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Cross-linked SecA dimers are not functional in protein translocation

Eran Or^{a,b} and Tom Rapoport^{a,*}

^a Department of Cell Biology, Harvard Medical School, 240 Longwood Avenue, Boston, MA 02115

Abstract

The ATPase SecA is involved in post-translational protein translocation through the SecY channel across the bacterial inner membrane. SecA is a dimer that can dissociate into monomers with translocation activity. Here, we have addressed whether dissociation of the SecA dimer is required for translocation. We show that a dimer in which the two subunits are cross-linked by disulfide bridges is inactive in protein translocation, translocation ATPase, and binding to a lipid bilayer. In contrast, upon reduction of the disulfide bridges, the resulting monomers regain these activities. These data support the notion that dissociation of SecA dimers into monomers occurs during protein translocation.

Keywords

Protein translocation; SecA; cross-linking; dimer

1. Introduction

Many proteins are transported through the bacterial inner membrane after completion of their synthesis, i.e. post-translationally. The machinery involved in protein transport consists of two major components [1,2], the SecY complex and SecA. The SecY complex is a heterotrimeric protein complex, comprised of SecY, SecE, and SecG, which forms a protein-conducting channel in the membrane [3]. The SecA protein is a cytosolic ATPase, which moves polypeptides through the SecY channel [4]. SecA interacts with several factors, including the SecY complex[5], acidic phospholipids[6,7] and the chaperone SecB[8]. During translocation SecA undergoes repeated cycles of ATP-hydrolysis dependent conformational changes, which are linked to the movement of successive segments of the polypeptide chain through the protein-conducting channel [9,10]. However the exact mechanism by which SecA translocates substrate proteins through the SecY channel is still unknown.

The oligomeric state of SecA during translocation is an important issue and has been the subject of considerable controversy. SecA is a dimer in solution, and early work suggested that this is its functional state [11–13]. The first crystal structure of *B. subtilis* SecA showed a dimer[4], and both mutagenesis[14] and FRET studies[15] indicated that this dimer corresponds to the species in solution. However, more recent crystallographic and biochemical work has shown

*Corresponding author. Fax: (1)-617-4321190. E-Mail: tom_rapoport@hms.harvard.edu.

^bPresent address: Merck Research Laboratories, 33 Avenue Louis Pasteur, Boston MA 02115.

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that SecA can form at least two other dimeric species, and that SecA dimers are in rapid equilibrium with monomers [16–18]. Although dimers dominate in solution, the equilibrium is shifted towards monomers upon addition of membranes or phospholipids [14,19]. The crystal structure of monomeric *B. subtilis* SecA showed a drastically different conformation with a deep groove into which a polypeptide chain might bind [20]. The opening of the groove correlated with the dissociation of the SecA dimer into monomers. Some findings suggest that the monomer is active in protein translocation. For example, a detergent-solubilized translocation intermediate contained the translocation substrate associated with one copy of the SecY complex and a single copy of SecA [21]. In addition, a SecA derivative missing residues 2–11 (and also the last 70 residues) was almost completely monomeric, yet it retained significant activity in vitro and in vivo, particularly when tested with the SecY mutant prlA4 [22]. However two other studies reported that the same 2–11 deletion in the context of full-length SecA renders the protein inactive, suggesting that dimeric SecA is essential for translocation [23,24]. Here, we have tested the activity of a SecA dimer in which the two subunits are covalently linked by a disulfide bridge. We show that the cross-linked protein is inactive, but regains activity after reduction, suggesting that dissociation of dimers into monomers is required for SecA activity in protein translocation.

2. Materials and methods

Proteins

The construction, expression, and purification of SecY_{His6}G and of His-tagged N95(CC), N95(0 Cys), and SecA have been described [14,22,25].

In vitro Assays

Liposomes containing reconstituted SecYEG were prepared as before[25]. ATPase and translocation assays were carried out at 30°C and 37°C, respectively, as described[14].

Oxidation with diamide

N95(CC), N95(0 Cys), or SecA (0.31 mg/ml) in 50mM K-HEPES pH 7.5, 50mM NaCl, 4mM MgCl₂, 2mM ATP were treated with 1mM diamide (or a lower concentration, where indicated) for 15 min at 22°C and then put on ice. Samples to be analyzed by non-reducing SDS-PAGE were mixed with one volume of 40mM iodoacetamide, 100mM Tris-Cl pH 8.0, 2%SDS and incubated for an hour at 22°C. Then one volume of 30% glycerol, 4% SDS, 0.01% bromophenol-blue was added.

Liposome binding studies

Diamide-treated N95(CC), N95(0 Cys) or SecA (3 µg each) were incubated in 50mM K-HEPES pH 7.5, 50mM KCl, 4mM MgCl₂, 0.15mM diamide with (‘+’) or without (‘-’) 10mM DTT either in the absence (‘-’) or presence of 8µl liposomes (‘Lipid’) for 15 min at 22°C. Samples were transferred to ice and layered onto 100µl 0.2M sucrose in binding buffer (w/o 10mM DTT) and spun at 75,000 rpm for 10 min at 4°C in a table-top ultracentrifuge (OPTIMA™ TLA100.3). Supernatants were removed and pellets were dissolved in 30µl reducing sample buffer, resolved on a 4–20% gel and stained with coomassie.

3. Results

We have previously identified two positions that are in close proximity to one another across the interface between the two subunits in dimeric *B. subtilis* SecA. We introduced two cysteines into the corresponding positions (636 and 801) in a truncated version of *E. coli* SecA, termed N95. This derivative lacks the last 70 residues, but it is dimeric and fully functional. The double-

cysteine mutant N95(CC) [22] was converted almost quantitatively into disulfide-linked products following the addition of the oxidant diamide (Fig. 1, lane 1). The two prominent cross-linked bands correspond to SecA dimers linked by disulfide bridges between cysteine 801 of one subunit and either cysteine 636 or cysteine 801 of the other subunit [22]. Both bands disappeared upon addition of high concentrations of β -mercaptoethanol (Fig. 1, lane 4). Two additional bands in the 130kDa region, marked with asterisks (Fig. 1, lane 1), are probably cross-linked dimers of a N95(CC) truncation (cf. Fig. 5 lane 2). Treatment of a cysteine-free control mutant, N95(0 Cys), with diamide did not result in cross-linked bands (Fig. 1, lane 2). When wild-type SecA was treated with diamide, about 25% of the protein was converted into dimers that were only partially sensitive to reduction by β -mercaptoethanol (Fig. 1 lane 3 versus 6). These dimers were generated through disulfide bridge formation of their C-terminal cysteines [26].

Next we tested whether disulfide cross-linking of the N95(CC) dimer has an effect on its translocation ATPase activity (Fig. 2A). Under non-reducing conditions, the ATPase activity of diamide-treated N95(CC) in the presence of proOmpA (filled triangles) was the same as its activity in the absence of proOmpA (open symbols). A large stimulation of the ATPase activity was only observed when both proOmpA and DTT were present (filled circles). As a control, we measured the ATPase activity of diamide-treated N95(0 Cys), which has no cysteines to be cross-linked (Fig. 2B). In this case stimulation of translocation ATPase activity by proOmpA was observed under both reducing and oxidizing conditions (closed symbols). The same was observed with wild-type SecA (Fig. 2C). Taken together, these experiments show that the loss of translocation ATPase of N95(CC) following treatment with diamide can be reversed by reduction of the inter-subunit disulfide bridges.

To test whether cross-linking of N95(CC) affects ATP catalysis we treated N95(CC) with diamide and measured the kinetic parameters of its basal ATPase activity in the absence or presence of DTT (Fig. 3, filled and open circles, respectively). The presence of DTT reduced V_{max} from 32.0 ± 5.3 to 26.2 ± 1.4 pmole Pi/min and the K_m from $263 \pm 125 \mu M$ to $143 \pm 27 \mu M$. Thus, oxidation of N95(CC) had little effect on its basal ATPase activity. Similar results were obtained with wild-type SecA (not shown). These data show that cross-linked N95(CC) is defective in the stimulation of the basal ATPase activity.

Next we tested the cross-linked N95(CC) dimer for its ability to translocate proOmpA into proteoliposomes containing reconstituted SecY complex (Fig. 4). We found that diamide-treated N95(CC) essentially lacked translocation activity; the residual signal was as low as in the absence of ATP (lanes 3 and 5). However, upon addition of DTT, which reduces the disulfide-bridges linking the monomers, N95(CC) exhibited significant translocation activity (lane 4). Controls showed that both N95(0 Cys), the cysteine-less derivative, and wild-type SecA, had the same translocation activity, whether or not DTT was present (lanes 7, 8, 10, 11). Together, these results establish that the cross-linked N95(CC) dimer has no translocation activity and that reversal of the cross-links by reduction of the disulfide bridges restores activity.

Translocation of precursor proteins by SecA depends on its interaction with membrane lipids. When incubated with liposomes, a significant fraction of wild type SecA co-sedimented with the liposomes (Fig. 5 lanes 15, 16). In the absence of liposomes only negligible amounts of SecA were recovered in the pellet (lanes 12, 13). As expected, the presence of DTT had no effect on the binding of SecA to lipids (lane 16 versus lane 15). On the other hand, diamide-treated N95(CC) interacted only weakly with liposomes (lane 5); the amounts found in the pellet were similar to those seen in the absence of liposomes (lanes 3, 4). However, upon addition of DTT, a significantly larger portion of N95(CC) co-sedimented with the liposomes (Fig. 5 lane 6). The cysteine-less mutant N95(0 Cys) bound to liposomes both in the presence

or absence of DTT (lanes 9, 10). We conclude that cross-linking of N95(CC) inhibits its ability to translocate precursor proteins and disrupts its interaction with membrane lipids. Interestingly, the small amount of dimers that remained after treatment of oxidized wild type SecA with DTT could bind to liposomes (Fig. 5, lanes 15, 16), in contrast to the dimers formed by oxidation of N95(CC). Apparently, cross-linking of SecA molecules through their C-terminal cysteines does not prevent lipid interaction.

4. Discussion

We have generated a SecA derivative, N95(CC), which upon treatment with an oxidative agent produced disulfide-bridged dimers with an efficiency that approached 100%. Two major cross-linked products were observed (Figs. 1 and 2); the upper band corresponds to a dimer held together by an inter-subunit disulfide bridge between cysteine 801 and cysteine 636, in agreement with expectations based on the crystal structure of the anti-parallel dimer of *B. subtilis* SecA [4]. The lower band corresponds to a dimer containing a disulfide bond between cysteines 801 of both subunits [22]. This dimer likely corresponds to the recent structure of *T. thermophilus* SecA in which the two SecA molecules are in a parallel arrangement with the position equivalent to cysteine 801 at the interface [18]. It should be noted that the deletion of the ten N-terminal residues of SecA would be expected to destabilize both dimers, and in fact, all dimer cross-links are lost [22].

The disulfide cross-linked N95(CC) dimer had normal ATPase activity when tested on its own or in the presence of lipids, but absence of substrate (membrane ATPase activity) (Figs 2A and 3). However, it had no translocation ATPase activity (Figs. 2A) and failed to translocate proOmpA into proteoliposomes (Fig. 4 lane 3). In addition, binding of cross-linked N95(CC) to liposomes was impaired (Fig. 5 lane 5). Importantly, upon disruption of the disulfide cross-links with a reducing agent, N95(CC) regained all three activities (Fig. 2A, Fig. 4 lane 4, Fig. 5 lane 6). Our results are in partial disagreement with those of Jilaveanu and Oliver [27]. These authors reported that a cross-linked SecA dimer with cysteines at positions 11 and 661 is inactive, consistent with our conclusions. However, they also found that a cross-linked dimer with cysteines at positions 637 and 801 retains translocation activity. While we have no good explanation for the discrepancy, we note that the cross-linked double-cysteine mutant SecA (637/801) was also found to be inactive by Vassilyev et al. (personal communication).

At first sight, our results also appear to contradict those of de Keyzer et al. [26], who reported that a dimer of wild-type SecA, in which the two subunits were covalently linked, is functional in protein translocation. In fact, we obtained similar results although the cross-linking efficiency was lower (25%) (Fig. 1): The translocation and ATPase activities of the cross-linked SecA were the same as those of untreated SecA (Figs. 3C and 4). The apparent discrepancy is likely explained by the fact that the cysteines at the C-terminus of SecA lie at the flexible end of SecA [4], which, apart from interaction with SecB, is not required for activity [14,28]. Cross-linked C-termini thus probably make a long and flexible linker that may not interfere with the separation of the SecA subunits at their functionally important domains, a conclusion that is supported by our lipid binding experiments. In contrast, the disulfides that provide the cross-links in N95(CC) generate a tight linkage that most likely precludes the dissociation of these domains.

The simplest interpretation of our observations is that the SecA dimer has to dissociate into monomers for protein translocation to occur. However, it is conceivable that cross-linking of the two subunits prevents some conformational change that is required during protein translocation. While this possibility cannot be excluded, it is interesting that the basal ATPase activity of cross-linked N95(CC) remained unchanged, suggesting that at least the basic conformational changes required for ATP hydrolysis are not perturbed by cross-linking. The

liposome binding experiments also argue in favor of a requirement for dimer dissociation. The interaction of SecA with membrane phospholipids should not depend on subtle conformational changes and probably requires the exposure of a hydrophobic binding surface. This surface might be buried in the dimer but could be accessible in the monomer. In the anti-parallel dimer the interface between the two subunits is indeed hydrophobic. Dissociation of the dimer would be consistent with the suggestion that protein translocation involves the cycling of SecA between dimeric and monomeric states [19].

The present results are in agreement with our previous work suggesting that monomeric SecA is the active species in translocation. We found that a constitutively monomeric derivative of SecA retained significant translocation activity and supported the growth of *E. coli* [22]. Although the mutant was not as active as the wild type protein, these results led us to suggest that a dimer is not required for protein translocation. It is in fact possible that SecA dimers do not form under normal conditions, since cross-linking *in vivo* did not reveal any dimers [22]. Our ‘monomer hypothesis’ is at odds with the ideas of Jilaveanu et al. [23], who suggested that a dimer is essential for protein translocation. These authors made a similar N-terminal deletion in full-length SecA and found that it lacked translocation activity. Surprisingly, however, the mutant had translocation ATPase activity, in agreement with our results. In fact a close look at their data suggests that monomeric wild-type SecA has translocation activity: At 5 μ M and 300 mM NaCl SecA was largely monomeric ([23] Fig. 2B), and yet under these conditions it retained about 50% translocation activity ([23] Fig. 3C, lane 6). The SecA mutant protein may be less stable at higher temperature than the wild type protein, which could explain why it does not complement a temperature-sensitive SecA mutant [23].

The reasons for dimerization of SecA remain unclear. Perhaps, when SecA is upregulated, such as when secretion is blocked, dimerization may serve to prevent monomeric SecA from promiscuous interactions with cytoplasmic proteins.

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Figure 1.

Oxidation of N95(CC) produces disulfide cross-linked dimers with high efficiency. Samples (3 μ g) of N95(CC), N95(0 Cys) (a control protein lacking cysteines), and of wild type SecA were treated with diamide. In controls, 480mM β -mercaptoethanol was added. All samples were separated by 5% SDS-PAGE.

Figure 2. Cross-linked N95(CC) exhibits low translocation ATPase activity

A, Aliquots (1.5 μ g) of diamide-treated N95(CC) were tested for ATPase activity in the presence of reconstituted proteoliposomes containing purified SecYEG with or without proOmpA. Where indicated, 10mM DTT was added to reduce the disulfide-bridged dimer.

B, As in **A**, except that wild type SecA was used. **C**, As in **A**, except that N95(0 Cys) was used.

Figure 3. Cross-linking does not affect basal ATPase activity of N95(CC)

Diamide-treated N95(CC) was assayed for ATPase activity in the presence of increasing concentrations of ATP in the absence (closed circles) or presence (open circles) of 10mM DTT. Data were fitted to Michaelis-Menten kinetics. Parameters for cross-linked and reduced N95 (CC) are, respectively: $V_{\max} = 32.0 \pm 5.3$ and 26.2 ± 1.4 pmole Pi/min, $K_m = 263 \pm 125$ and $143 \pm 27 \mu\text{M}$.

Figure 4. Disulfide cross-linked N95(CC) has low translocation activity

Diamide-treated N95(CC), N95(0 Cys) and wild type SecA were assayed for translocation of proOmpA into liposomes containing reconstituted SecYEG. Translocated material was assayed after addition of protease. Where indicated, 5 mM DTT was added. In lane 6 (*), DTT was added 5 min after the onset of incubation, which was continued for further 15 min. Samples marked as '-ATP', received hexokinase/glucose instead of ATP. 'None', no SecA was added.

Figure 5.

Disulfide cross-linked N95(CC) binds to liposomes only when reduced with DTT. Presence or absence of DTT during incubation is indicated by '–' or '+', respectively. Absence or presence of liposomes during the incubation is indicated by '–' and 'Lipid', respectively. For more details see 'Methods'. 'T' – total amount of respective proteins (3μg) added to the incubation. Arrow points to bands of N95 and SecA. Asterisk marks residual cross-linked SecA. 'Percent' – fraction of proOmpA translocated using 10% of 'Total' as a reference.