The high affinity ATP binding site modulates the SecA–precursor interaction

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Abstract SecA is the central component of the protein-translocation machinery of *Escherichia coli*. It is able to interact with the precursor protein, the chaperone SecB, the integral membrane protein complex SecYEG, acidic phospholipids and its own mRNA. We studied the interaction between prePhoE and SecA by using a site-specific photocrosslinking strategy. We found that SecA is able to interact with both the signal sequence and the mature domain of prePhoE. Furthermore, this interaction was dependent on the type of nucleotide bound. SecA in the ADP-bound conformation was unable to crosslink with the precursor, whereas the ATP-bound conformation was active in precursor crosslinking. The SecA–precursor interaction was maintained in the presence of *E. coli* phospholipids but was loosened by the presence of phosphatidylglycerol bilayers. Examining SecA ATP binding site mutants demonstrated that ATP hydrolysis at the N-terminal high affinity binding site is responsible for the changed interaction with the pre-protein.

Key words: Phospholipid; Protein translocation; Signal sequence; Crosslinking; SecA

1. Introduction

Many proteins destined for the periplasm or outer membrane of *Escherichia coli* are transported across the inner membrane by a multicomponent protein complex called pre-protein translocase [1]. This machinery also catalyzes insertion of some proteins into the inner membrane [2]. The core of the translocase is formed by SecYEG which forms a large pore through which proteins are transported [3]. SecA is a dimeric ATPase bound to the cytoplasmic site of the pore actively driving translocation of precursor proteins.

Proteins that have to be transported by this machinery are synthesized with an N-terminal extension called the signal sequence. The N-terminus usually contains one to three basic amino acid residues followed by a hydrophobic stretch of 10–15 amino acid residues often containing a helix-breaking residue near the center. The C-terminal part defines the cleavage site for leader peptidase, which allows removal of the signal after translocation. The signal sequence is thought to insert into the membrane via interactions with phospholipids [4]. In the homologous eukaryotic translocation machinery crosslinking experiments have demonstrated that the signal sequence is in contact with phospholipids during translocation [5]. Recently, it was demonstrated that the signal sequence in this system binds to the translocase at the lipid–protein interface [6].

SecA is the key component of the translocation machinery interacting with almost all other components of the secretion machinery. SecA binds to the *E. coli* inner membrane at high affinity binding sites consisting of SecYE protein and acidic phospholipids [7]. Interaction with both acidic phospholipids and the signal sequence stimulates its ATPase activity [8]. Upon arrival of precursors at the translocase SecA inserts in the membrane pushing 20–30 amino acid residues to the other side [9]. ATP hydrolysis allows deinsertion and completes the cycle [10]. SecA contains two ATP binding sites per monomer [11]. The N-terminal site (NDBI) binds ATP with high affinity. A mutation in this ATP binding site blocks SecA in the membrane-inserted state [12,13]. The C-terminal ATP binding site (nucleotide binding domain (NBD)II) binds ATP with low affinity. A mutation in this ATP binding site does not interfere with the insertion-deinsertion cycle. However, NDBII is essential for translocation and translocation ATPase activity. This suggests that NDBII couples SecA-membrane cycling to forward movement of the precursor.

To investigate the role of the two ATP binding sites in the SecA–precursor interaction we made use of the precursor of the outer membrane pore protein PhoE containing single cysteines in either the signal sequence or the mature part of the protein. These cysteines were modified with a photoactivatable crosslinker, whereafter the precursor–SecA interaction was studied under a variety of conditions. UV irradiation of mixtures containing these precursors and SecA and SecB resulted in a nucleotide-dependent labeling of SecA. This interaction was unaffected by the presence of *E. coli* phospholipids but was prevented by phosphatidylglycerol. A mutation in the high affinity ATP binding domain of SecA interferes with the nucleotide dependency of crosslinking demonstrating that this site is involved in modulating the interaction with the precursor.

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Abbreviations: NBD, nucleotide binding domain; APDP, *N*-[4-(*p*-azidosalicylamido)butyl]-3′-(2′-pyridyldithio)propionamide; AMP-PNP, 5-adenylylimidodiphosphate; DTNB, 5,5′-dithio-bis(2-nitrobenzoic acid); TCA, trichloroacetic acid; DMSO, dimethylsulfoxide; PBS, phosphate-buffered saline; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis

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2. Materials and methods

SecA [14], SecB [15], phoE [16] and the prePhoE single-cysteine mutant A-5C [17] were purified as described. Plasmid pNN105, a derivative of pNN100 [17], in which the lysine codon 125 of prePhoE is changed into a cysteine codon by site-directed mutagenesis, was used to overexpress prePhoE K125C. prePhoE K125C was further purified as described. Processing with purified leader peptidase was done as described [18]. Protein concentrations were determined according to [19]. Inverted inner-membrane vesicles were prepared from *E. coli* strain MC4100 according to [20]. *N*-[4-(*p*-Azidosalicylamido)butyl]-3′-(2′-pyridyldithio)propionamide (APDP) and iodogen were obtained from Pierce chemical Co. (Rockford, IL, USA). ATP, ADP, 5-adenylylimidodiphosphate (AMP-PNP) and 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB) were purchased from Sigma chemical Co. (St. Louis, MO, USA). $Na_{125}I$ (IMS300, 350–600 mCi/ml) was from Amersham (Buckinghamshire, UK). Sephadex G25 Columns were from Pharmacia (Uppsala, Sweden). DOPG and DOPC were from Avanti polar Lipids (Birmingham, AL, USA). *E. coli* phospholipids were purified as described [21]. TID-maleimide tin-based precursor TTD-maleimide was a kind gift of Dr. J. Brunner and iodinated and conjugated as described [22]. All other chemicals were of analytical grade or better.

2.1. Preparation of large unilamellar vesicles

A lipid film consisting of 0.5 μmol of *E. coli* phospholipid, DOPC or DOPG was dried under vacuum. Films were hydrated in 50 mM HEPES–NaOH pH 7.9, 50 mM KCl and subsequently extruded through a 400 nm polycarbonate filter to obtain large unilamellar vesicles [23].

2.2. Reduction of precursors prior to conjugation with APDP

100 μg of each precursor was precipitated by addition of an equal volume of 10% trichloroacetic acid (TCA). Pellets were washed with ice-cold acetone and subsequently dissolved in 8 M Urea, 50 mM Tris–HCl pH 7.9, 10 mM DTT and incubated at room temperature for 30 min. Subsequently, the precursors were again precipitated with 10% TCA and washed with acetone. Pellets were now dissolved in 100 μl 8 M urea, 50 mM Tris pH 7.9 and used immediately for conjugation.

2.3. Iodination of APDP and conjugation to prePhoE

In an iodogen-coated glass tube 0.75 μl APDP in dimethylsulfoxide (DMSO) (6 mg/ml), 6.7 μl phosphate-buffered saline (PBS) (20 mM HPO_4/H_2PO_4 pH 7.4, 150 mM NaCl) and 1 mCi of ^{125} I were combined. After 2 min at room temperature, the reaction mixture was transferred to an Eppendorf tube containing 1 μl 100 mM unlabeled NaI to prevent remaining radioactive iodine to react with the preprotein. Subsequently, the freshly reduced precursor was added and allowed to react with APDP for 1 h at room temperature. The [^{125}I]APDP-labeled precursors were separated from free label on a Sephadex G25 gelfiltration column eluted with 8 M urea, 50 mM Tris–HCl pH 7.9. Fractions of 0.5 ml were collected and analyzed by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and autoradiography.

2.4. Protein-translocation reactions

Reaction mixtures (50 μl) contained SecA (20 μg/ml), SecB (34 μg/ml), ATP (2 mM), and inner membrane vesicles (1 mg protein) in translocation buffer (50 mM HEPES–KOH, pH 7.9, 30 mM KCl, 2 mM DTT, 20 mM MgAc, 100 μM DTNB). Sodium azide was used at 10 mM concentration to inhibit translocation. Precursor proteins were diluted 50-fold from a solution containing 8 M urea and 50 mM Tris–HCl, pH 7.9, and incubated in the reaction mixture at 37°C for 20 min. 45 μl of the reaction mixture was added to 1 μl of proteinase K (5 mg/ml) and incubated for 10 min on ice. Reactions were stopped by the addition of an equal volume of 20% (w/v) TCA. Samples were centrifuged for 10 min at 13 krpm in an Eppendorf centrifuge, washed with acetone, and analyzed on 15% SDS–PAGE [24]. DTNB was included in the reactions to prevent reduction of the label [25]. Translocation ATPase assays were performed as described [26].

2.5. Crosslinking reactions

SecA (20 μg/ml), SecB (33 μg/ml), labeled prePhoE (2 μg/ml), phospholipids (100 μM), azide (10 mM) and nucleotides (2 mM) as indicated in the figures were incubated in 50 μl translocation buffer for 10 min at 37°C. Subsequently, samples were irradiated for 5 min with a 15 W UV light source at ~254 nm wavelength, precipitated by addition of an equal volume of 10% TCA and analyzed by SDS–PAGE and autoradiography. For competition experiments unlabeled prePhoE or mature PhoE diluted from 8 M urea/50 mM Tris–HCl pH 7.9 was included in the reaction mixture.

3. Results

To investigate the interactions between prePhoE and its signal sequence with components of the Sec machinery, two mutant proteins containing a cysteine in either the signal sequence or the mature part of the protein were purified (Fig. 1B). These precursors were modified with radioiodinated APDP (Fig. 1A). The resulting labeled prePhoE could be detected as a radioactive band by SDS–PAGE and autoradiography (Fig. 1C). Treatment of the labeled precursors with β-mercaptoethanol resulted in removal of more then 95% of the label demonstrating that most of the bound radioactivity was derived from APDP conjugated via a disulfide

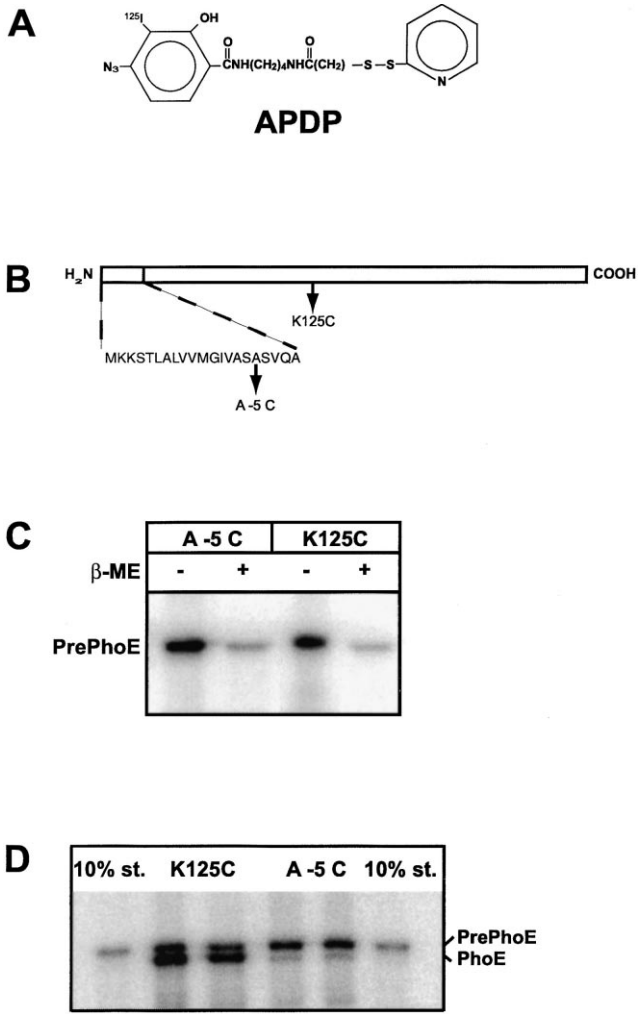

Fig. 1. The structure of APDP (A), the single cysteine mutants used (B), labeling of both mutants with [^{125}I]APDP and removal of the label after treatment with β-mercaptoethanol (β-ME) (C) and the translocation activity of both mutants (D) 10% st represents 10% of the material that was not treated with proteinase K.

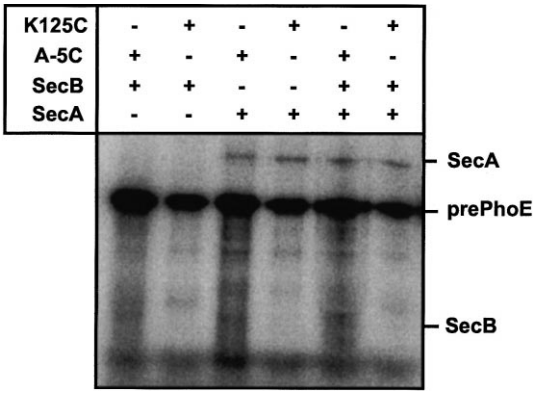

Fig. 2. Crosslinking of SecA with prePhoE K125C or A-5C in the presence or absence of SecA and SecB as indicated, analyzed by 15% SDS–PAGE and autoradiography.

bridge (Fig. 1C). The labeled precursors were used in in vitro translocation reactions using purified SecA, SecB and inverted inner-membrane vesicles. Translocation of prePhoE-K125C resulted in the appearance of two protease-protected bands corresponding to precursor and mature PhoE (Fig. 1D). Translocation of prePhoE-A-5C resulted in a protease-protected precursor band and a faint mature-sized band. In this case, processing leads to removal of the radioactive label, therefore the processed precursors can not be detected by autoradiography. Processing was not abolished by the modification of the signal sequence, since the labeled signal sequence could still be detected after in vitro processing with purified leader peptidase (not shown). The low amount of label in the mature-sized band was most likely derived from some iodination in the mature part of the precursor. Translocation efficiencies typically ranged from 60 to 90% for prePhoE-K125C and 30 to 40% for A-5C. Together these data demonstrate that [^{125}I]APDP-modified prePhoE A-5C and K125C are valid model proteins for studying interactions with components of the translocation machinery.

These precursors were used to investigate the interaction with SecA. Labeled prePhoE was incubated in the presence or absence of a five-fold excess SecB tetramers to ensure optimal formation of the SecB–prePhoE complex [16,27] and in the presence or absence of a five-fold excess of SecA dimers. In the absence of SecB, prePhoE rapidly loses its translocation competence [28]. Translocation of [^{125}I]APDP-modified precursors was dependent on SecB as well (not shown). Samples were irradiated and proteins were precipitated with TCA and APDP was subsequently removed from prePhoE by addition of sample buffer with β-mercaptoethanol. Crosslink products contained only a covalently linked APDP moiety and therefore run with the same electrophoretic mobility on SDS–PAGE gels. This resulted in labeling of a band with the electrophoretic mobility of SecA irrespective whether the label originated from the signal sequence or the mature part of the precursor (Fig. 2). Surprisingly, no band was observed at the position in the gel where SecB was present. This suggests that the precursor, at the positions where the crosslinker is attached, is not in direct contact with SecB. PrePhoE appears as a heavily labeled band.

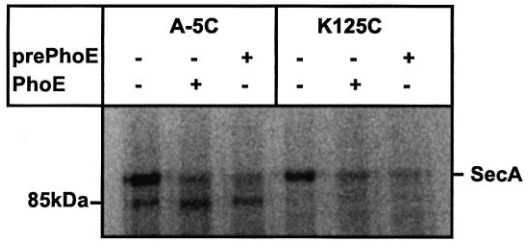

Fig. 3. Labeling of SecA in the presence or absence of competing precursor (20 μg/ml) or mature PhoE (20 μg/ml).

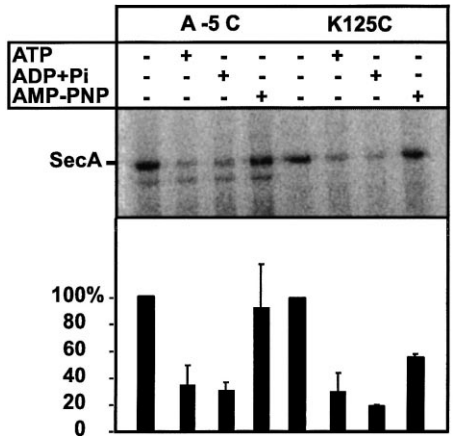

Fig. 4. Crosslinking of prePhoE to SecA in the presence or absence of nucleotides. Quantifications were done using molecular dynamics Image QuaNT software and represent the mean of two experiments. Error bars represent the standard deviation.

SecA labeling occurred from both the signal sequence and the mature part of the precursor protein. To get more information on the specificity of the SecA labeling from the precursor, we investigated whether unlabeled precursor and mature PhoE were able to compete with the [^{125}I]APDP-labeled prePhoE. Inclusion of a 10-fold excess of unlabeled precursor or mature PhoE both resulted in a strong reduction of the labeling indicating that SecA does not require the signal sequence for prePhoE binding (Fig. 3).

In this experiment, labeling from the signal sequence also resulted in the appearance of a faint 85 kDa band (Fig. 3). This protein was apparently not separated from SecA in the previous gel. This band was not dependent on addition of other proteins, so it must be derived from a minor impurity of the purified prePhoE-A-5C. This protein was most likely labeled with APDP during the labeling of prePhoE and does not lose all its label after irradiation and treatment with β-mercaptoethanol. Since it was not a crosslink product it was not further characterized.

Crosslinking from both the signal sequence and the mature domain was dependent on the presence of nucleotides. Addition of 2 mM ATP almost completely inhibited SecA labeling (Fig. 4). Addition of 2 mM ADP and phosphate also inhibited the SecA labeling. Addition of 2 mM AMP-PNP a non-hydrolyzable ATP analogue resulted in strong labeling of SecA. Apparently, ATP-bound SecA has a high affinity for the precursor which is strongly reduced under ADP-bound conditions.

Azide is a strong inhibitor of translocation and ATPases in general and leads to complete block in translocation of the labeled precursors (Fig. 5A) [29]. Therefore, we analyzed the crosslinking reactions in the absence and presence of 10 mM azide. Surprisingly, even in the presence of 10 mM azide ATP

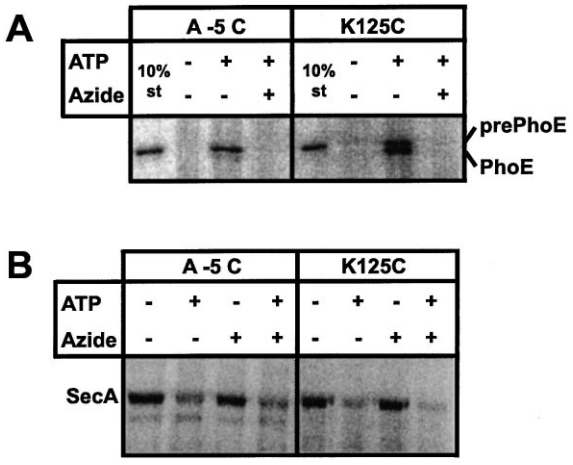

Fig. 5. Translocation of [^{125}I]APDP modified precursors is inhibited in the presence of azide (A) 10% st represents 10% of the material that was not treated with proteinase K. Azide does not influence the ATP modulated interaction with the precursor (B).

was still able to reduce the labeling efficiency of SecA (Fig. 5B).

SecA contains two ATP binding sites which differ in their affinity for ATP [11]. To get insight into which binding site was involved in modulating the SecA–precursor interaction, we purified two mutants SecAD209N and SecAR509K which contain mutations in NDBI (high affinity) and NDBII (low affinity) respectively, resulting in an inability to bind ADP at the mutated binding site [11]. Both mutants displayed no translocation ATPase activity and did not support protein translocation (not shown). Crosslinking reactions in the presence of SecAD209N resulted in labeling irrespective of the presence of ATP (Fig. 6). Crosslinking in the presence of SecAR509K also results in labeling of the SecA mutant. Addition of ATP reduced the amount of labeling to 35–50% indicating that binding and hydrolysis of ATP at NBD I is modulating the interaction with the precursor protein.

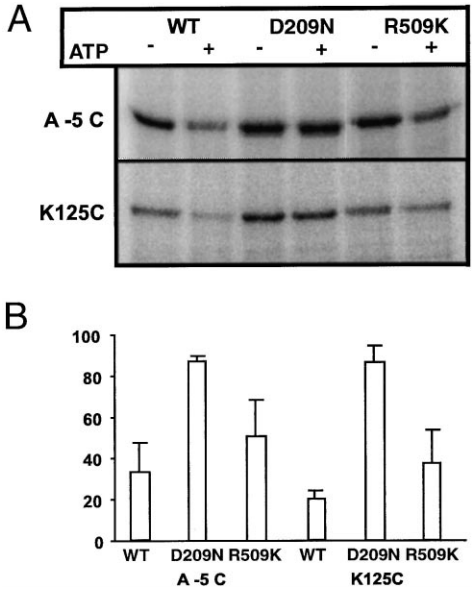

Fig. 6. Crosslinking of prePhoE with SecA, SecAD209N and SecAR509K in the presence of ATP (A). Quantitative analysis of the autoradiograms of three experiments (B). The relative level of labeling in the presence of ATP is plotted respective to the level of labeling in the absence of ATP. Error bars represent the standard deviation.

SecA inserts in phospholipid mono- and bilayers in a nucleotide-dependent way [30,31]. Furthermore, both the signal sequence and the mature domain interact with phospholipid monolayers [4,16]. Therefore, we were interested whether phospholipids affected the SecA–precursor interaction. First, SecA, SecB and prePhoE were incubated for 10 min to establish complex formation then liposomes were added and the mixtures were further incubated for another 5 min before photolysis. Addition of liposomes consisting of pure DOPG led to a strong reduction of the crosslink as compared to the situation without vesicles (Fig. 7). Addition of ATP in the presence of DOPG liposomes still had a small reducing effect on the crosslink efficiency. Addition of liposomes composed of the zwitterionic DOPC or purified *E. coli* phospholipids did not affect the crosslinking with SecA. This demonstrates that the presence of *E. coli* phospholipids does not disturb the complex, whereas highly charged membranes loosen the SecA–precursor interaction. Crosslinks between the precursor and phospholipids could not be detected. Arylazide-based photocrosslinkers like APDP tend to undergo rapid intra molecular ring expansion after photoactivation and are therefore less reactive with lipid acyl-chains [32]. Using a carbene-generating reagent it was shown that the signal sequence interacts with phospholipids during translocation [5]. Modification of prePhoE A-5C with [^{125}I]TID-maleimide [22], containing a similar reactive group, abolished translocation activity and could therefore not be used.

4. Discussion

To investigate the interaction between the precursor and components of the translocation machinery in more detail we made use of two prePhoE single cysteine mutants. One with a cysteine in the signal sequence and one with a cysteine in the mature part of the protein. These precursors were labeled with the photoactivatable crosslinker APDP. In in vitro translocation experiments these modified precursors behaved like wild type prePhoE. Therefore, these proteins could be used to assess interactions between prePhoE and other components during the translocation process.

Surprisingly, labeling of SecB was not observed under conditions that result in formation of the SecB–prePhoE complex. This may indicate that both the signal sequence and the res-

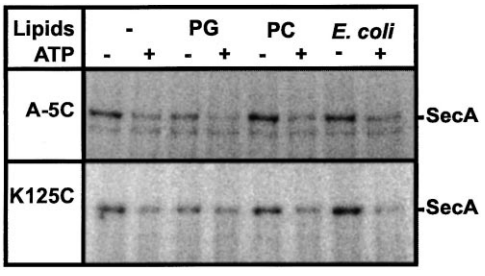

Fig. 7. Crosslinking of prePhoE in the presence of liposomes composed of different types of phospholipids. SecA, SecB and [^{125}I]APDP-labeled prePhoE where incubated in translocation buffer and subsequently liposomes were added. Samples were photolysed and treated as described in Section 2.

idues around residue 125 do not directly interact with SecB. Interaction between SecB and prePhoE was suggested to occur via β-structures [27]. Residue 125 is part of a loop and may therefore not be in direct contact with SecB. Furthermore, residue 125 was not reported to be part of the SecB binding frame which is located in four parts, namely residues 25–93, 129–174, 204–250, and 316–330 of the mature part of the precursor [33].

SecA was clearly labeled from both the signal sequence and the mature domain of prePhoE. Crosslinking was markedly reduced after addition of either ATP or ADP and phosphate, the products of ATP hydrolysis. Addition of the non-hydrolyzable ATP analogue AMP-PNP did not result in a reduction of SecA labeling. This indicates that SecA in the ADP-bound form is no longer in close proximity of the APDP-moiety suggesting that ADP-bound SecA has a less tight interaction with the precursor. To discriminate which of the two ATP binding sites was involved in changing the interaction with prePhoE we made use of two SecA ATP binding site mutants. The mutant in the high affinity ATP binding site showed no response to ATP addition. Therefore, it was concluded that this site was involved in modulating the precursor interaction.

SecA consists of two independently folding domains [34]. The N-terminal domain contains both ATP binding sites and the precursor binding site [35]. The C-domain regulates the SecA ATPase activity by binding to the N-domain [36]. Binding of ADP leads to formation of a more compact conformation [34]. We demonstrate that the ADP-bound conformation has a less tight interaction with prePhoE then the ATP-bound conformation. Possibly, the precursor binding site is buried in the more compact conformation of the N-domain. Alternatively, the precursor binding site may be inaccessible by binding of the C-domain [36].

Azide is a very potent inhibitor of protein translocation [29]. The ATP-dependent change in interaction between prePhoE and SecA, however, was not inhibited in the presence of azide. Similarly also the nucleotide-dependent insertion of SecA into phospholipid monolayers was unaffected by the presence of azide [30]. SecA was shown to possess some remaining ATPase activity in the presence of azide [37]. This remaining activity may be enough for SecA to acquire an ADP-bound conformation during the timescale of the experiment, and thereby loosen its interaction with the precursor.

A synthetic signal peptide was shown to enhance SecA-lipid ATPase activity of *E. coli* phospholipids [8]. Furthermore, interaction between SecA and a signal sequence promoted SecA insertion into the bilayer [38]. This suggests that SecA and the signal sequence co-insert into the bilayer. In the presence of liposomes composed of *E. coli* phospholipids the nucleotide-dependent interaction between SecA and the signal sequence was still found. This demonstrates that in the natural lipid environment the SecA–precursor interaction is maintained. However, crosslinking in the presence of phospholipid vesicles composed of pure negatively-charged DOPG reduced the crosslinking between SecA and prePhoE, demonstrating that the presence of a high amount of negative charges at the membrane surface disrupts the complex. Inner-membrane vesicles of *E. coli* strains unable to synthesize PE contain a high concentration of the acidic phospholipids PG and cardiolipin [21,39]. Our results suggest that under these conditions the SecA–precursor interaction would be disturbed which could compromise translocation in this strain. This suggestion is supported by the observation that SecA expression is increased, apparently to compensate for a reduced translocation efficiency [39].

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