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Covalently Dimerized SecA Is Functional in Protein Translocation*

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Jeanine de Keyzer[‡], Eli O. van der Sluis[‡], Robin E. J. Spelbrink[§], Niels Nijstad[‡], Ben de Kruijff[§], Nico Nouwen[‡], Chris van der Does[¶1], and Arnold J. M. Driessen[‡2]

From the [‡]Department of Molecular Microbiology, Groningen Biomolecular Sciences and Biotechnology Institute and Materials and Science Centre Plus, University of Groningen, Kerklaan 30, 9751 NN Haren, The Netherlands, the [§]Department of Biochemistry of Membranes, Institute of Biomembranes and Bijvoet Centre for Biomolecular Research, Utrecht University, Padualaan 8, 3584 CH Utrecht, The Netherlands, and the [¶]Institute of Biochemistry, Biozentrum Frankfurt, Johann Wolfgang Goethe University, Marie-Curie-Strasse 9, 60439 Frankfurt am Main, Germany

The ATPase SecA provides the driving force for the transport of secretory proteins across the cytoplasmic membrane of *Escherichia coli*. SecA exists as a dimer in solution, but the exact oligomeric state of SecA during membrane binding and preprotein translocation is a topic of debate. To study the requirements of oligomeric changes in SecA during protein translocation, a non-dissociable SecA dimer was formed by oxidation of the carboxyl-terminal cysteines. The cross-linked SecA dimer interacts with the SecYEG complex with a similar stoichiometry as non-cross-linked SecA. Cross-linking reversibly disrupts the SecB binding site on SecA. However, in the absence of SecB, the activity of the disulfide-bonded SecA dimer is indistinguishable from wild-type SecA. Moreover, SecYEG binding stabilizes a cold sodium dodecylsulfate-resistant dimeric state of SecA. The results demonstrate that dissociation of the SecA dimer is not an essential feature of the protein translocation reaction.

Translocase mediates the translocation of precursor proteins (preproteins) across the cytoplasmic membrane of *Escherichia coli* and other bacteria. The ATPase SecA (1) is the peripheral motor domain of the translocase. The SecYEG complex constitutes a membrane-embedded protein-conducting channel (reviewed in Ref. 2) and a high affinity binding site for SecA (3). When bound to SecYEG, SecA functions as a high affinity membrane receptor for the molecular chaperone SecB with associated preproteins (3). The movement of the preprotein through the protein-conducting channel is driven by multiple cycles of ATP binding and hydrolysis at SecA (4) and the proton motive force.

In solution, SecA exist as a homodimer (5) that equilibrates with its monomeric form in a temperature-, salt- and protein concentration-dependent manner (6). Under physiological conditions, the dissociation constant (K_D) for the monomer-dimer equilibrium is ~0.1 μM (6), and because the cellular concentration of SecA is ~8 μM (5), SecA is mostly dimeric *in vivo*. Because heterodimers formed of active and inactive SecA subunits were shown to be inactive, SecA has been proposed to remain dimeric during preprotein translocation (7). SecA needs to be dimeric to functionally interact with SecB (8, 9), and the structural basis for this requirement was recently solved showing an interaction of the homotetrameric SecB protein with two carboxyl-terminal SecB binding domains of SecA (10). These observations lend strong support for a catalytic role of the SecA dimer during at least the initial preprotein targeting events by SecB and the consecutive preprotein transfer mechanism from SecB to SecA. Nevertheless, the oligomeric state of SecA during preprotein translocation has become a topic of controversy. *Bacillus subtilis* SecA has been crystallized both as a dimer (11) and a monomer (12). In addition, various *in vitro* studies suggest that the oligomeric state of SecA changes upon interaction with its ligands. Negatively charged lipids induce monomerization of SecA (13, 14), although in one report this phenomenon appeared nucleotide dependent (15). Synthetic signal peptides, on the other hand, were found to induce either monomerization (13) or oligomerization (14) of SecA. Covalently linked or antibody-stabilized solubilized SecY(EG) dimers were found to associate with either SecA monomers (16) or a mixture of SecA dimers and monomers (17), whereas a complex of a preprotein and SecYEG associated with monomeric SecA has been detected on Blue Native PAGE (17). Together, these observations have led to the proposal that the oligomeric organization of SecA may change during its reaction cycle (14, 16, 17).

The requirement for a dimeric state of SecA during translocation was further challenged by studies with SecA mutants that are predominantly monomeric in solution. One of these mutants contained 6 point mutations and a truncation of the carboxyl-terminal 70 amino acids (13). In another variant, both the amino-terminal 11 amino acids and the carboxyl-terminal 70 amino acids were removed (SecA(Δ2–11)-831) (18). These monomeric mutants exhibited a very low translocation activity *in vitro*. The SecA(Δ2–11)-831 mutant was found to partially complement the growth defect of a SecA temperature-sensitive strain. This has led to the suggestion that SecA is functional as a monomer (13, 18). In contrast, recent studies with a monomeric SecA mutant carrying a deletion of only the first 11 amino-terminal amino acids (SecA(Δ2–11)) show that this protein is entirely inactive for *in vitro* preprotein translocation (9, 19) and unable to complement the growth defect of a SecA temperature-sensitive strain (19). Evidently, monomerization of SecA by mutation and/or truncation is associated with a major, if not a complete, loss of the activity.

We have studied the functional requirement for the postulated dissociation of the SecA dimer by making use of covalently linked SecA dimers. Although this covalently linked SecA dimer no longer supports SecB-dependent protein translocation, it still binds the SecYEG complex with high affinity and displays normal preprotein-stimulated ATPase and translocation activity in the absence of SecB. These results demonstrate that SecA can bind SecYEG as a functional dimer.

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[1] Present address: Dept. of Molecular Microbiology, Groningen Biomolecular Sciences and Biotechnology Institute, University of Groningen, 9751 NN Haren, The Netherlands.

[2] To whom correspondence should be addressed. Tel.: 31-50-3632164; Fax: 31-50-3632154; E-mail: a.j.m.driessen@rug.nl.

EXPERIMENTAL PROCEDURES

Materials—Isolation of inner membrane vesicles (IMVs)[3] containing overproduced levels of SecYEG and purification of SecYEG, SecA, SecB, and proOmpA was performed as described (20). SecYEG was reconstituted into liposomes of *E. coli* phospholipids (Avanti Polar Lipids, Alabaster, AL) by detergent dilution (20). proOmpA(C290S) was labeled with fluorescein maleimide (Molecular Probes, Eugene, OR) (21). A stock solution of Cu^{2+}(phenanthroline)$_3$ (Sigma) was prepared as described previously (22). SecA antibodies were a kind gift from Dr. Hans de Cock.

Oxidation of SecA—Prior to the oxidation, SecA (5 μM) was incubated for 30 min on ice in the presence of 2 mM EDTA to remove the zinc ion that stabilizes the carboxyl-terminal SecB binding site. EDTA was removed using a Micro Biospin P-6 column (Bio-Rad), and 0.4 mM Cu^{2+}(phenanthroline)$_3$ was added. After 30 min of incubation at room temperature, the reaction was quenched by the addition of 5 mM neocuproine (Sigma). Cu^{2+}(phenanthroline)$_3$ and neocuproine were removed using a Micro Biospin P-6 column.

Surface Plasmon Resonance—SPR measurements were performed on a Biacore 2000 SPR system (Biacore AB) essentially as described (23). SecA binding to IMVs containing overexpressed SecYEG was measured at 25 °C in 50 mM Hepes-NaOH, pH 7, 150 mM KCl, 5 mM $MgCl_2$, 0.5 mg/ml bovine serum albumin in the presence or absence of 1 mM dithiothreitol. Data were corrected for background binding to IMVs containing wild-type (wt) levels of SecYEG. The data were analyzed using the BIAevaluation 2.2.4 and 3.2 software (Biacore).

Other Techniques—Translocation of fluorescently labeled proOmpA was performed as described and visualized in SDS-PAGE gel with a Roche Lumi-imager F1 (Roche Applied Science) (21). Fluorescent and Coomassie-stained bands were quantified with Roche Lumi-analyst 3.1 software. The SecA-stimulated ATPase activity was assayed as described (24). Solubilization of inner membranes at room temperature with SDS and analysis of the monomeric and dimeric forms of SecA by SDS-PAGE were performed as described before (25).

RESULTS

Oxidation of SecA Results in Intradimeric Cross-links—The carboxyl terminus of *E. coli* SecA contains 3 cysteine residues that are essential for coordination of a zinc ion and SecB binding (10, 26). This region of SecA is not resolved in any of the available crystal structures of full-length SecA (11, 12), but the structure of the carboxyl-terminal SecA peptide in complex with SecB has been reported showing the coordination of the zinc ion by the cysteines and a histidine (10). Fluorescence resonance energy transfer studies (7) indicate that the carboxyl termini of the two protomers of a SecA dimer are in close proximity. To induce disulfide bridge formation between these cysteines, the zinc ion was removed by incubation with the chelator EDTA, whereupon SecA was oxidized with Cu^{2+}(phenanthroline)$_3$. Under physiological salt conditions, the dissociation constant (K_D) for the monomer-dimer equilibrium is ~0.1 μM (6). Oxidation was done at a SecA concentration of 5 μM, where the majority of SecA is expected to be dimeric. The cross-linking efficiency was analyzed by SDS-PAGE (Fig. 1). Up to 80% of SecA could be oxidized into a covalently linked dimer that migrates with an apparent molecular size of ~200 kDa (cx-SecA) on non-reducing SDS-PAGE. Oxidation strictly required the addition of Cu^{2+}(phenanthroline)$_3$ (Fig. 1) and was completely reversed upon the addition of dithiothreitol (data not shown).

Oxidation can result in disulfide bridges within a SecA dimer (intradimer) but could also give rise to cross-links between two SecA monomers (inter-monomer) or two different SecA dimers (inter-dimer). A SecA tetramer linked by one inter-dimeric disulfide bridge will dissociate in one SecA dimer and two SecA monomers on SDS-PAGE. To address the nature of the cross-links, oxidation was performed at varying SecA concentrations with a fixed concentration of Cu^{2+}(phenanthroline)$_3$ in the absence or presence of high salt. If the covalently linked dimer resulted from intra-dimeric disulfide bridges, the cross-linking efficiency should not be influenced by the SecA concentration. Analysis of the cross-linking efficiency on SDS-PAGE showed that, in the absence of salt, dimer formation was independent of the SecA concentration used (0.25–5 μM, Fig. 1*B*), When cross-linking was done in the presence of high salt (500 mM) to shift the dimer-monomer equilibrium toward the monomer (6), cross-linking became less efficient and more dependent on the SecA concentration (Fig. 1*B*). Taken together, these data indicate that, in the absence of high salt, oxidation by Cu^{2+}phenanthroline results predominantly in the formation of intra-dimeric SecA disulfide bridges.

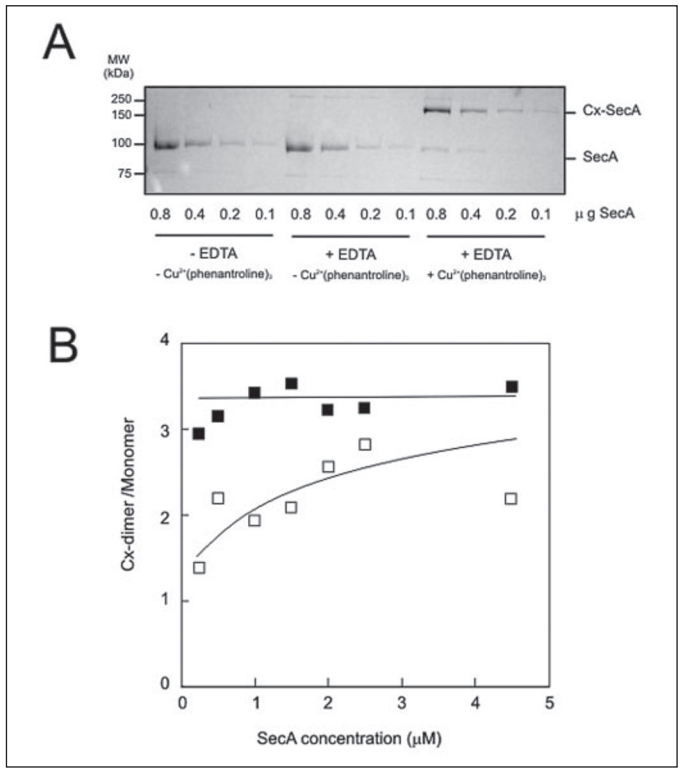

FIGURE 1. **Oxidation of SecA results in the formation of covalently linked dimers.** *A*, SecA was incubated on ice at a concentration of 5 μM in the presence or absence of 2 mM EDTA and 0.4 mM Cu^{2+}phenanthroline. 0.8, 0.4, 0.2, and 0.1 μg of SecA were analyzed on non-reducing Coomassie-stained SDS-PAGE. *B*, SecA (0.25–1 μM) was incubated with 2 mM EDTA and 0.4 mM Cu^{2+}phenanthroline in the presence (□) or absence (■) of 500 mM NaCl. The cross-link efficiency was determined after quantification of the monomeric and cross-linked (*Cx*) dimer bands on non-reducing SDS-PAGE stained with Coomassie Fluor Orange protein gel stain (Molecular Probes).

Cross-linked SecA Dimers Bind with High Affinity to SecYEG—To test whether the high affinity interaction between SecA and SecYEG is affected by the intra-dimeric cross-link, binding of cx-SecA to the SecYEG complex was assayed by SPR. Binding and dissociation of SecA to IMVs containing overexpressed SecYEG was followed in time, and the kinetic parameters of the SecA-SecYEG interaction were determined after correction for background binding to IMVs with endogenous SecYEG levels (23). As was observed previously (23), dissociation of SecA from the SecYEG complex could be described by a parallel dissociation model (TABLE ONE), but the association phase did not fit to this model (data not shown) (23). Therefore, the data were fitted to a model assuming a simple single site (A + B ↔ AB) interaction (23). The specific binding of wt-SecA and cx-SecA both reached saturation at a

[3] The abbreviations used are: IMV, inner membrane vesicle; SPR, surface plasmon resonance; cx, cross-linked; wt, wild-type.

TABLE ONE

Kinetic constants of the SecA-SecYEG interaction

Kinetic constants were determined at a SecA concentration of 50 nM. Data were fitted assuming that SecA was present as a dimer. The fractional contribution of the two k_{off} values is indicated in parentheses. The K_D was determined by the ratio k_{off}/k_{on}.

	k_{on}	k_{off}	k_{off1}	k_{off2}	K_D
	$M^{-1}\,s^{-1}$	s^{-1}	s^{-1}	s^{-1}	M
wt-SecA	$1.8 \pm 0.1\times10^{6}$	$8.0 \pm 0.1\times10^{-3}$	$6.0 \pm 0.2\times10^{-2}$(37%)	$5.5 \pm 0.3\times10^{-3}$(63%)	$4.3 \pm 0.1\times10^{-9}$
cx-SecA	$9.6 \pm 0.9\times10^{5}$	$5.3 \pm 1.2\times10^{-3}$	$5.2 \pm 0.3\times10^{-2}$(25%)	$3.2 \pm 0.9\times10^{-3}$(75%)	$5.5 \pm 0.8\times10^{-9}$

(dimeric) SecA concentration of 50 nM (data not shown) (23). The interaction of cx-SecA with the SecYEG complex was comparable with wt-SecA, although the binding kinetics were slower for cx-SecA (TABLE ONE). The SPR response of cx-SecA was comparable with the wt-control (Fig. 2). To exclude that the cx-SecA response was due to the small fraction of non-cross-linked SecA, binding was also monitored for a 5× lower wt-SecA concentration. This concentration corresponds to a maximal possible level of non-cross-linked SecA present in the cx-SecA sample (see also Fig. 1*A*). At this concentration, a much lower binding level was recorded (Fig. 2), which excludes the possibility that the binding response of cx-SecA is primarily due to the non-cross-linked fraction. SPR measures changes in the refractive index at the IMV-coated sensor chip. Because the SPR response is proportional to the mass bound to the SecYEG complex, these results suggest that the stoichiometry of the SecA-SecYEG interaction is similar for wt-SecA and cx-SecA. If wt-SecA would bind as a monomer or dissociate upon interaction with the SecYEG complex, its binding should result in a lower SPR response as compared with cx-SecA. Likewise, binding of cx-SecA as a "dimer of cross-linked dimers" should result in an increased SPR response compared with wt-SecA. Because the responses of cx-SecA and wt-SecA are in the same range, the SPR data indicate that both SecA proteins associate with the SecYEG complex as dimers.

SecYEG Overexpression Results in an Increased Level of Dimeric SecA—After solubilization of IMVs at room temperature with SDS, membrane-associated SecA migrates on SDS-PAGE as a mixture of monomers and dimers (25). Because purified SecA solubilized in SDS migrates as a monomer irrespective of the presence or absence of lipids under these conditions, the room temperature SDS-resistant SecA dimer could represent the SecYEG-associated SecA fraction (25). If this is the case, overexpression of SecYEG should result in an increased level of SDS-resistant SecA dimers. IMVs containing endogenous or overexpression levels of SecYEG were solubilized in SDS at room temperature and analyzed by SDS-PAGE and immunoblotting. Membrane-associated SecA migrated as two bands with apparent molecular masses of ~100 and ~200 kDa (Fig. 3). The high molecular mass band was only recognized by antibodies against SecA and its position corresponded to the position of the cross-linked SecA-dimer (25), suggesting that it represents the dimeric form of SecA. Evidently, overexpression of SecYEG results in a dramatic increase in the level of SecA dimers (Fig. 3, *lane YEG*$^{+}$), demonstrating that SecYEG stabilizes a dimeric state of SecA that is resistant to room temperature SDS. These data further suggest that the SecYEG-bound state of SecA is dimeric.

Cross-linked SecA Dimers Are as Active as Wild-type SecA—The ATPase activity of wt-SecA and cx-SecA was assayed in an *in vitro* system containing urea-treated IMVs harboring overexpressed SecYEG. Both in the presence or absence of preprotein, the ATPase activity of cx-SecA and non-cross-linked SecA were identical (Fig. 4*A*). Likewise, cx-SecA and wt-SecA were equally effective in supporting the translocation of fluorescein maleimide-labeled proOmpA(C290S) into IMVs (Fig. 4*B*) and proteoliposomes reconstituted with purified SecYEG complex (Fig. 4*C*). Furthermore, their activities were equally enhanced by the *prlA* mutation I408N in SecY (Fig. 4*C*).

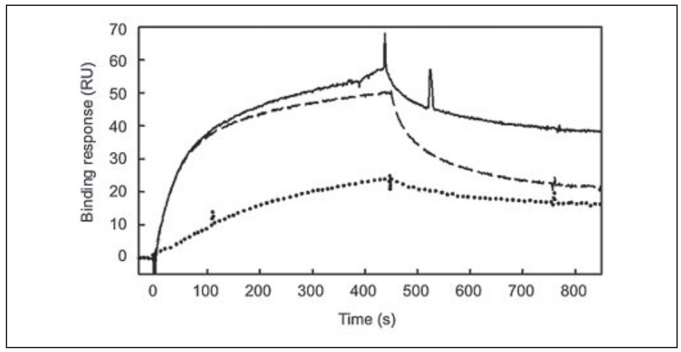

FIGURE 2. **Covalently dimerized SecA binds the SecYEG complex with high affinity.** Binding of 5 nM cx-SecA (*solid*) and wt-SecA (*dashed*) or 1 nM wt-SecA (*dotted line*) to IMVs containing overexpressed SecYEG. IMVs were immobilized on a Pioneer L1 chip as described under "Experimental Procedures." Binding was analyzed by surface plasmon resonance. Binding curves were corrected for background binding to a control cell containing IMVs with endogenous SecYEG levels. SecA binding was measured at 25 °C at a flow rate of 20 μl/min.

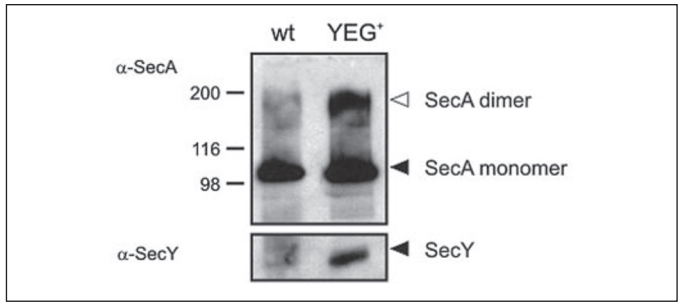

FIGURE 3. **SecYEG overexpression results in an increased level of membrane-associated SDS-resistant SecA dimers.** *E. coli* BL21 IMVs (6 μg) containing either endogenous (*wt*) or overexpression levels of SecYEG (*YEG*$^{+}$) were solubilized at room temperature with SDS and analyzed by immunoblotting with antibodies against SecA or SecY.

To exclude the possibility that the activity of cx-SecA was caused by the small fraction of non-cross-linked SecA present in the sample, translocation was assayed as a function of the SecA concentration. The preprotein-stimulated ATPase (Fig. 4*D*) and preprotein translocation (Fig. 4*E*) activity showed a similar dependence on the concentration of wt-SecA and cx-SecA. This further demonstrates that cx-SecA is as active as the non-cross-linked SecA.

Cross-linked SecA Dimers Are Defective in SecB-mediated Protein Translocation—Although biochemical data indicate that the extreme carboxyl terminus is not essential for the activity of SecA *per se* (27–29), this region is needed for the functional interaction with SecB (8). Therefore, we tested whether cross-linking of the SecA carboxyl termini affects the SecB-dependent translocation of proOmpA. With wt-SecA, addition of SecB enhanced the translocation of proOmpA (Fig. 5). In contrast, with cx-SecA, addition of SecB resulted in a slight but reproducible inhibition of the proOmpA translocation (Fig. 5). This effect could be restored after reduction of

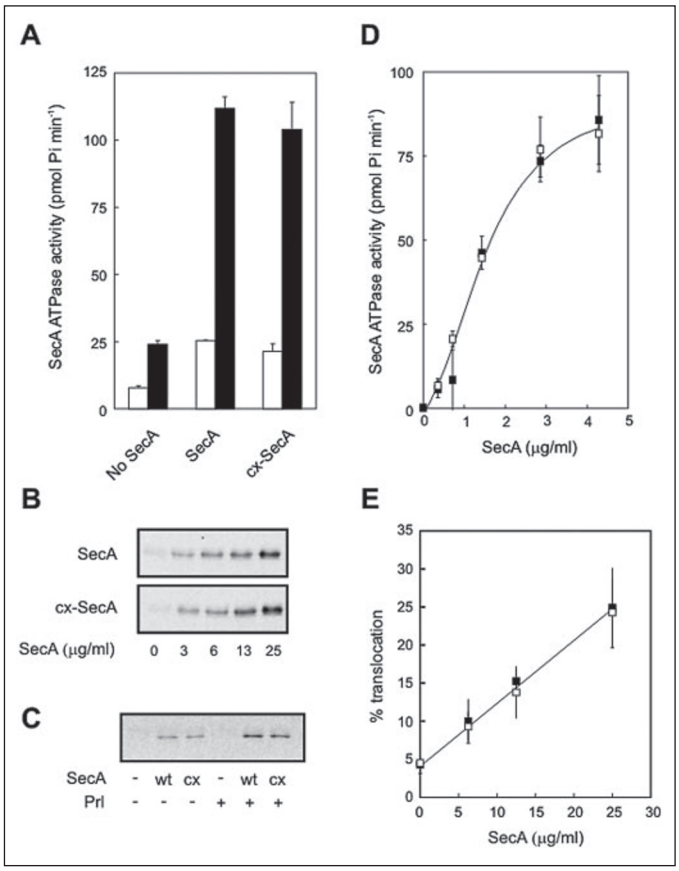

FIGURE 4. **Covalently dimerized SecA is fully active in its ATPase and translocation activity.** *A*, ATPase activity of (cx)-SecA (2 μg/ml) in the presence of urea-treated SecYEG$^+$ inner membrane vesicles (15 μg/ml). *Black columns* indicate incubation in the presence of proOmpA (40 μg/ml), *white columns* in the presence of solvent only. The background ATPase activity (*no SecA*) is due to a SecA population that could not removed during urea treatment of the IMVs. *B*, translocation of fluorescein-labeled proOmpA(C290S) (4 μg/ml) into urea-treated IMVs containing overexpressed SecYEG (60 μg/ml) in the presence of increasing SecA concentrations. *C*, translocation of fluorescein maleimide-labeled proOmpA(C290S) (4 μg/ml) into proteoliposomes (5 μg/ml) containing purified SecYEG or SecY(I408N)EG complex. *D*, proOmpA-stimulated ATPase activity in the presence of urea-stripped IMVs containing overexpressed SecYEG (15 μg/ml) and increasing concentrations of wt-SecA (■) or cx-SecA (□). Data were corrected for both the background ATPase activity in the absence of proOmpA and the endogenous ATPase activity in the absence of added SecA. *E*, quantification of the translocation of fluorescein-labeled proOmpA(C290S) (4 μg/ml) into urea-treated IMVs containing overexpressed SecYEG (60 μg/ml) in the presence of increasing concentrations of wt-SecA (■) or cx-SecA (□).

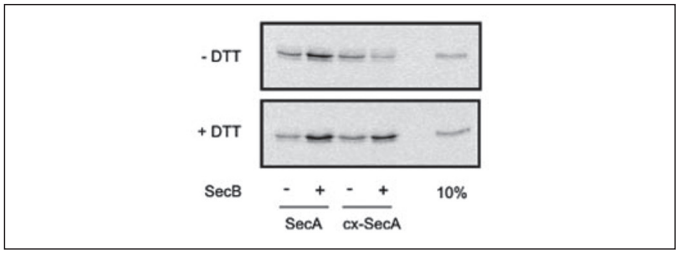

FIGURE 5. **Oxidation of SecA interferes with SecB-mediated proOmpA translocation.** Translocation of fluorescein-labeled proOmpA(C290S) into urea-treated IMVs containing overexpressed SecYEG in the presence or absence of SecB and 10 mM dithiothreitol.

the carboxyl-terminal disulfide bridges of cx-SecA by the addition of dithiothreitol (Fig. 5). These data suggest that the cross-linking of the carboxyl-terminal cysteines reversibly disturbs the functional SecA-SecB interaction, while it does not affect SecA-dependent preprotein translocation in the absence of SecB.

DISCUSSION

SecA is a motor protein that fulfills a key role in preprotein translocation. It interacts with the SecYEG complex, anionic phospholipids, nucleotides, signal peptides, the mature domain of preproteins, and the molecular chaperone SecB. *In vitro* studies with purified SecA have shown that SecA forms a dynamic dimer whose monomer-dimer equilibrium is altered upon interaction with some of these ligands (13–15). This has raised questions about the functional significance of the dimeric state of SecA during protein translocation. In this study, we have analyzed the functional requirement for dimer dissociation by means of a covalently linked SecA dimer. Although the covalently linked SecA dimer was defective in SecB-mediated translocation, its catalytic properties were indistinguishable from wt-SecA in the absence of SecB. These data demonstrate that complete dissociation of the SecA dimer is not an essential feature of the translocation reaction.

Covalently linked SecA dimers were obtained by oxidation with $Cu^{2+}(phenanthroline)_3$. This treatment results in disulfide bridge formation between the carboxyl-terminal cysteines of the two protomers of a SecA dimer. Cross-linking of the carboxyl termini alone cannot rule out the possibility that other, non-covalent interactions at the SecA dimer interface dissociate during catalysis or that only one of the protomers of the SecA dimer is actively involved in translocation at a given time. However, the activity of the SecA dimer was previously shown to be abolished upon inactivation of one of its subunits (7). Active participation of both protomers thus appears to be required for efficient preprotein translocation. Moreover, the activity of cx-SecA was essentially identical to wt-SecA under conditions where SecA was limiting for translocation. This strongly suggests that the covalently linked dimer acts similarly to the wild-type SecA dimer and does not merely function as a monomer with a non-functional SecA protomer attached to its carboxyl terminus.

The carboxyl-terminal cysteine residues of SecA are located in the region that forms the high affinity binding site for SecB (8). Without the carboxyl-terminal 21 amino acids, SecA no longer binds SecB in a functional manner but is still able to catalyze SecB-independent protein translocation (8, 31). We now observe that the addition of SecB no longer stimulates, but rather inhibits, preprotein translocation when the SecA dimer is cross-linked via its carboxyl-terminal cysteines. Recently, the structure of the extreme carboxyl terminus of *Haemophilus influenzae* SecA in association with SecB was solved (10). In agreement with biochemical studies (26), the crystal structure confirmed the presence of a zinc ion coordinated by 3 cysteines and 1 histidine residue (10). Zinc stabilizes a fold that exposes the basic amino acids of the carboxyl terminus toward the anionic binding surface on SecB. It is therefore likely that oxidation of the cysteines results in a disruption of the SecB binding site. Because efficient transfer of SecB-bound preproteins to SecA requires a functional SecB binding site (31), this would explain why the oxidized SecA no longer supports SecB-dependent translocation. Crystallographic (10) and biochemical (9, 26) studies lend strong support for the hypothesis that tetrameric SecB with bound preprotein associates with dimeric SecA via the two SecA carboxyl termini that embrace the SecB tetramer. Taken together these data point at a critical role of the SecA dimer, at least in the initial stages of SecB-mediated preprotein targeting and the preprotein transfer reaction.

Analysis of membrane-associated SecA on SDS-PAGE suggests that there are at least two SecA populations associated with the inner membrane (25). One of the populations corresponds to a stable dimeric form that resists solubilization in room temperature SDS, whereas the other population is either monomeric or dissociates into monomers under the conditions of solubilization. The presence of membrane-associated

SecA dimers is consistent with recent cross-linking experiments on IMVs that also identified a significant population of SecA dimers (19). In addition, wt-SecA was shown to be trapped in a membrane-bound heterodimer upon overproduction of SecA mutants that stabilize SecA at the membrane (19). Because the level of room temperature SDS-resistant dimers increased dramatically upon overexpression of SecYEG (this study), these data strongly suggest that the SecYEG-bound state of SecA is dimeric.

Or *et al.* (13, 18) proposed that SecA binds to the SecYEG complex as a monomer, because chemical cross-linking experiments failed to detect SecA dimers in the presence of proteoliposomes containing purified SecYEG. Their failure to detect dimers by means of cross-linking is, however, most likely due to a large fraction of lipid-bound SecA, as the lipid- and SecYEG-bound SecA populations were not separated. Our SPR experiments show that association of wt-SecA or cx-SecA with the SecYEG complex results in comparable SPR responses. This is only possible when these SecA species bind to the SecYEG complex with the same stoichiometry; *i.e.* as a SecA dimer. We therefore conclude that the dimeric SecA is the native binding partner for the SecYEG complex. The affinity of cx-SecA for SecYEG was comparable with that of wt-SecA, although the kinetics of both the association and dissociation phase were slower. The slower kinetics of cx-SecA may be due to physical constraints caused by the covalent linkage between the SecA carboxyl termini. In our SPR experiments wild-type SecA does not dissociate from the SecYEG complex according to a simple one-to-one model. Instead, there are two SecA populations that dissociate at different rates. The covalently linked SecA dimer dissociates according to the same model, but the contribution of the slow dissociating population is larger than for wild-type SecA. The mechanism that underlies the parallel dissociation has not been firmly established yet. It is possible that SecA is able to dissociate from the SecYEG complex either as a dimer or as individual monomers. If the dissociation of a SecA monomer is faster than the dissociation of a SecA dimer, this could provide an explanation for the parallel dissociation model as well as for the observation that the cx-SecA dissociates slower from the SecYEG complex than wt-SecA. Future studies should give more insight into the physiological relevance of the parallel dissociation model. Because both the translocation and the ATPase activity of cx-SecA were indistinguishable from wt-SecA, it appears that the kinetics of SecYEG binding are not rate-determining for the translocation reaction.

Blue Native Page analysis indicated that a preprotein is associated with at least the monomeric SecA and SecYEG complex during translocation (17). However, it cannot be ruled out that the preprotein-translocase complex partly dissociated during membrane solubilization and isolation of the complex. In this respect, co-immunoprecipitation, cross-linking, and fluorescence resonance energy transfer experiments indicate that the detergent used in the Blue Native Page study, dodecylmaltoside, dissociates the SecA dimer (13), although analytical ultracentrifugation suggests that dodecylmaltoside does not interfere with SecA dimerization (14). SecA variants that are predominantly monomeric due to deletion of the amino-terminal 11 amino acids (18) or multiple mutations in combination with a truncation of the carboxyl-terminal domain (13) were found to display translocation activity, but their activity was dramatically reduced as compared with wild-type SecA. To convincingly detect the activity of these monomeric SecA proteins, the authors employed the PrlA4 mutant of SecY in their assays. This mutant already shows a marked elevated activity with wt-SecA (32, 33), which implies that the SecA monomers will be essentially inactive for preprotein translocation with the wild-type SecYEG complex. Recent investigations with monomeric SecA variants similar to the ones used by Or *et al.* (18) demonstrate that, even though they are able to interact with the SecYEG complex (19), these monomers are completely inactive for translocation activity (9, 19). Furthermore, a SecA variant that lacks the 9 amino- and 40 carboxyl-terminal residues is fully active and dimeric, whereas further truncation of the amino terminus yielded unstable SecA mutants unsuitable for further analysis (34). Taken together, these data lend strong support for the notion that the monomer does not represent a physiologically active state of SecA and support the hypothesis that the SecA dimer is the active species.

So far, the oligomeric state of SecA has been addressed mainly in solution or in studies using (truncated) SecA mutants with reduced activity. We have re-investigated the catalytic role of the SecA dimer under conditions where it is actively involved in preprotein translocation. Stabilization of the SecA dimer by cross-linking of the carboxyl-terminal cysteine residues had no effect on the translocation activity under conditions that SecA was limiting for translocation. This strongly suggests that SecA is dimeric in its physiologically active state. Furthermore, our data show that complete dissociation of the SecA dimer is not a critical feature of the catalytic cycle and that SecA binds to the SecYEG complex as a dimer. Further studies should address the question of how the two protomers mechanistically participate in preprotein translocation.

REFERENCES

1. Cabelli, R. J., Chen, L., Tai, P. C., and Oliver, D. B. (1988) *Cell* **55,** 683–692
2. Veenendaal, A. K. J., van der Does, C., and Driessen, A. J. M. (2004) *Biochim. Biophys. Acta* **1694,** 81–95
3. Hartl, F. U., Lecker, S., Schiebel, E., Hendrick, J. P., and Wickner, W. (1990) *Cell* **63,** 269–279
4. Schiebel, E., Driessen, A. J. M., Hartl, F. U., and Wickner, W. (1991) *Cell* **64,** 927–939
5. Akita, M., Shinaki, A., Matsuyama, S., and Mizushima, S. (1991) *Biochem. Biophys. Res. Commun.* **174,** 211–216
6. Woodbury, R. L., Hardy, S. J., and Randall, L. L. (2002) *Protein Sci.* **11,** 875–882
7. Driessen, A. J. M. (1993) *Biochemistry* **32,** 13190–13197
8. Fekkes, P., van der Does, C., and Driessen, A. J. M. (1997) *EMBO J.* **16,** 6105–6113
9. Randall, L. L., Crane, J. M., Lilly, A. A., Liu, G., Mao, C., Patel, C. N., and Hardy, S. J. (2005) *J. Mol. Biol.* **348,** 479–489
10. Zhou, J., and Xu, Z. (2003) *Nat. Struct. Biol.* **10,** 942–947
11. Hunt, J. F., Weinkauf, S., Henry, L., Fak, J. J., McNicholas, P., Oliver, D. B., and Deisenhofer, J. (2002) *Science* **297,** 2018–2026
12. Osborne, A. R., Clemons, W. M., Jr., and Rapoport, T. A. (2004) *Proc. Natl. Acad. Sci. U. S. A.* **101,** 10937–10942
13. Or, E., Navon, A., and Rapoport, T. (2002) *EMBO J.* **21,** 4470–4479
14. Benach, J., Chou, Y. T., Fak, J. J., Itkin, A., Nicolae, D. D., Smith, P. C., Wittrock, G., Floyd, D. L., Golsaz, C. M., Gierasch, L. M., and Hunt, J. F. (2003) *J. Biol. Chem.* **278,** 3628–3638
15. Bu, Z., Wang, L., and Kendall, D. A. (2003) *J. Mol. Biol.* **332,** 23–30
16. Tziatzios, C., Schubert, D., Lotz, M., Gundogan, D., Betz, H., Schagger, H., Haase, W., Duong, F., and Collinson, I. (2004) *J. Mol. Biol.* **340,** 513–524
17. Duong, F. (2003) *EMBO J.* **22,** 4375–4384
18. Or, E., Boyd, D., Gon, S., Beckwith, J., and Rapoport, T. (2005) *J. Biol. Chem.* **280,** 9097–9105
19. Jilaveanu, L. B., Zito, C. R., and Oliver, D. (2005) *Proc. Natl. Acad. Sci. U. S. A.* **102,** 7511–7516
20. van der Does, C., de Keyzer, J., van der Laan, M., and Driessen, A. J. M. (2003) *Methods Enzymol.* **372,** 86–98
21. de Keyzer, J., van der Does, C., and Driessen, A. J. M. (2002) *J. Biol. Chem.* **277,** 46059–46065
22. Kaufmann, A., Manting, E. H., Veenendaal, A. K. J., Driessen, A. J. M., and van der Does, C. (1999) *Biochemistry* **38,** 9115–9125
23. de Keyzer, J., van der Does, C., Kloosterman, T. G., and Driessen, A. J. M. (2003) *J. Biol. Chem.* **278,** 29581–29586
24. Lill, R., Cunningham, K., Brundage, L. A., Ito, K., Oliver, D., and Wickner, W. (1989) *EMBO J.* **8,** 961–966
25. Spelbrink, R. E., Kolkman, A., Slijper, M., Killian, J. A., and de Kruijff, B. (2005) *J. Biol. Chem.* **280,** 28742–28748
26. Fekkes, P., de Wit, J. G., Boorsma, A., Friesen, R. H., and Driessen, A. J. M. (1999) *Biochemistry* **38,** 5111–5116
27. Matsuyama, S., Kimura, E., and Mizushima, S. (1990) *J. Biol. Chem.* **265,** 8760–8765

28. Rajapandi, T., and Oliver, D. (1994) *Biochem. Biophys. Res. Commun.* **200,** 1477–1483
29. Breukink, E., Nouwen, N., van Raalte, A., Mizushima, S., Tommassen, J., and de Kruijff, B. (1995) *J. Biol. Chem.* **270,** 7902–7907
30. Deleted in proof
31. Fekkes, P., de Wit, J. G., van der Wolk, J. P., Kimsey, H. H., Kumamoto, C. A., and Driessen, A. J. M. (1998) *Mol. Microbiol.* **29,** 1179–1190
32. de Keyzer, J., van der Does, C., Swaving, J., and Driessen, A. J. M. (2002) *FEBS Lett.* **510,** 17–21
33. van der Wolk, J. P., Fekkes, P., Boorsma, A., Huie, J. L., Silhavy, T. J., and Driessen, A. J. M. (1998) *EMBO J.* **17,** 3631–3639
34. Karamanou, S., Sianidis, G., Gouridis, G., Pozidis, C., Papanikolau, Y., Papanikou, E., and Economou, A. (2005) *FEBS Lett.* **579,** 1267–1271