The Low-Affinity ATP Binding Site of the *Escherichia coli* SecA Dimer Is Localized at the Subunit Interface[†]

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ABSTRACT: The homodimeric SecA protein is the ATP-dependent force generator in the *Escherichia coli* precursor protein translocation cascade. SecA contains two essential nucleotide binding sites (NBSs), i.e., NBS1 and NBS2 that bind ATP with high and low affinity, respectively. The photoactivatable bifunctional cross-linking agent 3′-arylazido-8-azidoadenosine 5′-triphosphate (diN$_3$ATP) was used to investigate the spatial arrangement of the nucleotide binding sites of SecA. DiN$_3$ATP is an authentic ATP analogue as it supports SecA-dependent precursor protein translocation and translocation ATPase. UV-induced photo-cross-linking of the diN$_3$ATP-bound SecA results in the formation of stable dimeric species of SecA. D209N SecA, a mutant unable to bind nucleotides at NBS1, was also photo-cross-linked by diN$_3$ATP, whereas no cross-linking occurred with the NBS2 mutant R509K SecA. We concluded that the low-affinity NBS2, which is located in the carboxyl-terminal half of SecA, is the site of cross-linking and that NBS2 binds nucleotides at or near the subunit interface of the SecA dimer.

SecA is a homodimeric ATPase (*1*, *2*) that functions as a dissociable subunit (*3*, *4*) of the *Escherichia coli* preprotein translocase (*5*, *6*), a multimeric membrane protein complex with SecY, SecE, and SecG as integral subunits (*7*−*10*) and SecD and SecF as associated components (*11*). Preprotein translocation requires two forms of energy, ATP and the proton motive force (*12*−*15*). SecA binds preproteins by virtue of its affinity for both the signal sequence and mature domain (*16*, *17*). The ATPase activity of SecYEG-bound SecA is stimulated by preproteins (*2*, *4*). SecA utilizes the energy of ATP binding to initiate preprotein translocation, and hydrolyzes the ATP to release the preprotein (*18*).

The mechanism by which the SecA dimer couples the energy of ATP binding and hydrolysis to preprotein translocation has been the subject of intensive investigation. SecA not only exists (*19*) but also functions as a dimer (*20*). Each of the SecA subunits contains two independently folding domains, the N-domain and the C-domain (*21*). Each of these domains harbors a nucleotide binding site (NBS),[1] i.e., the high-affinity ($K_{d,ADP} \sim 150$ nM) NBS1 located in the N-domain and the low-affinity ($K_{d,ADP} \sim 340$ μM) NBS2 which is contained in the C-domain (*22*−*24*). The SecY-interacting region of SecA is located in its C-domain (*25*). The SecYEG-associated form of SecA protrudes the membrane (*26*) with its carboxyl terminus (*10*). Binding of ATP to both NBSs of SecA elicits a large conformational change (*21*, *27*) and promotes the membrane insertion of a large part of the C-domain (*28*−*30*). Concomitantly, 20−30 amino acyl residues of the preprotein are translocated through the membrane by co-insertion (*18*, *31*). Hydrolysis of ATP dissociates the preprotein from its SecA-bound state and allows the C-domain to de-insert from the membrane (*29*). Studies with the soluble form of SecA suggest that the protein in its ADP-liganded state resembles a compact globular conformation with extensive contact between the N- and C-domains, and between the two subunits of SecA (*21*, *27*). In the ATP-bound form, the protein adopts a nonglobular, more elongated conformation with little interaction between the two domains (*21*). These two states may resemble the surface-bound and membrane-inserted states, respectively. Consequently, preprotein translocation may be driven by the spatial disposition of the N- and C-domains of SecA (*21*), while part of the C-domain is anchored to the SecYEG complex. Studies with NBS mutants of SecA indicate that both sites are needed for activity. Hydrolysis of ATP at NBS1 is required for SecA membrane de-insertion, whereas the function of NBS2 is unknown (*29*). Multiple cycles of ATP binding and hydrolysis drive the stepwise translocation of preproteins across the cytoplasmic membrane (*18*), while the proton motive force accelerates the rate of the translocation reaction (*15*, *32*).

In this study, the spatial arrangement of the nucleotide binding sites of the SecA dimer was investigated by the use of the bifunctional photoactivatable nucleotide analogue 3′-arylazido-8-azidoadenosine 5′-triphosphate (diN$_3$ATP). UV irradiation of SecA in the presence of diN$_3$ATP results in photo-cross-linking of dimeric SecA species. Cross-linking occurs at the low-affinity NBS2, indicating that this site binds nucleotides at or in the vicinity of the subunit interface of the SecA dimer.

EXPERIMENTAL PROCEDURES

Biochemicals. SecA (*3*), D209N SecA and R509K SecA (*24*), SecB (*33*), proOmpA (*34*), and SecYEG (*7, 10*) were purified from overproducing strains as described. SecYEG was reconstituted into proteoliposomes by detergent dilution (*7*). [^{35}S]proOmpA was synthesized using an *in vitro* transcription/translation reaction (*35*), and affinity purified as described (*36*). DiN$_3$ATP was synthesized and purified as described (*37*), and the concentration was determined spectroscopically using a molar extinction coefficient (ϵ) of 4200 at 480 nm.

Photoaffinity Labeling and Cross-Linking. Photoaffinity labeling and cross-linking experiments (in 200 μg/mL) were performed as described (*38*). SecA (100 μg/mL) was incubated in buffer A [50 mM Tris-HCl (pH 8.0) and 0.5 mM MgCl$_2$] in the presence or absence of varying concentrations of diN$_3$ATP (as indicated) for 5 min at 0 °C. Samples were subsequently irradiated for 5 min at 0 °C with a 254 nm lamp (model UVG-54, UVP Live Sciences Inc., Cambridge, U.K.) placed at a distance of 1 cm. Samples were used for *in vitro* translocation reactions or analyzed by SDS−PAGE on 6% acrylamide gels (*39*) and immunoblotting using a SecA oligoclonal antibody (mixture of monoclonal antibodies) (*27*).

In Vitro Translocation of proOmpA. Translocation of ^{35}S-labeled proOmpA (in 50 μL) into SecYEG proteoliposomes was performed essentially as described (*40*). Reaction mixtures contained 20 μg/mL SecA, 32 μg/mL SecB, 1 μL of urea-denatured [^{35}S]proOmpA, 10 mM phosphocreatine, and 50 μg/mL creatine kinase in buffer B [50 mM HEPES-KOH (pH 7.5), 30 mM KCl, 0.5 mg/mL bovine serum albumin, 10 mM DTT, and 2 mM Mg(OAc)$_2$] and 20 μg/mL SecYEG-reconstituted proteoliposomes. Reactions were initiated at 37 °C by the addition of 2 mM ATP or diN$_3$ATP (as indicated), and after 30 min, translocation was terminated by chilling on ice. Samples were treated with proteinase K (0.1 mg/mL) for 15 min on ice, precipitated with 7.5% (w/v) TCA, washed with ice cold acetone, and analyzed by 12% SDS−PAGE (*41*) and autoradiography using Kodak Biomax MR film.

ATP Binding. Photoaffinity [α-^{32}P]ATP cross-linking experiments were conducted as described by Matsuyama et al. (*22*). SecA (30 pmol) was incubated in buffer C [50 mM TrisOAc (pH 7.5), 100 mM KOAc, 2 mM MgOAc, and 1 mM DTT] with 100 nM [α-^{32}P]ATP for 15 min at 0 °C and subsequently illuminated for 10 min at 0 °C with a 254 nm lamp (model UVG-54) placed at a distance of 2 cm. Reactions were carried out in the presence or absence of nonlabeled ATP or diN$_3$ATP at the indicated concentrations. Samples were analyzed by SDS−PAGE on 10% acrylamide gels. The gels were dried and exposed to Kodak Biomax MR film.

Other Techniques. Protein determination was performed according to Lowry et al. (*42*) with BSA as the standard. The nucleotide occupancy of NBS1 and NBS2 was calculated as described by den Blaauwen et al. (*21*) using K_d values of 0.15 and 340 μM, respectively. The translocation ATPase activity of SecA was assayed according to the method of Lill et al. (*2*).
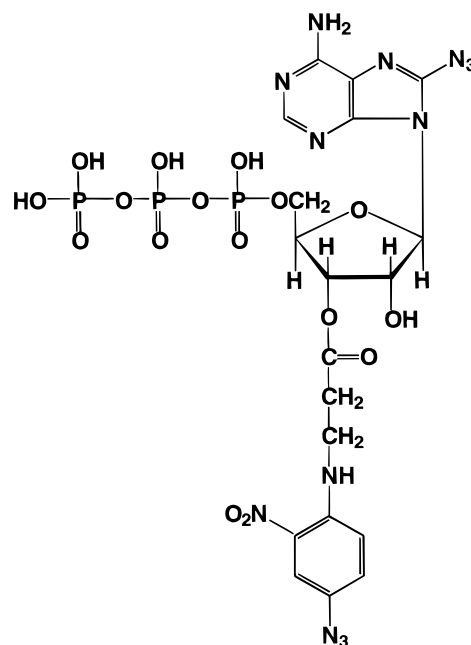

FIGURE 1: Structure of the photoactivatable ATP analogue diN$_3$ATP.

RESULTS

DiN$_3$ATP Is an Authentic ATP Analogue and Promotes SecA-Dependent Preprotein Translocation. To investigate the spatial arrangement of the nucleotide binding sites of SecA, the bifunctional photo-cross-linking ATP analogue diN$_3$ATP was used. This molecule bears azido groups both at the 8-position of the adenine ring and at the 3′-position of the ribose (Figure 1). To test if diN$_3$ATP is a genuine ATP analogue, *in vitro* preprotein translocation reactions were performed. Proteoliposomes reconstituted with purified SecYEG were incubated with SecA (20 μg/mL), and the translocation of ^{35}S-labeled proOmpA was analyzed in the presence of ATP or diN$_3$ATP. DiN$_3$ATP supported the translocation of proOmpA, although with a lower efficiency than ATP (Figure 2A, compare lanes 2 and 3). In the absence of nucleotides (lanes 1) or SecA (data not shown), no translocation was observed.

The lower efficiency of preprotein translocation with diN$_3$ATP as compared to that with ATP may result from a reduced turnover of the SecA ATPase and/or altered binding affinity. Therefore, preprotein-stimulated SecA translocation ATPase assays (*2*) were performed with diN$_3$ATP as the substrate (Figure 2B). DiN$_3$ATP is hydrolyzed by SecA in the presence of proOmpA and SecYEG proteoliposomes, although at a lower rate.

To test whether the reduced SecA translocation ATPase activity is due to a lowered affinity of SecA for diN$_3$ATP, competition experiments were performed using the photo-cross-linking of [α-^{32}P]ATP at NBS1 as an assay (*22, 24, 43*). Cross-linking can be efficiently prevented by preincubation with ATP at a concentration as low as 3 μM (Figure 2C, lane 4). In contrast, about 50 μM diN$_3$ATP was needed to obtain the same level of inhibition (lane 7). These data suggest that NBS1 binds diN$_3$ATP with an affinity at least 16-fold lower than that of ATP. The K_d for diN$_3$ATP binding to NBS1 must be on the order of 2.5 μM. Although binding of diN$_3$ATP to NBS2 was not assayed, a similar reduction of binding affinity will yield a K_d of greater than 5 mM.
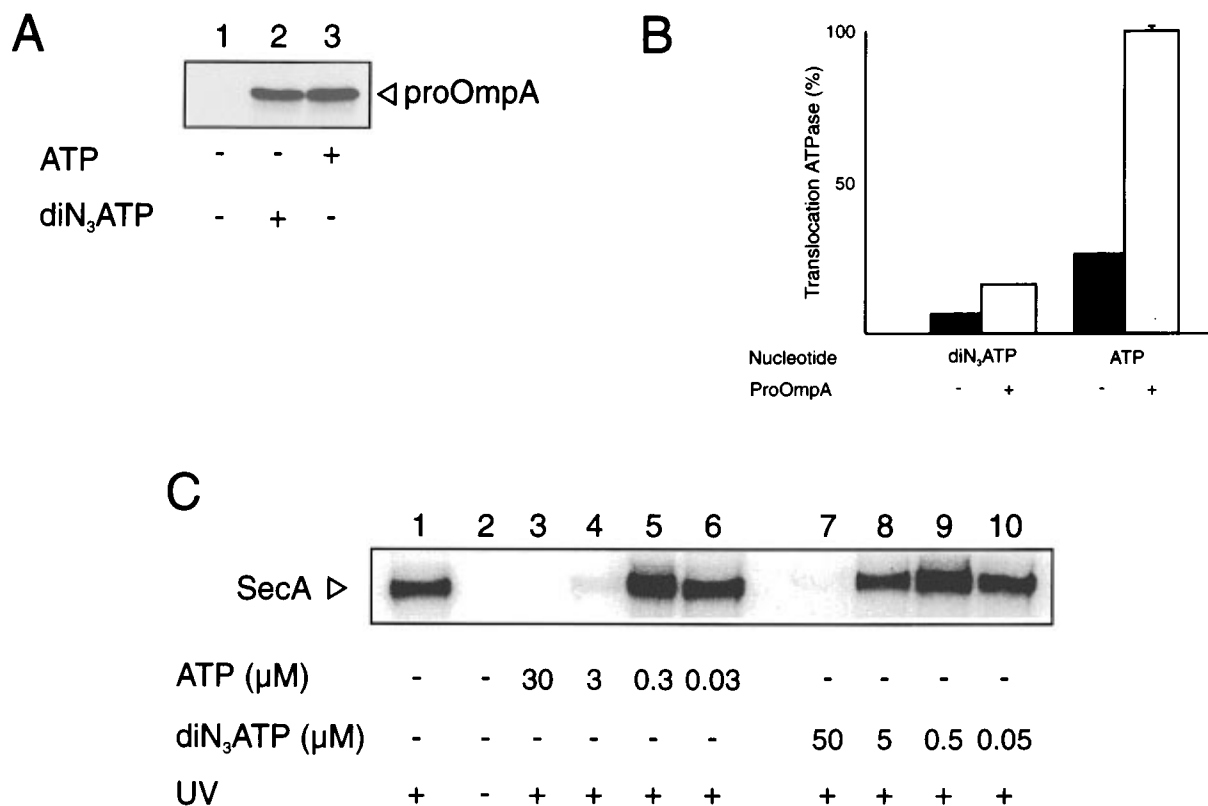
FIGURE 2: DiN$_3$ATP is a hydrolyzable ATP analogue that supports SecA-mediated preprotein translocation. (A) Translocation of [^{35}S]proOmpA into SecYEG-containing proteoliposomes (20 μg/mL) was assayed in the absence (lane 1) or the presence of 2 mM ATP (lane 2) or diN$_3$ATP (lane 3). (B) ATPase activity of SecA in the presence of SecYEG-containing proteoliposomes and in the absence and presence of proOmpA. Maximal activity with ATP and proOmpA was set to 100%. (C) UV photo-cross-linking of NBS1 of SecA by 100 nM [α-^{32}P]ATP in the presence or absence of increasing concentrations of ATP or diN$_3$ATP.

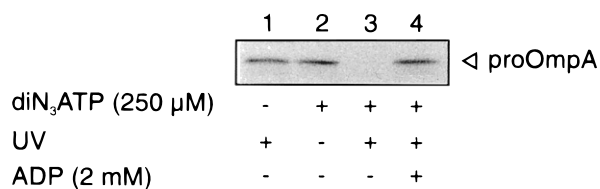
FIGURE 3: SecA is inactivated by diN$_3$ATP upon UV irradiation. As indicated, SecA (100 μg/mL) was incubated in the presence or absence of UV, diN$_3$ATP, and ADP. After cross-linking, the concentration of ADP in each sample was adjusted to 2 mM, and aliquots were used in translocation reactions (40 μg/mL SecA, final concentration) of [^{35}S]proOmpA into proteoliposomes (20 μg/mL) in the presence of 2 mM ATP. Translocation was terminated after 30 min and analyzed as described in Experimental Procedures.

This would explain the reduced translocation and SecA ATPase activity with diN$_3$ATP compared to that with ATP. We concluded that diN$_3$ATP is a hydrolyzable ATP analogue that is completely functional as an energy source for the SecA ATPase and translocation.

Ultraviolet Light-Induced Inactivation of SecA by DiN$_3$ATP. Since diN$_3$ATP is a genuine substrate for the SecA translocation ATPase, the effect of photo-cross-linking on the activity of SecA was examined. For this purpose, SecA was irradiated with UV light in the presence and absence of diN$_3$ATP, and subsequently tested for functionality in an *in vitro* preprotein translocation reaction. UV cross-linking of SecA with diN$_3$ATP completely inactivates the protein for translocation (Figure 3, lane 3), whereas inactivation does not occur when SecA is incubated with diN$_3$ATP in the absence of UV (lane 1) or when the protein is irradiated with UV in the absence of the nucleotide analogue (lane 2). When

added prior to UV treatment, ADP effectively prevented the inactivation of SecA (lane 4). These data demonstrate that SecA can be photoinactivated by diN$_3$ATP via a reaction that involves the specific binding of this nucleotide analogue to the NBSs of SecA.

DiN$_3$ATP Photoaffinity Cross-Links the SecA as Dimeric Species. Analysis of the diN$_3$ATP-cross-linked SecA on SDS−PAGE revealed the formation of additional protein bands that migrate in the gel at positions that correspond to an apparent molecular mass of about 203 and 238 kDa, respectively (Figure 4A, lane 3). The 238 kDa cross-linked product typically appears as a doublet. The efficiency of cross-linking of the 203 kDa protein is about 5%, whereas the 238 kDa product is formed with a 0.5−2% efficiency. The additional protein bands were not observed when SecA was UV irradiated in the absence of diN$_3$ATP (lane 1) or when the protein is incubated with diN$_3$ATP without UV treatment (lane 2). Moreover, preincubation of SecA with a 10-fold excess (500 μM) of ATPγS, a nonhydrolyzable ATP analogue, prior to UV cross-linking by diN$_3$ATP effectively prevented the formation of cross-linked products (lanes 6 and 7), while a 10-fold lower amount of ATPγS (5 μM) does not prevent the cross-linking (lanes 4 and 5). Immunostaining of the samples with an oligoclonal antibody directed against SecA (*27*) demonstrates that the cross-linked products are indeed derived from SecA (Figure 4B, lane 3). The migration behavior of the 203 kDa polypeptide band on SDS−PAGE is consistent with a cross-linked product that corresponds to a stable dimer of SecA. The quaternary structure of the 238 kDa cross-linked product is less certain, but likely represents a stable dimeric SecA protein that
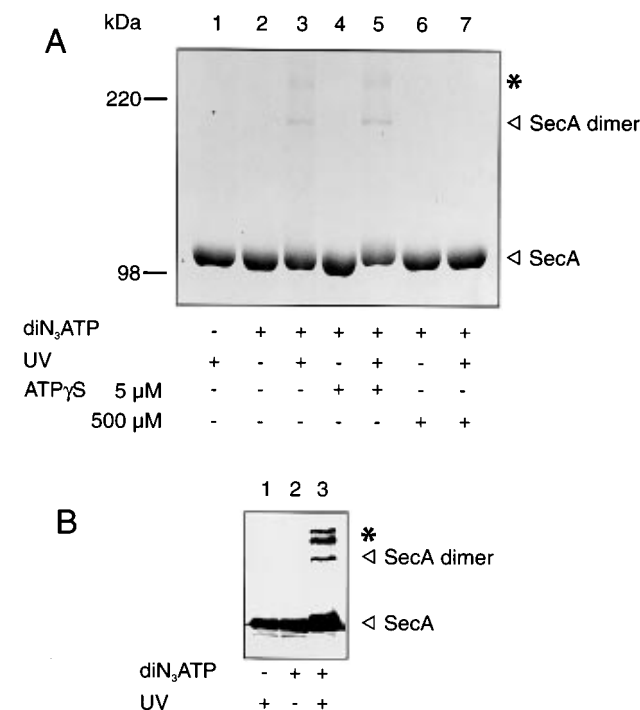
FIGURE 4: SecA is photoaffinity cross-linked to stable dimers in the presence of diN$_3$ATP. (A) SecA (100 μg/mL) was incubated with diN$_3$ATP and UV irradiated as indicated. Samples were TCA precipitated and analyzed by 6% SDS−PAGE and CBB staining. (B) Aliquots (30 μg/mL SecA) of cross-link reactions 1−3 were subjected to immunoblot analysis using a SecA oligoclonal antibody. SecA monomers and dimers are indicated by arrows. The asterisk denotes the 238 kDa cross-linked product.
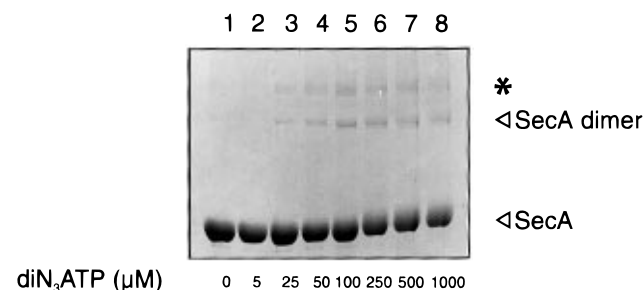

FIGURE 5: Concentration dependence of diN$_3$ATP photoaffinity cross-linking of SecA dimeric species. SecA (100 μg/mL) was cross-linked with increasing concentrations of diN$_3$ATP and analyzed by 6% SDS−PAGE and CBB staining. The SecA monomers and dimer are indicated by the arrows; the 238 kDa cross-linked product is indicated by the asterisk.

exhibits an aberrant migration behavior on SDS−PAGE due to a different conformational state and/or multiple cross-links.

Occupation of Both NBSs by Nucleotides Is Necessary for the Cross-Linking of the SecA Dimer. To address the question if only one or both of the NBSs are involved in the formation of the cross-linked products, a range of diN$_3$ATP concentrations was used (Figure 5). Cross-linking of SecA dimeric species required at least 25 μM diN$_3$ATP (lane 3). At this concentration, about 91% of the NBS1 is occupied by diN$_3$ATP, assuming a K_d value of 2.5 μM. At 5 μM diN$_3$ATP, no detectable cross-linked products are formed, while at this concentration, about 63% of the NBS1 is expected to be occupied (lane 2). Since the cross-linking efficiency increased with the amount of diN$_3$ATP, even at concentrations that by far saturate NBS1 (up to 100 μM), it appears that both NBS1 and NBS2 need to be occupied for
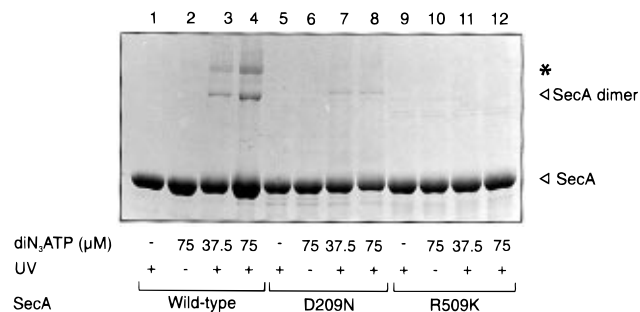

FIGURE 6: Photoaffinity cross-linking of wild type and mutant SecA proteins by diN$_3$ATP. Wild type, D209N, and R509K SecA proteins (75 μg/mL) were cross-linked with diN$_3$ATP, and the samples were analyzed by 6% SDS−PAGE and CBB staining. The positions of the SecA monomer and dimer are indicated by an arrow; the asterisk indicates the 238 kDa product. Other CBB stainable bands in the purified R509K SecA protein are contaminants.

efficient cross-linking. At very high concentrations of diN$_3$ATP (100 μM and above), the amount of cross-linked products diminished and aggregates appear in the very high molecular mass range (data not shown). These products are most likely the result of the cross-linking of SecA with polymerization products of the bifunctional compound (*37*).

The SecA Dimer Is Photo-Cross-Linked by DiN$_3$ATP at NBS2. To establish whether NBS2 is indeed involved in the cross-linking of SecA by diN$_3$ATP, SecA proteins bearing mutations in their ATP binding sites (*24*) were used for cross-linking studies. In D209N SecA, the aspartyl residue at position 209 in NBS1 has been replaced by an asparaginyl residue. This mutant is impaired in ATP binding at NBS1, but not at NBS2 (*24*), and still inserts into the membrane at SecYEG under preprotein translocating conditions (*29*). UV cross-linking with diN$_3$ATP of D209N SecA (Figure 6, lanes 7 and 8) yielded cross-linked products similar to the wild type (lanes 3 and 4), although the efficiency of cross-linking was reduced. Next, R509K SecA was examined. This mutant is impaired in ATP binding to NBS2, but still binds ATP at the high-affinity NBS1. R509K SecA is defective in preprotein translocation and ATPase activity (*24*), but not in the ATP-dependent membrane cycling (*29*). Strikingly, R509K SecA is unable to form stable dimers upon UV irradiation in the presence of diN$_3$ATP (Figure 6, lanes 11 and 12). These data demonstrate that the SecA dimer is cross-linked by the diN$_3$ATP bound at NBS2. Therefore, the low-affinity ATP binding site NBS2 binds nucleotide at or in the vicinity of the subunit interface of the SecA dimer.

DISCUSSION

In this study, we have examined the spatial arrangement of the nucleotide binding sites in the SecA dimer by using a bifunctional cross-linking agent, the ATP analogue diN$_3$ATP. Compared to ATP, diN$_3$ATP binds NBS1 with a reduced affinity. Although we have not analyzed binding of diN$_3$ATP to NBS2 by direct means, its ability to bind at this site is evident from the observation that diN$_3$ATP supports the SecA-dependent translocation of proOmpA into SecYEG proteoliposomes. These studies therefore identify diN$_3$ATP as a true ATP analogue as was shown earlier for the F$_1$ATPase from *Micrococcus luteus* (*37*) and spinach chloroplasts (*44*). Low-affinity binding of the monoreactive N$_3$ATP to SecA was shown before (*2, 45*).

UV irradiation of SecA in the presence of diN$_3$ATP inactivates the protein for *in vitro* preprotein translocation,

while the presence of an excess ADP [or ATPγS (unpublished data)] during irradiation protects the enzyme against inactivation. This demonstrates that the cross-linker interacts with SecA via its nucleotide binding sites. Photoaffinity cross-linking of SecA with diN$_3$ATP results in the formation of stable cross-linked products that migrate on SDS−PAGE with apparent molecular masses of about 203 and 238 kDa, respectively. The 203 kDa cross-linked product most likely corresponds to a stable SecA dimer as it runs at the same position on SDS−PAGE as the chemically cross-linked SecA dimer (*19*; data not shown). The quaternary structure of the 238 kDa product is less well-resolved. It clearly does not correspond to a higher-order SecA oligomer, such as trimers or tetramers that migrate slower on SDS−PAGE (*19*). It is well possible that the 238 kDa doublet represents a different conformation of the cross-linked SecA dimer. We should emphasize that the introduction of stable tertiary structures in a protein may affect its mobility on SDS−PAGE. Strikingly, both cross-linked products are formed at the same diN$_3$ATP concentrations, while higher-order structures (tetramers) that would require two or more consecutive cross-linking events are expected to be formed at much higher diN$_3$ATP concentrations. This is indeed observed, as in the very high diN$_3$ATP concentration range SecA aggregates were also formed. Under those conditions, the specificity of cross-linking is also lost due to the formation of diN$_3$ATP polymers than have a wider span than the monomeric compound (*37*).

The appearance of stable dimers of SecA upon cross-linking with diN$_3$ATP indicates that one of the NBSs binds the nucleotide in the neighborhood of the subunit interface. For stable cross-linking, the two monomers must not be more than 20 Å apart (*38*). Previous studies have shown that the α and β subunits of the F$_1$-part of the F$_1$F$_0$ATPase can be cross-linked by means of a bifunctional photoaffinity nucleotide analogue, and it has been suggested that these subunits are connected via an interfacial localization of the nucleotide binding sites (*44*, *46*). This observation was substantiated by the high-resolution atomic structure of the F$_1$-domain of the ATPase (*47*) showing that the NBSs are indeed located at the αβ subunit interface. On the basis of titration with diN$_3$ATP, it appears that both NBSs of SecA have to be occupied before cross-linked dimers are observed. Evidence that cross-linking occurs via NBS2 is provided by the study with the site-directed mutants of SecA that are disturbed in nucleotide binding at NBS1 and NBS2, respectively. DiN$_3$ATP is unable to photoaffinity cross-link the NBS2 mutant R509K as a dimeric species. This mutant is completely defective in nucleotide binding at NBS2, but not at NBS1 (*24*). On the other hand, the NBS1 mutant D209N that binds nucleotides at NBS2 still forms stable cross-linked dimers in the presence of diN$_3$ATP, although with lower efficiency as compared to the wild type SecA. The decreased cross-linking efficiency is likely the result of the disturbed cooperativity between both ATP binding sites in the D209N SecA (*21*, *24*, *29*). These data therefore strongly favor an interfacial localization of NBS2. In conjunction with the likelihood that UV irradiation of diN$_3$ATP bound to SecA will not always result in the formation of a cross-link with protein but also with the surrounding water molecules (*38*), the efficiency of cross-linking is further reduced by incomplete occupancy of NBS2. Nonspecific cross-linking events will dominate at the diN$_3$ATP concentrations needed to

obtain complete saturation of the low-affinity NBS2 (*21*). We should emphasize that both mutants exhibit an elution pattern on size exclusion chromatography identical with that of the wild type SecA, demonstrating that they have maintained their dimeric organization.

Our results are consistent with recent data of Hirano et al. (*48*), who showed that carboxyl-terminal parts of SecA, located just after NBS2, can be cross-linked with a nonspecific chemical cross-linker. Although the effects of ATP binding on the formation of the cross-linked products have not been investigated, the same study shows that monomeric amino-terminal fragments of SecA bind ATP with high affinity, indicating that NBS1, at least functionally, is not located at the subunit interface. Thermodynamic studies of the unfolding characteristics of the two domains of SecA are also consistent with an interfacial localization of NBS2 (*21*). In the presence of NBS2-saturating concentrations of ADP, SecA undergoes large conformational changes. ADP binding to NBS1 increases the interaction between the N- and C-domains of SecA, while NBS2-saturating concentrations of ADP cause a major reduction in the amount of solvent accessible protein surface. The latter could be due to a global increase in the amount of solvent shielded residues and/or result from a considerable increase in the amount of shared protein surface of the monomers in the dimer. We have previously shown that SecA not only exists but also functions as a dimer (*20*). The interfacial localization of NBS2 may provide a mechanistic explanation for why the dimer acts as the functional unit. Hydrolysis of ATP at NBS2 could thus promote the more compact conformation of SecA by increasing the interfacial contact between the subunits of the SecA protomer. This conformational state of SecA might be employed during preprotein translocation to de-insert the C-domain from its membrane-integrated state at the SecYEG protein (*28*−*30*). Alternatively, binding and hydrolysis of ATP at NBS2 may modulate the relative spatial position of the two C-domains of the SecA dimer. Such nucleotide-driven movements of SecA domains (and subunits) may provide a physical mechanism for converting chemical energy into mechanical energy, and thus allow the SecA to function as a molecular motor.

In conclusion, UV irradiation of SecA in the presence of the bifunctional photoaffinity and hydrolyzable ATP analogue diN$_3$ATP results in the formation of cross-linked SecA dimeric species. Cross-linking takes place at NBS2, which is located in the C-domain of SecA. Thus, nucleotides bound at this site are localized at or near the subunit interface of the SecA dimer.

ACKNOWLEDGMENT

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