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Structure of the GMPPNP-stabilized NG domain complex of the SRP GTPases Ffh and FtsY

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Abstract

Ffh and FtsY are GTPase components of the signal recognition particle co-translational targeting complex that assemble during the SRP cycle to form a GTP-dependent and pseudo two-fold symmetric heterodimer. Previously the SRP GTPase heterodimer has been stabilized and purified for crystallographic studies using both the non-hydrolysable GTP analog GMPPCP and the pseudo-transition state analog GDP:AlF_4, revealing in both cases a buried nucleotide pair that bridges and forms a key element of the heterodimer interface. A complex of Ffh and FtsY from *T. aquaticus* formed in the presence of the analog GMPPNP could not be obtained, however. The origin of this failure was previously unclear, and it was thought to have arisen from either instability of the analog, or, alternatively, from differences in its interactions within the tightly conscribed composite active site chamber of the complex. Using insights gained from the previous structure determinations, we have now determined the structure of the SRP GTPase targeting heterodimer stabilized by the non-hydrolysable GTP analog GMPPNP. The structure demonstrates how the different GTP analogs are accommodated within the active site chamber despite slight differences in the geometry of the phosphate chain. It also reveals a K^+ coordination site at the highly conserved DARGG loop at the N/G interdomain interface.

The Signal Recognition Particle (SRP) mediates signal peptide recognition and co-translational targeting of secreted and membrane proteins to the membrane translocon (Walter and Johnson, 1994; Keenan et al., 2001). SRP is a phylogenetically conserved ribonucleoprotein that comprises, in prokaryotes, Ffh, the SRP GTPase, and the 4.5S RNA (Luirink and Dobberstein, 1994). The primary structure of Ffh includes three domains, the N and G domains, and the M domain (Bernstein et al., 1989; Römisch et al., 1989). The M domain provides sites for signal sequence recognition and for interaction with the RNA (Zopf et al., 1990; Luirink et al., 1992; Lütcke et al., 1992). The N and G domains of the SRP GTPase, together the 'NG' domain, form a structural and functional unit (Freymann et al., 1997). The membrane associated receptor for SRP is also phylogenetically conserved, and its primary structure includes an NG GTPase as well (Montoya et al., 1997). The two SRP NG GTPases interact directly with each other forming a GTP-dependent heterodimeric targeting complex that plays a central role in co-translational protein targeting to the membrane (Powers and Walter, 1995; Powers and Walter, 1997; Rapiejko and Gilmore, 1997; Song et al., 2000; Mandon et al., 2003).

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Author Contributions

J.G.S. purified the GMPPNP-stabilized complex and setup the crystallization screen; D.M.F. carried out the crystallographic work and wrote the manuscript.

The structure of the SRP GTPase complex in the GMPPCP and GDP:AlF_4 stabilized forms have been determined (Egea et al., 2004; Focia et al., 2004; Focia et al., 2006). The structure is a remarkably pseudo-symmetric heterodimer, in which the two NG domains assemble across their respective GTP binding sites to generate a composite active site chamber that is shared between them. Hydrogen bonding between a γ-phosphate oxygen of each nucleotide analog and the ribose 3′ hydroxyl of the nucleotide analog across the interface means that the two nucleotides contribute directly to the interface, and in the absence of that interaction, the heterodimer does not form (Egea et al., 2004). With GDP:AlF_4 bound, the interaction is with the fluorine atom of the AlF_4 group (Focia et al., 2006), and the putative transition state analog is accommodated within a 'ground state' structure of the active site chamber. The structures of the heterodimer explain key features of the assembly of the SRP GTPase targeting complex, and have revealed coordinated structural changes that occur on assembly that provide insight into the mechanisms by which its assembly, or disassembly by GTP hydrolysis, may be regulated (Shan et al., 2004; Focia et al., 2006).

Interestingly, initial attempts to assemble the SRP GTPase heterodimer for crystallization using the commonly used non-hydrolysable GTP analog GMPPNP failed (Shepotinovskaya and Freymann, 2002), despite its being successfully used in a number of biochemical studies for trapping the interaction between the SRP GTPases (Rapiejko and Gilmore, 1992; Miller et al., 1994; Peluso et al., 2000; Shan et al., 2004). Preliminary data in our laboratory suggested that this failure to stabilize the complex arose from hydrolysis of the nucleotide analog during the long incubation time required for an endogenous proteolysis found to be essential for assembly of the stable complex to occur (Shepotinovskaya and Freymann, 2002). However, the question of whether different nucleotides or nucleotide analogs exhibit different properties for assembly of the SRP GTPase complex has not been fully explored. Interaction specific to the analog GMPPNP in a different GTPase was suggested by a structure of an EF-G complex in which a peptide flip within the P-loop directs a carbonyl oxygen towards the β–γ amido group of the analog (Hansson et al., 2005). A peptide flip of the corresponding residue of the P-loop in Ffh is observed as a minor population in structures of the Ffh NG domain determined ultra-high resolution (Ramirez and Freymann, 2006). Further, the presence of the β–γ methylene bridging group of GMPPCP (substituting for the amido and ester groups of GMPPNP or GTP, respectively) raises the possibility that the GMPPCP-stabilized complex structure might obscure polar or hydrogen bonding interactions present in the native complex. We used insight gained from the structure of the GMPPCP-stabilized *T. aquaticus* Ffh:FtsY complex to generate a fast-assembly deletion construct of FtsY (Focia et al., 2006; Gawronski-Salerno et al., 2006), and have exploited it here to determine the structure of the GMPPNP-stabilized SRP GTPase complex.

Crystallization of the GMPPNP-stabilized complex

The crystal structure of the SRP GTPase heterodimer revealed the nature of a 20 amino acid N-terminal truncation of *T. aquaticus* FtsY that removed an apparent barrier to formation of a stable complex (Shepotinovskaya and Freymann, 2002). The uncharacterized proteolysis of FtsY occurred slowly, over days, but it was necessary for the successful purification (and crystallization) of the complex (Shepotinovskaya and Freymann, 2002; Shepotinovskaya et al., 2003; Egea et al., 2004; Focia et al., 2004). A construct of *T. aquaticus* FtsY in which the first 20 amino acids were deleted (FtsY NGd20) was subsequently expressed and purified, and it allowed us to readily assemble and purify the SRP GTPase complex stabilized using GMPPNP. Ffh NG and FtsY NGd20 were purified as described previously (Shepotinovskaya et al., 2003; Focia et al., 2006). Formation of the FtsY NGd20:Ffh NG complex was assayed using gel filtration over a Sephadex 75 HR 10/30 column, monitoring the A_{280}/A_{260} ratio to distinguish the nucleotide-bound from unbound species, and was found to be essentially complete within minutes, rather than days (Shepotinovskaya and Freymann, 2002). For

crystallization trials the complex was purified by ion exchange chromatography from a reaction mix of 16 μM FtsY NGd20, 21 μM Ffh NG, 1 mM GMPPNP (CalBiochem) in 50 mM HEPES pH 7.5, 2 mM $MgCl_2$, and 50 mM NaCl, incubated at 37°C for 60′. The mix was desalted to remove unbound nucleotide and the complex purified over Q Sepharose using 50 mM Tris, pH 8.0 and a linear gradient to 0.5 M NaCl; subsequently the eluted complex peak was desalted and purified from unbound protein as the flow-through fraction over an SP Sepharose column in 10 mM HEPES 7.5. The purified complex was concentrated to 6 mg/ml using a YM 30 Centricon, yielding 73% of the starting material in the GMPPNP-stabilized heterodimer. The protein was used directly for crystallization trials in a Nextal PEGS screen, using 1μl drops equilibrated against 100μl mother liquor wells assembled with an Apogent Discoveries Hydra II Microdispenser. Blade-like crystals were obtained under the condition: 0.2 M KI, 20% PEG 3350.

A 100 μ × 250 μ crystal was harvested to mother liquor supplemented by 15% ethylene glycol, mounted using a nylon loop, and frozen in LN_2. Data were measured at SBC beamline 19ID, using a wavelength of 1.011 Å, and an exposure time of 12s/0.7° oscillation for 175° rotation at a distance of 160 mm. Data were integrated using HKL2000 (Otwinowski and Minor, 1997), yielding an overall R_{sym} of 0.067 and 99.2% completeness to 1.97 Å (Table 1). The crystal had space group $P2_1$, with two Ffh:FtsY NG heterodimers in the asymmetric unit. The structure was determined by molecular replacement with PHASER (Storoni et al., 2004), using the structure of the GMPPCP-stabilized complex (1OKK) with all ligands and waters removed as the search model. Water molecules were built using ARP/wARP (Lamzin et al., 2001) and the model subsequently refined with REFMAC (Murshudov et al., 1997). The final model has an R_{cryst} of 18.4% and R_{free} 22.7% (Table 1), with 98.7% of residues in the favored region of the Ramachandran plot (Richardson et al., 2003). The N-terminal four residues of both Ffh molecules in the asymmetric unit are disordered, as was observed previously (Focia et al., 2004), due to displacement by the C-terminal helix on formation of the complex. For both FtsY molecules the linker between the N and G domains (residues 79–95) could not be built; however, the linker was not cleaved during complex formation (as there was no long incubation (Shepotinovskaya and Freymann, 2002)) suggesting that it is disordered in this structure. Persistent negative peaks at 28 (of 112 total) glutamate residues were interpreted as decarboxylation caused by radiation damage and were modeled by setting the terminal carboxylate occupancies to 0.5 (Burmeister, 2000). Similarly, radiation damage was found to affect the terminal methylthio groups of two (of 20) methionine sidechains; in addition, the sidechain of Ffh Met^{39} exhibited continuous density suggesting a covalent modification, but the nature of the modification was unclear and it was modeled as an ‘unknown’ atom (data not shown). Finally, the four Mg^{2+}GMPPNP groups, and several well defined solute features - six molecules of ethylene glycol, sixteen I^- atoms and two K^+ ions (see below) - could readily be interpreted from the electron density map. A ribbon diagram of the overall heterodimer structure is shown in Fig. 1A.

Crystal structure of FfhNG:FtsYNGd20:GMPPNP

The structure of the GMPPNP stabilized complex is very similar to that of the GMPPCP complex (PDB 1OKK), and comprises a pseudo-twofold interaction between the N and GTPase domains of Ffh and FtsY that buries a large interface between them that includes the two bound nucleotides (Fig. 1A) (Focia et al., 2004). The nucleotides form the center of a tripartite interaction surface - to one side (left in Fig. 1A) the ‘latch’ region is formed by extensive van der Waals and hydrogen bonding interactions that extend from the N domains of each protein to the adjacent loops of their G domains; on the other side (right in Fig. 1A) the IBD subdomain, an extension of the core GTPase fold unique to the SRP GTPases that contributes much of the catalytic machinery, packs against its partner across the interface and interacts with the bound nucleotide pair. When superimposed over the whole complex, the rmsd between the GMPPNP

and GMPPCP stabilized complexes is 0.65 Å for 548 Cαs, with all large deviations located at the distal loops of the N domain (which tend to be disordered). When superimposed over either the G domain of Ffh or the G domain of FtsY (i.e. the GTPase fold that circumscribes the buried catalytic chamber) the rmsd over Cαs for both is only ~0.22 Å.

Despite the slight differences in configuration of the terminal groups of the two nucleotide analogs GMPPNP and GMPPCP, the structure of the nucleotide binding subunits is essentially identical. The bond and angle parameters for GMPPNP and GMPPCP were monitored carefully during the refinement, based on data from very high resolution protein and small molecule structures (Saenger, 1984; Scheidig et al., 1999). The target β–γ bridging (P-x-P) bond angles and lengths are slightly different (GMPPNP: 122°, 1.70Å; GMPPCP 109.5°, 1.81Å), but these differences result in no striking change in the structures in the active site, or in the adjacent water structure (Fig. 1B). The remarkable symmetric hydrogen bonding interaction between nucleotides across the interface is present (Focia et al., 2004), with symmetric 2.6–2.7Å H-bonds formed between each ribose 3′ hydroxyl and the γphosphate oxygen of GMPPNP bound across the heterodimer interface (Fig. 1C). The water structure between the two is essentially identical as well - the shared (and completely sequestered) 'central' water is present, as are the two corresponding 'nucleophilic' waters at each active center (Focia et al., 2004) (Fig. 1B, 1C). However, the position of the Glu274 sidechain is different between the two structures, which introduces an asymmetry in the water structure at the edge of the active site chamber.

As with the GMPPCP complex (Focia et al., 2004), the GMPPNP-stabilized structure is very similar to that of the GDP:AlF4 complex (PDB 2CNW) (overall rmsd 0.72 Å for 549 atoms, G-domain rmsds of ~0.25 Å over both Ffh and FtsY), although there is also a very slight rotational shift between the two domains along their interface, perhaps to accommodate the larger AlF_4 group and opening of the P-loop buried at the center of their interaction (Focia et al., 2006).

A potassium ion binding site at a conserved loop

The crystal was obtained in the presence of 0.2 M KI, and sixteen iodine atoms were located in the structure, generally associated with nearby arginine and lysine sidechains. The iodine density peaks were typically large, poorly defined, and nonspherical, and so were modeled using anisotropic temperature factors, and their occupancies adjusted manually as necessary to minimize residual negative density. None affects the structure of the latch interface or active site chamber. Two K^+ ions were readily identified in the map by their residual positive density, the coordination bond lengths (~2.8 Å), and the identity of the coordinating groups (i.e. three carbonyl oxygens, three water molecules) (Harding, 2002) (Fig. 2A). The K^+ site is adjacent to the functionally important DARGG loop at the interface between the N and G domains of FtsY (Fig. 2B) and occurs in both of the non-crystallographic symmetry-related FtsY monomers in the asymmetric unit. Both the coordination bond distances and the residual difference density allow us to exclude Mg^{2+} ion at the position, and, indeed, the arrangement is quite typical of potassium - K^+ is generally observed in 6, 7, or 8 coordinate configurations with the most common coordinating group being backbone carbonyl oxygens at a coordination distance of 2.84Å (Harding, 2002). All water molecules in the structure were screened for evidence of similar coordination geometry, but no other K^+ ions could be identified.

The location of the potassium coordination interaction is interesting, because the sequence of the DARGG loop is highly conserved in both Ffh and FtsY and, although it adopts various conformations in the monomeric proteins (Ramirez and Freymann, 2006), on assembly of the complex it adopts one conformation in both Ffh and FtsY (Fig. 2A). The loop links GTPase motif IV, which mediates nucleotide binding specificity (Freymann et al., 1997), to the α4

interface helix, which mediates the interaction between the N and G domains (Ramirez et al., 2002) (Fig. 2B). In Ffh (but not FtsY) the DARGG loop acquires a very well defined interaction with a completely conserved arginine sidechain that extends from the C-terminal helix and that may play a role in positioning the helix on assembly of the complex (Gawronski-Salerno et al., 2006) (Fig. 2A). In FtsY there is no such interaction; however, that the particular conformation of the loop generates a unique and stable (i.e. crystallizable) K^+ coordination site suggests that there may be a functional significance to the interaction there. The crystal structure of the complex obtained after substituting NaI for KI during the crystallization is similar (rmsd over the heterodimer of 0.34 Å, 550 Cαs, over G-domains, ~0.17Å for both proteins), and in that structure there is no coordination of the FtsY DARGG loop (data not shown).

Implications of the structure

The structure of the GMPPNP complex of the SRP GTPases Ffh and FtsY highlights two issues. First, we now have obtained a similar heterodimer structure using three different GTP or transition state analogs - GMPPNP, GMPPCP, and GDP:AlF_4 - and in each case the GTPase heterodimer accommodates the analog pair without significant disruption. This suggests that the stability of the heterodimer is driven by the protein:protein interaction across the interface, particularly along the extensive 'latch' interface that bridges the bound nucleotide pair and the N domains of the two proteins. The K_D measured for both GMPPCP and GMPPNP-mediated assembly of the *T. aquaticus* Ffh NG:FtsY NGd20 heterodimer is ~10 nM (Focia et al., 2006). Further, there is sufficient plasticity in the interface to readily accommodate each of the nucleotide analogs, despite small shifts in the positions of the γphosphate group (to ~0.25 Å between GMPPNP and GMPPCP). We see no evidence for interaction with the β–γ bridging group of the bound nucleotide analogs in this or in previous structures, suggesting that if interactions with the bridging group arise, they must be mediated by the catalytic machinery contributed by the IBD at a subsequent step, during disengagement of the complex rather than its assembly. The only moieties that after slight rotation might be in position to interact with the β–γ bridging groups are the buried sidechains of the conserved arginine pair Arg^{138}/Arg^{142} (Fig. 1B, 1C), and the sidechain of Ffh Gln^{107} (but not the corresponding Asn^{111} in FtsY). There is as of yet no structural evidence that allows us to understand the roles of these residues in the two GTPases.

Second, the structure suggests that different solution ion compositions might affect the structure and biochemistry of the SRP GTPase FtsY due to differential stabilization of a functionally important DARGG loop conformation (Freymann et al., 1997; Ramirez et al., 2002). Preliminary assays for assembly of the Ffh NG/FtsY NG complex in the presence of different cations, however, have not revealed a clear difference in our laboratory (data not shown), and careful quantitative biochemistry of the solution behavior of the complex may be required to discern an effect, if such exists. We note, however, that the standard salt conditions for many reports of SRP GTPase biochemistry includes potassium acetate (Miller and Walter, 1993; Miller et al., 1993; Powers and Walter, 1995). And, interestingly, it has been shown recently that K^+ ion can function as a GTPase activating element for the dimerization-dependent GTPase MnmE (Scrima and Wittinghofer, 2006). That in Ffh, in contrast to FtsY, an arginine at position 290 of the C-terminal helix is present in all sequences suggests that the residue may play a role either in stabilizing the conformation of the DARGG loop in Ffh, or in orienting the C-terminal helix on assembly of the complex. In the heterodimer, conserved Ffh sidechains Arg^{290}, Arg^{286} and Arg^{252} are arranged as a basic 'ladder' along the helix, which may allow the M domain/4.5S RNA component of SRP to regulate assembly (Gawronski-Salerno et al., 2006). Similar interactions do not occur in FtsY, as there are functionally distinct packing interactions between the C-terminal helix and the N/G domain interface (Gawronski-Salerno et al., 2006).

In summary, we have reported the structure of the GMPPNP-stabilized complex of *T. aquaticus* Ffh and FtsY NG domains. The structure is similar to that of the GMPPCP and GDP:AlF_4 stabilized complexes, but demonstrates that the GMPPNP complex can be readily obtained under certain conditions - here, the removal of the 20 amino acid N-terminal peptide of FtsY – and that the three different nucleotide analogs are readily accommodated in similar ways within the shared active site chamber of the SRP GTPase heterodimer. These structures lay the groundwork for subsequent kinetic, thermodynamic, and mutagenesis-based analyses of the mechanism of assembly and regulation of the SRP GTPase targeting heterodimer.

Database Accession

The atomic coordinates and structure factors have been deposited at the Protein Data Bank with ids: 2j7p and r2j7psf, respectively.

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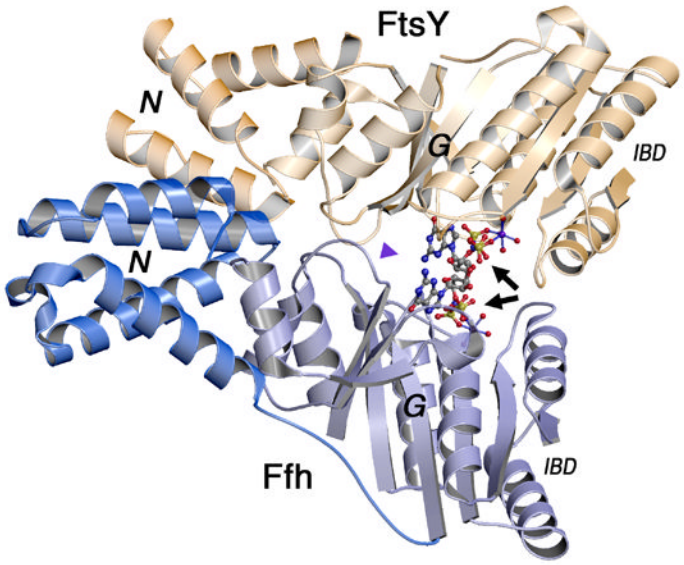

Figure 1. Comparison of the active site structure in Ffh NG:FtsY NG complexes

(**A**) Overall structure of the GMPPNP-stabilized SRP GTPase heterodimer. The two non-hydrolysable GTP analogs are buried within a composite active site chamber at the interface of the two proteins (arrows). The 'N' and 'G' domains of Ffh and FtsY are indicated. The extensive 'latch' interface is at left of the nucleotide pair, and much of the catalytic machinery is provided by the IBD subdomain, at right. The blue arrowhead indicates the point of view in (C). (**B**) The nucleotide analogs bound to Ffh within the active site chambers of the GMPPNP- and GMPPCP- stabilized complexes are superimposed (over the ribose and α-phosphate groups, bracketed). There are only small relative shifts of the two analogs, although the orientations of the terminal phosphate moieties change due to the different β–γ bridging group (-CH2- or -NH-). In neither structure do the bridging methylene or amido groups make direct interactions with sidechain or mainchain atoms or with water molecules. (**C**) Stereo diagram of the sidechain and water interactions of the buried GMPPNP pair. All buried waters and all sidechains that interact with the nucleotides are shown - note that the sidechains are exclusively arranged on the 'IBD' face of the nucleotide pair, as all interactions on the opposite face are mediated by mainchain atoms (and not shown in the figure). The nucleophilic (**n**), auxiliary (**a**), and shared central (**c**) waters are indicated. Figures were made with O, Molscript and Raster3D (Jones et al., 1991; Kraulis, 1991; Merritt and Bacon, 1997).

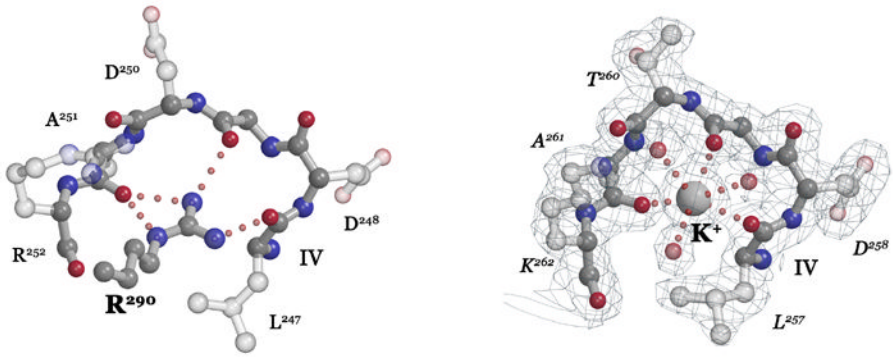

Figure 2. Coordination of the DARGG loop carbonyl crown

(**A**) In Ffh (left), Arg^{290} forms an extensive set of hydrogen bonding interactions with the carbonyl oxygens of the Ffh DARGG loop (which has the sequence Asp^{250}AlaArgGlyGly^{254} in *T. aquaticus* Ffh). In FtsY (right), a potassium ion (large ball) is coordinated by the carbonyl crown contributed by motif IV/DARGG residues *Leu*^{257}, *Gly*^{259} and *Ala*^{261}, and by three water molecules (small red balls). The 2Fo−Fc electron density map is shown with the backbone atoms of residues 257–262 in ball-and-stick. The 'DARGG' loop has the sequence *Thr*^{260}*AlaLysGlyGly*^{264} in *T. aquaticus* FtsY. (**B**) The context of the DARGG loop interactions in (A) are shown in two orientations of a surface representation of the heterodimer. The interactions are at the junction of the N and G domains of each protein, and in Ffh, couple to the position of the C-terminal helix (which in the intact protein is linked to the signal sequence recognition subunit, the M-domain). The C-terminus is indicated (*C*), and the DARGG loop indicated in each case by an *arc*. The figure was made with PYMOL (DeLano, 2002).

Table 1

Crystallographic statistics

Data collection			
Spacegroup	$P2_1$		
Unit Cell	51.03 Å, 129.56 Å, 88.96 Å, β=91.66°		
Resolution	50.00-1.97 Å	*(2.04-1.97)*	
R_{sym}	0.067	*(0.411)*	
Redundancy	3.7	*(3.7)*	
Completeness	99.2	*(98.9)*	
I/σ(I)	17.1	*(2.8)*	
Refinement			
R_{cryst}	0.184	*(0.240)*	
R_{free}	0.227	*(0.290)*	
Protein atoms (#, <B>)	8781		29.1 $Å^2$
GMPPNP atoms (#, <B>)	128		17.7 $Å^2$
Potassium atoms (#, <B>)	2		29.7 $Å^2$
Water molecules (#, <B>)	536		36.3 $Å^2$
rms Bonds	0.011Å		
rms Angles	1.486°		

There are two Ffh:FtsY NG heterodimers in the asymmetric unit. Data in parentheses are for the high resolution shell. <B> is the mean isotropic temperature factor for the atom set.

$R_{sym} = \Sigma|I_h - \langle I_h \rangle| / \Sigma I_h$, where $\langle I_h \rangle$ is the average intensity over symmetry equivalents

$R_{cryst} = \Sigma|F_o - F_c| / \Sigma F_o$. R_{free} is for 5% of the data omitted from the refinement.