Potassium-activated GTPase Reaction in the G Protein-coupled Ferrous Iron Transporter B*

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FeoB is a prokaryotic membrane protein responsible for the import of ferrous iron (Fe²⁺). A defining feature of FeoB is that it includes an N-terminal 30-kDa soluble domain with GTPase activity, which is required for iron transport. However, the slow intrinsic GTP hydrolysis rate of this domain appears to be too slow for FeoB either to function as a channel or to possess an active Fe²⁺ transporter. The atomic coordinates and structure factors (codes 3LX5 and 3LX8) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1–S5.

Small regulatory GTPases (G proteins) are key binary switches in cellular processes such as differentiation, proliferation, and cell motility (1, 2). As such, their aberrant functions result in a number of pathophysiological disorders, such as asthma and cancer (3, 4). G proteins cycle between a GDP-bound inactive state and a GTP-bound active state. When bound to GTP, they interact with effector proteins through active conformations of certain polypeptide segments designated Switch I and Switch II (5). The active GTP-bound conformation of the Switch segments are stabilized by their interactions with the nucleotide γ-phosphate and a Mg²⁺ ion at the nucleotide-binding site. Hydrolysis of GTP to GDP releases the γ-phosphate and the Mg²⁺ ion, leading to the relaxation of the Switch I and II segments to their inactive conformations (5).

The prokaryotic G protein-coupled ferrous iron (Fe²⁺) transporter B (FeoB) is unique in that its cytoplasmic G protein domain is directly tethered to a polytopic membrane domain (6). The G protein domain belongs to the TrmE-Era-EngA-YihA-Septin-like (TEES) superfamily of bacterial GTPases, which are recognized by sequence conservation between the G₁ and G₂ motifs. (See Fig. 1A) (7). GTP binding to the G protein domain (FeoGP) initiates the transport of Fe²⁺ across the membrane, which is halted by the hydrolysis of GTP to GDP. Previous studies have shown that FeoGP has slow GTPase activity (Escherichia coli kcat = 0.0015 s⁻¹, equivalent to the hydrolysis of one GTP molecule in 11 min) (6, 8, 9). A slow intrinsic GTPase rate is not uncommon for G proteins, because their activity can be accelerated by GTPase-activating proteins (GAPs), which can stimulate GTP hydrolysis by several orders of magnitude (10). One mode of activation by many GAPs is through the insertion of an arginine residue (arginine finger) into the active site of the G protein, which neutralizes the developing negative charge on the nucleotide during hydrolysis (11, 12). Such GAPs are an essential aspect of the function and regulation of G protein-coupled processes, yet despite extensive efforts, no such GAP or other activating factor has been identified for FeoB. The slow intrinsic hydrolysis rate of FeoB has thus remained somewhat puzzling, being too slow to support an active Fe²⁺ transport mechanism (8). Yet should it rather function as a G protein-coupled channel, the channel would remain in an “open” state for an unfeasible period of time after GTP binding.

To gain further insight into the mechanism of FeoB, we and other groups have recently structurally characterized the soluble G protein domain from four different organisms: E. coli, Methanococcus jannaschii, Thermotoga maritima, and Legionella pneumophila (9, 13–15). The structures revealed a canonical G protein similar to Ras, followed by a helical domain with a proposed effector (9) or GDP dissociation inhibitor role (8, 13). However, in all FeoB structures of the G protein complexed with GTP analogues, the crucial Switch I loop was either disordered (9, 14) or situated far from the nucleotide-binding site, leading to the relaxation of the Switch I and II segments to their inactive conformations (5).
site (13). This indicated that the Switch I region was not essential for nucleotide or Mg\(^{2+}\) binding, consistent with mutational analysis of conserved residues in Switch I of *E. coli* FeoB, which did not significantly affect its GTPase activity (8). Conversely, studies upon full-length FeoB harboring the same mutations in Switch I were unable to restore Fe\(^{2+}\) uptake in a *feoB*-deficient background (6). This apparent disparity has left the role played by the Switch I region in FeoB open.

To probe the function of Switch I in FeoB and to extend the current structural information, we have determined the structure of the 30-kDa soluble domain of FeoB from *Streptococcus thermophilus* (NFeoB\(^{St}\), residues 1–270) complexed with GDP and with the nonhydrolyzable GTP analogue (GDP-\([\beta,\gamma\text{-imido}]\) triphosphate) derivative, mant-GMPPNP. The latter structure reveals an ordered Switch I motif in the canonical, active conformation, in which the loop hydrogen bonds with the nucleotide γ-phosphate and coordinates the Mg\(^{2+}\) ion. Hydrolysis measurements conducted in the presence of K\(^{+}\) show FeoB to be a potassium-activated G protein, with the cation increasing the rate of GTP hydrolysis by up to 20-fold. Furthermore, from structural comparison and mutational analysis, we propose that two conserved asparagine residues (Asn11 and Asn19) facilitate the activation by potassium and that these residues confer the general characteristic of potassium-dependent activation in the TEES superfamily of G proteins.

**EXPERIMENTAL PROCEDURES**

**Protein Preparation and Crystallization**—DNA encoding residues 1–270 from FeoB was amplified from *S. thermophilus* genomic DNA (strain LMG 18311) and cloned into a pGEX-4T1/NFeoB assay method. The concentrated protein was stored at 24 h at 25 °C. NFeoB\(^{St}\) (NFeoB\(^{St}\)-deficient background) was then applied to a Superdex 75 size exclusion column (GE Healthcare Life Sciences), and the eluted protein concentrated to exclusion column (GE Healthcare Life Sciences), and the eluted protein concentrated to 0.2 mM GDP, 2 mM NaF, and 0.2 mM AlCl\(_3\). Crystallization conditions were screened at room temperature using the hanging drop vapor diffusion method with Qiagen JCSG plates (Greiner Bio-One) using a Mosquito nanolitre liquid handling robot (Molecular Dimensions) and contained equal volumes of reservoir solution (150 nl) and protein solution. For mant-GMPPNP-bound NFeoB\(^{St}\), a large crystal plate appeared after 1 week in PACT screen condition D5 (0.1 M MMT buffer, pH 8.0, 25% (w/v) polyethylene glycol 1500), which had the approximate dimensions 350 \(\times\) 100 \(\times\) 20 \(\mu\)m. GDP-bound NFeoB\(^{St}\) crystallized after 3 days in the JCSG + condition H8 (0.2 M NaCl, 0.1 M bis-Tris, pH 5.5, 25% (w/v) polyethylene glycol 3350). The crystal had the approximate dimensions 100 \(\times\) 100 \(\times\) 10 \(\mu\)m.

**Data Collection, Structure Determination, and Refinement**—Single crystals were flash frozen directly into a cold N\(_2\) gas stream (100 K) using 20% (v/v) glycerol in reservoir solution as cryoprotectant. Diffraction data for each crystal were recorded on a Mar345 image plate detector using copper Ka X-rays from either a Rigaku RU-200 (mant-GMPPNP-bound NFeoB\(^{St}\)), or a Rigaku MicroMax-007 HF (GDP-bound NFeoB\(^{St}\)) rotating anode generator. The data were processed and scaled using HKL2000 (16). The structure of NFeoB\(^{St}\) complexed with mant-GMPPNP was solved by molecular replacement using the structure of the G domain of *E. coli* NFeoB as a search model (Protein Data Bank accession code 3HYR, residues 1–168). The waters were removed prior to molecular replacement, and all of the selenomethionine residues were mutated to methionine. CHAINSAW (17) from the CCP4 program suite (18) was used to truncate nonconserved side chains to the gamma atom. Molecular replacement was performed using the program Phaser (19), using diffraction data to 2.5 Å. The G domain was unambiguously positioned with a single unique solution, which had a log likelihood gain of 84 and a Z-score of 7.4. Molecular replacement was then attempted using the helical domain of *E. coli* FeoB (residues 171–260); however, no clear solution could be found. Refinement thus commenced with the G domain only, which represented ~60% of the NFeoB\(^{St}\) structure. Refinement of all structures was carried out using REFMAC5.5 (20) with TLS (21), and all model building was performed in COOT (22). No or cut-off was employed during refinement. After several rounds of refinement of mant-GMPPNP-bound NFeoB\(^{St}\), the electron density was of sufficient quality to manually position the C-terminal helix of the helical domain (residues 242–259), and iterative rounds of manual building and refinement produced a model of 199 residues. Model phases were then input into ARP/warp (23), which built a further 59 residues. The final model contains protein residues 2–161 and 167–265. The structure of GDP-bound NFeoB\(^{St}\) was solved by molecular replacement using Phaser, with the coordinates of mant-GMPPNP-bound NFeoB\(^{St}\) as a search model. Molecular replacement identified a single solution with a log likelihood gain of 174 and a Z-score of 16.5. The final model consists of protein residues 1–258.

**Measurement of GTPase Activity by Malachite Green**—A Malachite Green phosphate assay kit (BioAssay Systems) was used to measure the GTPase activity of wild-type and mutant NFeoB\(^{St}\) in the presence of various monovalent cations. NFeoB\(^{St}\) (0.3 \(\mu\)M) was incubated with 250 \(\mu\)M GTP and 5 \(\mu\)M MgCl\(_2\) at 37 °C in 20 mM Tris, pH 8, and 100 or 200 mM salt (either LiCl, NaCl, KCl, NH\(_4\)Cl, RbCl, or CsCl). After the addition of protein, aliquots were removed at frequent intervals for up to 3 h and mixed in a 1:4 ratio with the Malachite Green.
**K+-activated GTPase Reaction**

Reagent according to the manufacturer’s instructions. For each aliquot, color was allowed to develop for 30 min at room temperature before measuring the absorbance at 620 nm in a 96-well plate (Greiner Bio-One) on a POLARstar Omega microplate reader (BMG LABTECH). The turnover number ($k_{cat}$), was calculated from the slope of each time course. All of the hydrolysis assays were performed in triplicate.

**Measurement of GTPase Activity by Intrinsic Tryptophan Fluorescence**—The rate of GTP hydrolysis by wild-type and mutant NFeoB$^{52}$ in NaCl and KCl were determined by intrinsic tryptophan fluorescence in single-turnover experiments. All of the experiments contained 70 μM NFeoB$^{52}$ and 30 μM GTP and were conducted on a POLARstar Omega microplate reader (BMG LABTECH) in black flat-bottomed 96-well plates (Greiner Bio-One). A 280 ± 5-nm filter was used for excitation, and fluorescence was measured using a 355 ± 5-nm emission filter. NFeoB$^{52}$ was preincubated for 5 min at 37 °C in buffer containing 20 mM Tris, pH 8, and either NaCl (100 or 200 mM) or KCl (50–200 mM). This solution was used to set the relative gain of the plate reader, which was adjusted to give a fluorescence signal of 90% of the maximum possible value. GTP was then added to the mixture, and the fluorescence was monitored for 1 min. The GTPase reaction was initiated with the addition of 1 mM MgCl₂ using the POLARstar reagent injection apparatus. After the addition of MgCl₂, hydrolysis was monitored every 5 s for 4 min or every 20 s for 30 min for reactions performed in KCl and NaCl, respectively. The data were fit to a single-exponential association function using GraphPad Prism 5.0 (GraphPad Software, San Diego, CA), where the rate constant is equal to $k_{cat}$. Because of the presence of a 60-s lag phase in the NaCl reactions, these data were excluded in the calculation of $k_{cat}$ in NaCl. All of the experiments were performed in triplicate.

**Determination of $K_M$ for GTP**—Intrinsic tryptophan fluorescence was also used to determine the binding affinity of wild-type and mutant NFeoB$^{52}$ for GTP. Different concentrations of GTP (0.02–0.8 mM) were added to protein (15 μM) in 20 mM Tris, pH 8, with either 100 mM NaCl or 100 mM KCl at 23 °C. The gain of each sample in the POLARstar plate reader was adjusted to give a signal of 90% of the maximum value. The fluorescence was monitored every 10 s for 1 min, after which time nucleotide binding was initiated with the addition of 3 mM MgCl₂ using the POLARstar reagent injectors. For wild-type measurements conducted in NaCl and for mutant measurements, the slow rate of nucleotide hydrolysis precluded any hydrolysis-related increase in fluorescence on the time scale of the experiment. Therefore, the fluorescence of the samples after MgCl₂ addition was recorded every 10 s for a further minute, and the steady-state fluorescence values (both before and after nucleotide binding) were individually averaged. The relative change in fluorescence for each nucleotide concentration was used to plot a binding isotherm. For wild-type measurements in the presence of KCl, the data were collected every 5 s for 3 min after the addition of MgCl₂. At lower nucleotide concentrations, rapid hydrolysis caused a significant increase in fluorescence after MgCl₂ addition. Therefore, the data were fit to an exponential association function, and the calculated fluorescence at 0 s was used to determine the change in fluorescence upon nucleotide binding. Binding curves were fit to a rectangular hyperbola using GraphPad Prism 5.0, and all of the experiments were performed in triplicate.

**Stopped Flow Fluorescence Assays**—The rates of mant-GMPPNP and mant-GTP binding to mutant and wild-type NFeoB$^{52}$ were measured using a stopped flow apparatus (SF-61; Hi-Tech Scientific Ltd., Salisbury, UK) as previously described (6, 9). Briefly, protein (10 μM in 20 mM Tris, pH 8.0, 100 mM MgCl₂, and either 100 mM NaCl or KCl) was rapidly mixed with 500 μM mant-GMPPNP or mant-GTP (in 20 mM Tris, pH 8.0, and 100 mM of the corresponding salt). To measure the rate of mant-GDP release, protein (10 μM in 20 mM Tris, pH 8.0, and either 100 mM NaCl or KCl) was incubated for 10 min with 0.5 μM mant-GDP and then rapidly mixed with 500 μM GTP in the corresponding buffer. All of the measurements were conducted at 25 °C. The mant fluorophore was excited at 355 nm using a mercury lamp (Osram, Berlin, Germany), and the fluorescence was collected at wavelengths of ≈400 nm using a GG400 glass cut-off filter (Schott, Mainz, Germany). All of the experiments were performed 7–10 times, and the data were averaged before calculating the final $k_{obs}$ or $k_{cat}$ values.

**RESULTS**

**Overall Structure of NFeoB from S. thermophilus**—NFeoB$^{52}$ (residues 1–270) co-crystallized with the nonhydrolyzable GTP analogue mant-GMPPNP in space group C222₁, with one molecule in the asymmetric unit. The crystal diffracted to 1.9 Å, and refinement converged with residuals $R_{work} = 0.197$ and $R_{free} = 0.241$. The final model contains residues 2–161 and 167–265, a mant-GMPPNP molecule, a Mg²⁺ ion, and a glycerol molecule. The mant group from the nucleotide is disordered in the structure and makes no contacts with the protein (supplemental Fig. S1). The GDP-bound form of NFeoB$^{52}$ crystallized in space group P4₁2₁2, with one molecule in the asymmetric unit. The structure was determined and refined to 2.9-Å resolution with $R_{work} = 0.239$ and $R_{free} = 0.298$. Coordinates and structure factors have been deposited under Protein Data Bank codes 3LX5 and 3LX8 for the mant-GMPPNP- and GDP-bound forms, respectively. The data collection and refinement statistics are summarized in Table 1.

Both structures of NFeoB$^{52}$ show the G protein domain (residues 1–170) followed by the helical domain (residues 171–270) (Fig. 1B). The GTPase domain forms the archetypal G protein fold, which consists of a six-stranded β-sheet surrounded by six α-helices. The helical domain is composed of a five-helix bundle (h1–h5), which contacts the G domain via helices h2, h3, and h5. Previous structures of the soluble domain of FeoB have indicated putative biological dimer (13, 14) and trimer (9) configurations, whereas gel filtration indicates NFeoB$^{52}$ is monomeric in solution. In the crystal of mant-GMPPNP-bound NFeoB$^{52}$, a symmetric dimer (generated by symmetry operation $-x, y, -z + 1/2$) lies across a crystallographic 2-fold axis. The dimer interface is formed from the Switch I loop, yet its low total buried surface area (409 Å²/protomer) suggests that it is most likely a product of crystal packing (24). For GDP-bound NFeoB$^{52}$, there are likewise no significant contacts within the crystal that are suggestive of any biologically relevant higher order oligomer, with the largest buried interface area being 376 Å²/protomer.
The Structure of NFeoB<sup>St</sup> Bound to GDP—GDP-bound NFeoB<sup>St</sup> was crystallized from a solution containing the components necessary for formation of the transition-state analogue of GTP (25). Despite intrinsic tryptophan fluorescence indicating that the transition state was successfully formed in solution (supplemental Fig. S2), the resultant crystals contained only GDP-bound protein. The GDP molecule is contacted by a number of residues at the nucleotide-binding site, including Asn<sup>113</sup>, Asn<sup>116</sup>, Ser<sup>16</sup>, and the backbone amides of Asn<sup>11</sup>–Gly<sup>13</sup>. The Switch I loop (residues 24–36) does not interact with the GDP and is instead oriented away from the nucleotide-binding site in its inactive conformation. The loop forms an additional anti-parallel β-strand against the β-sheet core of the protein (Fig. 1B), as has been observed for the GDP-bound forms of NFeoB from other organisms (13, 14). Following the nomenclature of the Ras-related protein Ran (26) and ADP-ribosylation factor 1 (27), where the additional β-strand in Switch I has also been observed, we refer to this strand as β<sub>S</sub>. The Switch Movement in mant-GMPPNP-NFeoB<sup>St</sup>—The core structures of GDP-NFeoB<sup>St</sup> and mant-GMPPNP-NFeoB<sup>St</sup> are highly similar, and superposition of the two structures, excluding the Switch regions, gives a root mean square deviation of 1.2 Å in 218 C<sub>α</sub> positions. However, three principal regions have undergone structural rearrangement between the two forms: the Switch I motif, the Switch II motif, and the helical domain. The most dramatic of these changes is in Switch I, where the β<sub>S</sub> strand has been broken and the loop has been shifted almost 30 Å toward the nucleotide-binding site (Fig. 1B). Now in its GTP-bound orientation, Switch I forms a lid over the nucleotide, the apex of which is comprised of residues 29–33. This lid leaves the nucleotide base and ribose solvent-exposed yet completely buries all three of its phosphate groups (supplemental Fig. S3). There is a distinctive bend in the Switch I backbone near residue 26, which lies at the base of the loop. This bend is caused by two hydrogen bonds between both side chain groups of a conserved asparagine residue, Asn<sup>19</sup>, and the backbone amide and carbonyl groups of Asn<sup>26</sup> from the Switch I loop (Fig. 2A). Not only is Asn<sup>26</sup> fully conserved in FeoB, but it is also a distinguishing feature of all members of the TEES family (Fig. 1A).

The movement of Switch I upon GTP binding is associated with a shift in the orientation of Switch II. The Switch II motif (residues 56–77) consists of a loop and a helix, which lie at the interface with the helical domain. When the protein is bound to GDP, the Switch II region must accommodate the side chains of Trp<sup>31</sup>, Val<sup>34</sup>, and Val<sup>36</sup> from the Switch I loop. Therefore GTP binding and the reorientation of Switch I toward the nucleotide-binding site causes the Switch II helix to shift ~2 Å toward the β-sheet core of the protein. Finally, the helical domains in GDP-NFeoB<sup>S</sup> and mant-GMPPNP-NFeoB<sup>S</sup> are offset between the two structures. There has been a small rigid body movement of the helical domain, which, relative to its position in the GDP-bound structure, has moved up to 4.5 Å toward the Switch II region.

Details of the Nucleotide-binding Site of mant-GMPPNP-NFeoB<sup>S</sup>—The nucleotide-binding site in mant-GMPPNP-bound NFeoB<sup>S</sup> is formed by the G protein signature motifs (G<sub>1</sub>–G<sub>5</sub>), as in other GTP-binding proteins (5). The contacts between the G motifs and the nucleotide are analogous to those observed in the E. coli, M. jannaschii, and T. maritima FeoGP structures (9, 13, 14). The sole exception is the ordered, nucleotide-bound Switch I motif seen in NFeoB<sup>S</sup>, which interacts with both the mant-GMPPNP molecule and the Mg<sup>2+</sup> ion (Fig. 2A and supplemental Fig. S1). This active, GTP-bound conformation of Switch I is mediated by the following protein–nucleotide interactions not observed in the other NFeoB structures: the nucleotide γ-phosphate is hydrogen-bonded with the backbone amides of residues Gly<sup>33</sup>, Val<sup>34</sup>, and Thr<sup>35</sup>, and the backbone amide of Gly<sup>39</sup> forms a long hydrogen bond (3.3 Å) with the α-phosphate. In addition, the side chain of Thr<sup>35</sup> is one of six ligands to the Mg<sup>2+</sup> ion, which is also coordinated by Thr<sup>35</sup> from the G<sub>1</sub> motif, two trans-waters, and one oxygen atom each from the β- and γ-phosphates of the nucleotide (Fig. 2A). Finally, the main chain carbonyls of Gly<sup>29</sup> and Trp<sup>31</sup> indirectly hydrogen bond with the nucleotide via a water molecule (Wat<sup>32</sup>), which acts as a bridge between the Switch I residues, the α-phosphate, and the imido nitrogen from the nucleotide (Fig. 2A).
**K⁺-activated GTPase Reaction**

**A**

|   | FeoB_STRT | E. coli FeoB | MnmE | E. coli | TrmE | Ena_A thermo | EngA | ERA | ERA帮助 |
|---|-----------|--------------|------|---------|------|--------------|------|-----|--------|
| G1 | MTEIALUGNGSNGKLSLLETGH-PRQNGNPGTUSVRKSG-LVKKK | 46 | | | | | | | |
| G2 | MTEIALUGNGSNGKLSLLETGH-PRQNGNPGTUSVRKSG-LVKKK | 46 | | | | | | | |

**B**

**FIGURE 1. Primary sequence and overall structure of NFeoB.** A. Sequence alignment of representative members of the TEES superfamily: *S. thermophilus* FeoB (OSM586), *E. coli* FeoB (P33650), *E. coli* MnmE (P25522), *S. pneumoniae* TrmE (C1CE09), *T. maritima* EnaA (Q09148), *E. coli* EnaA (P0A6PS), *E. coli* ERA (P06616), and *Helicobacter pylori* ERA (P56059). For all of the TEES protein sequences, only the region covering the G1 and G2 motifs and Switch I are included in the alignment. Residues characteristic of the TEES superfamily are highlighted in purple. Other residues discussed in the text are highlighted in blue (Mg²⁺ coordination) and orange (Trp fluorescence). B. Superposition of the GDP-bound (purple) and mant-GMPPNP-bound (gray) structures of NFeoB. Inactive (blue) and active (red) conformations of Switch I and Switch II are highlighted. For black, mant-GMPPNP is shown in a stick representation (green), and the Mg²⁺ ion is shown in black. All of the structural figures were made using PyMOL.

Different from that seen in the GTP-bound forms of the eukaryotic Ras family of G proteins (Fig. 2B). Unlike FeoB, in which Switch I forms four hydrogen bonds with the nucleotide phosphates, members of the Ras family possess only one such hydrogen bond. As a result, the Switch I lid seen in FeoB (supplemental Fig. S3) is absent in the Ras family, instead leaving the GTP moiety at the active site exposed to the solvent (supplemental Fig. S3). Furthermore, Ras family members, which lack an equivalent to the conserved Asn₁⁹ residue, do not exhibit the distinctive bend at the base of the Switch I loop that is present in our NFeoB structure. However, there is almost perfect structural agreement between the active Switch I loop in NFeoB and the GTP-bound Switch I loops from members of the bacterial TEES superfamily, that have been crystallized (supplemental Fig. S3). All of the key interactions that give the Switch I loop its distinctive conformation are conserved between other members of the family and NFeoB, including the four hydrogen bonds between Switch I and the nucleotide phosphates, the Thr⁵⁰-Mg²⁺ interaction, and the hydrogen bonds between Asn₁⁹ and the backbone of Switch I.

**Monovalent Cation Acceleration of the GTPase Activity of FeoB**—The structural superposition of NFeoB with MnmE and other members of the TEES superfamily revealed a high similarity, particularly in the conformation of the Switch I segment (Fig. 2C). Recently it has been reported that the GTPase activity of MnmE is activated via the binding of a potassium ion (28–30). Structural studies revealed the K⁺ ion to be coordinated by carboxyl oxygens from the Switch I motif, the bound GTP molecule, and an asparagine residue that is fully conserved in the TEES superfamily (Asn₁¹ in NFeoB⁵). In the structure of mant-GMPPNP-NFeoB, there is a tightly bound water occupying the same position as the K⁺ ion in MnmE (Fig. 2A). This, in addition to the conserved Switch I conformation between the two proteins, prompted us to explore whether K⁺ or other monovalent cations might also activate the GTPase activity of NFeoB. For the initial GTPase activity measurements, we used a colorimetric assay based on quantification of a green complex formed between Malachite Green, molybdate, and free orthophosphate. The measurements were performed under steady-state maximum velocity (Vmax) conditions, using an excess of GTP. The measurements in the presence of 100 and 200 mM NaCl at 37 °C demonstrated slow reaction rates (kcat = 0.0023 ± 0.0004 and 0.0023 ± 0.0004 s⁻¹, respectively), slightly faster than what has previously been reported for the E. coli protein (kcat = 0.0015 s⁻¹) (6, 9).

We then performed the same measurements in the presence of 200 mM Li⁺, Cs⁺, NH₄⁺, Rb⁺, or K⁺. Interestingly, GTPase activity in the presence of K⁺ was accelerated almost 20-fold (kcat = 0.043 ± 0.003 s⁻¹) relative to the activity in NaCl. Rb⁺ (kcat = 0.205 ± 0.002 s⁻¹) and NH₄⁺ (kcat = 0.034 ± 0.002 s⁻¹), which have similar ionic radii to K⁺, also significantly activated the GTPase activity of NFeoB. Larger (Cs⁺, kcat = 0.003 ± 0.001 s⁻¹) or smaller (Li⁺, kcat = 0.009 ± 0.001 s⁻¹) cations gave no such significant acceleration (Fig. 3A).

**Monitoring GTPase Activity by Intrinsic Tryptophan Fluorescence**—To corroborate the K⁺-dependent activation observed under Vmax conditions, intrinsic tryptophan fluorescence was used to measure GTPase activity under conditions of single turnover. NFeoB contains a single Trp residue (Trp₃¹), which is situated in Switch I. Because Switch I undergoes a large structural rearrangement upon MgGTP₂⁻ binding and GTP hydrolysis (Fig. 1B), it is expected that these structural changes will be reflected in the quantum yield of the Trp fluorophore. In its apo or GDP-bound state, Trp₃¹ is buried within a hydrophobic pocket between the β-sheet core of the protein and the Switch II helix. Upon MgGTP₂⁻ binding, the reorientation of Switch I toward the nucleotide-binding site exposes Trp₃¹ to the solvent (supplemental Fig. S4). One cycle of GTP binding and hydrolysis will thus exhibit two distinct fluorescence.
FIGURE 2. The active Switch I loop structure in NFeoB^{St}. A, stereo view of the GTP binding site. Shown in stick representation are the mant-GMPPNP molecule, the carbonyl oxygens of Gly^{32} and Trp^{31} from the K-loop, and those residues involved in water (red spheres) and Mg^{2+} (dark gray sphere) coordination. The mant group from the nucleotide is disordered in the structure and has been removed for clarity. The water molecule Wat^{33} is situated in the same position as K^{+} in the structure of MnmE. The K^{+} ion, after superposition of NFeoB^{St} and MnmE (Protein Data Bank code 2GJ8), is shown as a transparent yellow sphere. B, the active Switch I loop in Ras-type GTPases. The G domain of NFeoB^{St} is shown in gray, and the mant-GMPPNP-bound Switch I loop in color. red. The active Switch I loops from Ras (Protein Data Bank code 1QRA), Ran (Protein Data Bank code 1RRP), Arf1 (Protein Data Bank code 1J2J), Arf3 (Protein Data Bank code 3BH6), and RhoA (Protein Data Bank code 1A2B) are shown in blue. C, the structure of the GTP-bound Switch I loop in the TEES superfamily of GTPases. Coloring as for B, with the active Switch I loops from MnmE (Protein Data Bank code 2GJ8), EngB (Protein Data Bank code 1SVW), Aquifex aeolicus Era (Protein Data Bank code 3IEV), and Thermus thermophilus Era (Protein Data Bank code 1WF3) shown in blue.

phases, as seen in Fig. 3B. First, MgGTP^{2+} binding induces a sudden drop in fluorescence, because the quantum yield of the tryptophan, now in its GTP-bound conformation, is quenched by the solvent. Hydrolysis of GTP to GDP then causes a gradual increase in fluorescence as Trp^{31} returns to the hydrophobic cavity. The rate of return to maximal fluorescence, via a first order exponential association function, is thus indicative of the rate of GTP hydrolysis. Intrinsic tryptophan fluorescence measurements gave $k_{cat}$ values of 0.0021 ± 0.0003 and 0.0028 ± 0.0002 s^{-1} in 100 and 200 mM NaCl, respectively, agreeing well with the values obtained from the Malachite Green assays. It should be noted that after the addition of MgCl_{2}, the fluorescence time course in NaCl displayed a lag phase of approximately 1 min before hydrolysis commenced and the fluorescence began to increase. This lag phase was absent in the reactions conducted in KCl, in which hydrolysis began immediately after MgGTP^{2+} binding (Fig. 3B). GTPase turnover rates measured in KCl were maximal in 200 mM salt and pH 8.0, with a $k_{cat}$ of 0.044 ± 0.002 s^{-1}. Even in 50 mM KCl, activity was increased ~5-fold ($k_{cat} = 0.013 ± 0.002$ s^{-1}) relative to NaCl.

Potassium Does Not Affect Nucleotide Binding—To ascertain whether K^{+} affected the binding of GTP in addition to its hydrolysis, we performed stopped flow experiments to compare the rate of GTP binding in the presence of either Na^{+} or K^{+}. Because previous studies with E. coli NFeoB have shown that mant-GMPPNP and mant-GTP do not bind with equal affinities (8), we determined the observed association rate constants for both of these GTP analogues. The $k_{obs}$ rates for mant-GMPPNP were 6.2 and 6.0 s^{-1} in Na^{+} and K^{+}-containing buffers, respectively. The $k_{obs}$ rates for mant-GTP were higher, with rate constants of 24.7 s^{-1} in Na^{+} and 24.0 s^{-1} in K^{+} (Table 2). To extend the findings from the stopped flow experiments, the overall affinity of NFeoB^{St} for GTP was then investigated using intrinsic tryptophan fluorescence. GTP binding causes a decrease in the quantum yield of Trp^{31}, and for a given protein concentration, the magnitude of this change is directly proportional to the amount of bound substrate. This may be exploited to construct a binding curve for different concentrations of GTP, and the results in Na^{+} and K^{+} can be compared.
**K⁺-activated GTPase Reaction**

wild-type protein (Table 2), and the dissociation constants for the mutant N11A protein even suggested a slightly tighter GTP binding relative to wild type, with values of 24 ± 2 and 22 ± 2 μM in Na⁺- and K⁺-containing buffer, respectively (Table 2 and supplemental Fig. S5). Although these results show that the mutation did not compromise nucleotide binding, intrinsic tryptophan fluorescence measurements suggest that the K⁺ no longer binds at the nucleotide-binding site. For wild-type protein, the quantum yield of the tryptophan upon GTP binding is quenched ~1.5 times more in the presence of K⁺ than in Na⁺, consistent with a positive charge in the proximity of the fluorophore (supplemental Fig. S5) (31). However, this phenomenon is abolished for N11A, with GTP binding in Na⁺ and K⁺ causing equal fluorescence quenching (supplemental Fig. S5).

Activity studies upon N11A showed a total loss of cation-dependent activation, regardless of the nature of the cation (Fig. 3A). Intrinsic tryptophan fluorescence measurements in 200 mM Na⁺ and K⁺ gave $k_{cat}$ values of 0.0014 ± 0.0002 and 0.0015 ± 0.0002 s⁻¹, respectively. Taken together, these results suggest that Asn¹¹ is critical for K⁺ binding and K⁺-dependent activation.

**DISCUSSION**

FeoB functions as a G protein-coupled Fe²⁺ transporter, with its tethered G protein domain structurally homologous to the small Ras-type family of GTPases. Previous crystal structures of the G protein domain of FeoB have lacked essential details of the GTP coordination sphere, because of disorder or unfavorable crystal contacts in the Switch I loop (9, 13, 14). This shortcoming presented an incomplete picture of, in particular, the involvement of the Switch I region in GTP hydrolysis. Disordered Switch regions are not unusual, in that their conformations are inherently unstable; both solution and solid state NMR experiments have shown that Ras exhibits conformational dynamics of the Switch I loop in its GTP-bound form (32, 33). It is only upon interaction with effectors that the loop exists exclusively in the ordered, active conformation. This could in part explain the disorder observed in the previously published NFeoB-GTP analogue structures, because in the absence of any effectors or the membrane domain, the Switch I loop may exist in two or more conformations in dynamic exchange. However, without further studies, the involvement of the Switch I region in GTP hydrolysis and FeoB function remains unclear. In an attempt to resolve this, we determined the structures of *S. thermophilus* NFeoB complexed with both GDP and mant-GMPPNP and found ordered Switch I and Switch II motifs in both structures. This is the first set of FeoB structures in which both Switch regions have been resolved in both their active and inactive conformations. Comparison of the two structures reveals large conformational changes in the Switch motifs (Fig. 1B), changes that are similar to those that have been observed for Ran and ADP-ribosylation factor 1 (26, 27). In addition, the structure of the mant-GMPPNP complex clearly illustrates the involvement of the Switch I region in GTP and Mg²⁺ coordination. These structural rearrangements indicate that the Switch I and II motifs are likely to be involved in relaying the state of the G protein to the membrane domain. Furthermore, helices h2 and h3 of the helical domain are situated next to Switch II (Fig. 1B) and are in a position where they could act as conveyors of signaling. GTP binding and the structural rearrangements of the Switch motifs are indeed accompanied by a

**TABLE 2**

GTP hydrolysis rates and nucleotide binding properties of wild-type and N11A NFeoB

The nucleotide hydrolysis rates were measured by intrinsic tryptophan fluorescence in 200 mM salt. The errors in the $k_{cat}$ and $K_D$ values are the standard deviations of three independent experiments. The $k_{cat}$ values are the averages of 7–10 measurements. The values are given for NaCl/KCl.

|       | $k_{cat}$ | $K_D$ | $k_{cat}$, mant-GTP | $k_{cat}$, mant-GMPPNP | $k_{cat}$, mant-GDP |
|-------|----------|-------|--------------------|------------------------|-------------------|
| Wild-type | 0.0028 ± 0.0002/0.0044 ± 0.0002 | 38 ± 6/40 ± 7 | 24.7/24.0 | 6.2/6.0 | 12.2/14.1 |
| N11A | 0.0014 ± 0.0001/0.0015 ± 0.00002 | 24 ± 3/22 ± 2 | 16.8/16.8 | 4.3/4.8 | 9.9/11.6 |

**FIGURE 3. Activation of NFeoB by monovalent cations.** A, $k_{cat}$ values for wild-type and N11A NFeoB in the presence of various monovalent cations. All of the GTP hydrolysis rates were measured in 200 mM salt under $V_{max}$ conditions using a colorimetric-based phosphate detection assay. Cations are listed in order of increasing ionic radius: Li⁺ (76 pm), Na⁺ (102 pm), K⁺ (138 pm), NH₄⁺ (144 pm), Rb⁺ (152 pm), and Cs⁺ (169 pm). The errors bars are the standard deviations from three independent experiments, with those for N11A too small to be visualized. B, single-turnover hydrolysis of GTP in the presence of K⁺ and Na⁺, as measured by intrinsic tryptophan fluorescence from Trp³¹. 70 μM NFeoB was incubated with 30 μM GTP, and then nucleotide binding and hydrolysis was initiated with 1 mM MgCl₂ (time 0, dotted line). The drop in fluorescence upon MgCl₂ addition is caused by the reorientation of Trp³¹ in Switch I. As GTP is hydrolyzed, the fluorescence increases as Trp³¹ adopts its GDP-bound conformation. The $k_{cat}$ values are calculated by fitting the data to a first order exponential association function.
movement in the helical domain (Fig. 1B). Although it is difficult to discern whether the movements observed in the helical domain are due to crystal contacts or are a direct consequence of GTP binding, these rearrangements clearly indicate an inherent structural flexibility in the domain.

The intrinsic GTPase activity of FeoB is very slow, which has prompted many questions regarding the mechanism by which it is coupled to Fe\(^{2+}\) transport. In particular, the rate is too slow to be compatible with the active transport of ferrous iron, which led to the suggestion that transport instead occurs via a G protein-regulated channel (8, 9). FeoB has previously been classified into the TEES superfamily of bacterial GTPases, based on high sequence similarity in residues between the G\(_1\) and G\(_2\) motifs (Fig. 1A) (7). This group also includes the evolutionary conserved MnmE protein, which was recently shown to have GTP hydrolysis activity that was drastically accelerated by K\(^+\) ions (29, 30). Here, we similarly show that potassium is required for the acceleration of GTP hydrolysis in NFeoB\(^{52}\). As with MnmE, maximal acceleration (20-fold) was observed at 200 mM K\(^+\), which is close to physiological concentrations (34). A wider monovalent cation screen also illustrated significant GTPase acceleration in the presence of NH\(_4\)^+ or Rb\(^+\) ions, an attribute that is generally observed for K\(^+\)-activated enzymes because of the similar ionic radii of these ions (K\(^+\) = 138 pm, NH\(_4\)^+ = 144 pm, Rb\(^+\) = 152 pm) (35). Larger (Cs\(^+\)) or smaller (Na\(^+\), Li\(^+\)) cations showed no significant acceleration (Fig. 3A). Apart from NFeoB\(^{52}\) and MnmE, K\(^+\)-dependent stimulation has been shown for the ATPase activity of several kinases and chaperones (35). Of these, the chaperone GroEL has the highest K\(^+\) affinity (K\(_d\) = 80 \mu M) and a very large K\(^+\)-dependent ATP hydrolysis activation (10\(^4\)-fold) (36). The crystal structure of GroEL bound to ATP and Mg\(^{2+}\) reveals the K\(^+\) ion to be situated next to the ATP molecule (37).

Structural characterization of MnmE showed that the K\(^+\) responsible for activation was situated close to the GTP molecule, in an identical position to the positive charge provided by the arginine finger in the structure of the Ras-RasGAP complex (29). An asparagine residue situated in the G\(_1\) motif (Asn\(^{226}\) in MnmE) was shown to be critical for the coordination of the K\(^+\) ion. This residue is conserved in all members of the TEES superfamily and corresponds to Asn\(^{11}\) in NFeoB\(^{52}\). We mutated Asn\(^{11}\) in NFeoB\(^{52}\), which abolished the K\(^+\)-dependent activation. In addition, the mutant showed no activation by any of the M\(^+\) ions screened yet retained a similar reaction rate as the wild-type protein in the presence of Na\(^+\) ions. These results strongly indicate that the K\(^+\)-binding site is conserved between MnmE and FeoB and that Asn\(^{11}\) is critical for the coordination of the cation in NFeoB\(^{52}\). Although attempts were made to crystallize NFeoB\(^{52}\) in the presence of Rb\(^+\) or K\(^+\) ions and either mant-GMPPNP or a GTP transition state analogue GDP-AlF\(_4^\text{--}\), crystals could only be grown from mant-GMPPNP-bound protein. Unfortunately, these crystals did not contain any monovalent cation at the nucleotide-binding site, most likely because cation binding is precluded by the derivative imido group between the \(\beta\) - and \(\gamma\)-phosphates. Nonetheless, the current results provide strong evidence that there are direct relationships between: (i) two conserved asparagine residues in the TEES superfamily, (ii) the distinct GTP-bound Switch I conformation associated with the family, and (iii) the propensity to be activated by K\(^+\) ions. These relationships present themselves in the following ways. First, a conserved asparagine residue (Asn\(^{19}\) in FeoB) induces a structurally conserved bend in the active Switch I loop (Fig. 2), forcing the apex of the loop (residues 29–33 in FeoB, also called the K-loop) to cap the nucleotide-binding site and form multiple hydrogen bonds with bound GTP. This nucleotide cap places two backbone carbonyls of the K-loop in the correct orientation to potentially coordinate a K\(^+\) ion (Fig. 2A). Second, another asparagine residue (Asn\(^{11}\) in FeoB), positioned near the K-loop (Fig. 2A), then acts as another ligand for the K\(^+\) ion. This residue facilitates K\(^+\) binding and the concomitant GTPase activation, with three oxygen atoms from the nucleotide phosphates completing the K\(^+\)-binding site (29). Therefore, these combined attributes (Asn\(^{11}\), Asn\(^{19}\), and a conserved K-loop cap) distinguish the TEES superfamily from other G proteins and together suggest that other members of the family, such as Era and EngA, are also likely to show K\(^+\)-dependent activation.

Given the results from the current study, it now appears that the GTPase activity measurements from E. coli FeoB have been carried out under suboptimal conditions in the absence of its activating factor, potassium. This would explain the observation that the E. coli NFeoB T37A mutant protein (equivalent to NFeoB\(^{52}\) T35A) was able to catalyze GTP hydrolysis nearly as efficiently as wild-type protein (8); without any activating element, the measurements reveal only the intrinsic, unstimulated hydrolysis rate, which is likely to be independent of the involvement of the Mg\(^{2+}\)-coordinating threonine residue. Indeed, this has already been demonstrated for the Ras T35A mutant, which hydrolyzed GTP only slightly more slowly than wild-type protein yet was unresponsive to stimulation by a Ras-specific GAP (38, 39).

The results presented here reveal a means of identifying G proteins that possess K\(^+\)-dependent activation. For FeoB in particular, the K\(^+\)-dependent acceleration of GTP hydrolysis brings the maximal rate tantalizingly close to that of some ATP-binding cassette transporters, which use ATP hydrolysis to drive the transport of molecules across the membrane (40–42). The nucleotide hydrolysis turnover numbers for this family of transporters, such as HlyB, MalK, and HisP, are in the range of 0.05–0.9 s\(^{-1}\) (compared with 0.044 ± 0.002 s\(^{-1}\) for NFeoB\(^{52}\)) (40–42). With the GTP turnover rate of FeoB now being close to the hydrolysis rate of the ATP-binding cassette transporters, it opens up the possibility that FeoB could similarly possess an active transport (pumping) mechanism. However, the precise mechanism of coupling between the G protein activity of FeoB and Fe\(^{2+}\) transport will remain an interesting question for future studies.

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