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Conformational change of the N-domain on formation of the complex between the GTPase domains of *Thermus aquaticus* Ffh and FtsY

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Abstract

The structural basis for the GTP-dependent co-translational targeting complex between the signal recognition particle (SRP) and its receptor is unknown. The complex has been shown to have unusual kinetics of formation, and association in vivo is likely to be dependent on catalysis by the SRP RNA. We have determined conditions for RNA-independent association of the 'NG' GTPase domains of the prokaryotic homologs of the SRP components, Ffh and FtsY, from *Thermus aquaticus*. Consistent with previous studies of the *Escherichia coli* proteins, the kinetics of association and dissociation are slow. The *T. aquaticus* FtsY is sensitive to an endogenous proteolytic activity that cleaves at two sites—the first in a lengthy linker peptide that spans the interface between the N and G domains, and the second near the N-terminus of the N domain of FtsY. Remarkably, this second cleavage occurs only on formation of the Ffh/FtsY complex. The change in protease sensitivity of this region, which is relatively unstructured in the FtsY but not in the Ffh NG domain, implies that it undergoes conformational change on formation of the complex between the two proteins. The N domain, therefore, participates in the interactions that mediate the GTP-dependent formation of the targeting complex.

Keywords

SRP; Ffh; FtsY; GTPase

1. Introduction

The signal recognition particle (SRP) and its receptor (SR) function in co-translational targeting of nascent proteins to the endoplasmic reticulum of eukaryotic cells [1]. The SRP is a ribonucleoprotein that comprises a number of protein components, one of which, SRP54, both recognizes the signal sequence of the nascent polypeptide and interacts directly with the membrane receptor [2]. SRP54 and SR α are GTPases, with the GTPase activity of each mediated by structurally homologous 'NG' domains in each protein [3–5]. In SRP54 the NG domain occurs N-terminally to the methionine-rich M-domain which mediates signal sequence recognition, and in SR, the NG domain occurs C-terminally to an 'A' domain which appears to mediate membrane association of the receptor [6–8]. The GTP-dependent formation of the complex of SRP54 and SR at the membrane provides a key intermediate in the targeting pathway [1,9–12]. However, the structural basis for the GTP-dependent interaction remains unknown.

Several components of the SRP machinery are universally conserved [2], and homologs of SRP54 (Ffh) and SR (FtsY) are found in prokaryotes [3,13]. The interaction in vitro between the purified components of the prokaryotic targeting complex, Ffh, FtsY, and 4.5S RNA has been characterized extensively [14–17]. It has been shown that the complex can be trapped in the presence of the nonhydrolyzable analog GMPPNP [14], and it has been shown that the 4.5S RNA, which is bound by the C-terminal M-domain of Ffh, plays a catalytic role in the assembly of the complex [16,17]. Assembly of the complex, however, does not require the RNA [16], and, indeed, it appears that assembly requires neither the M-domain of Ffh nor the A domain (when present) of the FtsY, as the interaction between the two proteins is mediated entirely by the two NG GTPase domains [2,4,5]. A number of crystallographic studies have shown that the NG domains of Ffh and FtsY form a structural and functional unit [18–20].

Because the biochemical data implied that a minimal system comprising the two NG domains should exhibit interactions similar to those of the intact proteins and that the kinetics of the association in the absence of the RNA would be slow, we reasoned that a minimal complex of the Ffh and FtsY NG domains from *Thermus aquaticus* could be readily purified and would provide a useful substrate for further studies. The task was simplified because the FtsY from *T. aquaticus* comprises only the NG domain (as has been observed in a number of species [21,22]). We found that, consistent with the behavior of the *Escherichia coli* Ffh/FtsY complex [16], both formation and dissociation of the NG domain complex proceeded slowly, allowing us to establish conditions for purification and stabilization of the complex in the presence of a nonhydrolyzable GTP analog, GMPPCP. However, we also found that the *T. aquaticus* FtsY is susceptible to specific cleavage by an endogenous protease activity at two sites. The first is located in a linker peptide between the N and G domains that is predicted to be relatively disordered in the *T. aquaticus* protein. The second is near the N-terminus, and, remarkably, cleavage at this site appears to require formation of the Ffh/FtsY complex. When placed in the context of the crystallographic structures of the Ffh and FtsY NG domains these data provide direct evidence for re-arrangement of the N domain of FtsY on formation of the Ffh/FtsY NG complex.

2. Materials and methods

2.1. Purification of the NG domains

The NG domain of *T. aquaticus* Ffh was expressed and purified as described previously [18]. The *T. aquaticus* FtsY, which comprises only the 304 residue NG domain, was expressed from a pET3-derived expression plasmid, pTP88 (obtained from C. Reyes and P. Walter, UCSF), maintained in the BL21(DE3)/RP strain of *E. coli* (Stratagene). A starter culture of 50 ml LB medium containing carbenicillin (100 µg/ml) and chloramphenicol (34 µg/ml) was inoculated from an isolated colony and the culture grown to an OD₆₀₀ of 0.2 before being resuspended in 2 ml of media. This was then used to inoculate 4 l of LB with antibiotics, as above, and growth was continued at 37 °C until OD₆₀₀ = 0.3, when expression was induced using IPTG (1 mM). Cells were harvested at OD₆₀₀ = 0.8 and resuspended in 30 ml of buffer containing 20 mM EPPS (pH = 8.0), 250 mM NaCl, 2 mM EDTA, 10% glycerol, and 0.1 mg/ml lysozyme. After three liquid nitrogen freeze/thaw cycles, the cell paste was disrupted by sonication and then placed at 70 °C for 20 min. Cell debris and precipitated proteins were removed by centrifugation at 14,000 × *g* for 30 min at 4 °C. The supernatant was passed over a 50-ml DE53 column equilibrated with 20 mM Tris, pH 8, 250 mM NaCl, and the flow-through dialyzed against 50 mM HEPES, pH 7.5, 2 mM MgCl₂ overnight. The dialysate was loaded on a 60 ml column of S-sepharose FF equilibrated with the same buffer, and the protein eluted using a linear gradient to 1 M NaCl. Fractions containing protein were pooled, aliquoted and stored at –20 °C. Samples were

evaluated by SDS-PAGE under reducing conditions, and were estimated to be at least 95% pure. Approximately 30 mg of protein were obtained from 4 l of culture.

2.2. Complex formation reactions

Ffh and FtsY NG domains were concentrated and the buffer exchanged using Centricon 30 devices (Millipore). Incubations for complex formation were carried out using 10 μ M FtsY and 15 μ M Ffh NG in 50 mM HEPES (pH 7.5), 2 mM MgCl_2 , and 50 mM NaCl ('low-salt' conditions) or 300 mM NaCl ('high-salt' conditions). GMPPCP (β - γ -methyleneguanosine 5' triphosphate (Sigma)) was added to a final concentration of 1 mM. The K_d for the GMPPNP-dependent complex of the *E. coli* Ffh and FtsY is on the order of 50 nM [16]; we assumed similar affinities between the *T. aquaticus* proteins and therefore maintained a 5- μ M excess of Ffh NG in all complex association reactions. During the initial characterization of the behavior of complex formation, test reactions (100 μ l) were incubated over a range of conditions, temperatures (25–70 °C) and times (1 h–7 days). For production of the complex, a 5-ml reaction was incubated at 37 °C for 4 days. Protein concentrations were determined using a Bradford protein assay (Bio-Rad) standardized by quantitative amino acid analysis. Nucleotide concentration was determined by absorbance at 252 nm.

2.3. Purification of the Ffh/FtsY NG complex

Complex formation was monitored using gel filtration chromatography on a Superdex 200 HR 10/30 column (Amersham Pharmacia). The column was equilibrated with 50 mM HEPES (7.5), 2 mM MgCl_2 , and 50 mM NaCl. On this column the Ffh/FtsY NG complex is resolved from the two monomeric species, Ffh NG and FtsY NG (which elute together), and from unbound nucleotide analog. The complex peak could be distinguished as well by a change in the A_{280}/A_{254} ratio due to the presence of bound nucleotide. The components present in the peak fractions were analyzed by SDS-PAGE under reducing conditions.

The Ffh/FtsY NG complex was purified for preparative purposes by anion exchange chromatography. The 5-ml reaction mixture was loaded directly onto a 1.6-ml Poros HQ column (Perseptive) equilibrated with 50 mM Tris, pH 8.0, and the complex eluted using a linear gradient of 1 M NaCl in the same buffer. The Ffh/FtsY NG complex eluted after the NG domain of Ffh, at approximately 200 mM NaCl. The yield of the purified complex was ~ 75% of the maximum theoretical yield of the reaction (i.e., corresponding to 10 μ M complex in the mixture). Peaks containing the complex (at ~ 0.4 mg/ml) were pooled, and the complex stabilized by addition of MgCl_2 and GMPPCP to a final concentration of 1 mM. Complex was aliquoted, frozen, and stored at –20 °C.

2.4. Proteolysis studies

For studies of proteolytic degradation the FtsY NG domain was incubated at 37 °C for 4 days in parallel with the complex formation reactions. The incubation mixture contained 10 μ M FtsY in 50 mM HEPES, pH 7.5, 2 mM MgCl_2 , and 50 mM NaCl ('low-salt' condition) or 300 mM NaCl ('high-salt' condition). GDP or GMPPCP were added to a final concentration of 1 mM. Protease Inhibitor Cocktail containing 4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF) (at 0.2 mM final concentration), *trans*-epoxysuccinyl-L-leucylamido(4-guanidino) butane (E-64) (0.14 μ M), bestatin (0.13 μ M), leupeptin (0.1 μ M), aprotinin (0.03 μ M) and sodium EDTA (0.1 mM) was obtained from Sigma. Samples were analyzed by SDS-PAGE on a 10% Bis-Tris NuPAGE gel (Novex) and visualized by Colloidal Blue staining (Invitrogen). Cleavage products were blotted onto PVDF membrane (Novex) using standard methods and submitted for 5–7 cycles of N-terminal amino acid sequencing.

2.5. Mutagenesis of the FtsY NG domain

A double mutant of FtsY (R17Q/L18M and R87Q/K88Q/L89I) was constructed using PCR site-directed mutagenesis (QuikChange, Stratagene). Two mutagenic oligonucleotides were obtained from Operon: the first was 5'-

GCCAAGACCCGCGAGCAGATGCTGAAGGCCATTCCCTGG-3', and the second, 5'-CGCCGGGCCACCCTTCAGCAGATTGGCTTCAACCC-3'. The presence of the mutations in the expression clone was confirmed by DNA sequencing. The FtsY double mutant was expressed and purified in the same manner as the wild-type protein.

3. Results

3.1. Slow formation of the complex

In our initial trials we assumed a K_d for the complex of ~ 50 nM based on studies of the *E. coli* proteins [16], and maintained a 5- μ M excess ($100 \times K_d$) of one component during all reactions. Formation and dissociation of the *E. coli* Ffh/FtsY complex was found to be very slow in the absence of the 4.5S RNA [16] so in our trials samples were incubated for a wide range of times (to 7 days). The test samples contained 10 μ M FtsY, 15 μ M Ffh NG, and 1 mM GMPPCP, in 50 mM HEPES (pH 7.5), 2 mM $MgCl_2$, 50 mM NaCl. The two ~ 32-kDa proteins were expected to form an ~ 65-kDa complex which could be readily distinguished by gel filtration using a Superdex 200 chromatography column. When samples were incubated for a short time interval (5 h at 70 °C, overnight at 42 °C and RT) no complex was detected (Fig. 1a). However, after 3 days' incubation at 37 °C, a peak migrating with a higher molecular weight was detected on the chromatogram, and the relative ratio of the peak heights continued to increase after 4 days' incubation time (Fig. 1b,c). The two peaks were well resolved, with apparent molecular weights of 36 and 64 kDa based on the positions of protein standards, consistent with the predicted sizes of the monomeric proteins and the heterodimeric complex. Peak fractions were analyzed by SDS-PAGE, and revealed that both the slower eluting species (the two monomeric proteins) and faster eluting species (the Ffh/FtsY NG complex) contained both the Ffh and FtsY NG domains, as expected (see Fig. 3).

3.2. Complex formation is nucleotide triphosphate and Mg^{2+} dependent

Formation of a stable Ffh/FtsY complex was dependent on the presence of the nonhydrolyzable nucleotide triphosphate analog as no complex was observed when the two proteins were incubated in the absence of added nucleotide, or in the presence of GDP (Fig. 1e). (Note that intrinsic GTPase activity of the thermophilic proteins was sufficient to obviate use of GTP in these assays.) In order to eliminate the possibility of homodimer formation during the incubation, the two proteins were incubated individually in the presence of GMPPCP. Under these conditions no new peak was detected (Fig. 1d). Finally, while both Mg^{2+} -dependent and Mg^{2+} -independent nucleotide binding modes have been demonstrated for the Ffh NG domain [23,24], the GTP hydrolysis activity of Ffh and SRP54, like other GTPases, requires the presence of magnesium [25–28]. That Ffh and FtsY each reciprocally stimulates the other's GTPase activity [29] implies that Mg^{2+} , which binds at a specific site, should be required for assembly of the Ffh/FtsY NG complex. This appears to be the case, as complex formation is dependent on the presence of magnesium, and addition of EDTA to the reaction mixture causes disappearance of the complex peak (Fig. 1f).

3.3. Slow dissociation kinetics

In order to evaluate the kinetics of dissociation of the Ffh/FtsY NG complex, reaction mixtures were incubated at room temperature for different time intervals in the presence of

excess GDP (Fig. 2). The experiment exploits the observations that nucleotide exchange is rapid in the SRP GTPases and the affinities for GDP are five to 10-fold higher than for GTP [14,15,30,31]. Therefore, we expected that binding of GDP to the monomeric NG domains would make dissociation of the complex irreversible. This conversion of complex to monomers was then monitored using anion exchange chromatography. We found that after 18 h of incubation with GDP, 50% of the initial complex remained intact (Fig. 2). These data are consistent with the studies of the *E. coli* Ffh/FtsY complex [16] where the half-life for the GMPPNP-stabilized complex in the absence of 4.5S RNA was found to be approximately 15 h.

3.4. Cleavage of the FtsY NG domain

During our characterization of the FtsY NG domain, we noticed a specific proteolysis event upon prolonged storage that yielded two products of apparent molecular weights ~ 22 and ~ 9 kDa on SDS-PAGE. During the 37 °C incubations required for complex formation the protein proved to be susceptible to this proteolysis as well, both in the absence of the Ffh NG domain (Fig. 3a, lane 4), and in its presence (Fig. 3a, lane 5). Thus, when the complex obtained under 'low-salt' (50 mM NaCl) conditions was purified, it was found to contain the intact Ffh NG domain, but only the two products of cleavage of the FtsY NG domain (Fig. 3a, lane 5) migrating at ~ 22 and ~ 6 kDa (see below). The cleavage was not affected by the presence or absence of GDP or GMPPCP and it was not prevented by addition of a protease inhibitor cocktail (Sigma) (data not shown). Subsequently, however, we found that incubation under 'high-salt' conditions (300 mM NaCl) inhibited this proteolysis event (which we term cleavage at site '1'). Thus, after incubation at 37 °C for 4 days in high salt buffer, samples that contained the FtsY NG alone remained intact (Fig. 3b).

3.5. An alternate pattern of cleavage in the complex

From these results, we expected that under 'high-salt' conditions we would be able to purify complex containing intact FtsY. However, when we carried out complex formation reactions with buffer containing 300 mM NaCl we observed a new species that migrated between the intact FtsY NG domain and the Ffh NG domain on SDS-PAGE (Fig. 3b, lane 4). In some of our reactions, complex formation did not go to completion, and in these cases, the unpurified reaction mixture included both the intact FtsY NG domain and its clipped product. Remarkably, however, only the clipped species co-purified with the complex on column chromatography (Fig. 3b, lane 5). Incubation of the two proteins in the absence of nucleotide or in the presence of GDP under the same conditions did not yield the clipped product (Fig. 3b, lane 3). Consistent with these observations, a closer examination of the purified complex obtained under 'low-salt' conditions reveals that the smaller of the two products of FtsY proteolysis is shifted relative to that obtained in the absence of the complex (Fig. 3a, lane 5). This result suggests that cleavage at a second site (which must be near the N or C terminus) is associated with formation of the complex. We term this second site '2'. Cleavage at site 1 occurs in the free FtsY NG domain and presumably prior to or independently of complex formation, and it is inhibited by salt. Cleavage at site 2 is insensitive to salt concentration and occurs only in association with formation of complex. Therefore, protein recovered from purified complex is clipped at position 2, but depending on the salt concentration, may or may not be cleaved at position 1.

3.6. Sequences locate two cleavage sites

In order to map the two cleavage sites, the products of proteolysis of the FtsY NG domain were blotted for N-terminal amino acid sequencing (Fig. 4). Two cleavage positions were identified unambiguously, but, interestingly, each sequence revealed a set of two staggered cleavage sites. Position 1 was determined from the sequence of the ~ 22-kDa band (see Fig.

3a), which indicated that cleavage occurs between Arg87 and Lys88 or between Leu89 and Gly90 at approximately equal frequency (based on their relative presence in the sequencing data). Position **2**, which occurs only in the presence of the Ffh/FtsY NG complex, was determined from the sequence of the clipped ~ 30-kDa FtsY NG fragment obtained under 'high-salt' conditions, and subsequently from the sequence of the ~ 6-kDa product obtained after cleavages at sites **1** and **2** under 'low-salt' conditions (see Fig. 3). The sequences indicated cleavage between Leu18 and Leu19 or between Lys20 and Ala21 depending on whether the sample was prepared under 'low-salt' (predominantly 18/19) or 'high-salt' (predominantly 20/21) conditions.

3.7. Mapping the cleavage sites on the three-dimensional structure

Because there is substantial sequence conservation between the members of the SRP GTPase subfamily (e.g., the sequences of the *E. coli* and *T. aquaticus* Ffh NG domains are 48% identical) we can locate the cleavage positions in the protein fold by using the available structures of the *T. aquaticus* Ffh NG domain [18], and the *E. coli* FtsY NG domain [19] (Fig. 5). The first site, **1**, maps to a linker region between the N and G domains in both structures. This region shows substantial sequence variation between members of the SRP GTPase family [32], and it is relatively mobile and solvent-exposed in the crystal structures of the SRP GTPases [18,19]. In the sequence of the *T. aquaticus* FtsY the linker is particularly long, and it is not unreasonable to propose that the cleavage site **1** maps to a large disordered loop that is a solvent exposed and flexible. The second site, **2**, maps in the structure of Ffh to a turn between the α N1 and α N2 helices of the N domain. Interestingly, however, it is here that the arrangement of the polypeptide is quite different between the structures of the Ffh NG and the FtsY NG. In FtsY, there is no long α N1 helix and the region is much less regular, such that the polypeptide is displaced towards its N terminus (Fig. 5). Cleavage site **2** maps to a region of the polypeptide that is packed in an irregular conformation against the hydrophobic core of the N-domain, at a position corresponding to that occupied by the α N1 helix in Ffh NG. That cleavage occurs there suggests that the polypeptide is conformationally variable in the different states of the protein, as we propose below.

Despite cleavage of the linker peptide between the N and G domains of FtsY, the proteolytic fragments co-purify during gel filtration and anion-exchange chromatography, demonstrating that after cleavage at site **1** the FtsY NG domain remains intact. This behavior is consistent with the presence of numerous of tight packing interactions between the N and G domains of Ffh and FtsY [19,23]. Furthermore, gel filtration chromatography of the complexes generated under 'low-salt' and 'high-salt' conditions (and cleaved at both positions **1** and **2**, or at position **2** alone (see above)) showed that the two complex species migrate almost identically. This observation suggests that there is no further degradation of FtsY after cleavage at site **1**, a point which we cannot establish by the sequencing data alone because we do not define the C-terminal sequence of either of the products of that cleavage. This conclusion is supported as well by the fact that complex formation is dependent on the presence of the nucleoside triphosphate analog, implying that there is a functional and intact interaction between the two proteins under both 'low-salt' and 'high-salt' conditions.

3.8. Mutations suggest proteolysis is sequence-independent

Because susceptibility to cleavage appeared to be associated with a pattern of basic residues we generated neutral mutants at each of the susceptible sites (Fig. 4). The mutation introduced at site **1** was R87Q/K88Q/L89I, and the mutation introduced at site **2** was R17Q/L18M. Studies of the SRP54 in *S. pombe* [33] indicate that mutations introduced into regions of the yeast SRP54 close to these sites had little effect on growth rates in yeast, and so we expected the mutations to be tolerated in the *T. aquaticus* protein. Indeed, the proteins

expressed from plasmids containing the mutations were stable, and could be purified using the 70 °C incubation that was used for the wild-type protein. Remarkably, however, despite mutation of the cleavage sites, we observed the same pattern of proteolysis at sites **1** and **2** under low- and 'high-salt' conditions. The cleavage product corresponding to site **1** was submitted for N-terminal sequencing, which revealed the site of the cleavage, between residues 87 and 88, to be identical to that seen in the wild-type protein despite the presence of the new sequence.

4. Discussion

The GTP dependent interaction between SRP and its receptor is mediated primarily through their NG domains [4,5]. In this work, we have demonstrated that the NG domains of the two proteins Ffh and FtsY are sufficient for stable interaction in the presence of the nonhydrolyzable GTP analog GMPPCP, and that 4.5S RNA and the M domain of the *T. aquaticus* Ffh are not strictly required for their association. We find that the association and dissociation of the complex are slow, consistent with and supporting the conclusions of previous studies of the *E. coli* Ffh/FtsY complex [16]. Finally, we demonstrate changes in the pattern of proteolytic susceptibility of the *T. aquaticus* FtsY that imply that a conformational change of the N-domain of FtsY accompanies the GTP-dependent formation of its complex with Ffh.

In Ffh, the N-terminal α N1 helix extends the full length of the domain, packing against three other helices to form a well-defined four-helical bundle. In FtsY, although there is clear structural correspondence with helices α N2, α N3 and α N4 (see Fig. 5), the N-terminal polypeptide exhibits an irregular packing arrangement such that the N-terminus of the protein (which in the *E. coli* FtsY structure is truncated from the additional 'A' domain) is packed around the C-terminal helix and displaced from the N domain. Cleavage site **2** maps to a region of irregular conformation, but this region does not appear to be disordered in the crystal structure, consistent with the fact that the peptide is stable to proteolysis in the absence of formation of the Ffh/FtsY complex.

A specific cleavage event that occurs only in the presence of the Ffh/FtsY NG complex implies strongly that there is a conformational change that makes site **2** vulnerable to proteolysis, and therefore that the N domain must be involved, directly or indirectly, in the structural rearrangements that occur during nucleotide dependent complex formation. Interestingly, mapping the cleavage site **2** onto the structure of Ffh NG places it at a terminal loop known to be highly flexible (and accessible to exogenous proteolysis [34]), suggesting that the conformational change might correspond to movement of the FtsY N domain helical structure towards that of the Ffh N domain. However, this is certainly not the only mechanism by which cleavage could be enabled—an alternative that simply exposes the cleavage site to solvent by displacement of the N terminal peptide might be equally appealing, so that the small peptide product does not remain associated in the complex, or is degraded. Whether this might be of physiological significance cannot yet be addressed. However, we note that the first 20–30 amino acids of the sequences of the FtsY NG domains diverge between species and from the sequences of the Ffh NG domain [32], raising the possibility that the region has few, or different, functional and structural constraints.

Interestingly, a membrane-dependent cleavage in the linker peptide between N and G domains of *E. coli* FtsY has been reported previously [7]. In that case, a novel membrane-bound isoform of FtsY composed of only the A and N domains was observed, and proteolysis was proposed to be due the action of a membrane-associated protease, although a candidate for this membrane-associated protease remains uncertain. Changes in the pattern of proteolysis of *M. mycoides* Ffh on binding nucleotide have been demonstrated as well

[34]. In that study, binding was accompanied by a change in susceptibility due to a conformational change that can be inferred, from comparison of the corresponding crystal structures [23], to reflect the movement of the 'closing loop' against the bound nucleotide. In the *T. aquaticus* Ffh and FtsY, in the absence of added proteases, we do not see a similar pattern of cleavage.

The nature of the proteolytic activity seen in our experiments remains unclear. Cleavage appears to be sequence independent, as mutations of the cleavage sites failed to prevent proteolysis at the same position. As noted above, the cleavage sites may be distinguished instead by being particularly disordered and solvent exposed, such that the peptide backbone could become accessible to cleavage by a relatively nonspecific protease or proteolytic contaminant. However, we have not excluded the possibility of a self-catalyzed protein rearrangement, as occurs in protein splicing and autoproteolysis [35,36], and these questions, therefore, remain for further study. Nevertheless, although not yet fully understood, the proteolytic susceptibility of *T. aquaticus* FtsY provides structural insight, because only on forming complex does FtsY become susceptible to cleavage close to its N-terminus.

The G domains of the Ffh and FtsY must be directly involved in complex formation, of course, because not only is association dependent on nucleotide binding, but in the complex the two proteins reciprocally stimulate the other's GTPase activity [29]. This function is likely to be mediated by interactions local to the active site of the GTPase, as is seen in the structures of other GTPases complexed with their activating proteins [37–40]. Furthermore, evidence from fluorescence studies suggests that a tryptophan located in the effector subdomain (the 'IBD') of *E. coli* FtsY, at the opposite end of the G domain from the N/G interface, is in a significantly different environment on formation of the GTP-dependent complex with *E. coli* SRP (i.e., Ffh and 4.5S RNA) [15].

However, a number of lines of evidence suggest that the region of interaction between the two proteins may be more extensive [41,42]. Recent studies have identified mutations of Ffh likely to disrupt the conformation of the N/G interface that are not defective in substrate binding or intrinsic GTPase activity, but that are defective specifically in the formation of the complex with receptor, FtsY [42]. A corresponding mutant in FtsY was found to have a similar phenotype. Thus, complex formation requires that the N/G interface be intact [42]. It was also found that mutations of the *E. coli* Ffh that eliminate its intrinsic GTPase activity do not necessarily abrogate stimulation of GTP hydrolysis in the complex (presumably by FtsY) [42]. Conformational change in the N domain of *T. aquaticus* FtsY on formation of its complex with Ffh is consistent with these observations, which together raise the intriguing possibility that the N domains of each protein might play a direct role in their interaction. These results also argue against models of the targeting complex that limit the interaction to the GTPase active sites of the two proteins [20].

The purification of the Ffh/FtsY NG complex will allow us to more precisely examine the kinetics of the association and dissociation of the complex, and it provides the groundwork for biochemical characterization of the structural basis for the interaction between SRP and its receptor. The role of the N-domain in the SRP GTPases has long remained a puzzle. The irregular conformation of the N-terminus of the N domain in the structure of the *E. coli* FtsY, and the conformational change of the *T. aquaticus* FtsY N domain implied by its transition to a protease sensitive state in the Ffh/FtsY complex, suggest that perhaps the N-terminal region of the FtsY NG domain is quite dynamic. Thus, conformational changes of the N domain could play a fundamental role in the formation of the GTP-dependent SRP/SR complex during co-translational protein targeting.

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Fig. 1.

Ffh/FtsY NG complex formation demonstrated by gel filtration chromatography. The 100- μ l reactions were prepared with 10 μ M FtsY, 15 μ M Ffh NG and 1 mM GMPPCP in 50 mM HEPES (pH 7.5), 2 mM MgCl_2 , 50 mM NaCl and were incubated for the indicated time interval and temperature. The full reaction mixture was injected onto a Superdex 200 HR 10/30 column equilibrated with 50 mM HEPES (pH 7.5), 2 mM MgCl_2 , 50 mM NaCl. HPLC traces (280 nm) correspond to: (a) 42 °C overnight, (b) 3 days 37 °C, and (c) 4 days at 37 °C. No complex was observed after short incubations (1 h to overnight) over temperatures ranging from RT to 70 °C. Results from control reactions (all carried out at 37 °C, 4 days) are shown: (d) FtsY alone with GMPPCP, (e) Ffh/FtsY complex reaction with

GDP substituted for GMPPCP; and (f) the reaction mixture plus 2 mM EDTA. The position of the complex peak is indicated (asterisk), and the elution volumes of 29 and 150 kDa standards are shown. The expected ratio of the total absorbance at 280 nm for the complex peak relative to the monomer peak assuming 100% complex yield under these conditions is ~ 8.3–1.

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Fig. 2.

A slow dissociation rate for the complex. After a preincubation of a reaction mixture at 37 °C (100 μ l, containing 10 μ M FtsY, 15 μ M Ffh NG, 50 mM HEPES, pH 7.5, 2 mM MgCl_2 , 50 mM NaCl, 1 mM GMPPCP), GDP was added to 5 mM final concentration and the incubation continued at RT. Samples were taken at the indicated times (0–48 h).

Dissociation of the complex in the presence of excess GDP was monitored using anion exchange chromatography on a Poros HQ column equilibrated with 50 mM Tris (pH 8.0), 2 mM MgCl_2 and eluted with linear gradient of 1 M NaCl. After 18 h, ~ 50% of the initial complex was still intact.

Fig. 3.

Different patterns of cleavage of FtsY free in solution and in the Ffh/FtsY complex. Protein samples (10 μ M FtsY and 15 μ M NG) were incubated in 'low-salt' conditions (50 mM HEPES (pH 7.5), 2 mM MgCl_2 , 50 mM NaCl) and 'high-salt' conditions (50 mM HEPES (pH 7.5), 2 mM MgCl_2 , 300 mM NaCl) at 37 °C. GMPPCP or GDP were used at a final concentration of 1 mM. (a) 'Low-salt' experiments. Lane 1—Mark 12 MW standard; lanes 2 and 3—Ffh and FtsY NG stored at -20 °C; lane 4—FtsY, nucleotide-free, 4 days at 37 °C; lane 5—Ffh/FtsY complex purified after 4 days at 37 °C. (b) 'High-salt' experiments. Lane 1—Standard; lane 2—Ffh and FtsY NG nucleotide-free control, co-purified by gel filtration; lane 3—Ffh and FtsY NG incubated with GDP at 37 °C for 4 days; lane 4—Ffh/FtsY NG

complex formation reaction mix, incubated with GMPPCP 37 °C for 4 days; lane 5—Ffh/FtsY complex purified by gel filtration. Note the appearance of the FtsY cleavage product during the GMPPCP incubation (lane 4) but not the GDP incubation (lane 3). After blotting to PVDF membranes, the fragments marked by *arrowheads* were cut out and sequenced. The bands are labeled (N-terminus and sites **1** and **2**) corresponding to the N-terminal sequences obtained (see Fig. 4). Note that on this gel system FtsY and its larger cleavage products migrate slow relative to the markers and its smaller cleavage products migrate anomalously fast.

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Fig. 4.

Mapping the sites of proteolysis of FtsY. The sequences of the cleavage products of FtsY (indicated in Fig. 3) are shown on a linear map of the N domain of FtsY NG. Sequences identified the N-terminus (GFFDR), overlapping sites at position 1 (KLGFN and GFNPQ), and overlapping sites at position 2 (LKAIPWG and AIPxG). The secondary structure of the domain is diagrammed based on the published structure of the *E. coli* FtsY NG domain [19], patterns of sequence identity, and the position of the conserved 'ALLEADV' motif between helices $\alpha N2$ and $\alpha N3$ (indicated by asterisks). The N domain comprises the first ~ 82 residues of the protein, the 'linker' peptide, residues ~ 83–101, and the G domain, residues ~ 102–304. The three helices of the N domain that can be identified with confidence and are indicated ($\alpha N2$, $\alpha N3$, $\alpha N4$). The N-terminal helix $\alpha N1$ does not appear as such in the *E. coli* FtsY structure (see Fig. 5); the approximate position of the helix in Ffh NG is indicated ('~ $\alpha N1$ ~'). The diagram is shaded by the percentage of sequence conservation at each position in the sequence (white, < 30%; light grey, 30–60%; grey 60–100%).

Fig. 5.

A structural model for the locations of the cleavage sites. The positions of the two cleavage sites **1** and **2** are indicated relative to the structure of the *E. coli* FtsY NG domain (the G domain is truncated at right). Cleavage position **1** can be mapped to the linker peptide that extends between the N and G domains. This region varies widely in sequence and structure between different members of the SRP GTPase family, and may well be disordered and accessible to proteolysis in *T. aquaticus* FtsY. Cleavage position **2** maps to an N-terminal region that has somewhat irregular conformation and packs against the hydrophobic core of the N domain. The polypeptide backbone at position **2** is presumably inaccessible to

proteolysis in the *E. coli* FtsY conformation, but would become accessible if the region underwent conformational change or if it became displaced in the complex. The position of the conserved 'ALLEADV' motif is indicated (asterisks). This figure was generated using Molscript and Raster3d [43,44].

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