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Structure of Dimeric SecA, the *Escherichia coli* Preprotein Translocase Motor

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**Running Title:** *E. coli SecA structure*

**Subject Category:** Protein and nucleic acid structure, function and interactions
Summary

SecA is the preprotein translocase ATPase subunit and a Superfamily 2 (SF2) RNA helicase. Here we present the 2 Å crystal structures of the *Escherichia coli* SecA homodimer in the apo form and in complex with ATP, ADP and adenosine 5′-[β,γ-imido]triphosphate (AMP-PNP). Each monomer contains the SF2 ATPase core (DEAD motor) built of two domains (Nucleotide Binding Domain, NBD and Intramolecular Regulator of ATPase 2, IRA2), the Preprotein Binding Domain (PBD), which is inserted in NBD and a carboxy-terminal domain (C-domain) linked to IRA2. The structures of the nucleotide complexes of SecA identify an interfacial nucleotide-binding cleft located between the two DEAD motor domains and residues critical for ATP catalysis. The dimer comprises two virtually identical protomers associating in an anti-parallel fashion. Dimerization is mediated solely through extensive contacts of the DEAD motor domains leaving the C-domain facing outwards from the dimerization core. This dimerization mode explains the effect of functionally important mutations and is completely different from the dimerization models proposed for other SecA structures. The repercussion of these findings on translocase assembly and catalysis is discussed.

Keywords: ATPase; Crystal structure; DNA-RNA helicase; Protein translocation; SecA

Abbreviations: *ec*SecA, *Escherichia coli* SecA protein; *bs*SecA, *Bacillus subtilis* SecA protein; *mt*SecA, *Mycobacterium tuberculosis* SecA protein; NBD, Nucleotide Binding Domain; IRA2, Intramolecular Regulator of ATPase 2 domain; DEAD-motor, NBD and IRA2 domains; PBD, Preprotein Binding Domain; C-domain; Carboxy-domain; SD; Scaffold sub-Domain; WD; Wing sub-Domain; IRA1, Intramolecular Regulator of ATPase 1 sub-domain; CTD; Carboxy Terminal sub-Domain.
Introduction

Most extracytoplasmic proteins are translocated into and across the bacterial inner membrane by a complex membrane transporter called the translocase or translocon.\textsuperscript{1,2} The translocase is comprised of the core heterotrimeric SecY/SecE/SecG transmembrane protein conducting channel and a peripheral ATPase motor called SecA. The \textit{Escherichia coli} translocase has been fully reconstituted in a functional state \textit{in vitro}\textsuperscript{3} and the high resolution structures of an archaeal SecYEG,\textsuperscript{4} the SecA from \textit{Bacillus subtilis} (hereafter bsSecA)\textsuperscript{5-7} and a SecA from \textit{Mycobacterium tuberculosis} (hereafter mtSecA)\textsuperscript{8} have been previously reported.

Secreted proteins are synthesized with N-terminal signal peptides and interact with export-specific piloting factors and chaperones. These interactions target the preprotein to the membrane where they bind to SecA.\textsuperscript{3} Preprotein binding triggers ATP-driven cycles of conformational changes in SecA bound at SecYEG, described as “insertion/de-insertion” cycles.\textsuperscript{9,10} During SecA membrane cycling, 20-30 residue segments of the preprotein are consecutively moved into the membrane\textsuperscript{10,11} through SecYEG.\textsuperscript{12} These results suggest that SecA acts as a processive machine\textsuperscript{13} and that specific co-ordinated motions in SecYEG take place.\textsuperscript{4}

The functional oligomeric state of SecYEG and of SecA is a matter of controversy. \textit{E. coli} SecA (hereafter ecSecA) forms a stable dimer in solution at concentrations comparable to those in the cell cytoplasm.\textsuperscript{14-16} ecSecA is functional as a homodimer during protein translocation.\textsuperscript{17-19} However, other studies propose that SecA is functional as a monomer,\textsuperscript{20,21} since SecA can bind to SecYEG as a monomer or dimer\textsuperscript{22-23} and appears to monomerize upon binding to lipids\textsuperscript{21} or to detergent-solubilized SecYEG.\textsuperscript{24}

SecA is large (102 kDa) and elongated.\textsuperscript{25} Each protomer comprises four structural domains (see Results). Two of these comprise a “RecA-like” fold and associate to build a structure widespread in nucleic acid helicases.\textsuperscript{26,27} This structure is the ATPase engine of these enzymes and contains characteristic sequences such as “Asp-Glu-Ala-Asp” or “DEAD” and
derivatives thereof. Sequence homology places SecA in the DExD/H helicase Superfamily 2 (SF2).\textsuperscript{28} Inserted in each of the SecA DEAD motor domains is a “specificity domain”. Despite the vast body of mutagenic and biochemical data, exactly how preprotein segments are translocated and which parts of SecA are involved remains unresolved.

To gain an insight into the molecular basis of SecA-mediated catalysis and oligomerization we determined the structure of ecSecA at 2 Å resolution in the apo state and complexed with the nucleotides ADP, ATP and its non-hydrolyzable analogue AMP-PNP. Our data reveal that: \textbf{a.} ecSecA crystallizes as an antiparallel dimer; \textbf{b.} Dimerization occurs exclusively between the DEAD motors; \textbf{c.} The extreme N-terminus of ecSecA does not participate in dimerization; \textbf{d.} The DEAD motor and the two “specificity domains” can undergo significant rigid body motions; \textbf{e.} Several residues identified by genetic and biochemical studies to be important for either preprotein translocation or/and catalysis in nucleic acid helicases participate in or are located in the immediate vicinity of the ecSecA dimerization interface.
Results

Structure determination

In this work, the recombinant ecSecA[9-861] protein was used. ecSecA[9-861] is practically indistinguishable from wild type SecA (901 residues) as far as its in vitro and in vivo activity and dimerization are concerned. For simplicity ecSecA[9-861] will be referred hereafter as ecSecA. The full length protein only ever produced crystals that diffracted to less than 8 Å resolution. These results and the steps taken to obtain suitable crystals for structure analysis of ecSecA, will be reported elsewhere (Y. Papanikolau, in preparation).

The structure of ecSecA was determined in the apoprotein and nucleotide-bound states from crystals soaked with ATP, ADP.Mg$^{2+}$ and AMP-PNP.Mg$^{2+}$. In all cases, two protein molecules were identified in the asymmetric unit. The structure of ecSecA apoprotein was built into electron density maps calculated to 2.0 Å. The structures of the ATP, ADP and AMP-PNP complexes were determined to 2.2, 2.1 and 2.0 Å resolution, respectively. The structure was solved using multiple and single wavelength anomalous diffraction data collected from crystals of selenomethionylated protein (Table 1). There was insufficient electron density to confidently build some residues at the amino and carboxy termini and most of the Preprotein Binding Domain (PBD; residues 221-376$^{30,31}$ Figures 1(b) and S1 of Supplementary Material). The missing residues from the final refined model are the chain α residues: 9-12, 229-364 and 835-861 and the chain β residues: 9-11, 233-279, 314-364 and 833-861. The determined structure of ecSecA represents ~84% of the crystallized protein. Nevertheless, the experimentally determined part of the PBD structure was enough to determine its overall orientation. Modelling of the unresolved parts of ecSecA PBD was based on bsSecA(1TF5)-PBD$^5$ as described in Materials and Methods. The modelled PBD contains in addition helices α10, α13 and strands β7-β11 (Figure S1).
Structure of the ecSecA protomer

ecSecA consists of 29 α-helices and 23 β-strands organized into four structural domains (Figure 1(a) and S1 of Supplementary Material). Two of these, termed the Nucleotide Binding Domain (NBD; residues 1–220 and 377-416; dark blue) and the Intramolecular Regulator of ATPase 2 (IRA2; residues 417–621; light blue), are α/β domains that assemble to form an SF2 DEAD motor.

NBD consists of a β-sheet made from seven parallel β-strands (strands β1-β5 and β13-β14; Figure S1A) that is surrounded by eleven α-helices (α1-α9 and α14-α15). PBD is inserted between helices α9 and α14 of NBD without disturbing the structural integrity of the RecA fold. PBD comprises characteristic Stem and Bulb sub-structures. The Stem is formed by two antiparallel β-strands (β6 and β12; Figure S1) and inserts in NBD. The Bulb comprises two lobes (Bulb1, extending to the end of helix α12 and Bulb2). In some helicases, e.g. UvrB, an unrelated “specificity domain” is inserted in exactly the same location, while in others (e.g. the Drosophila development protein Vasa) the corresponding α9 and α14 are fused. The position and length of secondary structural elements in different PBDs varies (Figure S1B).

IRA2 is composed of a central β-sheet made from seven parallel β-strands (β15-β19 and β21-β22; Figure S1A) and an antiparallel β-strand (β20) and, is surrounded by seven α-helices (α16-α22). IRA2 contains two characteristic sub-structures: the variable region (VAR; a hair pin of helices α19-α20 comprising residues Gly516 to Gly553; Figure S1A; red) that shows significant length and sequence variability in different SecA proteins (Figure 2) and the “Joint” comprising residues 591-621 (Figure S1A; grey) that connects IRA2 to the C-domain (see below). A similar, albeit differently oriented, structure is also seen in other helicases such as Vasa (Figure 3).

ecSecA has a Carboxy-domain (C-domain) which consists of residues 622–901 (Figure 1(b); green) and can be subdivided into four sub-domains (Figure 1(a) and S1A): the Scaffold...
Domain (SD) that comprises the longest helix ($\alpha_{23}$) of the structure (residues 622-668); the Wing Domain (WD; residues 669-755) that comprises four helices ($\alpha_{24}$-$\alpha_{27}$) and one short $\beta$-strand ($\beta_{23}$); the IRA1 domain (residues 756-829) that comprises helices $\alpha_{28}$ and $\alpha_{29}$ connected by an extended loop; the extreme C-terminal domain (CTD) (residues 830-901) of which only residues 830-834 can be modeled (Figure 1(a); orange). The PBD Stem crosses loosely over SD/IRA1 bundle, forming only minor contacts. In contrast, significant contacts are made by helix $\alpha_{12}$ from the Bulb, whose N-terminal residues bind the concave surface underneath the tip of the IRA1 hairpin. Five IRA1 residues that are important for SecA catalysis line the $\alpha_{12}$ binding site (Figure 4).

Average residue temperature factors (Figure S2C) reveal that the most ordered domains of *ec*SecA is NBD followed by IRA2, while the two “specificity domains” (C-domain and PBD) show increasing flexibility.

**Structure of the *ec*SecA dimer**

In the asymmetric unit of the *ec*SecA crystals there is a molecular dimer (Figure 5) of approximate 2-fold symmetry (179.4°). The area of the dimerization interface for *ec*SecA is 3292 Å² (7.9% of the monomer surface). The two antiparallel molecules of *ec*SecA are virtually identical (rms-deviation of 684 C$_\alpha$-atoms 1.3 Å) with minor conformational differences (Figure S2A-B). Nonetheless, the relative flexibility between the two protomers differs (Figure S2C).

Dimerization is mediated exclusively by DEAD motor residues of the two juxtaposed protomers (Figure 6(a)). Most of the interfacial contacts are provided by the same residue from each protomer located in NBD (N132, Y134, Q137, P159 and G160), IRA2 (T470, K471, H476, N477, F483, H484, N486 and A489) (Figure 6(b)) and its VAR region (Q520, A524, E527, N528 and K538) (Figure S4B). Additional contacts are provided only by protomer $\alpha$ (K475, W519 and P529) and protomer $\beta$ (E141, M161, A525 and L526) residues. The contacts are
primarily hydrophobic (Figure S3A) and further stabilised by a total of 15 hydrogen bonds (Figure S4 and Table S1 of Supplementary Material). Residues N132, Y134, Q137 and E141 of helix α6 and its preceding loop all belong to the helicase Motif Ia of SecA (see below). Additionally, residues F263-V265 of PBD are in hydrophobic contact between the two protomers and could contribute to dimerization. In the dimer, helix α18 from IRA2 of one protomer penetrates into the ATP cleft of the DEAD motor of the other protomer (Figure 6(b) and S1A). α18 lies ~10 Å from the nucleotide, at the opposite side of the cleft (Figure S3C), and hence it does not sterically interfere with nucleotide binding.

Structure of ecSecA in complex with nucleotides

Nucleotides were soaked into the protein crystals and a single nucleotide molecule was observed in each protomer (Figure 7(a-d)), corroborating previous evidence that NBD and IRA2 form a mononucleotide binding cleft\(^ {28}\) in common with other helicases.\(^ {26}\) Interactions of the bound adenine nucleotides with the DEAD motor (Figure 7(b-d)) are almost exclusively located on the NBD. The nucleotide binding sites are solvent-exposed on the outer rim of the dimer. The adenine ring is sandwiched between two hydrophobic residues of the Q motif (M81, F84) and additional polar contacts with Q motif residues (R82 and Q87; Figure S5-S6), ensure a tight association. These interactions suggest that the Q motif is of importance in SecA catalysis, as shown in other SF2 RNA helicases.\(^ {36}\)

Several NBD residues in Motif I (T104, G105, E106, G107, K108, T109 and L110), are involved in direct contact with the nucleotides (Figures 7(b-d), S5 and S6 of Supplementary Material). Additional residues from Motifs Ia (R138, D139, N142 and N143) and II (D209) make water-mediated interactions with the nucleotide (Figures 7(b), S5 and S6). Only one residue from Motif V (R509) of IRA2 makes a direct contact (Figures 7(c-d), S5 and S6) and this is the first structural evidence that R509 of the helicase Motif V interacts directly with ATP,
underlining its essential contribution to SecA ATP hydrolysis. Motif VI residues are not involved in nucleotide binding, making instead specific contacts (Table S2) to Motif V residues located in IRA2 (e.g. R574 with M506, R577 with R509 and D512) and to Motif II residues located in NBD (R566 with D212). Two of the Motif VI residues that are essential for catalysis, R574 being the presumed “arginine finger” residue and R577 face away from the nucleotide, their side chains being rotated by at least 45°. We must therefore assume that the ecSecA in the crystal structure is catalytically inactive.

While soaking of nucleotides into the protein crystals does not result in major structural changes (rms-deviation of 2716 main-chain atoms ~0.5 Å) (Table S3), there are distinct conformational changes in the side chains of residues that line the nucleotide cleft. For example, R509, which contacts E210 in the apoprotein, reorients to interact with ADP or AMP-PNP in both protomers, but only with ATP in protomer β (Figures S5 and S6). Moreover, R509 in protomer α, its neighbours in the Motif V loop (G508 and G510) and residues in VAR (A531 and E532) show a significant conformational difference (rms-deviation of Ca atoms ~1 Å) between the ATP and ADP-bound structures. In protomer β, R509 does not undergo a significant structural change but residues in VAR (Q520, A521, A524, A525 and A531) do (rms-deviation of Ca atoms >1 Å). Moreover, some Motif VI residues also become rearranged in the presence of ATP (Table S2), forming contacts with Motif I (Q578 with E106) and Motif III (N569 with T393).

Comparison of ecSecA with the other SecA structures

Superimposition of ecSecA with bsSecA (pdb codes 1M6N and 1TF5) and mtSecA (pdb code 1NL3) indicates that they share a similar overall fold (Figure 8(a-c) and Table S4). Significant sequence and structural variation is only seen in the VAR regions (Figures 2 and 8).
A comparison of the four structures reveals the remarkable repertoire of large-scale and localized domain motions possible, mainly involving the DEAD motor and “specificity domains”. The most extensive conformational difference involves a rigid body movement of the PBD. In the \( bs\text{SecA}(1M6N) \) and \( mt\text{SecA} \) structures the PBD is almost attached to WD via its Bulb1 lobe (Figure 9(a)). In the \( ec\text{SecA} \) and \( bs\text{SecA}(1TF5) \) structures the PBD Bulb swivels around its Stem by \( \sim90^\circ \), such that Bulb1 moves away from WD to interact, from below, with the IRA1 sub-domain (Figures 4 and 9(a)). This motion creates a considerable solvent-accessible space between PBD and WD. It is clear that this rigid body movement is accomplished by some significant secondary structure rearrangements. For example, helix \( \alpha_12 \) of Bulb1 undergoes a significant reduction in secondary structure and shortens by six residues (Figures 4 and S1B).

Another striking difference is that the DEAD motor is more “open” in \( ec\text{SecA} \), allowing the second protomer to dock in an antiparallel fashion (see below). This occurs because IRA2 undergoes a significant lateral rigid body movement away from NBD (Figure 9(b)) and was not observed in the other SecA structures. Nevertheless, IRA2 is not completely detached from NBD and remains bound through a salt-bridge between D217 and R566. The rigid body motion of IRA2 away from NBD in a “clam-shell” fashion that mediates “opening” of the DEAD motor is possible because of the linker connecting NBD to IRA2 (residues 413-420; Figure S1A). This linker was shown to be very flexible.\(^{30,34,38}\) Lastly, there is significant variation in the orientation of the SD and WD sub-domains between \( ec\text{SecA} \) and the other SecAs (Figure 8).

While the protomer folds are similar, the quaternary organization of the four SecA structures is widely different. These differences are readily visualized when the most conserved and rigid NBD domain of the \( ec\text{SecA} \) protomer \( \alpha \) is superimposed pairwise with the corresponding domain from the other structures. This analysis reveals that protomer \( \beta \) (Figure
10; pink) assumes a completely different orientation in the three structures. bsSecA(**1TF5**) structure exists as a monomer in the crystals and was proposed to represent a monomeric form. 

**ecSecA** forms a dimer in the asymmetric unit of the crystals and the DEAD motor is the only region involved in dimerization (Figures 5, 6, S3A and S4A, Table S1). Only **ecSecA** makes use of its VAR sub-structure in dimer formation. The VAR region is essentially missing in **bsSecA** (Figure 2) and the **mtSecA** DEAD motor assumes a “closed” conformation (with the VAR region coming close to NBD), prohibiting the monomers from dimerising in a fashion similar to the **ecSecA** structure. **bsSecA**(**1M6N**) crystallized as a monomer in the asymmetric unit and was proposed to form a dimer with a symmetry related molecule (Figure 10(a)). This proposed dimerisation interface is mediated through associations between the extreme N-terminal NBD residues (G3, I4 and L5 of **bsSecA**) and the C-domain IRA1 hairpin of the symmetry-related chain, and buries 5432 Å² or 14.7% of the monomer surface. The resulting crystal-contact dimer was proposed to also exist in solution and to be physiological. 

The **mtSecA** also exists as a dimer in the asymmetric unit with a buried surface area of 2739 Å². The dimer interface involves PBD, IRA2 and C-domain (Figure 10(b)).

**Several PrlD mutations line the ecSecA dimer interface**

Protein localization (PrlD) mutant SecAs can secrete preproteins with defective or deleted signal peptides. Most of the PrlD mutations lie within the dimerization interface of the **ecSecA** structure. **prlD21,22,23** involve mutations of residue Y134 that participates in both polar and apolar interactions at the dimerisation interface of **ecSecA** (Figure S3B and Table S1), or NBD-IRA2 contacts in the other SecA structures (Figure 9(b-c)). **prlD43** affects H484 and **prlD2,3** mutations alter A488. These residues participate in apolar interactions between the protomers in **ecSecA** (Figure S3B and Table S1) or are involved in NBD-IRA2 contacts in the other SecA structures (Figure 9(c)). Interestingly, the helicase Motif
V (Figure S1A) that is essential for catalysis lies directly behind H484 and A488 near the dimerization interface of \textit{ec}SecA (Figure 9(c)) and A507 of Motif V is a hotspot for \textit{prlD} mutations.

**Discussion**

We present the high resolution structure of SecA, the dimeric preprotein translocase motor, from \textit{E. coli}. The overall protein fold and domain assembly of the monomer is basically conserved among the previously determined structures of SecA proteins,\textsuperscript{5,6,8} as expected from their high sequence identity. The fundamental organization of the protein involves a core helicase DEAD motor, from which protrude the PBD and C-domain or “specificity domains”. A single mononucleotide cleft is formed between the DEAD motor domains. A comparison of the SecA structures reveals that the DEAD motor and the two “specificity domains” participate in a repertoire of significant rigid body motions (Figures 8 and 9) that are discussed below.

The DEAD motor of \textit{ec}SecA assumes a more “open” conformation (Figure 9(b-c)) when compared to the other SecA structures. When detached from NBD, IRA2 can undergo a remarkable unfolding transition,\textsuperscript{42} allowing the release of ADP from the cleft.\textsuperscript{43} IRA2 restructuring, involving Motifs V and VI, is believed to be necessary for ATP hydrolysis and presumably allows optimal positioning of IRA2 residues essential for catalysis. These include R509 of the helicase Motif V,\textsuperscript{28} and R574/R577 of Motif VI. IRA2-like domains in SF2 helicases are very mobile and have even been crystallized in complete dissociation from the corresponding NBD-like domains.\textsuperscript{26,27} An important contributor to enhanced flexibility of IRA2 domains is the reduced number of $\alpha$-helices that enfold the inherently unstable parallel $\beta$-sheet in their core. However, while complete detachment of IRA2-like domains may be an essential adaptation for the unwinding of nucleic acids, the IRA2 mobility is clearly under tighter control in SecA. Thus SecA is unique among SF2 proteins in having acquired the C-domain that crosses...
the DEAD motor longitudinally from below and latches onto both IRA2 and NBD through the SD helix (Figure 1(a)). This effectively allows SD to act as a molecular staple that controls the IRA2 detachment from NBD via the Joint region. In other helicases, the role of a weaker and transient molecular staple may be played by the nucleic acid, clearly seen in Vasa, to bind to both DEAD motor domains (Figure 3). Additional factors may act directly on IRA2 to regulate its structure and mobility, as is the case with eIF4G acting on eIF4A. Obviously, tightly-regulated cycles of IRA2 detachment and rebinding to NBD are a common and fundamental feature of SF2 catalysis.

IRA2 motions and nucleotide occupancy of the DEAD motor are transmitted to PBD, causing both conformational and dynamical residue changes. The position of the PBD Stem region is similar in all four SecA structures, in contrast with the Bulb moiety that occupies distinctly altered states (Figure 9(a)), of presumably similar energy values. The “open” PBD state in *bsSecA(1TF5)* is postulated to be coincident with the active monomeric state of SecA. However, our observation of an identical conformational state in PBD of dimeric *ecSecA* excludes the possibility that this PBD conformation is related to monomerization. PBD conformation is probably dependent on crystallization conditions because it can adopt either the “open” or “closed” conformation in the crystal lattice without the presence of any translocation ligands. Nevertheless, we anticipate that the “open” PBD state is physiologically relevant, since SecA derivatives with mutations of highly conserved IRA1 residues in the binding region to “open” PBD (Figure 4) are non-functional. It is well known that PBD is prone to conformational alterations that are regulated by the preprotein and nucleotide. These PBD motions, i.e. swivelling around its stem that leads to ‘opening’ and ‘closing’ of the PBD-WD interface, could provide a cog-wheel mechanism that pushes extended preprotein segments into the membrane and the two adjacent PBDs in the dimer would allow a hand over hand mechanism for such a preprotein chain relay. The alternative possibility is that a PBD movement
allows for the optimal docking of SecA to SecY where the opening between WD and PBD is wide enough (~16 Å) to accommodate SecY preprotein secondary structure elements in the “open” state, thereby facilitating SecA docking.

\textit{ec}SecA forms a dimer exclusively \textit{via} DEAD motor residues and is distinct from the dimers of \textit{bs}SecA and \textit{mt}SecA (Figure 10).\textsuperscript{5,8} We anticipate that the \textit{ec}SecA dimer is physiologically relevant since it is in agreement with extensive hydrodynamic analyses that reveal the propensity of the isolated SecAΔC to dimerize and tetramerize.\textsuperscript{45} More importantly, the mode of \textit{ec}SecA dimerization is consistent with the front-to-front SecYEG dimer.\textsuperscript{46} The dimensions of this SecYEG dimer are in good agreement with the dimensions of \textit{ec}SecA DEAD motor, the region of \textit{ec}SecA that binds to SecYEG.\textsuperscript{35,47} Only the SecA dimer presented here places both DEAD motor domains on the same side of the structure for SecYEG binding. Another exciting possibility is offered by the \textit{ec}SecA dimer and the differences observed between nucleotide interactions in the two protomers. This structural arrangement actually allows a direct nucleotide-regulated allosteric communication between the two protomers. Perhaps this could explain the existence of a second, kinetically determined but structurally elusive, low-affinity nucleotide binding site in SecA.\textsuperscript{37,43}

Extreme N-terminal residues were proposed to be important for \textit{ec}SecA function and dimerization.\textsuperscript{19,20,48} However, these residues are clearly unrelated to the determined \textit{ec}SecA dimerization interface and can be removed in \textit{ec}SecA[9-861] without affecting dimerization or function \textit{in vitro} and \textit{in vivo}.\textsuperscript{29} Similar N-terminal truncations also result in functional \textit{ec}SecA proteins.\textsuperscript{20,49} Several SecA sequences are naturally devoid of these residues.

The mode of SecA oligomerization still remains a conundrum. All the available SecA structures have a different quaternary organization. Clearly, SecA dimerization is highly dynamic\textsuperscript{16} and SecA may acquire different, monomeric,\textsuperscript{20,21,24} dimeric\textsuperscript{17,19} and perhaps, even
higher order states when associated with its cognate ligands. Whether all or even some of these are physiologically relevant remains to be determined.

It has not escaped our attention that the ecSecA structure allows a significant body of genetic and biochemical evidence to now be rationalized. Several of the isolated SecA PrlD mutants, whose molecular basis remains elusive, map to the dimerization interface of ecSecA. Some residues altered by PrlD mutations (e.g. Y134 from NBD and, H484 and A488 from \( \alpha_{18} \) of IRA2) are closely associated when the DEAD motor is in the “closed” state (e.g. bsSecA and mtSecA; Figure 9(c)). These residues dissociate and form new critical contacts in the ecSecA dimer (Figures 6(b), S3 and S4). Obviously, PrlD mutations could either pry open the dimer interface by introducing slightly bulkier side chains (e.g. A488V) or weaken it by rendering large residues shorter, e.g. Y134C/S/N. PrlD mutations occurring at the dimer interface promote SecA monomerization, which would then allow the preprotein to be exported without a signal peptide. Preproteins alter the dimer-monomer equilibrium\(^21,22\) and PrlD mutations could mimic preprotein-driven “loosening” of the physiological SecA dimer during translocation, thus rendering functional signal peptides redundant. We consider two possibilities: a. preproteins could act as allosteric modulators of the dimerization interface\(^30\) through their determined binding to the adjacent Stem region of PBD.\(^31\) b. PrlD residues that affect dimerization may reveal a previously unsuspected pathway along the DEAD motor, where preproteins displace the interfacial helix, \( \alpha_{18} \), thus possibly leading to monomerization. Regions in the Vasa helicase\(^33\) that correspond to the ecSecA dimerization interface (e.g. helix \( \alpha_{6} \) and the \( \beta_{3}/\alpha_{7} \) connecting loop of NBD and helix \( \alpha_{18} \) of IRA2) form an extended ridge that mediates RNA substrate binding (Figure 3). The binding surface is lined with fifteen RNA binding residues, only three of which are conserved in SecA. If preproteins and RNA bind to similar surfaces this lack of conservation could be an adaptation that underpins substrate specificity.
Further understanding of how SecA catalyzes preprotein translocation and how it adapted nucleic-acid helicase chemistry will require the determination of co-crystallized complexes with preproteins and translocase subunits as well as detailed \textit{in vitro} dissection of the reaction using mutagenesis and biochemical assays. The structure presented here allows us to formulate testable hypotheses to achieve this dissection.

\textbf{Materials and Methods}

\textbf{Bacterial strains and protein purification}

Strains and DNA manipulation were as previously described\textsuperscript{29,34,37}. Transformed \textit{E. coli} BL21/pLysS cells with plasmid pET3a-pIMBB272 (N9-861)\textsuperscript{29} were grown in 2 l LB growth medium at 37 °C. The culture was induced for expression of \textit{ecSecA}[9-861] at 30 °C with 0.3 mM IPTG when the absorbance OD\textsubscript{600}= 0.8. It was centrifuged after 5 h of induction yielding 3-8 g of cell paste. All following steps were performed at 23 °C unless it is mentioned otherwise. 3 g of cell paste were thawed in 20 ml of 25 mM Tris-HCl pH 7.6, 10% glycerol, 50 mM NaCl (buffer A). 1 mM of Phenyl-methyl-sulfonyl fluoride (PMSF) was added. The cells were disrupted by passing twice through a French Press cell. In the resulting lysate 0.5 mM PMSF was added and it was centrifuged for 1 h at 18000 g at 4 °C. The supernatant was loaded onto an in-house prepared Cibacron-Blue Sepharose\textsuperscript{TM} CL-6B (Pharmacia), equilibrated with buffer A. A linear gradient was applied from buffer A to 25 mM Tris-HCl, pH 7.6, 10% glycerol, 2.6 M KCl. \textit{ecSecA} eluted at \textasciitilde{}0.6 M KCl. The protein containing fractions were diluted 1:1 with water and loaded onto Hydroxyapatite\textsuperscript{TM} (BioRad) equilibrated with 1 mM Tris-HCl pH 7.6, 100 mM KCl (buffer B). A linear gradient was applied from buffer B to 0.1 M potassium phosphate buffer pH 6.8. \textit{ecSecA} eluted at 20 mM potassium phosphate. The protein was dialysed against 50 mM potassium phosphate buffer pH 7.5, 10% glycerol, concentrated with an Ultrafree-15 centrifugal filter device (Millipore) and loaded onto Sephacryl\textsuperscript{TM} S-300
(Pharmacia). Protein containing fractions were passed once more through the latter column with 25 mM Tris-HCl pH 7.6, 100 mM KCl. Protein purity was assessed by denaturing gel electrophoresis and protein amounts were determined by the Bradford photometric method. ~3 g of cell paste yields 20-38 mg of chemically homogeneous protein suitable for crystallization. The same purification protocol was applied for both native and selenomethionylated ecSecA.

**Crystallization, data collection and structure determination**

Crystals of both native and selenomethionylated ecSecA were obtained at 18 °C employing the hanging drop technique of vapour diffusion. Each hanging droplet contained 6 µl aliquot of 25 mg/ml protein in water and 3 µl reservoir solution (50 mM sodium citrate pH 5.8, 6-9 % polyethylene glycol 35000, 6-10 % glycerol and 50 mM ammonium sulphate) and was equilibrated against 1 ml of reservoir solution. The diffraction of the crystals was improved from 3.5 to 2 Å resolution limit by the method of dehydration with 2 M KCl solutions, which reduced the crystal solvent content by ~9 % (v/v). The resulting monoclinic crystals (space group \(P2_1\)) used for the structure determination, contained an estimated 56 % (v/v) solvent corresponding to one molecular dimer per asymmetric unit. The molecular packing of these crystals is shown in Figure S7A-B of the Supplementary Material. The buried surface area between the closest dimeric neighbours varied from 2.1 to 5.6 % of their solvent accessible surface. High-resolution native, MAD and SAD data were collected from single cryo-cooled crystals at the European Synchrotron Radiation Facility (ESRF) beamline ID14-4 using a Quantum 4R (ADSC) detector. The Multiwavelength Anomalous Dispersion (MAD) phasing method was used with data processed to 2.4 Å resolution from a selenomethionylated ecSecA crystal. Three different wavelengths were chosen such that the dispersive and Bijvoet differences due to Se atoms were maximized (Table 1). A combination of computational methods, including \(SHELXD^{53}\), \(SOLVE^{54}\) and \(SHARP^{55}\) were used to locate, refine 58 of the 64
Se sites and phase calculations. An initial protein model was constructed using ARP/wARP\textsuperscript{56} and refined against the MAD (0.97939 Å wavelength) data using REFMAC5\textsuperscript{57} as implemented in CCP4.\textsuperscript{58} These coordinates were then refined against native diffraction data extending to 2.0 Å resolution and manually modelled using 2mF\textsubscript{obs}-DF\textsubscript{calc} and mF\textsubscript{obs} - DF\textsubscript{calc} electron density maps. The final models converged after several cycles of model building using XtalView\textsuperscript{59} with REFMAC5. The structures of the nucleotide complexes were refined with the respective data starting from the native coordinates. The results of all refinements are summarized in Table 2.

Only residues 280-313 of protomer β could be fitted into the calculated electron density maps of ecSecA. This area of the Bulb is bounded by helices α11 and α12. Modelling of the unresolved parts of ecSecA PBD was based on bsSecA(1TF5)-PBD\textsuperscript{6}, which assumes approximately the same orientation. Residues 264-271 and 276-293 of bsSecA were superposed onto the crystallographically determined residues of ecSecA (280-287 and 296-313). The resulting PBD structure was anchored to the respective ends of the Stems (residues 228 and 365) and the whole structure refined against the experimental native data. Molecular areas were calculated using the program CNS\textsuperscript{60} with probe radius 1.4 Å. The Figures were produced with PyMol (http://pymol.sourceforge.net/).

The coordinates of the reported structures have been deposited in the Protein Data Bank with accession codes: 2FSF, ecSecA apoprotein; 2FSG, ecSecA:ATP; 2FSH, ecSecA:AMP-PNP; 2FSI, ecSecA:ADP.

Acknowledgements

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References


Figure Legends

**Figure 1** Crystal structure of ecSecA monomer. (a) Ribbon diagram showing chain α. Color coding of domains: NBD; dark blue, IRA2; light blue, IRA2-VAR; red, IRA2-Joint; grey, PBD; magenta, and sub-domains of the C-domain: SD; green, WD; lime, IRA1; yellow, CTD; orange. (b) Protomer α surface diagram showing the nomenclature and color coding of the main domains. NBD; dark blue, IRA2; light blue, IRA2-VAR; red, IRA2-Joint; grey, PBD; magenta, C-domain; green.

**Figure 2** Map of the VAR region’s α-helices of selected SecAs. Sequence alignments are based on structural comparisons. *Synechocystis* sp. SecA (SECA_SYNY3: Q55709) (labelled as *Syn* sp) has one of the longest VAR regions (145 residues). Its IRA2-VAR structure was predicted by PROF. The α-helices are shown as cylinders. The ecSecA helices are numbered. The crystallographically determined SecAs are labeled according to Figure S1A of Supplementary Material. Conserved residues are highlighted in yellow.

**Figure 3** The *Drosophila* development protein Vasa on the DEAD motor of bsSecA. Top view of the superimposed DEAD-box helicase Vasa (PDB accession code; 2DB3, beige, residues 253-616) and *B. subtilis* SecA DEAD motor (PDB accession code; 1M6N, NBD; dark blue, residues 59-218 and 357-395, IRA2; light blue, residues 396-567). *B* sSecA was chosen because, like Vasa, its DEAD motor assumes a “closed” conformation. Beside the nine conserved SF2 motifs, two regions of conservation in some DEAD-box helicases are: GG and QxxR corresponding to ecSecA residues, 159–PG–160 and 484–HANE–487, respectively (colored on both structures). The respective helices near or at these motifs of ecSecA/Vasa structures as well as helix α6 of motif Ia are drawn in ribbon presentation. The N- and C-
terminal helices of SecA are indicated (ecSecA numbering). The red surface shows the RNA heptamer 5′-p-(Up)6-U bound to Vasa.

**Figure 4** IRA1, SD and helix α12 of PBD of ecSecA. The N-terminal residues of PBD Bulb helix α12 bind to the concave surface underneath the tip of the IRA1 hairpin. Five IRA1 residues that are important for SecA catalysis\(^{35}\) and line the α12 binding site are labelled. In order to make I789 visible, only the surface of the backbone atoms of R792 is shown.

**Figure 5** Crystal structure of ecSecA dimer. Surface of protomer α is colored as in Figure 1(b). Protomer β is shown uniformly colored beige. Front (a) and top view (b) of the dimer. The direction of the near 2-fold axis relating the protomers is clearly marked.

**Figure 6** Map of interfacial residues. (a) Close up view of the dimerization interface. For simplicity, the ribbon diagram shows only the DEAD motors of protomers α and β. Coloring is as in Figure 5. The approximate direction of the near 2-fold axis is clearly marked. (b) Dimerization interface as in panel (a) with the IRA2-VAR sub-domain removed. Residues involved in contacts are shown labelled in the α-protomer. The ordered solvent molecules participating in inter-subunit interactions are shown as violet dots with green dashed lines indicating the polar interactions.

**Figure 7** ecSecA adenine nucleotide complexes. (a) Ribbon diagram of the DEAD motor of ecSecA:ATP complex. Identification of the Helicase motifs in and around the nucleotide binding cleft: Q-motif; light blue, Motif I (Walker Box A); violet, Motif Ia; magenta, Motif Ib; grey, Motif II (Walker Box B); red, Motif III; orange, Motif IV; yellow-green, Motif V; light green, Motif VI; dark green. NBD; dark-blue, IRA2; light-blue. The bound ATP molecule is
shown in stick representation. (b)-(d) Atomic interactions in the nucleotide cleft of the α-protomer. Close up views of the pocket with the three nucleotides ecSecA:ATP (b) ecSecA:ADP (c) and ecSecA:AMP-PNP (d). Nucleotide binding residues of NBD are colored red, and Arg509 of IRA2 is colored yellow. Solvent molecules interacting with the nucleotides are shown as violet dots and H-bonds are indicated with dashed lines. The electron density around the nucleotides and the ordered solvent is colored dark blue and contoured at 1σ level.

**Figure 8** Comparison of one ecSecA protomer with other SecA structures. Ribbon diagrams showing superimpositions based on the structural alignments of the NBD domains of ecSecA with the other known SecA structures. ecSecA is colored as in Figure 1(b). The VAR subdomains of the other SecAs are colored yellow. (a) bsSecA(1M6N) is colored beige. (b) bsSecA(1TF5) is colored pink. (c) mtSecA(1NL3) is colored grey. mtSecA corresponds to SECA1_MYCTU, one of the two SecAs in this bacterium.

**Figure 9** Variations of subdomain orientations in ecSecA and bsSecA. The superimposition is based on the structural alignments of the NBD domains with ecSecA colored as in Figure 1(a) and bsSecA(1M6N) in beige. (a) The orientation of the PBD (space filling) in ecSecA and bsSecA. (b) The "open" arrangement of the ecSecA DEAD motor, as revealed by superimposition with the B. subtilis DEAD motor. The conserved residue H484 of helix α18 lies over 12 Å away (green dashed line) from the corresponding H464 of bsSecA. The conserved Tyr134/Tyr132 are shown on the NBD side. The VAR subdomains have been removed to allow a clear view into the nucleotide cleft. (c) Relative locations of prlD mutation sites in the SecA structures. The critical R509 of Motif V lies behind H484 and A488. Only residues of ecSecA are labelled.
Figure 10 Comparison of ecSecA with the other SecA dimers. The superimposed ecSecA dimer (α-chain; ribbon presentation, colored as in Figure 5, β-chain; surface presentation, beige) with the other SecA dimers is based on the structural alignments of the α-chain NBD domains. Only protomers β of the other SecAs are shown for clarity, colored pink and their IRA2-VAR region yellow. (a) bsSecA(1M6N) symmetry related (1-y, 1-x, 2/3-z) molecule β proposed to be the second protomer of the physiological dimer. (b) mtSecA(1NL3) protomer β. The mtVAR, lying on the opposite side of mtSecA(β) is invisible.
Table 1

Data collection and processing statistics for the synchrotron data sets

Values in parentheses refer to the highest resolution shell.

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\[ aR_{\text{anom}} = \Sigma |I^+| - |I^-|/\Sigma (|I^+| + |I^-|), \text{ where } I^+ \text{ and } I^- \text{ are the integrated intensities of Bijvoet pairs of reflections.} \]

\[ bR_{\text{merge}} = \Sigma |I| - |<I>|/\Sigma I, \text{ where } I \text{ is the integrated intensity of a given reflection. } I^+ \text{ and } I^- \text{ are scaled separately.} \]

**Table 2**

Refinement and Stereochemical Statistics

Values in parentheses refer to the highest resolution shell.

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$^a$As defined by PROCHECK$^{62}$
Ecoli 516-GGSWQAEVALEN------------------PTAEQIEKIKADWQRHDAVLIEAGG-553
Bacsu 496-GEG--------------------------------------VKEELGG-504
Myctu 497-GGNVDFLTDQLRERGLDPVETPEYEAAWHSELFIVKEEASKEAKEVIEAGG-549
Synsp 502-GGNSDYMARLKREY-98aa-VILKIREVNYQIRREYEVLTSAEHKEVVELGG-646

Consensus G---------------------------V-E-GG
(a) ecSecA-bsSecA(1m6n)

(b) ecSecA-bsSecA(1tf5)

(c) ecSecA-mtSecA