Molecular Crosstalk between the Nucleotide Specificity Determinant of the SRP GTPase and the SRP Receptor†

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ABSTRACT: In signal recognition particle (SRP)-dependent targeting of proteins to the bacterial plasma membrane, two GTPases, Ffh (the SRP GTPase) and FtsY (the receptor GTPase), form a complex in which both proteins reciprocally stimulate each other's GTPase activities. We mutated Asp251 in the Ffh active site to Asn (D251N), converting Ffh to a xanthosine 5′-triphosphate (XTP)-specific protein as has been observed in many other GTPases. Unexpectedly, mutant SRP(D251N) is severely compromised in the formation of an active SRP·FtsY complex when bound with cognate XTP, and even more surprisingly, mutant SRP(D251N) works better when bound with noncognate GTP. These paradoxical results are explained by a model in which Ffh Asp251 forms a bidentate interaction with not only the bound GTP but also the receptor FtsY across the dimer interface. These interactions form part of the network that seals the lateral entrance to the composite active site at the dimer interface, thereby ensuring the electrostatic and/or structural integrity of the active site and contributing to the formation of an active SRP·FtsY complex.

Two interacting GTPases, Ffh and FtsY, mediate the signal recognition particle (SRP[1])-dependent targeting of nascent polypeptide chains to the bacterial plasma membrane (*1*, *2*). Ffh, in complex with the bacterial 4.5S SRP RNA, constitutes a minimal bacterial SRP that recognizes N-terminal signal sequences as nascent polypeptide chains emerge from the ribosome. The complex of the SRP, ribosome, and nascent chain is then targeted to the plasma membrane (or to the ER membrane in eukaryotic cells) via the interaction of SRP with the SRP receptor, FtsY. The SRP·FtsY interaction releases the ribosome·nascent chain complex from SRP and transfers it to the translocation machinery, where the protein is either integrated into the membrane or secreted.

Ffh and FtsY define a unique subgroup in the GTPase superfamily (*3*). Like those of other GTPases, the targeting cycle carried out by Ffh and FtsY is tightly coupled to the cycle of guanosine 5′-triphosphate (GTP) binding and hydrolysis on these proteins. The interaction of SRP with FtsY requires both proteins to be in their GTP-bound form, and hydrolysis of bound GTP results in dissociation of the SRP·FtsY complex, allowing the SRP components to be regenerated for subsequent rounds of targeting (*4*, *5*). The regulatory mechanism for conversion between their GTP- and GDP-bound forms, however, is unique among the GTPase superfamily. Unlike many other GTPases, to date no nucleotide exchange factors have been identified to help release bound GDP from Ffh and FtsY. Presumably, such enzymes are not required because both Ffh and FtsY exhibit low GDP affinity and fast GDP dissociation rates (*5*−*8*). Likewise, conversion from their GTP- to GDP-bound forms does not require exogenous GTPase activating proteins. Rather, GTP hydrolysis is catalyzed within the SRP·FtsY complex, in which both proteins reciprocally act as GTPase activating factors for one another (*5*, *9*). The structure of the Ffh·FtsY complex reveals that two GTPs, one bound to Ffh and one to FtsY, are sequestered away from solvent in a composite active site, where they form contacts both with each other and with active site residues of their respective enzymes (*10*, *11*). These interactions lead to the mutual stimulation of GTP hydrolysis on both proteins.

A major challenge in delineating the mechanism of SRP-dependent targeting is to define the role of GTP binding and hydrolysis on individual steps of the targeting cycle. Such analyses are complicated, however, by the presence of two GTPases in the targeting reaction. We therefore altered the nucleotide specificity of one of the proteins by mutation of a conserved aspartate residue in the GTP-binding consensus motif (NXKD). This aspartate residue, conserved among the GTPase superfamily of proteins including Ffh and FtsY, has been observed to form a hydrogen bond with the exocyclic amino group of the guanine ring (*12*, *13*). In Ras, EF-Tu, and many other GTPases, substitution of this aspartate residue with asparagine converts the GTPase into a protein specific for xanthosine 5′-triphosphate (*14*−*23*).

We previously employed an analogous approach to switch the GTPase specificity of FtsY by mutating the corresponding Asp residue. These studies unveiled the reciprocal GTPase

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[1] Abbreviations: SRP, signal recognition particle; ER, endoplasmic reticulum; GTP, guanosine 5′-triphosphate; GDP, guanosine 5′-diphosphate; XTP, xanthosine 5′-triphosphate; GppNHp, 5′-guanylylimidodiphosphate; XppNHp, 5′-xanthylylimidodiphosphate.

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activation of Ffh and FtsY (*9*), and later led to the discovery that FtsY becomes specific for its cognate nucleotide only upon interaction with SRP (*24*). Here we show that mutant Ffh(D251N) behaves as predicted for classical GTPases, but only when assayed as a purified protein in isolation. In contrast, when assayed in the presence of its interaction partner FtsY, Ffh(D251N) proves to be severely compromised in complex formation and reciprocal GTPase activation, allowing us to identify functionally important interactions across the Ffh−FtsY interface.

MATERIALS AND METHODS

Protein Purification. Mutants Ffh(D251N) and FtsY(K390E) were constructed using the QuickChange site-directed mutagenesis procedure (Stratagene). The construction of mutant FtsY(E475K) has been described previously (*11*). Mutant Ffh and FtsY were expressed and purified by the same procedure as wild-type proteins (*5*, *25*).

Kinetics. GTP and XTP hydrolysis reactions were carried out at 25 °C using [γ-^{32}P]GTP (GTP*) and [γ-^{32}P]XTP [XTP* (Amersham)], respectively, and were followed and analyzed as described previously (*5*). The data shown in each figure are obtained side by side in the same experiment and are representative of three to five independent measurements; the results of the measurements differ by <20% from experiment to experiment.

The basal GTPase and XTPase reactions of Ffh or Ffh(D251N) were measured in single-turnover reactions with a trace amount of GTP* or XTP* (<0.1 nM) and excess protein (*5*). The protein concentration dependence of the reaction rates was analyzed as described to obtain K_m and k_{cat} values (*5*).

The affinity of Ffh for GTP and the affinity of Ffh(D251N) for XTP are the same as their respective K_m values in the basal GTPase and XTPase reactions, because the chemical step is rate-limiting (*5*). The affinities of Ffh and Ffh(D251N) for other nucleotides were determined using these nucleotides as competitive inhibitors of the basal GTPase and XTPase reactions, respectively (*5*).

Stimulated GTPase reactions were measured in multiple-turnover experiments as described previously (*5*); the concentration of nucleotides is specified in the figure legend. Alternatively, the SRP(D251N)-stimulated GTPase reaction of FtsY was determined in single-turnover experiments with trace amounts (<0.1 nM) of GTP*, 1−10 μM FtsY, and varying concentrations of SRP(D251N). XTP (50 μM) was present to selectively occupy the active site of SRP(D251N) [K_d^{XTP} = 0.37 and 460 μM for Ffh(D251N) and FtsY, respectively (Table 2 and ref *5*)]. The concentration dependence of the observed GTPase rate constant is fit to eq 1

$$k_{obsd} = k_{max} \times \frac{[SRP(D251N)]}{[SRP(D251N)] + K_{1/2}} \quad (1)$$

in which k_{max} is the maximal rate constant with saturating SRP(D251N) and $K_{1/2}$ is the concentration of SRP(D251N) required to reach half-saturation. The FtsY-stimulated XTPase reaction of SRP(D251N) was analogously determined in single-turnover experiments with trace XTP* (<0.1 nM), 2 μM SRP(D251N), and varying concentrations of FtsY. GTP (50 μM) was present to ensure that FtsY is selectively bound with GTP [K_d^{GTP} = 15 and 101 μM for FtsY and Ffh(D251N), respectively (Table 2 and ref *24*)]. The FtsY concentration dependence is fit to eq 2

$$k_{obsd} = k_{max} \times \frac{[FtsY]}{[FtsY] + K_{1/2}} \quad (2)$$

in which k_{max} is the maximal rate constant with saturating FtsY and $K_{1/2}$ is the concentration of FtsY required to reach half-saturation.

XTP Inhibition of the Stimulated GTPase Reaction. The inhibitory effect of XTP on the SRP(D251N)-stimulated GTPase reaction of FtsY is consistent with the model of eq 3a

$$\begin{array}{ccc} GTP + SRP(D251N) + FtsY^{\bullet GTP} & \xrightarrow{k_0} & products \quad (3a) \\ K_i \Big\updownarrow \pm XTP & & \\ {}^{XTP}{}^{\bullet}SRP(D251N) + FtsY^{\bullet GTP} & \xrightarrow{k'} & products \quad (3b) \end{array}$$

$$k_{obsd} = k_0 \times \frac{K_i}{[XTP] + K_i} + k' \times \frac{[XTP]}{[XTP] + K_i}$$

in which XTP binds to SRP(D251N) and forms a less reactive complex ($k' < k_0$). The data were fit to eq 3b, derived from eq 3a, in which k_0 is the reaction rate in the absence of added XTP, k' is the rate at which the reaction levels off at saturating XTP, and K_i is the inhibition constant of XTP. Because subsaturating concentrations of GTP and FtsY were used, K_i is equal to K_d, the dissociation constant of XTP for free SRP(D251N).

Fluorescence Measurements. Fluorescence measurements were performed at 25 °C using a photon-counting SLM 8100 spectrofluorometer. Fluorescence emission spectra were acquired using an excitation wavelength of 290 nm in the presence of 1 μM FtsY and varying amounts of SRP(D251N). Complex formation was initiated by addition of Mg^{2+} (*24*).[2] The SRP(D251N) concentration dependence of the observed fluorescence change ΔF_{obsd} was fit to eq 4

$$\Delta F_{obsd} = \Delta F_{max} \times \frac{[SRP(D251N)]}{K_d + [SRP(D251N)]} \quad (4)$$

in which ΔF_{max} is the fraction of fluorescence change observed with saturating SRP(D251N) and K_d is the equilibrium dissociation constant of the SRP(D251N)·FtsY complex. The concentration of nucleotides is specified in the figure legend.

[2] Mg^{2+} is not required for nucleotide binding by Ffh and FtsY, but is required for GTP hydrolysis and for formation of an active SRP·FtsY complex (*10*, *11*; S. Shan and P. Walter, unpublished results). Thus, the addition of Mg^{2+} provides a convenient way to initiate complex formation while all other components in the reaction are held constant; this provides an internal control for the internal filter effects of 4.5S RNA and nucleotides on the fluorescence of FtsY (e.g., the lower fluorescence of FtsY in Figure 3b than in Figure 3a in the absence of Mg^{2+} is due to the presence of larger amounts of 4.5S RNA; however, as the same amount of 4.5S RNA is present in samples with and without Mg^{2+}, the internal filter effect of the RNA is controlled). The addition of Mg^{2+} does not alter the fluorescence spectra of FtsY in the absence of Ffh or Ffh(D251N) (not shown), suggesting that the interactions of Mg^{2+} with FtsY, with 4.5S RNA, or with nucleotides do not contribute to the fluorescence shift of FtsY observed here.

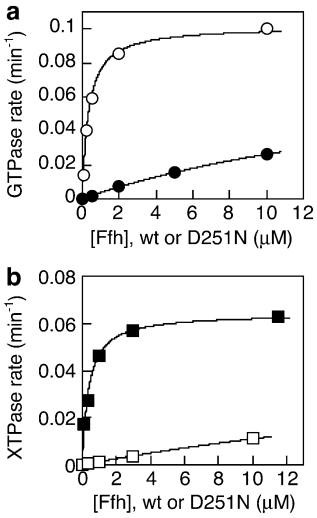

FIGURE 1: The D251N mutation converts Ffh into an XTP-specific protein. The rate constants of basal GTPase (a) and XTPase (b) reactions of wild-type Ffh (○ and □) and mutant Ffh(D251N) (● and ■) were determined in single-turnover experiments as described in Materials and Methods. The lines are fits of the data to a single binding curve (*5*), and gave a K_m value of 0.39 μM and a k_{cat} value of 0.092 min^{-1} for the GTPase reaction of wild-type Ffh, and a K_m value of 0.37 μM and a k_{cat} value of 0.062 min^{-1} for the XTPase reaction of mutant Ffh(D251N).

Table 1: Rate Constants for GTP and XTP Hydrolysis by Wild-Type Ffh and Mutant Ffh(D251N)[a]

Ffh construct	$(k_c/K_m)^{GTP}$ (M^{-1} min^{-1})	$(k_c/K_m)^{XTP}$ (M^{-1} min^{-1})	GTP specificity[b]
wild type	2.9×10^5	1.3×10^3	2.2×10^2
D251N	3.6×10^3	1.7×10^5	2.1×10^{-2}
relative (k_{WT}/k_{D251N})	81	0.0076	not applicable

[a] Second-order rate constants $(k_c/K_m)^{NTP}$ (N is G or X) were determined from the slope of the initial linear portion of the protein concentration dependence in Figure 1 as described previously (*5*). [b] GTP specificity is the preference of the Ffh constructs to hydrolyze GTP relative to XTP, obtained from $(k_c/K_m)^{GTP}/(k_c/K_m)^{XTP}$.

RESULTS

The D251N Mutation Converts Ffh to an XTP-Specific Protein. To dissect the mechanistic contribution of the two GTPases in the SRP and SRP receptor to the protein targeting reaction, we changed the nucleotide specificity of Ffh. To this end, we mutated the conserved Asp251 to Asn, a change that has been successfully applied in many other GTPases to change their specificity from GTP to XTP (*14*, *17*, *23* and references therein).

To characterize the enzymatic properties of the mutant protein, we first determined the rate constants for GTP and XTP hydrolysis by wild-type Ffh and mutant Ffh(D251N). As shown in Figure 1 and summarized in Table 1, wild-type Ffh hydrolyzes GTP with a rate constant (k_c/K_m) that is 220-fold faster than that for XTP. In contrast, mutant Ffh(D251N) hydrolyzes XTP 200-fold faster than GTP (Table 1), reflecting an 80-fold slower rate of GTP hydrolysis (Figure 1a) and a 120-fold faster rate of XTP hydrolysis compared to wild-type Ffh (Figure 1b).

In agreement with these results, we found that guanine-based nucleotides, such as GTP, GDP, GTPγS, and GppNHp, bind 200−400-fold more strongly to wild-type Ffh than to mutant Ffh(D251N) (Table 2), whereas xanthine-based nucleotides, such as XTP, XDP, and XppNHp, bind ~10^2-fold more strongly to mutant Ffh(D251N) than to wild-type Ffh. Thus, taken together, these results show that the mutation results in a 10^4-fold switch in the nucleotide specificity of Ffh from GTP to XTP, and that mutant Ffh(D251N) binds and hydrolyzes XTP with the same affinity and kinetics with which wild-type Ffh binds and hydrolyzes GTP. The rationally engineered switch in nucleotide specificity therefore appeared to be successful.

Table 2: Nucleotide Affinities of Wild-Type Ffh and Mutant Ffh(D251N)

	K_d (μM)		
nucleotide	wild type	D251N	K^{rel} [c]
GTP	0.39[b]	101[a]	0.0040
GDP	0.36[a]	55[a]	0.0065
GTPγS	0.44[a]	154[a]	0.0029
GppNHp	3.5[a]	655[a]	0.0053
XTP	30[a]	0.37[b]	81
XDP	22[a]	0.25[a]	88
XppNHp	180[a]	2.6[a]	69
ITP	49[a]	126[a]	0.39

[a] Determined by inhibition methods as described previously (*5*). [b] Determined from Ffh concentration dependencies as described previously (*5*). [c] K^{rel} is the binding affinity of mutant Ffh(D251N) relative to wild-type Ffh for the nucleotide of interest, obtained from $K^{rel} = K_d[Ffh(wt)]/K_d[Ffh(D251N)]$.

The D251N Mutation Impairs the Reciprocal GTPase Stimulation between SRP and FtsY. We previously used an analogously engineered XTP-specific mutant of FtsY [FtsY-(D449N)] to show that SRP (composed of Ffh and the 4.5S SRP RNA) and FtsY act as reciprocal GTPase activating proteins for one another (*9*). We therefore tested the prediction that mutant SRP(D251N) would analogously stimulate the reciprocal nucleotide hydrolysis reaction with FtsY. To this end, we compared the ability of wild-type SRP and mutant SRP(D251N), bound to either GTP or XTP, to stimulate the GTPase reaction of FtsY (Figure 2a). To ensure that each protein was bound with the desired nucleotide, a high concentration of GTP was used to saturate the active sites of both SRP (wild type or D251N) and FtsY, and for the reaction of SRP(D251N) bound with XTP, a sufficient amount of XTP was present to compete with GTP and bind selectively at the active site of SRP(D251N).

In contrast to our expectation, SRP(D251N) bound with XTP fails to stimulate the GTP hydrolysis reaction of FtsY efficiently, reaching a maximal rate of only 1.2 min^{-1} [Figure 2a (▲), enlarged in the inset]. This rate is 40-fold slower than the maximal stimulation observed with wild-type SRP (○), but since only one GTP is hydrolyzed in this reaction (vs two GTPs in the case of wild-type SRP), the amount of stimulation of FtsY's GTPase reaction is actually reduced by 20-fold. The reciprocal reaction, FtsY-stimulated XTPase reaction from SRP(D251N), is analogously slowed by 20-fold (see the Supporting Information). To our further surprise, SRP(D251N) bound with GTP (i.e., its noncognate nucleotide forced into the binding site by a high concentration) stimulates the GTPase reaction with FtsY, reaching a maximal GTP hydrolysis rate of 11 min^{-1}, which is only 4-fold slower than the stimulation obtained with wild-type SRP [Figure 2a (● and ○, respectively)]. Thus paradoxically, mutant SRP(D251N) bound with the *noncognate* nucleotide GTP works 5-fold faster than when bound with *cognate* XTP.

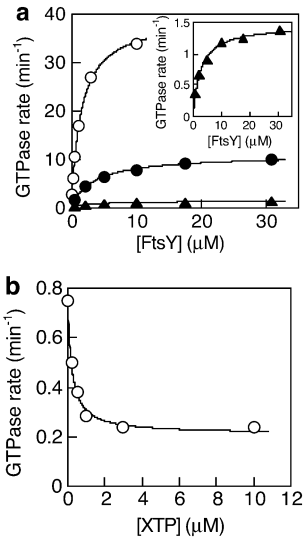

FIGURE 2: SRP(D251N) is defective in stimulating FtsY's GTPase reaction. (a) Rate constant of stimulated GTP hydrolysis in the presence of FtsY and wild-type SRP (○), SRP(D251N) bound with XTP (▲), or SRP(D251N) bound with GTP (●), determined as described in Materials and Methods. The following nucleotide concentrations were used: 100 μM GTP for wild-type SRP, 500 μM GTP for SRP(D251N) with GTP bound, and 50 μM XTP and 100 μM GTP for SRP(D251N) with XTP bound [see Table 2 for the nucleotide affinities of Ffh and Ffh(D251N); the nucleotide affinities are unaffected by the presence of the SRP RNA (*5*)]. The lines are fits of the data to eq 2, and gave maximal rate constants of 42, 11, and 1.2 min^{-1} for the reactions of wild-type SRP, SRP(D251N) with GTP bound, and SRP(D251N) with XTP bound, respectively. The inset shows the reaction of SRP(D251N) with XTP bound on an expanded scale. (b) XTP inhibits the SRP(D251N)-stimulated GTPase reaction of FtsY. Observed GTPase rate constants were determined in the presence of 0.1 μM SRP(D251N), 0.5 μM FtsY, and 50 μM GTP, as described in Materials and Methods. The data were fit to eq 3 (see Materials and Methods), and gave an XTP inhibition constant of 0.31 μM.

To provide independent evidence for this surprising result, we determined the effect of increasing XTP concentrations on the ability of mutant SRP(D251N) to stimulate the GTPase reaction of FtsY. For this experiment, we used a GTP concentration lower than that shown in Figure 2a so that FtsY would be selectively occupied and SRP(D251N) largely unoccupied by GTP. This was possible because GTP binds with a higher affinity to FtsY than to mutant SRP(D251N) [K_d = 15 and 101 μM for FtsY and SRP(D251N), respectively (ref *5* and Table 2)]. Given these affinities, we calculated that at 50 μM GTP, FtsY molecules should be 80% occupied, whereas SRP(D251N) should be 70% unoccupied by GTP. This allowed us to monitor the effect of XTP on the reaction SRP(D251N) + FtsY$^{\bullet GTP}$ → products.

As shown in Figure 2b, in the absence of XTP, a small amount of stimulated GTP hydrolysis is observed (GTPase rate = 0.78 min^{-1}) due to partially GTP-occupied SRP(D251N). Remarkably, addition of XTP progressively inhibits this GTPase reaction. The inhibition constant (K_i) of 0.31 μM is the same, within error, as the binding affinity of XTP for Ffh(D251N) determined independently [0.37 μM (Table 2)], strongly suggesting that the binding of XTP to the active site of SRP(D251N) is responsible for the observed inhibition. At saturating XTP concentrations, the reaction levels off at a new, reduced rate, reflecting the slower reaction when the active site of SRP(D251N) is occupied by XTP. This inhibitory effect is in marked contrast to the

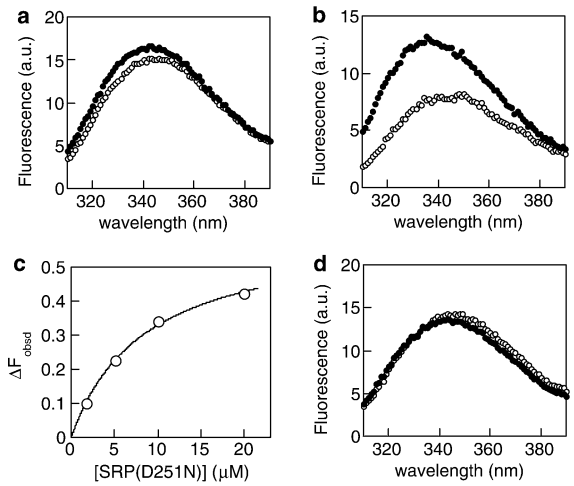

FIGURE 3: Fluorescence measurement of complex formation between SRP(D251N) and FtsY. The fluorescence emission spectrum of a mixture of FtsY and SRP(D251N) was determined in the presence of EDTA (○), and complex formation was initiated by addition of Mg^{2+} (●) as described previously (*24*). The spectra were determined in the presence of 1 mM GppNHp and 2 μM (a) or 20 μM (b) SRP(D251N). (c) The fluorescence change at 335 nm (parts a and b and data not shown) was quantitated as a function of SRP(D251N) concentration. The line is a fit of the data to eq 4, and gave a dissociation constant of 8.5 μM. (d) Fluorescence emission spectra of FtsY determined as described for panel a, except that 120 μM XTP was present in addition to GppNHp.

results obtained previously with the analogous XTP-specific mutant FtsY(D449N), for which the addition of cognate XTP *stimulated* the rate of GTP hydrolysis from SRP (*9*). Taken together, our results show that for the stimulation of FtsY's GTPase reaction, mutant SRP(D251N) with XTP bound is less active than wild-type SRP, or than SRP(D251N) with GTP bound.

The SRP(D251N) Mutation Also Weakens the Complex between SRP and FtsY. The analyses described above compared the maximal rate constants at saturating protein concentrations, which reflect the reactivity of the fully formed SRP(D251N)·FtsY complex. Further, the data in Figure 2a indicate that higher protein concentrations are required to reach maximal stimulation with mutant SRP(D251N) than with wild-type SRP, suggesting that the affinity of the two binding partners is also compromised by the SRP(D251N) mutation. To test this possibility directly, we examined complex formation using a fluorescence assay that monitors a late step during formation of an active complex. Formation of the wild-type SRP·FtsY complex gives rise to a blue shift and an ~2-fold increase in the tryptophan fluorescence of FtsY (*7*, *8*). The tryptophan residue responsible for this effect is sandwiched by the IBD loop from Ffh and the closing loop from FtsY at the dimer interface in the SRP·FtsY complex; both of these loops rearrange after a stable complex has formed (*11*, *26*). In the presence of GppNHp as the sole nucleotide, addition of mutant SRP(D251N) produces a change in the fluorescence spectrum of FtsY similar to that observed for wild-type SRP (Figure 3b). However, a higher concentration of mutant SRP(D251N) than wild-type SRP was required to observe the full extent of the fluorescence change (Figure 3a−c). Quantitation of the fluorescence change as a function of SRP(D251N) concentration gives a dissociation constant of 8.5 μM for formation of the $^{GppNHp\bullet}$SRP(D251N)·FtsY$^{\bullet GppNHp}$ complex (Figure 3c), more than

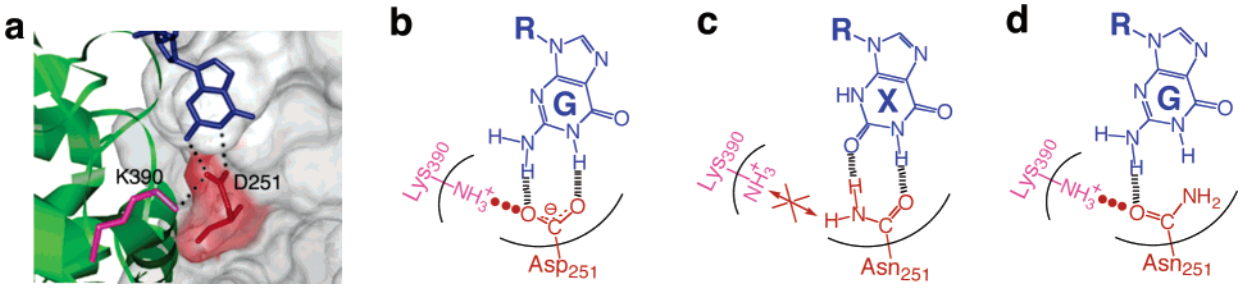

FIGURE 4: Model for the interactions of Lys390 of FtsY with the side chain of Ffh residue 251. (a) Interaction network observed in the crystal structure of the Ffh·FtsY complex. Ffh is in a surface representation, FtsY is depicted as a green ribbon, Ffh Asp251 is colored red, FtsY Lys390 is colored magenta, and the nucleotide bound to the Ffh active site is colored dark blue. (b−d) Schematic drawing of the interactions for the wild-type SRP·FtsY complex (b) and the SRP(D251N)·FtsY complex with XTP (c) or GTP (d) bound at the SRP(D251N) active site. The dots (···) highlight the interaction with Lys390, and the dashed lines depict the hydrogen bonding interactions between residue 251 and the purine ring.

200-fold weaker than the dissociation constant of 36 nM determined for the wild-type ^{GppNHp}•SRP•FtsY•^{GppNHp} complex (*27*). Note that under these conditions, the *noncognate* guanine nucleotide is bound to the mutant SRP engineered to bind xanthine nucleotides.

In contrast to the results obtained with GppNHp-bound SRP(D251N), with XTP-bound SRP(D251N) no fluorescence change was observed even in the presence of 20 μM SRP(D251N) (Figure 3d). Thus, either this high concentration of SRP(D251N) is not sufficient to drive complex formation, or the conformational rearrangements that lead to the fluorescence change upon complex formation are inhibited when SRP(D251N) is bound with XTP.

Taken together, the results presented so far show that the SRP(D251N) mutation has deleterious effects on the formation of the SRP·FtsY complex and/or the conformational changes once a complex has formed. In addition, the reactivity of the formed complex is compromised. Paradoxically, both effects are more deleterious with the cognate nucleotide XTP bound at the active site of SRP(D251N) than with the noncognate nucleotide GTP bound (Figures 2 and 3).

SRP(D251N) Relieves the Defect of the FtsY(K390E) Mutation. The results described above indicate that Asp251 in SRP and the interacting nucleotide make essential contributions to the conformation of the active complex. One possibility is that Asp251 directly interacts with FtsY at the interface. We reasoned that a residue on FtsY that donates a hydrogen bond to Asp251 upon complex formation could be responsible for the observed effect (Figure 4; below we show that the residue responsible is Lys390 of FtsY). According to this notion, one of the carboxylate oxygens of Asp251 forms a bidentate interaction both with the exocyclic amino group of guanine and, upon complex formation, with an H-bond donor from FtsY (Lys390) at the interface (Figure 4a,b). This interaction is disrupted by substitution of the carboxylate oxygen with an amino group (Figure 4c), thereby impairing the SRP−FtsY crosstalk when SRP(D251N) is bound with cognate XTP. XTP forms two hydrogen bonds with Asp251, thereby locking it in the position depicted. By contrast, when SRP(D251N) is bound with noncognate GTP, the side chain of Asn251 is less constrained and can reorient so that the carbonyl oxygen of Asn251 retains its interaction with the exocyclic amino group of the noncognate guanine base (Figure 4d). In this orientation, the carbonyl oxygen can also interact with Lys390 of FtsY, thereby restoring an active SRP(D251N)·FtsY complex (Figure 4d). This model explains why the interaction between SRP(D251N) and FtsY is more efficient with noncognate GTP than with XTP bound at the active site of SRP(D251N).

The structure of the Ffh·FtsY complex (*10*, *11*), determined shortly after this model was developed, identifies Lys390 as the only residue that could function as the proposed hydrogen bond donor in FtsY (Figure 4a). We therefore tested this hypothesis by mutagenizing Lys390 of FtsY and characterized the enzymatic activity of mutant FtsY(K390E). The basal GTP binding and hydrolysis reactions of FtsY are unaffected by the K390E mutation (not shown), suggesting that this mutation does not grossly alter the folding or active site of the protein. However, when assayed in the presence of SRP, mutant FtsY(K390E) exhibits a deleterious effect on the reciprocally stimulated GTPase reaction, reducing the maximal rate constant of the stimulated reaction by 18-fold (Figure 5a). In contrast, when assayed in the presence of mutant SRP(D251N) with XTP bound, the FtsY(K390E) mutation no longer has a deleterious effect and is even slightly stimulatory (Figure 5b). Thus in agreement with our proposed model (Figure 4), these results provide biochemical evidence for a direct and functionally important interaction between Asp251 of Ffh and Lys390 of FtsY at the interface.

SRP(D251N) Displays a Synergistic Defect with the FtsY(E475K) Mutation. In the course of this study, we found that another mutation in FtsY, FtsY(E475K), displays a strongly synergistic defect with the SRP(D251N) mutation. Glu475 is situated on the closing loop near the guanine-binding site of FtsY. By itself, this mutation does not significantly affect the basal GTP binding and hydrolysis activity (not shown) and has an only less than 2-fold effect on the maximal amount of GTPase stimulation between wild-type SRP and FtsY (Figure 6a). In contrast, when mixed with SRP(D251N), FtsY(E475K) is more than 100-fold less efficient than wild-type FtsY in the maximal rate of the stimulated XTPase reaction of SRP(D251N) (Figure 6b). Analogously, the reciprocal reaction, stimulation of the GTPase reaction of FtsY by XTP-bound SRP(D251N), is also 100-fold slower with mutant FtsY(E475K) than with wild-type FtsY (Figure 6c). The strong synergistic defect suggests that both the SRP(D251N) and FtsY(E475K) mutations effect a common step during formation of the active SRP·FtsY complex (see the Discussion).

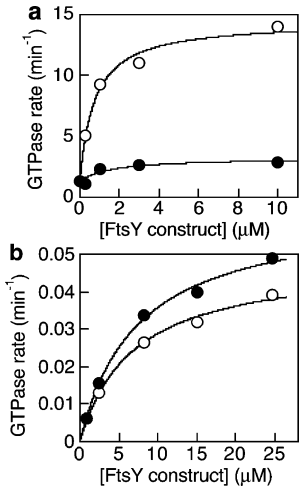

FIGURE 5: Cooperative effect of the FtsY(K390E) mutation with the SRP(D251N) mutation. (a) The FtsY(K390E) mutation compromises the reactivity of the SRP·FtsY complex. The SRP-stimulated GTPase reactions of wild-type FtsY (○) and mutant FtsY(K390E) (●) were determined in the presence of 0.1 μM wild-type SRP and 100 μM GTP, as described previously (*24*). The lines are fits of the data to eq 2, and gave k_{max} values of 12 and 0.66 min^{-1} for wild-type FtsY and mutant FtsY(E475K), respectively. (b) The FtsY(K390E) mutation does not compromise the reaction with mutant SRP(D251N). SRP(D251N)-stimulated GTPase reactions of wild-type FtsY (○) and mutant FtsY(K390E) (●) were assessed in the presence of 1 μM FtsY and 50 μM XTP as described in Materials and Methods. The lines are fits of the data to eq 2, and gave maximal rate constants of 0.49 and 0.62 min^{-1} for wild-type FtsY and mutant FtsY(K390E), respectively.

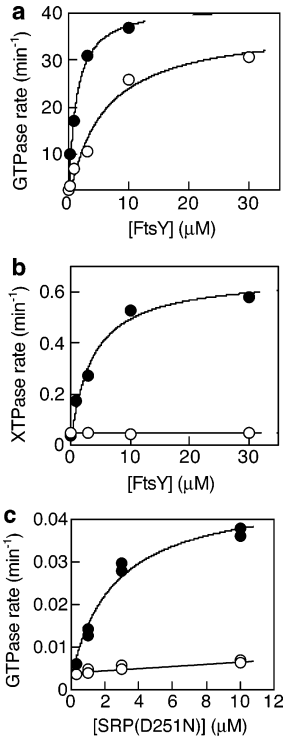

FIGURE 6: Synergistic defect of the FtsY(E475K) mutation with the SRP(D251N) mutation. (a) The FtsY(E475K) mutation has no significant effect on the reactivity of the SRP·FtsY complex. The SRP-stimulated GTPase reactions of wild-type FtsY (●) and mutant FtsY(E475K) (○) were determined as described in the legend of Figure 5a. The lines are fits of the data to eq 2. (b) Stimulation of the XTPase reaction of SRP(D251N) by wild-type FtsY (●) and mutant FtsY(E475K) (○), determined in the presence of 1 μM SRP-(D251N) and 50 μM GTP as described in Materials and Methods. The lines are fits of the data to eq 2. (c) SRP(D251N)-stimulated GTPase reaction of wild-type FtsY (●) and mutant FtsY(E475K) (○), determined in the presence of 1 μM FtsY and 50 μM XTP as described in Materials and Methods. The lines are fits of the data to eq 1.

DISCUSSION

Engineered GTPases with altered nucleotide specificity have been used extensively to decipher the roles that individual GTPases play in complex biological processes where multiple GTPases are present (*9, 14*−*23*). Here we have introduced a single-amino acid substitution into the GTP binding site of Ffh to alter its nucleotide specificity. When analyzed in isolation, Ffh(D251N) behaved just as expected by analogy to other GTPases engineered similarly, switching the specificity of Ffh from GTP to that for XTP by 10^4-fold. When analyzed in the context of its receptor FtsY, however, this mutation displayed unexpected effects, severely disrupting the interaction of SRP with FtsY and their reciprocal activation. Thus, the in-depth analysis of the behavior of the mutant Ffh reveals an unanticipated role of the nucleotide specificity determinant in the interaction of SRP with the SRP receptor, and defines an important part of the interaction network that is essential for the formation and activation of the SRP·FtsY complex.

Beyond the novel insights into the molecular details of the SRP−SRP receptor interaction discussed below, our results demonstrate the complexity of effects that can arise from nucleotide specificity switch experiments in GTPases (or ATPases) and emphasize the vigilance that is necessary in the interpretation of such experiments. Residues at the active site of GTPases are often involved in interaction networks with other components that read out their nucleotide-bound state. As demonstrated here, a minor alteration that disrupts a supposedly "local" interaction can have unforeseen consequences when assayed in a larger biological context. Currently, we do not know the extent to which the results presented here pertain to other GTPases. Nevertheless, our findings suggest that in-depth mechanistic analyses of the behavior of the wild type and mutant GTPases, not only as isolated enzymes but also in the context of their biological interaction partners, are required to decipher the effects of the mutation and to gain insights into the role of these proteins.

Mutation of the Conserved Asp Produces an XTP-Specific Ffh Protein. The high specificity of the SRP GTPase Ffh and its mutant form Ffh(D251N) for their respective cognate nucleotides is in marked contrast to the low nucleotide specificity of the homologous SRP receptor GTPase, FtsY, and FtsY(D449N) (*24*). Our previous analyses suggested an open nucleotide-binding pocket in free FtsY, which then tightens up and becomes nucleotide-specific upon complex formation. The higher nucleotide affinity and specificity of Ffh for GTP and its higher basal GTPase activity (*5*) suggest that the guanine nucleotide is better positioned in the Ffh than the FtsY GTPase site. These results are consistent with the crystal structures of the GppNHp-bound forms of individual Ffh and FtsY proteins, which showed that Asp251 is already within hydrogen bonding distance of the guanine base in the Ffh, but not the FtsY active site (*28*; C. Reyes and R. Stroud, manuscript in preparation). The mechanistic details by which free Ffh and FtsY bind GTP therefore differ,

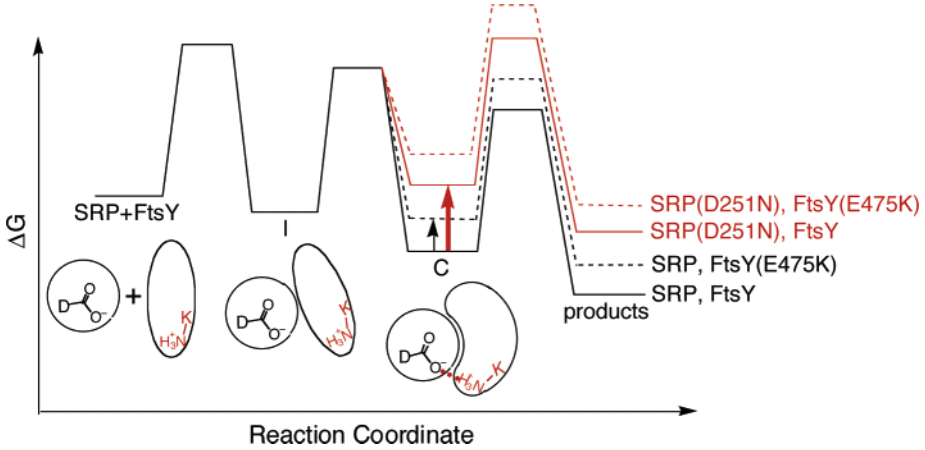

FIGURE 7: Model for a conformational change during formation of an active SRP·FtsY complex. I represents an inactive intermediate stage during SRP·FtsY complex formation, and C represents a closed SRP·FtsY complex that can be activated for GTP hydrolysis. The change in the shape of FtsY depicts a conformational change to the closed state during formation of a stable complex, as suggested from previous studies (*24*, *26*); an analogous conformational change may occur in SRP but is not depicted for simplicity. We assume the simplest scenario in this model. (i) The sole effect of the Ffh(D251N) and FtsY(E475K) mutations is to destabilize C relative to I (depicted by the red and black arrows, respectively). (ii) The magnitudes of the destabilizing effects from the two mutations are independent of each other. (iii) The mutations do not affect the reaction rate from the C complex once it is formed.

despite their strong structural homology and functional symmetry.

A number of observations suggest that the perturbations introduced by the Ffh(D251N) mutation and nucleotide substitution are local to the purine binding part of the active site, with minimal disruption to other active site interactions. First, the affinities of mutant Ffh(D251N) for xanthine-containing nucleotides are similar to the affinities of wild-type Ffh for guanine-containing nucleotides. Second, XDP and XTP bind to mutant Ffh(D251N) with similar affinities, analogous to the similar binding affinities of GDP and GTP for wild-type Ffh. Finally, the GTP analogue, GppNHp, binds to wild-type Ffh ~10-fold more weakly than GTP; an analogous 10-fold effect was observed in the binding of GppNHp relative to GTP to mutant Ffh(D251N), or in the binding of XppNHp relative to XTP to either wild-type Ffh or mutant Ffh(D251N), indicating that the active site interaction with the bridging oxygen between the β- and γ-phosphate groups of GTP is preserved. Together, these observations strongly suggest that besides the interaction between Asp251 and the N2 amino group of the guanine base, other active site interactions with the nucleotide are unperturbed by the D251N mutation.

Asp251 Plays a Central Role in Formation of an Active SRP·FtsY Complex. As the perturbations introduced by the D251N mutation appeared to be confined to direct interactions of Asp251 with the purine ring of the nucleotide, it came as a complete surprise that mutant SRP(D251N) is substantially compromised in both complex formation with FtsY and their reciprocal GTPase activation. Even more surprisingly, mutant SRP(D251N) is less active in both of these processes when bound with the *cognate* XTP than with the *noncognate* GTP nucleotide. Thus, the function of the Asp251 residue is not restricted to conferring GTP specificity to SRP. Indeed, both crystallographic analysis (*10*, *11*) and the functional coupling between the Ffh(D251N) and FtsY-(K390E) mutations shown here suggest that the Asp251 residue directly participates in a hydrogen bonding interaction with Lys390 of FtsY across the interface, as depicted in the model shown in Figure 4 and described above.

Even when the SRP(D251N)·FtsY complex is formed, reciprocal GTPase activation in the complex is significantly compromised. More remarkable still, the single D251N mutation at the Ffh GTP binding site disrupts the activation of *both* the Ffh and FtsY GTPase sites. These results suggest that the SRP(D251N)·FtsY complex is trapped in an intermediate, inactive conformation (I) depicted in the free energy profile in Figure 7. In contrast, wild-type SRP can form a more stable, "closed" complex with FtsY (Figure 7, C), which can be further activated to lead to rapid GTP hydrolysis (Figure 7, solid black line; *26*). It is plausible that the closed complex is stabilized in part by the interaction between Asp251 of Ffh and Lys390 of FtsY (*10*, *11*); disruption of this interaction would destabilize the closed state (red arrow), causing the mutant SRP(D251N)·FtsY complex to reside predominantly in the I state (solid red line). Therefore, mutant SRP(D251N) forms a weaker complex with FtsY. To react, however, the I complex still needs to rearrange to the closed state (solid red line); thus, the need to overcome the additional energy barrier imposed by this unfavorable I → C rearrangement also explains the slower observed reaction rate from the SRP(D251N)·FtsY complex.

We also observed a strong synergistic defect of the FtsY-(E475K) mutation with the SRP(D251N) mutation. In light of the model presented in Figure 7, this effect is most simply explained by a "threshold" model (*29*, *30*) in which both of these residues are involved in the I → C rearrangement, and depending on whether the I or C state is more populated, the full magnitude of the mutational effects on reactivity can be observed or masked (Figure 7). In the wild-type SRP·FtsY complex, there is sufficient interaction energy so that C is much more stable than the I state (solid black line). The destabilizing effect of a single FtsY(E475K) mutation (Figure 7, small black arrow) is not sufficient to render I more stable than the C complex (dashed black line). Thus, the SRP·FtsY(E475K) complex still reacts primarily from the C state, explaining why the FtsY(E475K) mutation by itself has little effect on the rate of the stimulated GTPase reaction; the effect of the FtsY(E475K) mutation is exhibited primarily on the complex formation step (*26*). By contrast,

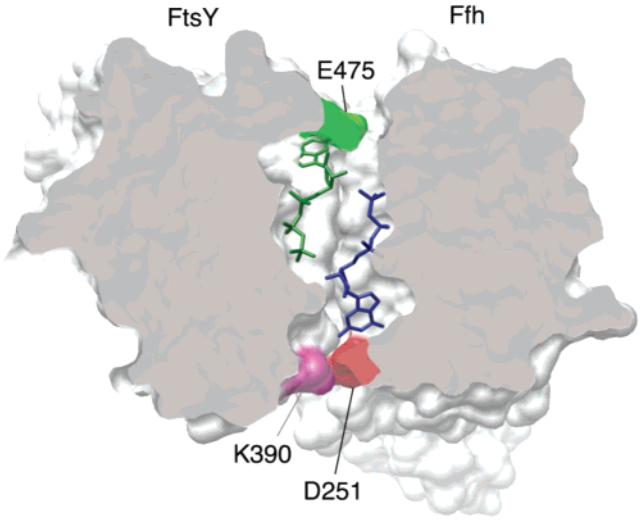

FIGURE 8: The Ffh Asp251−FtsY Lys390 ion pair and FtsY Glu475 form part of the networks that seal the lateral entrance to the composite active site in the Ffh·FtsY complex (*10*, *11*). The structure of the complex is viewed from the top, with the Ffh and FtsY molecules in surface representation, Ffh Asp251 colored red, the FtsY Lys390 residue colored magenta, and the FtsY Glu475 residue colored green. Note that the structure above the plane has been removed (shaded areas) to allow visualization of the bound nucleotides and the residues of interest.

in the mutant SRP(D251N)·FtsY complex, the C complex is already sufficiently destabilized so that the SRP(D251N)·FtsY complex is predominantly in the I state (solid red line); the additional FtsY(E475K) mutation thus increases the barrier for the I → C rearrangement and in this way exhibits its deleterious effect on the GTPase reaction step (Figure 7, dashed red line), even though this mutation has the same destabilizing effect in the context of both wild-type and mutant SRP.

In the crystal structure of the Ffh·FtsY complex, the FtsY Lys390−Ffh Asp251 pair form part of the interaction network that seals the lateral entrance to a composite active site at the interface between the two GTPases (Figure 8, red and magenta; *10*, *11*). Remarkably, the FtsY Glu475 residue, whose mutation exhibits a synergistic defect with the Ffh D251N mutation, seals the lateral entrance to the active site at the opposite side of the Ffh·FtsY complex (Figure 8, green). Thus, these residues may help exclude solvent from the active site, thus facilitating electrostatic interactions with the β- and γ-phosphate groups of GTP that contribute to the hydrolysis reaction. Alternatively or in addition, they may act as "clamps" that confer rigidity to the interaction network within the composite active site, so that the two GTP molecules are better aligned with respect to the catalytic residues and with respect to each other. Thus, our analyses have revealed two essential parts of the molecular interaction networks that ensure the integrity of the composite active site in the Ffh·FtsY complex.

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SUPPORTING INFORMATION AVAILABLE

Symmetry of the two hydrolysis reactions from both active sites in the SRP(D251N)·FtsY complex. This material is available free of charge via the Internet at http://pubs.acs.org.

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