The Long α-Helix of SecA Is Important for the ATPase Coupling of Translocation

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SecA contains two ATPase folds (NBF1 and NBF2) and other interaction/regulatory domains, all of which are connected by a long helical scaffold domain (HSD) running along the molecule. Here we identified a functionally important and spatially adjacent pair of SecA residues, Arg-642 on HSD and Glu-400 on NBF1. A charge-reversing substitution at either position as well as disulfide tethering of these positions inactivated the translocation activity. Interestingly, however, the translocation-inactive SecA variants fully retained the ability to up-regulate the ATPase activity. Moreover, a charge-reversing substitution at one position inactivated the translocation activity, whereas a charge-reversing substitution at the other position retained the ability to translocate in response to a preprotein and the SecYEG translocon. The translocation defect was suppressible by second site alterations at the hinge-forming boundary of NBF2 and HSD. Based on these results, we propose that the motor function of SecA is realized by ligand-activated ATPase activity and its HSD-mediated conversion into the mechanical work of preprotein translocation.

Although the crystal structures of the translocation components have provided an important basis for our understanding of the translocation molecular mechanisms, the static structures alone are insufficient to reveal the dynamic processes of translocation and the regulatory domains. On the basis of this finding, we propose that HSD plays a crucial role in the ATPase translocation coupling.

EXPERIMENTAL PROCEDURES

Escherichia coli Strains—JM109 (22) was used as a host for plasmid engineering. KD1087 (mutDS) (23) was used for plasmid mutagenesis. MM52 (secA51(Ts)) (24) and its ara- deriv-
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FIGURE 1. The long α-helix (HSD) in SecA. A, a ribbon diagram representation of the SecA main chain. The structure of B. subtilis SecA (Protein Data Bank accession number 1M6N) is shown with the residue numbering modified to match the E. coli SecA sequence. The SecA domains are colored according to Hunt et al. (6) except for HSD, which is shown in black. Its residue 642 and a NBF1 residue 400 are space-filled in red and yellow, respectively, for the side chains. The positions of second-site alterations identified as E400R suppressors are space-filled in magenta. HWD, helical wing domain. B, HSD contains evolutionarily conserved residues. HSD segment 624–660 is shown in which those highly conserved among SecA orthologs from 33 bacterial species are indicated in boldface letters. Arg-642 is highlighted in red.

Plasmids—Plasmids used are listed in Table 1. pAN31 (28) and pAN55 (16) carried secA and secM-secA, respectively, under the ara promoter. Plasmid pKY173 was an SecA overproducer (29), whereas pNA83 was its derivative encoding cysteine-less SecA. pAN5 was another SecA overproducer, which was constructed on a vector compatible with pKY173 (28). Site-directed mutations were introduced by the QuiChang method.

Isolation of Suppressors That Alleviate the Defect of the E400R Alteration in SecA—Plasmid pHM574, a pAN55-based plasmid encoding SecA(E400R), was allowed to propagate in the mutant (mutD5) strain, KD1087. Resulting plasmid mixtures were then transformed into HM1802 (secA51(ts) ara-) with selection on l-chloramphenicol-arabinose agar at 42 °C. Colonies that appeared at frequencies of \( \sim 10^{-8} \) as compared with those that appeared at 30 °C were then purified and subjected to the characterization of plasmids that they carried. Suppressor mutations thus identified and confirmed by back cloning are listed in Table 2.

Preparation of Biochemical Materials—Inverted membrane vesicles (IMVs) were prepared from the SecYEG-overproducing strain (30, 31). ProOmpA (C290G) was purified from its overproducing strain by the published procedures (29). Wild-type SecA and mutated variants were overproduced in strain CK4706 and purified as described previously (30). Concentration of SecA was determined from \( A_{280} \) assuming \( e = 1 \times 10^5 \).

Assays for in Vivo and in Vitro SecA-related Events—Export of OmpA in vivo was assessed from pulse-labeling ratios of its mature and precursor forms as described previously (30). In vitro activities of SecA preparations to drive preprotein translocation were assayed using urea-washed SecYEG IMVs and purified proOmpA as described previously (30).

RESULTS

Importance of Arg-642 on HSD and Its Possible Ionic Partner, Glu-400—On NBF1—The HSD part of E. coli SecA contains a number of evolutionarily conserved residues (Fig. 1B, boldface), which were individually mutated to cysteine. We thus found that cysteine substitutions at residues Glu-635, Arg-642, and Arg-656 were partially deleterious for the SecA function (supplemental Fig. S1). Glu-635 and Arg-642 are close to Arg-566 in

PLASMAID

| Encoded SecA variant | Ara promoter-controlled \(^*\) | Lac promoter-controlled \(^*\) |
|----------------------|-----------------------------|-----------------------------|
|                      | secA | secM-secA | secA | secM-secA |
| Wild-type            | pAN31 | pAN55   | pKY173 |
| E635C                | pHM557 | pHM560 | pHM550 |
| R642C                | pHM556 | pHM561 | pHM550 |
| R656C                | pHM555 | pHM559 | pHM550 |
| E642E                | pHM614 | pHM565 | pHM568 |
| E400C                | pHM613 | pHM574 | pHM572 |
| E400R, R642C         | pHM580 | pHM583 | pHM583 |
| R400R, R656C         | pHM615 | pHM587 | pHM587 |
| Cys-less             | pNA83 |

\(^{*}\) This series of plasmids carried either secA or secM-secA under the ara promoter control and were used for in vivo complementation analyses. In pNA31 and its derivatives, secA was fused to an idealized Shine-Dalgarno (SD) sequence, whereas in pAN55 and its derivatives the native SD sequence for secM was used. The SecA expression level is higher for pAN51 than for pAN55.

\(^{*}\) This series of plasmids carried secA under the lac promoter control and were used for purification of SecA derivatives. pAN31, pAN55, and pKY173 were described by Murakami et al. (28), Nakatogawa et al. (16), and Yoshihisa & Ito (29), respectively. Others were constructed by site-directed mutagenesis. Plasmid pHM544, not included in this list, was a pAN5 (28)-based plasmid encoding SecA(E400C) under the lac promoter control and compatible with the pKY173-based plasmids.
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TABLE 2
Effects of second site mutations on the NBF1 and HSD alterations

| Second-site mutation* | Primary mutation |
|-----------------------|------------------|
| Nucleotide change     | Amino acid change | Complemented ΔsecM-secAc | OmpA export† |
| T1820C                | M607T            | Cs                      | 70 |
| T1829C                | L610P            | +                       | 61 |
| G1855A                | E619K            | Ts, Cs                  | 61 |
| A1889C                | H620P            | +                       | 66 |
| T1880C                | I627T            | +                       | 70 |
| G1882A                | A628T            | Ts, Cs                  | 63 |
| A1885G                | N629D            | +                       | 68 |
|                        |                  |                         |    |
| Complemented ΔsecM-secAcA | OmpA export† |
| spdAc                  | Cs, Ts           | 69 |
| spdAc                  | Cs, Ts (37)      | 54 |
| spdAc                  | Cs, Ts (37)      | 69 |
| spdAc                  | Ts               | 73 |
| spdAc                  | Ts (37)          | 73 |

* These mutations were originally selected as suppressors against the E400R defect. They were also combined with the R642E mutation.

† Derivatives of pAN55 (pseAMF-secM-secAc) having E400R or R642E mutation in combination with an indicated second site mutation were further combined by transduction with the ΔsecM-secAc-tetA. The transductants were obtained in the presence of either of the original mutant plasmids.

We found further that charge-reversing substitutions, R642E in HSD and E400R in NBF1, abolish the functional activity of SecA since the mutant proteins failed to complement the secA51(Ts) growth and protein export defects (Fig. 2A). Moreover, higher level overproduction of SecA(E400R) or that of SecA(R642E) from plasmid was found to impair growth of secA+ cells on minimal-arabinose medium (Fig. 2B). Neither wild-type SecA nor a “suppressed” variant, SecA(E400R,M607T) (see the last section of “Results”), exhibited such growth interference (Fig. 2B). The dominant-negative characters observed for SecA(E400R) and for SecA(R642E) are consistent with the functional importance of this pair of HSD and NBF1 residues. On the three-dimensional structure of SecA, the oxygen atom of the Glu-400 side chain and the nitrogen atom of the Arg-642 side chain are united only separated by 3.1 Å (Fig. 1A). Taken together, it seems possible that they form a salt bridge, although the double charge reversal, R642E,E400C, remained non-functional (data not shown).

Cysteines at Positions 400 and 642

Form an Intramolecular Disulfide Bond—To verify the close physical proximity of Glu-400 and Arg-642, we mutated both of these residues to cysteine. The double cysteine variant, SecA(E400C,R642C), was functional in vivo (data not shown).

Upon purification and non-reducing SDS-PAGE, it predominantly migrated at an apparent molecular mass of ~130 kDa, slower than normal SecA (Fig. 3A, lane 9; band indicated by “*”). This electrophoretic shift was

NBF2 and Glu-400 in NBF1, respectively, which are also conserved in bacterial species. Thus, these HSD residues appear to interact with the ATPase elements.
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Cysteines at positions 400 and 642 form an intramolecular disulfide bond. A, SDS-PAGE mobilities of SecA variants. Purified cysteine-less SecA (lanes 1 and 6), SecA(E400C) (lanes 2 and 7), SecA(R642C) (lanes 3 and 8), and SecA(E400C,R642C) (lanes 4 and 9) were dissolved in SDS sample buffer containing 5% β-mercaptoethanol (lanes 1–4) or that containing 10 mM iodoacetamide in place of β-mercaptoethanol (lanes 6–9), separated by 7.5% SDS-PAGE, and stained with Coomassie Brilliant blue. Lanes 5 and 10 were for size markers. *, **, and *** indicate the positions of SecA protomer, SecA having Cys-400–Cys-642 intramolecular disulfide bond, and the covalently linked dimer of SecA having Cys-642–Cys-642 intermolecular disulfide bond, respectively. B, the Cys-400–Cys-642 disulfide bond is formed within a single polypeptide chain. Cells expressing both SecA(E400C) and SecA(R642C) (lane 1), those expressing SecA(E400C) (lane 2), and those expressing SecA(E400C,R642C) (lane 3) were cultivated. Crude lysates prepared by sonication were treated with iodoacetamide and subjected to 7.5% non-reducing SDS-PAGE, and proteins were visualized by anti-SecA immunoblotting. Lanes 4 and 5 received 25 ng of purified SecA(E400C,R642C) and SecA(R642C), respectively. Vec, vector. C, disulfide bond formation of SecA(E400C,R642C) in the redox mutant cell. The trxA arhC mutant cells (27) carrying plasmid for cysteine-less SecA (lane 1), SecA(E400C) (lane 2), SecA(R642C) (lane 3), or SecA(E400C,R642C) (lane 4) were grown until a mid-log phase, and cultures were directly treated with 5% trichloroacetic acid to denature cellular proteins, which were then dissolved in non-reducing SDS sample buffer containing 10 mM iodoacetamide, separated by 7.5% SDS-PAGE, and visualized by anti-SecA immunoblotting. Note that the host cells also expressed chromosome-encoded wild-type SecA, which overlapped the non-oxidized SecA expressed from plasmid. D, sedimentation behaviors of SecA variants. SecA(E400C,R642C) (upper panels) or SecA(R642C) (lower panels), 10 µg of each, was mixed with bovine serum albumin (BSA) (60 µg). Samples were further supplemented with 0.05% dodecyl maltoside (DDM) for the right-hand panels. They were centrifuged through a 5–20% sucrose gradient in 50 mM Tris-HCl (pH 7.5) containing 0.05% DDM for the right-hand panels, at 100,000 × g for 13 h using a Hitachi S55S rotor. The gradients were fractionated into 10 fractions, and each fraction was examined by non-reducing SDS-PAGE and Coomassie Brilliant blue staining.

canceled by β-mercaptoethanol treatment (Fig. 3A, lane 4), indicating that a disulfide bond was responsible for it. Since neither of the single cysteine variants, SecA(E400C) nor SecA(R642C), generated this ~130-kDa band (Fig. 3A, lanes 7 and 8), the disulfide bond that was responsible for the mobility shift must have been formed specifically between cysteines at positions 400 and 642 of either the same polypeptide chain (for the formation of an intramolecular disulfide bond) or different polypeptide chains (for the formation of a disulfide-bonded dimer).

When two single cysteine variants, SecA(E400C) and SecA(R642C), were co-expressed in the same cell, they did not generate the ~130-kDa species (Fig. 3B, lane 1), suggesting that this band was accounted for by the intramolecular disulfide bond between Cys-400 and Cys-642. It was noted that the double mutant, SecA(E400C,R642C), also contained a significant amount of a reductant-sensitive species that migrated more slowly than the 220-kDa molecular mass marker (Fig. 3A, lane 9; band indicated by ***). Since as much as ~50% of the purified SecA(R642C) single cysteine variant also formed this >220-kDa species (Fig. 3A, lane 8; band indicated by **), it must have represented a disulfide-bonded dimer. This observation agrees excellently with the recent observation by Zimmer and Rapoport (8) that SecA can assume an intertwined dimer, in which residue 642 (in the case of E. coli SecA) from each subunit is located adjacent each other. Thus, SecA(E400C,R642C) molecules acquire two different sets of disulfide bonds, one (major product) between these cysteines on the same polypeptide chain and the other (minor product) between Cys-642 of different polypeptide chains.

Upon sedimentation fractionation, SecA(E400C,R642C) behaved identically with wild-type SecA whether or not they were treated with DTT (data not shown). Like wild-type SecA (data not shown) and non-disulfide-bonded SecA(R642C) (Fig. 3D, lower panels; band indicated by SecA†), SecA(E400C,R642C) exhibited a significantly lower sedimentation speed in the presence of a detergent, dodecyl maltoside (Fig. 3D, compare upper left and right columns for the band indicated by SecA†). On the other hand, the disulfide bond-linked SecA(R642C) dimer retained the same sedimentation speed in the presence and the absence of the detergent (Fig. 3D, compare lower left and right panels for the band indicated by SecA**). Also, the detergent did not affect the sedimentation rate of bovine serum albumin added to the same centrifugation tubes (Fig. 3D, BSA). The detergent-
induced shift in sedimentation agrees with the known property of SecA, which undergoes detergent-induced monomerization (15). These results, taken together, establish that SecA(E400C,R642C) contained an intramolecular Cys-400–Cys-642 linkage despite its unexpected effect on SDS-PAGE mobility.

The 130-kDa species containing the intramolecular disulfide was also observed when the E400C,R642C double cysteine mutant of SecA was expressed in the trxgor ahpC mutant host having an oxidizing cytoplasmic environment (27) and examined directly by immunoblotting (Fig. 3C, lane 4). Thus, the disulfide bond between these positions of SecA should represent one of the native features that SecA(E400C,R642C) possesses in vivo.

Both the Charge-reversing Mutations and the Disulfide Tethering of Position 642 and Position 400 Impair Translocation Drive but Not Ligand Activation of SecA ATPase—The R642E and the E400R variants of SecA were overproduced stably and purified by standard procedures. Both of them proved severely defective in the in vitro assay of proOmpA translocation into urea-washed IMVs from SecYEG-overproducing cells (Fig. 2C). The mutant proteins nevertheless exhibited higher than normal intrinsic ATPase activities (Fig. 2D, open columns), which were up-regulated further in the presence of IMVs and more pronouncedly in the presence of proOmpA and IMVs. The ATPase stimulation over the background was almost the same for wild-type SecA, SecA(R642E), and SecA(E400R) (Fig. 2D, striped portions of the columns).

We then characterized the functionality of the SecA(E400C,R642C) variant when it contained the intramolecular disulfide bond and when the disulfide bond was reduced with DTT. In vitro assays of proOmpA translocation in the presence and the absence of DTT were carried out using different concentrations of SecA and a reaction time of 7 min. Although the activity of SecA(E400C,R642C) was markedly lower in the absence of DTT than in its presence (Fig. 4A), that of the cysteine-less SecA was unaffected by DTT (Fig. 4A). The low level activity observed with the non-reduced SecA(E400C,R642C) preparation could be ascribed to the small fraction that remained non-oxidized. Thus, SecA is inactivated by the intramolecular disulfide linkage between positions 400 and 642. Like the SecA variants with charge-reversing mutations, the internally disulfide-tethered SecA possessed an increased level of intrinsic ATPase activity (Fig. 4B, open columns), which was accelerated markedly in the presence of both proOmpA and SecYEG membrane vesicles (Fig. 4B, striped portions of the columns). Thus, the disulfide tethering of NBF1 and HSD does not compromise the allosteric activation of the ATPase by a preprotein and the SecYEG translocon.

The E400R Defect Is Suppressible by Alterations at the ATPase-HSD Boundary—The importance of the Glu-400 residue in SecA was suggested further by reversion analysis of the SecA(E400R) mutant. Plasmids encoding SecA(E400R) was mutagenized, and those that regained the ability to complement the secAS1(Ts) growth defect were selected as described under “Experimental Procedures.” We thus isolated seven suppressor mutations, shown in Fig. 5 and Table 2. They all retained the original E400R alteration and con-
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![Diagram](image)

**FIGURE 5. Intragenic suppressors of SecA(E400R).** A, *in vivo* functionality of the suppressed SecA proteins. Amino acid substitutions of the suppressed forms of SecA(E400R) are summarized at the bottom. They were examined for growth and export complementation as shown in the legend for Fig. 2. Precursor; m, mature; Vec, vector. B, *in vitro* translocation activity of a suppressed SecA variant. Translocation of proOmpA(C290G)-His\(_6\)-Myc (0.5 ng/\( \mu \)l) was assayed using indicated and purified SecA variants (5 ng/\( \mu \)l) at 37 °C for indicated times. Translocation efficiencies (percentage of proteinase K-resistant anti-Myc signal) at 5 min are shown on the right. C, ATPase activity. ATPase activities were measured as described in the legend for Fig. 2.

**DISCUSSION**

We have shown that Arg-642 in HSD and Glu-400 in NBF1 are functionally important. They are located in close mutual proximity such that they could form a salt bridge. Mutational reversal of the charge at either position as well as their disulfide-tethering impaired the translocase function of SecA but not the ability of SecA to up-regulate ATPase activity in response to SecYEG and proOmpA. This novel phenotype of the SecA alterations should be contrasted to the previously characterized translocation-negative SecA variants that are all compromised for the translocation ATPase activity as well. Conversely, none of previously described ATPase-enhancing mutations adversely affects the translocase function.

When an ATP-utilizing enzyme is coupled with mechanical work, in which substrate ligands activate the enzyme, two general coupling mechanisms are conceivable. First, the coupling is obligatory in such a way that ligand-induced ATPase activation is mechanistically coupled with the final outcome of the system. In this case, ATPase activation is always accompanied by the concurrent execution of the mechanical work; if the latter is non-functional, ATPase is no longer up-regulated. In the second case, the enzyme is installed with separate modules: a ligand-induced ATPase activation module, a mechanical work module, and a coupling device. Our present results suggest that SecA uses the latter mechanism; the mutational impairment of the proper interplay between NBF1 and HSD allows the ligand-dependent activation of ATPase without ongoing active protein translocation. By analogy, the ATPase, HSD, and the C-terminal translocation domain could perform the respective jobs of a power-controllable engine, a torque converter, and wheels.

In the SecA charge-reversed variants, Glu-Glu or Arg-Arg electrostatic repulsion could increase the average distance between positions 400 and 642, whereas the disulfide tethering will keep them positioned closely. The fact that both of these alterations gave the same uncoupled phenotype suggests that this part of the NBF1-HSD interface must be both closely associated and flexible, probably undergoing time-dependent fluctuation in their geometrical arrangements, such as the swinging motion discussed below.

In our suppression studies, the second site mutations at the hinge-forming NBF2-HSD boundary alleviated the E400R defect. It is tempting to speculate that the angle of this hinge is designed in wild-type SecA to position HSD at proper proximity to NBF1 and that HSD might swing back and forth in response to the ATPase cycle to drive the preprotein movement. The suppressor mutations could affect the average angle of the hinge to compensate for the mutational Arg-Arg repulsion. Consistent with the importance of the hinge region, our suppressor mutations were themselves partially defective (data not shown).

The ATPase-uncoupled SecA variants still interact with
the SecYEG translocon and a preprotein, thereby activating the futile ATP hydrolysis. Overexpression of SecA(R642E) or SecA(E400R) in secA− cells interfered with cell growth, consistent with the inactive but interacting nature of the variant SecA. The ATP-binding pocket of SecA is formed between the two NBFs, but nucleotide binding to the isolated soluble form of SecA does not induce a large conformational change, and the catalytic arginine finger remains far from the γ-phosphate of ATP (10), explaining the low intrinsic ATPase activity. In contrast, ATP-dependent conformational change is detectable for SecA in association with the SecYEG channel (33).

We propose that SecA interacts with a preprotein at least in two different modes, one for the ATPase regulation and the other for translocation. PPXD might be recruited for the first purpose as it is connected to NBF1 via two anti-parallel β-strand loops, through which a signal of preprotein binding could be transmitted to activate NBF1. A recent report showing that PPXD binds the mature portion of preprotein (34) is consistent with this notion. In contrast to the sensing of an ATPase-activating signal by the PPXD globular domain, physical translocation by the assumed movement of HSD would require that a preprotein be captured more firmly. The crystal structure of the monomeric form of Bacillus subtilis SecA revealed an open groove similar to the substrate-binding grooves of molecular chaperones (10). In addition, SecA has another and shallower groove located more closely to NBF1 and HSD (6, 10) with possible complementarity to a signal peptide (35). It is conceivable that such substrate-binding cavities are used for the second mode of preprotein binding used for the actual movement of the preprotein.

Further, it is conceivable that SecA interacts with SecYEG in two different ways, again for ATPase activation and for translocation drive. Recently, we have carried out site-directed SecY-SecA photocross-linking in vivo and in vitro (36), revealing different modes of SecY-SecA interactions. In particular, the fifth cytosolic domain of SecY interacts stably with the ATPase-PPXD region of SecA. In contrast, the C-terminal tail of SecY presumably associates with other region of SecA, and this latter interaction is transient and coupled with active translocation reaction. These results as well as the results of Dapic and Oliver (37) that a N-terminal fragment of SecA is sufficient for the high affinity binding to SecYEG are consistent with our proposal made in this work that the ATPase itself can sense SecYEG as an activating ligand. The maximum ATPase up-regulation, which requires preprotein binding as well, must be accompanied by a substantial conformational change of the ATPase domain (32) to facilitate the arginine finger access to the ATP γ-phosphate and possibly to induce the swinging movement of HSD.

The disulfide tethering of positions 400 and 642 seems to prevent SecA from undergoing transition from an ATPase-activated state to a preprotein-driving state. Our genetic analyses presented here lead to the proposal that the ligand-controlled conformational change of the ATPase domain is converted to the mechanical work of preprotein movement by the action of the long α-helical scaffold. This working hypothesis deserves rigorous tests, including structural determination of SecA that is associated with SecYEG and a preprotein.

Acknowledgments—We thank N. Shimokawa and A. Murakami for constructing some plasmids, J. Beckwith for the E. coli redox mutant, Y. Akiyama, K. Inaba, and S. Chiba for stimulating discussion, and T. Saika, K. Mochizuki, K. Yoshikai, and M. Sano for technical support.

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