

# The Multifunctional Preprotein Binding Domain of SecA

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Sec-pathway is the main protein secretion pathway in prokaryotes and is essential for their survival. The motor protein SecA is the main coordinator of the pathway in bacteria as it has evolved to perform multiple tasks, acting like a "swiss army knife", from binding pre-proteins to altering its oligomeric and conformational states. This study focuses on the role of its Preprotein Binding Domain (PBD), which is a key protein module that identified in three conformational states (Wide-Open (WO), Open (O) and Closed (C)). A thorough analysis was conducted to identify PBD's inter- and intra-protomeric interactions, highlighting the most significant and conserved ones.

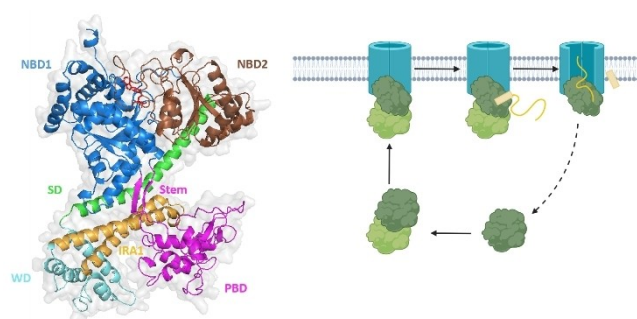
Both crystallographic and biophysical data indicate that the WO state is the main during dimerization, while the monomeric structure can adopt all three states. C-tail, Stem<sub>PBD</sub> and 3β-tip<sub>PBD</sub> are important elements for the stabilization of different oligomeric and conformational states, as they offer specific interactions. Alterations in the lipophilicity of the Stem<sub>PBD</sub> causes increased proteins dynamics or/and Prl phenotype. In the C state, 3β-tip<sub>PBD</sub> interacts and opens the ATPase motor. We hypothesize that this partial opening of the motor with the increased dynamics describes the Prl phenotype.

## Introduction

More than a third of cellular proteins are exported from the cytoplasm, where they are synthesized, into and across cellular membranes.<sup>[1,2]</sup> Migrating proteins include insulin, antibodies, channels and toxins. Protein secretion is an essential cellular process. Notably, over 2/3 of drugs target membrane proteins.<sup>[3]</sup> How do exported proteins cross cell membranes in such a sophisticated and efficient manner?

To understand this, a strong focus has been placed on the ubiquitous and essential Sec pathway.<sup>[1,2]</sup> It takes proteins across the eukaryotic endoplasmatic reticulum (ER) or the prokaryotic plasma membrane. *Escherichia coli* secretion system (Sec) has been widely used as a model because: (i) functional reconstitution *in vitro* using purified components, (ii) dissection in stages, (iii) combined *in vivo* and *in vitro* approaches of enzymology, structural biology and biophysics.<sup>[1,2]</sup> Sec-routed preproteins commonly carry N-terminal, ~20–30 residue-long signal peptides,<sup>[4]</sup> that are cleaved off after translocation.<sup>[5]</sup> Various cellular factors are thought to facilitate preprotein targeting, i.e. delivery to membrane. Post-translational translocation is used by the ~500 secretory preproteins that fully cross the inner membrane of *E.coli* through SecYEG and are released at the *trans* periplasmic side.

SecA, a dimeric 4-domain ATPase (Figure 1A), is the primary translocase receptor recognizing targeting signals whether it acts as an exported protein receptor in the cytoplasm (with low



**Figure 1.** A) Structure of SecA. NBD1: Nucleotide Binding Domain (blue), NBD2 (or IRA2): Nucleotide Binding Domain 2 (brown), PBD: Preprotein Binding Domain (magenta), IRA1: Intramolecular regulator of the ATPase (orange). WD: Wing domain (cyan), SD: Scaffold domain (green). B) Model depicting stages in the SecA (green) mediated preprotein (yellow) translocation. SecA dimers associate with SecYEG dimers (cyan) and initiate preprotein docking and translocation. During the course of the translocation, SecA monomerizes and the mature domain is translocated.

$\mu\text{M}$   $K_d$ s) or bound to the membrane embedded protein-conducting channel (with high nM  $K_d$ s).<sup>[6]</sup> In a second step at the membrane SecA undergoes quaternary metaphorphoses and converts from a preprotein-binding dimeric receptor to a monomeric translocation motor (Figure 1B).<sup>[6]</sup> This dimer to monomer transition lies at the core of translocase,<sup>[7]</sup> and recent studies have suggested that it relies on the dynamics of the Preprotein Binding Domain (PBD).<sup>[8,9]</sup>

This review summarizes the recent findings and analyzes the contribution of SecA's PBD to protein's dynamics and functionality. Observing the available multiple SecA structures (X-ray crystallography and Cryo-EM, Table 1), we have reported the most significant inter-/intra- protomeric interactions of PBD. Our analysis demonstrates that the PBD can acquire three specific conformers, each important for different steps of the translocation mechanism, including dimerization, binding to the SecYEG channel, and activation of the ATPase motor (described as Prl phenotype). Trying to characterize this gain of

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PBD state	WO	O	C
Dimers	1M6N	–	–
	1M74		
	1NKT		
	1NL3		
	2IBM		
	3IQM		
	3IQY		
Monomers	2IPC	1TF2	3DIN
	4UAQ	1TF5	3DL8
	4YSO	2VDA	5EUL
	6GOX	3JV2	6ITC
	3JUX	6SOK	7XHA
			7XHB

function phenotype, we have recognized key elements, such as the C-tail, Stem<sub>PBD</sub>, and 3β-tip<sub>PBD</sub>, which are crucial for stabilizing various states, providing specific interactions.

## The Swiss Army Knife of Proteins

In the translocation mechanism, SecA plays a vital role, as it is capable to perform various tasks. SecA is able to a) change its oligomeric state, b) bind tightly to the SecYEG channel, c) bind different substrates and protein clients and d) acquire different conformational states and intrinsic dynamics to perform the coordination of the translocation work. Only a very advanced evolutionary protein would be capable of doing all the above.<sup>[10,11]</sup> There are examples of sophisticated protein super-families, such as G protein-coupled receptors (GPCRs) involved in biased signaling,<sup>[12]</sup> and P450 oxidoreductases involved in biased metabolism.<sup>[13]</sup> Being evolved from the helicases,<sup>[14,15]</sup> SecA kept some important structural domains as core, such as the ATPase motor, but most importantly, adopt new modules,

such as the PBD domain, that allow the protein to acquire the missing abilities for client specificity and coordination in the translocation work.<sup>[16]</sup> In soluble SecA, its PBD acquires three distinct conformations by slowly rotating its bilobate globular domain (Bulb) in tens of msec, for up to ~70°, around an antiparallel β-stranded Stem that connects the Bulb to the ATPase motor (Nucleotide Binding Domain 1,2 (NBD1,2)).<sup>[8,16,17]</sup> These states resemble structures that have been resolved crystallographically<sup>[18,19]</sup> (Figure 2). PBD rotation may be an important mechanical event for translocation as it binds signal peptides<sup>[18]</sup> and SecYEG<sup>[19,20]</sup> and restricts its motion and hinders secretion,<sup>[21,22]</sup> preprotein recognition<sup>[23]</sup> and ATP hydrolysis<sup>[24]</sup>. Moreover, in one state the PBD closes in towards the NBD2 domain of the SecA ATPase motor<sup>[18]</sup> (Figure 2, Closed), forming a clamp that is expected to trap the exiting polypeptide chain.<sup>[25]</sup> Also, SecA interacts with specific nucleotides and preproteins in solution.<sup>[26,27]</sup> Preproteins bind as bivalent ligands with their signal peptides on the PBD and their mature domains on the Stem and other adjacent regions of SecA.<sup>[17,23]</sup>

## Observing the Structures of SecA

Summary information on the available crystal structures of SecA homologues used in this study is provided in order to identify and classify the different protein states. In our analysis, we have selected the oligomeric state of the protein, either monomeric or dimeric state, that is proposed by EPPIC to be physiologically relevant (<http://www.eppic-web.org/ewui/>). Comparing all structures, PBD is the main element that is captured in different states. It is identified in the wide open (WO), open (O) and closed state (C) (Figure 2). These different conformers must play an important role and contribute significantly in the multiple roles of SecA, including the above described dimerization, binding to SecYEG channel, binding of different substrates and translocation work.<sup>[8,19,24,28–31]</sup>



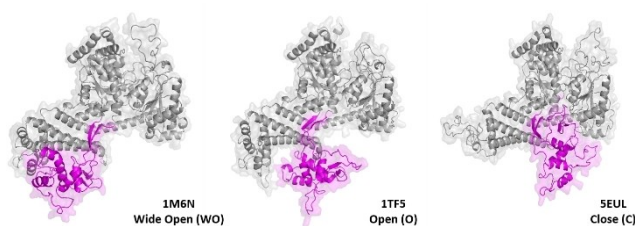
Emmanouil Giotas completed his BSc in Chemistry at Aristotle University of Thessaloniki in 2021, specializing in Organic Chemistry for his BSc thesis. He pursued his MSc in Biochemistry at the University of Crete under the supervision of Prof. Nikolaos Eleftheriadis, graduating in 2024. His MSc thesis focused on SecA as an antibiotic target, employing various biochemical and biophysical techniques.



Stavroula-Aikaterini Kaplani earned her BSc in Chemistry from the University of Crete, graduating in 2023. She is now pursuing an MSc in Biochemistry at the same institution under the supervision of Prof. Nikolaos Eleftheriadis. Her MSc research focuses on unraveling the protein dynamics of SecA by employing single-molecule FRET techniques.



Nikolaos Eleftheriadis obtained his BSc/MSc degrees (with honors) at the Aristotle University of Thessaloniki in 2012. Next, he moved to the University of Groningen (RUG) and enrolled for a doctoral degree (cum laude) in the Division of Chemical and Pharmaceutical Biology. Postdoctoral training in Molecular Microscopy (RUG) and Molecular Bacteriology (KU Leuven) followed. In 2021, he became an Assistant Professor in the Chemistry Department at the University of Crete. Prof. Eleftheriadis is a “modern” chemical biologist who utilizes innovative tools from biochemistry and biophysics. His research is published in top journals, and he has received numerous awards for scientific excellence.



**Figure 2.** Structures of SecA protomers (1M6N, 1TF5, 5EUL) in three conformational states according to PBD position (magenta).

## The PBD States of SecA

We have observed that all the physiologically relevant dimeric structures have the PBD domain located in the WO state, while in the monomeric structures the PBD is captured equally in all three states (Table 1). These observations are confirmed by smFRET studies (timescale of milliseconds), that reveal three isoenergetic states in the monomeric SecA and a huge enrichment of the WO state after dimerization.<sup>[31]</sup> Moreover, it is worth mentioning that the closed PBD conformation is observed only in the crystal structures where SecY is present (3DIN, 3DL8, 5EUL, 6ITC, 7XHA, 7XHB).

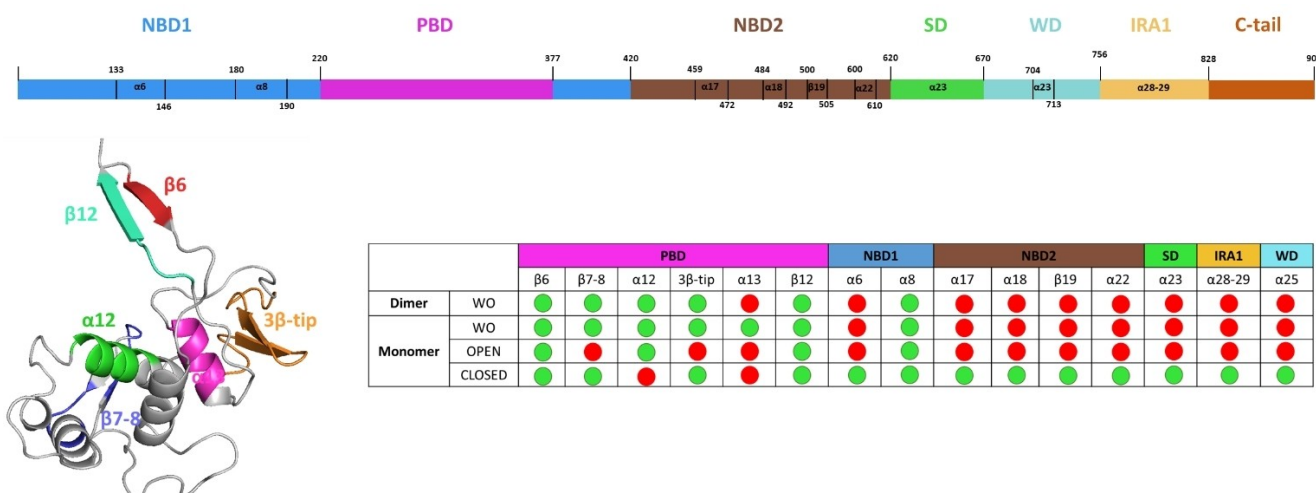
## Signature PBD Interactions

Next, we reported and summarized all of the interdomain PBD interactions identified in different crystal structures.<sup>[31]</sup> Initially, homology models of *E. coli* SecA were created based on the available crystal structures, using the Swiss-Model web tool (<https://swissmodel.expasy.org>). Then, for every structure, all the interactions were calculated using the PIC webserver (<http://pic.mbu.iisc.ernet.in>) and only the interdomain PBD interactions (with acceptable criteria)<sup>[32]</sup> were reported (Figure 3). In the dimeric state of the protein, inter-protomeric

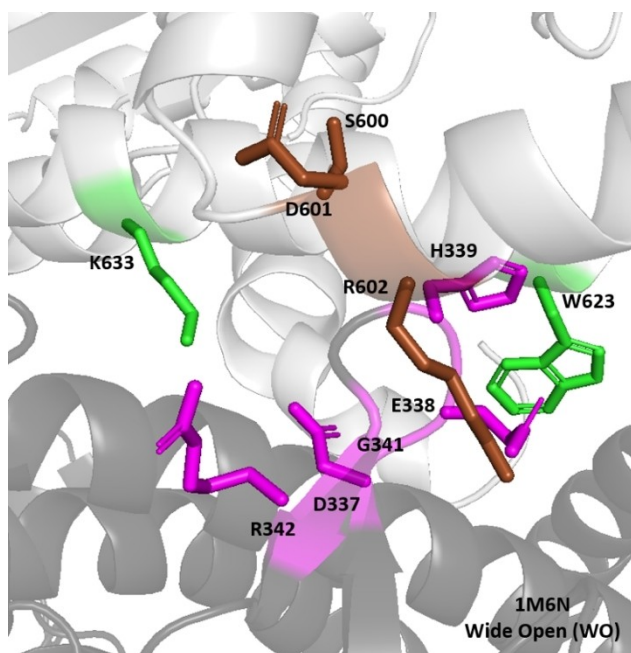
interactions are also reported. The elements of PBD that offer interactions are the  $\beta_{6\text{PBD}}$ ,  $\beta_{7\text{PBD}}$ ,  $\beta_{8\text{PBD}}$ ,  $\alpha_{12\text{PBD}}$ ,  $3\beta\text{-tip}_{\text{PBD}}$  and Stem( $\beta_{6,12}$ )<sub>PBD</sub> (Figure 3B), while the elements of other domains that interact with PBD are the 640–650 region of  $\alpha_{23\text{SD}}$ ,  $\alpha_{25\text{WD}}$ ,  $\alpha_{28-29\text{IRA1}}$ ,  $\alpha_{6\text{NBD1}}$ ,  $\alpha_{8\text{NBD1}}$ ,  $\alpha_{17\text{NBD2}}$ ,  $\alpha_{18\text{NBD2}}$ ,  $\beta_{19\text{NBD2}}$  and  $\alpha_{22\text{NBD2}}$  (Figure 3A,C). Specifically, the 337–343 part from the  $3\beta\text{-tip}$  is a main player in the PBD state while it forms hydrogen bonds and ionic interactions either with IRA1 in WO or with NBD2 in C state. The other part of the  $3\beta\text{-tip}_{\text{PBD}}$  (321–336) contributes only to the C state. Moreover, only in the WO state, 347–352 and  $\alpha_{13}$  (354,355) are involved with hydrogen bonds and ionic interactions with IRA1, possibly offering extra stabilization of that state.

## Dimerization and C-Tail Stabilizes the WO State

As mentioned before, in all dimeric SecA structures, PBD is observed in the WO state. This can be justified by the extra inter-protomeric interactions of PBD (Figure 4). From these identified interactions, the  $3\beta\text{-tip}_{\text{PBD}}$  is the only element of PBD that interacts with the other protomer. More specifically, in almost all dimers (except 1NL3) the  $3\beta\text{-tip}_{\text{PBD}}$  forms hydrogen bonds and ionic interactions with the other protomer at  $\alpha_{23\text{SD}}$  as well as at  $\alpha_{22\text{NBD2}}$  (Figure 4). However, these inter-protomeric interactions are not essential for PBD to be located in the WO state, since that state can also be found in the dimer 1NL3 as well as in some other monomeric structures (2IPC, 4UAQ, 4YSO, 6GOX). Though, it seems that these interactions offer extra stabilization for the PBD to be located in the WO state. Finally, from the observed intra-protomeric interaction of PBD, it seems that the C-tail probably contributes to the stabilization of the WO state, while the interactions between PBD and C-tail are only present in the WO state (resolved as an extra third  $\beta$ -sheet aligning with the Stem<sub>PBD</sub>, however, due to its high dynamics, the C-tails' structure is absent in most SecA structures).<sup>[31]</sup> The smFRET and HDX-MS data support this, as the SecA <sub>$\Delta$ Ctail</sub>



**Figure 3.** A) Linear map of the domain organization of SecA. B) The key elements of PBD, contributing with inter- and intra-protomeric interactions. C) The elements that participate with intra-protomeric interactions per different PBD state (green circle: yes, red circle: no participation).



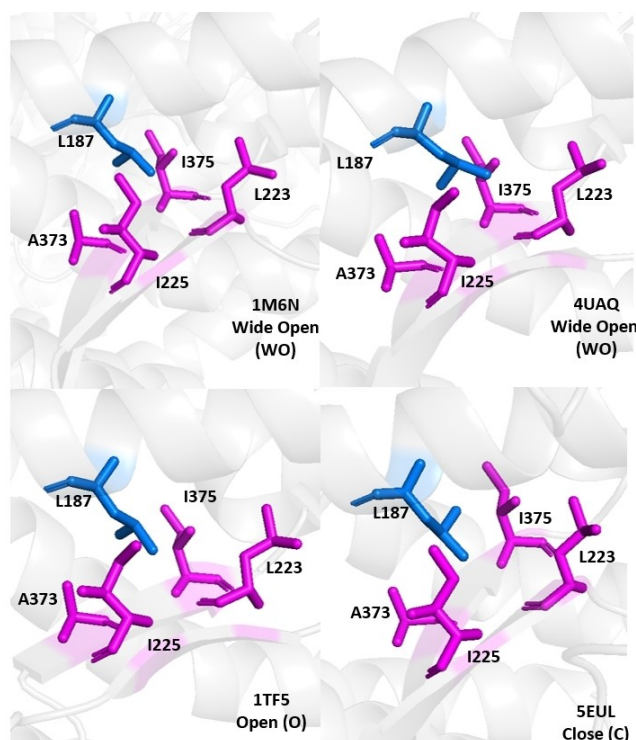
**Figure 4.** The residues of PBD (magenta) that contribute with hydrogen bonds and ionic interaction with the residues from the other protomer (SD: green, NDB2: brown) of the dimeric SecA (1M6N). The two protomers are shown with light and dark grey ribbons respectively.

derivative has demonstrated increased PBD dynamics and a partial loss of the WO state and consequently PBD closing.<sup>[31]</sup>

### The Lipophilic Role of Stem<sub>PBD</sub>

The Stem<sub>PBD</sub> and  $\alpha 8_{\text{NBD1}}$  share a conserved hydrophobic interface, formed primarily by residues L187 and M191 and A373. This hydrophobic interface might be important for the stem to regulate local and PBD dynamics of SecA. In particular, the O state forms the least number of the interactions with the majority of them being hydrophobics. That indicates a not very favorable thermodynamic state but a more favorable entropic driven state. Specifically, the structures suggest that the lipophilic interactions of the Stem<sub>PBD</sub> with NBD1 and IRA1 are crucial for the PBD motions (Figure 5). We hypothesize that because of these interactions, the domain can slide and take multiple states. It is a very sensitive balance since enhancement or reduction of these lipophilic interactions can affect dramatically PBD motions and therefore increase/decrease protein dynamics.<sup>[33,34]</sup> Eventually, this can cause allosteric responses, through the intrinsic dynamics networks across SecA, as the protein derivatives SecA<sub>L187A</sub> and SecA<sub>A373V</sub> displayed elevated ATPase compared to the wild type SecA.<sup>[24]</sup>

**Reducing the Lipophilicity:** The L187 interacts with L223, I225, A373 and I375, monitoring the Stem<sub>PBD</sub> in all PBD states (Figure 5). Protein mutations such as L187 A have reduced lipophilic interactions and that leads to destabilization of the PBD, which can reflect to increased protein dynamics (observed by HDX-MS data).<sup>[31]</sup> Moreover, smFRET experiments of the



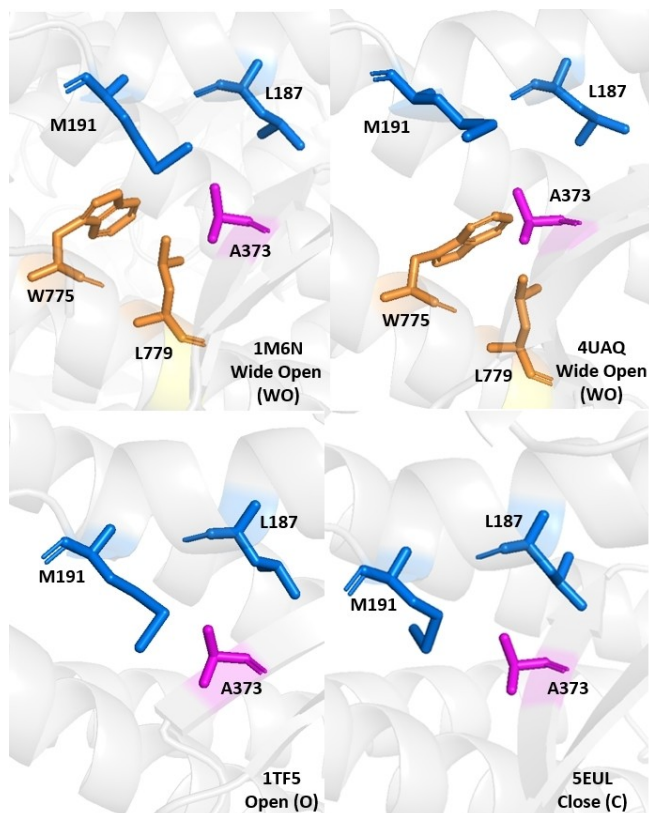
**Figure 5.** Residues that participate in interactions of StemPBD in WO, O and C PBD state in different structures of SecA (1M6N, 4UAQ, 1TF5, 5EUL).

SecA<sub>L187A</sub> reveal partial (not significant) loss of the WO state, compared to the WT in the monomeric/dimeric oligomeric state.<sup>[24,31]</sup> This could be explained by the fact that these lipophilic interactions are present in all the PBD states, so all the states are affected in the same way.

**Enhancing the Lipophilicity:** The A373 interacts with L187 and M191 in all PBD states while only in the WO state also interacts with W775 and L779 (Figure 6). When the hydrophobic packing at the Stem<sub>PBD</sub> was altered, by the substitution of the A373<sub>Stem</sub> with V, which is a larger hydrophobic residue, the derivative SecA<sub>A373V</sub> displayed a PrI phenotype (gain of function)<sup>[35]</sup> *in vitro* or *in vivo*.<sup>[24]</sup> Notably, the same was not observed for the SecA<sub>M191A</sub>, which is located at the end of the Stem<sub>PBD</sub>/ $\alpha 8_{\text{NBD1}}$  interface and was not found to be a PrI mutant.<sup>[24]</sup> This could mean that the mutant SecA<sub>A373V</sub> could provide more crucial lipophilic interactions and promote the stabilization. It seems that the balance of clamp dynamics it is very sensitive and even small differences in lipophilicity can be favorable towards specific PBD states.

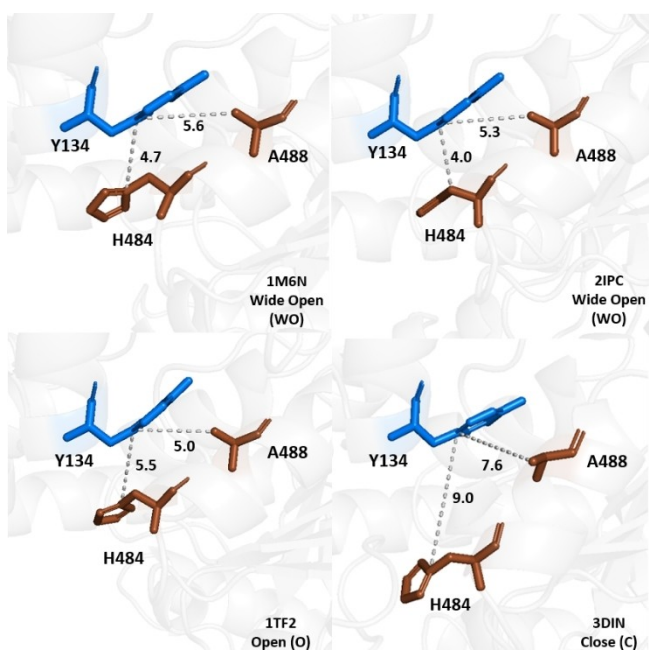
### The C State of PBD Opens the Motor

PBD interacts with the NBD2 from the motor only in C state (Figure 3), acting like a signature contact. In line with that, it was observed that enrichment of the C state equals enhanced motor dynamics.<sup>[24,31]</sup> Remarkably, from all the crystal structures, it is obvious that the motor is closed when PBD is located in WO and O position while it opens when PBD is in the C state



**Figure 6.** Other residues that participate in interactions of StemPBD in WO, O and C PBD state in different structures of SecA (1M6N, 4UAQ, 1TF5, 5EUL).

(Figure 7). The well-studied key elements of the motor, the residues Y134, H484 and A488, seems to participate mainly with lipophilic interactions.<sup>[36]</sup> Minor side chain changes in Y134 or



**Figure 7.** Key residues that participate in interactions of the motor in WO, O and C PBD state in different structures of SecA (1M6N, 2IPC, 1TF2, 3DIN).

H484 mimic the binary effect of channel plus signal peptide binding, in the absence of either.<sup>[24]</sup> In all structures, the geometry and the position of the Y134 are the same, with the benzene ring facing the other two residues. That indicates a pi interaction provided by the ring of Y134. The same interaction cannot be offered by the C, S and N residues, yielding SecA derivatives that can secrete preproteins with defective signal peptides.<sup>[6,37]</sup> Observing the structures, we cannot identify any participation in interactions from the hydroxyl group of Y134, concluding that, once again, mainly lipophilic interactions keep the motor closed. In the derivatives SecA<sub>Y134C/S/N</sub> these lipophilic interactions cannot be provided. The same is true for the residue H484, which its side chain also provides similar interactions. The SecA<sub>H484Q</sub> derivative most likely offers more interactions to the 3β-tip<sub>PBD</sub> (especially T340, G341 and R342), so it stabilizes the C state, something which is confirmed by smFRET studies.<sup>[24]</sup> Finally, in the WO and O structures, the methyl side chain group of A488 seems to point to the center of the benzene ring of Y134, satisfying the necessary geometry for the pi stacking. We assume that when this correct geometry is lost, like in the case of SecA<sub>A488V</sub> or SecA<sub>Y134C/S/N</sub>, the pi stacking is disrupted, yielding the PrI phenotype.<sup>[38]</sup> According to this analysis and the observations of biophysical methods, we can assume that PrI means partial opening of motor with increased dynamics.

## Summary and Outlook

In bacteria, the ATPase motor protein SecA plays a central role in Sec-pathway, which is the primary protein secretion pathway and is crucial for their survival. SecA performs a variety of functions, including binding to SecYEG channel and translocations of pre-proteins, changing at the same time its oligomeric and conformational states. A strategic module for that is SecA's Preprotein Binding Domain (PBD), which can be observed in three conformers: Wide-Open, Open, and Closed. Observing the available SecA structures, we reported the PBD inter- and intra-protomeric interactions, emphasizing the most significant and conserved ones. Both crystallographic and biophysical data indicate that the WO state predominates during dimerization, while the monomeric structure can adopt all three states. Trying to characterize the gain of function phenotype (PrI), we have highlighted key elements, such as the C-tail, Stem<sub>PBD</sub>, and 3β-tip<sub>PBD</sub>, which are crucial for stabilizing various states, providing specific interactions. Specifically, changes in the lipophilicity of the Stem<sub>PBD</sub> lead to increased protein dynamics. Also, we highlight that only in the C state, 3β-tip<sub>PBD</sub> interacts and opens the ATPase motor. We hypothesize that this partial motor opening, combined with increased dynamics, characterizes the PrI phenotype. In the future, the acquired knowledge could be employed in fundamentally explaining the bacterial secretion mechanism as well as in innovative drug discovery efforts for the development of novel antibiotics. Moreover, our approach to explaining the dynamics of the complex SecA system could be applied to other protein systems, offering a step-by-step methodology.

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## Conflict of Interests

The authors declare no conflict of interest.

**Keywords:** Protein secretion · Sec-pathway · SecA · Preprotein binding domain · Conformational states

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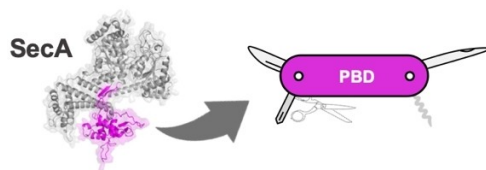
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## CONCEPT

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SecA protein has evolved to perform multiple tasks. Its Preprotein Binding Domain (PBD) is a key module, adopting three conformational states. The PBD's inter- and intra-protomeric interactions, along with biophysical

data, reveal the influence of these states on the translocation process. Key elements provide stabilization and promote the activation of the ATPase motor, described as the Prl phenotype.

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### **The Multifunctional Preprotein Binding Domain of SecA**