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18 Domain Dynamics of the *Bacillus subtilis* Peripheral Preprotein *translocase* Subunit SecA

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18.1 OUTLINE

The homodimeric SecA protein is the peripheral subunit of the preprotein *translocase* in bacteria. It promotes the preprotein translocation across the cytoplasmic membrane by nucleotide-modulated co-insertion and de-insertion into the integral domain of the *translocase*. SecA has two essential nucleotide binding sites (NBS): the high-affinity NBS-I resides in the amino-terminal domain of the protein and the low-affinity NBS-II is localized at 2/3 of the protein sequence. The nucleotide bound states of soluble SecA were studied by differential scanning calorimetry (DSC). Thermal unfolding reveals that the amino- and carboxy-terminal halves of SecA unfold independently with a transition midpoint of 49 and 40 °C, respectively. Binding of ADP to NBS-I increased the interaction between the two domains, whereas binding of AMP-PNP does not influence this interaction. When ADP binds both NBS-I and NBS-II, SecA seems to have a more compact globular conformation, whereas binding of AMP-PNP seems to cause a more extended conformation. It is concluded that SecA is a two-domain protein and that the interaction between both domains is modulated by nucleotides. The compact ADP-bound conformation may resemble the membrane-de-inserted state of SecA, while the more extended ATP bound conformation may correspond to the membrane-inserted form of SecA.

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18.2 INTRODUCTION

SecA, the peripheral subunit of the preprotein *translocase* in *Bacillus subtilis* has been identified in Gram-negative and Gram-positive bacteria, primitive algae and higher plants. The *B. subtilis* SecA shares an overall 65% homology with *Escherichia coli* SecA[1,2] and appears to have a similar function.[3–6] Although most studies involve *E. coli* SecA, we assume that the general observations are also applicable for *B. subtilis* SecA. The homodimeric SecA[7] associates in a binary complex with preproteins or in a ternary complex with the cytosolic chaperone SecB-bound preprotein, and guides them to the translocation sites at the cytoplasmic membrane[8] due to its affinity for negatively charged phospholipids and the integral membrane subunits of the *translocase*: SecY, SecE and SecG (for a recent review see ref. 9). Preprotein translocation is initiated by the ATP-dependent co-insertion of SecA and the preprotein in the integral domain of the *translocase*. Interaction of SecA with SecYEG stimulates the ATPase activity of SecA[10] and allows the de-insertion of SecA.[11] The Δp drives the completion of the preprotein translocation.[12,13] In the absence of a Δp, multiple cycles of nucleotide-modulated SecA insertion and de-insertion can also drive the complete translocation.[13] SecA has two essential nucleotide binding sites:[4–6,14,15] a high-affinity binding site ($K_D \approx 0.15$ μM) is confined to the amino-terminal domain of the protein (NBS-I), and a low-affinity binding site ($K_D \approx 340$ μM) is located in the second half of the protein sequence (NBS-II: Figure 18.1). Both NBS-I and NBS-II are indispensable for the translocation activity of SecA.[5,6,15] Although they are able to bind nucleotides independently, they somehow seem to hydrolyse ATP in a cooperative manner.[15] To elucidate the mechanism underlying the nucleotide-induced SecA movements, we investigated the conformation of the ADP and non-hydrolysable ATP analogue AMP-PNP bound state of soluble SecA by differential scanning calorimetry (DSC).

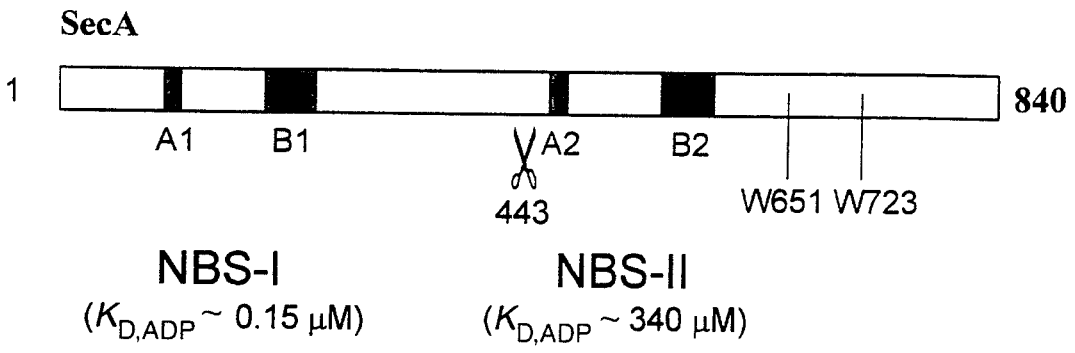

Figure 18.1. Schematic representation of the amino acid sequence *B. subtilis* SecA in which NBS-I, NBS-II, and the position of the tryptophans are indicated. The scissor indicates an accessible V8 protease cleavage site in the absence of nucleotides

18.3 RESULTS AND DISCUSSION

18.3.1 SECA HAS TWO INDEPENDENTLY UNFOLDING DOMAINS

The DSC profile of *B. subtilis* SecA (192 kDa as a dimer) shows two endothermic reactions with transition midpoints (T_m1 and T_m2) of 40 and 49 °C, respectively (Figure 18.2A). This is followed by an exothermic reaction (T_m3) with an onset temperature of 60 °C at pH 7.5 (Figure 18.2B). Since aggregation of proteins is largely exothermic,[16] it seems most likely that this exothermic transition corresponds to the aggregation of SecA immediately after or perhaps partly during the thermal denaturation.

The irreversibility of unfolding was evaluated by re-scanning the samples after cooling at a rate of 1 °C min^{-1} and a resting period of 15 min at 25 °C. No transitions were observed during the re-scan, demonstrating that the thermally induced denaturation of SecA is irreversible. To assess if both endothermic transitions were irreversible a new SecA sample was scanned from 30 to 43 °C (just past the first transition), cooled as described and re-scanned from 25 up to 55 °C. The first transition appeared to be completely reversible (Figure 18.2A) and is therefore independent from the second transition. Changing the scan rate from 1 to 1.5 °C min^{-1} up-shifted the T_m of the second transition 1 °C, but not the T_m of the first transition. This indicates that unfolding of the domain of the first transition is thermally controlled whereas that of the second transition is kinetically controlled. The onset of the exothermic reaction appeared to be dependent on the buffer composition. In the buffers used at pH 6.5, 7.5 and 8.0, the onset of the exothermic reaction was at 5, 10 and 20 °C past the second transition, respectively. At pH 8.0, where the exothermic reaction did not contribute to the heat of the second transition, this transition also appeared to be also irreversible (results not shown).

The baseline problem is always a source of uncertainty in DSC, especially when the process of denaturation is irreversible. We have followed the procedure described by Takahashi *et al.* and carried out deconvolutions of the DSC curves in two-state transitions, characterized by the equivalence of the ΔH_{cal} (calorimetric enthalpy change) and ΔH_{vH} (van't Hoff enthalpy change).[17] This approach is formally allowed only in the case of reversible phenomena, but shown to be admissible even on many systems having little or no reversibility.[18,19] Since the first transition is reversible, the deconvolution of the DSC data of this transition will be relatively reliable whereas the thermodynamic parameters derived from deconvolution of the second transition will be tentative. The first transition can be simulated by a two-state transition with a ΔH_{vH} approximately identical to the calorimetric enthalpy indicating the simultaneous unfolding of a subdomain in the SecA dimer (see below and Table 18.1). The $\Delta H_{vH}/\Delta H_{cal}$ ratio of the second transition

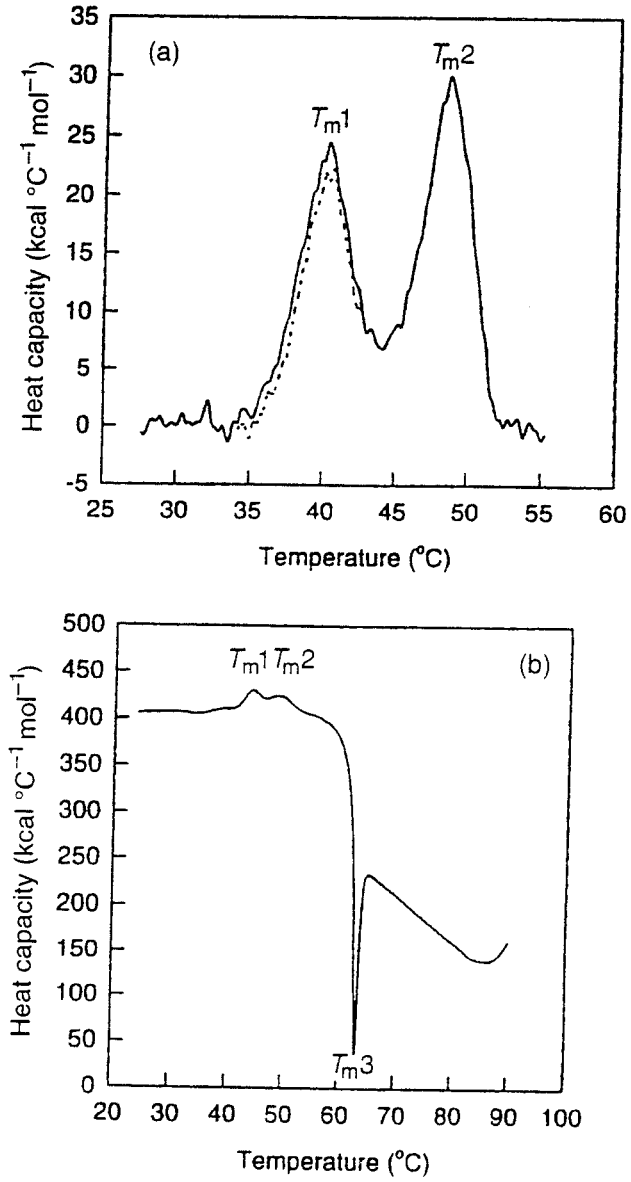

Figure 18.2. Temperature dependency of the excess molar heat capacity of *B. subtilis* SecA in 50 mM KP_i, pH 7.5, 50 mM KCl. (a) The dotted line represents a scan from 30 to 43 °C and the solid line represents a re-scan of the same sample from 25 to 55 °C. Indicated are the transition midpoints of T_m1 and T_m2. (b) Thermoscan from 20 to 95 °C. Indicated are the midpoints of T_m1, T_m2, and T_m3

is dependent on the onset temperature of the exothermic reaction, which in turn depends on the buffer composition in which the protein was denatured (Table 18.1). If the irreversible step occurs at temperatures significantly higher than the denaturation temperature, then application of equilibrium thermodynamics is valid. Therefore, the most reliable thermoscan was

obtained by the sample wherein the exothermic reaction was postponed by 20 °C (see above). Using this sample, the second transition could also be simulated by a two-state transition. The ΔC_p was about 2000 cal $°C^{-1}mol^{-1}$ dimer for the complete unfolding of SecA in 50 mM Tris–HCl, pH 8.0 (Figure 18.4A). We conclude that SecA has at least two thermally independent folding domains.

18.3.2 THE INTERACTIONS BETWEEN THE TWO DOMAINS OF SECA INCREASE IN THE PRESENCE OF ADP

The thermal denaturation of *B. subtilis* SecA was studied in the presence of increasing concentration of ADP in 50 mM KP_i, pH 7.5 supplemented with 50 mM KCl and 5 mM $MgCl_2$ (Figure 18.3A, Table 18.2). The thermoscans in Figure 18.3A are all concentration normalized as dimer and baseline subtracted as described (experimental procedures). Again two unfolding transitions can be discriminated followed by the exothermic aggregation reaction onset at 60 °C. This latter reaction is partly compensated for by an endothermic hydrolysis of the ADP at 80 °C. This is particularly evident in the thermoscan in the presence of 0.2 mM ADP (trace 3). Under all conditions the transition profile deconvolutes approximately as two-state transitions (Table 18.2). At the low ADP concentrations of 0.02–0.2 mM, which saturate NBS-I but not NBS-II, an up-shift in T_m1 from 40 to 46 °C with no significant change in T_m2 is observed. The shift in T_m1 saturates, and T_m2 seems to up-shift slightly at higher ADP concentrations up to 2 mM (Figure 18.3B), unfortunately this latter shift is obscured by the simultaneous binding of ADP to NBS-II (see below). ADP binding to NBS-I stabilizes the SecA domain of the first transition, but not that of the second transition. Such a pattern is frequently observed in two-domain proteins, which will bind a ligand at the more stable domain (T_m2), whereas the ligand interaction with this domain is influenced by ligand-dependent changes in the interactions between the binding domain (T_m2) and the regulatory domain (T_m1).[20] NBS-I could be situated at the interface of both domains. This is supported by the observation that ADP binding to NBS-I causes a change in the environment of the carboxy-terminal tryptophans of SecA[21] and that cross-linking of [α^{32}P]ATP to amino-terminal peptides containing NBS-I of *E. coli* SecA is only possible in the presence of the carboxy-terminal counterpart.[14] These data seem to support a model in which the interaction between the two domains of SecA increases upon ADP binding to NBS-I.

Apart from, perhaps, a slight destabilization of both transitions, the thermal unfolding of SecA was not altered significantly by 0.02 mM of the non-hydrolysable ATP analogue adenylyl-imido-diphosphate (AMP-PNP) compared with that of the nucleotide-free protein (Table 18.2). The instability of AMP-PNP at high temperatures does not allow us to use higher

Table 18.1. Thermodynamics of the unfolding of SecA and N-SecA[a]

Buffer 50 mM	T_m1 (°C)	$\Delta H_{cal}1$ (kcal mol^{-1})	$\Delta H_{vH}1$ (kcal mol^{-1})	$\Delta H_{vH}1/\Delta H_{cal}1$	T_m2 (°C)	$\Delta H_{cal}2$ (kcal mol^{-1})	$\Delta H_{vH}2$ (kcal mol^{-1})	$\Delta H_{vH}2/\Delta H_{cal}2$
KP_i, 6.5	38.3 ± 0.5	131 ± 19.5	133 ± 20	1.01	45.8 ± 1.0	107 ± 32	171± 26	1.60
KP_i, 7.5	40.5 ± 0.5	154 ± 23	133 ± 20	0.86	48.6 ± 0.5	175 ± 26	163 ± 24	0.93
Tris, 8.0	39.6 ± 0.5	150 ± 22.5	152 ± 20	1.01	47.3 ± 0.5	160 ± 24	161 ± 24	1.01
Tris, 8.0[a]					50.3 ± 0.5	167 ± 25	172 ± 26	0.97

[a] N-SecA, fragment of *B. subtilis* SecA corresponding to amino acids 1–443

Table 18.2. Thermodynamics of the unfolding of SecA in the presence of ADP or AMP-PNP

[Ligand]	T_m1 (°C)	$\Delta H_{cal}1$ (kcal mol^{-1})	$\Delta H_{vH}1$ (kcal mol^{-1})	$\Delta H_{vH}1/\Delta H_{cal}1$	T_m2 (°C)	$\Delta H_{cal}2$ (kcal mol^{-1})	$\Delta H_{vH}2$ (kcal mol^{-1})	$\Delta H_{vH}2/\Delta H_{cal}2$
ADP mM								
0	40.5 ± 0.5	154 ± 23	133 ± 20	0.86	48.6 ± 0.5	175 ± 26	163 ± 24	0.93
0.02	43.8 ± 0.5	176 ± 26	185 ± 28	1.05	48.6 ± 0.5	172 ± 26	155 ± 23	0.90
0.2	46.3 ± 0.2	197 ± 29	235 ± 35	1.20	49.1 ± 0.2	112 ± 17	218 ± 33	1.94
2	49.3 ± 0.5	705 ± 106	129 ± 19	0.18				
AMP-PNP mM								
0.02	39.7 ± 0.5	142 ± 21	146 ± 22	1.03	48.5 ± 0.5	186 ± 56	145 ± 44	0.78

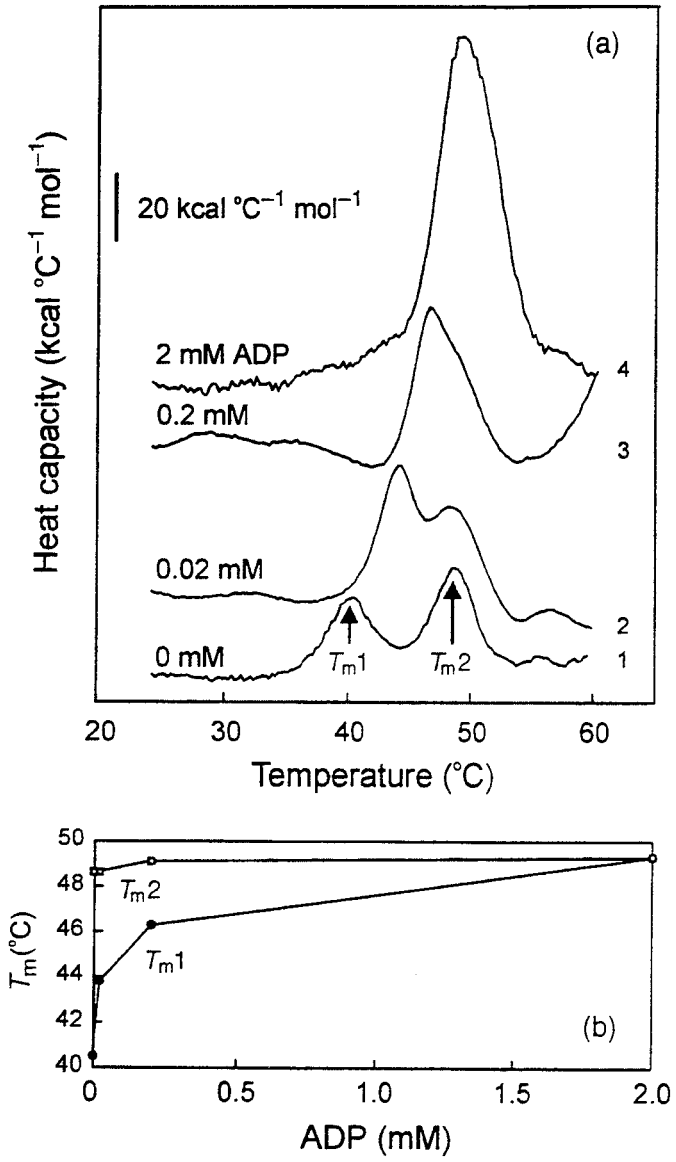

Figure 18.3. Temperature dependency of the excess molar heat capacity of *B subtilis* SecA in 50 mM KP_i, pH 7.5, 50 mM KCl in the absence and presence of ADP. (a) Thermal unfolding of SecA in the absence of ADP (1), in the presence of 0.02 mM ADP (2), 0.2 mM ADP (3), and 2 mM ADP (4). Indicated are the transition midpoints of T_m1 and T_m2. (b) The dependency of the midpoints of transitions 1 and 2 on the ADP concentration

concentrations of AMP-PNP in the DSC measurements. At the concentration used, AMP-PNP does not seem to induce changes in the interaction of the two domains of SecA.

18.3.3 THE DOMAINS COMPRISE THE AMINO- AND CARBOXY-TERMINAL HALF OF SECA

Since the enthalpy of the unfolding is similar for both domains, they could possibly be of similar size. In such a case, it is to be expected that each domain can be expressed and isolated as a separate domain which has retained its thermal unfolding characteristics. Based on the thermal behaviour of both domains in the presence of ADP, NBS-I is expected to be located in the most stable domain (T_m2). Therefore, an amino-terminal domain, containing amino acids 1 up to 443 (N-SecA) with an amino-terminal tag of six histidines, was cloned in an expression vector. The N-SecA protein was over-expressed in *E. coli*, purified by Ni^{2+}-NTA affinity and ion-exchange chromatography, and analysed by Coomassie-stained SDS–PAGE and immunoblotting using polyclonal antisera directed against *E. coli* or *B. subtilis* SecA. Immunoblots confirmed that the protein samples were free of residual *E. coli* SecA. The N-SecA protein migrates on SDS–PAGE as a 50 kDa protein, confirming its predicted molecular weight (results not shown). Since the N-SecA was prone to aggregation, its thermal unfolding was studied in the most stabilizing buffer (Tris–HCl, pH 8.0, 50 mM KCl and 5 mM $MgCl_2$). The DSC profile of the N-SecA protein shows only one endothermic transition at 50 °C and subsequently an exothermic transition indicating aggregation similar to the second transition of intact SecA (Figure 18.4B). This confirms the hypothesis that SecA consists of an independently folding amino- and carboxy-terminal half and that binding of ADP to the amino-terminal domain increases its interaction with the carboxy-terminal domain. The transition of N-SecA could also be simulated with a two-state transition assuming the protein to be a dimer of 100 kDa (Table 18.1). Consequently, the amino-terminal domain must be at least partly responsible for the dimerization of SecA.

18.3.4 THE APPARENT MOLECULAR MASS CHANGES DRAMATICALLY IN NUCLEOTIDE-BOUND SECA

At 2 mM ADP the enthalpy of unfolding of SecA increases from 350 kcal mol^{-1} dimer to 700 kcal mol^{-1} dimer (Figure 18.3A, Table 18.2). At this concentration NBS-II will be occupied by ADP for 86%, using the reported K_D at 25 °C of 340 μM.[15] The overall unfolding enthalpy of SecA in the absence of ADP or in the presence of NBS-I saturating ADP concentration is unusually small, 329 ± 49 kcal mol^{-1} dimer or 1.71 cal g^{-1} protein, but not unprecedented.[22] In the presence of 2 mM ADP the specific enthalpy of unfolding increases to 3.67 cal g^{-1}, which is in the range normally found for globular proteins.[17,19] The doubling in enthalpy cannot be attributed to the enthalpy of the dissociation of the bound ADP since this has never been reported to be more than *ca.* 30 kcal mol^{-1} ADP (for example, see ref. 19).

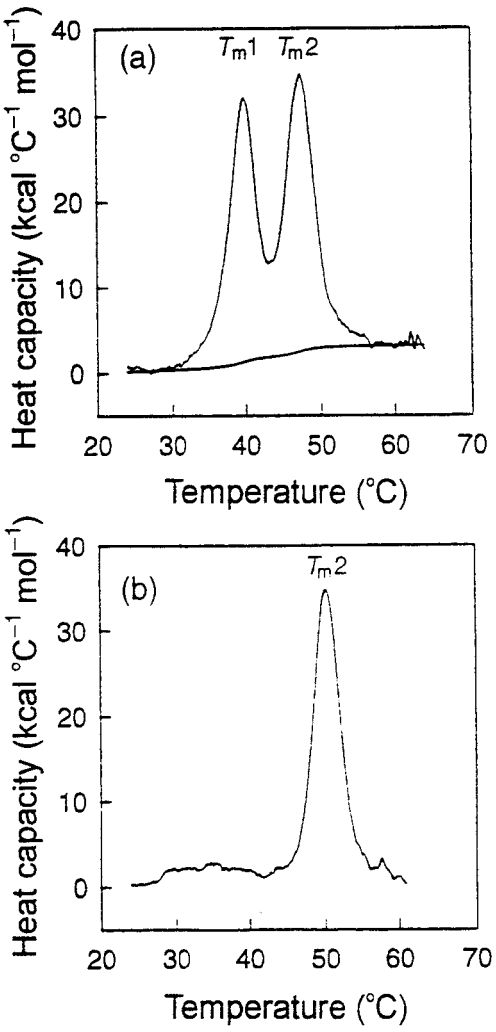

Figure 18.4. Temperature dependency of the molar heat capacity of *B.subtilis* SecA in 50 mM Tris-HCl, pH 8.0, 50 mM KCl. (a) SecA (showing the baseline). (b) N-SecA in the same buffer with an additional 5 mM $MgCl_2$ for stabilization. Indicated are the transition midpoints of T_m1 and T_m2

The enthalpy of unfolding is largely dependent on the number of amino acid residues which is inaccessible to the solvent.[23] The increase in enthalpy upon ADP binding to NBS-I and NBS-II suggests an increase in the amount of solvent-shielded residues or a more compact conformation. Alternatively, a considerable increase in the amount of shared protein surface of the monomers in the dimer could result in an increase in the enthalpy of unfolding. For instance, if NBS-II were to be located at the interface of the monomers. In the presence of NBS-II saturating concentrations of the slowly hydrolysable ATP analogue ATP-γ-S, or ADP, the proteolytic site at Glu-443 of *B. subtilis* SecA is protected against V8 proteolysis.[6] The *B. subtilis*

SecAD215N mutant defective in NBS-I is permanently resistant against V8 proteolysis even in the absence of nucleotides.[6] These data support our observation that binding of ADP to both NBSs increases the interaction between both domains and promotes a more compact conformation of SecA.

Experiments in which the apparent size of SecA undergoing Brownian motion in solution was studied by dynamic light scattering (DLS, for a recent review see ref. 24) showed an increase of 16% and 40% in apparent mass of SecA upon saturation of NBS-I and -II with ADP or AMP-PNP, respectively.[21] These data were used to calculated the hydration shell of the protein assuming it to be spherical. This was 1.47 and 2.02 g g^{-1} for ADP and AMP-PNP-bound SecA, respectively, which clearly indicates that the protein contains more water-accessible surface than expected for a spherical protein. The 25% increase in solvation shell in the presence of AMP-PNP suggests an increase in the amount of solvent-accessible protein surface, and indicates an asymmetry in the shape of the protein. Based on the DSC measurements, the ADP-bound conformation seemed to be relatively compact. In combination with the DLS results, the ADP-bound conformation of SecA could possibly be visualized as irregular spherical, whereas in the AMP-PNP-bound conformation, the protein could have a more elongated shape.

The nucleotide-free form of SecA is likely to be non-existent *in vivo*, while the soluble form of SecA will be predominantly in the ADP-bound state. For membrane association of SecA with SecYEG, either ATP and preprotein or AMP-PNP are needed.[25] Mutants of *E. coli* and *B. subtilis* SecA which are defective in ATP hydrolysis but not in ATP binding at NBS-I are able to insert into the membrane but are not able to de-insert or to translocate preproteins.[5,25] The increased interaction between the amino- and carboxy-terminal domain upon ADP binding to NBS-I could be interpreted as movement that conceals the membrane insertion site of SecA, whereas ATP binding could reveal this site. Mutants in NBS-II which still have 4% of the wild type activity in ATP binding and hydrolysis[15] are able to insert and de-insert in the membrane, but cannot translocate preproteins.[25] ATP binding and hydrolysis at NBS-II may be required for a more stable insertion, since AMP-PNP binding to both sites protects a membrane-bound 30-kDa fragment against protease K digestion.[25] The NBSs cannot hydrolyse ATP independently[15] and after ATP-induced insertion, additional ATP binding and hydrolysis seems to be required for the de-insertion of SecA.[11] This indicates that ATP hydrolysis at both sites might be required for complete de-insertion. Signal sequence repressing mutations are also found in both NBS regions, indicating that both sites are involved in the recognition and presentation of the signal sequence to the *translocase*.[26] The observation that ADP and AMP-PNP binding to NBS-I as well as to NBS-II cause considerable changes in conformation and shape of SecA, shows that both sites are functionally involved in the process of preprotein trans-

location. We propose that the initiation of preprotein translocation is a two-step process in which ATP binding to NBS-I and -II allows integration of SecA into the membrane presenting the preprotein to SecYEG. ATP hydrolysis at NBS-II could promote a more compact conformation, which causes the release of the preprotein, while hydrolysis at NBS-I would be needed for the de-insertion of SecA.

18.4 MATERIALS AND METHODS

18.4.1 BACTERIAL STRAINS. GROWTH MEDIA AND BIOCHEMICALS

Unless indicated otherwise, strains were grown in Luria Bertani (LB) broth or on LB-agar[27] supplemented with 50 μg of ampicillin ml^{-1}, 0.5% (w/v) glucose, or 1 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG), as required. *B. subtilis* SecA was over-expressed in JM109[28] and NO2947[29] containing pMKL4[4] and purified as previously described,[5] and N-SecA was expressed in *E. coli* SF100.[30] Protein concentration was determined by the method of Bradford.[31]

18.4.2 CONSTRUCTION AND EXPRESSION OF THE SECA DOMAIN

To express the amino-terminal domain of *B. subtilis* SecA (amino acids 1–443) in *E. coli* under control of the *trc* promoter, the individual domain was amplified from the wild type *B.subtilis* SecA containing plasmid pMKL4[4] by PCR using the primers N1 forward and N1 reverse (5′ GCGCCATG-GTTGGAATTTTAAATA AA and 5′ CAGCTTAGTCGACTTTGCAGC) for the synthesis of nucleotides 1 to 691 and the primers N2 forward and N2 reverse (5′ GCTGCAAA GTCGACTAAGCTG and 5′ GCCCTCTAGAC-TACTATTCAGATGTTTCAACGGC) for the synthesis of nucleotides 691 to 1325. The introduced restriction sites are underlined and the base pair changes are in bold. This introduced a *Nco*I site at the start codon (replacing a leucine by a valine) and a *Sal*I site at nucleotide 691 in the first half, and two stop codons and a *Xba*I site at the end of the second half of the amino-terminal domain. All PCR reactions were carried out with *Pwo* polymerase (Boehringer Mannheim, Germany) using a Biometra triothermoblock (Biometra, Göttingen, Germany) employing the manufacturer's recommendations. The PCR products containing fragments of SecA were cloned in pET400 (K. H. M. van Wely, University of Groningen, NL) using the introduced restriction sites and completely sequenced on a Vistra DNA sequencer 725 (Amersham, Buckinghamshire, England) using the automated

Δtag sequencing kit of Amersham. The synthetic gene N-SecA under control of the *trc* promoter in pET302 was derived from the N1 and N2 PCR fragments by standard cloning techniques (Sambrook *et al.*, 1989).[32] PET302 is a pTRC99A derivative[33] with a six histidine tag preceding the *Nco*I site in the multiple cloning site (C. van der Does, University of Groningen, NL). Cells over-expressing N-SecA were harvested by centrifugation, and resuspended in 50 mM HEPES-KOH, pH 7.5, 50 mM KCl, 20% sucrose and lysed twice by French-pressure treatment at 8000 psi.[34] Unbroken cells were removed by centrifugation (15 min, 7600 × *g*, 4 °C) and membranes were removed by ultra-centrifugation (Ti 70, 40 min, 120 000 × *g*, 4 °C). The supernatant was incubated with 5 mM imidazole, 50 mM HEPES-KOH, pH 7.5, 50 mM KCl pre-washed Ni^{2+}-NTA agarose beads (Qiagen, Chatsworth, CA, USA) for 1 h at 4 °C. Subsequently, the Ni^{2+}-NTA was washed with 20 mM imidazole and the protein eluted with 100 mM imidazole. The eluted protein was further purified on a MonoQ ion-exchange column (Pharmacia, Upsalla, Sweden) using a 0–1 M NaCl gradient in 50 mM Tris–HCl pH 7.6, 1 mM DTT, 10% glycerol.

18.4.3 CALORIMETRIC MEASUREMENTS

DSC experiments were performed using an MC-2 microcalorimeter (Microcal, Amherst, MA), with a constant pressure of 2 atm over the liquids in the cells. Unless otherwise stated a differential scanning rate of 1 °C min^{-1} was employed. The DSC experiments were carried out with a *B. subtilis* SecA concentration of 1–2 mg/ml in 50 mM KP_i, pH 7.5, 50 mM KCl or SecA in the same buffer which was supplemented with 5 mM $MgCl_2$ and ADP or AMP-PNP. The reversibility of the DSC transitions were checked by re-heating the solution in the calorimeter cell after cooling (1 °C min^{-1}) from the first run. A thermogram corresponding to a water against water run was used as the instrumental baseline.

18.4.4 ANALYSIS OF CALORIMETRIC RESULTS

The dependence of molar heat capacity on temperature was analysed using the ORIGIN software (Microcal Ltd). Analysis of the data involved fitting and subtraction of an instrumental baseline, as previously described.[22] The data were normalized, assuming that the SecA dimer dissociated after or during the most stable transition.

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