Biochemical and Biophysical Research Communications 447 (2014) 250–254

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

# SecAAA trimer is fully functional as SecAA dimer in the membrane: Existence of higher oligomers?

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ARTICLE INFO

*Article history:*
Received 7 March 2014
Available online 1 April 2014

*Keywords:*
SecA
Trimer
Hexamer
AFM

ABSTRACT

SecA is an essential ATPase in bacterial Sec-dependent protein translocation pathway, and equilibrates between monomers and dimers in solution. The question of whether SecA functions as monomers or dimers in membranes during the protein translocation is controversial. We previously constructed a tail-to-head SecAA tandem dimer, and showed it is fully functional by complementation *in vivo* and protein translocation *in vitro*, indicating that SecA can function at least as a dimer in the membrane without dissociating into monomers. In this study, we further constructed genetically a tail-to-head SecAAA trimer, which is functional in complementing a temperature-sensitive *secA* mutant. The purified SecAAA trimer per protomer is fully active as SecAA tandem dimers in ATPase activity, in protein translocation *in vitro* and in ion channel activities in the oocytes. With these functional tail-to-head trimer SecAAA and tandem SecAA, we examined their surface topology in the presence of liposomes using AFM. As expected, the soluble SecAAA without lipids are larger than SecAA. However, the ring/pore structures of SecAAA trimers were, surprisingly, almost identical to the SecA 2-monomers and SecAA dimers, raising the intriguing possibility that the SecA may exist and function as hexamer ring-structures in membranes. Cross-linking with formaldehyde showed that SecA, SecAA and SecAAA could form larger oligomers, including the hexamers. The molecular modeling simulation shows that both tail-to-head and tail-to-tail hexamers in the membranes are possible.



## 1. Introduction

In bacteria, many secreted proteins are transported post-translationally across the cytoplasmic membranes by the general secretary pathway (Sec-pathway). SecA, SecYEG and other Sec proteins are key components in this process [1–5]. The prevailing model depicts that SecYEG-SecDF·YajC complexes form the essential translocation core channel; and motor protein SecA undergoes ATP-driven cycles of membrane insertion/de-insertion, pushes the preprotein through the channel in a step-wise fashion [3,6–8]. We have recently shown that in addition to this high-affinity SecA-SecYEG-SecDF·YajC protein-conducting channel, there is an additional SecA-alone low-affinity protein-conducting channel [9] with lower specificity and less efficiency that can be transformed into the high-affinity channels [10]. This additional SecA-alone channel has been supported by the observations that SecA integrates into membranes [4,11–14] and forms ring-like pore structures in the presence of phospholipids bilayers as observed by transmission electron microscopy and atomic force microscopy (AFM). This ring-like pore structures are not formed in solution or in uncharged phosphatidylcholine [15]. Such SecA-liposomes are functional in promoting *in vitro* protein translocation and eliciting ion channel activity in the oocytes and in the patch-clamp electrophysiological recordings [9]. These results indicate that SecA may also form the core and can play a structural role in the protein translocation process. A model of SecA-alone channel has been presented [14].

SecA is distributed between the inner membrane and cytosol [11,16], and functions in the membrane. The soluble SecA equilibrates between dimer and monomer [17,18]. However, the functional oligomeric state of SecA in the membrane has been

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http://dx.doi.org/10.1016/j.bbrc.2014.03.116
0006-291X/

the subject of considerable controversy. There were reports that monomer is the functional status of SecA in the membrane [19–21], while others showed that SecA functions as a dimer. We earlier showed that SecA tail-to-head tandem dimer, SecAA, has similar *in vivo* and *in vitro* activities as wild-type SecA [22]. Moreover, SecAA and SecA can form ring-like pore structures with similar sizes upon interaction with anionic phospholipids [22], suggesting that SecA can function at least as a dimer without dissociating into monomers. These results also show that SecA can function in a tail-to-head configuration.

In this study, we further constructed a genetically tail-to-head SecAAA trimer and show that the trimers are fully functional *in vivo* and *in vitro* per protomer as SecA monomers and SecAA dimers. Interestingly, SecAAA upon interaction with lipids also forms a ring structure similar to SecA and SecAA in sizes and shapes as observed by AFM, suggesting the possible existence of functional hexamers. Crosslinking and molecular modeling data further support the possible existence of SecA hexamers.

## 2. Materials and methods

### 2.1. Bacterial strains, plasmids, and media

*Escherichia coli* DH5α was used for plasmid isolation and for subcloning DNA fragments. pET5a*secA*$^*$ which harbors a mutation of A to G at nucleotide #2098 [23], resulting in SecAM700V that has no effect on SecA activity, was used for all SecA constructions. *E. coli* BL21.19 [24] harboring pET5a*secAA* and pET5a/*secAAA* were used for complementation assays and overproduction of SecA derivatives. To construct pET5a/*secAA* with linker from tail-to-head, an entire *secA* was PCR amplified from pET5a*secA* using 5′ primer containing NdeI, and 3′ primer including the linker sequences (BamHI and XhoI) and NdeI. DNA fragment was then digested with NdeI and inserted into the NdeI site of pET5a/*secA* to yield pET5a/*secAA* containing tail-to-head linker (TCA GGA TCC ATT CTC GAG CAT) sequences between two *secA*s.

pET5a/*secAAA* was constructed from pET5a/*secAA* by PCR amplification of an entire *secA* using primers containing BamHI and XhoI at 5′ and 3′, respectively. The PCR generated DNA fragment was digested with BamHI and XhoI and inserted into the same sites of pET5a/*secAA*. The resulting pET5a/*secAAA* contains two linkers (Ser-Gly-Ser-Thr-Ser-Ile-His and Pro-Gly-Ser-Ile-Leu-Glu-His). All the plasmid constructions were verified at the DNA Sequencing Core Facility (Department of Biology in Georgia State University). Strains for SecAA and SecAAA overproduction were cultured in TAG medium (10 g/L Tryptone, 5 g/L NaCl, A salts [22] and 0.5% glucose) supplemented with 100 μg/ml ampicillin.

### 2.2. Complementation test

Overnight BL21.19 cultures harboring vector pET5a alone or pET5a*secAA* and pET5a/*secAAA* were inoculated into fresh LB/Amp and grow at 30 °C. Log-phase growing cells were then adjusted to the same density of 0.5 O.D. 600 nm. 100 μl volumes of serial 10-fold dilutions were applied onto the plates, incubated at 42 °C and plates containing 30 to 300 colonies were used to obtain colony forming units. Duplicate control plates were incubated at 30 °C as described previously [22].

### 2.3. Expression and purification of SecA tandem trimer and dimer

All protein purification steps and centrifugations were performed at 4 °C. SecAA and SecAAA overexpressed cells were cultured at 30 °C until O.D. 600 nm of 1.2 and induced with 0.5 mM IPTG. Proteins were overexpressed at 30 °C for 1.5 h. Cells were harvested by centrifugation in a Beckman JA-10 rotor at 5000 rpm for 15 min and resuspended in 3 ml/g wet cells of TKMD buffer (10 mM Tris–HCl, pH 7.6, 50 mM KCl, 10 mM MgOAc and 1 mM DTT) with 20% $(NH_4)_2SO_4$ to prevent SecAAA degradation. The protease inhibitor cocktail (Roche) and PMSF was added before cell suspensions were passed through a French Press cell (SLM-Aminco) at 15,000 psi. Cell lysates were centrifuged at 90,000 rpm for 30 min using a Beckman TLA-100.3 rotor to separate membrane fractions from supernatant. SecAA and SecAAA were more stable in the membrane fractions which were used for purification as follows. Membrane fractions were dissolved in DTKM buffer containing 1.25% *n*-octyl-D-glucoside (OG) for 2 h at 4 °C followed by centrifugation at 90,000 rpm for 30 min to remove insoluble materials. Solubilized proteins were precipitated by 35% $(NH_4)_2SO_4$ and dissolved in 0.5 ml DTKM. After centrifugation to remove the debris, the supernatant was applied onto a Sephacryl-300 (50/100) column using DTKM buffer containing 1 M NaCl, 1 mM DTT, 0.5 mM EDTA; the high salt was used to dissociate SecA monomers during purification [18]. Fractions containing SecAA or SecAAA were combined and precipitated by 60% $(NH_4)_2SO_4$. Protein pellets were dissolved in 200 μl 100 mM $NH_4HCO_3$ and loaded onto a Superose 6 HR gel-filtration column (Amersham Bioscience Corp) and eluted in 100 mM $(NH_4)HCO_3$ buffer containing 1 mM DTT at a flow rate of 0.4 ml/min. Fractions contained SecAA and SecAAA were combined, aliquoted and stored at −80 °C. Protein concentration was determined by the Bradford assay (Bio-Rad) using BSA as a standard.

### 2.4. ATPase assay

The ATPase activity assays were carried out as reported previously [25]. Liposomes were prepared from *E. coli* total lipid mixture (Avanti, Polar Lipids, Inc) by sonication in an ice-cold water bath.

### 2.5. In vitro protein translocation assays

Protein translocations were carried out as previously described [26], using SecA-depleted BA13 membrane vesicles [1]. Protein synthesis was performed with an S30 extract of SecA-depleted BL15.5 as previously described [1]. The messenger was total RNA from strain HJM114 containing plasmids p*Omp9* and pCI857 [27]. The membrane vesicles containing the $^{35}$S-labelled OmpA protein after proteinase K treatment were isolated, analyzed in 12% SDS–PAGE, autoradiographed and quantified by densitometer [1,22]. The input amount of $^{35}$S-labelled proOmpA used in the translocation assay was used as 100%. The results were averages from four separated experiments.

### 2.6. Xenopus oocyte preparation and channel activity recording

The preparation of oocytes from *Xenopus laevis*, injection of the *E. coli* membranes and precursors, and the recording of ion channel activities were performed as described previously [9,28]. 50 nl of solution containing 60 ng of BA-13 membranes depleted of SecA, and 17.5 ng proOmpA with indicated amounts of purified SecAA and SecAAA were injected into oocytes. The channel activities were determined after the injected oocytes were incubated for 3 h at 23 °C and recorded for 1 min as described [9,28].

### 2.7. Cross-linking assay

Cross-linkings with purified SecA, SecAA and SecAAA *in vitro* with 1-ethyl-3-(3-dimethyl-aminopropyl) carbodiimide (EDC) was performed as described [19,23] or with formaldehyde (Sigma Chemical Co) [29] *in vitro* at indicated concentrations. Cross-linked