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Synthetic peptides identify a second periplasmic site for the plug of the SecYEG protein translocation complex

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ABSTRACT

A short helix in the centre of the SecY subunit serves as a 'plug' blocking the protein channel. This site must be vacated if the channel is to open and accommodate translocating protein. We have synthesised a peptide mimic of this plug, and show that it binds to E. coli SecYEG, identifying a distinct and peripheral binding site. We propose that during active translocation the plug moves to this second discrete site and chart its position. Deletion of the plug in SecY increases the stoichiometry of the peptide–SecYEG interaction by also exposing the location it occupies in the channel. Binding of the plug peptide to the channel is unaffected by SecA.

1. Introduction

Protein secretion in bacteria usually occurs by means of the SecYEG complex that forms a conduit through which polypeptides are threaded [1]. The driving force for the reaction is derived by coupling the reaction to ATP hydrolysis by the cytosolic ATPase SecA. The exact movements and motivation for channel opening and protein passage have yet to be described.

Our understanding of protein transport is in a large part due to the results of a number of structural studies of the channel (SecYEG) and motor (SecA) components. An X-ray structure of the channel revealed the protein pore to be formed between two pseudo-symmetrical halves of the SecY subunit [2,3]. SecE embraces the two domains, holds them together and the channel closed [2]. The pore itself viewed from the side has an hourglass configuration, with a ring of hydrophobic amino acid side-chains at its constriction point. The outwardly facing funnel of the channel accommodates a small helix, 2a, formed by a re-inserted periplasmic loop. This 'plug' nestles against the oily seal and together they ensure that the channel is closed. There must be a series of significant conformational changes during the transport process as the substrate is very large. Presumably, it happens by the separation of the two halves of SecY, disruption of the centre ring and a dislocation of helix 2a to vacate the periplasmic vestibule; these events are proposed to be initiated by binding of the signal peptide in the lateral gate between helices 2b and 7 [2].

The structure reveals that the channel is formed through the centre of the monomer of the complex [2]. However, in the membrane it forms a dimer [4]. A lower resolution crystal structure of this oligomeric form, solved by electron cryo-microscopy, reveals that both channels are closed, however, the plug helix has been displaced by 6.5 Å towards the periplasm [5]. Similarly, a new high-resolution crystal structure of the SecYEG–SecA complex also shows the plug shifted away from the waist of the hourglass. In this state the channel may be primed for translocation as the plug is closer to the position it might occupy when the channel is open.

Biochemical and genetic studies also have implications on the dynamics of the plug during translocation. Combining point mutations in the plug and at the C-terminus of SecE creates a synthetic lethality phenotype even though they are 20 Å apart in the (closed) structure [2,6]. This genetic interaction was subsequently confirmed biochemically; cross-links between helix 2a and SecE were enhanced in the dimer of SecYEG and during active translocation [6,7]. When the plug is locked in this open position, in the 'plug pocket', the channel is overactive and leaky [7,8] and this state is lethal in vivo [6]. Conversely, locking it in the closed position, in the 'plug hole', inactivates it [9]. Surprisingly, mutants that have had their plug removed retain their activity, due to the reformation of a functional mini-plug [8–11]. However, the 'plugless' mutants

Abbreviations: BPA, *p*-benzoylphenylalanine; DDM, dodecyl maltoside; PL, proteoliposomes.

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have been compromised with respect to leakage of small ions, implicating a role in channel sealing [8,9].

The nature and timing of the conformations adopted by SecYEG complex during protein translocation underpin a detailed understanding of the molecular events of translocation. The subject of this study concerns the structure of the open state of the channel, specifically with respect to the plug. The experiments employed a synthetic peptide mimic of the plug to probe the vacant pocket that houses the plug during protein translocation. The results detail this interaction in SecYEG and the complex of the channel with partner SecA.

2. Materials and methods

2.1. Materials

Detergents were purchased from Glycon. Chromatographic items were from GE Healthcare except where indicated otherwise. Sequencing grade modified trypsin was from Promega. All other biochemicals were from Sigma–Aldrich.

2.2. Peptide synthesis and labelling

Peptides were made by Dr G. Bloomberg (University of Bristol). Fluorescein was covalently attached to the peptides on the amino-terminal amine group. Concentrations were calculated according to fluorescein ($\varepsilon = 78\,000\ M^{-1}\ cm^{-1}$). The standard plug peptide was based on $SecY_{61-73}$ (Table 1). Successful formation of the cyclic peptide was confirmed by mass spectrometry, showing a reduction of the molecular weight by two atomic mass units after oxidation by stirring open to the air for 2 days.

2.3. Protein purification

SecA, SecYEG (in dodecyl maltoside, DDM[1]) and YidC were expressed and purified by established protocols [12–15].

2.4. Fluorescence spectroscopy

Fluorescence spectroscopy was performed on a Jobin Yvon Horiba Flourolog-3, using a 150 μl volume cuvette (Hellma). The fluorescence polarisation of fluorescein-labelled peptides was measured with excitation and emission wavelengths of 494 nm and 523 nm, respectively. Each measurement was the mean of three readings with an integration time of 10 s. Polarisation (P) was calculated according to Eq. (1):

$$P = \frac{\frac{VV}{VH} - G}{\frac{VV}{VH} + G} \tag{1}$$

where $G = \frac{HV}{HH}$; VV, VH, HV and HH are the fluorescence intensities measured with the excitation and emission polarisers, respectively, in vertical and vertical, vertical and horizontal, horizontal and vertical and horizontal and horizontal positions.

SecYEG was titrated into a solution of peptide in a buffer containing 20 mM Tris–HCl pH 8.0, 100 mM NaCl, 10% (v/v) glycerol, 0.02% DDM (SecYEG buffer) and the polarisation was plotted against the concentration of SecYEG.

Table 1
Sequences of the peptides used in this study. The cyclic peptide was cyclised by oxidative formation of an intramolecular disulphide bond.

Peptide	Sequence
Linear peptide	(fluorescein)-IEMFNMFSGGALS
Cyclic peptide	(fluorescein)-CIEMFNMFSGGALSC
Scrambled peptide	(fluorescein)-SMGAGFILENMSF
BPA peptide	(fluorescein)-IEM(BPA)NMFSGGALS

2.5. Data analysis

All data were analyzed using Grafit (Erithacus Software). Weak-binding curves (measured where the peptide concentration was lower than the K_d) were fitted according to Eq. (2):

$$P = \frac{B_{max} \cdot L}{K_d + L} + \text{offset} + m \cdot L \tag{2}$$

where B_{max} is the maximum polarisation change (binding capacity), L is the concentration of protein being titrated in (SecYEG or YidC), m is a linear gradient resulting from a non-saturable background and the offset is the initial polarisation value.

When the concentration of peptide was higher than the K_d, the binding curves were fitted to a tight-binding model according to Eq. (3):

$$P = B_{max} \cdot \frac{L + E_0 + K_d - \sqrt{[L + E_0 + K_d)^2 - 4E_0 \cdot L}}{2.E_0} + \text{offset} + m \cdot L \tag{3}$$

where E_0 is the total concentration of binding site equivalents for SecYEG.

2.6. In vitro protein translocation assay

Assays for the translocation of the preprotein proOmpA into protoliposomes containing SecYEG and *E. coli* polar lipid have been described previously [12].

2.7. Cross-linking and tryptic fragment analysis

A peptide containing *p*-benzoylphenylalanine (BPA) in the place of a phenylalanine (Table 1) was mixed with a 5-fold molar excess over SecYEG and irradiated with a Blak-Ray 365 nm lamp (UVP) for 30 min. Excess peptide was removed by gel filtration on a Superose-6 column equilibrated in SecYEG buffer and the cross-linked protein was digested with 1/100th the weight of trypsin at 37 °C for 15 h. The resultant fragments were separated on a C8 reverse phase column (Grace-Vydac) using a linear gradient of 5–95% acetonitrile and 0.1% trifluoroacetic acid. Fractions containing fluorescein absorbed light at 444 nm and were analysed by N-terminal sequencing.

2.8. Molecular model building

A model of the *E. coli* SecYEG complex bound to the linear plug peptide was made using the homology model of the *E. coli* SecYEG structure generated using the coordinates from the X-ray crystal structure of the *M. jannaschii* channel [5]. A copy of the plug helix from this structure was fitted into the plug pocket manually, accounting for the experimentally observed cross-link between F67C on the plug and S120C on SecE [6,7]. The whole complex was soaked in a 10 Å layer of water and energy minimised using Discover 2.98 (Accelrys) and the CVFF forcefield.

3. Results and discussion

3.1. Synthetic peptides of helix 2a ($SecY_{61-73}$) bind to the SecYEG complex with high affinity

Protein translocation requires that helix 2a of SecY (the plug) moves away from the centre of the channel to a second peripheral site, the plug pocket. In order to probe this site, a fluorescently