The Conformation of a Signal Peptide Bound by *Escherichia coli* Preprotein Translocase SecA*

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To understand the structural nature of signal sequence recognition by the preprotein translocase SecA, we have characterized the interactions of a signal peptide corresponding to a LamB signal sequence (modified to enhance aqueous solubility) with SecA by NMR methods. One-dimensional NMR studies showed that the signal peptide binds SecA with a moderately fast exchange rate ($K_d \sim 10^{-3}$ M). The line-broadening effects observed from one-dimensional and two-dimensional NMR spectra indicated that the binding mode does not equally immobilize all segments of this peptide. The positively charged arginine residues of the n-region and the hydrophobic residues of the h-region had less mobility than the polar residues of the c-region in the SecA-bound state, suggesting that this peptide has both electrostatic and hydrophobic interactions with the binding pocket of SecA. Transferred nuclear Overhauser experiments revealed that the h-region and part of the c-region of the signal peptide form an α-helical conformation upon binding to SecA. One side of the hydrophobic core of the helical h-region appeared to be more strongly bound in the binding pocket, whereas the extreme C terminus of the peptide was not intimately involved. These results argue that the positive charges at the n-region and the hydrophobic helical h-region are the selective features for recognition of signal sequences by SecA and that the signal peptide-binding site on SecA is not fully buried within its structure.

Every organism must specifically and efficiently translocate newly synthesized proteins to extracytoplasmic locations. The signal sequence, a 15–30-residue N-terminal extension on the mature protein, is generally required for the selective targeting of secretory and membrane proteins in both eukaryotes and prokaryotes (1). Although there is no conserved primary sequence homology between signal sequences apart from the cleavage site for signal peptidase (2), recognition still occurs with high fidelity. Although variable, signal sequences share similar properties: the net positively charged N-terminal n-region, the hydrophobic h-region, followed by a polar c-region (2). That these regions function in a concerted manner is suggested by the observation that mutations in one region can be restored by changes in another (3–6). The interactions between signal sequences and their receptors appear to be selective for these physical characteristics rather than the primary amino acid sequence.

SecA, the unique preprotein translocating ATPase in bacterial cells, is a dimer of 100 kDa subunits that recognizes both the signal sequence and parts of the mature region of the preprotein/SecB complex (7, 8) and then targets them to the membrane/SecYEG translocon complex (8, 9). Utilizing energy from ATP binding and hydrolysis, SecA dramatically changes its conformation to insert and retract from the SecYEG channel and translocate the preprotein stepwise across the membrane (10–13).

The efficiency of preprotein translocation is dependent on the properties of the signal sequence. Genetic and biochemical studies have shown that mutations or deletions in the signal sequence significantly reduce or eliminate its translocation activity (5, 14–17). Studies of *prl* mutations, which can suppress defective signal sequences to allow the translocation of mutant secretory proteins (18), suggest that SecA, SecY, SecE, and SecG have direct interaction with signal sequences. However, recent studies raise the possibility that *prl* mutations may be due to a variety of factors (19) as follows: tighter binding between SecA and SecY (20), a loosened association among the SecYEG subunits that might improve SecA membrane insertion (21), and a more relaxed SecA conformation that has higher affinity for the translocation channel than SecA, as well as a higher ATPase activity (22). SecA has been biochemically shown to interact directly with signal peptides by cross-linking (24–27), by signal peptide modulation of the ATPase activity of SecA (23, 24, 26, 28), and by signal peptide-induced changes in the state of oligomerization of SecA (29, 30). Therefore, signal sequence recognition by SecA is a key entry point to the protein targeting and translocation pathway in bacteria.

Despite considerable research directed at SecA function, the basis for its recognition of signal sequences is not clear. Previous studies of synthetic signal peptides suggested that the capacity to take up an α-helical conformation is important for their function (17, 31–37). Recent structural determinations (38–40) have revealed a unique multidomain architecture for soluble SecA. The N-terminal region resembles superfamily II DEAD helicases with two nucleotide-binding folds in intimate contact forming a regulated binding site for nucleotide at their interface. The first nucleotide-binding fold has an insertion (from residues 220 to 373 in *Escherichia coli* SecA), which has been proposed to bind the signal sequence based on cross-linking (25), direct binding to truncated constructs (27), and mutational analysis (41, 42). The helicase domain is followed by a long α-helical scaffold and then by a C-terminal domain that has been shown biochemically to interact with the membrane (43, 44). Considerable evidence suggests that SecA undergoes a large conformational change upon interaction with SecY and the surrounding membrane lipids (8). There are no direct data regarding the mode of recognition of the signal sequence by SecA, although deletional analysis and assessment of the ability of various fragments to bind signal sequences, as well as inspection of crystal structures, have led to various models (8, 38, 40). Understanding the mode of recognition of signal peptides by SecA and assessment of the likelihood of any of the proposed models would be greatly helped by observation of the SecA-bound conformation of a signal peptide. Transferred nuclear Over-

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The Conformation of a SecA-bound Signal Sequence

Hausser effect (trNOE) spectroscopy offers a promising approach to accomplishing this; we have successfully used trNOEs in the past to characterize chaperone-bound peptides (45, 46). We report the results of trNOE experiments on the water-soluble modified LamB signal peptide (KRR-LambB) (36) upon interaction with E. coli SecA. We find that the signal peptide binds SecA with a moderately fast exchange rate. The signal peptide forms an α-helical conformation upon binding to SecA. The positively charged arginine residues of the n-region and the hydrophobic residues of the h-region are more immobilized than the polar residues of the c-region in the bound state. These results suggest that electrostatic interaction at the n-region and hydrophobic interactions of the α-helical h-region are important for signal sequence recognition by SecA.

Materials and Methods

Reagents—Unless specifically mentioned, standard laboratory reagents were purchased from Sigma or VWR Scientific.

SecA Preparation—SecA was purified from overexpressing E. coli strains BL21.19 (pT7-SecA2) kindly provided by Don Oliver (Wesleyan University, Middletown, CT), and proteins were purified as described previously (23) with minor modifications. ATPase activity of purified SecA was verified to be comparable with literature values (23). Protein samples were concentrated using Centricon-30 or -50 (Millipore) to achieve millimolar concentration and then co-mixed with the peptides to the desired concentration for NMR. Protein concentrations were measured via the Bradford assay.

Signal Peptide Synthesis and Purification—The KRR-Lamb signal peptide (H2N-MMITLRRKLPLAVAAVGMSAQAMA-CO2H) and a deletion mutant that is defective for export, the Δ78 variant (H2N-MMITLRRKLPVAAGVMSAQAMA-CO2H) were synthesized and purified as described previously (36). Signal peptide concentrations were determined via the Bradford assay, which was calibrated by quantitative amino acid analysis.

NMR Studies—Samples for NMR spectrometry consisted of 0.8–1.0 mM peptide and 0–20 μM SecA in 5 mM KCl, 25 mM potassium phosphate buffer (pH 6.3–7.0), and 10% D2O with 0.02% azide. Spectra were obtained using a Bruker Avance 600 NMR spectrometer. 1H chemical shifts were referenced with respect to the methyl protons of 3-(trimethylsilyl)propionate (0 parts/million). Data were processed within the FELIX 2000 program (Acce loss Inc., San Diego, CA) running on a Silicon Graphics Indigo workstation (Mountain View, CA). For all experiments, the spectral widths were 6000 Hz. All two-dimensional experiments were recorded using States-time proportional phase incrementation (47) with 16–64 scans. For the total correlated spectroscopy (TOCSY) experiment, the mixing time was 70 ms. Nuclear Overhauser effect spectroscopy (NOESY) spectra were obtained using mixing times of 75–300 ms. The H2O resonance was suppressed by 3–9–19 WATERGATE field-gradient pulses (47). Two-dimensional spectra were composed of 2048 or 4096 complex points in the F2 dimension and 1024 real points in the F1 dimension with 16–64 scans per t1 increment. All free induction decays were zero-filled, apodized with Gaussian or sine–bell window functions, and Fourier-transformed, yielding matrices of 2048 × 2048 or 2048 × 4096 real points for TOCSY or NOESY spectra.

Results

Conformation and Sequential Assignment of the Free KRR-Lamb Signal Peptide—KRR-modified Lamb signal peptides free in solution have been shown to adopt largely random structures at room temperature, judged by circular dichroism spectroscopy (Ref. 36 and data not shown). They self-associate at high concentration (>millimolar), which increases their helical content (Ref. 36 and data not shown). Due to the presence of several identical residues in tandem in the sequence, the tumbling rate of the signal peptide, and the lack of a preferred conformation at low concentration in aqueous solution, spectra of the free signal peptide had severe spectral overlap and relatively few NOEs, making sequential assignment difficult. The assignment process was facilitated by comparison to previous assignments of a highly similar peptide (36) and by adjustment of solution conditions (lowering pH and/or temperature or adding a small amount (2.5 mol%) of trifluoroethanol, which promotes formation of a helical conformation and disperses the proton resonances). Sequential assignments of the KRR-Lamb signal peptide under different conditions were successfully completed using standard methods (48); the resulting chemical shifts are listed in TABLE 1. Only very small shifts (generally <0.02 parts/million) were observed upon the addition of 15 μM SecA and obtaining spectra at 25 °C, causing no difficulty in confirming assignments made on the free peptide.

Line-broadening Effects Reveal the Binding Mode of a Signal Peptide with E. coli SecA—When a rapidly tumbling peptide binds to a slowly rotating protein, characteristically sharp resonances for the free peptide will be broadened due to the shortened relaxation time T2, induced by the binding (48). If the peptide resonances broaden as the protein concentration increases, no new resonances appear, and no significant chemical shifts occur, the system can be identified as undergoing moderately fast exchange (49).

The one-dimensional 1H NMR spectra in Fig. 1A show that the KRR-LambB signal peptide was bound by SecA with a moderately fast exchange rate, as indicated by line-broadening to a degree that was dependent on the SecA concentration. The internal standard (3-(trimethylsilyl) propionate) was not affected by the addition of SecA, indicating that the observed broadening was not a viscosity effect. Some peaks showed weaker broadening, specifically Lys-7/10 εH and Gln-25 δH. This suggests that the binding mode does not equally immobilize all segments of the peptide. Lys-7/10 εH and Gln-25 δH were weakly broadened (Δνε/δH = 3.5 and 1.7 Hz, respectively) after the addition of 20 μM SecA. In contrast, under the same conditions, Arg-6/8/9 εH were strongly broadened (Δνε/δH = 12.6 Hz). These large differences suggest that one or more of the Arg-6, -8, and -9 side chains are more immobilized, most likely via direct contact with SecA. In contrast, Lys-7, Lys-10, and Gln-25 side chains are less immobilized and may not have direct contact with the protein when the peptide binds to SecA.

The one-dimensional 1H spectrum of the non-functional KRR-LambB Δ78 signal peptide, which has four residues deleted from the h-region (37), is not affected by the addition of SecA (data not shown). This indicates that there is no interaction between the non-functional signal peptide KRR-LambB Δ78 and SecA and suggests that the hydrophobic residues of the h-region are essential for signal sequence recognition by SecA.

To understand the detailed binding mode between the KRR-LambB signal peptide and SecA, we examined TOCSY spectra in both the absence and in the presence of 15 μM SecA protein (Fig. 1, B and C). The line width and intensity changes of the cross-peaks in the backbone amide proton and α proton (NH-αH) region of the TOCSY spectrum reported the loss of mobility of the backbone of the corresponding residue, whereas that of the amide proton and side chain proton (NH-
βγδH) region were also influenced by the mobility of the side chain of the corresponding residue. Although most of the cross-peaks experienced significant broadening upon the addition of SecA (Fig. 1, B and C), judged by increased line width and decreased intensity of the cross-peaks, resonances from the c-region residues (Ala-26 NH-δ, Ala-28 NH-δ) were less affected (~20% loss of intensity in contrast to intensity decreases of ~40–50% for the other cross-peaks). The intensity changes of the cross-peaks in the NH-βγδH regions further revealed that the side chains of the residues in the n-region (except lysine) and the hydrophobic residues of the h-region were most directly involved in the binding to SecA. Their intensities decreased significantly, between 30 and 55%, whereas the NH-γδH, NH-δδH, and NH-δδH all were significantly broadened upon the addition of SecA. On the other hand, the cross-peaks of Lys-7 and -10 only had ~20% loss of intensity in contrast to intensity decreases of ~40–50% for the other cross-peaks).

The Conformation of the SecA-bound KRR-LamB Signal Peptide Based on trNOEs—In the unbound form, the KRR-LamB signal peptide is characterized by a short correlation time (τc ~ 1 ns), which is near the inverse of the Larmor precession frequency of protons in the NMR spectrometer; therefore, the magnitudes of NOEs are close to zero (Fig. 2A). When KRR-LamB signal peptide is bound to SecA (τc ~ 100 ns), magnetization transfer between different NMR-active nuclei within the signal peptide becomes more efficient because of its slower tumbling. Under conditions of a moderately fast exchange rate, where the dissociation rate constant koff is faster than the cross-relaxation rate, we can then observe in the free state of the peptide the trNOEs that reflect distance relationships of the bound state (50–52). As shown in Fig. 2B, numerous cross-peaks were observed in the NOESY spectra of the SecA-bound KRR-LamB signal peptide. The intensities of these inter-residue NOEs increased as the concentration of SecA increased, indicating that the binding between the signal peptide and SecA protein accounts for the trNOEs (data not shown).

The inter-residue NH(i)/NH(i + 1) trNOE cross-peaks observed in the amide-amide region (such as Ala-14/Ala-15, Val-15/Ala-16, Ala-16/Ala-17, Ala-17/Ala-18, Ala-18/Ala-19, Ala-19/Gly-20, Val-20/Val-21, Met-21/Ser-23, Ala-23/Ala-24, Ala-24/Val-25, Ala-25/Ala-26, Ala-26/Met-27, Ala-27/Ala-28) were also significantly broadened upon the addition of SecA (Fig. 2A, B). The stack plots of the major α proton and side chain proton region (Fig. 1C) also showed similar results. The cross-peaks of Met-3 αH/γH, Leu-13 αH/γH, Thr-4 βH/γH, Val-15 and -17 αH/γH, and Pro-12 αH/βH all were significantly broadened upon the addition of SecA. The comparison suggests that the side chain residues of the n- and h-regions (except the lysine) are immobilized more by SecA than those in the c-region, indicating these two regions bind more tightly with SecA than the c-region of the signal peptide.

### TABLE ONE

**Proton chemical shifts for the KRR-LamB signal peptide (ppm)**

| Residue | NH   | Hα  | Hβ  | Hy   | Hδ  | Other |
|---------|------|-----|-----|------|-----|-------|
| Met-1   | 4.48 | 2.14| 2.01| 2.64| 2.57|
| Met-2   | 4.56 | 2.11| 2.04| 2.64| 2.55|
| Ile-3   | 4.24 | 1.86| 1.21| 0.92| 0.86|
| Thr-4   | 4.32 | 1.95|     |     |     |
| Leu-5   | 4.37 | 1.62| 1.58| 0.87| 0.92|
| Arg-6   | 4.30 | 1.83| 1.78| 1.70| 3.21|
| Lys-7   | 4.28 | 1.81| 1.76| 1.47| 3.00|
| Arg-8   | 4.29 | 1.84| 1.77| 1.67| 3.21|
| Arg-9   | 4.31 | 1.83| 1.77| 1.62| 3.19|
| Lys-10  | 4.30 | 1.80| 1.77| 1.47| 3.00|
| Leu-11  | 4.64 | 1.65| 1.59| 1.55| 0.92| 0.90|
| Pro-12  | 4.41 | 2.29| 1.88| 2.03| 3.86| 3.62|
| Leu-13  | 4.29 | 1.65| 1.60| 1.59| 0.95| 0.90|
| Ala-14  | 4.34 | 1.41|     |     |     |
| Val-15  | 4.05 | 2.04| 0.94|     |     |
| Ala-16  | 4.33 | 1.37|     |     |     |
| Val-17  | 4.04 | 2.04| 0.95|     |     |
| Ala-18  | 4.29 | 1.39|     |     |     |
| Ala-19  | 4.31 | 1.43|     |     |     |
| Gly-20  | 3.97 |     |     |     |     |
| Val-21  | 4.08 | 2.10| 0.93|     |     |
| Met-22  | 4.55 | 2.09| 2.55| 2.64| 2.55|
| Ser-23  | 4.42 | 3.18| 7.63| 6.95|
| Ala-24  | 4.30 | 1.43|     |     |     |
| Gln-25  | 4.26 | 2.11| 2.02| 2.41| 7.63| 6.95|
| Ala-26  | 4.28 | 1.43|     |     |     |
| Met-27  | 4.44 | 2.14| 2.04| 2.67| 2.59|
| Ala-28  | 4.28 | 1.43|     |     |     |

All data were obtained in phosphate buffer at pH 6.3 and 5 °C, with 1.0 mM peptide concentration, as described under “Materials and Methods.”
Val-17, Val-17/Ala-18, Ala-18/Ala-19 and Gly-20/Val-21) suggest an α-helical conformation from Ala-14 to Ala-18, and the helix may extend to residues Leu-13 and Met-22 (Fig. 2C) (48). Some NH(i)/NH(i + 1) cross-peaks cannot be clearly seen because of the similarity of the ith and (i + 1)th amide proton chemical shifts causing them to be too close to the diagonal peaks (such as Leu-13/Ala-14 and Ala-19/Gly-20). Although Ala-16/Val-17 and Val-17/Ala-18 overlap with each other in this spectrum (the mixing time is 150 ms), they can be separately identified when the mixing time is 75 ms (data not shown). Other characteristic α-helical inter-residue trNOE cross-peaks were found and also support the conclusion that the bound signal peptide adopts an α-helical conformation from Leu-13 to Val-21, specifically the αH(i)/NH(i + 3,4) interaction between Leu-13/Val-17 and possibly between Ala-18/Val-21 (Fig. 2B). Also, several significant side chain inter-residue

FIGURE 1. Effects of SecA on line-broadening of one-dimensional and two-dimensional proton NMR spectra of the KRR-LamB signal peptide. A, selected regions of the one-dimensional 1H NMR spectra of 1 mM KRR-LamB signal peptide alone, signal peptide with 10 μM SecA, and signal peptide with 20 μM SecA. B and C, stack plots of TOCSY spectra for the KRR-LamB signal peptide alone and in the presence of 15 μM SecA, showing the region containing cross-peaks between the amide protons and upfield protons (both α and side chain protons) (B) and the region containing cross-peaks between the α protons and side chain protons (C). Red labels indicate that the peak intensity diminished >35% upon the addition of SecA. ppm, parts/million.
trNOE cross-peaks argue that the segment within the h-region and the first residue of the c-region in the KRR-LamB signal peptide both form an \(\alpha\)-helical conformation. For example, \(\alpha H(i)/\beta H(i+3)\) cross-peaks of Val-15/Ala-18 and of Ala-18/Val-21 (Fig. 2, B and C) lend further support to the observation that the SecA-bound KRR-LamB signal peptide adopts an \(\alpha\)-helical conformation in the h-region. Among the above telltale signatures of \(\alpha\)-helical conformation, \(\alpha H(i)/\alpha H(i+3)\), \(\alpha H(i)/\beta H(i+3)\) cross-peaks and \(\alpha H(i)/\beta H(i+3)\) cross-peaks were not observed between Leu-13/Ala-16 or between Ala-14/Val-17 and also no Val-15/Ala-19 or Val-17/Val-21 \(\alpha H(i)/\alpha H(i+4)\) cross-peak was seen, all of which might have been expected to appear. The trNOE results are consistent with a model in which at least one side of the peptide helix interacts with the hydrophobic binding groove of SecA and in which the SecA-contacting side includes Val-15/Ala-18/Val-21 in the KRR-LamB signal peptide. The direct contacts between the hydrophobic side chain protons of the residues on this side and the hydrophobic binding groove of SecA significantly reduce the mobility of these side chain protons, thus enhancing their inter-residue trNOEs, even at a very low protein/peptide ratio (\(10^{-2}\)) and relatively long distances (2.5–4.4 Å). The TOCSY \(\alpha/\beta\) cross-peak intensity change of Ala-18 is the greatest among all the alanines in the KRR-LamB signal peptide (data not shown), which also supports the trNOE results (TOCSY \(\alpha/\beta\) cross-peaks of Val-15 and Val-17 were overlapped, therefore we could not compare the changes between values). The n- and c-regions lacked diagnostic \(\alpha\)-helical inter-residue trNOEs, and this result, together with the strong sequential inter-residue trNOEs of these two regions in the \(\alpha H/\alpha H\) region (Fig. 2C), argue that these two regions adopt extended conformations in the SecA-bound state.
DISCUSSION

In this study, we directly observed conformational changes in an unstructured free signal sequence induced by SecA binding. Previous studies show that signal sequences have a high propensity to adopt \( \alpha \)-helical conformation in interfacial environments, such as when inserted into a membrane (31, 36, 53–55). In vivo studies suggest that hydrophobicity and helical conformation of signal peptides are important for preprotein translocation (2, 4, 17, 37, 56). Our NMR results demonstrated that helical propensity and hydrophobicity are required of a signal sequence at the stage of recognition by SecA. Also, observation of direct binding by NMR confirmed the interpretation of previous work based on inhibition of SecA ATPase activity (23), namely that a four-residue deletion from the \( h \)-region of the non-functional signal peptide removes its binding ability to SecA, even though the net positive charge in the \( n \)-region and the polar \( c \)-region are still conserved. Our studies suggest that the \( \alpha \)-helical conformation in the \( h \)-region is required to create an adequately hydrophobic structure for signal sequences to fit into the binding pocket of SecA, and that this pocket, together with suitably placed residues providing electrostatic complementarity to the \( n \)-region positive charges of the signal peptide, make up the crucial elements for the specificity of signal sequence recognition in the Sec-translocation pathway.

Our NMR results provide a detailed picture of the mode of binding of a signal peptide to SecA, which in turn offers insight into the nature of the signal peptide-binding site on SecA. The line-broadening effects of one-dimensional proton NMR and two-dimensional TOCSY spectra show that signal peptide binding to SecA does not equally immobilize the whole peptide segment. Surprisingly, the side chain protons of the lysine residues at positions 7 and 10 in the \( n \)-region are significantly less immobilized than the side chain protons of the neighboring arginine residues (positions 8 and 9) within the signal peptide, suggesting that the signal peptide may lie on the binding site with the arginines oriented toward interacting residues on SecA. Our NMR study provides further evidence for the orientation of the signal peptide on SecA; one side of the helical \( h \)-region interacts more strongly with the binding site of SecA than the rest of the helix in the peptide, as shown by line-broadening and strength of side chain trNOEs. The interacting side, Val-15/Ala-18/Val-21, presents a highly hydrophobic face of the helix in the signal peptide \( h \)-region, consistent with optimization of binding to a hydrophobic groove on SecA. Our data also argue that the binding site of the signal peptide on SecA is not deeply buried, but instead resides on the surface, such that only one side of the signal peptide is intimately contacting SecA. Genetic studies of LamB signal sequence mutations find that alterations at Val-15 and Ala-18 result in severe kinetic defects in the processing of precursor LamB into mature protein (17), consistent with the model for SecA binding emerging from our NMR data. Although several other mutations, such as changes at Ala-16, Val-17, and Met-22, also give rise to kinetic defects, we cannot exclude the possibility that the changes of these nonpolar residues to charged residues could disrupt the \( \alpha \)-helical conformation or other factors affecting binding.

Therefore, more detailed study is necessary to further support our observations, such as changing these three residues to less hydrophobic amino acids that will not disrupt the helix.

It is of interest to consider our data for the SecA-bound conformation of a signal sequence in light of recently published crystal structures of SecA (38–40) and earlier biochemical and cross-linking studies of SecA with signal sequences (27). Hunt et al. (40) identify a groove between nucleotide-binding fold (NBF)-1 and the \( \beta \)-strand connecting it to the PPXD(N), which in their structure of *Bacillus subtilis* SecA is occupied by the C-terminal linker (CTL), as a likely signal sequence-binding pocket because of its highly conserved and largely hydrophobic surface.

This \( \beta \)-strand (\( \beta \)-PPXD) is also part of the region determined by Economou and co-workers (27) to be essential for signal sequence binding. The proposed binding groove is \(~30 \text{ Å}\) long and \( 15 \text{ Å} \) wide. There are at least nine conserved hydrophobic residues distributed along the interface (Fig. 3), which is \(~10 \text{ Å} \) wide at the \( \beta \)-PPXD and \( 15 \text{ Å} \) wide at the NBF-1. Therefore, an \( \alpha \)-helical conformation in the \( h \)-region of the signal sequence could readily be accommodated in hydrophobic contact with SecA in this region.

In this proposed signal sequence binding groove of SecA, the distance between the last two available negatively charged residues, Asp-215/Glu-216 (\( \beta \)-subtilis SecA numbering), and the first available hydrophobic residue, either Leu-221 in the \( \beta \)-PPXD or Leu-180 in the NBF-1 (Fig. 3), is \(~7–10 \text{ Å} \). This distance, which is about three residues long for an extended conformation or six residues long for a helical conformation, matches the 2–5 intervening residues between the first residue of hydrophobic core and positively charged residues in the \( n \)-region of the KRR-LamB signal peptide. This overlay is consistent with the requirement for the net positive charge of the \( n \)-region in enhancing the binding between signal peptides and SecA. Our one- and two-dimensional NMR data implicate Arg-6, -8, and -9 of the signal sequence as being likely residues to contact the highly conserved Asp-215 and Glu-216 of SecA. Asp-215/Glu-216 occur sequentially after the Walker-B motif or so-called DEAD box (DEV\_ in SecA) that is interacting with Mg\(^{2+}\). In the crystal structure, Asp-215 forms an electrostatic interaction with Arg-517 (helicase motif VI, mobile region 1, intramolecular regulator of ATP hydrolysis 2 (IRA2)) (57, 58). Therefore, if the \( n \)-region of the signal sequence interacts with Asp-215, it is reasonable that the binding of the signal sequence could inhibit the ATPase activity of SecA, as we reported based on the signal peptide interaction with an N-terminal 64-kDa fragment of SecA (23), because binding could interfere with the interaction between the Walker-B motif and Mg\(^{2+}\). The observed high mobility (57) of motif VI could allow this part of the binding pocket more flexibility for differently sized signal sequences with net positively...
charged n-regions. There are also several nearby hydrophobic residues in SecA, Ile-212, Leu-213, and Ile-214, which might explain how a longer hydrophobic stretch of signal sequence could compensate for a lack of positive charge in the n-region (59). The polar residues of SecA that may contact the c-region of the signal sequence are Ser-224 and Gin-226 in the β-PPXD. Ala-227 and Ala-228 may form a shallow hydrophobic binding pocket similar to that found during signal peptidase recognition; however, these two residues are only partially conserved in SecA. This might explain the high mobility observed in this part of the peptide by trNOE.

The fact that the CTL occupies part of this proposed signal sequence binding region offers the possibility of the regulation of signal sequence binding. Several lines of evidence have shown that the CTL is highly mobile. We observed this region to be mobile by NMR (57), and the CTL is disordered and not observed in the structure of SecA from Mycobacterium tuberculosis (39) as well as the recent structure of monomeric SecA from B. subtilis (38). This region could protect the signal sequence binding pocket when SecB/preprotein is not present to avoid nonspecific binding. Consistent with a role of the CTL in regulated occupancy of the signal sequence binding site is the finding that mutations in this region (termed intramolecular regulator of ATP hydrolysis 1 (IRA1) by the laboratory of Economou) also enhance signal sequence binding affinity (27).

An alternative potential polypeptide-binding site was identified by Rapoport and co-workers (38) based on their recent structure of B. subtilis SecA in a monomeric state. This structure revealed a substantial rearrangement of the PPXD with respect to the C-terminal helical domain, opening up a large cleft between these two domains. These authors propose that this site could represent the precursor-binding site, and the regulation of access to the precursor could be mediated by the movements they observed in the PPXD relative to the rest of the SecA structure. This is a large cleft, and several features could be consistent with polypeptide binding. However, this site is more likely to bind the mature region of a precursor, or even SecY, and not the signal sequence, based on several factors. First, recognition of the signal sequence by this site is inconsistent with the observation that signal peptides bind to the N-terminal region of SecA (shown by us using a 64-kDa fragment (23) and by the laboratory of Economou using deletions (27)). Moreover, the level of conservation and the match to the 64-kDa fragment (23) and by the laboratory of Economou using deletions (27) of the helical propensity of the h-region and the charge of the n-region (59). The polar residues of SecA that might explain how a longer hydrophobic stretch of signal sequence could compensate for a lack of positive charge in the n-region (59). The polar residues of SecA that may contact the c-region of the signal sequence are Ser-224 and Gin-226 in the β-PPXD. Ala-227 and Ala-228 may form a shallow hydrophobic binding pocket similar to that found during signal peptidase recognition; however, these two residues are only partially conserved in SecA. This might explain the high mobility observed in this part of the peptide by trNOE.

The fact that the CTL occupies part of this proposed signal sequence binding region offers the possibility of the regulation of signal sequence binding. Several lines of evidence have shown that the CTL is highly mobile. We observed this region to be mobile by NMR (57), and the CTL is disordered and not observed in the structure of SecA from Mycobacterium tuberculosis (39) as well as the recent structure of monomeric SecA from B. subtilis (38). This region could protect the signal sequence binding pocket when SecB/preprotein is not present to avoid nonspecific binding. Consistent with a role of the CTL in regulated occupancy of the signal sequence binding site is the finding that mutations in this region (termed intramolecular regulator of ATP hydrolysis 1 (IRA1) by the laboratory of Economou) also enhance signal sequence binding affinity (27).

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Clearly, additional experiments will be needed to pin down the signal peptide-binding site on SecA. A combination of cross-linking and mutagenesis strategies is being deployed in our laboratory to accomplish this goal and to enable deeper understanding of how a single preprotein translocase can guide a wide range of substrates with variable signal sequences into the protein export pathway. The studies reported here foreshadow the mode of signal sequence binding and provide explanations for features that are essential for signal peptide function. Our results indicate that two features shared by all prokaryotic signal sequences, the helical propensity of the h-region and the charge of the n-region, are required for SecA recognition.

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The Conformation of a SecA-bound Signal Sequence

Enzymol. 239, 657–700
50. Clore, G. M., and Gronenborn, A. M. (1982) J. Mag. Res. 48, 402–417
51. Clore, G. M., and Gronenborn, A. M. (1983) J. Mag. Res. 53, 423–442
52. Campbell, A. P., and Sykes, B. D. (1993) Annu. Rev. Biophys. Biomol. Struct. 22, 99–122
53. Briggs, M. S., and Gierasch, L. M. (1984) Biochemistry 23, 3111–3114
54. McKnight, C. J., Stradley, S. J., Jones, J. D., and Gierasch, L. M. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 5799–5803
55. Hoyt, D. W., and Gierasch, L. M. (1991) J. Biol. Chem. 266, 14406–14412
56. Goldstein, J., Lehnhardt, S., and Inouye, M. (1991) J. Biol. Chem. 266, 14413–14417
57. Chou, Y. T., Swain, J. F., and Gierasch, L. M. (2002) J. Biol. Chem. 277, 50985–50990
58. Sianidis, G., Karamanou, S., Vrontou, E., Boulias, K., Repanas, K., Kyrpides, N., Politou, A. S., and Economou, A. (2001) EMBO J. 20, 961–970
59. Mori, H., Araki, M., Hakita, C., Tagaya, M., and Mizushima, S. (1997) Biochim. Biophys. Acta 1326, 23–36