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Regional Discrimination and Propagation of Local Rearrangements along the Ribosomal Exit Tunnel

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Abstract

All proteins, from bacteria to man, are made in the ribosome and are elongated, one residue at a time, at the peptidyl transferase center. This growing peptide chain wends its way through the ribosomal tunnel to the exit port, ~100 Å from the peptidyl transferase center. We have identified locations in the tunnel that sense and respond to single side chains of the nascent peptide to induce local conformational changes. Moreover, side-chain sterics and rearrangements deep in the tunnel influence the disposition of residues 45 Å away at the exit port and are consistent with side-chain-induced axial retraction of the peptide backbone. These coupled responses are neither haphazard nor uniform along the tunnel. Rather, they are confined to discriminating zones in the tunnel and are sequence specific. Such discerning communication may contribute to folding events and mechanisms governing sequence-specific signaling between different regions of the tunnel during translation.

Keywords

photocrosslinking; cysteine accessibility; ribosomal nascent peptide interactions; translation; allosteric rearrangements

Introduction

Communication within or between molecules is an essential attribute of living systems. The properties of this type of signaling are optimized for the particular needs of the system. While some are designed for ultrafast responses (picoseconds to microseconds) or supersensitivity, for example, one photon, others are tailored for life in the slow lane, for example, transporters (milliseconds to minutes). The ribosome–peptide complex is no less in need of signaling devices during translation, a process that is precisely honed for decoding, peptide bond formation, and movement of the elongating peptide chain over a distance of 100 Å. The molecular mechanisms mediating such communications are largely unknown but must involve crosstalk between the nascent peptide and its host, the ribosome exit tunnel.

This molecular corridor is heterogeneous (rRNA, ribosomal proteins, water, ions) and constitutes a specialized microenvironment for executing functions (e.g., peptide transit, folding, targeting, co-factor recruitment, and degradation), in accordance with the chemistry

of the nascent peptide and needs of the cell. To do this, the ribosome evolved sensors and coupling mechanisms. The nascent peptides themselves contain molecular barcodes, sequences whose order, precise spacing, and nature of specific amino acids can be used to transmit information to an appropriately matched molecular reader, thus providing the basis for diverse signaling [1–7]. Barcodes, however, are only as good as their co-evolved ribosomal sensors, which can be exquisitely discriminating [8–10]. Moreover, sensor–peptide interactions within the tunnel induce conformational changes at the peptidyl transferase center (PTC) and modulate peptide elongation, termination, recruitment of factors mediating targeting, folding, translocase activity, and mRNA degradation [1–4,9,11–26].

Since there is little wiggle room for a nascent peptide along much of the length of the ribosomal tunnel, each amino acid side chain may have a preferred microenvironment [10,27]. In the mid-tunnel region (18–22 residues from the PTC), the solvent-accessible volume in the immediate vicinity of a given peptide residue increases with the van der Waals volume of the adjacent side chain [10], consistent with a relocation and/or reorientation of the peptide relative to the tunnel that depends on the nature of the primary sequence of the nascent peptide. The tunnel walls may rearrange locally in response to a long side chain on the nascent peptide, and/or the peptide itself may alter its disposition and torsional angles to compensate for the confined space. Such gyrations are consistent with structural implications that a nascent peptide, in some locations in the tunnel, might populate a multiplicity of conformations [28], thus modulating a peptide's microscopic trajectory along the tunnel [29]. Here, we explore these possibilities using photocrosslinking and pegylation techniques to demonstrate that side-chain-dependent rearrangements, both short range and long range, occur but only at certain regions of the tunnel. This regional discrimination likely plays a critical role in signaling events during chain elongation and can explain differences in the length dependence of folding of different nascent chains [30–32].

Results

Local peptide rearrangement

In general, modification rates of an introduced cysteine report on accessibility. Previously, we showed that the reactivity (accessibility) of a cysteine engineered into a nascent peptide housed within the ribosomal exit tunnel depends on the volume of the adjacent residue's side chain [10]. Larger adjacent side chains enhance the modification rate of the cysteine, as if they increase the solvent-accessible volume around the cysteine side chain. These results lead to the prediction that larger adjacent side chains in the tunnel would decrease the proximity of neighboring components of the ribosomal tunnel to an introduced cysteine. To test this prediction, we used a photocrosslinking approach. We initially engineered a cysteine at a distance 24 amino acids from the PTC (i.e., PTC 24; we designate distance from the PTC as PTC) in the background of a cysteine-free construct and translated it in a cell-free rabbit reticulocyte lysate system. The construct was also engineered to lack a stop codon and thus remains attached to the tRNA and the ribosome as a stable biogenic intermediate. The peptide we used is an all-extended molecular tape measure previously demonstrated to accurately predict the distance along the tunnel [33]. We reacted the introduced cysteine of

this biogenic intermediate (nascent chain–ribosome complex) with azidophenacyl bromide (APB; Fig. 1), which generates a reactive nitrene upon irradiation with UV light and will crosslink to nearby proteins and/or ribosomal RNA. Subsequent treatment of the irradiation products with RNase removes all RNA, and fractionation by LDS-PAGE then reveals the parent tape measure and any crosslinked protein products. Since only the nascent peptide is radioactive, protein bands of higher apparent mass than the nascent peptide must include nascent peptide crosslinked to ribosomal protein. To assess the efficiency of APB labeling, we used a mass-tagging strategy, pegylation [34], and evaluated the ability of the cysteine to be labeled by PEG-MAL. In the absence of APB, the nascent chain cysteine is available and gets pegylated, manifested a gel shift of ~10 kDa. The fraction pegylated (F_{peg}) is 0.86, which reflects maximal pegylation efficiency. However, following APB labeling, F_{peg} is <2%, consistent with nearly complete prior labeling of cysteine with APB. Crosslinking thus reflects the microenvironment and not APB-labeling efficiency. Figure 1 shows that UV irradiation of the cysteine (PTC 24) produces three crosslinked products, the masses of which are consistent with crosslinking to three specific ribosomal proteins, L39 (5.5 kDa), L17 (20 kDa), and L4 (47 kDa) [9,35,36]. In the absence of APB (with irradiation), in the absence of irradiation of an APB-modified peptide, or in the presence of a non-azido phenacyl bromide (PB), <2% crosslinking bands were detected. APB treatment and UV irradiation of a cysteine-free tape measure gave <5% background bands, which did not overlap with L4, L17, or L39, indicating a minimum contribution from endogenous ribosomal cysteines crosslinking to our radioactive nascent peptide at this tunnel location. In all experiments for PTC 18, PTC 20, and PTC 24, a corresponding cysteine-free control construct was simultaneously tested in a side-by-side comparison and subtracted from experimental values. Two additional controls were documented. First, total nascent peptide in samples irradiated in the presence or absence of APB was unchanged. Second, the integrity of the ribosome after irradiation is not disturbed, as assessed by accessibility of a reporter cysteine (pegylation) at PTC 33 and PTC 27.

We compared the crosslinking results for the APB-labeled reporter cysteine adjacent to either alanine (A) or tryptophan (W). If the larger W leads to rearrangement of the peptide relative to the tunnel [10], then we predict that this will be manifested as a change in location of the reporter cysteine and therefore its ability to crosslink to ribosomal proteins. Because an introduced W apparently increases reaction volume around an adjacent cysteine [10], we predict that it will decrease total crosslinking (P_{xlink}) and might also cause a change in mass of crosslinked peptide, suggesting that the nitrene has found another protein target. Besides an increase in distance between the nitrene and its potential targets, an increase in aqueous accessible volume in the vicinity of the cysteine will also more efficiently quench the reactive nitrene and decrease the extent of crosslinking.

For constructs with reporter cysteines at PTC 18, PTC 20, PTC 24, PTC 27, and PTC 32, with adjacent small (A, S) or large (W) side chains (Fig. 2a), irradiation of APB-modified cysteines produces location-dependent photocrosslinking along the tunnel (Fig. 2b). As predicted, larger adjacent side chains reduce total crosslinking, especially at locations deeper within the tunnel. Ribosomal proteins L4 and L17 project into the tunnel at the “constriction”, which is located deep in the tunnel, ~25–30 Å from the PTC; L39 is more distally located near the exit port [37]. The relative amounts of crosslinked peptide that are

crosslinked to L4, L17, and L39 at the indicated PTC locations (Fig. 2c) suggest that there is a decrease in crosslinking to L17 with increasing distance from the PTC, whereas crosslinking to L39 is increased. These crosslinking results are consistent with the cysteine being less accessible to the L17 and more accessible to L39 as the cysteine is positioned further from the PTC and closer to the exit port. Crosslinking to L4 changes little along the tunnel until the cysteine reaches the folding vestibule, the exit port and beyond (PTC 27 and PTC 32). UV-induced crosslinking to L4 has previously been shown to be relatively insensitive to repositioning of an azido-bearing residue [9,35,36]. Short-range consequences of adjacent side chain (W *versus* A) are manifested at PTC 18 and PTC 20 and agree with results obtained from kinetics of modification with small cysteine-modifying reagents [10], consistent with a local peptide rearrangement relative to the tunnel.

Long-range peptide rearrangements

Do these local rearrangements propagate to more distal locations, that is, can differences in side-chain sterics deep in the tunnel influence the disposition of residues at the exit port? Such a mechanism could provide a basis for communication along the tunnel. To explore this possibility, we moved the adjacent reporter cysteine from a PTC 20 to a PTC 33 (Fig. 3a), thereby situating our reporter cysteine at a location near the exit port that is sensitive to altered axial distance in the tunnel [33,38]. Downstream, at PTC 19, we introduced either an A or a W. The approximate distance between the side chain at PTC 19 and PTC 33 is 45 Å. Pegylation of each of these peptides demonstrates that the reporter cysteine is maximally modified ($F_{\text{peg}} > 0.75$) when A is present but less so in the W-containing peptide and intermediate between these for the native phenylalanine (F)-containing peptide (Fig. 3b), suggesting that peptide rearrangements are transmitted at least as far as 45 Å along the tunnel. Moreover, our results indicate a high sensitivity to a single W side chain because, although multiple consecutive glycines can reduce the persistence length of a peptide or nascent chain [38], a single W alone is capable of achieving a similar end. To accentuate these side-chain-dependent changes in accessibility, we substituted three consecutive tryptophans (3W) at locations PTC 17–19 and compared the F_{peg} to that for three consecutive alanines (3A; Fig. 3c). There is a dramatic decrease in accessibility for the reporter cysteine when 3W are present. F_{peg} is 0.49 ± 0.03 ($n = 3$) for 3W and is 0.79 ± 0.03 ($n = 3$) for 3A.

The two most likely mechanisms for these long-range effects are (i) relocation of the peptide backbone and (ii) reorientation of the cysteine side chain, in response to mutations of the residue at PTC 19. The first category includes a kink-induced axial retraction (or tilt) of the peptide backbone (Fig. 4a) or backbone rotation. Evidence for a kink has been implicated from previous kinetic studies [10] and from the decrease in photocrosslinking at PTC 20 when the C-terminal adjacent residues are mutated to bulky side chains, for example, P_{link} for 1S, 1W at PTC 19, and 3W at PTC 17–19 are 0.56 ± 0.01 , 0.39 ± 0.01 , and 0.12 ± 0.01 , respectively ($n = 2$). Assuming that the decreased P_{link} is due to a change in proximity to ribosomal protein, these values are consistent with a propagated change in conformation. The second possible mechanism underlying effects at PTC 33 includes a change in isomer angle, which also changes the distance between the thiol side-chain group and the PTC. To distinguish between these two scenarios, we compared pegylation, one at a

time, of consecutive cysteines engineered a distance of 31–36 residues from the PTC, in constructs containing either 1A at location PTC 19 or 3W at location PTC 17–19. Backbone axial retraction predicts that each of these cysteines will exhibit a decreased F_{peg} [33] for 3W at PTC 17–19, whereas the other scenarios would not be expected to have the same effect for each consecutive cysteine reporter. F_{peg} is reduced significantly at each location ($P < 0.004$; Fig. 4b), consistent with a kink-induced axial retraction (or tilt) of the peptide backbone. Moreover, the plot of pegylation *versus* PTC suggests a parallel shift of the curve to the right when 3W is present downstream in the nascent chain, and the slopes of the two regression lines are not significantly different (t -test, $P = 0.19$). Thus, at least 36 amino acids are required for a modifiable cysteine in 3W to fully emerge from the ribosomal tunnel (maximum $F_{\text{peg}} \sim 0.8$). This is consistent with an axial retraction of ~ 6 – 9 Å. The outlier, residue 34, in the 3W construct likely indicates additional side-chain rearrangement (cysteine rotamer) at the new position in the tunnel.

To investigate whether W *versus* A induces distal rearrangement at all locations along the tunnel, we repositioned W and A side chains at three locations in the tunnel (Fig. 5a) that were previously shown to differentially discriminate side chains (locally) in the nascent peptide [10]. Substitution of 1W into a nascent peptide at its mid-tunnel location (PTC 19) causes more dramatic distal rearrangements than substitution into regions either closer to the exit port (PTC 27) or at the constriction site (PTC 12; Fig. 5b). At all locations, single alanine substitution yields near maximal pegylation of the tape measure cysteine at PTC 33 ($F_{\text{peg}} = 0.77 \pm 0.02$, $n = 11$, for all three tunnel locations; Fig. 5c). In contrast, a single W at location PTC 19 causes a significant decrease in F_{peg} compared to A ($P < 0.001$; asterisks). Consecutive 3W, regardless of tunnel location, produces a marked decrease in pegylation ($F_{\text{peg}} = 0.51 \pm 0.03$, 0.49 ± 0.03 , and 0.58 ± 0.03 , respectively, at PTC 10–12, PTC 17–19, and PTC 25–27).

What are the boundaries for sensitivity? A W/A swap at 19 is sensitive, yet a swap at 27 is not. Tryptophan substitution at the intervening position PTC 22 gives F_{peg} for the cysteine reporter at PTC 33 of 0.65 ± 0.02 ($n = 3$) compared to F_{peg} of 0.80 ± 0.02 ($n = 3$) for alanine. These results suggest that midgut sensitivity extends at least to residue 22, thus giving a long-range profile that mimics side-chain-induced local rearrangement in the tunnel midgut [10] (Fig. 2).

Our data show a long-distance communication between a cysteine at PTC 33 and residues at the mid-tunnel location. The propagation of this information along the tunnel likely depends on the conformation of the nascent peptide in this region of the tunnel and thus on peptide residues. We therefore examined the roles of peptide residues between PTC 33 and PTC 19. To this end, we created three chimeric constructs (Fig. 6a; compare with Fig. 5a). The first construct, a “Relocation” construct, addresses whether sensitivity/insensitivity is due to specific amino acid sequences. In the Relocation construct, eight residues were inserted into the C-terminus of the nascent peptide (Fig. 6a, boldfaced residues in the “Relocation” construct; compare with Fig. 5a), thereby shifting the sequence distally toward the exit port. The consequence of this insertion is that residues at the sensitive mid-tunnel location were relocated nearer to the exit port, while residues at the insensitive location near the constriction were relocated to the mid-tunnel. In addition, we re-engineered the single

cysteine to maintain its reporter location at PTC 33. Pegylation of Cys 33 for a construct with a W *versus* A at PTC 19 yields F_{peg} values of 0.56 ± 0.01 ($n = 3$) and 0.69 ± 0.04 ($n = 3$), respectively ($P = 0.004$). This region remains sensitive to a W/A swap, suggesting that, here at the midgut, tunnel location and not sequence dominates. However, a different scenario obtains at PTC 27. Pegylation of this peptide yields F_{peg} of 0.74 ± 0.03 ($n = 3$) when the residue at PTC 27 is an alanine and of 0.65 ± 0.02 ($n = 3$; $P = 0.017$) when PTC 27 is a tryptophan (Fig. 6b). This region of the tunnel, previously insensitive to W *versus* A at PTC 27 (Fig. 5c), is now sensitive to a W/A swap, which highlights the importance of the specific residues at this location of the tunnel. Therefore, to investigate sequence-dependent determinants of long-range communication, we focused on the consequences of a W/A swap at PTC 27.

Communication could be mediated by a propagated rearrangement of the intervening residues between W/A at PTC 27 and the reporter cysteine at PTC 33 or via rearrangements of tunnel components or both. We investigated these possibilities by swapping the five intervening residues in the sensitive “Relocation” construct (ISGLQ) for those located between the reporter cysteine and W/A at PTC 27 (SGERV) in the original insensitive construct shown in Fig. 5a to produce a chimeric sequence (“Adjacent Swap” construct; Fig. 6a, ISGLQ highlighted in blue). The five intervening residues are adjacent to both the W/A at PTC 27 and the reporter cysteine. Pegylation of this construct indicates that this construct remains insensitive to side chain at PTC 27 [F_{peg} is 0.77 ± 0.02 ($n = 3$) and 0.80 ± 0.02 ($n = 3$), respectively, for W and A at PTC 27]. This suggests that permissive sequences lie further downstream from the residue at PTC 27.

Two downstream motifs were candidates for cooperative modulators of side-chain recognition. The first motif is the proline flanked by large side chains (FPE), which is five residues downstream from the W/A site at PTC 19 in the sensitive construct (Fig. 5a). The second motif is the Q-repeat motif (LQFQTQ), which surrounds position PTC 19. In the former case, proline may be able to kink the peptide in certain locations in the tunnel, thereby influencing the orientation of the nascent chain and nearby side chains. In the latter case, Q has been shown in some sequences to increase the solvent-accessible volume of nearby cysteines in the ribosomal exit tunnel [10,39]. Substitution of FPETLL into the original insensitive construct (tape measure shown in Fig. 5a), five residues away from W/A at PTC 27 (“Distal Swap” construct; Fig. 6A), produced a decreased F_{peg} of Cys 33 when W, rather than A, is present at PTC 27, consistent with conferring side-chain sensitivity at position PTC 27. Mutation of this proline to alanine (FPETLL $\rightarrow$ FAETLL), however, left this construct still sensitive to W/A at PTC 27 (data not shown). It is possible that conferral of side-chain sensitivity by FPETLL substitution was not due to insertion of this sequence but rather to deletion of the Q-repeat sequence at this location. We therefore made a series of mutations in the inserted FPETLL sequence (Fig. 7a, highlighted in blue) to diagnose critical interacting residues. First, we restored the original three Qs (FQEQLQ), which left the construct sensitive for a W/A switch at PTC 27 (Fig. 7b). Therefore the three-Q repeat alone has no effect on the sensitivity for W/A at PTC 27 manifested in the construct containing the downstream LQAQTQ sequence. Thus, for the three remaining non-Q residues, we mutated them pairwise (underlined residues in Fig. 7a) and evaluated the

accessibility of Cys 33. Only LQEQTQ restores the insensitivity for W/A at PTC 27 (Fig. 7b).

These results elucidate two factors that play a role in facilitating a long-range allosteric communication along the exit tunnel, namely, the location of a W/A switch, and the residues further C-terminal to the W/A switch at PTC 27.

Discussion

All life is supported by intricate networks of interactions, within and between molecules. These networks allow for both short-range and long-distance communication on the atomic scale, and these are essential for normal function of living matter. We have focused our attention on such interactions along the ribosomal tunnel. Our results show that mutating a single side chain in a nascent peptide within the tunnel has consequences for the environment around side chains both adjacent to and 45 Å from the mutation.

We used two distinctly different assays of the tunnel–peptide regions, namely, photocrosslinking and accessibility of introduced cysteine residues. Photocrosslinking produces self-consistent results with previous kinetic measurements of accessibility at the same midgut location [10], thus strongly supporting the idea that both assays report on the proximity of the nascent peptide to components of the ribosomal tunnel. We show that local rearrangements may have long-range consequences in the tunnel as individual residues navigate toward the exit port. These side-chain-induced rearrangements are not indiscriminant. Rather, certain regions of the tunnel are more sensitive than others. Moreover, regions of the tunnel both upstream and downstream of a W/A switch can affect the reactivity (accessibility) of a cysteine residue near the exit port.

It is worth considering the unique properties of tryptophans that might contribute to the conformational changes underlying our experimental observations. Besides being the largest natural amino acid, tryptophans prefer to be at aqueous hydrophobic interfaces [40–44]. They may form π – π stacking and π –cation interactions in the ribosome–peptide complex [45] and contribute energetically to the attractive interactions between the peptide and the tunnel walls. The observation that 3W, regardless of its location in the tunnel, decreases F_{peg} of a reporter at the exit port (Fig. 5) may be explained as follows. Perhaps 3W, by contrast to 1W, has an aggregate increased affinity for ribosomal protein and/or RNA. This would provide more driving force for rearrangement, which leaves more solvent surrounding a neighboring cysteine and decreased accessibility for a cysteine at the exit port. This possibility may help explain the potency of 3W *versus* 1W at any location in the tunnel.

What is the nature of the conformational changes induced by a W/A swap? This swap increases solvent-accessible volume in the vicinity of the neighboring residue at PTC 20 and also causes a decreased accessibility of cysteine residues far upstream of the introduced W, at every location between PTC 31 and PTC 36. This is consistent with the tryptophan-induced local rearrangement causing axial retraction of the peptide backbone. The exact nature of the rearrangement is not known, but an attractive possibility is the formation of either a small angle (“kink”) or a large angle (“curving”) folding of the peptide in the

vicinity of the introduced W residues. Besides retraction, other possibilities could underlie the decreased accessibility of residues at PTC 31–36, including a collapse of the tunnel near the exit port, a rotation of the backbone, and/or rotamer switching. The latter two candidates seem less likely than axial retraction because all residues between PTC 31 and PTC 36 exhibit decreased accessibility in response to insertion of 3W at locations PTC 17–19.

Molecular dynamics simulations suggest that disembodied amino acid side chains have different free-energy profiles at different tunnel locations [27]. All regions of the tunnel are not equal, and this has been demonstrated experimentally [10]. In the mid-tunnel region (a distance of 18–22 residues from the PTC, the “midgut”), the location and/or orientation of the peptide relative to the tunnel depends on the nature of the primary sequence of the nascent peptide [10]. However, neither the constriction nor the distal regions closer to the exit port are locally sensitive to W/A swaps (Ref. [10] and this paper). This regional specificity of side-chain sensitivity at short range appears to be recapitulated at long range, over a distance of ~45 Å, as we show here. Short-range effects are propagated, either along the peptide itself and/or in collaboration with tunnel components, to produce the long-range effects detected at the exit port.

Why are there sensitive and insensitive regions of the tunnel vis-à-vis a tunnel of uniform sensitivity or a tunnel that is oblivious to passing side chains? At least two possibilities seem likely. First, regional diversity may provide gates for modulating transit rates along the tunnel, which in turn could be a mechanism contributing to folding events. Second, regional diversity and sequence-dependent gyrations of the peptide/tunnel components may be exploited as signaling devices. Regarding the first possibility, it has long been suggested that nucleotides and ribosomal protein side chains pointing into the lumen of the tunnel could serve as discriminating gates [2,27,46–48]. Both functional measurements and cryo-electron microscopy structures of a variety of arrested peptides reveal direct interactions between the nascent chain and discrete tunnel locations [28,29,37,49,50]. These sites may undergo small conformational changes in response to peptide side chains moving along the tunnel.

Regarding the second possibility, the ribosome–nascent chain complex uses signaling to regulate rates of translation. Precise positioning of A-site and P-site tRNA at the PTC is necessary for efficient peptide bond formation [51,52]. If peptide–tunnel interactions (at a distance from the PTC) propagate to induce slight shifts in relative A-tRNA and/or P-tRNA positions, then this could alter the efficiency of peptide bond formation and underlie the mechanism of translational arrest [25,29,37,52]. Such relay mechanisms appear to occur for several arrest peptides and for antibiotic action [4,25,36,37,50,53]. For one arrest peptide, SecM, the interacting residues, which are not necessarily contiguous, must be displayed at specific positions in three-dimensional space and/or acquire secondary structure to instigate a relay signal and inhibit peptide elongation [3,54].

Small movements can have profound effects. This is especially true for protein domain movements involved in catalysis where fast transitions cannot involve high-energy barriers [55]. Thus, a series of low-energy conformational changes occur during catalysis [56], and asynchronous succession of protein motions occurring in a wave-like manner can produce

dramatic functional consequences [57,58]. Analogous small domino effects are likely along the ribosomal tunnel so that an accumulation of small movements of ribosomal protein, nucleotides, and nascent chain relay significant shifts to the PTC or exit port. Such movements may be initiated and/or propagated by side-chain-dependent rearrangements in the tunnel.

Axial retraction of the nascent chain backbone is one such relay signal. It can modulate efficiency of folding for a given chain length [30,31,59] and thus delay folding until more of the C-terminal segment has been synthesized. Retraction may also inhibit chaperone activity and recruitment of factors involved in peptide modification.

This study reveals a previously undocumented prototype in allosteric communication along the ribosomal exit tunnel. These allosteric effects are neither haphazard nor uniform; rather, they depend specifically on the primary sequence of the nascent peptide and on the location of given side chains within the tunnel. Thus, specific amino acids of the nascent peptide collaborate with tunnel components to transmit information along the tunnel. Our results mainly focus on communication from the middle of the tunnel to a region ~ 50 Å upstream, near the exit port, a region where folding begins in earnest [30,31,59]. This raises the tantalizing possibility that specific downstream amino acids could influence secondary and tertiary folding. Our data support this possibility in that single point mutations can result in retraction of the nascent peptide back into the tunnel.

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 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 [33]. The cysteines engineered one at a time for all experiments are 47C, 48C, 49C, 50C, 51C, 52C, 57C, and 62C in the native Kv1.3. Mutants (A, S, W, C) and chimeras were made using Stratagene's QuikChange site-directed mutagenesis kit. To move 62C closer to the PTC site, we deleted residues from the C-terminus of the 62C 34 tape measure constructs using PCR methods. 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; Amersham) for 1 h at 22 °C in rabbit reticulocyte lysate according to the Promega Protocol and Application Guide.

Pegylation

As described previously [33], 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), and 50 µM 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 (critical requirement) and the pellet was resuspended on ice in 50 µl of buffer containing 100

mM NaCl, 2.5–5 mM Mg²⁺, 50 μM DTT and 20 mM Hepes (pH 7.3). Pegylation of a reporter cysteine (e.g., at PTC 31–36) in a ribosome-attached nascent peptide was carried out by adding an equal volume of 2 mM PEG-MAL and incubating the sample on ice for 3 h. The reaction was quenched by addition of 50 mM DTT [38].

Photocrosslinking

The translation reaction mixture was centrifuged through a sucrose cushion as described above but with no added DTT and the pellet was resuspended in 200–500 μl of 20 mM Hepes buffer (pH 7.3) + 110 mM KOAc + 2.5–5 mM Mg²⁺ and vortexed. In dim light, APB (from stock solution of 200 mM in methanol) was added to a final concentration of 1 mM. These samples were incubated on ice, in the dark for ~30 min. Open tubes, placed 3–4 inches from the light source, were irradiated for 30 min (predetermined as the optimal time/conditions for crosslinking) with UV light (Stratalinker). Samples were centrifuged through 120 μl of sucrose cushion containing 1 mM DTT for 20 min at 70,000 rpm at 4 °C. The pellet was resuspended in 20 μl of 20 mM Hepes buffer with 1% SDS, 50 μg/ml RNase, 100 mM NaCl, and 50 μM DTT and was then incubated for 1 h at room temperature.

Gel electrophoresis and fluorography

All final samples were heated at 70 °C for 10 min in 1× 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 (4-morpholineethanesulfonic acid) running buffer. Gels were soaked in Amplify (Amersham) to enhance ³⁵S fluorography and were 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.

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Abbreviations

PTC	peptidyl transferase center
APB	azidophenacyl bromide

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Fig. 1.

Experimental strategy. Labeling of engineered cysteine [yellow circle; background construct is the all-extended molecular tape measure (red line)] with APB to form an azidophenacyl cysteine. Modified peptides were either pegylated with polyethyleneglycol maleimide (PEG-MAL, molecular weight 5000) to assess APB labeling (bottom gel) or UV irradiated to produce crosslinked adducts (top gel). In the former case, the gel-shifted band represents peptide that had an available thiol (no APB labeling) and was pegylated whereas the lower-molecular-weight band represents unpegylated peptide that lacked an available thiol (APB-labeled). In the gel for the crosslinking assay, the bands in lane 1 display similar mobilities described previously for ribosomal proteins L39, L17, and L4 [9,35,36]. Lane 2, derived from APB-treated peptide in the absence of UV irradiation, shows no crosslinking bands.

Fig. 2.

Crosslinking at different tunnel locations. (a) Experimental design. Left: A series of cysteines was engineered one at a time into an all-extended molecular tape measure (red line) at the indicated PTC distances (in amino acids). Constructs were made by shortening the C-terminus of the peptide to reposition the peptide and decrease PTC. Right: The residue C-terminally adjacent to each cysteine is either a small (alanine, serine, blue circle) or a large (tryptophan, red circle) amino acid. (b) Probability of photocrosslinking, $h\nu P_{xlink}$, which is calculated as $1 - F_0$, where F_0 is the fraction uncrosslinked that is derived from the intensity of the lowermost band on the gel, for example, Fig. 1 (top gel). Asterisks indicate $P < 0.01$ (t -test) for W versus A/S for the indicated PTC. Symbols are means $\pm$ average deviations ($n = 2$). (c) Conditional probabilities. Relative amounts of crosslinked peptide that are crosslinked to L4, L17, and L39 at the indicated locations in the tunnel (ΔPTC). Asterisks indicate $P < 0.03$ (t -test) for W versus A/S for the indicated PTC. Symbols are means $\pm$ average deviations ($n = 2$), except PTC 32 (means $\pm$ SD for triplicates).

Fig. 3.

Long-range side-chain-dependent accessibility in the exit tunnel. (a) Cartoon of experimental strategy. An all-extended tape measure containing A (blue), F (black, native residue), or W (red) at PTC 19 was engineered to have a single cysteine at PTC 33 (S33C), which serves as a reporter of accessibility in the pegylation assay. The fraction pegylated (F_{peg}) decreases monotonically with distance into the tunnel for the last 20 Å near the exit port [33]. Thus, changes in F_{peg} upon substitution at PTC 19 (~45 Å away) indicate long-range rearrangements at PTC 33. (b) Pegylation of cysteine at PTC 33 for 1A, 1F, or 1W at PTC 19. The peptide was synthesized, pegylated, and fractionated on LDS-NuPAGE gels. The lower band (0) in each gel represents unpegylated peptide and the upper band (1) represents singly pegylated peptide at PTC 33. Quantification of F_{peg} is shown as a bar graph. (c) As in (b), for 3W or 3A at PTC 17–19 (replaced residues are T, Q, and F, respectively). Bar graphs in (b) and (c) are color-coded as in (a). Data are means $\pm$ SD for $n = 6, 4$, and 5 for 1W, 1F, and 1A, respectively, and $n = 3$ for 3W and 3A.

Fig. 4.

Cysteine scan. (a) Hypothesis for side-chain-dependent rearrangement in the tunnel. NB: Kink could be anywhere between the cysteine (yellow circle) and the PTC (black bar). There are alternative possibilities (see Discussion). (b) Fraction pegylated at locations PTC 31–36 in the vestibule for nascent peptides containing 1A (blue circles), 3A (white hexagon), and 3W (red circles), as described in Fig. 3. Cysteines for pegylation were substituted one at a time in each construct (G31C, S32C, S33C, D34C, Q35C, and E36C). Samples were treated as described in Fig. 3b. Symbols represent means $\pm$ SD for triplicate samples. The red and blue lines are linear regression fits to the data ($R = 0.8$ and 1.0 , respectively). We used a standard t -test to compare slopes of the two regression lines. The P -value is 0.19 , an underestimate relative to nonparametric tests. Thus, the slopes are not significantly different.

Fig. 5.

Regional sensitivity. (a) Amino acid sequence of the nascent chain (all-extended tape measure) that resides within the tunnel. Boldfaced residues indicate positions substituted with the indicated amino acids. The numbers above each residue indicate the distance from the PTC in amino acids. The reporter cysteine at PTC 33 is highlighted in red. (b) Pegylation of reporter cysteine PTC 33. Peptides with the indicated number of consecutive W or A at indicated positions from the PTC (PTC 10–12, PTC 17–19, or PTC 25–27) were engineered into tape measure backgrounds containing a single cysteine at PTC 33, synthesized, pegylated, and fractionated on NuPAGE gels (upper and lower bands as described in Fig. 3b). (c) Quantification of F_{peg} at PTC 33. Bar graph is color-coded as in Fig. 3a. Data are means $\pm$ SD for $n = 3$ each for 3W, 1W, and 1A at PTC 10–12; $n = 3, 6, 5$, and 3 for 3W, 1W, 1A, and 3A, respectively, at PTC 17–19; and $n = 3$ for 3W, 1W, and 1A, respectively, at PTC 25–27. Asterisks indicate that F_{peg} values for 1W and 1A at PTC 17–19 are significantly different ($P = 0.001$, two-tailed t -test). F_{peg} for 3W at PTC 17–19 is significantly different ($P = 0.02$, two-tailed t -test) from 3W at PTC 25–27.

Fig. 6.

Sequence-dependent sensitivity to side chain. (a) Amino acid sequence of nascent chain (all-extended tape measure) that resides within the tunnel. Boldfaced residues indicate residues inserted between the PTC and the C-terminus of the original tape measure construct shown in Fig. 5a. The numbers above each residue indicate the distance from the PTC in amino acids. A reporter cysteine has been engineered at PTC 33 and is highlighted in red. This construct is referred to as a “Relocation” construct, in order to emphasize that the sequence previously occupying the tunnel as in Fig. 5a has been repositioned to a more distal (toward the exit port) location. The “Adjacent Swap” construct is identical to the original tape measure (Fig. 5a) but with five consecutive adjacent residues (SGERV) substituted with ISGLQ (boldfaced light blue) to mimic the flanking sequence found in the “Relocation” construct, which is sensitive to W/A at location PTC 27. The “Distal Swap” construct is identical to the original tape measure (Fig. 5a) but with six consecutive adjacent residues (LQAQTQ) substituted with FPETLL (boldfaced light blue) to mimic the downstream sequence found in the “Relocation”. (b) Fraction pegylated. Each of the constructs depicted in (a) was synthesized, pegylated at PTC 33, fractionated on NuPAGE gels, and F_{peg} quantified. Bar graph is color-coded as in Fig. 3a. Data are means $\pm$ SD for $n = 3$ for each W/A set of constructs.

Fig. 7.

Sequence-dependent sensitivity of the Distal Swap construct. (a) Amino acid sequence of the Distal Swap construct mutated to the residues indicated in boldface. Boldfaced black residues indicate those found in the original tape measure with an alanine at position 19 (Fig. 5a). Boldfaced blue residues indicate residual amino acids that are identical with those in the Distal Swap construct (Fig. 6a). Underlined residues indicate pairwise mutations back to the original tape measure. (b) Fraction pegylated. Each of the constructs depicted in (a) was synthesized, pegylated at PTC 33, fractionated on NuPAGE gels, and F_{peg} quantified. Bar graph is color-coded as in Fig. 3a. Data are means $\pm$ SD for $n = 3$ for each W/A set of constructs.