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STRUCTURAL FOLD, CONSERVATION AND FE(II) BINDING OF THE INTRACELLULAR DOMAIN OF PROKARYOTE FEOB

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Abstract

FeoB is a G-protein coupled membrane protein essential for Fe(II) uptake in prokaryotes. Here, we report the crystal structures of the intracellular domain of FeoB (NFeoB) from *Klebsiella pneumoniae* (KpNFeoB) and *Pyrococcus furiosus* (PfNFeoB) with and without bound ligands. In the structures, a canonical G-protein domain (G domain) is followed by a helical bundle domain (S-domain), which despite its lack of sequence similarity between species is structurally conserved. In the nucleotide-free state, the G-domain's two switch regions point away from the binding site. This gives rise to an open binding pocket whose shallowness is likely to be responsible for the low nucleotide binding affinity. Nucleotide binding induced significant conformational changes in the G5 motif which in the case of GMPPNP binding was accompanied by destabilization of the switch I region. In addition to the structural data, we demonstrate that Fe(II)-induced foot printing cleaves the protein close to a putative Fe(II)-binding site at the tip of switch I, and we identify functionally important regions within the S-domain. Moreover, we show that NFeoB exists as a monomer in solution, and that its two constituent domains can undergo large conformational changes. The data show that the S-domain plays important roles in FeoB function.

Keywords

G protein; ferrous iron transport; *Klebsiella pneumoniae*; *Pyrococcus furiosus*; Feo; FeoB

Introduction

Eukaryotic GTPases are transducers of information utilized in a variety of cellular processes including growth, differentiation, translation, signal transduction and transport (Sprang,

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1997; Sprang et al., 2007). They are known as molecular switches that can be turned on (GTP-bound) or off (GDP-bound) and provide targets for regulatory interactions with other proteins that accelerate nucleotide hydrolysis or modulate the rate of GDP release. Likewise, prokaryotic G proteins are involved in essential cellular pathways. However, with the exception of the bacterial GTP-binding proteins involved in protein synthesis and secretion they are poorly characterized (Caldon and March, 2003; Caldon et al., 2001).

Within the past decade several near atomic resolution structures of prokaryotic small GTPases have been solved (Buglino et al., 2002; Chen et al., 1999; Montoya et al., 1997; Robinson et al., 2002), revealing their high structural similarity with eukaryotic G proteins. However, the structures failed to explain the low nucleotide binding affinities typically found for prokaryotic G proteins, leaving unanswered the question whether prokaryotic G protein play regulatory roles or function as plain enzymes. Given their fast nucleotide off rates (several nucleotides per second (Chen et al., 1990; Moser et al., 1997; Sullivan et al., 2000)), the ready exchange of GTP/GDP would result in low signal fidelity if these modules were involved in signal transduction. In contrast, any function relying on the enzymatic turnover of GTP would be limited by the intrinsically slow rates of hydrolysis (one turnover on the timescale of minutes (Hwang and Inouye, 2001; Welsh et al., 1994; Yamanaka et al., 2000)). Adding to the mystery, the structures of several prokaryotic G proteins revealed additional domains that either precede or follow the G protein core. While in some cases the functions of these additional domains are known like in the case of Era (Inoue et al., 2006; Sharma et al., 2005), none of these domains affect the basic biochemical properties of the G proteins themselves. Adding to this lack of regulatory interplay, no prokaryotic specific factors, such as guanine nucleotide exchange factors, GTPase-activating proteins, or guanine nucleotide dissociation inhibitors, have been identified to create a bacterial version of a guanine nucleotide signaling pathway, thus causing a roadblock towards understanding the mechanisms by which the majority of prokaryotic G proteins function in their respective contexts.

The integral membrane protein FeoB has emerged as a notable exception to the poorly defined biological functions of prokaryotic G proteins. Being involved in G protein dependent high-affinity Fe(II)-uptake, the FeoB family of G proteins couple a GTP binding domain with a polytopic transmembrane domain (Cartron et al., 2006). Recently, and unprecedented among prokaryotic G proteins, FeoB was shown to contain a novel regulatory spacer domain (S-domain) that follows the G protein core in the sequence and interacts with the switch regions to inhibit guanine nucleotide exchange (Eng et al., 2008; Marlovits et al., 2002). The possible existence of a blue print for a G protein regulatory cycle in a bacterial membrane protein spawned much interest, which over the past year has led to the release of several crystal structures (Guilfoyle et al., 2009; Hattori et al., 2009; Köster et al., 2009). While informative, none of the crystal structures shed light on the function of the S-domain, nor did it address how well this domain is conserved in light of large sequence divergence. In the present study we report the crystal structures of the intracellular domain of *Klebsiella pneumoniae* FeoB (KpNFeoB), with and without bound guanine nucleotides, and the nucleotide free *Pyrococcus furiosus* FeoB (PfNFeoB). Besides the canonical G-domain signature fold, the remarkable structural similarity of the S-domains in both species replicates what was observed in recently determined structures for the intracellular domain of *E. coli* FeoB (EcNFeoB) (Guilfoyle et al., 2009) and *Thermotoga maritima* FeoB (TmNFeoB) (Hattori et al., 2009). Given the high degree of divergence at the sequence level, the structural conservation of the S-domain was unexpected (Figure 1). Just as surprising, the observed behavior of the intracellular domain in solution is at odds with the emerging crystallographic consensus, emphasizing that understanding of this system is still in its infancy. Nevertheless, our studies suggest that the S-domain may functionally couple the GTP-binding and transmembrane domains, raising the possibility that FeoB represents an archetypal template for modern G protein regulated receptors, transporters and channels.

Material and Methods

Expression and Purification of NFeoB Proteins

The genes encoding the NFeoB proteins from *K. pneumoniae* and *P. furiosus* were amplified by polymerase chain reaction (PCR) and the authenticities of the constructs were confirmed by nucleotide sequencing. C-terminally hexa-histidine tagged constructs were expressed from pET24a- and ptac-based vectors as described previously (Marlovits et al., 2002). *E. coli* cells harboring the recombinant expression vectors were grown at 37 °C in Luria Broth (LB) medium with 100 µg/mL ampicillin. Protein expression was induced by the addition of 1.0 mM Isopropylthio-β-D- galactoside (IPTG) when the culture reached OD₆₀₀ of ~ 0.6. The culture was incubated for additional 4 hrs and harvested by centrifugation at 6000 rpm for 20 min. The cell pellets were resuspended, lysed with Microfluidizer, and the protein purified through a Ni-NTA affinity column (Qiagen, CA, USA). The protein was further purified through size exclusion on a HiLoad 16/60 Superdex 75 column (Amersham Pharmacia Biotech, Sweden). Complete Protease Inhibitor cocktail (Roche, Germany) was added to the purified protein. Protein concentration was determined with the Bio-Rad Protein Assay kit as per instructions from the manufacturer (Bio-Rad, CA, USA). The sizes of all protein products were checked by SDS-PAGE and confirmed with mass spectrometry (Voyager-DE STR, PerSeptive Biosystems, MA).

Seleno-methionine (SeMet) substituted *Kp*NFeoB and *Pf*NFeoB used for diffraction studies were expressed in *E. coli* B834(DE3) and grown in modified M9 media containing all amino acids except Met at concentrations of 50mµg/ml, 0.4% glucose, 1 mM MgSO₄, 4.2mµg/ml Fe₂SO₄, 1 µg/ml vitamin B mixture (B1, B2, B3, B6, B12), and 50 µg/ml SeMet. Protein purification was performed as described above. After purification, SeMet-substituted *Kp*NFeoB was dialyzed against 20 mM Tris-HCl buffer at pH 7.5, containing 50 mM NaCl, 50 mM Glu, 50 mM Arg, and 10 mM MgCl₂. For producing the complex of *Kp*NFeoB with GDP or GMPPNP the purified protein was concentrated to 6.5 mg/ml and incubated with the molecules at molar ratio of 1:10.

Characterization of the enzyme activity

The intrinsic GTPase activity of *Kp*NFeoB was assayed using EnzChek phosphate kit (Invitrogen). In the presence of inorganic phosphate generated by enzymatic reaction of GTPase, purine nucleoside phosphorylase (PNP) converts the substrate 2-amino-6-mercapto-7-methylpurine ribonucleoside (MESG) to ribose-1-phosphate and 2-amino-6-mercapto-7-methylpurine with strong absorption at 360nm, suitable for quantification of the G protein activity. 20 µM *Kp*NFeoB was first mixed with assay reagents containing 50 mM Tris-HCl, 0.1 mM MESG, 1U PNP, 1 mM MgCl₂ and 0.1 mM NaN₃ and incubated at pH 7.5 and 298 K for 10 min. Various concentrations of GTP were loaded into the pre-incubated reaction solution and the UV_{360nm} absorbance was monitored immediately with a VersaMax microplate reader at desired temperatures. Data were fitted to the Michaelis-Menten equation using SoftMax® Pro software to obtain kinetic and thermodynamic parameters of the enzymatic reaction.

Measurement of nucleotide-binding affinity

Guanine nucleotide binding equilibrium constants (K_D) were determined from the changes in fluorescence intensities of the 2'(3')-O-(*N*-methylantraniloyl) (mant)-substituted analogs. Assay reagents containing 20 µM mant-nucleotide, 50 mM Tris-HCl and 50 mM NaCl at pH 7.5 were mixed with various concentrations of *Kp*NFeoB in the presence or absence of 1 mM Mg²⁺ for 3 min incubation at 298 K. The fluorescence measurements at 298 K were performed on a Gemini M microplate spectrofluorometer with an excitation wavelength of 356 nm and

fluorescence was detected at 440 nm. The K_D values were extracted following the protocol given by Herrmann *et al.* (2002)(Ahmadian *et al.*, 2002)

Stopped-Flow Measurements

Stopped-flow measurements were used to determine fast binding and the release of fluorescent nucleotides for *PfNFeoB* as described previously (Marlovits *et al.*, 2002). A detailed description is provided in the Supplemental text.

Fe(II)-Uptake Complementation

Complementation assays allow the function of full-length *E. coli* FeoB mutants to be assessed through rescue of iron uptake defects in a FeoB deficient host. Complementation experiments were carried out as described previously (Marlovits *et al.*, 2002). A more detailed description can be found in the Supplemental Information.

Sulfhydryl Reactivity

The reaction of Aldrithiol-4 (4-pyridyl-disulfide, 4-PDS) (Sigma-Aldrich, St. Louis, MO) with the thiol groups of globin proteins to form 4-thiopyridone (Grassetti and Murray, 1967) was employed to measure free cysteines. FeoB constructs were incubated in 10-fold excess of the theoretical maximum cysteine concentration and the release of 4-thiopyridone was monitored at 324 nm. The content of free thiol groups was determined using an extinction coefficient for 4-thiopyridone of $21.3 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$.

Gel-Filtration Chromatography

Size-exclusion chromatography experiments were performed at 4°C on an AKTA FPLC equipped with a Superdex-75 column (GE Healthcare). 25μM of protein was incubated without or with 50μM GDP, or 50μM GTP at 4°C for 10min in 20mM Tris, pH7.5 buffer containing 200μM Mg^{2+} , 50mM NaCl before being loaded onto column. Protein was eluted with the same buffer at a flow rate of 0.5ml/min. Protein mass was calibrated with protein standards of molecular mass range of 6.5-75 kDa.

Sedimentation Velocity Experiments

A Beckman-Coulter Optima XL-A analytical ultracentrifuge equipped with An60 Ti analytical rotor was employed for sedimentation velocity experiments. 25μM of protein in Tris buffer at pH7.5 containing 50mM NaCl and 200uM Mg^{2+} was incubated at 20°C without or with 50uM GDP, or 50uM GMPPNP for 1 hr. All sedimentation velocity experiments were performed at 20°C and 60,000rpm for 20hr. AUC data were analyzed using software Sedfit with nonlinear regression algorithm.

Crystallization and structure determination of *KpNFeoB*-GMPPNP

Crystals of SeMet-substituted *KpNFeoB*-GMPPNP were obtained at 298 K using the hanging-drop vapor-diffusion method by mixing 1μl of *KpNFeoB*-GMPPNP mixture with an equal volume of reservoir containing 80 mM MES sodium salt pH 6.5, 160 mM magnesium acetate, 16% (w/v) PEG8000, and 20% (v/v) glycerol. Cubic-like crystals of diffraction quality appeared after five days. The crystals were flash-frozen in liquid nitrogen and data were collected at Beam line BL13B1 of the National Synchrotron Radiation Research Center in Taiwan. The data was collected with the ADSC Quantum-315 charge-coupled device detector. The crystals belong to space group $P2_13$ with cell dimensions $a = b = c = 106.897 \text{ \AA}$ and were diffracted to $2.05 \text{ \AA}$. There is only one *KpNFeoB*-GMPPNP molecule per asymmetric unit. The structure was determined using multi-wavelength anomalous diffraction (MAD) phasing applied to selenium. Two energies, $0.97869 \text{ \AA}$ and $0.97888 \text{ \AA}$, were chosen near the absorption peak and

edge of the selenium atoms in *KpNFeoB*·GMPPNP, which correspond to the maximum f'' and the minimum f' , respectively. A remote energy was selected as reference wavelength in 0.96365 Å. Data integration and scaling were performed using the *HKL2000* package (Otwinowski and Minor, 1997). *Solve* (Terwilliger and Berendzen, 1999) was used to locate the four selenomethionine sites and generate the initial MAD phases at 3.0 Å resolution. The initial phases were extended and further improved to 2.05 Å by *Resolve* (Terwilliger, 2000). *XtalView* (McRee, 1999) was used to examine electron density maps and molecular models. Further refinement was performed by using *CNS* (Brunger et al., 1998) and *Refmac* (Murshudov et al., 1997). The final model of *KpNFeoB*·GMPPNP contains amino acid residues from Met¹ to Thr²⁶⁴ with two poorly ordered loops encompassing residues Arg²⁹ to Arg⁴⁰ and Gln⁶⁹ to Leu⁷², 60 water molecules, a GMPPNP molecule, and two Mg²⁺ ions. The *R*-factor of this model for all reflections above 2 σ between 25.9 and 2.05 Å resolution was refined to 21.5% and a *R*-free value of 21.8% was obtained using 5% randomly distributed reflections. The overall root-mean-square deviations from ideal geometry are 0.021 Å and 2.3° for bond lengths and bond angles, respectively. A Ramachandran plot showed 90.4% of residues lie within the most favored regions, 9.6% in the additional allowed region; no residues were found in generally or disallowed regions. The average temperature factor of the refined model for all atoms is 45.9 Å². The atomic coordinates of *KpNFeoB*·GMPPNP has been deposited in the Protein Data Bank under accession code: 2wic.

Crystallization and structure determination of Apo *KpNFeoB* and *KpNFeoB*-GDP

Crystals of apo *KpNFeoB* and *KpNFeoB*·GDP were also obtained at 298 K using the hanging-drop vapor-diffusion method, similar to those described for *KpNFeoB*·GMPPNP. More detailed descriptions are given in the supplemental text. The atomic coordinates of Apo and *KpNFeoB*·GDP have been deposited in the Protein Data Bank under accession codes: 2wia and 2wib for Apo *KpNFeoB* and *KpNFeoB*·GDP, respectively.

Crystallization and structure determination of *PfNFeoB*

Crystals of the seleno-methionine substituted *NFeoB* were grown at 23°C by the hanging drop method against a well liquor containing 100 mM sodium acetate (pH 5.0), 10 mM manganese chloride, 15 % (v/v) PEG 1000, and 1 mM DTT. The crystals belong to space group $P2_12_12_1$, with unit cell dimensions of $a=55.26$ Å, $b=106.11$ Å and $c=253.89$ Å. They diffracted to ~ 2.6 Å at beam line 24ID (NE-CAT) at the Advanced Photon Source, Argonne National Laboratory. An anomalous data set collected at the selenium edge was used in phasing. Data were integrated and scaled using the program *HKL2000* (Otwinowski and Minor, 1997). Selenium sites were located using *SHELXD* (Schneider and Sheldrick, 2002) and refined with *CNS* software suite (Brunger et al., 1998). Two independent phase calculations and refinements were run using the *CNS* for each hand of the Se site configurations. Using *CNS*, the SAD phases together with solvent flipping produced interpretable experimental maps at 2.7 Å. A solvent content of 55% was used. The initial model was built in *Coot* (Emsley and Cowtan, 2004). The position of seleno-methionine side chains guided aligning the sequence of *NFeoB* to the model of its polyalanine backbone. The model was further improved by cycles of torsion angle dynamics and B-factor refinement, as implemented in the *CNS* software suite (Brunger et al., 1998), and manual rebuilding followed by simulated annealing. Non-crystallographic symmetry restraints were applied to the four copies of *PfNFeoB* in the asymmetric unit and omitted the last round of refinement. For the final model, $R=23.7\%$ and $R_{\text{free}}=27.6\%$ for all data between 50–2.7 Å. It contains four copies of *PfNFeoB* (chain A: residues 1–267; chain B: residues 1–222, 227–267; chain C: residues 1–223, 227–267; chain D: residues 1–267). Side chains without density in 2FoI–|FcI maps were modeled as an alanine. The statistical data of data collection and structure refinement of *PfNFeoB* structures are shown in Supplemental Table SII. The atomic coordinates of *KpNFeoB*·GMPPNP has been deposited in the Protein Data Bank under accession code: 3K35.

Results

Nucleotide binding affinity, kinetics and catalytic activity of KpNFeoB

To confirm that KpNFeoB's biochemical properties were similar to other NFeoBs that have been studied, we determined the nucleotide binding affinities following the procedure described by Lenzen *et al.* (Lenzen *et al.*, 1995) (Table I). Using fluorescently labeled nucleotides 2'(3')-*O*-(*N*-methylantraniloyl) (mant)-GDP, mant-GTP and mant-GMPPNP at 25°C, titration of the purified KpNFeoB resulted in binding constants that were comparable to other reported values ($K_D = 4.1 \mu\text{M}$ mant-GMPPNP $\cdot\text{Mg}^{2+}$, $31.4 \mu\text{M}$ mant-GTP $\cdot\text{Mg}^{2+}$, and $28.1 \mu\text{M}$ mant-GDP $\cdot\text{Mg}^{2+}$ respectively) (Eng *et al.*, 2008). Concerning KpNFeoB GTPase activity (assayed at 25 °C and 37°C and using MESG/PNP system (Table I)), a k_m of 167 μM , k_{cat} of 0.00052 S^{-1} and an absolute requirement for Mg^{2+} -ions all were similar to those reported for the *E. coli* enzyme, suggesting that mechanistic clues from the structure would be generally applicable to other NFeoBs.

Structural overview of KpNFeoB and PfNFeoB

The crystals of nucleotide-free KpNFeoB diffracted to 2.45 Å (Supplemental Table SI), and revealed a protein that folded into an apparent single globular structure consisting of eleven α -helices and seven β -strands (Fig. 2A and supplemental figure S1). Subdividing the structure, the N-terminal ~170 residues (G-domain) assume a G protein fold, which was highly homologous to the structure of the canonical G protein. The C-terminal five helices (H7-H11, residues 171-270) (S-domain) assumed a compact hammer-shaped helix bundle structure, with the long H11 helix positioned as the handle. The S-domain interacted extensively (interfacial area of ~1,256 Å²) with G-domain residues opposite to the nucleotide binding pocket, and near the switch II region via both hydrophobic interaction (grey lines, 16 pairs) and hydrogen bond interactions (Direct H-bond: red lines, 10 pairs; water-mediated H-bond: red dotted lines, four pairs) (Figure 3). Despite these interactions, all elements of the S-domain fragment had higher B-factors than were observed for the G-protein domain. This indicated a higher flexibility of the S-domain (Figure 2B) and suggested that the motion/disorder of the S-domain was independent of that of the G-domain. Further support for this idea came from the observation that elevated B-factors were also observed in the structures of the nucleotide bound forms KpNFeoB-GDP and KpNFeoB-GMPPNP (Data not shown).

To test whether and how sequence divergence in the S-domains of different species would affect the structure of the domain, we also solved the crystal structure of the intracellular domain of *Pyrococcus furiosus* FeoB (PfNFeoB) (PDB ID: 3K35). The crystals of nucleotide-free PfNFeoB diffracted to 2.7 Å (Supplemental Table SII) and revealed that despite large sequence divergence, the structure of the S-domain was strikingly similar to the S-domain of KpNFeoB (rms deviation ~ 1.148 Å) (Fig. 2C). At the level of individual domains, the rms deviations were 0.906 Å and 1.973 Å for the G-domain and S-domain, respectively, which reflected that, like its counterpart in KpNFeoB, the S-domain of PfNFeoB displayed higher B-factors compared to that of the G-domain (Fig. 2D). The remarkable structural similarity between the evolutionarily separated S-domains of these two species agreed well with the previously reported functional importance of the domain.

The G-domain assumes an “open” conformation and switch I has no direct interaction with nucleotides

Inspection of the structures of the G-domain of apo KpNFeoB (Fig. 4A), KpNFeoB-GDP (Fig. 4B) and KpNFeoB-GMPPNP (Fig. 4C) showed that the G-protein signature motifs were located in the expected spatial locations and the loop segment ¹⁴⁹VSTRG¹⁵³ was located in the position normally occupied by the G5 motif. In both KpNFeoB-GDP and KpNFeoB-GMPPNP the α -phosphate was coordinated to NH of Thr¹⁷ and the hydroxyl group of Thr¹⁸ (Fig. 4D). The β -

phosphate was coordinated to the P-loop residues, ¹³NSGKT¹⁷ in both structures and further hydrogen bonded to the Mg²⁺ ion and two more water molecules in the GMPPNP-bound form. The γ -phosphate of GMPPNP was coordinated to NH of Asn¹³, the N⁵ of Lys¹⁶, Mg²⁺ and three water molecules. It further interacted with Asp⁵⁶ of the switch-II region (G3) through a water-mediated hydrogen bond. The Mg²⁺ ion was hexacoordinated to the following six molecules: three water molecules, the β - phosphates, the γ -phosphates, and the NH of Thr¹⁷. The three water molecules formed part of an extensive hydrogen bonding network among Mg²⁺, β - and γ -phosphates, Thr¹⁷ and Asp⁵⁶. Thus, both Thr¹⁷ and Asp⁵⁶ were responsible for coordinating the Mg²⁺ ion. The guanine ring was recognized by the protein through specific hydrogen bonding to the side chains of Asn¹²⁰ and Asp¹²³, as predicted previously (Marlovits et al., 2002). The ring was further stabilized through hydrophobic interaction with Met¹²¹ and Ile¹²⁴ on one side and the G5 motif (¹⁴⁹VSTRG¹⁵³) on the other side. Thus, the structure of the guanine nucleotide binding pocket is mostly conserved. One unique feature of the structure of the *KpNFeoB* G-domain was the total absence of the switch I region in direct guanine nucleotide interaction. In Ras p21 Thr³⁵ in the switch I region binds to the γ -phosphate and the Mg²⁺ ion whilst the corresponding Thr³⁷ in G-domain is far away from the binding site. In fact the whole switch I loop had no contact with the nucleotide at all. The role of Thr³⁷ in the switch I region was taken up by Thr¹⁷ and one water molecule. Significantly, both switch I and switch II loops pointed away from the nucleotide-binding site such that the binding site appeared to be very “open”. As in other G proteins, the switch I and switch II regions were much more flexible/disordered in all three structures.

Conformational changes induced by guanine nucleotide binding

The most surprising finding was that nucleotide binding left the spatial relationships between the G- and S-domains unaltered. This was unexpected based on the reported biochemical properties of NFeoB (Eng et al., 2008). In fact, the crystal structures did not provide an explanation for the observed effects of the S-domain on nucleotide binding. However, nucleotide binding did cause minor structural changes in the G-domain. Specifically, binding of guanine nucleotide stabilized two regions, residues Thr⁶⁵-Ser⁶⁸ in the switch II region and Val¹⁴⁹-Arg¹⁵² in the G5 motif. These two regions were unobservable in the electron density map of the nucleotide-free *KpNFeoB* (Fig. 4A and Fig. 4B). The structural change in the G5 motif seemed particularly significant as direct interactions of this region with the guanidine ring have been proposed to play an important role in GDP release from the G _{α} subunit of heterotrimeric G-proteins (Oldham and Hamm, 2008; Oldham et al., 2006).

The structural difference between the GDP-bound and the GTP-bound forms is of particular interest for its role in signal transduction. The *KpNFeoB*·GMPPNP complex contained three structured water molecules and a Mg²⁺ ion in the binding site that were absent in the GDP-bound form (Fig. 4C). These extra molecules formed extensive hydrogen bonds with β -phosphate, γ -phosphate, Thr¹⁷ and Asp⁵⁶ (Fig. 4D). Interestingly, no direct interaction was observed between GMPPNP and the conserved Gly⁵⁹, whose equivalent (Gly⁶⁰) provides a critical pivot point for the reorientation and partial refolding of the helical region of switch II in Ras p21. Instead, water mediated interactions of Asp⁵⁶ with the γ -phosphate and the Mg²⁺ ion in *KpNFeoB*·GMPPNP, but not in *KpNFeoB*·GDP, pulled the B4 strand and switch II loop closer to the guanine nucleotide binding site. This destabilized the β -sheet and, in contrast to Ras p21, caused disordering of four residues (Gln⁶⁹-Leu⁷²) in the switch II loop. Thus Asp⁵⁶ appears to play a pivotal role in mediating the guanine nucleotide induced conformational changes in *KpNFeoB*.

Identification of S-domain regions required for function

The remarkable conservation in the structures of the S-domain from *E. coli* (Guilfoyle et al., 2009), *K. pneumoniae*, *P. furiosus* and *T. maritime* (Hattori et al., 2009) provided an

opportunity for directed biochemical characterization of the S-domain, which has been difficult in the past because the S-domain is poorly conserved across the FeoB family (Fig. 1B). A ConSurf (Landau et al., 2005) analysis of phylogenetic information revealed three regions of sequence similarity that may be important for function (Supplemental Fig. S2). Mapping of the ConSurf information onto the structure showed that the three weakly conserved motifs lie on α -helical faces: (1) Exxx Φ xxx Φ on H7, (2) R(W/Y) Φ x Φ xx Φ ExD on H8, and (3) Φ x Φ Axx (R/K)Yxx Φ xx Φ on H11, where Φ , hydrophobic residues, are typically leucine or isoleucine (Fig. 1B and Fig. 5A).

The functional significance of the second motif, which is the most conserved of the three, had previously been discovered because a E210A mutation in *EcFeoB* (Gln212 in *P. furiosus*) impaired Fe(II)-uptake *in vivo*, negatively affected GDP-binding *in vitro*, and reduced the apparent binding affinity between G-domain and S-domain as determined by *in vitro* pull-down assays using individual domain constructs (Eng et al., 2008). In the structures, E210 interacts with the G-domain, which may explain some of the observed biochemical properties (Fig. 5A). To test the functional significance of the remaining two motifs, we generated point mutations in *EcFeoB*, which is amenable to genetic complementation assays (Kammler et al., 1993). In the first motif residue Glu¹⁸⁴ was targeted because it was the most highly conserved residue within the motif. A Glu184Ala mutation had a wild type-like phenotype. In contrast, charge reversal (E184R) impaired but did not completely abolish Fe(II)-uptake (Fig. 5B) suggesting that motif 2 was functionally significant. Interestingly, in the structure, motif 2 faces into a direction opposite from the interaction interface with the G-domain (H8, the following loop, and the loop between H10 and H11), which suggested that motif 2 may mediate interactions with the membrane embedded region.

The third, moderately conserved motif in S-domain - Φ x Φ Axx(R/K)Yxx Φ xx Φ - was located on H11, which was the last of S-domain helices and in the structure did not seem to contribute to the core of S-domain helical bundle. However, this motif was located in the G-domain/S-domain interface and mutation in this motif was expected to have strong effects on domain interaction. Three residues were targeted in the third motif to test its involvement in FeoB function: Ala²⁴⁵, Arg²⁴⁸, and Tyr²⁴⁹ (Fig. 5A). Mutations in all three positions reduced Fe(II) uptake or conferred a non-functional phenotype, attesting to the functional importance of this region (Fig. 5B). The small side chain of Ala²⁴⁵ was suspected to allow the helix to make close contacts with an opposing binding surface. Consistent with this idea, an A245G mutation conferred wild type-like phenotype, while an A245W mutation yielded an iron deficiency phenotype. In the case of R248, charge removal was compatible with function (as was the case for E184 within motif 1), while reversing the charge (R248E) yielded a pronounced non-functional phenotype. Even less tolerant to changes, both Y249A and a phosphomimetic Y249E mutation of the conserved Tyr²⁴⁹ compromised function *in vivo*. While the precise mechanisms causing these impairments will need to be determined in future studies, it is interesting to note that the surfaces for the three moderately conserved motifs pointed in different directions, suggesting that in addition to its role as GDP-dissociation inhibitor, S-domain may serve as a molecular adaptor that participates in intramolecular signal integration.

Identification of an iron binding site within in the GTP-binding domain

Having obtained evidence that the S-domain harbors several functionally important regions, at least one of which (R(W/Y) Φ x Φ xx Φ ExD on H8 helix) affects the domain's ability to stabilize GDP-binding by the G-domain, the question arose whether the functional state of the G-domain may also be subject to control by Fe(II) whose uptake is FeoB-dependent. One way for Fe(II) to modulate the property of the G-domain would be through interference with some aspect of nucleotide binding and/or hydrolysis. Therefore, it seemed reasonable to investigate whether NFeoB contained putative Fe(II)-binding sites. Specifically, we wondered whether

the presence of short ExxE motifs in the sequences of NFeoB were functionally significant since such motifs had previously been shown to function as an iron recognition/signaling motif found in several iron binding proteins (Große et al., 2006; Stearman et al., 1996; Wosten et al., 2000). Notably, an ExxE-motif is conserved within the switch I region of NFeoB of many bacterial species, including all six species listed in Fig. 1B: ³⁹ExxE⁴² (Figure 1B and Figure 5A). To test whether this site could coordinate Fe(II), we exploited the ability of Fe(II) to engage in Fenton-Haber-Weiss reactions that generate highly reactive hydroxyl radicals (•OH) from H₂O₂. As has been shown in other cases, the presence of an Fe(II)-binding site can cause site specific cleavage of the protein within or near the metal binding site (Hlavaty et al., 2000). Performing this reaction on *Ec*NFeoB in the absence and presence of GMPPNP, GTP or GDP showed that cleavage of the polypeptide backbone occurred in the presence of nucleotides (Fig. 6B), with GDP being more efficient in promoting a cleavage competent state of NFeoB. Interestingly, presence of GDP resulted in the formation of two major cleavage products while only one major product was observed in the presence of GTP or GMPPNP. N-terminal sequencing identified cleavage sites within the G1 (asterisk) and G3-motifs (dot) respectively (data not shown), which based on the crystal structure are spatially close to the putative Fe(II)-binding ExxE-motif in switch I.

To obtain further evidence that Glu39 and Glu42 may participate in the formation of a Fe(II)-binding site these residues were mutated to alanine and examined in the Fenton reaction. While removal of either glutamic acid reduced the efficiency of Fenton cleavage of *Ec*NFeoB (Fig. 6A), some cleavage was still observed even in the double mutant *Ec*NFeoBE39A,E42A. This indicated that additional residues may have been involved or that the reactive Fe(II) was bound to the nucleotide's phosphate groups. To address this latter possibility, we asked whether adding increasing amounts of Mg(II) would interfere with cleavage. This would be expected because Mg(II) makes for a better phosphate ligand and therefore should competitively exclude Fe(II) from the nucleotide-binding site. As shown in Fig 6B, this was not the case even at physiological ratios of the two metal ions (mM Mg(II), μM Fe(II)) suggesting that at least some of the Fe(II)-induced cleavage may have been due to interaction of Fe(II) with one or both of the Glu-residues in the ExxE motif. Moreover, genetic complementation assays for E39A and E42A mutations in the context of full-length FeoB caused moderate Fe(II)-deficiency phenotypes, showing that these residues were relevant for the function of FeoB *in vivo* (Fig. 6C). The observation of some protein fragmentation in the AXXA double mutant complicates the unambiguous identification of the Fe²⁺ binding site. Clearly, there are aspects that we do not yet understand. Nevertheless, our data for the first time provide evidence (both *in vitro* and *in vivo*) that iron is likely to modulate the function of the G-domain and thus, these experiments provide an intriguing new perspective for future mechanistic studies.

Discussion

Molecular mechanism for low nucleotide binding affinity of FeoB G-domain

While understudied for over a decade, a recent avalanche of crystal structures of NFeoB (Guilfoyle et al., 2009; Hattori et al., 2009) for the first time allows detailed comparison of these elusive regulators. Based on the sequence identities of the G-domains (ranging from 33-92% between *Kp*NFeoB, *Pf*NFeoB and *Ec*NFeoB (Fig. 1B)) it does not surprise that their structures are virtually identical (rms deviations between the G-domain of *Kp*NFeoB (PDB ID: 2WIB) and the other three species are 0.464 Å, 0.840 Å and 0.964 Å for the *E. coli* (PDB ID: 3I8X), *M. jannaschii* (PDB ID: 2WJG) and *P. furiosus*, respectively). Marking what accounts for the largest difference compared to eukaryotic G proteins, the switch regions point away from the nucleotide-binding pocket, which lessens their interactions with the nucleotide. In the case of switch I, it does not interact with the nucleotide at all, which starkly contrasts with G proteins like p21-Ras (Fig. 7A,C) where switch I obstructs a nucleotide-binding pocket that in

addition is deeper than that observed in FeoB's G-domain (Figure 7B,D). In the case of switch II, we only observe a water-mediated interaction between Asp⁵⁶ the γ -phosphate and the Mg²⁺ ion in *KpNFeoB*•GMPPNP. The failure of the switch regions to extensively engage the nucleotides contributes to a wide open and shallow binding site that explains the much lower nucleotide-binding affinities compared to eukaryotic G proteins.

Interactions of the switch regions with S-domain

In contrast to the G-domain, the S-domain of prokaryotic FeoB is highly variable, with sequence identities as low as 12% between *K. pneumoniae* and *P. furiosus* (Figure 1B). Remarkably, all S-domains assume the same, hammer-shaped five-helix bundle fold. The rms deviations between the S-domain structures of *KpNFeoB* and those of *E. coli* and *P. furiosus* are 0.518 Å, and 1.973 Å, respectively, suggesting that the hammer-shaped five-helix bundle structure is likely to be conserved in all prokaryote FeoBs. Interestingly, the G-domain of *MjFeoB* was determined in the absence of the S-domain. The high structural similarity of *MjFeoB*'s G-domain to those of the *EcNFeoB*, *KpNFeoB* and *PfNFeoB* suggests that the S-domain does not affect the structure of the G-domain with or without bound guanine nucleotide.

Structurally reminiscent of other known G protein regulators, the S-domain functions as a GDP dissociation inhibitor and had previously been shown to interact with both of the G protein's switch regions (Eng et al., 2008). Surprisingly, the biochemically identified interactions between Cys²³⁴ (S-domain) with Gly³⁵ (switch I) and Asp⁶⁷ (switch II) in FeoB from *V. cholerae* (*VcNFeoB*) were not recognizable in the crystal structures as the corresponding residues in *PfNFeoB* (Gly³⁵, Asp⁶⁹, Tyr²⁴⁴) and *KpNFeoB* (Gly³⁵, Asp⁷², Glu²³⁶) were >30Å apart. Regardless of whether this domain arrangement was caused by the absence of nucleotide, or crystal packing interactions, significant changes in the spatial organization of the two domains would be required to implement the previously observed interactions. To address this issue, we repeated the biochemical experiments for *PfNFeoB* and confirmed that in solution, the S-domain of *PfNFeoB* is capable of physically interacting with the switch regions of the G protein (Table II). These findings are puzzling and cannot currently be explained by the crystal structures. However, we did observe that the S-domains of both *KpNFeoB* and *PfNFeoB* showed higher B-factors, suggesting that these domains are more flexible. Hence, it cannot categorically be ruled out that despite their extensive interactions, the G- and S-domains may become unhinged from each other to explore other molecular interaction schemes.

Does NFeoB form a trimeric pore for gating of Fe²⁺ transport ?

As structural information finally begins to emerge for the FeoB system, new hypotheses have emerged proposing that the N-terminal domain forms a regulated intracellular pore for Fe²⁺ (Guilfoyle et al., 2009). Based on the observation that *EcNFeoB* crystallized in a trimeric form with or without bound mant-GTP (Guilfoyle et al., 2009), the hypothesis of a G-protein regulated gating mechanism is tantalizing. Seemingly in support of the hypothesis, the center of the reported trimer forms a pore about 20Å in length and with a diameter of ~1.2Å in the narrowest point. In the apo form (closed conformation), Glu¹³³ effectively blocks the pore, while a slight opening is observed in the mant-GTP-bound form. Interestingly, both apo *KpNFeoB* (Figure 7A) and *KpNFeoB*•GMPPNP (Figure 7B) also formed trimers similar to *EcNFeoB* (Figure 8). Moreover, we observed a Mg²⁺ ion at the center of the pore in both structures and in both cases, the ion was tightly coordinated by each of the crystallographically related subunits (Figure 8D). Overall, these observations are consistent with the trimer Fe(II) gating model (Guilfoyle et al., 2009).

In contrast, *KpNFeoB*•GDP crystallized as dimers and without bound Mg²⁺ even though there were 200 mM MgCl₂ present in the crystallization buffer (Fig. 8E,F). The dimer packed with the nucleotide binding pocket of each protomer facing each other. There are three inter-

molecular hydrogen bond found, two between the O2' and O3' of the GDP ribose with the hydroxyl group of Thr¹⁵¹ and one between O3' and the carboxyl group of Asp¹²³. In addition, intermolecular interactions between helix H5 and G5 motif also contributed to stabilize the dimer. The dimer structure bears some resemblance to those observed for *Mj*NFeoB (Köster et al., 2009) and these features have been compared to those of the ABC transporter (Dawson and Locher, 2006) and the Toc34 GTPase (Koenig et al., 2008) but the exact role of the dimer is not clear at present. Likewise, *Pf*NFeoB crystallized with four independent monomers in the asymmetric unit. Notably, *Pf*NFeoB is evolutionary the most removed of the NFeoB's whose structures have so far been analyzed. The fact that despite a low 12% sequence identity, the structure of the S-domain is virtually the same as that of *Kp*NFeoB strongly suggests that at a mechanistic level, both molecules function in the same way. Therefore, if trimerization and formation of a G-protein regulated intracellular gate were a part of this mechanism, one would expect this mechanistic requirement to dominate the crystallization behavior of these molecules. The inability of *Mj*NFeoB, *Kp*NFeoB•GDP and *Pf*NFeoB to crystallize in the trimeric form, and our redundant observation that *Kp*NFeoB behaves as a monomer in solution by two independent approaches (size exclusion and sedimentation, Supplemental Figure S3 and S4) therefore raise questions about the proposed trimeric pore model and emphasize the need for future studies to resolve this and other questions such as the potentially regulatory role of Fe²⁺ on FeoB function. It is imperative to emphasize that all the above experiments were conducted with the intracellular domain separated from the transmembrane domain of FeoB. A thorough understanding of the function of FeoB will only be accomplished by solving the structures of the entire FeoB protein with and without nucleotides bound.

Conclusion

In conclusion, we report four new FeoB intracellular domain structures (*Kp*NFeoB, *Kp*NFeoB•GDP, *Kp*NFeoB•GMPPNP and *Pf*NFeoB) from two species (*Klebsiella pneumoniae* and *Pyrococcus furiosus*). The results show that there is an extraordinary degree of conservation at the structural level, and identify functionally important regions in the S-domain beyond what has been studied previously. These studies build a case for interactions of the S-domain with both the G-domain and the membrane embedded domain. Moreover, the various crystal forms that were observed cast doubt on the trimer-pore model, and indeed our biophysical studies show that there is no evidence of oligomerization in solution. We further show the presence of a putative Fe(II)-binding motif in the switch I region and that there is nucleotide dependent cleavage. The perspectives raised by our studies show that this story has only begun to emerge, and that a combination of approaches will be necessary to answer the open questions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

GDI	guanine nucleotide dissociation inhibitor
GEF	guanine nucleotide exchange factor
NFeoB	the intracellular domain of FeoB
G-domain	a fragment of FeoB with the G protein fold
S-domain	a fragment of FeoB containing residues between G-domain and the TM-domain
TM-domain	the C-terminal transmembrane domain of FeoB
<i>Ec</i> FeoB	FeoB from <i>Escherichia coli</i>
<i>Kp</i> FeoB	FeoB from <i>Klebsiella pneumoniae</i> , <i>Mj</i> FeoB, FeoB from <i>Methanococcus jannaschii</i>
<i>Pf</i> FeoB	FeoB from <i>Pyrococcus furiosus</i>
<i>Vc</i> FeoB	FeoB from <i>Vibrio cholerae</i>
<i>EcN</i> FeoB	NFeoB from <i>Escherichia coli</i>
<i>KpN</i> FeoB	NFeoB from <i>Klebsiella pneumoniae</i>
<i>MjN</i> FeoB	NFeoB from <i>Methanococcus jannaschii</i>
<i>PfN</i> FeoB	NFeoB from <i>Pyrococcus furiosus</i>
GMPPNP	guanosine-5'[(β,γ)-imido]triphosphate
mant	2'(3')-O-(<i>N</i> -methylantraniloyl)

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Figure 1.

(A) Domain organization of FeoB. (B) Sequence alignment of *Kp*NFeoB (Kp), *Ec*NFeoB (Ec), *Mj*NFeoB (Mj), *Pf*NFeoB (Pf), *Tm*NFeoB (Tm) and *Vc*NFeoB (Vc). Dashed lines represent deletions. Residues in the G protein motifs are colored red. The switch regions are shaded in magenta. Residues in the S-domain are shaded with light grey and the three putative functional motifs are shaded with blue. The secondary structure assignments are also indicated on top of the sequence. Strands are shown as blue arrows and helices are shown as yellow rods.

Figure 2.

(A) and (C): Ribbon representations of the tertiary folding of *KpNFeoB* (A) and *PfNFeoB* (C). The designations of the secondary elements are given and the two domains, G-domain and S-domain are boxed. (B) and (D): Sausage representations of the b-factor of the crystal structures of *KpNFeoB* (C) and *PfNFeoB* (D). The diameter of the sausage is proportional to the b-factor. The structures are generated with the program PyMOL.

Figure 3.

(A) Mapping of the interaction between G-domain and S-domain domains of apo *KpNFeoB*. The secondary elements involved in domain interaction are labeled. Residues involved in domain interactions are connected by grey lines (hydrophobic interactions), red lines (direct hydrogen bonds) or red dotted lines (water-mediated hydrogen bonds). The contact information is analyzed using LIGPLOT (Wallace et al., 1995) based on hydrophobic interaction distance within 2.9 – 3.9 Å and hydrogen bond length within 2.5 – 3.5 Å. (B) Views of the domain interaction interface. The ribbon diagram of the *KpNFeoB*-GDP structure is colored blue and orange for G-domain and S-domain domain, respectively. The residues involved in the hydrogen-bond interactions between G-domain and S-domain domains are indicated on the bottom panels. Wherein, the residues participating in the interactions between switch II region and S-domain are labeled red. The detail interaction between switch II region and S-domain are further highlighted on the upper panels.

Figure 4.

Ribbon representations of the tertiary fold of: (A) Apo *KpNFeoB*; (B) *KpNFeoB*-GDP; (C) *KpNFeoB*-GMPPNP; The hallmark G protein motifs are color-coded as: G1, blue; G2, cyan; G3, magenta; G4, orange; and G5, gold. The nucleotides are represented by color-coded sticks; (D) Close up structure of the nucleotide binding pocket of *KpNFeoB*-GMPPNP. Residues which are hydrogen bonded to GMPPNP are labeled.

Figure 5.

(A) Spatial locations of the putative functional motifs and the mutated residues in the S-domain of *EcNFeoB*. The three motifs are color coded with magenta for motif I, green for motif II and blue for motif III. The putative Fe(II) binding site residues (³⁸ERKE⁴¹) within switch I are also shown in gold. The GDP molecule was shown to label the guanine nucleotide binding pocket. (B) *In vivo* complementation of motif 1- and motif 3-mutant FeoBs. Three 10-fold serial dilutions were plated for each mutant, and results were compared to wild-type FeoB and a FeoB^{P58A}-mutant that had previously been shown to be non-functional. Dark blue colonies indicate severely impaired iron acquisition. Light blue underlay indicates partially functional phenotypes.

Figure 6.

Fenton-Cleavage of NFeoB. Wild type and mutant proteins were subjected to Fe^{2+} -dependent foot printing in the presence and absence of nucleotides, and reaction products were separated by SDS-Page (Panel A). In the presence of nucleotides, two major cleavage products were observed that were generated by cleavage within the G1 motif (asterisk) or G3 motif (dot). Mutations in putatively Fe^{2+} -binding residues reduced cleavage efficiency. The effects of Fe^{2+} were specific and could not be suppressed by increasing amounts of Mg^{2+} (B). *In vivo* complementation showed that mutations in the putative Fe^{2+} -binding site resulted in impaired iron uptake (C).

Figure 7. Nucleotide binding pockets of the GMPPNP-bound p21-Ras (A and C) and *KpNFeoB* (B and D). The switch I regions are colored cyan and nucleotides are represented by colored sticks.

Figure 8.

Crystal packing of the trimeric apo *KpNFeoB* (A) and *KpNFeoB*-GMPPNP (B). The three protomers are shown in different colors. The Mg(II) ion is coordinated to Asp¹³³ in the center pore and is colored magenta. C and D show the close up of the coordination pattern of the Mg(II) ion to Asp¹³³ and water molecules in the pore of *KpNFeoB* (C) and *KpNFeoB*-GMPPNP (D). The electron density in the pore is also shown. (E): Two views of the dimeric crystal packing of *KpNFeoB*-GDP.

Table I

Nucleotide binding and catalytic activities of KpNFeoB

Nucleotide binding affinities of KpNFeoB (Determined by fluorescence titration)					
Ligand	mant-GDP	mant-GDP+Mg(II)	mant-GTP	mant-GTP+Mg(II)	mant-GMPpNP+Mg(II)
K _d (μM)	11.8 ± 1.48	28.1 ± 3.23	196.3 ± 72.49	31.4 ± 4.20	4.1 ± 0.73
GTPase activity of KpNFeoB (Measured by MESG/PNP assays)					
Temperature (°C)		25°C		37°C	
k _m (μM)		141 ± 21.2		167 ± 9.6	
k _{cat} (S ⁻¹)		0.00023 ± 2.7E-5		0.00052 ± 1.8E-5	

Table IICross linking data of *Pf*NFeoB

<i>Pf</i> NFeoB	Cysteine accessibility	Expected	Actual
wt	0.95±0.29	1	1
C93A	0.07±0.06	0	0
C93A, Y244C	1.14±0.07	1	1
C93A, G35C	0.93±0.04	1	1
C93A, G35C, Y244C	0.32±0.17	2	0
C93A, A65C	1.10±0.14	1	1
C93A, A65C, Y244C	1.75±0.17	2	2
C93A, D69C	0.90±0.06	1	1
C93A, D69C, Y244C	0.33±0.09	2	0
C93A, E70C	1.03±0.02	1	1
C93A, E70C, Y244C	1.99±0.12	2	2