Published in final edited form as:

J Mol Biol. 2017 June 02; 429(11): 1722–1732. doi:10.1016/j.jmb.2017.04.022.

Determinants of Helix Formation for a Kv1.3 Transmembrane Segment inside the Ribosome Exit Tunnel

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Abstract

Proteins begin to fold in the ribosome and misfolding has pathological consequences. Among the earliest folding events in biogenesis is the formation of a helix, an elementary structure that is ubiquitously present and required for correct protein folding in all proteomes. The determinants underlying helix formation in the confined space of the ribosome exit tunnel are relatively unknown. We chose the second transmembrane segment, S2, of a voltage-gated potassium channel, Kv1.3, as a model to probe this issue. Since the N-terminus of S2 is initially in an extended conformation in the folding vestibule of the ribosome yet ultimately emerges at the exit port as a helix, S2 is ideally suited for delineating sequential events and folding determinants of helix formation inside the ribosome. We show that S2's extended N-terminus inside the tunnel is converted into a helix by a single, distant mutation in the nascent peptide. This transition depends on nascent peptide sequence at specific tunnel locations. Co-translational secondary folding of nascent chains inside the ribosome has profound physiological consequences that bear on correct membrane insertion, tertiary folding, oligomerization, and biochemical modification of the newborn protein during biogenesis.

Graphical Abstract

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Keywords

ribosome; translation; nascent peptide; secondary structure; helix formation

INTRODUCTION

Protein misfolding underlies many diseases, including Creutzfeldt-Jakob disease and neurodegenerative diseases such as Alzheimer's, Parkinson's, Huntington's, and amyotrophic lateral sclerosis¹. To avoid these perilous consequences, a protein must fold correctly, a process that includes multiple maturation events. The first step appears to be acquisition of local secondary structures, e.g., helices and turns, followed by folding to a more global native structure^{2, 3}. Helix formation depends on the protein's primary sequence, its solvent environment, and the combination of physicochemical properties that underlie intrahelical side-chain interactions, helical dipole (charge-dipole) interactions, and the effects of other residues flanking the helix^{4, 5}. Despite the importance and ubiquity of helical structures in mature proteins, the question of when/where/how these critical structures arise during early biogenesis is still largely unanswered. The first opportunity for helix folding is in the ribosome during translation and protein synthesis of the nascent peptide.

The large subunit of the ribosome is endowed with an exit tunnel, a heterogeneous microenvironment of confined space and non-bulk water^{6–8} through which the nascent peptide moves as it is elongated (Fig. 1B). This molecular corridor, which is lined predominantly by rRNA and some protein, is ~100Å long and 10–20Å wide, with distinctive variations and 'constrictions' along the way. At one end of the tunnel, the peptidyl transferase center (PTC) decodes mRNA and peptide bonds form. At the other end, the exit port, the nascent chain emerges. Despite the apparent structural constraints of a narrow exit tunnel, polypeptides may adopt distinctly different conformations in certain regions of the tunnel (e.g., the 'folding vestibule' in the last 20Å (Fig. 1B)) that are large enough to accommodate a nascent chain α -helix and even minimal tertiary structures^{9–26}.

The coupled coordination of helix formation and peptide synthesis is notably important in early biogenesis²⁷. First, the presence of an α -helix or a signal sequence in the vestibule can facilitate or inhibit chaperone recruitment^{27–30}. Second, the sequential nature of synthesis promotes sequential folding (N- to C-terminus) that obviates misfolding, which is particularly critical for multidomain proteins³¹. Third, coupling translation and folding permits each process to fine-tune the rates of the other, thereby regulating and optimizing membrane targeting and biochemical modifications as the nascent chain emerges from the tunnel^{31–35}. This is critical for correct protein maturation and function. Fourth, early generation of secondary and tertiary conformations in the folding vestibule ensures that i) proteolytic vulnerability of emerging non-native states is minimized until synthesis is complete, and ii) promiscuous interactions with more C-terminal sequences are prevented.

In solution, where the determinants of helix formation are well-defined, it has been shown that helix formation is enhanced by tertiary interactions with other parts of the protein³⁶. This compensates energetically for the unfavorable loss of both side-chain and backbone entropy in making a helix from an extended peptide³⁷. In the ribosome exit tunnel, however, peptide interactions with tunnel components may provide the equivalent compensatory tertiary interactions to inhibit/facilitate helix formation of the nascent chain. Thus far, such determinants responsible for conversion of the peptide from an extended to a helical conformation during early biogenesis have not been reported. Here, we address these issues and identify determinants of helix formation inside the ribosome exit tunnel for a specific helical segment, the second transmembrane segment, S2, of Kv1.3, a voltage-gated potassium (Kv) channel (Fig. 1A). This protein has six transmembrane segments and assembles as a tetramer to form the functional channel. Each transmembrane segment (S1–S6) forms a canonical α -helix in the mature Kv channel in the membrane³⁸, and each segment's helicity is manifest early in biogenesis of the nascent peptide¹² in the folding vestibule of the ribosome tunnel. Moreover, most of these transmembrane segments compact their N-terminal portions in the folding vestibule independently of the extended structure of their C-terminal portions that are closer to the PTC^{12,13} (Fig. 1B, right cartoon, S_x). The S2 segment, however, is distinctly different. It is entirely extended when its N-terminus resides in the folding vestibule¹² (Fig. 1B, left cartoon, S2), yet helical in the mature Kv channel³⁹. Thus S2 is a good candidate for identifying its determinants of helix initiation and propagation as it is elongated in the ribosome tunnel. We now demonstrate that conversion of S2 from an extended to helical conformation happens inside the tunnel, is sequence dependent, and occurs at specific tunnel locations.

RESULTS

To determine the conformational status of S2 nascent peptides in the ribosome exit tunnel, we used a mass-tagging assay, pegylation (polyethylene glycol maleimide, PEG-MAL), to modify single reporter cysteines (Cys) engineered into a nascent peptide. A decrease in the *extent of pegylation* (fraction of peptide pegylated, F_{peg}) and/or the slope of a Cys-scanned segment relative to a known extended nascent peptide ('tape measure') indicates compaction^{12,13}. The tape measure is a 95-amino acid segment derived from the T1 domain of Kv1.3 and has been modified to be completely extended. From previous studies of Kv1.3, the slope of pegylation curves (F_{peg} versus PTC-Cys) for the N-termini of S1, S3, S4, S5,

and S6 are in the range of 0.44–0.50 relative to the tape measure, consistent with their compaction in the tunnel vestibule¹² (Fig. 1B, S_x red line). In contrast, the extent of pegylation and the slope of the pegylation curve for S2 at this same tunnel location are similar to the extended tape measure (Fig. 2C, red circles), consistent with S2 being extended at this location. We propose that S2's N- and C-terminal regions, along with the local tunnel environment, determine compaction of S2's N-terminus in the tunnel.

Helix formation inside the ribosome tunnel

To test this hypothesis, we mutated the mid-region of the S2 sequence to residues designed to interrupt putative intrapeptide N- and C-terminal communication, and measured F_{peg} of the Cys-scanned N-terminal S2 sequence (Fig. 2A, dotted residues). The engineered cysteines are evaluated for their ability to be covalently modified by PEG-MAL. The adduct appears as a gel-shifted band 1 (Fig. 2B; ~ 10 kD shift in mass) and F_{peg} is quantitated as a ratio of band 1 pegylated protein to total protein for each lane. The extent and slope of pegylation of S2 (Fig. 2C, red circles) approximately coincide with the extended tape measure¹³ (black circles). F_{peg} for a Cys engineered 33 residues from the PTC is 0.7, compared to 0.8 for the control tape measure at this location. This small difference is likely due to a slightly altered side-chain position of the Cys rotamer and/or to a backbone kink due to the presence of a proline (residue 266) or a tryptophan (residue 253) in S2^{12, 14, 40}. By comparison, mutant S2 peptides substituted with either Pro, Gly, or Ser residues (for example, SPSGS) for WFSFE, the native sequence in the middle of S2 (Fig. 2A), exhibited less pegylation (Fig. 2B–C). We chose these three residues (P, G, and S) because they have been reported to alter the flexibility of a peptide backbone and therefore might disrupt communication between the N- and C-termini of S2^{41, 42}. F_{peg} for 244C in the native peptide (0.7) decreased to ~0.4 in the mutated peptide, suggesting that the N-terminus of the mutant S2 is now compact, equivalent to one helical turn and consistent with the 50% lower slope of the F_{peg} versus PTC curve^{12–14} (Fig. 2C). To assess which of the mid-region native residues is responsible for inhibiting compaction, we mutated just the WF residues of the native sequence to either SP or SV. Either mutation is sufficient to reduce F_{peg} . Importantly, the relative slope of mutant S2 compared to the tape measure is ~0.5. Thus, when the sequence WFSFE is mutated to SPSGS, SPSFE, or SVSFE, the N-terminus of S2 compacts. Three conclusions may be drawn. First, the mid-region of the peptide at this tunnel location is important for N-to-C communication. Second, this communication determines secondary structure formation of the S2 N-terminus. Third, the WF residues play a role in this scenario.

To further understand the contribution of mid-region residues to the interactions governing compaction of the N-terminus, we mutated W and F one at a time to residues with unique biophysical properties. Residues W and F are aromatic, hydrophobic, and sterically large, with helix propensities midway between alanine and glycine (maximum and minimum helix propensity, respectively). Each of these properties can lead to interactions with the tunnel or configure W and F to influence the N-terminal region of the peptide. We therefore varied these parameters at each residue position by selecting mutant side chains over a range of helix propensities, hydrophobicity, and van der Waals volume (Fig. 3B), and evaluated F_{peg} at residue 244C. The presence of WF at PTC 24 inhibits helicity (i.e., the peptide has a

high F_{peg} , red bar, Fig. 3A). Substitution of W with residues F, Q, R, S, V, or Y each induce compaction, i.e., $F_{\text{peg}} \sim 0.2$ (green bars), consistent with formation of a two-turn helix^{12–14}. This is the case regardless of whether a residue with similar (F, V, Y, S) versus lower (Q, R) helix propensity, or similar (F) versus dramatically different hydrophobicity (S, Q, R), or similar (F, R) versus markedly smaller (S, V) size was substituted for W. In contrast, F can be substituted (blue bars) with Y (similar helix propensity and size, but a different hydrophobicity) to full effect (i.e., F_{peg} is ~ 0.7 at Cys reporter 244), whereas A (markedly higher helix propensity, hydrophobicity, and size) and V (similar helix propensity and size, but different hydrophobicity) exhibit average F_{peg} values ~ 0.47 , consistent with one helical turn. Therefore, WF mutants can induce helix formation. The degree of extension does not appear to correlate with the inherent helix propensity, hydrophobicity, or van der Waals volume of the individual amino acid X substituted in $\underline{X}F$ or $\underline{W}X$ (Fig. 3B). From the results shown in Figures 2 and 3, we conclude that the native N-terminus of S2 itself can form a helix in the tunnel vestibule and that the peptide's WF, at this specific tunnel location (PTC 24–23), is an operational unit or inhibitory motif that prevents helix formation. These results lead to the hypothesis that there is a strong, and long-range, communication between WF and the S2 N-terminus that depends on both intrapeptide interactions and the tunnel environment. Each of these factors should vary with tunnel location, peptide sequence, and peptide conformation. Moreover, side-chain entropies influence helix formation^{5, 37} and this may be especially relevant in the narrow confines of the ribosome tunnel. We suggest that the conformational freedom of mid-region side chains, e.g., WF, might allosterically influence remote N-terminus compaction. Such peptide-tunnel constraints may also limit helix formation in the immediate neighborhood of WF, which consequently can inhibit or foster remote sequence compaction.

Does WF, at the specific tunnel location PTC 24–23, always inhibit compaction and constitute a dominant repressor-like motif in S2 folding? If so, then we propose that disruption of N-terminus and WF communication, or enhanced helical stability of the N-terminus, will shift the folding equilibrium to favor helix formation of S2's N-terminus. Inherent in these proposals are two testable predictions. First, experimental strategies that interrupt intrapeptide communication should promote helix formation of the N-terminus. Second, introduction of helix-stabilizing factors within the N-terminus helix should also enhance compaction. We tested the first prediction in two ways. First, we inserted a minimal linker, SV (blue, Fig. 4A, top), between WF and the N-terminus. Insertion of SV immediately upstream and adjacent to WF leaves WF in the same tunnel location while increasing the distance between WF and the N-terminus, and relocating the N-terminus two residues more proximal to the exit port. F_{peg} for Cys reporters located at PTC 35 is 0.40 ± 0.02 (Fig. 4B). Another Cys reporter, at PTC 38, gives F_{peg} of 0.57 ± 0.05 ($n=3$). Both Cys residues in the SV-peptide are more distally located along the tunnel, yet have less pegylation and a lower apparent slope (~ 0.5) compared to native S2. These results are consistent with N-terminal compaction in the SV-inserted peptide. This induced helix formation may be due to either (or both) of two possibilities. First, the increased distance between WF and the N-terminus will insulate the N-terminus, which has been shifted downstream by two residues, from the WFSFE segment. Second, relocated N-terminal amino acids will experience interactions with a different tunnel environment. Another way

to test interruption of the N-C communication is to simply substitute a proline residue between the mid-region and the N-terminus of S2. We chose proline because proline/glycine residues have been used as flanking residues to insulate a given sequence from other interactions, while themselves having a low probability of secondary structure formation⁴³. As such, we predicted that proline substitution would decrease N- and C-terminal communication and permit the N-terminus to compact. We introduced proline at position 249, intermediate between W253 (PTC 24) and the reporter Cys, 244C (PTC 33; magenta, Fig. 4A middle). Pegylation of the nascent peptide decreased F_{peg} at 244C to 0.23 ± 0.02 (n=3), significantly lower than 0.68 ± 0.05 (n=33) for the native S2 with residue 276 at the PTC (Fig. 4B), consistent with compaction of the N-terminus of S2 in the proline-substituted peptide, likely due to disruption of communication.

We tested the second prediction by introducing a charged side chain to form an intra-helical salt-bridge. Despite the unfavorable entropy loss and desolvation accompanying salt-bridge formation⁴⁴, the energetically favorable Coulomb interaction between the salt bridge's positive and negative charges promotes helix formation. We introduced a basic residue, arginine (R), at position 243 (green, Fig. 4A bottom), which is ideally situated to form a salt-bridge with native E247 (*i, i+4*)^{45–47}. We chose an R in preference to lysine (K) because properly spaced E-R residues form a helix inside the tunnel⁴⁸ and, in solution, have higher helix content than their E-K counterparts⁴. The 243R mutation yields a nascent chain that is compact (Fig. 4B). F_{peg} for reporter Cys at 244C (PTC 33) is 0.42 ± 0.03 (n= 3) compared with the native control at 244C (0.68 ± 0.05 , n= 33).

The results shown in Figure 4 are consistent with allosteric communication between the N- and C-terminal segments of S2. Interruption of this connection, whether by increasing the distance between the S2 N-terminus (SV insertion) or by disengaging N-C communication (proline substitution), permits compaction of the N-terminus. Another less likely possibility is that SV-insertion may shift the N-terminal sequence to a less constrained microenvironment that is permissive for helix formation. Additionally, a proline at residue 249 may disengage N- and C-terminal communication through a kink or bend in the intervening backbone that precludes transmission of the WF inhibitory signal. Finally, the free energy contribution of an additional salt-bridge is sufficient to overcome the inhibitory signal due to the presence of mid-region residues WF, and promotes helix formation. These findings suggest that, at this specific location, residues WF in the middle of S2 act in a repressor-like mode to postpone or prevent S2 helix formation in the folding vestibule and may involve specific interactions with tunnel components.

Thus far, we find that WF, at a specific tunnel location, is a dominant determinant of N-terminus helix formation, however, it remains to be evaluated where and when compaction begins during the natural process of elongation and co-translational folding. To address this issue, we chose to mimic the native process of S2 elongation and thereby identify the biogenic stage at which helix formation of the N-terminus of S2 is initiated. Elongation will also indicate the location specificity of the WF repressor-like activity, i.e., does WF inhibit at any other tunnel locations? For this purpose, we elongated native S2 by introducing new restriction sites in the DNA. Consequently, the nascent chain is lengthened at its C-terminus at the PTC (Fig. 5A) and S2 is repositioned in the tunnel. At each new location, we

measured F_{peg} of the N-terminus of S2 (Fig. 5B). Extension of the nascent chain by 2, 4, or 6 residues (restriction sites 278, 280, or 282, respectively) reduced pegylation (band 1) at reporter Cys residues to give lower F_{peg} . The slope of the pegylation versus PTC curve is likewise reduced and is ~ 0.5 compared to the shorter S2 nascent chain with residue 276 at the PTC. Both results are consistent with helix formation and suggest that elongation by just two amino acids situates the peptide in a region of the tunnel that is 6 Å closer to the exit port. In this new microenvironment, the communication between N- and C-terminal regions of the peptide may be altered, either or both of which can promote compaction of S2's N-terminus in the folding vestibule.

DISCUSSION

The process of helix formation in solution has been studied extensively and is well understood, yet this process has not been explored in the confined microenvironment of the ribosome exit tunnel. Although nascent chain helices do exist in the tunnel, especially in its folding vestibule^{10, 12–14, 16, 20, 21, 49}, elucidation of 'in-tunnel' principles that govern helix formation during biogenesis is lacking. Here we have focused on the S2 transmembrane segment of Kv1.3 to address this issue for a specific case. Like the other five transmembrane segments, S2 has unique functional roles in the mature channel, notably its electrostatic interactions with the S4 segment^{50–53}. We now provide mechanistic insights into the sequence dependence of S2 helix formation and the role of its tunnel location in promoting initiation and propagation of secondary structure. At specific tunnel locations, the N-terminus of S2 can be converted from its initial extended conformation to a compact conformation. Thus, S2's N-terminus becomes a helix before it leaves the folding vestibule and folding is vectorial in nature (N-to-C). This observed compaction of the N-terminus is consistent with secondary folding in aqueous solutions, where helical turns typically start from the N-terminal side of a helical sequence^{54–57}. The first α -helical turn (initiation) involves a discrete set of six dihedral angles among 3.6 residues to form a carbonyl-amide hydrogen bond between residues i and $i+4$. This first step thus constitutes an entropic penalty for initiation of helix formation. Extending or propagating the helix, i.e., an additional hydrogen bond with fixation of only two additional dihedral angles, is energetically less costly. Nonetheless, the entropic penalty associated with initiation suggests an energetic contribution likely comes from another component of the folding process to form a helix. Our studies suggest that this free energy component involves interactions between the mid-region of the S2 helical sequence, its N-terminus, and tunnel constituents at specific locations, which together subserve an operational function to inhibit helix formation. Although we introduce this model for the specific case of S2, analogous repressor-like motifs may exist, albeit with different peptide sequence dependence and tunnel locations, to govern helix formation in other nascent peptides. Our findings simply demonstrate a first precedent for determinants of in-tunnel helix formation. To establish whether the WF pair has repressor-like activity in other Kv segments, and which tunnel components mediate helix formation, will require more extensive studies.

At the heart of this inhibitory motif is WF. This WF motif is absolutely conserved in S2 segments in the Kv1, Kv2, and Kv3 subfamilies⁵⁸, including the Kv1.2/2.1 chimera and *Shaker*³⁸, but not across all structurally conserved K⁺ channel proteins. We might speculate

that these conserved WF residues modulate S2 helix formation in these Kv subfamilies as well. In our studies of Kv1.3 S2, WF are 24–23 amino acids, respectively, away from the PTC and proximal to ribosomal proteins. One such protein is eL39, which lines the tunnel, spans the distance between WF and the N-terminus of S2 near the exit port, can be crosslinked to nascent peptide, and makes contact with the translocon^{16, 40, 49, 59}. Another ribosomal protein is uL23. It is similar to its bacterial counterpart L23, which is proximal to elongating nascent peptide near the exit port and binds strongly to the signal recognition particle (SRP)⁶⁰ (see below). Each of these proteins may modulate the orientation of WF and mediate S2 folding. For example, eL39 could undergo a WF-induced rearrangement that allosterically disfavors helix formation at the S2 N-terminus, or eL39 could orient W and F side chains and/or the peptide backbone. Each possibility could shift the equilibrium between folded and extended N-terminus. Moreover, tunnel interactions that orient W can affect peptide solvation and consequently, helix formation. W, a bulky, nonpolar side chain, will shield the intrapeptide backbone hydrogen bonds from the solvent, reduce the interaction energy between the hydrogen-bond dipoles and water, thereby inhibiting helix formation⁶¹. So, if tunnel orientation of W favors desolvation and decreased helix formation of the mid-region sequence, then coupling of this mid-region to the N-terminus will leave the N-terminus extended. Alternatively, W may associate with an upstream intrapeptide residue, Leu, in the S2 helical sequence, LxxxW. At this spacing (corresponding to $(i, i+4)$ in a helix), the flexible side chain of Leu can interact with aromatic peptide residues to stabilize a helix^{62–64}. We might speculate that when the S2 nascent chain is elongated (e.g., when the PTC is at 278–282), the tunnel environment allows a Leu-Trp interaction that potentiates helix stability, whereas Leu and/or Trp in the 276 peptide are conformationally confined and therefore do not interact or contribute favorably to the free energy of helix formation.

The aforementioned scenarios describe putative mechanisms by which interactions between the nascent chain and the tunnel could shift the folding equilibrium of the S2 N-terminus. Note that the distance over which such interactions exert an influence on the folding equilibrium is ~10 nascent chain residues (~ 30Å), i.e., the number of amino acids between W and the beginning of the S2 N-terminus (P241). This suggests long-range modulation of helix initiation inside the ribosome tunnel.

This model of helix formation is supported by two additional lines of evidence. First, insertion of SV between WF and the N-terminus perturbs N-to-C-terminal communication and leads to helix formation. The two-residue insertion increases the distance between WF and the N-terminus and relocates the intervening residues to new tunnel microenvironments. Both consequences alter the free energy of helix formation. The second line of evidence is strategically different. Neither the location of WF relative to the N-terminus, nor the location of the N-terminus was changed. Only a proline was substituted halfway between WF and the N-terminus, which disrupts N-to-C terminal communication and produces compaction of the N-terminus. Due to its pyrrolidine ring, Pro has less conformational freedom than other amino acid side chains and imposes rigid constraints on a peptide backbone. This can produce conformational rearrangements or kinks in the peptide chain, either of which might interrupt the native S2 N-to-C-terminal communication that prohibits helix formation. Yet another strategy to overcome this prohibition of helix formation was to shift the folding equilibrium of the N-terminus by increasing its helix stability. Introduction of an arginine,

ideally situated to form an intrahelical salt-bridge ($i, i+4$) with a downstream Glu, was sufficient to override the inhibition generated by WF in its tunnel environment and permit helix formation.

The shift in folding equilibrium must occur during elongation of the nascent chain because S2 leaves the ribosome as a helix. Our results of S2 folding during chain elongation suggest that the biogenic transition between unfolded and folded conformations occurs just as WF enters the folding vestibule, a compartment of increased solvent-accessible volume where helices and small tertiary hairpins are permitted to form^{12, 23, 24}. Where and when co-translational folding takes place has physiological consequences. These consequences include association with chaperone proteins and biogenic factors, tertiary folding, biochemical modification, targeting, correct membrane insertion, and oligomerization. For example, we have shown previously that S2 functions as the initial signal sequence to establish Kv1.3 topology and target Kv1.3 to the endoplasmic reticulum (ER). In contrast, S1, the first transmembrane segment of Kv1.3, is unable to initiate targeting to the ER, translocation, or integration into the ER bilayer⁶⁵. It is thus critical that S2 assume its helical conformation at the correct time and location in the ribosome vestibule so that it can fulfill its role as the obligate signal sequence. We propose that the extent, timing, and tunnel location of S2 helix formation will modulate its binding to SRP, a biogenesis factor that recognizes hydrophobic signal sequences and transmembrane segments for efficient delivery of the peptide-ribosome complex to the secretory pathway. It is generally known that contact between the peptide and the tunnel wall increases the binding affinity of SRP (K_d in the nanomolar range)^{60, 66}, which can be kinetically regulated to enhance the specificity and timing of membrane targeting⁶⁷. Similar to the local slowdown of translation by nonoptimal codons that favors SRP binding⁶⁸, S2's compaction at distal tunnel locations could, by decreasing the end-to-end peptide length, increase the time for transmembrane recognition by SRP and binding. Moreover, since SRP binds to ribosomal proteins eL39/uL23⁶⁰, it is possible that SRP induces conformational changes in eL39/uL23 that orient S2's WF to coordinate helix formation, thereby conferring location specificity to the WF motif.

Helix formation is a fundamental process in the correct folding of all proteins and its co-translational occurrence inside the ribosome exit tunnel may even determine the fate of the newborn protein. Our findings for the transmembrane helix S2 set a precedent for analogous folding determinants at distinct tunnel locations that may underlie helix formation in other membrane proteins during early biogenesis in the ribosome.

MATERIALS and METHODS

Constructs and in vitro translation

Standard methods of bacterial transformation, plasmid DNA preparation and restriction enzyme analysis were used. In all experiments, we used either a molecular tape measure, which is the C-terminal 44 amino acids of the first 95 amino acids of the T1 domain of Kv1.3, made cysteine-free and all-extended from a Kv1.3 native transmembrane segment¹³. The cysteine engineered for all experiments is indicated in the native Kv1.3 in each figure. Mutants were made using Stratagene's QuikChange site-directed mutagenesis kit. Capped complementary RNA was synthesized *in vitro* from linearized templates or PCR fragments

using Sp6 RNA polymerase (Promega). Proteins were translated *in vitro* with [³⁵S]methionine Express (2 µl per 25 µl translation mixture; ~10 µCi µl⁻¹; Amersham) for 1h at 22 °C in rabbit reticulocyte lysate according to the Promega Protocol and Application Guide.

Pegylation

Translation product (5–20 µl) was centrifuged through a sucrose cushion (120 µl; 0.5 M sucrose, 100 mM KCl, 5 mM MgCl₂, 50 mM HEPES, (pH 7.3), no added DTT) for 20 min at 70,000 rpm with a TLA 100.3 Beckman ultra-centrifuge rotor at 4 °C to isolate ribosome-bound peptide. The supernatant was completely removed and the pellet was resuspended on ice in 100–500 µl of buffer containing 100 mM NaCl, 2.5–5mM Mg²⁺, 20 mM HEPES, (pH 7.3) and 500 µM DTT. Pegylation was started by adding a final concentration of 2 mM polyethylene glycol maleimide (PEG-MAL; SunBio, Korea) and continued for 3–5 hours at 4 °C in a refrigerator. The reaction was then terminated by adding 50 mM DTT and vortexing. Translation for all constructs (WT and mutants) is carried out for 1 hour, which produces full-length protein (band 0), which plateaus to a maximum value between 45 and 90 min. Fpeg, measured at 3 and 5 hours to ensure final extent of pegylation, is calculated as the ratio of band 1 to the sum of band 1 + band 0. Mutations do not affect either the rate of translation or the extent of pegylation.

Gel electrophoresis and fluorography

All final samples were heated at 70 °C for 10 min in 1 x of NuPAGE loading buffer (Invitrogen) before loading onto the NuPAGE gel (Invitrogen). Electrophoresis was performed using the NuPAGE system and precast Bis-Tris 10%, 12%, or 4–12% gradient gels and Mes or MOPS running buffer. Gels were soaked in Amplify (Amersham) to enhance ³⁵S fluorography, dried and exposed to Kodak X-AR film at –70 °C. Typical exposure times were 16–30 h. Quantification of gels was carried out directly using a Molecular Dynamics PhosphorImager.

Acknowledgments

We thank Dr. Richard Horn for critical reading of the manuscript. This work was supported by funds from NIH R01 GM052302 to CD.

References

1. Chaudhuri TK, Paul S. Protein-misfolding diseases and chaperone-based therapeutic approaches. *FEBS J.* 2006; 273:1331–1349. [PubMed: 16689923]
2. Hockenmaier J, Joshi AK, Dill KA. Routes are trees: the parsing perspective on protein folding. *Proteins.* 2007; 66:1–15. [PubMed: 17063473]
3. Voeltz VA, Dill KA. Exploring zipping and assembly as a protein folding principle. *Proteins.* 2007; 66:877–888. [PubMed: 17154424]
4. Sommesse RF, Sivaramakrishnan S, Baldwin RL, Spudich JA. Helicity of short E-R/K peptides. *Protein Sci.* 2010; 19:2001–2005. [PubMed: 20669185]
5. Scholtz JM, Baldwin RL. The mechanism of alpha-helix formation by peptides. *Annu Rev Biophys Biomol Struct.* 1992; 21:95–118. [PubMed: 1525475]
6. Ban N, Nissen P, Hansen J, Moore PB, Steitz TA. The complete atomic structure of the large ribosomal subunit at 2.4 Å resolution. *Science.* 2000; 289:905–920. [PubMed: 10937989]

7. Nissen P, Hansen J, Ban N, Moore PB, Steitz TA. The structural basis of ribosome activity in peptide bond synthesis. *Science*. 2000; 289:920–930. [PubMed: 10937990]
8. Lucent D, Snow CD, Aitken CE, Pande VS. Non-bulk-like solvent behavior in the ribosome exit tunnel. *PLoS Comput Biol*. 2010; 6:e1000963. [PubMed: 20975935]
9. Seidelt B, Innis CA, Wilson DN, Gartmann M, Armache JP, Villa E, Trabuco LG, Becker T, Mielke T, Schulten K, Steitz TA, Beckmann R. Structural insight into nascent polypeptide chain-mediated translational stalling. *Science*. 2009; 326:1412–1415. [PubMed: 19933110]
10. Bhushan S, Gartmann M, Halic M, Armache JP, Jarasch A, Mielke T, Berninghausen O, Wilson DN, Beckmann R. α -Helical nascent polypeptide chains visualized within distinct regions of the ribosomal exit tunnel. *Nat Struct Mol Biol*. 2010; 17:313–317. [PubMed: 20139981]
11. Bhushan S, Meyer H, Starosta AL, Becker T, Mielke T, Berninghausen O, Sattler M, Wilson DN, Beckmann R. Structural basis for translational stalling by human cytomegalovirus and fungal arginine attenuator peptide. *Mol Cell*. 2010; 40:138–146. [PubMed: 20932481]
12. Tu LW, Deutsch C. A folding zone in the ribosomal exit tunnel for Kv1.3 helix formation. *J Mol Biol*. 2010; 396:1346–1360. [PubMed: 20060838]
13. Lu J, Deutsch C. Secondary structure formation of a transmembrane segment in Kv channels. *Biochemistry*. 2005; 44:8230–8243. [PubMed: 15938612]
14. Lu J, Deutsch C. Folding zones inside the ribosomal exit tunnel. *Nat Struct Mol Biol*. 2005; 12:1123–1129. [PubMed: 16299515]
15. Lu J, Hua Z, Kobertz WR, Deutsch C. Nascent peptide side chains induce rearrangements in distinct locations of the ribosomal tunnel. *J Mol Biol*. 2011; 411:499–510. [PubMed: 21663746]
16. Woolhead CA, McCormick PJ, Johnson AE. Nascent membrane and secretory proteins differ in FRET-detected folding far inside the ribosome and in their exposure to ribosomal proteins. *Cell*. 2004; 116:725–736. [PubMed: 15006354]
17. Lin PJ, Jongsma CG, Liao S, Johnson AE. Transmembrane segments of nascent polytopic membrane proteins control cytosol/ER targeting during membrane integration. *J Cell Biol*. 2011; 195:41–54. [PubMed: 21949411]
18. Daniel CJ, Conti B, Johnson AE, Skach WR. Control of translocation through the Sec61 translocon by nascent polypeptide structure within the ribosome. *J Biol Chem*. 2008; 283:20864–20873. [PubMed: 18480044]
19. Woolhead CA, Johnson AE, Bernstein HD. Translation arrest requires two-way communication between a nascent polypeptide and the ribosome. *Mol Cell*. 2006; 22:587–598. [PubMed: 16762832]
20. Matheisl S, Berninghausen O, Becker T, Beckmann R. Structure of a human translation termination complex. *Nucleic Acids Res*. 2015; 43:8615–8626. [PubMed: 26384426]
21. Wilson DN, Arenz S, Beckmann R. Translation regulation via nascent polypeptide-mediated ribosome stalling. *Curr Opin Struct Biol*. 2016; 37:123–133. [PubMed: 26859868]
22. Deutsch C. Tunnel Vision: Insights from Biochemical and Biophysical Studies. 2014:61–86.
23. Kosolapov A, Deutsch C. Tertiary interactions within the ribosomal exit tunnel. *Nat Struct Mol Biol*. 2009; 16:405–411. [PubMed: 19270700]
24. Tu L, Khanna P, Deutsch C. Transmembrane segments form tertiary hairpins in the folding vestibule of the ribosome. *J Mol Biol*. 2014; 426:185–198. [PubMed: 24055377]
25. Nilsson OB, Hedman R, Marino J, Wickles S, Bischoff L, Johansson M, Muller-Lucks A, Trovato F, Puglisi JD, O'Brien EP, Beckmann R, von HG. Cotranslational Protein Folding inside the Ribosome Exit Tunnel. *Cell Rep*. 2015; 12:1533–1540. [PubMed: 26321634]
26. Marino J, von HG, Beckmann R. Small protein domains fold inside the ribosome exit tunnel. *FEBS Lett*. 2016; 590:655–660. [PubMed: 26879042]
27. Gloge F, Becker AH, Kramer G, Bukau B. Co-translational mechanisms of protein maturation. *Curr Opin Struct Biol*. 2014; 24:24–33. [PubMed: 24721450]
28. Lin KF, Sun CS, Huang YC, Chan SI, Koubek J, Wu TH, Huang JJ. Cotranslational protein folding within the ribosome tunnel influences trigger-factor recruitment. *Biophysical Journal*. 2012; 102:2818–2827. [PubMed: 22735532]
29. Ito, K., editor. *Regulatory Nascent Polypeptides*. Springer; Tokyo: 2014.

30. Pool MR. A trans-membrane segment inside the ribosome exit tunnel triggers RAMP4 recruitment to the Sec61p translocase. *J Cell Biol.* 2009; 185:889–902. [PubMed: 19468070]
31. Zhang G, Ignatova Z. Folding at the birth of the nascent chain: coordinating translation with co-translational folding. *Curr Opin Struct Biol.* 2011; 21:25–31. [PubMed: 21111607]
32. Robinson PJ, Findlay JE, Woolhead CA. Compaction of a prokaryotic signal-anchor transmembrane domain begins within the ribosome tunnel and is stabilized by SRP during targeting. *J Mol Biol.* 2012; 423:600–612. [PubMed: 22867705]
33. Zhang G, Hubalewska M, Ignatova Z. Transient ribosomal attenuation coordinates protein synthesis and co-translational folding. *Nat Struct Mol Biol.* 2009; 16:274–280. [PubMed: 19198590]
34. Zhou M, Guo J, Cha J, Chae M, Chen S, Barral JM, Sachs MS, Liu Y. Non-optimal codon usage affects expression, structure and function of clock protein FRQ. *Nature.* 2013; 495:111–115. [PubMed: 23417067]
35. Peterson JH, Woolhead CA, Bernstein HD. The conformation of a nascent polypeptide inside the ribosome tunnel affects protein targeting and protein folding. *Mol Microbiol.* 2010; 78:203–217. [PubMed: 20804452]
36. Daggett V, Fersht AR. Is there a unifying mechanism for protein folding? *Trends in Biochemical Sciences.* 2003; 28:18–25. [PubMed: 12517448]
37. Creamer TP, Rose GD. Side-chain entropy opposes alpha-helix formation but rationalizes experimentally determined helix-forming propensities. *Proc Natl Acad Sci U S A.* 1992; 89:5937–5941. [PubMed: 1631077]
38. Long SB, Tao X, Campbell EB, MacKinnon R. Atomic structure of a voltage-dependent K⁺ channel in a lipid membrane-like environment. *Nature.* 2007; 450:376–382. [PubMed: 18004376]
39. Long SB, Campbell EB, MacKinnon R. Crystal structure of a mammalian voltage-dependent Shaker family K⁺ channel. *Science.* 2005; 309:897–903. [PubMed: 16002581]
40. Lu J, Deutsch C. Regional discrimination and propagation of local rearrangements along the ribosomal exit tunnel. *J Mol Biol.* 2014; 426:4061–4073. [PubMed: 25308341]
41. Bright JN, Sansom MSP. The flexing/twirling helix: exploring the flexibility about molecular hinges formed by proline and glycine motifs in transmembrane helices. *J Phys Chem B.* 2003; 107:627–636.
42. Cordes FS, Bright JN, Sansom MS. Proline-induced distortions of transmembrane helices. *J Mol Biol.* 2002; 323:951–960. [PubMed: 12417206]
43. Hessa T, Kim H, Bihlmaier K, Lundin C, Boekel J, Andersson H, Nilsson I, White SH, von Heijne G. Recognition of transmembrane helices by the endoplasmic reticulum translocon. *Nature.* 2005; 433:377–381. [PubMed: 15674282]
44. Hendsch ZS, Tidor B. Do salt bridges stabilize proteins? A continuum electrostatic analysis. *Protein Sci.* 1994; 3:211–226. [PubMed: 8003958]
45. Marqusee S, Baldwin RL. Helix stabilization by Glu-... Lys⁺ salt bridges in short peptides of de novo design. *Proc Natl Acad Sci USA.* 1987; 84:8898–8902. [PubMed: 3122208]
46. Wang WZ, Lin T, Sun YC. Examination of the folding of a short alanine-based helical peptide with salt bridges using molecular dynamics simulation. *J Phys Chem B.* 2007; 111:3508–3514. [PubMed: 17388513]
47. Dzubielia J. Salt-specific stability and denaturation of a short salt-bridge-forming alpha-helix. *J Am Chem Soc.* 2008; 130:14000–14007. [PubMed: 18821757]
48. Tu L, Wang J, Deutsch C. Biogenesis of the T1-S1 linker of voltage-gated K⁺ channels. *Biochemistry.* 2007; 46:8075–8084. [PubMed: 17567042]
49. Wilson DN, Beckmann R. The ribosomal tunnel as a functional environment for nascent polypeptide folding and translational stalling. *Curr Opin Struct Biol.* 2011; 21:274–282. [PubMed: 21316217]
50. Yellen G. The voltage-gated potassium channels and their relatives. *Nature.* 2002; 419(6902):35–42. [PubMed: 12214225]
51. Tiwari-Woodruff SK, Schulteis CT, Mock AF, Papazian DM. Electrostatic interactions between transmembrane segments mediate folding of *Shaker* K⁺ channel subunits. *Biophysical Journal.* 1997; 72:1489–1500. [PubMed: 9083655]

52. Papazian DM, Shao XM, Seoh SA, Mock AF, Huang Y, Wainstock DH. Electrostatic interactions of S4 voltage sensor in Shaker K⁺ channel. *Neuron*. 1995; 14:1293–1301. [PubMed: 7605638]
53. Pless SA, Galpin JD, Niciforovic AP, Ahern CA. Contributions of countercharge in a potassium channel voltage-sensor domain. *Nat Chem Biol*. 2011; 7:617–623. [PubMed: 21785425]
54. Young WS, Brooks CL III. A microscopic view of helix propagation: N and C-terminal helix growth in alanine helices. *J Mol Biol*. 1996; 259:560–572. [PubMed: 8676388]
55. Hummer G, Garcia AE, Garde S. Conformational diffusion and helix formation kinetics. *Phys Rev Lett*. 2000; 85:2637–2640. [PubMed: 10978126]
56. Zangi R, Kovacs H, van Gunsteren WF, Johansson J, Mark AE. Free energy barrier estimation of unfolding the alpha-helical surfactant-associated polypeptide C. *Proteins*. 2001; 43:395–402. [PubMed: 11340656]
57. Monticelli L, Tieleman DP, Colombo G. Mechanism of helix nucleation and propagation: microscopic view from microsecond time scale MD simulations. *J Phys Chem B*. 2005; 109:20064–20067. [PubMed: 16853593]
58. Chandy KG, Gutman GA. Voltage-Gated Potassium Channel Genes. *Ligand- and Voltage-Gated Ion Channels*. 1995:1–71.
59. Voorhees RM, Fernandez IS, Scheres SH, Hegde RS. Structure of the mammalian ribosome-Sec61 complex to 3.4 Å resolution. *Cell*. 2014; 157:1632–1643. [PubMed: 24930395]
60. Bornemann T, Jockel J, Rodnina MV, Wintermeyer W. Signal sequence-independent membrane targeting of ribosomes containing short nascent peptides within the exit tunnel. *Nat Struct Mol Biol*. 2008; 15:494–499. [PubMed: 18391966]
61. Luo P, Baldwin RL. Interaction between water and polar groups of the helix backbone: an important determinant of helix propensities. *Proc Natl Acad Sci USA*. 1999; 96:4930–4935. [PubMed: 10220396]
62. Padmanabhan S, Baldwin RL. Tests for helix-stabilizing interactions between various nonpolar side chains in alanine-based peptides. *Protein Sci*. 1994; 3:1992–1997. [PubMed: 7703846]
63. Padmanabhan S, Baldwin RL. Helix-stabilizing interaction between tyrosine and leucine or valine when the spacing is $i, i + 4$. *J Mol Biol*. 1994; 241:706–713. [PubMed: 8071994]
64. Creamer TP, Rose GD. Interactions between hydrophobic side chains within alpha-helices. *Protein Sci*. 1995; 4:1305–1314. [PubMed: 7670373]
65. Tu L, Wang J, Helm A, Skach WR, Deutsch C. Transmembrane biogenesis of Kv1.3. *Biochemistry*. 2000; 39:824–836. [PubMed: 10651649]
66. Flanagan JJ, Chen JC, Miao Y, Shao Y, Lin J, Bock PE, Johnson AE. Signal recognition particle binds to ribosome-bound signal sequences with fluorescence-detected subnanomolar affinity that does not diminish as the nascent chain lengthens. *J Biol Chem*. 2003; 278:18628–18637. [PubMed: 12621052]
67. Noriega TR, Tsai A, Elvekrog MM, Petrov A, Neher SB, Chen J, Bradshaw N, Puglisi JD, Walter P. Signal recognition particle-ribosome binding is sensitive to nascent chain length. *J Biol Chem*. 2014; 289:19294–19305. [PubMed: 24808175]
68. Pechmann S, Chartron JW, Frydman J. Local slowdown of translation by nonoptimal codons promotes nascent-chain recognition by SRP in vivo. *Nat Struct Mol Biol*. 2014; 21:1100–1105. [PubMed: 25420103]
69. Pace CN, Scholtz JM. A helix propensity scale based on experimental studies of peptides and proteins. *Biophysical Journal*. 1998; 75:422–427. [PubMed: 9649402]
70. Creighton, TE. *Proteins*. W.H. Freeman and Company; New York: 1993. Conformational Properties of Polypeptide Chains; p. 171-199.

Highlights

1. The N-terminus of the transmembrane segment S2, initially an extended nascent peptide, can be induced to form a helix in the ribosome by a single remote S2 mutation.
2. The N-terminus of S2 forms a helix before it completely exits the ribosome folding vestibule.
3. Helix formation involves interactions between the mid-region of the S2 helical sequence, its N-terminus, and the tunnel at specific locations.
4. A Trp-Phe residue pair in the mid-region of the S2 helical sequence functions in a repressor-like mode to govern helix formation.

Figure 1.

Kv1.3 segments in the ribosome exit tunnel. **A.** Structure of the monomer protein of Kv1.2/2.1 channel (PDB ID 2R9R), taken from the crystal structure of the tetrameric mature channel³⁸. It has six trans-membrane segments, S1–S6, a pore helix (PH), a cytosolic tetramerization domain (T1), and an extended linker between T1 and S1 (T1-S1). This channel is highly sequence homologous to Kv1.3. **B.** Schematic of the secondary structure of transmembrane segments (red) of Kv nascent peptide in the ribosome exit tunnel (blue lines). The tunnel, delimited by a vertical red double-arrow, is 100Å long and contains the Kv nascent peptide. The peptidyl transferase center (PTC) is at the entrance to the tunnel and a folding vestibule (near the exit port) are indicated. The peptide's N-terminus is in the vestibule and its C-terminus resides in the middle of the tunnel. Transmembrane sequences S2 or S_x (where x is one of S1, S3–S6) are depicted at a tunnel location where the segment straddles the vestibule and a more downstream region of the tunnel. S2 is shown as extended, whereas S_x is compact (likely α-helical), each consistent with previously determined pegylation data¹².

Figure 3.

Tryptophan is a dominant determinant of helix formation in the N-terminus of S2. **A.** Fraction of nascent peptide pegylated at 244C for S2 peptides with mutated WF amino acids. Data are means $\pm$ SEM ($n = 3$). **B.** Fractional helix propensity, relative hydrophobicity, and side-chain volume of residues. P_{HP} , the average helix propensity scaled from 0 to 1 (Ala to Gly), was calculated from experimental studies of proteins and peptides⁶⁹. Relative hydrophobicity was calculated as free energy, ΔG (kcal/mol), using the Wimley-White scale (http://blanco.biomol.uci.edu/hydrophobicity_scales.html). Volume is given as the volume ($\text{\AA}^3$) enclosed by van der Waals radius⁷⁰. Glycine (G) is included as a calibration for the lower limits for helix propensity and van der Waals volume.

Figure 4. Perturbed communication and salt-bridge stabilization each induce helix formation. **A.** The primary sequence of nascent peptide attached to the PTC at residue 276 with either an inserted SV linker (blue **SV**, top), a proline mutation at residue 249 (magenta **P**, middle), or an arginine mutation at residue 243 (green **R**, bottom). The filled circles above selected amino acids represent residues mutated, one at a time, to cysteine. **B.** Fraction of nascent peptide pegylated with PEG-MAL for the indicated number of amino acids from the PTC site to (and including) the labeled cysteine (PTC-Cys Distance (aa)). Data were analyzed as described in Figure 2 and are means $\pm$ SEM (n = 3).

Figure 5.

Elongation-induced helix formation of the S2 N-terminus. **A.** The primary sequences of nascent peptides attached to the PTC at residues 276, 278, 280, or 282. Residues mutated to Cys are colored as indicated in C. **B.** Pegylation of nascent peptides. Nascent peptides were pegylated with PEG-MAL at the indicated reporter Cys (above gels) and indicated times and then fractionated on polyacrylamide gels, as described in Fig. 2B. **C.** Fraction of nascent peptide pegylated. As described in Figure 2, fraction pegylated is plotted for extended tape measure (black circles), S2 (red circles, restriction site 276), and relocated S2 in the tunnel (restriction sites at 278, 280, 282). Data are means $\pm$ SEM (n = 3).