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Bfd a New Class of [2Fe-2S]-Protein that Functions in Bacterial Iron Homeostasis Requires a Structural Anion Binding Site

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Abstract

Mobilization of iron from bacterioferritin (BfrB) requires specific interactions with a [2Fe-2S] ferredoxin (Bfd). Blocking the BfrB:Bfd interaction results in irreversible iron accumulation in BfrB and iron deficiency in the cytosol [Eshelman K. *et al.* (2017) *Metallomics* 9, 646–659]. The only known Bfd structure, which was obtained in complex with BfrB (PDB 4E6K), indicated a new fold and suggested that the stability of Bfd is aided by an anion binding site consisting of R26, R29 and K46. We investigated the Bfd fold using site-directed mutagenesis, X-ray crystallography and biochemistry in solution. The X-ray structure, which is nearly identical to that of Bfd in the BfrB:Bfd complex, shows that the [2Fe-2S] cluster pre-organizes residues at the BfrB:Bfd interface into a structure complementary of the Bfd-binding site on BfrB. Studies in solution showed rapid loss of the [2Fe-2S] cluster at low ionic strength, but higher stability with increasing ionic strength, thus supporting a structural anion binding site. Structures of the R26E and R26E/K46Y mutants are nearly identical to that of Bfd, except for a new network of hydrogen bonds stabilizing the region encompassing the former anion binding site. The stability of the R26E and R26E/K46Y mutants, which is weakly and completely independent of solution ionic strength, respectively, corroborates that Bfd requires an anion binding site. The mutations, which caused

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ASSOCIATED CONTENT

Accession Codes. Coordinates and structure factors were deposited to the Worldwide Protein Databank (www.PDB) with the accession codes 6E6Q (truncated Bfd), 6E6R (truncated R26E Bfd) and 6E6S (truncated R26E/K46Y Bfd).

Supporting information. The Supporting Information is available free of charge on the ACS Publications website. Sequence alignment of Bfd, electronic absorption of spectra of truncated and mutant Bfd, representative spectra obtained while monitoring the stability of truncated Bfd in tris + NaCl buffers, SPR data for the association between Bfd mutants and BfrB.

Notes. The authors declare no competing financial interest.

only small changes to the strength of the BfrB:Bfd interaction and iron mobilization from BfrB, indicate that the anion binding site in Bfd serves primarily a structural role.

Graphical abstract

INTRODUCTION

Iron sulfur clusters are versatile prosthetic groups present in a wide range of proteins and enzymes ubiquitous in all kingdoms of life, where they function to support important physiological processes such as respiration, photosynthesis, DNA repair, and gene regulation.^{1, 2} The most common clusters are [2Fe-2S], [3Fe-4S] and [4Fe-4S], in which Fe ions coordinated to protein ligands are linked to one another by bridging sulfide ions.^{3, 4} The [2Fe-2S] clusters contain two iron atoms coordinated in a distorted tetrahedral geometry by two inorganic sulfurs and four protein-provided ligands; these can be four cysteine thiolates, or a combination of cysteines and histidines.⁵ On the basis of amino acid and structural alignments, as well as the position of the iron coordinating ligands in the protein sequence, [2Fe-2S]-binding proteins have been classified in four main groups (Table 1):^{5–10} the [2Fe-2S] plant-, vertebrate- and bacterial-ferredoxins, the [2Fe-2S] thioredoxin-like ferredoxins, the [2Fe-2S] Rieske proteins, and the more recently discovered [2Fe-2S] NEET proteins. Plant-type ferredoxins function mainly in photosynthesis by shuttling electrons between photosystem I and several enzymes, whereas their bacterial and vertebrate homologs function by transferring electrons to hydroxylating enzymes. The thioredoxin-like [2Fe-2S] proteins have been observed mostly in nitrogen fixing bacteria, where they are thought to function in nitrogen metabolism. The [2Fe-2S] Rieske proteins function as subunits of photosynthetic and respiratory electron transfer complexes, as well as subunits or domains in water soluble oxidases. The [2Fe-2S] NEET proteins are thought to function in cluster transfer to other proteins and as regulators of iron and ROS homeostasis.¹⁰ The plant- and vertebrate-[2Fe-2S] ferredoxins share a common β -grasp fold, where a four stranded β -sheet is covered by an α -helix (Figure 1A). The [2Fe-2S] cluster is located near the surface coordinated by 4 Cys ligands arranged in a conserved sequence motif (see Table 1).^{6, 7} The thioredoxin-like ferredoxins assume a thioredoxin-like fold with the [2Fe-2S] cluster located between two loop regions (Figure 1B), coordinated by four Cys ligands arranged in a conserved motif. The [2Fe-2S] Rieske proteins share a highly conserved fold (Figure 1C) with the [2Fe-2S] cluster held between two loops, coordinated by 2Cys and 2 His ligands arranged in the conserved sequence motif shown in Table 1. The NEET proteins fold consists of a four-stranded β -cap residing above a structured loop and a turn of helix that harbors the [2Fe-2S] cluster, where the iron ions are coordinated by 3Cys and 1His ligands arranged in a conserved motif (Figure 1D).

Early studies with *E. coli* K-12 suggested that that mobilization of iron from bacterioferritin (Bfr) requires interactions with a [2Fe-2S] ferredoxin, dubbed Bfd,^{11–13} and pointed to a new function for [2Fe-2S] proteins, namely electron transfer in bacterial iron metabolism. This issue was later investigated in the opportunistic pathogen *Pseudomonas aeruginosa*. Capitalizing from the previously reported genetic response of *P. aeruginosa* to iron limiting conditions,¹⁴ it was proposed that Bfd, together with Fpr (ferredoxin NADP reductase), promote the mobilization of Fe³⁺ stored in bacterioferritin B (BfrB), as schematically illustrated in Figure 2, where electrons from Fpr are shuttled to the Fe³⁺ core in BfrB to promote the mobilization of Fe²⁺.^{15–17} Subsequently, it was demonstrated that the [2Fe-2S] cluster of Bfd is required to deliver electrons to the heme in BfrB, which in turn relays the electrons to the Fe³⁺ mineral in the bacterioferritin core, as shown in Figure 2,^{15, 18, 19} thus corroborating the participation of a [2Fe-2S] Bfd protein in bacterial iron metabolism.

The relatively small size of Bfd, which is approximately 40 residues shorter than other structurally characterized [2Fe-2S] proteins, and the unique arrangement of its Cys ligands (Table 1), suggested that the Bfd structure may exhibit a fold distinct from those of known [2Fe-2S] proteins. In agreement with these expectations, the first example of a Bfd structure, which was obtained in complex with BfrB,¹⁹ revealed a helix-turn-helix fold (Figure 1E) different from the fold of other types of [2Fe-2S] proteins. In fact, the Bfd fold revealed in the structure of the Bfd:BfrB complex had not been previously observed in a single domain protein, although close matches had been observed in portions of domains incorporated into proteins and enzymes with diverse function belonging to the Fer2_Bfd Pfam family (PF04324).¹⁹ Analysis of the information obtained from the BfrB:Bfd complex interface showed that the key residues from each protein participating at the protein-protein interface are conserved in Bfd and Bfr proteins from a number of pathogenic bacteria, thus suggesting that the protein-protein interaction is likely conserved in several pathogenic bacteria.^{18, 19} The structural information at the protein-protein interface was also exploited to identify the hot spot residues responsible for the stability of the complex and to demonstrate that a double mutation in BfrB (L68A/E81A) is sufficient to block the BfrB:Bfd interaction and inhibit iron mobilization from BfrB.²⁰ Building from these *in vitro* findings, it was subsequently established that BfrB is the main iron storage protein in *P. aeruginosa* cells and that Bfd is essential for the mobilization of iron from BfrB in the bacterial cytosol. In the absence of Bfd, or when the BfrB:Bfd interaction is blocked, iron accumulates irreversibly in BfrB and causes iron deficiency in the *P. aeruginosa* cytosol.²¹ These findings, which strongly suggest that electron transfer from Bfd is the only mechanism in *P. aeruginosa* that enables mobilization of iron stored in BfrB, highlight a central role for Bfd in bacterial iron homeostasis.

Despite the significant role played by Bfd in bacterial iron metabolism, there are significant gaps in the structural and biochemical understanding of this novel protein. For example, analysis of the 3 Bfd molecules in the asymmetric unit of the BfrB:Bfd complex led to the hypothesis that the stability of the Bfd protein and associated [2Fe-2S] cluster is dependent on the coordination of a phosphate ion by three positively charged residues R26, R29 and K46.¹⁹ In addition, although the structure of the BfrB:Bfd complex revealed small but crucial rearrangements in the BfrB structure upon binding to Bfd, it is not known whether Bfd also undergoes structural rearrangements upon binding Bfd, in order to enable electron

transfer between its [2Fe-2S] cluster and the heme in BfrB. These questions have now been investigated, and herein we report that the structure of Bfd is nearly identical to that in the BfrB:Bfd complex, which indicates that Bfd does not undergo structural reorganization upon binding to BfrB. We also found that the stability of Bfd is strongly influenced by a conserved anion binding site. In absence of a suitable anion, or at low ionic strength, Bfd loses its [2Fe-2S] cluster and unfolds. To further demonstrate the influence of the anion binding site on the structural stability of Bfd we used site-directed mutagenesis and X-ray crystallography to prepare mutant proteins with structures nearly identical to Bfd, but where the stabilizing interactions conferred by anion binding are replaced by intramolecular hydrogen bonding and packing interactions.

EXPERIMENTAL

As has been reported previously, the [2Fe-2S] cluster of wild type Bfd has a tendency to degrade during protein purification and storage. This problem was circumvented by replacing the non-conserved Cys43 with a Ser, as the C43S mutant is more stable to purification and storage but has the same spectroscopic properties as wild type Bfd.^{15, 19} Consequently, in this report the C43S Bfd mutant will be referred to as Bfd. Truncated Bfd and Bfd mutants R26E and R26E/K46Y were constructed in the background of the gene encoding Bfd.

Site-directed mutagenesis, protein expression and purification.

Primers were synthesized by Integrated DNA Technologies, Inc. and were used in conjunction with the QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). Primers used to install the R26E mutation are 5'-GAAGGCTGCTG-CAGCTATGAAGAAGTGC GCGAAGCGACCG-3' and 5'-CGGTCGCTTCGCGCACTTCTTCAT-AGCTGCAGCAGCCTTC-3', and those for the K46Y mutation are 5'-CAAGTGCGCAAGCCTGG-CCTATCAGGTGGTGC GCGAAACC-3' and 5'-GGTTTCGCGCACCACTGATAGGCCAGGCTT-GCGCACTTG-3'. Truncated Bfd was prepared by changing the codon encoding Q57 to a stop signal (TAA) using primers 5'-GAAACCCTGAACGACCTGTAAAGCGCGCAGCCGGTGCCGG-3' and 5'-CCGGCACCGGCTGCGCGCTTTACAGGTCGTTTCAGGGTTTC-3'. Each construct was transformed into XL1-Blue competent cells (Stratagene) for amplification, and the DNA sequence corroborated by SeqWright (Houston, TX). Each of the recombinant plasmids with the correct sequence was transformed into *Escherichia coli* Arctic express RIL competent cells (Agilent Technologies, Santa Clara, CA). Bfd, truncated Bfd and the R26E and R26E/K46Y mutants were expressed and purified using a previously reported protocol for the purification of Bfd, and stored in Bfd storage buffer (50 mM potassium phosphate buffer, 150 mM NaCl, 5 mM DTT, pH 7.0).^{15, 19}

Crystallization and X-ray diffraction data collection.

Crystallization screening was carried out in Compact Jr. (Emerald Biosystems) sitting drop vapor diffusion plates at 20 °C using 0.5 µL of protein (1.2 mM (except for truncated Bfd; see below) in Bfd storage buffer) and 0.5 µL of crystallization solution equilibrated against

100 μ L of the latter. Data were collected at the Advanced Photon Source beamline 17-ID using a Dectris Pilatus 6M pixel array detector.

Truncated Bfd formed needle-shaped crystals during concentration of the protein. The sample was spun to pellet the crystallized material and the supernatant was screened for crystallization in Compact Jr. (Emerald Biosystems) sitting drop vapor diffusion plates at 20 °C using 0.5 μ L of protein and 0.5 μ L of crystallization solution equilibrated against 100 μ L of the latter. Since small crystals were probably still present in the supernatant, these effectively served as seeds for the subsequent crystallization trials. Needle-shaped crystals were observed from various conditions within an hour and grew to their maximum size in approximately 24 hours. Samples used for X-ray data collection were obtained from Crystal Screen HT (Hampton Research) condition A1 (30% (v/v) 2-methyl-2,4-pentanediol, 100 mM sodium acetate pH 4.6, 20 mM CaCl_2). Crystals were transferred to a fresh drop of crystallization solution, which served as the cryoprotectant, before flash freezing in liquid nitrogen for data collection.

Truncated R26E Bfd formed red prisms in 1–2 days from Crystal Screen HT (Hampton Research) condition G4 (1.6 M sodium citrate tribasic pH 6.5). Crystals were transferred to a fresh drop containing 80% crystallization solution and 20% glycerol before flash freezing in liquid nitrogen for data collection.

Truncated R26E/K46Y Bfd formed red prisms in 1–2 days from Crystal Screen HT (Hampton Research) condition E8 (1.5 M NaCl, 10% (v/v) ethanol). Crystals were transferred to a fresh drop containing 80% crystallization solution and 20% ethylene glycol before flash freezing in liquid nitrogen.

Structure solution and refinement.

Intensities were integrated using XDS²² via AutoPROC²³ and the Laue check and data scaling were performed with Aimless.²⁴ Structure refinement with anisotropic atomic displacement parameters except for solvent molecules for R26E Bfd and R26E/K46Y Bfd was conducted with Phenix²⁵ and model building was carried out using Coot.²⁶ Structure validation was conducted with Molprobity²⁷ and figures were prepared using the CCP4MG package.²⁸ Structure superpositions were carried out using secondary structure matching²⁹ via the CCP4³⁰ interface. Crystallographic data for the truncated Bfd structures are provided in Table 2.

Truncated Bfd: The highest probability Laue class was $2/m$ and space group $P2_1$. The Matthew's coefficient (V_m)³¹ and solvent content were estimated to be $V_m=1.9 / 35.6$ % solvent for 2 molecules of Bfd in the asymmetric unit. Structure solution was conducted by molecular replacement in $P2_1$ and $P2$ with Molrep³² using a Bfd chain from the BfrB:Bfd complex structure (PDB: 4E6K)¹⁹ as the search model. The top solution was obtained in the space group $P2_1$.

Truncated R26E and R26E/K46Y Bfd: The highest probability Laue class was mmm and space group $P2_1$. The Matthew's coefficient (V_m)³¹ and solvent content were estimated to be $V_m=1.7 / 29$ % solvent for one molecule (R26E Bfd) in the asymmetric unit and

$V_m=2.0 / 38\%$ solvent for two molecules (R26E/K46Y) in the asymmetric unit. Structure solution was conducted by molecular replacement with Phaser³³ using Bfd C43S as the search model and the top solution was obtained in the space group $P2_12_12_1$ for each structure.

Effect of ionic strength on the stability of the [2Fe-2S] cluster.

To perform experiments in Tris buffers of different ionic strength, stock protein solutions (400 μM) of each, truncated Bfd, Bfd R26E and Bfd R26E/K46Y in Bfd storage buffer (50 mM phosphate, 150 mM NaCl, 5 mM DTT pH 7.0) were diluted to give a final protein concentration of 20 μM in Tris buffer + NaCl, which consists of Tris base, 3 mM TCEP, pH 7.1 and the required amount of NaCl to obtain solutions with ionic strengths $\mu = 150\text{ mM}$ (138 mM Tris + 12 mM NaCl), $\mu = 230\text{ mM}$ (138 mM Tris + 91 mM NaCl), and $\mu = 330\text{ mM}$ (138 mM Tris + 192 mM NaCl). To carry out experiments in phosphate buffer (pH 7.1) with different ionic strengths, stock protein solutions (400 μM) in Bfd storage buffer were diluted to a final protein concentration of 20 μM in phosphate solutions with ionic strength $\mu = 150\text{ mM}$ (64 mM phosphate ($\text{HPO}_4^{2-} + \text{H}_2\text{PO}_4^-$)), $\mu = 230\text{ mM}$ (100 mM phosphate ($\text{HPO}_4^{2-} + \text{H}_2\text{PO}_4^-$)), and $\mu = 330\text{ mM}$ (140 mM phosphate ($\text{HPO}_4^{2-} + \text{H}_2\text{PO}_4^-$)). A UV-vis spectrum was obtained immediately after dilution in the appropriate Tris or phosphate buffer and subsequently every five minutes in a Cary 50/60 UV-vis spectrophotometer (Agilent Technologies). The integrity of the [2Fe-2S] cluster in Bfd was monitored as a function of time by following the intensity of the absorbance at 465 nm.

Measurement of K_d by Surface Plasmon Resonance (SPR).

SPR experiments were performed at 25 °C using a Biacore X100 instrument (GE Healthcare) using a previously reported protocol.²⁰ In short, BfrB was immobilized on CM5 sensor chips using amine coupling chemistry. The surface of the sensor was preconditioned with 50 mM NaOH, 10 mM HCl, 0.1% SDS and 0.085% H_3PO_4 and then activated with 0.1 M N-hydroxysuccinimide (NHS) and 0.4 M N-ethyl-N'-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) in water. Immobilization of BfrB on the activated sensor surface was carried out by flowing (5 $\mu\text{L}/\text{min}$, 600 sec) a 100 nM solution of BfrB in 10 mM sodium acetate buffer (pH 5.0), followed by quenching activated sites not bound to BfrB by flowing (5 $\mu\text{L}/\text{min}$, 420 sec) 1.0 M aqueous ethanolamine-HCl (pH 8.5). A cell activated by EDC/NHS and quenched by ethanolamine was used as the reference surface. To measure K_d values, a solution of Bfd (or mutant Bfd) in SPR running buffer (PBS, pH 7.4 with 1.5 mM TCEP) was simultaneously flowed (25 $\mu\text{L}/\text{min}$) over the cell with immobilized BfrB and the reference cell. A binding curve was constructed by flowing Bfd (or mutant Bfd) of concentrations specified in Figures 5 and S4). The SPR responses were corrected by subtracting the response from the reference cell with contact time 120 sec and a dissociation time 120 sec. Experiments were conducted in triplicate.

Mobilization of iron from BfrB.

These experiments were conducted in an anaerobic glove box (Coy Lab Products, MI) following a previously described method¹⁵ with some modifications. A 1 cm path-length cuvette was filled with a solution containing BfrB (0.18 μM) reconstituted with $400 \pm 20\text{ Fe}^{3+}$ ions per BfrB molecule, Bfd or mutant Bfd (0.9 μM), Fpr (7.2 μM), and 2–2'–

bipyridine (bipy), 3 mM. The set of reactions conducting to Fe^{2+} mobilization from BfrB (see Figure 2) were started by the addition of NADPH, and the iron mobilization process was monitored by following the formation of the $[\text{Fe}(\text{bipy})_3]^{2+}$ complex, which exhibits an absorption maximum at 523 nm. The percentage of mobilized iron was calculated by normalizing the intensity of the 523 nm intensity to the intensity expected upon mobilization of the 400 Fe^{3+} ions stored in BfrB.

RESULTS AND DISCUSSION

The [2Fe-2S] cluster stabilizes the Bfd fold and organizes the structure to bind BfrB with minimal structural reorganization.

The structure of the BfrB:Bfd complex showed that the helix-turn-helix fold of Bfd is distinct from the fold exhibited by the four classes of well characterized [2Fe-2S] proteins (see Figure 1). In the BfrB:Bfd complex, electron density defining Bfd could not be traced beyond residue 56, suggesting that the remaining 16 residues in the C'-terminus are disordered. As part of this study, significant effort was directed, unsuccessfully, at crystallizing the stand-alone full-length Bfd. We hypothesized that our inability to grow crystals of full-length Bfd is a consequence of disorder in the C'-terminal domain. Inspection of the amino acid sequence alignment in Figure S1 reveals that compared to its counterparts, the *P. aeruginosa* Bfd sequence has approximately 10 extra residues in its C'-terminus. This observation suggests that these C'-terminal residues may not be important to function. Consequently, we made the decision to truncate Bfd after Leu56, effectively removing the last 17 residues, which do not show electron density in the structure of Bfd in complex with BfrB. The UV-vis spectrum of truncated Bfd (Figure S2A) is identical to that of the full-length protein, which indicates that removing the last 16 residues does not influence the electronic structure of the [2Fe-2S] cluster. Solutions of truncated Bfd afforded high quality crystals which diffracted to 1.20 Å resolution. The structure of truncated Bfd revealed a helix-turn-helix fold (Figure 3) identical to that obtained from full-length Bfd in the BfrB:Bfd complex: An N'-terminal hairpin loop (L-1), flanked at each end by two short β -sheets, harbors iron ligands Cys4 and Cys6. L-1 is followed by helix α -1, which in turn is connected to a two-turn helix (α -2) by loop L-2. A third loop (L-3) follows, which contains the other two iron ligands, Cys38 and Cys41, and is connected to a relatively long helix (α -3), which ends at residue 56. Superposition of the structure of truncated Bfd with that of Bfd in complex with BfrB reveals nearly identical structures (RMSD = 0.42 Å for 56 residues, Figure 4A), indicating that the Bfd structure is not affected significantly by binding to BfrB; the [2Fe-2S] cluster in the truncated protein is structurally nearly identical to the cluster of Bfd in the BfrB:Bfd complex.

A closer view of the two loops flanking the [2Fe-2S] cluster shows that Bfd binds to BfrB with remarkably small rearrangements, which are confined to a few side chain rearrangements (Figure 4B); the largest side chain conformational change is observed in Q37, which in the BfrB:Bfd complex forms a hydrogen bond with D73 in BfrB. To underscore the significance of this observation it is important to note that amino terminal residues Met1, Tyr2 and Leu5 in Bfd are three of the four residues with the largest contribution to the buried Bfd surface in the BfrB:Bfd complex.¹⁹ In addition, Met1, Tyr2

and Leu5 contribute the majority of the stabilization energy from Bfd (hot spot residues) to the BfrB:Bfd complex by forming a clustered network of H-bonding and packing interactions with hot spot residues in BfrB (Figure 4C).²⁰ Given their positions in the Bfd sequence, Met1, Tyr2 and Leu5 could be expected to experience conformational disorder in the stand alone Bfd structure. That this does not occur is most likely a consequence of the organizing influence of the [2Fe-2S] cluster and a network of hydrogen bonds, which stabilize the N'-terminal loop (L1) and organize the conformation of ligands Cys4 and Cys6 (Figure 4B). The structural role we attribute to the [2Fe-2S] cluster in the Bfd structure is in good agreement with the fact that apo-Bfd is largely unfolded in solution.¹⁹ Accordingly, we conclude that assembly of the [2Fe-2S] cluster in Bfd serves not only to stabilize the Bfd fold but also to organize the amino terminal hot spot residues Met1, Tyr2 and Leu5 into a structure that is nearly perfectly complementary of the Bfd binding surface on BfrB. As will be shown below, the stability of the Bfd fold also depends on the presence of an anion binding domain.

The disordered C-terminal tail in Bfd does not influence its biochemical or functional properties.

Our previous studies demonstrated that the mobilization of iron stored in BfrB requires the formation of a complex with Bfd, which enables electron transfer from the [2Fe-2S] cluster to the Fe³⁺ mineral in BfrB.^{15, 18, 19, 21} In both available structures (truncated stand-alone and full-length in complex with BfrB) Bfd is missing the last 16 residues in the C'-terminal domain. Although the structure of Bfd in the BfrB:Bfd complex suggests that the C'-terminal domain of Bfd does not participate in binding BfrB, it is not known whether the missing residues have an influence on modulating its binding affinity for BfrB, or its BfrB-iron-mobilization-function. To evaluate the consequences of truncating Bfd on its ability to interact with BfrB, we measured the dissociation constant (K_d) for the complex formed between truncated Bfd and BfrB, as well as the efficacy of truncated Bfd at promoting iron mobilization from BfrB. Surface plasmon resonance was utilized to measure the K_d , in a manner similar to that used previously to measure the K_d for the interaction between full-length Bfd and BfrB.²⁰ The studies conducted with full-length Bfd revealed that BfrB has 12 Bfd-binding sites that are equivalent and independent, and that the Bfd:BfrB association at each of these sites is characterized by a dissociation constant, $K_d = 3.4 \mu\text{M}$.²⁰ Results from flowing truncated Bfd over immobilized BfrB are summarized in Figure 5A, which shows the reference and baseline-subtracted responses obtained upon flowing truncated Bfd solutions of different concentrations over immobilized BfrB. Each of the sensograms reaches a plateau, indicating steady state equilibrium. Plotting each response at steady state as a function of Bfd concentration results in the hyperbolic binding curve defined by the black circles in Figure 5B. The interpretive model to fit these data, which has been reported in detail elsewhere,²⁰ is summarized as follows: Since the 12 binding sites in BfrB are identical and non-interacting, the expression for K_d can be written as in equation 1, where n is the stoichiometry, [BfrB:Bfd] is the concentration of the protein complex at the surface, [BfrB_f] is the concentration of immobilized BfrB not bound to Bfd, [Bfd_f] is the concentration of Bfd in the flowing solution (maintained constant by the flow system), and [BfrB_t] is the total concentration of immobilized BfrB, given by [BfrB_f] + [BfrB:Bfd].

At each of the Bfd concentrations shown in Figure 5A ($[Bfd_f]$), when the system reaches steady-state equilibrium (plateau), the concentration of the BfrB:Bfd complex at the surface ($[BfrB:Bfd]$) is proportional to the magnitude of the SPR response, which is termed R_{eq} . Consequently, equation 1 can be expressed as equation 2, where R_0 is the response when one Bfd molecule binds to every immobilized 24-mer BfrB molecule. The value of R_0 can be estimated from equation 3, where MW_{bfd} is the molecular mass of Bfd, MW_{BfrB} is the molecular mass of a 24-mer BfrB, and R_{immo} is the SPR response obtained upon immobilizing BfrB. Fitting the data to equation 2 shows that the K_d for the interaction between truncated Bfd and BfrB is similar to that obtained from the interaction between full-length Bfd and BfrB (Table 3), and indicates that as expected, the disordered C'-terminal tail on Bfd exerts a minimum effect on the strength of the BfrB:Bfd interaction.

$$[BfrB:Bfd] = \frac{n[BfrB_t][Bfd_f]}{K_d + [Bfd_f]} \quad (1)$$

$$R_{eq} = \frac{nR_0[Bfd_f]}{K_d + [Bfd_f]} \quad (2)$$

$$R_0 = \frac{MW_{Bfd}}{MW_{BfrB}} \times R_{immo} \quad (3)$$

The efficiency of the truncated protein at promoting the mobilization of Fe^{3+} stored in BfrB was evaluated with the aid of an assay developed in our laboratory which utilizes BfrB containing ~400 Fe atoms/BfrB molecule.^{18, 19} Mobilization of the iron core is monitored by UV-vis spectrophotometry following the addition of excess NADPH to a solution containing BfrB, Fpr, Bfd and an excess of the Fe^{2+} chelator, bipy. NADPH initiates the electron transfer reactions shown in Figure 2, and the release of iron is followed by changes in the intensity of a band at 523 nm (A_{523}), which tracks the time-dependent formation of the $[Fe(bipy)_3]^{2+}$ complex. The black circles in Figure 5C track the A_{523} normalized to the total absorbance change expected upon mobilization of the 400 iron atoms from BfrB when the solution contains full-length Bfd. Results obtained from a similar experiment conducted with truncated Bfd (red circles in Figure 5C), show that the truncated protein promotes mobilization of iron from BfrB at a rate that is nearly identical to that observed with full length protein. Taken together, these results indicate that the disordered residues in the C'-terminal of Bfd do not significantly influence the strength of its association with BrB, or its BfrB-iron-mobilization function. On the other hand, the expression yield of truncated Bfd is more than two times that of the full-length protein, which facilitates biochemical and biophysical investigations. Consequently, the studies presented below were conducted with truncated Bfd.

The Bfd fold is stabilized by an anion binding site.

There are 3 Bfd molecules in the asymmetric unit of the BfrB:Bfd structure (chains G, H and I), with average B-factors 25.8, 64.0 and 29.0 Å², respectively.¹⁹ Chain H, which does not experience crystal contacts, exhibits the larger B-factors and is missing electron density between residues 16 and 33, which comprise the C-terminal portion of helix α -1, the entirety of loop L2 and helix α -2, and the N-terminal portion of L3.¹⁹ In comparison, the corresponding sections of chains G and I are stabilized by crystal contacts and display well-defined electron density from Met1 to Leu56. Moreover, chain G binds a phosphate anion via the side chains of R26 and R29 located in α -2 and K46 on α -3, as shown in Figure 6. We proposed that the phosphate anion mediates otherwise repulsive interactions between R26, R29 and K46 side chains and also stabilizes the conformation of the hydrophobic portion of the K46 side chain to enable it to pack against the side chain of Y25. Consequently, we hypothesized that the side chains of R26, R29 and K46 constitute an anion binding site which is an important component of a network of interactions that contribute to stabilize the short helix α -2. In absence of a suitable anion coordinated at this anion binding site, the short helix α -2 is likely to experience significant folding/unfolding excursions that also affect loop L3, where iron ligands Cys38 and Cys41 are located (see Figure 6). Hence, the structural function of the anion binding site probably influences the stability of the [2Fe-2S] cluster.

If the electrostatic interactions at the putative anion binding site contribute significantly to the stability of the Bfd fold and integrity of the [2Fe-2S] cluster, as hypothesized above, then the stability of the protein in solution should be strongly dependent on the ionic strength of the buffer. To investigate this idea, we diluted a 400 μ M stock solution of Bfd in storage buffer to prepare 20 μ M Bfd solutions in Tris + NaCl buffers with different ionic strengths (μ = 150 mM, 230 mM and 330 mM). The stability of the protein in the resultant solutions was monitored by UV-vis spectrophotometry following the intensity of the peaks characteristic of the [2Fe-2S] cluster, as shown in Figure S3. The fraction of Bfd molecules binding a [2Fe-2S] cluster (f_{folded}) was estimated from the ratio $A_{465(t)}/A_{465(0)}$, where $A_{465(t)}$ is the intensity of the 465 nm peak at any time during the experiment and $A_{465(0)}$ is the intensity immediately after dilution in the corresponding Tris buffer. Plotting f_{folded} as a function of time shows that at relatively low ionic strength (150 mM) truncated Bfd completely loses the [2Fe-2S] cluster in approximately 2 h at 25 °C (black circles in Figure 7A). If the ionic strength is increased, however, the protein becomes progressively more resilient to cluster loss, as can be seen in the red and green traces of Figure 7A. Taken together, these observations support the idea that the anion binding site on Bfd contributes to the stability of the Bfd fold by mediating electrostatic interactions between R26, R29 and K46. When similar experiments are conducted by diluting the 400 μ M Bfd stock solution to prepare 20 μ M solutions of Bfd in phosphate buffer of different ionic strengths, the protein exhibits relative higher stability than in Tris buffer and a much shallower dependency on ionic strength (Figure 7B). Given that anion recognition at the anion binding site appears to be important to maintain stability, it is instructive to consider the concentrations of the chloride and phosphate anions in the Tris and phosphate buffers. The chloride concentrations at μ = 150, 230 and 330 mM are 12, 91, and 192 mM, respectively (see Experimental), whereas the respective concentrations of phosphate ($\text{HPO}_4^{2-} + \text{H}_2\text{PO}_4^-$) are 64, 100 and 140 mM.

Hence, comparing the stability of Bfd in Tris and phosphate buffers at $\mu = 230$ mM shows that at similar concentrations of the pertinent anions (90 mM chloride or 100 mM phosphate), Bfd is significantly more stable in the presence of phosphate. These observations indicate that the anion binding site recognizes phosphate with higher affinity than chloride.

To obtain more direct evidence for a structural role of the anion binding site in Bfd we prepared charge reversal mutants (R26E, R29E and K46E), with the intention of removing the electrostatic repulsion between the R26, R29 and K46 side chains. The R26E mutant was prepared successfully, but the R29E and K46E mutants could not be overexpressed, presumably because they are unstable. Moreover, as indicated above, the hydrophobic portion of the K46 side chain packs against the side chain of Y25 and mediates interactions between α -3 and the short helix α -2 (see Figure 6). Hence, to eliminate the charge on K46 while maintaining packing interactions with Y25, we also prepared the truncated double mutant R26E/K46Y. The structures of the truncated R26E and R26E/K46Y mutants are very similar to that of Bfd (rmsd = 0.59 Å, and 0.56 Å, for 56 residues aligned, respectively, Figure 8A), as are the [2Fe-2S] clusters. Inspection of the anion binding region in the R26E/K46Y mutant strongly suggests that the mutations introduced stabilizing interactions that eliminate the need of an anion to mediate otherwise repulsive interactions between R26, R29 and K46: These are evident in chain B, where a network of hydrogen bonds and electrostatic interactions involving the side chains of E26, R29 and E30 in α -2 stabilize the short helix (Figure 8B). In addition, the Y46 side chain packs against Y25, thus preserving the function of the Y25 side chain as mediator between helices α -2 and α -3. In the second molecule in the asymmetric cell (chain A), the R29 side chain is disordered, but the hydrogen bonding between E26 and E30 can be observed. These observations suggest that the stability of the R26E/K46Y mutant should be largely independent of the solution ionic strength. A zoomed-in view of the anion binding site in the R26E mutant (Figure 8C) shows that the side chain of R29 is involved in crystal contacts with a nearby molecule, and the side chain of E30 is disordered, thus preventing observation of stabilizing interactions along α -2 similar to those observed in the structure of the R26E/K46Y double mutant. It is possible, however, that when the protein is in solution the E26, R29 and E30 side chains experience the stabilizing interactions suggested in the structure of R26E/K46Y Bfd. This idea was probed by investigating the stability of the mutants relative to Bfd in Tris buffer with different ionic strength. Results from these investigations are presented below.

The electronic absorption spectrum of both mutants is indistinguishable from that of the wild type protein (Figure S2). These observations, which indicate that the mutations did not introduce significant alterations in the electronic structure of the [2Fe-2S] cluster, are in agreement with the nearly identical structure of the wild type and mutant proteins. Consequently, the UV-vis spectrum of the mutants was used to monitor the integrity of the cluster and the stability of the mutants in Tris buffer solutions with different ionic strength, as described above for the wild type protein. Results from experiments in which the R26E mutant in Bfd's storage solution is diluted in Tris + NaCl buffer solutions with different ionic strengths show that R26E Bfd is significantly more stable than its Bfd counterpart at low ionic strength (Figure 9A). In fact, the striking degree of stabilization of the R26E mutant at low ionic strength relative to Bfd (Figure 7A), strongly suggests that E26, R29 and

E30 experience the stabilizing interactions along α -2 suggested in the structure of the R26E/K46Y double mutant. It is possible that transient repulsions between R29 and K46 in the R26E mutant contribute to the observed mild dependency of protein stability on ionic strength. Along the same vein, it is remarkable that similar experiments conducted with R26E/K46Y Bfd demonstrate that the stability of the double mutant is independent of the solution ionic strength (Figure 9B). These findings indicate that when the double mutant is in solution, the side chains of E26, R29 and E30 interact as observed in its structure (Figure 8B), thus stabilizing the short α -2 helix. In addition, the replacement of K46 with a tyrosine eliminates the transient electrostatic repulsions between R29 and K46 thought to take place in the R26E mutant, therefore eliminating the destabilizing effect of low ionic strength on the Bfd fold. When the structural and biochemical observations are taken together, strong support emerges for the idea that the Bfd fold and the integrity of its associated [2Fe-2S] cluster require an anion binding site. Anion binding eliminates otherwise electrostatic repulsions at the site and minimizes large folding/unfolding excursions of the short helix α -2, which in turn also affect the cysteine ligands in loop L3 and compromise the stability of the [2Fe-2S] cluster.

The results presented above indicate a structural role for the anion binding site in Bfd. The mutations were carried out with the intention of eliminating the site while minimizing structural changes to Bfd. The X-ray crystal structures of the mutants, together with the results obtained from investigations in solution, indicate that as intended, the mutations did not alter the structure of the protein, but introduced new side chain interactions in helix α -2 which replace the anion binding requirement to stabilize this section of the structure. It is also important to note that the anion binding site in Bfd is removed from the portion of the protein involved in binding BfrB. Consequently, we predicted that the ability of the R26E and R26E/K46Y Bfd mutants to bind BfrB and promote iron mobilization from its core would not be largely affected. To investigate this issue, we evaluated the dissociation constants for the interaction between truncated R26E or R26E/K46Y Bfd with BfrB and also compared the efficiency of the Bfd mutants at promoting iron mobilization from BfrB relative to Bfd. The sensograms and binding curves obtained from the SPR experiments performed to study the protein-protein interactions are shown in Figure S4 and the K_d values are listed in Table 3. These data show that the mutations introduced to interrogate the anion binding site in Bfd caused a relatively small (~3-fold) increase in K_d . This small decrease in binding affinity cannot be explained on the basis of structural arguments, since the structures of the mutants and Bfd are nearly identical. It is therefore tempting to speculate that the mutations introduced subtle changes in protein dynamics which exert small effects on the strength of the interaction. In fact, we have previously shown that similarly small increases in the K_d for the BfrB:Bfd interaction brought about by replacing some residues at or near the protein-protein interface cause a near negligible effect on iron mobilization from BfrB. In contrast, replacement of hot spot residues in BfrB, such as L68, E81 and E85, which cause large increases in K_d (~100-fold) and substantial impairment of iron mobilization function, also result in significant loss of iron mobilization efficiency.²⁰ In agreement with these previous observations, the small increase in K_d resulting from mutating residues at the anion binding site also cause nearly negligible changes in iron mobilization efficiency

(Figure S4). Consequently, it is probable that the anion binding site in Bfd serves mainly a structural role.

Concluding Remarks.

Bfd is a new type of [2Fe-2S] protein whose function contributes to the iron homeostasis machinery in *P. aeruginosa* by enabling the mobilization of iron from BfrB. In the absence of Bfd or when the BfrB:Bfd interaction is blocked, *P. aeruginosa* cells irreversibly accumulate iron in BfrB, which causes iron deficiency in the cytosol.²¹ Hence, blocking the BfrB:Bfd interaction is probably a viable approach to hijack iron homeostasis in *P. aeruginosa* and probably in many other bacteria. Bfd is present in a number of pathogens with high degree of sequence conservation, which includes most of the residues participating at the BfrB:Bfd interface and all of the crucial hot spot residues which contribute the most to the binding energy (Figure S1).^{19, 20} In this report we showed that the stability of *P. aeruginosa* Bfd and the integrity of its [2Fe-2S] cluster depends on an anion binding site composed by the side chains of Arg26, Arg29 and Lys46 (Figure 6), whose function is most likely to stabilize the two-turn helix α -2. In the absence of a suitable anion helix α -2 probably experiences relatively large folding unfolding excursions that also affect the stability of loop L3, which harbors Fe ligands Cys38 and Cys41. We suggest that the anion binding site on Bfd recognizes phosphate and offer the following observations in support: Amino acid sequence alignments suggest that the anion binding site in *P. aeruginosa* Bfd is conserved in a relatively large number of the Bfd structures from different organisms (Figure S1); residues at positions 29 and 46 are invariably Lys or Arg and residue 26 is either Lys, Arg or Gln. Studies of phosphate recognition by proteins indicate that phosphate binding site residues are more conserved than other residues in the alignment, with Arg and Lys having the largest occurrence when the site recognizes phosphate via side chains.^{39, 40} Hence, these characteristics of phosphate binding sites are consistent with the exclusive presence of Arg, Lys or Gln at positions 26, 29 and 46 in the sequence alignment of Bfd proteins. In addition, it is important to note that the core iron mineral of bacterioferritins isolated from natural sources contain high levels of phosphate (Fe:P ~ 1:1),⁴¹ and that iron uptake and mobilization from bacterioferritin is accompanied by a corresponding flux of phosphate across the Bfr shell.^{18, 42} Given the effect of phosphate on the stability of Bfd, and the intriguing but not yet understood interplay between iron and phosphate metabolism in *P. aeruginosa*,⁴³ it is tempting to speculate that phosphate concentration in the cytosol may influence iron homeostasis by regulating iron mobilization from BfrB.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Crystal structures of **A)** spinach ferredoxin (PDB 1A70), **B)** thioredoxin-like ferredoxin from *Aquifex aeolicus* (PDB 1F37), **C)** Rieske protein from *Rhodobacter sphaeroides* (PDB 2NVE), **D)** Human mitochondrial inner NEET protein MiNT (PDB 6AVJ), and **E)** Bfd from the complex with BfrB (PDB 4E6K). The structures are colored by secondary structure showing the α -helices (green) and β -sheets (magenta). The 2Fe-2S atoms are rendered as spheres (Fe-coral and S-yellow) and coordinating residues are drawn as cylinders.

Figure 2.
Schematic representation of the model for iron mobilization from BfrB, where electrons originating in NADPH are delivered to the Fe^{3+} mineral in the core of BfrB.

Figure 3.

The structure of truncated Bfd. **(A)** Elements of secondary structure defining the Bfd fold are shown in different colors, with the α -helices in green, the β -sheets in magenta and the loops in gray. The Fe and S atoms in the [2Fe-2S] cluster are rendered as spheres (Fe-coral and S-yellow) and coordinating cysteine ligands are rendered as cylinders. **(B)** Zoomed-in view of the [2Fe-2S] cluster showing the 2Fo-Fc electron density map (blue mesh) contoured at 1 σ .

Figure 4.

Comparison of truncated Bfd with Bfd in the BfrB:Bfd complex (PDB 4E6K). (A) Superposition of truncated Bfd (magenta) onto Bfd (chain G, cyan) in the BfrB:Bfd complex (PDB 4E6K) showing their orientation relative to subunits A (green) and B (gray) of a BfrB subunit dimer. (B) Zoomed-in view of the two loops flanking the [2Fe-2S] cluster (L-1 and L-3). Loops L-1, spanning Met1–Asp11 are colored cyan in 4E6K and magenta in truncated Bfd, and loops L-3, spanning Gly33–Cys41 are gray in 4E6K and green in truncated Bfd. (C) Hot spot region at the Bfd:BfrB interface. Bfd is in cyan, whereas subunits A and B of BfrB are in green and gray, respectively.

Figure 5.

The disordered C'-terminal tail in Bfd does not influence its interaction with BfrB. **(A)** Overlay of reference- and baseline-subtracted sensograms obtained from flowing truncated Bfd solutions over immobilized BfrB. **(B)** Black circles are the responses at steady state concentrations in A plotted as a function of truncated Bfd concentration and fitted to the model described by equation 3 (solid line). **(C)** Mobilization of Fe^{3+} stored in BfrB, as indicated by the time-dependent increase in the normalized A_{523} upon addition of excess NADPH (1.0 mM) to 20 mM phosphate buffer (pH 7.6) containing 0.18 μM BfrB, 7.2 μM Fpr, and 0.9 μM of (black) full-length Bfd or (red) truncated Bfd.

Figure 6.
Interaction between phosphate and Bfd (chain G) in the BfrB:Bfd complex (PDB 4E6K).
The [2Fe-2S] cluster and the sulfur ligands are shown as spheres.

Figure 7.

Monitoring the stability of truncated Bfd in Tris + NaCl or phosphate buffers of different ionic strength; $\mu = 150$ mM (black), $\mu = 230$ mM (red) and $\mu = 330$ mM (green) at 25 °C. (A) The stability of truncated Bfd is strongly dependent on the solution ionic strength in Tris + NaCl buffer. (B) Bfd exhibits higher stability and a much shallower propensity to lose its [2Fe-2S] cluster in phosphate buffer. Every plot in the figure is the average of two experiments.

Figure 8. Comparison of truncated Bfd structures. (A) Superposition of Bfd (magenta), R26E (gold) and R26E/K46Y (blue) structures demonstrates minimal structure differences. (B) Zoomed-in view of the anion binding region in R26E/K46Y Bfd (chain B); the dotted lines represent the hydrogen bonding interactions thought to stabilize the short helix α -2. (C) The anion binding region in Bfd R26E.

Figure 9.

Monitoring the stability of mutant Bfd molecules in Tris + NaCl buffers of different ionic strength; $\mu = 150$ mM (black), $\mu = 230$ mM (red) and $\mu = 330$ mM (green) at 25 °C. (A) The stability of the R26E mutant exhibits a mild dependency on the ionic strength of the solution, which is in remarkable contrast with the steep dependency exhibited by Bfd (see Figure 7A). (B) The stability of the R26E/K46Y double mutant is nearly independent of the solution ionic strength.

Table 1.

Classes of [2Fe-2S] Proteins

[2Fe-2S]-protein class	Protein provided ligands	Ligand arrangement
Plant/vertebrate ferredoxin	4Cys	C-X ₄₋₅ -C-X ₂ -C-X ₃₀₋₃₇ -C
Thioredoxin-like ferredoxin	4Cys	C-X ₁₀₋₁₂ -C-X ₂₉₋₃₄ -C-X ₃ -C
Rieske centers	2Cys and 2His	C-X-H-X ₁₅₋₁₇ -C-X ₂ -H
NEET proteins	3Cys and 1His	C-X-C-X ₂ -(S/T)-X ₃ -P-X-CDG-S/A/T)-H
Bfd	4 Cys	C-X ₂ -C-X ₃₁₋₃₂ -C-X ₂ -C

Table 2.

Crystallographic data for Bfd structures

	Bfd (C43S)	Bfd (R26E, C43S)	Bfd (R26E, C43S, K46Y)
Data Collection			
Unit-cell parameters ($\text{\AA}, ^\circ$)	$a=23.95, b=49.71, c=39.90,$ $\beta=96.9$	$a=22.44, b=43.17, c=44.32$	$a=43.19, b=46.19, c=48.85$
Space group	$P2_1$	$P2_12_12_1$	$P2_12_12_1$
Resolution ($\text{\AA}$) ¹	39.61–1.20 (1.22–1.20)	43.17–1.50 (1.53–1.50)	48.85–1.45 (1.47–1.45)
Wavelength ($\text{\AA}$)	1.0000	1.0000	1.0000
Temperature (K)	100	100	100
Observed reflections	96,242	43,227	113,397
Unique reflections	28,905	7,316	17,875
$\langle I/\sigma(I) \rangle$ ¹	10.3 (2.1)	13.2 (1.8)	15.8 (1.9)
Completeness (%) ¹	99.5 (99.2)	99.8 (99.8)	99.6 (99.9)
Multiplicity ¹	3.3 (3.2)	5.9 (6.2)	6.3 (6.6)
R_{merge} (%) ^{1, 2}	6.5 (63.3)	7.0 (99.3)	5.7 (89.0)
R_{meas} (%) ^{1, 4}	7.7 (76.4)	7.7 (108.7)	6.2 (96.7)
R_{pim} (%) ^{1, 4}	4.1 (42.0)	3.1 (43.4)	2.4 (37.5)
$CC_{1/2}$ ^{1, 5}	0.997 (0.766)	0.999 (0.670)	0.999 (0.862)
Refinement			
Resolution ($\text{\AA}$)	39.61–1.20	30.93–1.50	33.56–1.45
Reflections (working/test)	27,391/1,483	6,907/374	17,044/762
$R_{\text{factor}} / R_{\text{free}}$ (%) ³	12.1/14.8	15.8/17.8	16.0/19.2
No. of atoms (Bfd/Fe-S/water)	863/8/105	420/4/36	844/8/89
Model Quality			
R.m.s deviations			
Bond lengths ($\text{\AA}$)	0.012	0.020	0.009
Bond angles ($^\circ$)	1.634	1.069	1.516
Average B factor ($\text{\AA}^2$)			
All Atoms	15.6	26.1	24.3
Bfd	14.0	25.5	23.4
Fe-S	10.9	19.5	24.6
Water	29.5	33.5	32.7
Coordinate error, maximum likelihood ($\text{\AA}$)	0.11	0.18	0.10
Ramachandran Plot			
Most favored (%)	98.2	96.3	100
Additionally allowed (%)	1.8	3.7	0

¹) Values in parenthesis are for the highest resolution shell.

²⁾ $R_{\text{merge}} = \sum_{hkl} \sum_i |I_f(hkl) - \langle I(hkl) \rangle| / \sum_{hkl} \sum_i I_f(hkl)$, where $I_f(hkl)$ is the intensity measured for the i th reflection and $\langle I(hkl) \rangle$ is the average intensity of all reflections with indices hkl .

³⁾ $R_{\text{factor}} = \sum_{hkl} ||F_{\text{obs}}(hkl) - |F_{\text{calc}}(hkl)|| / \sum_{hkl} |F_{\text{obs}}(hkl)|$; R_{free} is calculated in an identical manner using 5% of randomly selected reflections that were not included in the refinement.

⁴⁾ R_{meas} = redundancy-independent (multiplicity-weighted) R_{merge} .^{24, 34} R_{pim} = precision-indicating (multiplicity-weighted) R_{merge} .^{35, 36}

⁵⁾ $CC_{1/2}$ is the correlation coefficient of the mean intensities between two random half-sets of data.^{37, 38}

Table 3.

Dissociation constants for the BfrB:Bfd interaction involving BfrB and mutant Bfd molecules

Protein	K_d (pH 7.0)	Reference
Full-length Bfd	$3.4 \pm 0.5 \mu\text{M}$	²⁰
Truncated Bfd	$5.4 \pm 0.4 \mu\text{M}$	This work
R26E truncated Bfd	$10.9 \pm 2 \mu\text{M}$	This work
R26E/K46Y truncated Bfd	$9.5 \pm 2 \mu\text{M}$	This work