. Myoglobin (horse skeletal muscle) and soybean trypsin inhibitor were obtained from Sigma Aldrich. *N*,*N*′-Diethylpiperazine (DEPP)[2] was purchased from GFS Chemicals. Iron(III) citrate trihydrate was obtained from Avocado Research Chemicals. Potassium phosphate, monobasic, was purchased from United States Biochemical Corp.

General Methods and Instrumentation. MALDI mass spectra were acquired on either a Voyager DE biospectrometry workstation (Applied Biosystems) or a Bruker Ultraflex II mass spectrometer. Spectra were collected in the linear mode using nitrogen (337 nm) and Nd:YAG lasers. SA was the matrix used in all of the experiments. Positive ion mass spectra were collected manually using the following parameters: 25 kV acceleration voltage, 23.5 kV grid voltage, 75 V guide wire voltage, and 225 ns delay time. Each mass spectrum was the sum of 32 laser shots. Raw MALDI spectra were either processed with a Microsoft Excel macro that performed the following operations, a 19-point floating average smoothing of the data, a 2-point mass calibration using the protein's ion signals of the internal calibrants, and a center of mass determination for the protein's $[M + H]^+$ peak, or calibrated using the calibration software supplied by the instrument manufacturer.

GdmCl concentrations were determined with a Bausch & Lomb refractometer as previously described (*20*). pH measurements were performed with a Jenco 6072 pH meter equipped with a Futura calomel electrode from Beckmann Instruments. To correct for isotope effects, the measured pH of each D_2O solution was converted to pD by adding 0.4 to the measured pH value (*21*).

Isolation and Purification of FbpA. Purified apoFbpA, cloned from *N. gonorrheae* and expressed in *Escherichia coli*, was prepared as previously reported (*5, 22*). Briefly, *E. coli* cells overexpressing FbpA were subjected to extraction using cetyltrimethylammonium bromide in 0.1 M Tris base, pH 8.0, followed by binding to a carboxymethyl-Sepharose column. Iron-loaded FbpA was collected from the column, or while bound to the column, iron-loaded FbpA was converted to apoFbpA by the addition of 10 volumes of 0.1 M Tris base, pH 8.0, containing 1 mM citrate. Elution of apoFbpA was accomplished using a NaCl gradient in buffers rendered iron-free by exposure to Chelex 100 (Bio-Rad) prior to their addition to the column. Fractions were collected in acid-washed glassware, extensively dialyzed against Chelex 100-treated 0.05 M MES buffer containing 0.2 M NaCl, and concentrated using an Amicon filtration unit.

Preparation of FeFbpA-X Complexes. Purified samples of FeFbpA-X (X = PO_4^{3-}, citrate) were prepared as described previously (*4*). Briefly, for FeFbpA-Cit, 2 equiv of ferric citrate was added to 1 equiv of apoFbpA until saturation was achieved as measured spectrophotometrically. The FeFbpA-Cit sample was dialyzed overnight at 4 °C against three 200 mL volumes of 50 mM MES and 200 mM NaCl, pH 6.5. For FeFbpA-PO_4, the native holoprotein was dialyzed overnight at 4 °C against three 200 mL volumes of 50 mM MES and 200 mM NaCl, pH 6.5. The sample was then dialyzed for 3 h at 4 °C against 200 mL of 50 mM MES, 200 mM KCl, and 3 mM KH_2PO_4, pH 6.5. Excess iron in the form of insoluble $Fe(OH)_3$ was removed using a syringe-driven 0.20 μm filter unit (Corning). The final concentration and saturation of the iron-loaded protein stock solutions were

[1] Abbreviations: SUPREX, stability of unpurified proteins from rates of hydrogen/deuterium exchange; MALDI, matrix-assisted laser desorption/ionization; GdmCl, guanidinium chloride; SA, sinapinic acid; TFA, trifluoroacetic acid; MeOH, methanol; MeCN, acetonitrile; MES, 2-(4-morpholino)ethanesulfonic acid; DEPP, diethylpiperazine.

determined using the absorbance value at 280 nm ($\epsilon = 51100$ M^{-1} cm^{-1}) for holo-FbpA, at 474 nm ($\epsilon = 1770$ M^{-1} cm^{-1}) for FeFbpA-Cit, and at 481 nm ($\epsilon = 2430$ M^{-1} cm^{-1}) for FeFbpA-PO_4 (*4*). All spectra were obtained using a Cary 100 Bio UV/visible spectrophotometer (Varian) at 25.0 ± 0.1 °C. The final concentration of iron-loaded protein in the above stock solutions was diluted to 300 μM before use in SUPREX experiments.

The binary mixtures of FeFbpA-Cit and FeFbpA-PO_4 were prepared by combining appropriate amounts of the purified FeFbpA-Cit and FeFbpA-PO_4 samples to yield the desired 1:1, 10:1, and 1:10 ratios of FeFbpA-Cit and FeFbpA-PO_4 species. In all of the binary mixtures the final concentration of FbpA was 300 μM.

E. coli and N. gonorrheae Propagation. E. coli expressing recombinant FbpA (*23*) was propagated in Luria–Bertani (LB) broth containing ampicillin. The composition of LB broth is comprised of yeast extract, tryptone, and sodium chloride. For *N. gonorrheae*, a clinical isolate (i.e., strain FA19) was propagated on gonococcal (GC) broth agar supplemented with 25 μM desferral to reduce the iron content in the medium and to induce FbpA expression (*22, 24*). The composition of GC agar includes proteose peptone, glucose, sodium chloride, and phosphate. To this medium is added isoviataleX, a microbiological supplement containing amino acids and vitamins (especially rich in thiol-containing cysteine and glutathione) that has been engineered to support the growth of fastidious organisms.

Release of Periplasmic Extract by Chloroform. Periplasmic proteins were collected from *N. gonorrheae* and from *E. coli* as described previously (*25*). The method of periplasmic extraction was the chloroform treatment described by Judd and Porcella (*26*). Briefly, 2 mL bacterial cultures were grown to saturation overnight. Cells were collected by centrifugation for 10 min at 1100*g*. The supernatant was decanted and the cell pellet suspended in the culture tubes by brief vortexing in residual medium, and 20 μL of $CHCl_3$ was added. The tubes were then vortexed briefly and stored at room temperature for 15 min; then 0.2 mL of 10 mM Tris-HCl, pH 8.0, was added. The cells were separated by centrifugation at 6000*g* for 20 min after which the supernatant containing the periplasmic proteins was withdrawn.

SUPREX Sample Preparation and Data Collection. SUPREX data were collected and analyzed according to previously established protocols (*16*). SUPREX analyses were initiated by the 10-fold dilution of 1 μL aliquots of the appropriate FeFbpA-X stock solution into a series of H/D exchange buffers. The deuterated exchange buffers contained 50 mM DEPP, 200 mM KCl (pD = 4.5), and concentrations of deuterated GdmCl that ranged from 0 to 6 M. After dilution of the FeFbpA-X complex into the series of exchange buffers, the resulting solutions were incubated at room temperature (293 K) and allowed to exchange for a specified amount of time. After a specific exchange time, a 1 μL aliquot of each protein containing exchange buffer was combined with 9 μL of an ice-cold, MALDI matrix solution which included internal standards. The matrix solution consisted of a saturated solution of SA containing 45% MeCN and 0.1% TFA. The low temperature and low pH essentially quenched the H/D exchange reaction. Subsequently, 1 μL of the quenched solution was spotted on a stainless steel MALDI target and allowed to air-dry.

In SUPREX analyses of the FeFbpA-X species in the *E. coli* and *N. gonorrheae* periplasmic extracts, the H/D exchange reactions were quenched with the addition of TFA (final concentration 0.3% v/v), and the protein samples in each H/D exchange reaction were submitted to a concentration and desalting step using C4 ZipTips (Millipore, Inc.) prior to MALDI mass spectral analysis. The concentration and desalting step was performed as previously described (*27*). Because the concentration and desalting step was performed *after* the H/D exchange reaction was quenched, the measured biophysical properties of the protein (as defined by the H/D exchange behavior) are those of the protein in the periplasmic extracts.

Ten replicate spectra were collected to determine an average molecular mass of the deuterated protein at each denaturant concentration. The standard deviations of our average molecular mass measurements were typically ~20−30 Da. ΔMass values were determined by subtracting the molecular mass of the fully protonated FbpA protein (i.e., 33598 Da for *E. coli* FbpA and 33642 Da for gonococcal FbpA) from the average molecular mass of the protein ascertained in our MALDI analyses. These values were used to generate FbpA SUPREX curves: plots of ΔMass versus [GdmCl]. The plots were fit to a four-parameter sigmoidal equation using Sigma Plot to extract a $C^{1/2}_{SUPREX}$ value (the denaturant concentration at the transition midpoint). The $C^{1/2}_{SUPREX}$ was plotted against exchange time according to eq 1 as previously described (*16*).

$$-\Delta G_f^\circ = mC^{1/2}_{SUPREX} + RT \ln \frac{\frac{\langle k_{int} \rangle t}{0.693} - 1}{\frac{n^n}{2^{n-1}}[P]^{n-1}} \quad (1)$$

In eq 1, m is defined as $\delta\Delta G_f^\circ/\delta$[denaturant] in kcal mol^{-1} M^{-1}, R is the gas constant in kcal mol^{-1} K^{-1}, T is the temperature in kelvin, $\langle k_{int} \rangle$ is the average intrinsic rate of a backbone amide proton in s^{-1}, t is the H/D exchange time in seconds, and n is the multimeric state of the protein (n = 1 for FbpA). A linear least-squares analysis was used to determine the slope and y-intercept of the plots of $-RT \ln[(\langle k_{int} \rangle t/0.693) - 1]$ versus $C^{1/2}_{SUPREX}$ for each protein system. These values correspond to the protein folding m value and folding free energy value, ΔG_f°, respectively. Since all experiments were performed at room temperature, T was set at 293 K for all calculations involving eq 1. The $\langle k_{int} \rangle$ value used in our calculations was 29.4 h^{-1}. This value was determined using the SPHERE program and the entire primary amino acid sequence of FbpA (*28, 29*).

RESULTS AND DISCUSSION

SUPREX Analysis of Purified FbpA Samples. The periplasm is known to have a diverse anion composition that is variable and largely defined by the environment (*12*). This results from the porin-laced outer membrane of Gram-negative bacteria, through which ions of less than 600 Da diffuse. The periplasm represents the intervening space between the porous outer and the relatively impervious cytoplasmic membrane. This space is not insignificant, comprising about 30% of the bacterial total volume. It is this space through which low molecular mass anionic

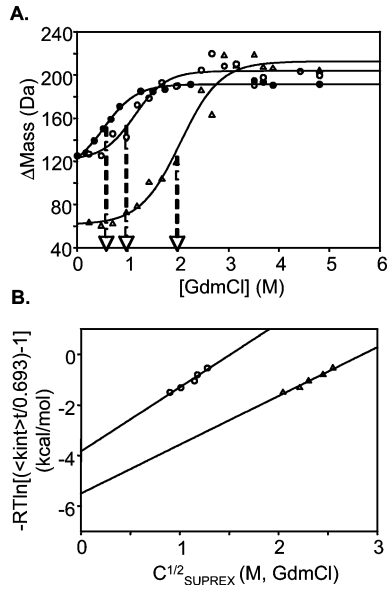

FIGURE 1: (A) SUPREX data obtained for purified samples of apoFbpA (closed circles), FeFbpA-Cit (open circles), and FeFbpA-PO_4 (open triangles). Each curve was generated using an H/D exchange time of 20 min. The line represents the best fit of the data to a four-parameter sigmoidal equation using Sigma Plot. (B) The $-RT\ \ln[(\langle k_{int}\rangle t/0.693) - 1]$ versus $C^{1/2}_{SUPREX}$ plots obtained for purified FeFbpA-Cit (open circles) and purified FeFbpA-PO_4 (open triangles). Correlation coefficients greater than 0.9600 were obtained in the linear least-squares analyses performed on each data set.

nutrients must transiently pass from the environment to the cytosol. Proteins such as FbpA and other high molecular mass molecules such as membrane-derived oligosaccharides, trapped within this space, facilitate the accumulation and uptake of nutrients. Commensal and pathogenic species of the genus *Neisseria* are obligate human parasites and, as such, are restricted to growth in biological fluids. Citrate and phosphate concentrations in human serum are approximately 0.1 and 1.0 mM, respectively, and therefore are expected to be normal constituents of the low molecular mass composition of the bacterial periplasm (*30, 31*). Consequently, phosphate and citrate are two main anions suspected to be bound by FbpA, and therefore purified samples of FeFbpA-PO_4, FeFbpA-Cit, and apoFbpA were each subjected to a SUPREX analysis as part of the control experiments in this work. These control experiments involved the acquisition of a series of different SUPREX curves using different H/D exchange times. Shown in Figure 1A are typical SUPREX curves obtained for apoFbpA, FeFbpA-PO_4, and FeFbpA-Cit using an H/D exchange time of 20 min. Summarized in Table 1 are the $C^{1/2}_{SUPREX}$ values obtained from the SUPREX curves in Figure 1 as well as those obtained at the other H/D exchange times used in this study. Ultimately, the $C^{1/2}_{SUPREX}$ values obtained at the different H/D exchange times in Table 1 were used to generate plots of $-RT\ \ln[(\langle k_{int}\rangle t/0.693) - 1]$ versus the observed $C^{1/2}_{SUPREX}$ values for FeFbpA-PO_4 and FeFbpA-Cit (Figure 1B). The lines in each plot represent the best linear fit of the data. According to eq 1 (Experimental Procedures section), the slope and *y*-intercept of each line were taken as the *m* value and ΔG_f, respectively, of FeFbpA-PO_4 and FeFbpA-Cit. These values are summarized in Table 2.

SUPREX curves of apoFbpA did not move with exchange time (data not shown), indicating there was no correlation between $-RT\ \ln[(\langle k_{int}\rangle t/0.693) - 1]$ and $C^{1/2}_{SUPREX}$. As a result, a $-RT\ \ln[(\langle k_{int}\rangle t/0.693) - 1]$ versus $C^{1/2}_{SUPREX}$ value plot is not shown for apoFbpA. This lack of correlation is likely due to the absence of a dominant global unfolding event in the protein's GdmCl-induced equilibrium unfolding/refolding reaction. In the absence of such global unfolding/refolding events, the $C^{1/2}_{SUPREX}$ value is not expected to change with H/D exchange times. This is because only the H/D exchange rates of globally protected amide protons have a strong denaturant dependence. The addition of iron to FbpA appears to make the protein's global unfolding reaction dominant. When global unfolding events provide the dominant mechanism of H/D exchange, the $C^{1/2}_{SUPREX}$ value of a protein is expected to move with H/D exchange time as such global unfolding events are highly dependent on the denaturant concentration.

We have previously reported on the SUPREX behavior of iron-loaded FbpA in the presence of different synergistic anions. While iron-loaded protein was a so-called "nonideal" protein in the SUPREX experiment because of its non-two-

Table 1: Transition Midpoints ($C^{1/2}_{SUPREX}$ Values) Obtained for FbpA Samples

	exchange time (min)	$C^{1/2}_{SUPREX}$,[a] [GdmCl] (M)
ApoFbpA (purified)	20	0.6
FeFbpA-PO_4 (purified)	5	2.5
	7	2.4
	10	2.3
	15	2.2
	20	2.0
FeFbpA-citrate (purified)	5	1.3
	7	1.2
	10	1.1
	15	1.0
	20	0.90
FeFbpA-PO_4/FeFbpA-citrate (1:1 binary mixture)	7	2.4 and 1.2
	10	2.3 and 1.1
	15	2.2 and 1.0
	20	2.1 and 0.9
FeFbpA-PO_4/FeFbpA-citrate (1:10 binary mixture)	20	1.8 and 0.4
FeFbpA-PO_4/FeFbpA-citrate (10:1 binary mixture)	20	1.9 and 0.8
FbpA in *E. coli* periplasmic preparation	20	2.1 and 0.6
FbpA in gonococcal periplasmic preparation	20	2.0

[a] Reported values were obtained from one SUPREX curve. Errors from the SUPREX curve fits were typically ~0.1 M.

Table 2: Summary of SUPREX-Derived Thermodynamic Parameters for FeFbpA-PO_4 and FeFbpA-Citrate

	m[a] (kcal mol^{-1} M^{-1})	ΔG_f[a] (kcal mol^{-1})	$\Delta\Delta G_f$ (kcal mol^{-1})
FeFbpA-PO_4 (purified)	1.9 ± 0.2	−5.5 ± 0.4	0[b]
FeFbpA-citrate (purified)	2.5 ± 0.3	−3.8 ± 0.3	1.7 ± 0.5[b]
FeFbp-PO_4 (1:1 binary mixture)	2.1 ± 0.2	−6.0 ± 0.3	0[c]
FeFbp-citrate (1:1 binary mixture)	2.5 ± 0.3	−3.8 ± 0.4	2.2 ± 0.5[c]

[a] Values obtained from the linear least-squares analysis of the data in Figures 1B and 2B. [b] Values obtained from the ΔG_f data in column 3 and are relative to the purified FeFbpA-PO_4. [c] Values obtained from the ΔG_f data in column 3 and are relative to FeFbpA-PO_4 in the binary mixture.

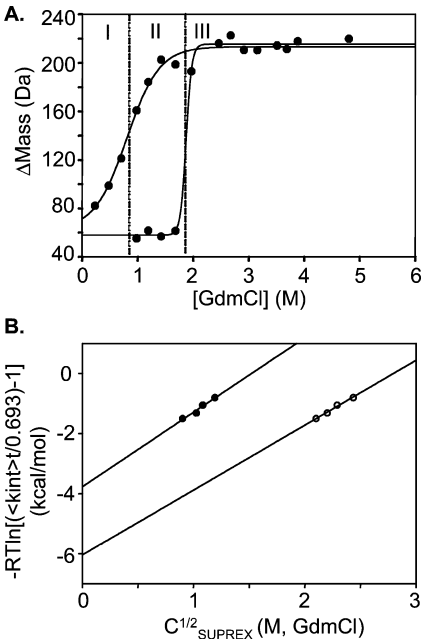

FIGURE 2: (A) SUPREX data obtained for a mock 1:1 mixture of FeFbpA-PO_4 and FeFbpA-Cit using an H/D exchange time of 20 min. The lines represent the best fit of the data to a four-parameter sigmoidal equation using Sigma Plot. (B) The $-RT\ln[(\langle k_{int}\rangle t/0.693) - 1]$ versus $C^{1/2}_{SUPREX}$ plots obtained for FeFbpA-Cit (closed circles) and FeFbp-PO_4 (open circles) in the mock mixtures. Correlation coefficients greater than 0.96 were obtained in the linear least-squares analyses performed on each data set.

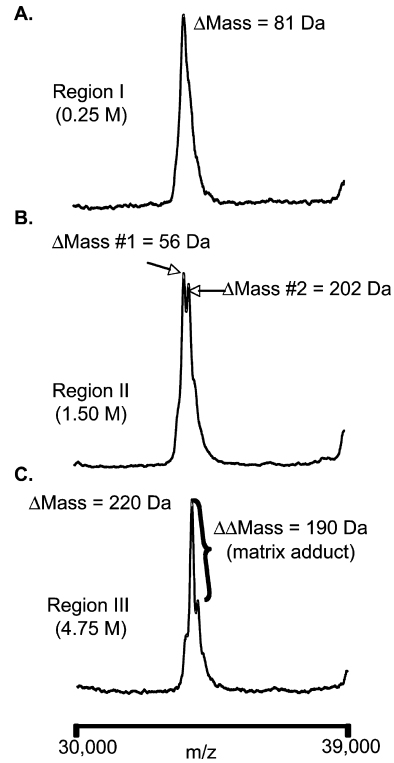

FIGURE 3: Representative MALDI-TOF mass spectra used to create the FeFbpA-PO_4 and FeFbpA-Cit SUPREX curves shown in Figure 2A. The spectra shown in (A), (B), and (C) are typical of those used to generate the data in regions I, II, and III, respectively, of the SUPREX curves shown in Figure 2A.

state folding behavior, it was still possible to determine ΔG_f and m values for the holoprotein by SUPREX (*9*). Significantly, the ΔG_f values could be used to determine accurate $\Delta\Delta G_f$ values associated with synergistic anion binding (*9*). We note that the $\Delta\Delta G_f$ values calculated for the FeFbpA-PO_4 and FeFbpA-Cit complexes in the purified and unpurified samples in this work (Table 2) are within experimental error of those values we previously determined on purified FbpA samples, 0 and 1.45 kcal/mol, respectively, albeit under slightly different solution conditions (*9*).

SUPREX Analysis of Binary Mixtures of FeFbpA-PO_4 and FeFbpA-Cit. Control experiments involving binary mixtures of purified proteins demonstrated the validity of the SUPREX technique in the identification of closely related protein species. Initially, a 1:1 binary mixture of FeFbpA-PO_4 and FeFbpA-Cit was subjected to a SUPREX analysis that included the acquisition of a series of different SUPREX curves using a range of H/D exchange times (Table 1). Shown in Figure 2A are typical SUPREX curves obtained in the analysis of the 1:1 mixture of FeFbpA-PO_4 and FeFbpA-Cit using an H/D exchange time of 20 min. Summarized in Table 1 are the $C^{1/2}_{SUPREX}$ values extracted from all the curves collected on the 1:1 mixture.

Shown in Figure 3 are MALDI mass spectra representative of the three major regions (i.e., I, II, and III) of the SUPREX curves shown in Figure 2A. Spectra collected in region I (Figure 3A) revealed a single ion signal for FbpA with a relatively low ΔMass value of ~80–120 Da. Spectra collected in region II (Figure 3B) revealed two ion signals for FbpA, one with a relatively low ΔMass value of ~60 Da and one with a relatively high ΔMass value of ~180−200 Da. Spectra collected in region III (see Figure 3C) revealed one major ion signal for FbpA with a relatively high ΔMass value of ~220 Da. On the basis of the SUPREX curves generated in Figure 1A, the ΔMass values expected for FeFbpA-PO_4 and FeFbpA-Cit species are similar at denaturant concentrations greater than 2.5 M (i.e., ~200 Da), whereas the ΔMass values expected for these two species are unique at denaturant concentrations less than 2.5 M. A difference in ΔMass values as great as ~80−100 Da is expected at denaturant concentrations between 1 and 2 M, and a smaller difference in ΔMass values (i.e., ~60 Da) is expected at denaturant concentrations less than 1 M.

The resolution of our MALDI-TOF mass spectrometer only enabled us to resolve the greatest difference in ΔMass values (i.e., the 80−100 Da difference at denaturant concentrations between 1 and 2 M). This is why the pretransition baseline of the FeFbpA-PO_4 curve generated in Figure 2 begins at 1 M. The resolving power of our MALDI-TOF mass spectrometer was not great enough to resolve the ~60 Da difference in ΔMass values expected at low denaturant concentrations. However, we note that the major FbpA ion signal observed in the mass spectra we collected at low denaturant concentrations (Figure 3A) is most likely a combination of two poorly resolved signals, one from FeFbpA-PO_4 species and one from FeFbpA-Cit species. Evidence for this comes from the increased widths of these ion signals that were consistently about twice as great as those in spectra used to generate the ΔMass values in region III, for example.

The $C^{1/2}_{SUPREX}$ values obtained at the different H/D exchange times in our SUPREX experiments on the 1:1 mixture of FeFbpA-PO_4 and FeFbpA-Cit were used to generate plots of $-RT\ln[(\langle k_{int}\rangle t/0.693) - 1]$ versus the observed $C^{1/2}_{SUPREX}$ value (Figure 2B). The lines in each plot

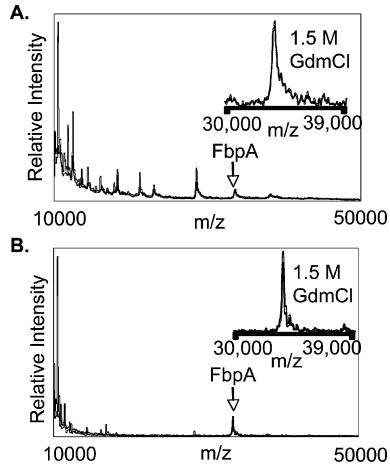

FIGURE 4: Representative MALDI-TOF mass spectra obtained in the SUPREX analysis of the unpurified FbpA samples. Typical spectra used to generate the 1.5 M data points in the FbpA SUPREX curves generated from the *E. coli* and gonococcal periplasmic extracts are shown in (A) and (B), respectively.

represent the best fit of the data to a straight line. According to eq 1, the slope and *y*-intercept of each line were taken as the *m* value and ΔG_f value, respectively, for each complex. The values obtained from the data in Figure 2B are summarized in Table 1. The ΔG_f and *m* values obtained on the FeFbpA-PO_4 and FeFbpA-Cit species in the mock mixture (Table 2) were within experimental error of the same values obtained on the species in the purified samples (Table 2).

SUPREX analyses were also conducted on 1:10 and 10:1 binary mixtures of FeFbpA-PO_4 and FeFbpA-Cit species. SUPREX curves like those shown in Figure 3 for the 1:1 mixture were recorded in each case. That is, two FbpA SUPREX curves were resolved in each case, and the $C^{1/2}_{SUPREX}$ values of the resulting curves were similar to those recorded for the 1:1 mixture. The results of these control experiments suggest that SUPREX can differentiate populations of FeFbpA-PO_4 and FeFbpA-Cit in the same sample, and they establish that a wide range of relative populations can be detected. However, we note that it was not possible to quantify the relative ratio of FeFbpA-PO_4 and FeFbpA-Cit species in the samples; only their presence (or absence) could be confirmed. The limited resolution of our mass spectrometer made it difficult to accurately integrate the peak areas of the FbpA ion signals in our MALDI mass spectra.

SUPREX Analysis of FbpA in a Preparation of E. coli Periplasmic Fluid. FbpA overexpressed in an *E. coli* periplasmic preparation was subjected to a SUPREX analysis using an H/D exchange time of 20 min. Shown in Figure 4A and 5 are representative MALDI mass spectra from the three major regions (i.e., regions I, II, and III) of the *E. coli* periplasmic extract SUPREX curves shown in Figure 6A. The ion signal(s) from FbpA was (were) well resolved from other protein ion signals detected in our MALDI analyses of the *E. coli* periplasmic fluid (Figure 4A). One major FbpA ion signal was detected in region I (see Figure 5A); two FbpA ion signals, though poorly resolved, were observed in region II (Figures 4A and 5B); and one FbpA ion signal was observed in region III (Figure 5C). The mass spectral results in regions I and III are indicative of one population of FbpA, while the mass spectral results of region II are indicative of two populations of FbpA.

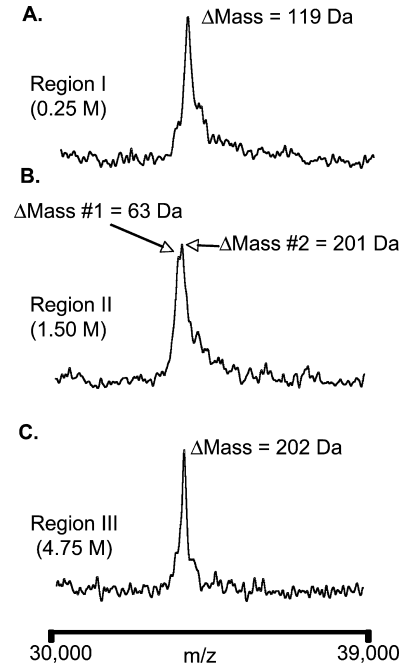

FIGURE 5: Representative MALDI-TOF mass spectra used to create the two SUPREX curves generated from the *E. coli* periplasmic fluid (Figure 6A). The spectra shown in (A), (B), and (C) are typical of those used to generate the data in regions I, II, and III, respectively, of the SUPREX curve (Figure 6A).

The one FbpA ion signal observed in Figure 5A (i.e., region I) yields a ΔMass value of 119 Da. This value is consistent with that expected for apoFbpA or FeFbpA-Cit species (see Figure 1A). The two ion signals in Figure 5B (i.e., region II) yield ΔMass values of 63 and 201 Da. These values are only consistent with those expected for the FeFbpA-PO_4 and apoFbpA species, respectively. It is noteworthy that the low ΔMass value of 63 is uniquely indicative of the FeFbpA-PO_4 species (i.e., it is not consistent with values expected for either the apoFbpA or FeFbpA-Cit species). The one ion signal in Figure 5C (i.e., region III) yields a ΔMass value that is consistent with that expected for apoFbpA, FeFbpA-Cit, or FeFbpA-PO_4 species.

In total, three separate SUPREX analyses were conducted on the FbpA from the preparation of *E. coli* periplasmic fluid. Results similar to those shown in Figure 5 were generated in each of the three replicate experiments. The average $C^{1/2}_{SUPREX}$ values for the two SUPREX curves generated in each case were 0.6 ± 0.1 and 2.1 ± 0.1 M GdmCl. These $C^{1/2}_{SUPREX}$ values are within experimental error of the $C^{1/2}_{SUPREX}$ values of apoFbpA and FeFbpA-PO_4 individually and are different than that determined for an FeFbpA-Cit species (see Table 1 and control experiments above).

The resolution and mass accuracy of our mass spectrometer were not sufficient to unambiguously identify the FbpA ion signal based solely on its *m/z* value. However, our assignment of the FbpA ion signals in the MALDI mass spectra shown in Figures 4A and 5 is based on five pieces of experimental evidence: (1) the molecular mass determination of the *E. coli* FbpA protein (in protonated solvents), 33598 Da, was within experimental error (<0.1%) of that previously reported for the molecular mass of this construct (i.e., 33606 Da) in an electrospray ionization mass spectral analysis of the purified protein (*32*); (2) FbpA is a major protein in this size range, as judged by the results of a gel-

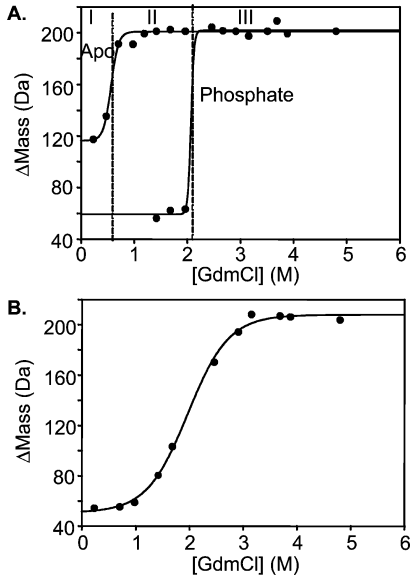

FIGURE 6: (A) SUPREX data obtained on unpurified FbpA from the *E. coli* periplasmic extract. (B) SUPREX data obtained on unpurified FbpA from the gonococcal periplasmic extract. In each case, the data were generated using an H/D exchange time of 20 min, and the lines represent the best fit of each data set to a four-parameter sigmoidal equation using Sigma Plot.

based separation of the proteins in the sample (see Figure S1 in Supporting Information); (3) no ion signals at *m/z* 33598 ± 30 were detected in the MALDI-TOF analysis of a periplasmic extract from an *E. coli* strain not expressing FbpA (see Figure S2 in Supporting Information); (4) no ion signals at *m/z* 33598 ± 30 were detected in the MALDI-TOF analysis of a periplasmic extract from an *E. coli* strain overexpressing FbpA after the FbpA was selectively removed by immunoprecipation (see Figure S3 in Supporting Information); and (5) the ion signals assigned to FbpA were used in the SUPREX experiment to generate SUPREX curves essentially identical to those generated in our control experiments on purified FbpA samples.

Our SUPREX analysis of the *E. coli* periplasmic fluid indicates that two distinct populations of FbpA are present in the periplasmic preparation samples including an apoFbpA population and a FeFbpA-PO_4 population. The presence of the apoFbpA species in the *E. coli* periplasmic preparations is not surprising because the bacteria were iron-starved to maximize FbpA expression levels. Of particular significance is the observation that the holoprotein population contains phosphate as the synergistic anion.

The H/D exchange reactions in our SUPREX experiments were performed directly on the periplasmic preparations (i.e., FbpA was not subjected to purification prior to H/D exchange). A concentration and desalting step utilizing reversed-phase chromatographic media was needed in our SUPREX analyses of FbpA from the *E. coli* periplasmic preparations (Experimental Procedures) in order to observe the FbpA ion signal in our MALDI readout. However, it is important to note that the concentration and desalting step was performed under conditions that preserved the deuterated state of FbpA. Thus, the SUPREX-derived thermodynamic parameters measured in this work (i.e., the $C^{1/2}_{SUPREX}$, *m*, and ΔG_f values in Table 2) are truly reflective of the unpurified FbpA species in the *E. coli* periplasmic preparation, and they indicate that phosphate is the synergistic anion *in vivo*. This observation is consistent with crystallographic studies on purified FbpA samples, which also found phosphate to be the synergistic anion (*3, 7*).

SUPREX Analysis of FbpA in a Preparation of Periplasmic Fluid from N. gonorrheae. A *N. gonorrheae* periplasmic preparation containing FbpA at endogenous levels was subjected to a SUPREX analysis using an H/D exchange time of 20 min. Unlike in the case of the SUPREX analysis of FbpA in the *E. coli* preparations, one FbpA ion signal, like that shown in the inset of Figure 4B, was detected at all denaturant concentrations. Thus, one SUPREX curve was generated from our SUPREX analysis of gonococcal periplasmic extract (Figure 6B). A $C^{1/2}_{SUPREX}$ value of 2.0 M GdmCl was obtained using a 20 min H/D exchange time in the SUPREX experiment. The $C^{1/2}_{SUPREX}$ value (i.e., 2.0 M) is consistent with that of a purified FeFbpA-PO_4 using the same 20 min H/D exchange time (Table 1).

Assignment of the FbpA ion signal in the MALDI mass spectra used to generate the SUPREX curve in Figure 6B was based on the same pieces of experimental evidence as noted above in the *E. coli* analysis, with the exception that a *N. gonorrheae* periplasmic preparation lacking FbpA was not analyzed because such a sample cannot be prepared. However, as in the case of the *E. coli* analysis, no ion signals at *m/z* 33639 ± 30 were detected in a MALDI-TOF analysis of the *N. gonorrheae* periplasmic preparation after the FbpA was selectively removed by immunoprecipation (see Figure S4 in Supporting Information). We also note that the expected mass of the FbpA in the *N. gonorrheae* periplasmic preparation was 33639 Da, which is based on the wild-type sequence given elsewhere (*33*). This is within experimental error of the molecular mass determined here for the gonococcal FbpA, 33642 Da.

SUPREX analysis of the gonococcal periplasmic preparation indicates both that one distinct population of FbpA is present in the sample and that phosphate is the *in vivo* anion bound to FbpA. The FbpA in the gonococcal periplasmic fluid was present at endogenous levels (i.e., there was no overexpression of the protein). Interestingly, our SUPREX results indicate that at such endogenous levels FbpA is fully saturated with iron. Although FbpA likely does not serve a storage role like that of bacterioferritin in the cytoplasm, the finding of iron-saturated FbpA in the periplasm represents a pool of available iron, which is deliverable on demand to the cytosol.

CONCLUSION

This work is the first direct determination of the identity of the *in vivo* anion, phosphate, bound to FbpA in the periplasm. It also represents the first example in which SUPREX was used to evaluate the thermodynamic properties of multiple populations of the same protein in a given protein sample. While our data cannot prove that FbpA may function *in vivo* without a synergistic anion as recently suggested from a study of mutant FbpAs (*11*), we have directly probed the periplasm and have shown that recombinant FbpA in *E. coli* and wild-type FbpA in gonococcal periplasmic fluid is bound to phosphate anion. The identification of phosphate as the *in vivo* anion bound to FbpA resolves the ambiguity of the crystallographic studies concerning the anion bound to FbpA. Saturation

of the protein in the gonococcal periplasmic preparation suggests an iron buffer as well as transport role where iron is delivered to the cytosol on demand.

SUPPORTING INFORMATION AVAILABLE

Four figures as described in the text. This material is available free of charge via the Internet at http://pubs.acs.org.

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