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Characterization of a novel prokaryotic GDP dissociation inhibitor domain from the G protein coupled membrane protein FeoB

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Summary

The FeoB family of membrane embedded G proteins are involved with high affinity Fe(II) uptake in prokaryotes. Here, we report that FeoB harbors a novel GDP dissociation inhibitor like domain that specifically stabilizes GDP-binding through an interaction with the switch I region of the G protein. We show that the stabilization of GDP-binding is conserved between species despite a high degree of sequence variability in their GDI-like domains, and demonstrate that the presence of the membrane embedded domain increases GDP-binding affinity roughly 150-fold over the level accomplished by action of the GDI-like domain alone. To our knowledge, this is the first example for a prokaryotic GDI, targeting a bacterial G protein-coupled membrane process. Our findings suggest that Fe(II) uptake in bacteria involves a G protein regulatory pathway reminiscent of signaling mechanisms found in higher order organisms.

Keywords

Bacterial Proteins; GTP-binding protein; Guanine nucleotide dissociation inhibitor; switch region; Fe(II)-uptake

Introduction

Small GTP-binding proteins are well known for their ubiquitous contribution to signal transduction in eukaryotic cells.^{1, 2} In contrast, the mechanistic contribution of prokaryotic G proteins to bacterial physiology remains largely unresolved because in many cases understanding of these G proteins has not advanced past association with global processes, such as ribosome biogenesis, tRNA-modification, progression through the cell cycle or DNA replication.³⁻⁶

The advent of several near atomic resolution structures of prokaryotic G proteins⁷⁻¹⁰ show that these G proteins are structural twins of their eukaryotic relatives. While very helpful in establishing commonalities between prokaryotic and eukaryotic G proteins, the structures have not been able to explain why the nucleotide binding affinities of the majority of prokaryotic

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GTPases are several orders lower than those observed in their eukaryotic counterparts, nor did the structures provide insights into the molecular mechanism by which most prokaryotic G proteins act within bacterial cells. To at least partially resolve these issues, work on the signal recognition particle protein FtsY suggested that prokaryotic G proteins are regulatory and that fast nucleotide release is the manifestation of a domain N-terminal to the GTPase that acts like an intrinsic guanine nucleotide exchange factor (GEF).^{11, 12} While very attractive, this hypothesis has not yet been experimentally verified, nor have distinct GEFs been identified. Similarly, no GTPase Activating Proteins (GAPs), which stimulate GTP hydrolysis, or Guanine Nucleotide Dissociation Inhibitor (GDI)-like interaction partners, which inhibit nucleotide dissociation, have been found that would establish a prokaryotic version of the regulatory G protein cycle, with exception of EF-Tu and EF-Ts that are involved in translation. Apart from the latter two, toxins that are secreted by pathogenic bacteria to diminish host cell resistance to infection through interference with eukaryotic G proteins are the only other documented examples of prokaryotic G protein effectors.^{13, 14}

Prokaryotic uptake of ferrous iron recently emerged as a G protein coupled process involving the membrane protein FeoB, whose soluble, intracellular N-terminal region combines a GTP-binding/GTPase domain and a spacer domain of unknown function.¹⁵ Compared to TrmE, Era and EngA, FeoB provides a narrowly defined physiological readout and thus can be exploited to address structure-function relationships in the TrmE-Era-EngA-YihA-Septin-like superfamily of G proteins, which includes several prokaryotic members of the TRAFAC class of G proteins.¹⁶

In this study we show that just like eukaryotic G proteins, integrity of the switch regions is important for function of the GTP-binding domain of FeoB. Moreover, we show that GDP-binding is stabilized through an interaction of the switch regions with a highly variable linker domain (IIFeoB) that connects the GTP-binding domain (IFeoB) to the membrane embedded part of FeoB (IIIFeoB). We furthermore provide evidence that the presence of the membrane embedded domain further increases GDP affinity, possibly by stabilizing IIFeoB-binding to the switch regions of the GTP-binding domain. Taken together, these findings suggest that the ancient process of ferrous iron uptake involves a crude blueprint for a G protein cycle that over time has evolved into a central regulatory element of cell function in higher organisms.

Results

NFeoB has a higher affinity for GDP than GTP

In our previous study we had used fluorescently labeled mant-GMPPNP, a non-hydrolyzable GTP analog, to estimate GTP binding affinity.¹⁵ When it became apparent that NFeoB (Figure 1(a), residues 1-274 in *Escherichia coli*) may serve a regulatory function, we decided to revisit our original nucleotide-binding experiments, and to explicitly measure mant-GTP-affinity instead of using mant-GMPPNP-affinity as a substitute. Given that the k_{cat} of GTP hydrolysis was 0.0015 s^{-1} at 37°C (Table 1), it seemed unlikely that GTP hydrolysis would constitute a problem during stopped-flow experiments whose duration was limited to a few seconds. To our surprise, we found that in the case of NFeoB, mant-GMPPNP was not a good indicator for GTP-affinity as its dissociation constant was about an order of magnitude lower than the K_d for mant-GTP, which was determined to be $12 \mu\text{M}$. Interestingly, the K_d for mant-GTP also was higher than the mant-GDP K_d previously reported (and confirmed here, of $4.5 \mu\text{M}$ at 10°C (Table 1)), thus reversing the relative binding affinity reported previously to mirror that of other small regulatory G proteins ($= K_{d(\text{GDP})} < K_{d(\text{GTP})}$). Nevertheless, NFeoB's nucleotide binding properties still resembled the Era family of bacterial GTPases in that it featured a fast and spontaneous release of GDP coupled to slow GTP hydrolysis.¹⁷ Notably, even the amended binding affinities would be consistent with the previous suggestion that under physiological conditions ($\sim 0.1 \text{ mM}$ for GDP, 1 mM for GTP¹⁸), NFeoB, but not full-length

FeoB as will be shown later (Table 2) would be mostly in its GTP-bound form. To further test this hypothesis, which relied on the assumption that the binding affinity roughly doubles every increase of 10°C¹⁵, and to ensure that stopped-flow studies of mant-modified guanine nucleotides are representative of the true binding affinities, we performed isothermal calorimetry to measure GDP binding directly (data not shown). The values determined for GDP were consistent with the interpolated values and suggested that the presence of the mant-group did not have a large impact on the nucleotide affinity (data not shown).

Switch I and II regions are important for function

The switch regions of GTP-binding proteins are important in processing guanine nucleotides by interacting with downstream effectors and adopting unique conformations dependent upon what nucleotide species is bound.¹⁹ To assess the significance of the switch regions, we tested whether targeted point mutations interfered with *feoB*-function *in vivo* and whether the phenotypic behavior of these mutants could be understood through changes in the nucleotide binding and enzymatic properties of NFeoB, the soluble N-terminal domain of FeoB (Figure 1(a)). Six mutants in the switch regions – two in switch I (N32A, T37A) and four in switch II (T60Q, Y61E, Y61A, D73A) – were analyzed (Figure 1(b)). The mutations targeted residues/regions known to play important roles at different stages within the nucleotide cycle (GTP/GDP-binding, catalysis, and effector interaction) of regulatory G proteins. In switch I, Asn32 is one of the most highly conserved residues and analogous mutations in p21ras are functionally important.²⁰ The choice of Thr37 was motivated by its contribution to the coordination of a Mg²⁺-ion that through interactions with the nucleotide as well as the switch regions brings together multiple parts of the nucleotide binding site.²¹ In switch II, Thr60 was targeted because the corresponding glutamine residue in p21ras is thought to contribute to catalysis, but not conserved in FeoB. As for the highly conserved Tyr61 and Asp73: the former is adjacent to the G3 motif that is involved in coordination of the phosphates, while Asp73 maps to the part of switch II that undergoes nucleotide dependent structural rearrangements in eukaryotic G proteins.¹⁹ Two additional mutations were also made in the G-motifs important for nucleotide handling: P12G in the G1-motif and “hotspot” for mutations in p21ras²² and P58A in the G3-motif.

Each mutation decreased GDP-affinity at least 2-fold while affecting GTP-binding to a lesser extent (Table 1). The sole exception was the NFeoB^{T37A} mutant, which by losing the coordination for the Mg²⁺-ion had a low affinity for GTP (56.6 μM), even at 10°C. This mutant was unable to restore Fe(II)-uptake in a *feoB*-deficient background and behaved like a mutant in the G4-motif whose binding site had been modified to recognize Xanthine nucleotides instead of GTP/GDP.¹⁵ However, at physiological temperature and substrate concentrations of ~1 mM GTP¹⁸ the NFeoB^{T37A} mutant catalyzed GTP-hydrolysis nearly as efficient as wild-type protein *in vitro* (Table 1), suggesting that Mg²⁺ plays a less important role for catalysis in FeoB than reported for p21-ras.²³ The NFeoB^{N32A} (switch I) and NFeoB^{D73A} (switch II) mutants bound and hydrolyzed GTP with near wild-type efficiency, but were nonfunctional *in vivo* (Table 1 and Supplementary Data, Figure 1(b)), ruling out that GTP-binding and/or hydrolysis alone accounted for the contribution of the GTP-binding domain to FeoB function. Conversely, the low affinity of the NFeoB^{P12G} for GDP *in vitro* did not impair function *in vivo* (Table 1 and Figure 1(b)), indicating that GDP-binding was not a sole determinant in mediating NFeoB function either. Moreover, the NFeoB^{T60Q} (switch II), and NFeoB^{Y61A} (switch II) mutants demonstrated that not every mutation within the switch region was detrimental. However, the opposite effects of the FeoB^{Y61A} and FeoB^{Y61E} mutations *in vivo* suggests that the type of residue at position 61 in the sequence affects G protein function through a mechanism different from nucleotide binding or hydrolysis (Table 1).

The spacer domain does not function as a GEF, but a GDP-binding regulator

Multiple sequence alignments of the highly variable spacer domain that separates the GTP-binding domain from the membrane embedded domain revealed a weakly conserved sequence motif, R(W/Y)Lx(L/I)xxLExD (Figure 1(b) and Supplementary Data, Figure 2), whose LxxxE-motif resembles active site residues observed in structures of GEFs, such as CDC25, DH-domains, Sec7, or RCC1.²⁴⁻²⁶ To rule out the possibility that Glu210 was responsible for the observed fast GDP-release *in vitro*, we generated NFeoB^{E210A} and FeoB^{E210A} mutants to determine the effect of this mutation on nucleotide binding, GTP-hydrolysis *in vitro*, as well as its impact on FeoB function *in vivo*. Similar to all but the NFeoB^{T37A} substitution, mutating Glu210 to Ala affected mant-GDP more strongly than mant-GTP binding. Notably, mant-GDP affinity was lowered from 4.5 μ M to 17 μ M while the mant-GTP affinity decreased from 12 μ M to 21.3 μ M (Table 1). Thus, the weakly conserved, putative GEF-motif found in the spacer domain did not act like a GEF, and was not responsible for the spontaneous and fast release of GDP *in vitro*. However, the failure of FeoB^{E210A} to restore ferrous iron uptake *in vivo* established that despite its lack in sequence conservation across species, the spacer domain plays an important role in FeoB function.

To obtain clues about the mechanistic role of the spacer domain in FeoB function, truncation constructs were made of the N-terminal, cytosolic domains: IFeoB, the core GTP-binding domain (residues 1-170 in *E. coli*); IIFeoB, the spacer region (residues 171-274 in *E. coli*); and NFeoB, IFeoB and IIFeoB together (Figure 1(a)). The constructs were analyzed by far-UV CD analysis to confirm that the core GTPase domain (IFeoB) was folded. Deconvolution of the spectra obtained for IFeoB conformed to the content of secondary structure expected for prokaryotic G proteins, such as Era, and eukaryotic G proteins like p21-ras (Supplemental Data, Table 1). The direct GDP off-rate was determined using competition experiments as an internal control to verify the calculated on- and off-rates from apparent rates of nucleotide binding (Table 2). Using direct GDP-off rates to calculate binding affinity, IFeoB from *E. coli*, *in vitro*, bound mant-GDP with a K_d of 64.7 μ M indicating that the presence of IIFeoB in cis increased mant-GDP affinity by 12-fold to 5.3 μ M (Figure 2(a), *black bars*, and Table 2). Albeit slightly less efficient, this enhancement of mant-GDP affinity was also observed when IIFeoB was added in trans to IFeoB (Figure 2(a), *white bars*). Interestingly, IIFeoB had no significant influence on GTP-binding or the GTPase activity (Figure 2(b), and Table 2). Notably, however an overall enhancement of guanine nucleotide binding affinity was observed in the presence of the membrane embedded domain (Table 2). Yet even in this case, GDP-binding was affected more strongly than the binding of GTP.

IIFeoB's GDP-binding specific effect is conserved in the FeoB family

IIFeoB domains from different species are highly variable in sequence, length and physical properties, such as the isoelectric point (Figure 1(b)) and Supplementary Data, Figure 2). This raised the question whether the GDP-specific function of IIFeoB from *E. coli* represented a property throughout the FeoB-family. To address this question, equivalent constructs from *E. coli*, *Vibrio cholerae* (similar length to that of *E. coli*, yet IIFeoB varies in sequence), and *Helicobacter pylori* (IIFeoB is both shorter and has lower sequence identity) were compared. Like for *E. coli*, the removal of IIFeoB from *V. cholerae* had a minimal effect on GTP affinity, but had a 7-fold effect on GDP affinity (Figure 3(a),(b)). While somewhat less specific and pronounced, IIFeoB from *H. pylori* still affected GDP-binding more strongly than binding of GTP. These results established that a GDI-like activity was a shared property of the IIFeoB domains in these organisms, and therefore seemed to present a genuine function throughout the FeoB-family. Moreover, chimeric full-length FeoB molecules in which the IFeoB and NFeoB domains of the *E. coli* protein was replaced by the corresponding pairs from *Salmonella typhimurium*, *V. cholerae*, and *H. pylori* were non-functional *in vivo* when tested in *E. coli*, with exception of the replacement by the *S. typhimurium* variant (Figure 3(c)) that was partially

functional. The high degree of identity at the sequence level of *S. typhimurium* IFeoB and IIFeoB with the corresponding domains from *E. coli* provides an explanation for the partial functionality of this chimera (Figure 1(b)). At the same time, the failure of the other chimeras to function *in vivo* suggested that the general lack of sequence conservation between IIFeoB domains from different species was accompanied by evolutionary adaptation allowing each pair to function in conjunction with the membrane embedded domain of its own species.

IIFeoB binds the G protein with low affinities *in vitro*

The notion that IIFeoB and the G protein may interact through a defined packing interface implied that this interaction should manifest itself as a measurable binding constant. To test this hypothesis, fully functional N-terminal maltose binding protein (MBP) fusions of the G protein were used as bait for pull-down assays (Figure 4(a)). Varying the concentration of isolated IIFeoB, the apparent steady state binding constant for the interaction was 1.1 ± 0.2 μ M. (Figure 4(b)). Notably, this affinity was comparable with other low affinity effector binding to G proteins,^{27, 28} although in some cases, high nanomolar affinities have been observed for interactions of G protein subunits with their receptors.²⁹ Mutation of the weakly conserved glutamate in this region (Glu210 in *E. coli*) decreased IIFeoB binding to IFeoB by two to three fold (Figure 4(c), *black bars*). This apparent decrease may provide an explanation for why the FeoB^{E210A} mutation was non-functional *in vivo* while, *in vitro*, it bound both nucleotides with affinities comparable to that of the other G protein mutants tested in this study. Regardless, the pull-down assays for both the wild-type and mutant domains suggested that the moderately conserved sequence motif around position 210, R(W/Y)Lx(L/I)xxLExD, forms a platform for interaction with the GTP-binding domain and raised the question whether specific residues could be identified that mediate these interactions.

IIFeoB engages in intramolecular interactions with the switch regions

The ability of IIFeoB to modulate guanine nucleotide affinity and interact with the G protein suggested that IIFeoB exerts its function through interactions with the switch regions. To test this hypothesis, we used spontaneous cross-linking of cysteines to identify regions/residues that are spatially close to each other within the conformational space sampled by the protein. Targeted cysteine mutations were generated in the background of NFeoB from *V. cholerae* because NFeoB of this species contains four accessible cysteines (Cys96, Cys142, Cys234 and Cys254; Figure 1(b) and Supplementary Data, Figure 3) that do not spontaneously form disulfide bonds with each other (Table 3). Single cysteine replacement mutants were created for Gly34 (switch I), Leu62 (switch II), and Asp67 (switch II) (Supplementary Data, Figure 3). If the added cysteine was able to spontaneously cross-link with native cysteine residues already present, then one would expect to measure the equivalent of three instead of five accessible sulfhydryls, even under denaturing conditions. When the NFeoB^{G34C} construct was assayed for cysteine accessibility, using adrithiol as an indicator, 2.6 cysteines were found to be accessible. This suggested that an internal cross-link had formed, but did not reveal which of the four native cysteines acted as partner (Table 3). A NFeoB^{G34C,C234A,C254A} triple mutant was generated to test whether the disulfide involved either of the two native cysteines in IIFeoB, Cys234 and Cys254. If neither of them were involved, then one would expect only one cysteine to remain accessible, as G34C would still disulfide bond with either Cys96 or Cys142 located within the GTP-binding domain. However, analysis of the triple mutant revealed that 3.1 cysteines were accessible ruling out Cys96 and Cys142 as possible interaction partners and demonstrated that NFeoB can adopt conformations that bring IIFeoB spatially close to the switch I region. To identify which specific cysteine in IIFeoB was involved, single Cys234A and Cys254A mutations were generated in the NFeoB^{G34C} background showing that Cys234 engaged in spontaneous disulfide formation with the cysteine placed into position 34. Similarly, the D67C mutation in switch II region was able to form a cross-link with Cys234, yet the second switch II mutant, L62C, failed to crosslink with any of the native four cysteines providing an

internal “negative” control. Taken together these disulfide mapping experiments were consistent with the idea that IIFeoB and IFeoB sample conformations that bring regions of IIFeoB in proximity of the G protein’s switch regions.

Interaction of IIFeoB with the switch I region is responsible for the stabilization of GDP-binding

The finding that IIFeoB can interact with both switch regions raised the question, which, if any, of these interactions were responsible for the selective stabilization of GDP-binding that IIFeoB displayed in stopped flow experiments. Answering this question would be important since the pull-down experiments suggested that the association of the two domains, if provided in trans, is nucleotide independent under steady state conditions (Figure 4(c)). If this apparent independence were caused by kinetic effects due to the low binding affinity of the two domains, then using the disulfide linked mutants not only should circumvent this problem, but also result in a higher affinity, for GDP in particular, than is observed with the wild-type domain. To test this hypothesis, mant-GDP was incubated with constructs derived from NFeoB of *V. cholerae*, and competition experiments were conducted with unlabeled GTP. Dissociation of mant-GDP for wild-type NFeoB from *V. cholerae* fit to a single exponential with an off rate of 18.2 s^{-1} (Table 3). Data obtained for the cysteine mutations fit better to a double exponential (Table 3), whose first and major component was similar to the wild type for the L62C and D67C mutants. In contrast, the G34C showed a 35-fold decrease in GDP-off rate, suggesting that IIFeoB stabilizes GDP-binding through an interaction with the switch I region (Table 3). In contrast, the apparent off-rate for binding of the GTP analog mant-GMPPNP increased 2-fold (from 0.34 s^{-1} for wild-type NFeoB to 0.67 s^{-1} for the mutant), which was consistent with the observation that GDP-binding was affected the most by the presence of IIFeoB.

Discussion

Previous studies from our laboratory established that ferrous iron uptake in bacteria is a GTP-dependent process, involving the unique membrane protein FeoB.¹⁵ However, the contribution of FeoB’s integrated G protein to Fe(II) uptake remained unclear, as well as the role that the membrane embedded domain of FeoB plays in this process. Seeking to understand the contribution of the G protein to ferrous iron uptake, we discovered that integrated into its structure, FeoB contains a novel, prokaryotic specific GDI-like domain in addition to the G protein, suggesting that the ancient process of ferrous iron uptake employs a rudimentary regulatory G protein cycle.

Low nucleotide binding affinity is a property of the GTP-binding domain of FeoB

Low nucleotide binding affinities have been documented for several prokaryotic G proteins, 5, 11, 15, 17, 30 raising questions how these proteins contribute to bacterial physiology. In the absence of interacting proteins, a regulatory role of bacterial G proteins would be restricted to sensing changes in the guanine nucleotide pools that have only been demonstrated to exist in *Bacillus subtilis*.³¹ While such a limited functional scope cannot be ruled out, the alternative – an enzymatic function – seems to falter at the mechanistic level because, similar to eukaryotic counterparts, most prokaryotic G proteins are poor GTPases. For the signal recognition particle receptor, FtsY, structural data provided some rationale for the low substrate affinity towards GTP: in its nucleotide free form, the critical G2-motif, Thr331, points away from the binding site and needs to be released from an interaction with the I-box to allow GTP-binding.⁷ The fast nucleotide release observed was postulated to be caused by an intrinsic guanine exchange factor activity because the nucleotide exchange rates were similar to regulatory G proteins in the presence of exchange factors.¹¹ The first clue that this may not be the case came from an analysis of the *Caulobacter crescentus* CgtA protein, a member of the Obg-family of prokaryotic GTPases. A separate domain located N-terminal of CgtA’s G protein domain was

proposed to serve as a GEF, but deletion of this domain did not significantly alter nucleotide-binding behavior.³¹ The authors concluded that the spontaneous and rapid nucleotide release might be an intrinsic property of the CgtA nucleotide binding-site. This conclusion is supported by the data reported here for FeoB of the TrmE-Era-EngA-YihA-Septin-like superfamily, extending the concept of a genuinely low-affinity G protein core beyond the Obg-family of bacterial G proteins (a titled member of the Obg-HflX superfamily of G proteins¹⁶). While in itself, this raises more questions than it provides answers, it is interesting to note that every mutation analyzed in this study diminished nucleotide binding even further. However, not in all cases did this abolish function *in vivo* (e.g. P12G mutant, Table 1). This suggests that, in some cases, low nucleotide affinity is not a limiting factor and points towards the existence of regulatory mechanisms reminiscent of those found in the regulatory G protein cycle of eukaryotic cells.

Switch regions may serve different purposes in different families of bacterial G proteins

In eukaryotic G proteins, the switch regions have long been known to play key roles in interactions with regulatory proteins. In contrast, the mechanistic contributions of the switch regions in prokaryotic G proteins are far less well understood. To our knowledge, there is only one other report in the literature that investigated the function of prokaryotic switch regions. In this previous study,³² Thr193, the G2 motif in CgtA, was found to be essential for CgtA function. Given the critical role of the G2-motif in nucleotide binding, this finding was not unexpected and, Thr37, the G2 motif in FeoB, also is essential for function. However, in addition to Thr37, Asn32 in switch I of FeoB and Asp73 in switch II, were critical for function *in vivo* as well. Moreover, the Tyr61 mutants suggested that switch II in general is required to produce a functional readout in the case of FeoB. These findings contrast with results obtained for CgtA, where a systematic scan of the switch I region and two targeted mutations in the switch II region did not return other residues as being of equal importance as Thr193. Given that the sequences of the switch regions differ for CgtA and FeoB, but are highly conserved within their respective families, this discrepancy suggests that the extent by which the switch regions contribute to G protein function may vary between superfamilies of bacterial G proteins, and that in the case of FeoB, the role is more closely aligned with the function of a regulatory module.

The presence of internal effectors of FeoB

Since conformational changes of the switch regions, and intra-or intermolecular interactions appear to be important for GTP-binding domain function in FeoB, what would potential interaction partners be? At the genome level, FeoB is flanked by two additional reading frames, *feoA* and *feoC*. However, the absence of *feoA* and *feoC* in many bacterial species and the fact that in *E. coli* complementation with FeoB alone can rescue the ferrous iron uptake defect of strain H1771^{33, 34} argue that FeoA and C do not play major roles in the GTP-binding domain dependent signal. Attempts to crosslink FeoB with other proteins *in vivo* so far have been unsuccessful (data not shown), and may explain the failure of genetic screens to identify other downstream targets of FeoB. In addition, the FeoB operon from *Vibrio cholerae* can complement FeoB-deficient *Shigella flexneri* and *Escherichia coli*³⁴ suggesting that no additional protein factors may be necessary for ferrous iron uptake. If that were true then any interaction partners for the switch regions would need to reside within the FeoB operon, if not FeoB itself. Such a design is intriguing because any such interactions would either have to mimic the action of a GAP in order to accelerate GTP-turnover to a level compatible with a transporter mechanism for ferrous iron uptake, or act like a GDI that would stabilize a putative “off/closed state” were FeoB to act as a receptor or nucleotide regulated iron channel respectively (see Supplementary Data, Figure 4). Consistent with the latter model that the G protein acts as a regulatory domain, the presence of Fe(II) increased the GDP/GTP binding affinity of NFeoB while at the same time, Fe(II) seemed to modestly lower GTPase activity

(Supplementary Data, Figure 5). The significance of these observations will need to be fully established by future work.

Following up on our initial hypothesis that IIFeoB may act as a putative internal GEF, explaining why prokaryotic G proteins bind nucleotides with low affinities (micromolar instead of nano/picomolar), we discovered that this domain actually functioned similar to a GDI. Our results show that *V. cholerae* IIFeoB associates with the GTP-binding core domain through an interaction with the *V. cholerae* switch I region of and increased GDP-affinity by primarily decreasing the dissociation rate for GDP. However, when analyzed *in vitro* the puzzling observation was made that under steady state conditions, association of *E. coli* IIFeoB with the GTP-binding core was not sensitive to the presence of nucleotide. At first sight, this was counterintuitive and seemed to contradict a possible role as regulatory domain. However, taken into account that both the nucleotide binding affinities and the apparent binding constants for the intramolecular domain interaction were in the micromolar range (i.e. fast off rates), the lack of nucleotide dependence in the domain interactions under steady state conditions was less surprising. To be physiologically meaningful, however, one would expect additional factors to exist that are able to modulate the intramolecular interactions. *In vitro*, we were able to mimic such a modulation by introducing a covalent crosslink between IIFeoB and the switch I region from *V. cholerae*, which resulted in a >30-fold decrease in the off-rate for GDP-binding (Table 3). Interestingly, the presence of the membrane domain had a similar effect by increasing affinity for guanine nucleotides while leaving the rate of nucleotide hydrolysis unaltered (data not shown). The relative shifts in nucleotide affinity (~140-fold for GDP, but only ~40-fold for GTP, Table 2) not only suggest that the membrane domain itself is involved in the regulation of the N-terminal G protein but also holds a significant physiological implication in that at physiological guanine nucleotide concentrations (~0.1mM for GDP, 1mM for GTP¹⁸), full-length FeoB would be expected to largely exist in its presumably inactive GDP-bound form. It should be noted, however, that these values are only approximate. This is the case because the restricted availability of protein allowed only collection of a limited number of data points, which in addition were subject to larger errors in the estimation of protein concentrations due to the presence of detergent. Nevertheless, the picture that begins to emerge is that the functionality of IIFeoB bears resemblance to classical G protein regulators such as the β subunit in heterotrimeric G-proteins or GoLoco sequences that act as an effector on G- α_i ^{35, 36} and that the regulatory interaction between the GTP-binding domain and IIFeoB is interrogated/modulated by the membrane embedded domain. Exactly how this cross-talk between the G protein, the GDI-like IIFeoB and the membrane embedded domain takes place remains to be elucidated. However, an intriguing evolutionary perspective is that FeoB may have served as a template for other G protein coupled membrane processes. Vestiges of G proteins involved with iron homeostasis may be found in Ggc1/Yhm1, which are guanine nucleotide exchangers in the mitochondrial inner membrane.³⁷ Moreover, the current study suggests that the integral membrane protein FeoB contains the “blueprint” for the regulatory G protein cycle that in higher organisms has become extremely important in the regulation of membrane associated processes.

Materials and Methods

Plasmids

pET24a-based expression vectors containing the hydrophilic amino-terminus of FeoB (NFeoB, amino acids 1-274) from *Escherichia coli* K-12 with a hexahistidine tag and ptac-based vectors containing FeoB (amino acids 1-773) from *E. coli* with a decahistidine tag (pET24a-NFeoBh₆ and ptac-FeoBh₁₀, respectively) were used as described previously.¹⁵ In the same study, addition of the histidine tag was shown not to interfere with nucleotide binding or impact hydrolysis. pET24a-based expression vectors containing the hydrophilic G protein region

within the N-terminus of FeoB (IFeoB, amino acids 1-170) from *E. coli* and the non-G protein region within the N terminus of FeoB (IIFeoB, amino acids 171-274) from *E. coli* (pET24a-IFeoB₆ and pET24a-IIFeoB₆, respectively) were generated from pET24a-NFeoB₆. NFeoB₆ and IFeoB₆ from *Helicobacter pylori* J99 FeoB (**NP 231709**) and *Vibrio cholerae* N16961 FeoB (**NP 223345**) were generated amplifying DNA from genomic DNA. The DNA was amplified from *V. cholerae* N16961 genomic DNA (ATCC number 39315D; ATCC, Manassas, VA) by PCR using the oligonucleotide primers, FeoB164 (5'-AAGTATCAAGTACTACCGTAGGCAACCCG-3') and FeoB125 (5'-AATCCCTTGTACCAGCAGCTTATGCAATTTTCTT-3') for IFeoB; and FeoB 164 and FeoB100 (5'-GGCTTTTTCGGTGAATTTATGGCTCAGTTTACCCT-3') for NFeoB. For *H. pylori* J99 genomic DNA (ATCC number 700824D) the oligonucleotide primers, FeoB165 (5'-AAAGAAATCATTGTGCGCCCTTGTGGGCCAGCC-3') and FeoB124 (5'-AGTGTTTTGAGAATAAAGCCTGACAATTTTCGTCTA-3') were used for IFeoB; and FeoB165 and FeoB101 (5'-AATCTTATAAGTGTGTTCAAACTCGCATTTTGTT-3') for NFeoB. Maltose binding protein IFeoB fusions were generated from pMAL-c2x vectors (New England Biolabs, Ipswich, MA). Plasmid mutagenesis was performed using the Quikchange™ site directed mutagenesis kit (Stratagene, La Jolla, CA) according to the manufacturer's instructions, and all constructs used in this study were verified by nucleotide sequencing.

Fe(II) Uptake Complementation

Iron uptake was monitored as described previously.¹⁵ Briefly, all measurements were done in H1771, a strain that carries mutations in both *feoB* as well as siderophore biosynthesis genes, and contains a β-galactosidase reporter under control of a Fur repressor.³³ The FeoB wild-type (wt) strain (H1771 carrying a plasmid-borne *feoB* gene, *ptac*-FeoB_{H10}) and FeoB gene mutants (H1771 carrying mutated derivatives of *ptac*-FeoB_{H10}) were grown at 37°C for 16 h on LB-agar; β-galactosidase activity was monitored by adding 0.2 mg/ml X-Gal (American Bioanalytical, Natick, MA) to the LB-agar.

Overexpression and Purification

For overexpression of FeoB constructs, the respective plasmids were transformed into BL21 (DE3) cells (Novagen, Madison, WI), grown in liquid culture under antibiotic selection, and induced for protein expression according to the manufacturer's protocol (Novagen). Cells were harvested, resuspended in 20 mM MOPS/140 mM NaCl, pH 7.5, and lysed by passing the cells through a Z Plus Series cell disrupter (Constant Systems, Ltd, Daventry, United Kingdom). FeoB_{H10} was solubilized from disrupted cells with 1% DDM (Anatrace, Maumee, OH), and 0.025% DDM was used in subsequent buffer systems. Cell debris was removed by centrifugation in a Ti-45 rotor (Beckman Coulter, Fullerton, CA), at 35,000 rpm, for 45 min, at 4°C. The histidine-tagged proteins were purified on a Ni-NTA Superflow agarose column (Qiagen, Valencia, CA) by using resuspension buffer supplemented with 0.3 M imidazole (25°C), as the elution buffer. The maltose binding protein tagged proteins were purified on an amylose column (New England Biolabs) by using 10 mM maltose in 20 mM MOPS/100 mM NaCl/0.5 mM EDTA (4°C) as the elution buffer. To remove imidazole, maltose and/or EDTA, and increase purity, size exclusion chromatography was performed using a Superdex™ 200 column (Amersham Biosciences, Uppsala, Sweden) and a running buffer of 20 mM MOPS/100 mM NaCl, pH 7.0.

Stopped-Flow Measurements

Stopped-flow measurements were used to determine fast binding and the release of fluorescent nucleotides as described previously.¹⁵ Briefly, fluorescent *N*-methylantraniloyl (mant)-GDP, mant-GTP and the non-hydrolyzable nucleoside 5'[(β, γ)-imido]triphosphate derivative mant-GMPPNP (all from Molecular Probes, Eugene, OR) were used as probes. Measurements were

carried out at 10°C in 20 mM MOPS/100 mM NaCl, pH 7.0, by using an Applied Photophysics SX.18MV stopped-flow instrument (Leatherhead, United Kingdom). The mant group was excited at 360 nm and the fluorescence was monitored through a 405-nm cut-off filter. Between 5 and 12 traces were averaged to determine k_{obs} at various concentrations. Nonlinear least-squares fitting of the data was done with software provided with the instrument or with KALEIDAGRAPH (Synergy Software, Reading, PA). To avoid operator specific bias in during data collection and analysis, mutants were analyzed at least twice in a “double blind” study where each mutant was given a number code and all steps (expression, purification, and stopped-flow measurements) were assigned randomly to Eng and Jalilian. Mutants that after decoding of the results showed significant discrepancies were analyzed at least one more time in a directed fashion to determine the cause for the apparent disagreement between the previous, independent determinations.

GTPase Assays

NFeoBh₆ and NFeoBh₆ mutants (5 μM) were added to various concentrations of GTP with 0.5 μCi GTP (γ -P-33) as a tracer. The reaction mixture was brought up to a total volume of 120 μL in buffer (20mM MOPS/100 mM NaCl/5 mM MgCl₂, pH 7.0) at 37°C. At several times after starting the hydrolysis reaction, samples were added to, and mixed with 750 μL of a 5% activated charcoal resuspension in 50 mM NaH₂PO₄, pH 7.0 in 1.5 mL microfuge tubes, prechilled on ice. Microfuge tubes were centrifuged for 8 min at full speed and 600 μL of supernatant was transferred into scintillation vials and mixed with 4ml scintillation cocktail. Samples were counted using a Beckman Coulter LS 6500 multipurpose scintillation counter (Fullerton, CA).

Maltose binding protein pulldown assays

Purified MBP or MBP-IFeoB and IIFeoBh₆ were incubated for 1 hour at 4°C with gentle mixing. To the mixture 30 μL of amylose beads (New England Biolabs) were added and shaken for 1 hour. The amylose resin was recovered by centrifugation and washed four times in wash buffer 20 mM MOPS/200 mM NaCl/4 mM TCEP (Pierce, Rockford, IL), pH 7.0, and were boiled briefly in SDS-PAGE sample buffer to elute proteins for subsequent electrophoresis. The bound proteins were separated by SDS-15% PAGE and coomassie stained. Binding saturation curves were determined using IMAGEJ³⁸ with a BSA standard for protein quantitation and graphed on KALEIDAGRAPH.

Sulphydryl 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-thiopyridone³⁹ 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 $21.3 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$.

Chimera Construction

Wild-type ptac-FeoBh₁₀ SS2 with a unique SacI site introduced after IFeoB and a unique SalI site introduced after IIFeoB served as control construct. The SacI site was introduced by mutating AAT (ptac-FeoBh₁₀ nucleotide region 2038-2040) to CTC introducing a SacI site (gagtc). Forward primer FeoB 146 (5'-GCTATAAAGCTAACGAGctcGTGGAAGTGGTGC-3') and reverse primer FeoB 147 (5'-GCACCAGTTCCACgagCTCGTTAGCTTTATAGCG-3') were used in a QuikchangeTM reaction (Stratagene). The SalI site (gtcgac) was created by two point mutations, A (nucleotide 2349) to C, and T (nucleotide 2351) to C using forward primer FeoB 144 (5'-

CGTTTCACCACTGCGGTcGAcAAAATCGTGCTCAACC-3') and reverse primer FeoB 145 (5'-GGTTGAGCACGATTTTgTCgACCGCAGTGGTGAAACG-3'). To generate compatible fragments from other species, IFeoB and NFeoB primers for *V. cholerae* and *H. pylori* were used as described in the Plasmid section, but with appropriate restriction sites. *S. typhimurium* FeoB (NP_462409) constructs were cloned from *Salmonella enterica* subsp. *enterica* serovar *Typhimurium* LT2 genomic DNA (ATCC number 700720D-5). IFeoB primers were FeoB 158 (5'-AAAAAATTAACCATTGGTTTAATTGGTAATCC-3') and FeoB 159 (5'-GTTAGCGTTGTGACGATCAATGGCCAG-3') and NFeoB primers were FeoB 158 and FeoB 160 (5'-CGCCCTGGTGAAGCGGCTGGGTTTCGG-3') with appropriate restriction sites.

β -Galactosidase Assay

ptac-FeoB constructs were transformed into H1771 and grown to OD₆₀₀ = 0.6 in LB medium containing appropriate antibiotic at 37°C. 50 μ L of cells were used for the measurement of the β -galactosidase activity as described in Ref. 40, with a 5-10 min incubation time before stopping the reaction. Fe(II)-deplete conditions were simulated with 1 mM ferrozine (Sigma-Aldrich), an Fe(II) chelator.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations used

GEF	guanine nucleotide exchange factor activity
GAP	GTPase Activating Protein
GDI	Guanine Nucleotide Dissociation Inhibitor
TRAFAC	translation factor
mant	N-methylanthraniloyl
GMPPNP	guanosine-5'[(β,γ)-imido]triphosphate
MBP	maltose binding protein
X-gal	5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside
LB	Lauria Broth
MOPS	4-morpholinepropanesulfonic acid, 3-morpholinopropanesulfonic acid

Ni-NTA	nickel nitrilotriacetate
TCEP	Tris(2-Carboxyethyl) phosphine
4-PDS	4-pyridyl-disulfide
DDM	n-dodecyl- β -D-maltopyranoside

Figure 1.

Amino acid sequence alignment and structural comparison of representative G proteins. (a) Domain architecture of FeoB: IFeoB, the cytosolic, N-terminal GTP-binding domain, (residues 1-170 in *E. coli* FeoB); IIFeoB, the spacer domain with putative "GDI" function, (residues 171-274 in *E. coli* FeoB); and IIIFeoB the polytopic membrane domain (residues 275-773 in *E. coli* FeoB). NFeoB consists of IFeoB and IIFeoB. (b) Boxshade view of a CLUSTAL W 1.8 sequence alignment of NFeoB (residues 1-274 in *E. coli* FeoB) of FeoB from *E. coli* K12 (GenBank® accession number, [NP_417868](#)), *S. typhimurium* ([NP_462409](#)), *V. cholerae* ([NP_231709](#)), and *H. pylori* J99 ([NP_223345](#)) highlighting important motifs. Identities and >90% similarities between the sequences are indicated by black and grey boxes, respectively. Conserved G protein signature motifs (boxes with the canonical sequence underlined below), and functionally important switch regions (underlined with label) are highlighted. The G4 motif in FeoB differs from the canonical motif with a Met replacing Lys. The question mark for G5 indicates that it could not be unambiguously identified in FeoB, however the GCP (142-144

in *E. coli*) may be a likely candidate.⁴¹ The weakly conserved R(W/Y)Lx(L/I)xxLExD motif in the spacer domain is underlined and labeled.

Figure 2.

Effect of IIFeoB on nucleotide affinity. Bar graphs of (a) mant-GDP and (b) mant-GTP guanine nucleotide binding affinity determined by stopped-flow experiments. (*black bars*) Effect of IIFeoB in cis to the GTP-binding domain with C-terminal his-tagged proteins: “IFeoBh₆” (GTP-binding domain of FeoB) and “NFeoBh₆” (IFeoB with IIFeoB in cis). (*white bars*) Effect of IIFeoB in trans to the GTP-binding domain: “MBP-IFeoB” (GTP-binding domain tagged with a N-terminal maltose binding protein) and “MBP-IFeoB + IIFeoBh₆” (N-terminal maltose binding protein tagged GTP-binding domain with C-terminal his-tagged IIFeoB in trans). Error bars represent the standard deviation from 2-3 independent experiments each with 5-12 trials.

Figure 3.

Nucleotide binding affinity of FeoB constructs and function of FeoB chimeras from various species. Bar graphs of mant-GDP (a) and mant-GTP (b) guanine nucleotide binding affinity. (black bars) Nucleotide binding data of IFeoB, and (white bars) binding data of NFeoB (IFeoB with IIFeoB in cis). Error bars represent the standard deviation from 2-4 independent experiments each with 5-12 trials. (c) Ability of FeoB chimeras to mediate iron uptake in *E. coli* based on the response of a Fe(II)-dependent β -galactose reporter construct (see Supplemental Data, Figure 1(a)). Chimeras were constructed using *E. coli* FeoB as template, and replacing its IFeoB or entire NFeoB by their equivalents from *V. cholerae* or *H. pylori* from various species as described in Materials and Methods, and as indicated in the figure (e.g.

IFeoB chimera *V. cholera* was: *Vc*IFeoB+*Ec*IIFeoB+*Ec*IIIFeoB; NFeoB chimera *V. cholera* was: *Vc*IFeoB+*Vc*IIFeoB+*Ec*IIIFeoB) Increased Fe(II)-uptake represses β -galactosidase activity (see Supplemental Data, Figure 1(a)). *E. coli* FeoB (wild-type) typically has an activity of below 400 Miller units in iron replete conditions and over 1800 Miller units if the reporter strain is grown in presence of ferrozine, an Fe(II) chelator. Error bars represent the standard deviation from 3-6 independent trials.

Figure 4.

Identification of a putative packing interface in IIFeoB under steady-state conditions. (a) Pull down assays with an MBP-IFeoB fusion and IIFeoB. Lanes are input control, “I”; washed out, unbound protein, “W”; and eluted, bound protein, “E”. (b) Binding saturation curve of IIFeoB to IFeoB. (c) Bar graph of saturation curve assays in the absence and presence of nucleotide using an MBP-IFeoB fusion to pull down IIFeoB wt (*white bars*) and E210A (*black bars*). Affinities were calculated from binding curves applying quantitative densitometry on pull-downs with varying amounts of IIFeoB. Error bars represent the standard deviation from 2-5 independent trials.

Summary of nucleotide binding and enzymatic activities of soluble NFeoB mutants, as well as complementation behavior of the corresponding mutations in the context of full length FeoB.

Region	Protein	<u>mant-GTP</u>	<u>Kd, μM^d</u>	<u>Km, μM^b</u>	<u>Kcat, $\text{s}^{-1}b$</u>	Function ^c
-	NFeoB wt	11.7 $\pm$ 3.2	4.54 $\pm$ 0.6	130 $\pm$ 47.8	0.0015 $\pm$ 1.7E-4	Yes
G1	NFeoB P12G	20.9 $\pm$ 1.4	42.1 $\pm$ 4.8	281 $\pm$ 104	0.0009 $\pm$ 0.8E-4	Yes
Switch I	NFeoB N32A	12.7 $\pm$ 2.4	13.2 $\pm$ 0.2	235 $\pm$ 32.9	0.0021 $\pm$ 0.7E-4	No
Switch I, G2	NFeoB T37A	56.6 $\pm$ 6.1	22.5 $\pm$ 0.8	804 $\pm$ 240	0.0020 $\pm$ 1.8E-4	No
G3	NFeoB P58A	23.6 $\pm$ 3.1	34.8 $\pm$ 4.2	153 $\pm$ 36.5	0.0015 $\pm$ 1.1E-4	No
Switch II	NFeoB T60Q	19.7 $\pm$ 1.9	20.2 $\pm$ 0.8	230 $\pm$ 51.2	0.0020 $\pm$ 1.0E-4	Yes
Switch II	NFeoB Y61A	16.2 $\pm$ 1.9	16.5 $\pm$ 3.0	240 $\pm$ 40.1	0.0019 $\pm$ 0.7E-4	Yes
Switch II	NFeoB Y61E	25.8 $\pm$ 3.4	15.2 $\pm$ 1.1	302 $\pm$ 41.2	0.0023 $\pm$ 1.4E-4	No
Switch II	NFeoB D73A	13.3 $\pm$ 0.1	16.6 $\pm$ 0.6	145 $\pm$ 23.5	0.0019 $\pm$ 0.6E-4	No
IIIFeoB	NFeoB E210A	21.3 $\pm$ 1.4	17.0 $\pm$ 1.8	181 $\pm$ 20.6	0.0019 $\pm$ 0.5E-4	No

^a Affinity constants were determined using mant-GTP and mant-GDP at 10°C.

^b GTPase assays were determined using GTP(γ -P-33) at 37°C

^c “Function” was assayed by genetic complementation of the HI771 reporter strain harboring plasmids containing FeoB wt or FeoB gene mutants. “Yes” indicates the mutant complemented and “No” indicates the mutant did not complement. See Supplementary Data, Figure 1 for examples of the genetic complementation.

Table 2

Summary of rate constants and nucleotide binding affinities of wild-type NFeoB.

Substrate	Constant ^a	IFeoB	NFeoB	FLFeoB
mant-GDP ^b	k_{on1} , $\mu\text{M}^{-1}\text{s}^{-1}$	0.94	7.07	20.1
	k_{off1} , s^{-1}	61.2	32.1	16.3
	k_{off1} -direct, s^{-1}	60.8	37.7	0.266
	Kd, μM ^c	64.7	5.3	0.013
mant-GTP	k_{on2} , $\mu\text{M}^{-1}\text{s}^{-1}$	0.93	0.76	32.2
	k_{off2} , s^{-1}	8.41	8.94	9.13
	Kd, μM	9.0	11.7	0.284
GTP ^d	Km, μM	129	130	ND
	kcat, s^{-1}	0.0019	0.0015	ND

^a Binding data with mant-nucleotides were collected at 10°C.^b The mant-GDP data were taken from Ref. 15, but also were independently during this study.^c Binding affinity constants were determined using the directly measured off-rate, which is a concentration independent measurement.^d GTPase assays were determined using GTP(γ P-33) at 37°C

Table 3

V. cholerae NFeoB cysteine mutation cysteine accessibility and nucleotide competition experiments.

NFeoB Mutation	<u>Max</u>	<u>Accessible cysteines</u>	<u>k_{off} (direct, s^{-1})</u>	<u>mant-GDP</u>
		<u>Experimental</u>		<u>k_{off} (direct, s^{-1})</u>
wt	4.0	3.8 + 0.18	18.21 ± 2.16	NA
C234A, C254A	2.0	2.1 + 0.31	ND	ND
G34C	5.0	2.6 + 0.15	0.52 ± 0.25	0.04 ± 0.03
G34C, C234A	4.0	3.5 + 0.24	ND	ND
G34C, C254A	4.0	2.0 + 0.09	ND	ND
G34C, C234A, C254A	3.0	3.1 + 0.22	ND	ND
L62C	5.0	4.7 + 0.13	34.1 ± 1.70	3.67 ± 0.21
D67C	5.0	3.2 + 0.08	19.4 ± 1.79	1.91 ± 0.16
D67C, C234A	4.0	3.8 + 0.13	ND	ND
D67C, C254A	4.0	2.2 + 0.04	ND	ND
D67C, C234A, C254A	3.0	2.7 + 0.32	ND	ND