Two Independent Mechanisms Down-regulate the Intrinsic SecA ATPase Activity*

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SecA initiates protein translocation by interacting with ATP, preprotein, and the SecYEG membrane components. Under such conditions, it undergoes a conformational change characterized as membrane insertion, which is then followed by hydrolysis of ATP, enabling the release of the preprotein and deinsertion of SecA itself for the next cycle of reactions. Without ongoing translocation, the ATPase activity of SecA is kept very low. Previously, it was shown that the C-terminal 34-kDa domain of SecA interacts with the N-terminal 68-kDa ATPase domain to down-regulate the ATPase. Here, we show, using a deregulated SecA mutant, that the intrinsic ATPase activity is subject to dual inhibitory mechanisms. Thus, the proposed second ATP-binding domain down-regulates the ATPase activity executed by the primary ATPase domain. This regulation, within the N-terminal ATPase domain, operates independently of the C-terminal domain-mediated regulation. The absence of both the mechanisms resulted in a 50-folding elevation of translocation-uncoupled ATP hydrolysis.

Translocation of newly synthesized preproteins across the Escherichia coli cytoplasmic membrane is facilitated by the Sec translocase. The membrane-integrated SecYEG component provides a translocation pathway, and SecA drives the movement of the preprotein. SecA is a dimeric ATPase, containing 901 amino acid residues in each subunit (1), which consists of a C-terminal 34 kDa domain and an N-terminal ATPase domain (68 kDa). The ATPase domain has two proposed ATP-binding sites (2), the high affinity site (NBS I) and the low affinity site (NBS II), whereas the NBS I acts as the primary ATPase domain, the role of the NBS II region is less clear (2, 3).

The reaction cycle of SecA is accompanied by its striking conformational changes, in which the SecA-preprotein complex inserts into the membrane in response to ATP binding followed by deinserterion of SecA in response to ATP hydrolysis (3, 4). In this way, SecA seems to drive the movement of an ~20-aminoo

acid segment of preprotein into the membrane (5). The insertion of SecA was originally defined by the in vitro generation of a 30-kDa C-terminal fragment that was protected by membrane from proteolysis (4, 6). It was shown later that some N-terminal portions of SecA insert as well, because they were also protected from an external protease (7) or accessible from the periplasmic side (8, 9).

SecA exhibits three levels of ATPase activities (10). Although its intrinsic activity is very low, it is activated significantly by membranes or anionic phospholipids; the latter activity is called “membrane ATPase.” In the presence of both a preprotein and membrane vesicles containing functional SecYEG complexes, ATPase activity is enhanced markedly. This activity, referred to as “translocation ATPase,” should result from the SecA reaction cycles outlined above. The present work was aimed at elucidating the mechanisms by which the intrinsic ATPase activity of SecA is kept extremely low. Previous studies (6, 11, 12) indicate that SecA ATPase is down-regulated by interdomain interactions between the C-terminal regulatory domain (34 kDa) and the N-terminal ATPase domain (68 kDa). A region in the C-terminal domain responsible for this interaction is called the intramolecular regulator of ATP hydrolysis (IRA)† (12).

We have identified secY mutations that do not sufficiently support the SecA functions (13-15).2 Suppressor mutations in secA have been isolated using one of these secY mutations as the primary mutation (14, 16). Many of the SecA variants thus isolated proved to be “super-active” in that they suppressed a number of different sec mutations (16). Here, we characterized one such deregulated SecA variants biochemically. Our results show that, in addition to the IRA-mediated regulation, there is an independent regulatory mechanism within the N-terminal ATPase domain in which the NBS II region acts to down-regulate the ATPase activity of the NBS I region.

EXPERIMENTAL PROCEDURES

Plasmids—Plasmid pKY173 carried wild-type secA under the control of the lac promoter (14). pHM348 was a similar plasmid with the secA348 mutation, causing an Asp-580 to Val alteration in SecA. This mutant SecA protein is called SecAD580V in this paper. pNH14 encoded SecAD209N (with an Asp-209 to Asn alteration) and was constructed by site-directed mutagenesis (QuickChange mutagenesis kit, Stratagene) using the mutagenic primers 5′-GCATCTGCTGCTGTG-GAACGAGGAGCTC-3′ and its complementary strand (mutation to be introduced is underlined). pNH15 encoded SecAD580V-D209N (Asp-209 to Asn and Asp-580 to Val double mutant); an ~800-base pair BglII-SphI segment of pHM348 was replaced by the corresponding fragment from pNH14. pNH11 encoded the N86 fragment of SecA in which the Leu-610 codon (CTG) was mutated to UAG; a 9 XbaI-SphI fragment from pNH14 was cloned into a derivative of pBluescript. pHM348 was a similar plasmid with the secA548 mutation as

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2 H. Mori and K. Ito, manuscript in preparation.
Purification of SecA Proteins—Wild-type SecA was overproduced from pKY173 in strain GN45 (a MC1400 derivative carrying leu-B::Tn10 and F’ lacIq lacPl8 lacZ’ Y’ A’ ) as described previously (14). SecAD580V was similarly overproduced from pHM348 in a GN45 equivalent strain with the chromosomal secA348 and secY205 mutations. SecAD209N and SecAD580V-D209N were overproduced from pNH13 and pNH14, respectively, in strain CK4706 (F-lacU araD rpsL relA thi zmb::Tn10 secA65-128) (17) harboring pSTD343 (a pACYC184-derived plasmid carrying lacI). Thus, the mutant forms of SecA were overproduced either in cells having the identical secA allele both on the chromosome and on plasmid or in the presence of a chromosomally encoded variant that was easily distinguishable from the SecA species to be purified. SecA proteins were purified as described previously by Mitchell and Oliver (2).

N68 domains from wild-type SecA and from SecAD580V were overproduced from pNH11 and pNH12, respectively, in strain AD16 (Apro-lac thi f' lacIq ZM15S pro') (18) and purified essentially as described by Karamano et al. (12), except that a phenyl-Sepharose column was used in place of phenyl-Sepharose and a MonoQ 5/5 column was used in place of Fast Flow Q-Sepharose. The His6-tagged C34 domain of SecA was overproduced from pNH13 in strain AD16 by culturing the cells in the presence of 1 mM impropyl-1-thio-p-galactoside for 2 h. Cells were suspended in 20 mM Tris-HCl (pH 8.0) containing 0.5 mM NaCl, 5 mM imidazole, 10 mM 2-mercaptoethanol, and 0.1 mM phenylmethylsulfonylfluoride and disrupted by sonication. The sample was then ultracentrifuged at 45,000 rpm for 30 min (Beckman, Ti-70 rotor), and supernatant was loaded onto a nickel-nitrilotriacetic acid-agarose column, which was washed with 50 mM Tris-HCl (pH 8.0), 60 mM imidazole, 0.5 mM NaCl and then eluted with 50 mM Tris-HCl (pH 8.0), 1 mM imidazole, 0.5 mM NaCl.

Analysis of the Intrinsic SecA ATPase Activity—Intrinsic ATPase activity of SecA was assayed by means of the coupled enzyme reactions (19). In this assay, ADP, the product of ATP hydrolysis, was recycled back to ATP by the coupled reactions of pyruvate kinase and lactate dehydrogenase, in which the accompanying NADH consumption was followed spectrophotometrically. The reaction at 37 °C was started by the addition of a purified preparation of SecA (2–10 μg) to 200 μl of reaction mixture that consisted of 50 mM Tris-HCl (pH 7.5), 3 mM MgSO4, 3 mM phosphoenolpyruvate, 0.25 mM NADH, 5 units of pyruvate kinase, 7.5 units of lactate dehydrogenase, and 1 mM (or other indicated concentrations) ATP. Absorbance at 340 nm due to NADH was monitored in real time. The rate of ATP hydrolysis was calculated from the linear phase of the decrease in A340.

RESULTS

SecAD580V with an Alteration in the Minor ATP-binding Domain (NBS II) Exhibits Enhanced Activity of Intrinsic ATPase—We isolated a number of secA mutations as suppressors against cold-sensitive SecY defect caused by either the secY205 (14–16) or the secY39 (20) mutation. Many of these mutations are proved to be omnipotent in that they suppressed a number of different secY and other sec mutations. This class of mutant SecA proteins that we examined all possessed increased intrinsic and membrane ATPase activities; they are called super-active variants. The suppressor secA alterations were found to cluster within or adjacent to NBS II, the proposed low affinity ATP-binding domain of SecA. We characterized one such secA mutant, secA348, to understand the mechanisms of SecA regulation. This mutant had an amino acid alteration at Asp-209 (to Val), a hot spot for the super-active suppressor mutations (16). The wild-type and the mutant forms of SecA were overproduced and purified to characterize their enzymatic activities. Measurements of ATP hydrolysis in the presence of varying concentrations of the substrate (Fig. 1) showed that the Vmax of the intrinsic ATPase reaction was more than 10 times higher for SecAD580V than for the wild-type SecA (27.5 versus 2.2 nmol of ATP hydrolyzed/min/nmol of SecA-monomer). In contrast, the mutant enzyme had an ~3-fold higher apparent Km value than the wild type (25.9 versus 8.4 μM). Thus, the mutational alteration only slightly affects the affinity for ATP but greatly enhances the rate of translocation-uncoupled ATP hydrolysis in the presence of sufficient concentrations of ATP.

The Primary ATPase Domain (NBS I) Catalyzes Elevated ATP Hydrolysis—Although ATPase catalytic activity of SecA is supposed to be carried out by NBS I, the presence of the residue altered by the secA348 mutation within NBS II raised a question of whether the mutationally enhanced ATPase activity was executed by NBS I or by NBS II itself. To examine this point, a known alteration in NBS I, Asp-209 to Asn (D209N), was combined with the D580V alteration. A previous study by Mitchell and Oliver (2) showed that the D209N alteration inactivated the translocation ATPase activity but did not crucially affect the binding of the nucleotide. However, the intrinsic and membrane ATPase activities were unchanged or apparently enhanced, respectively, by this mutation (2). The D209N form of SecA as well as the D580V-D209N double mutant form were purified, and their intrinsic ATPase activities were measured. As shown in Fig. 2, the introduction of the D209N alteration into SecAD580V strikingly lowered the activity (Fig. 2, compare open circles and crosses). Now, the activity was identical with that of the D209N single mutant protein (Fig. 2, open triangles). Thus, the D580V effect was suppressed completely by the D209N amino acid change. As observed previously (2), the intrinsic ATPase activity of the wild-type protein was no higher than that observed for the D209N mutant protein. These results indicate that the NBS I domain function is required for the enhanced ATPase activity observed in the SecAD580V mutant form of SecA with alteration in the NBS II region. The latter domain may have a regulatory role against the ATPase activity executed by the former domain.

Mutational Enhancement of the ATPase Activity Persists in the Isolated N-terminal 68-kDa Fragment—Previous studies show that the SecA ATPase is down-regulated by an intramolecular domain interaction (6, 11, 12), in which the C-terminal 34-kDa domain acts as a negative regulatory element, termed IBA (12). This regulation was reconstituted by combining separately the purified N-terminal 68-kDa domain and the C-terminal 34-kDa domain (12). Given this mechanism, two pos-

3 Y. Akiyama, personal communication.
sibilities are conceivable for the mechanism responsible for the D580V enhancement of the ATPase activity. First, the NBS II region normally down-regulates the NBS I activity, and the mutation impairs this regulation. Second, the IRA action is mediated by its interaction with the NBS II region, leading to the inhibition of the NBS I ATPase activity, and the mutation abolishes the IRA-NBS II interaction.

As reported by Price et al. (6), mild trypsin treatment activates the intrinsic ATPase activity of SecA by cleaving it at a boundary between the N-terminal ATPase and the C-terminal regulatory domains. We observed about 6-fold elevation of the wild-type ATPase activity upon trypsin treatment (data not shown). When the SecAD580V mutant protein was similarly treated, the activity, which was already ~8-fold higher than the wild-type enzyme, was further stimulated ~6-fold (data not shown). This result suggested that the negative regulation by the C-terminal domain was still operating for the full-length mutant enzyme. It in turn suggested that the altered N-terminal domain itself had the increased ATPase activity to hydrolyze ATP. To substantiate this point, we constructed clones encoding the N-terminal 68-kDa fragment (SecA-N68) or with the D580V alteration (SecAD580V-N68), as well as the C-terminal 34-kDa fragment (C34). These fragments were purified using published procedures (12). SecA-N68 had ATPase activity that was ~10-fold higher than the intact protein (Fig. 3A). Whereas the full-length SecAD580V preparation used in Fig. 3 was already ~8-fold higher in the ATPase activity than in the wild type, SecAD580V-N68 showed a further 5.6-fold elevation over the SecAD580V full-length molecule (Fig. 3A). Thus, SecAD580V-N68 was 4.5-fold higher than SecA-N68 and 47-fold higher than the intact wild-type SecA in its activity to hydrolyze ATP.

Negative Regulation by the C-terminal Domain is Superimposed on the NBS II-mediated Regulation—We then examined whether the inhibitory action of the C34 fragment was still observed against SecAD580V-N68. As shown in Fig. 3B, the addition of increasing concentrations of C34 resulted in increasing extents of inhibition of the N68 ATPase activity (solid circles) as reported previously (12). When ATPase activity of SecAD580V-N68 was examined similarly, it was also inhibited by C34 (Fig. 3B, open circles). The dose-response curves of C34 against the wild-type N68 and SecAD580V-N68 were nearly identical (Fig. 3B). Physical interactions between C34 and N68 were examined by mixing either wild-type N68 or SecAD580V-N68 with C34 having a C-terminally attached hexahistidine tag (Fig. 3C). Upon nickel-nitriolitriacetic acid column chromatography, not only N68 but also SecAD580V-N68 was co-eluted with C34-His6 with imidazole. From these results, we conclude that the inter-domain interaction remained unimpaired in the SecAD580V mutant form of SecA. Thus, in the normal SecA protein, the C-terminal domain-dependent regulation is superimposed on the regulation within the N68 ATPase domain.

FIG. 2. Disruption of NBS I inactivates the ATPase activity of SecAD580V. Intrinsic ATPase activities of wild-type SecA (solid circles), SecAD580V (open circles) D209N (open triangles), and SecAD580V-D209N (crosses) were assayed at 37 °C using 5 μg each of proteins and 1 mM ATP. Values represent the amounts of ATP hydrolyzed per 1 nmol of SecA monomer.

FIG. 3. Down-regulation by the C-terminal domain remains unaltered in SecAD580V. A, ATPase activities were measured for intact SecA, isolated N68 domain, and their D580V counterparts (SecAD580V and SecAD580V-N68). WT, wild type. B, Inhibitory action of isolated C34-His on ATPase activities of wild-type N68-domain (solid circles) and SecAD580V-N68 (open circles). Twenty pmol of the N68 preparations were mixed with increasing amounts of the His6-tagged C34 preparation. After 15 min on ice, reactions with 1 mM ATP were started at 37 °C. The rates of ATP hydrolysis were calculated from linear phases of the reactions and reported as values relative to those in the absence of C34-His6. C, mutational effect on the interaction between N68 and C34-His6 was examined by nickel affinity fractionation. 50 pmol of wild-type N68 (lanes 1–5) and SecAD580V-N68 (lanes 6–10) were mixed with 500 pmol of C34-His6 in 50 μl of 20 mM Tris-HCl (pH 8.0). After 90 min on ice, the mixture was loaded onto a nickel-nitriolitriacetic acid spin column (Qiagen) that had been equilibrated with 20 mM Tris-HCl (pH 8.0). Flow-through fractions (lane 1 and 6), two successive wash fractions with 300 μl of the same buffer (lanes 2, 3, 7, and 8) and two successive eluates with 200 μl of 20 mM Tris-HCl (pH 8.0), 1 mM imidazole (lanes 4, 5, 9, and 10) were collected. Proteins in each fraction were precipitated with 5% trichloroacetic acid, subjected to SDS-polyacrylamide gel electrophoresis, and visualized by immunoblotting using anti-SecA antiserum (15).

According to the insertion/deinsertion model (4), ATP binding induces the membrane insertion of the SecA-preprotein complex, whereas hydrolysis of ATP occurs only after the above process. Consistent with this model, a nonhydrolyzable ATP analog can drive insertion of about 20 residues of preprotein into the membrane (5). Thus, initiation of translocation is a prerequisite for the SecA-catalyzed ATP hydrolysis. Indeed, intrinsic ATPase of SecA, in the absence of preprotein and membrane, is kept very low. Although the SecA ATPase activity is stimulated significantly by the presence of membranes or anionic phospholipids (membrane ATPase), it is enhanced dramatically by the presence of both preprotein and SecYEG membrane vesicles (translocation ATPase). The present study has focused on the problem of how the intrinsic ATPase activity was kept extremely low in the normal SecA protein. Such information will then be directly relevant to the problem of how this ATPase is activated in the presence of preproteins and the SecYEG integral membrane channel components.

We have shown in this paper that SecA ATPase is down-
regulated by dual regulatory mechanisms that work independently. The SecAD580V alteration of Asp580 strikingly enhances the translocation-uncoupled ATP hydrolysis activity of SecA. The enhanced activity can be ascribed to the catalysis carried out by the NBS I ATPase site, because the Asp-209 to Asn mutation of the Walker motif in NBS I abolishes it. Because the D580V mutational effect was observed with the isolated N68 fragment, the NBS II region appears to have a direct role in down-regulating the NBS I activity. According to Ramamurthy and Oliver (8), the NBS II region is included in or close to the regions that are accessible from the periplasmic side of the membrane under certain conditions. It is possible that, when SecA is in the resting state, the NBS II region interacts with the NBS I regulatory domain to suppress the intrinsic ATPase of SecA. Our results indicate that the IRA-mediated regulatory mechanisms, intrinsic SecA ATPase activity, and the resulting release of the inhibition of ATP hydrolytic activity.

In the SecAD580V mutant protein, the alteration in the central region in NBS II may disturb the NBS II-NBS I interaction that is required for down-regulating the NBS I ATPase. Thus, in the super-active class of SecA mutants, the ATPase activation step is bypassed, which may make the mutant SecA work better than wild-type SecA in combination with a partially defective channel component that only poorly activates SecA.

The regulatory mechanism that operates within the N68 ATPase domain is not the sole mechanism that regulates the intrinsic ATPase of SecA. Our results indicate that the IRA-mediated regulation works independently. The 34-kDa C-terminal regulatory domain largely overlaps the 30-kDa membrane insertion domain of SecA (6). Thus, a mechanism similar to that discussed above for the regulatory function of NBS II can be considered for the down-regulation exerted by the C-terminal domain as well; only under the active translocation conditions, the IRA region dissociates from the ATPase domain because of membrane insertion of the 30-kDa domain. SecA ATPase is fully activated under the conditions in which both the C-terminal region and the central NBS II region are engaged in the translocation-driving reactions. Our results demonstrate that in the absence of both the NBS II-mediated and the IRA-mediated regulatory mechanisms, intrinsic SecA ATPase activity is ~50-fold as high as the wild-type resting activity. The dual regulatory mechanisms may have evolved to avoid such futile consumption of ATP and to couple ATP hydrolysis effectively with polypeptide movement across the membrane.

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