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Nascent Peptide Side-chains Induce Rearrangements in Distinct Locations of the Ribosomal Tunnel

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Abstract

Although we have numerous structures of ribosomes, none disclose side-chain rearrangements of the nascent peptide during chain elongation. This study reports for the first time that rearrangement of the peptide and/or tunnel occurs in distinct regions of the tunnel and is directed by the unique primary sequence of each nascent peptide. In the tunnel mid-region, the accessibility of an introduced cysteine to a series of novel hydrophilic maleimide reagents increases with increasing volume of the adjacent chain residue, a sensitivity not manifest at the constriction and exit port. This surprising result reveals molecular movements not yet resolvable from structural studies. These findings map solvent accessible volumes along the tunnel and provide novel insights critical to our understanding of allosteric communication within the ribosomal tunnel, translational arrest, chaperone interaction, folding, and rates of elongation.

Keywords

cysteine accessibility; ribosomal-nascent peptide interactions; translation; allosteric rearrangements; solvent accessible volumes

INTRODUCTION

Protein folding begins in the ribosomal exit tunnel^{1–8}, which is ~80–100Å in length and 10–20 Å in width^{1,9–12}. Because a resident nascent peptide co-occupies the narrow tunnel volume with water and ions, it is a tight squeeze for a nascent peptide along most of the length of the tunnel. Thus the nature of peptide-tunnel interactions may depend on specific peptide side-chains and the tunnel environments they encounter. Side-chains vary with respect to size, hydrophobicity, charge, aromaticity, and flexibility. Molecular dynamics simulations suggest that disembodied amino acid side-chains may have different free energy profiles at different locations in the ribosomal tunnel¹³. To experimentally probe interactions between side-chains in a nascent peptide and the tunnel, we have engineered different side-chains adjacent to a cysteine introduced into specific positions of an all-extended peptide previously shown to accurately measure the operational length of the ribosomal tunnel⁵. We refer to this peptide as a molecular tape measure. The location of this

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target cysteine, either inside or outside the tunnel, was set by engineering the length of the nascent peptide chain between the peptidyl transferase center (PTC) and the cysteine. In each case, we measured the kinetics of cysteine modification with a series of reagents of increasing size: trimethyl-, triethyl-, tripropyl-, and tributylammonium ethylmaleimides. From the kinetics of modification, we calculated the relative accessibility of the target cysteine in specific tunnel locations compared to its accessibility at a location outside the tunnel. Moreover, we have assessed the relative accessibility of each cysteine as a function of the side-chain on the adjacent residue (C-terminal to the cysteine) in the nascent peptide. Our experimental findings suggest that reorientations of either tunnel wall components and/or nascent peptide occur at specific tunnel locations in response to exposure to different side-chains. These rearrangements are tied to the unique primary sequence of each sojourning peptide and begin to map the microscopic tunnel events involved in protein translation. The functional approach used here to probe tunnel-peptide interactions reveals physico-chemical properties of the tunnel-peptide complex not yet available from structural studies and will guide our understanding of the molecular events accompanying chain elongation.

RESULTS

To investigate the accessibility of nascent peptide side-chains in the tunnel, we used a molecular tape measure (attached to the ribosome, Figure 1A), which has been used for mapping the electrostatic potential in the tunnel¹⁴ and assessing secondary structure formation⁵⁻⁷. This molecular tape measure, 95-amino acids long, is composed of the N-terminus of a voltage-gated potassium (Kv) channel, Kv1.3. This N-terminus includes a portion of the T1 sequence that is known to be all-extended in the mature Kv protein^{15, 16} and has been engineered to reside within the ribosomal exit tunnel⁵. We engineered a cysteine into the tape measure (residue 62 according to the native sequence of the tape measure derived from the T1 domain of Kv1.3) and determined the change in accessibility by measuring the kinetics of cysteine modification by a small hydrophilic sulfhydryl reagent with a valence of +1¹⁴. We repeated this measurement at several locations along the tape measure by making a deletion from the C-terminus to shorten the chain by the appropriate number of amino acids, thereby positioning the engineered cysteine a specified number of residues from the PTC (Δ PTC). This series of constructs is shown in Figure 1B. For those cysteines located near the exit port and/or inside the tunnel, the distance in Å can be estimated because the extended tape measure alone constitutes the intervening segment between the cysteine and the PTC⁵.

The small, singly charged (+1) sulfhydryl reagents we chose are maleimides because they have the ideal kinetic and hydrolytic properties for the conditions of our experiments. Maleimides are Michael acceptors that react with an ionized SH group to produce a stable carbon-sulfur bond. We synthesized and characterized trimethyl (TMA)¹⁴, triethyl (TEA), tripropyl (TPA), and tributyl (TBA) ammonium ethylmaleimide, whose structures and volumes are shown in Figure 1C. We began by using the smallest probe, TMA, and a peptide chain length that locates the engineered cysteine outside the tunnel, 74 residues from the PTC (Δ PTC 74). The adjacent C-terminal residue, which is closer to the PTC, was engineered to alanine (Cys(A)). This construct was translated in a rabbit reticulocyte lysate, in the presence of ³⁵S-methionine to produce a radioactively-labeled nascent peptide that remained attached to the ribosome complex as a peptidyl-tRNA¹⁷ and can be detected on a protein gel. The peptide-ribosome complex was isolated and treated with TMA (3 μ M) for durations of 1 to 120 min, quenched, denatured and released from the ribosome, and then treated with a high molecular weight polyethylene glycol maleimide (PEG-MAL, 5kD) for detection on a protein gel (Figure 2A: top row, left gel). The unpegylated peptide migrates as a 17kD protein (0 band) whereas the pegylated peptide is shifted by ~10kD and migrates

more slowly (band 1). With increasing incubation time with TMA, the cysteine becomes modified, thereby rendering it unavailable for subsequent pegylation. This is manifest as a disappearance of band 1 and appearance of band 0. Quantification of the band intensities gives the fraction pegylated (F_{peg}), which is plotted as a function of time (right panel). The data are well fit with a single decaying exponential to give a modification rate constant of $3172 \text{ M}^{-1}\text{s}^{-1}$. A similar experiment was carried out for a Cys(A) construct engineered with a chain length of 20 residues from the PTC. To monitor the kinetics over a similar time period, we used a higher concentration of $20 \mu\text{M}$ TMA. Again, a well-behaved time course was obtained (Figure 2A: bottom row), which gave a modification rate constant of $287 \text{ M}^{-1}\text{s}^{-1}$. This is consistent with our expectation that the environment inside the tunnel is less conducive to cysteine modification^{5, 14}. Proceeding from the exit port into the tunnel, the peptide will encounter narrower surroundings¹ and less water than bulk solution¹⁸. A peptide chain might therefore be entropically constrained in confined regions inside the tunnel. Each of these factors is likely to contribute to a decrease in reactivity of a peptide cysteine to TMA. A comparison of the modification rate inside ($\Delta\text{PTC } 20$, $287 \text{ M}^{-1}\text{s}^{-1}$) to outside ($\Delta\text{PTC } 74$, $3172 \text{ M}^{-1}\text{s}^{-1}$) will approximate the n -fold change in accessibility^{14, 19} (see Materials and Methods) for this residue inside the tunnel. Thus, the accessibility at a location $\sim 60 \text{ \AA}$ from the PTC decreases by 11-fold compared to outside the tunnel, consistent with previous measurements of accessibility inside the tunnel^{5, 14}. We carried out similar measurements using the largest probe molecule, TBA, which has a volume of $\sim 388 \text{ \AA}^3$. As shown in Figure 2B, TBA modifies at a similar rate as TMA outside the tunnel, but at a slower rate inside the tunnel compared to TMA, thus requiring higher reagent concentrations for modification of residues inside the tunnel to obtain similar time courses. The rate constants for TBA modification of Cys(A) were 3067 and $78 \text{ M}^{-1}\text{s}^{-1}$, respectively, for locations $\Delta\text{PTC } 74$ and $\Delta\text{PTC } 20$. The relative accessibility for TBA at a tunnel location that is $60 \text{ \AA}$ from the PTC decreases 35-fold compared to outside the tunnel.

A more extensive study was carried out using both TMA and TBA for several additional locations along the tunnel: 27, 24, 22, 18, 13, 10, and 6 amino acids from the PTC. They are shown in Figure 3A. Likewise, these locations were probed with TMA and TBA for cysteines with an adjacent C-terminal tryptophan (Cys(W)), instead of alanine (Figure 3B). We chose to change the adjacent side-chain on the C-terminal side of the cysteine to avoid the complication of blocking access of the cysteine to the modifying reagent. Regardless of which probe was used, or which neighboring side-chain was present in the nascent peptide, the modification rates decrease with increasing distance into the tunnel from the exit port, i.e., with decreasing ΔPTC . This suggests that the accessibility of a cysteine decreases from exit port to positions deeper in the tunnel. Moreover, the decrease in rate is more marked (curve shifted to the right) for the larger TBA than for TMA, regardless of whether there is an alanine or a tryptophan adjacent to the cysteine. These results suggest a steric component to the rate of modification. Both TMA and TBA exhibit a linear dependence of rates on reagent concentration in the range of concentrations used (data not shown).

The difference between TMA and TBA kinetics can be evaluated more directly by comparing the ratio of rate constants, $k_{\text{TMA}}/k_{\text{TBA}}$, which is shown in Figure 3C for both alanine and tryptophan mutants. A ratio of 1 (dashed line) indicates no significant difference between the two rate constants, which is the case outside the tunnel at a ΔPTC of 74. Due to the preferential slowing of TBA relative to TMA, the ratio increases as the cysteine is relocated to ΔPTCs of 27, 24, 22, 20, 18, and 13. At location $\Delta\text{PTC } 13$, the ratio is almost an order of magnitude greater than that measured outside the tunnel. Upon relocation to position 10, near the constriction in the tunnel^{1, 9–12}, the ratio decreases due to a larger decrease in the rate constant for the smaller reagent, TMA. Possible explanations include sterics, electrostatics, flexibility, and hydrophobicity of the two reagents (see Supplementary Figures 2 and 4). If steric factors are the major contributors to the rate of modification, then

a comparison of the reagents for a given location should reveal a dependence on the volume of the probe. As shown in Figure 3D, there is a rank-order dependence, from smallest to largest, on probe size. These results indicate that when a nascent peptide is in the tunnel, the tunnel is functionally small enough to discriminate probes with volumes of $\sim 200\text{--}400\text{ \AA}^3$.

Are there regions in the tunnel that can discriminate between different side-chains? For example, can the tunnel detect if a bulky tryptophan or a small alanine is passing by? To investigate this possibility, we compared the relative rates of cysteine modification (inside-to-outside the tunnel) for adjacent tryptophan and alanine, and plotted this as relative accessibility ($(k_W/k_{W74})/(k_A/k_{A74})$) for each tunnel location (Figure 4). The dashed line at a relative accessibility of 1 indicates that regardless of whether there is an adjacent tryptophan or an alanine, the relative accessibility of the neighboring cysteine is the same. A value >1 indicates that cysteine accessibility increases when a tryptophan is at the adjacent position. A value of <1 indicates that cysteine accessibility decreases when a tryptophan is at the adjacent position. It is clear from the data in Figure 4 that different tunnel locations have different sensitivities. The relative accessibility at locations ΔPTC 22, 20, and 18 is increased when an adjacent tryptophan is present compared to an adjacent alanine, regardless of which reagent is used to modify the cysteine. In contrast, the constriction and vestibule near the exit port do not discriminate. This is especially unexpected given the prevailing dogma implicating the constriction as a site at which the nascent chains are “read”. We do not know which properties of the tryptophan (versus alanine) are responsible for these phenomena. Tryptophan is larger than alanine, more hydrophobic, and exhibits a substantial quadrupole moment with its electrostatic consequences. Any of these differences between tryptophan and alanine might contribute to the changes we observe in relative modification rates at the neighboring cysteine. Moreover, the different relative rates could be a consequence of the amino acid side-chains on either the nascent peptide itself or on the ribosomal tunnel.

To identify the relevant factors, we investigated a series of C-terminally adjacent residues: serine, glutamine, phenylalanine, and arginine, in addition to the tryptophan and alanine (Figure 5A). This series includes small hydrophilic residues, aromatic residues, and large side-chains that are either charged or uncharged. For each of these, we determined the modification rate constant using both TMA and TBA, and normalized the rate constant to that for alanine, i.e., we calculate k_X/k_A . If this ratio is plotted versus hydrophobicity²⁰, octanol partitioning (http://blanco.biomol.uci.edu/hydrophobicity_scales.html), or surface probability²⁰), there is no correlation (Supplementary Figure 1). However, k_X/k_A plotted against the volume of the adjacent side-chain yields a monotonic dependence on the side-chain volume (Figure 5B). Accessibility increases with increasing volume of the adjacent side-chain. As we observe, the larger TBA is more hindered in a confined space than the smaller TMA. We interpret these results to mean that a relocation and/or reorientation occurs that depends on the nature of the primary sequence of the nascent peptide at this location. Either the peptide (side-chain or backbone) rearranges and/or tunnel components rerearrange to increase accessibility in the tunnel.

DISCUSSION

Although we have numerous structures of ribosomes, systematic functional examinations of tunnel properties are scarce. Here, we report that at the mid-region of the ribosomal exit tunnel, accessibility of a given cysteine in a nascent peptide depends on the primary sequence of the nascent peptide. More specifically, the accessibility in the vicinity of the cysteine increases with increasing volume of the adjacent side-chain. This suggests a wave of altered conformations as the peptide is elongated and progresses through the mid-region of the tunnel. Each nascent peptide carves out its own idiosyncratic route through the tunnel

during chain elongation. Alternatively, we could interpret an increase in relative modification rate for a neighboring tryptophan versus alanine as an increase in affinity of the cysteine reagent for specific regions in the tunnel. Regardless of which of these scenarios we adopt, there is a side-chain dependent rearrangement of the peptide relative to the tunnel. Small peptide-induced localized movements of rRNA have been hypothesized to propagate from the exit tunnel throughout the ribosome to mediate signaling events during translation²¹. Moreover, stalling signals have been suggested to result from tunnel-peptide interactions that induce a specific conformation in the peptide and/or a series of subtle conformational changes in tunnel components¹. Our data provide experimental support for this idea.

Cysteine Reagents in the Tunnel

The rank-order dependence of kinetics on size of our alkylammonium reagents is apparent, but does this reflect their rank-order difference in sterics, distribution of surface electrostatic potential, or hydrophobicity? Three lines of evidence suggest that the distribution of surface electrostatic potential does not play a role, nor does hydrophobicity. First, outside the tunnel in free solution ($\Delta\text{PTC } 74$), the rates of modification by TMA and TBA are similar. The outside environment is maximally hydrophilic and there is no steric restriction. Yet the two probes with different surface potential distributions (Supplementary Figure 2), exhibit similar reactivities. At locations deeper in the tunnel, there is less water compared to bulk solution¹⁸, i.e., a decrease in dielectric constant, an environment that favors TBA over TMA because TBA is more hydrophobic and its surface electrostatic potential is more diffuse. This would predict a faster rate for TBA compared to TMA. The reverse is observed. Second, for both PEG-MAL, a noncharged cysteine reagent, and the charged alkylammonium maleimides, modification rates decrease by an order of magnitude at $\Delta\text{PTC } 20$ compared to outside the tunnel⁵. This argues against electrostatics being the dominant factor. Third, we previously showed that, along the ribosomal tunnel, a non-isopotential gradient of electrostatic potentials exists, which is not monotonic¹⁴, yet modification rates are monotonic along the tunnel between $\Delta\text{PTC } 74$ and $\Delta\text{PTC } 6$. Furthermore, hydrophobicity seems an unlikely cause of the rank-order dependence because the hydrophilic RNA lining dominates along the tunnel and it is difficult to imagine a monotonic decrease in hydrophobicity with increasing distance from the exit port.

Two additional arguments support accessibility, i.e., sterics, not the probability that the cysteine thiol is deprotonated (P_{S-}), as the dominant factor. P_{S-} is a function of the pKa of the thiol and local pH (see Supplementary Material and Data Analysis, Methods and Materials). First, the rank-order decrease in k_{mod} of a target cysteine with increasing size of the reagents conforms to an effect of sterics, not pKa. Second, the effect of the volumes of peptide side-chains on modification rates of a target cysteine is consistent with sterics and not pKa. This is especially true for the case of an adjacent arginine, which would be expected to increase the dielectric constant locally and produce a marked deviation from the curve shown in Figure 5B. This is not the case.

Regional discrimination

Along the 80–100 Å length of the ribosomal exit tunnel, different regions respond differently to peptide side-chains passing by. Two measured parameters, kinetics and final extent (see Supplementary Material) of modification, testify to this differential discrimination. First, the rates of cysteine modification at locations $\Delta\text{PTC } 18$, 20, and 22 increase with increasing volume of adjacent side-chains. This is a surprising observation, because a larger side-chain would displace more water than a smaller side-chain. This would effectively lower the dielectric constant and presumably restrict the volume within which the reagents and the cysteine thiol reside, both of which predict a slower modification rate. However, we

obtained exactly the opposite results. We interpret these findings as compelling evidence for side-chain dependent conformational changes in the nascent peptide and/or the tunnel components in the middle of the tunnel.

Regions at the constriction site (Δ PTC 10 and 13) and proximal to the constriction (Δ PTC 6) do not discriminate between large and small side-chains of the nascent peptide. Nor do more distal locations closer to the exit port or outside the tunnel at Δ PTC 74. These are provocative results because the constriction is thought to be a discriminating gate²². We speculate that regions in the last 20 Å of the tunnel, the folding vestibule previously identified as hosting secondary folding events and intrapeptide hairpin interactions^{7, 23}, are too large to discriminate differences in side-chain volume, and that the constriction is too narrow and rigid to accommodate rearrangements that alter accessibility. This may be functionally important to slow the movement of the elongating peptide and ensure its correct folding in the vestibule.

The second barometer of regional discrimination is the final extent of modification (see Supplementary Material). The fraction of inaccessible cysteines (F_{inacc}) for both TMA and TBA is ≤ 0.05 outside the tunnel and along most of the tunnel, except for a distribution of locations centered around the constriction. The F_{inacc} is 0.1, 0.36, and 0.2 for Δ PTC 13, 10 and 6, respectively (Supplementary Figure 3). Yet the modification rate constant at Δ PTC 10 is faster than that at Δ PTC 6, consistent with a hindered environment for the cysteine. Moreover, the rates of modification at Δ PTC 13, 10, and 6 are the same for tryptophan versus alanine at the adjacent residue, which suggests that a fraction of cysteines at constriction locations is hindered (or surrounded by a sufficiently low dielectric constant to inhibit generation of a reactive thiol) and does not equilibrate with accessible conformations in the time of our measurements.

Regional discrimination may also be related to water in the tunnel. Water will organize differently depending on its microenvironment. All-atom molecular dynamics simulations find that water does not behave as an isotropic continuous dielectric medium along the tunnel¹⁸. The water filling the tunnel is a structural component of the ribosome, "...like an amorphous skin that affects macromolecular dynamics in cramped areas..."¹⁸. Moreover, the constriction site at the intersection of ribosomal proteins L22 (L17) and L4, shown to be important for translation arrest²⁴, has complicated solvation behavior¹⁸. A layer of water at the constriction will shrink the apparent tunnel dimensions even more and define the "wall" at this location, something that cannot be detected in current cryo-EM structures. In prokaryotes, the stalling nascent peptides SecM and TnaC, contain a critical tryptophan positioned at this site during arrest. If conformational rearrangements are inhibited at this site, as our accessibility results suggest, then peptide movement through the tunnel could also be inhibited, which might contribute to the mechanism underlying stalling. Although there are some differences between prokaryotic and eukaryotic ribosomes used in our studies, we suggest similar considerations may be relevant in a eukaryotic system. Finally, our findings of side-chain dependent accessibility may be useful for MD simulations of peptide-tunnel interactions.

Cargo in the tunnel

Side-chain energetics in the tunnel have only been investigated using molecular dynamics simulations^{13, 25} but not experimentally. First, Petrone et al. derived free-energy maps for isolated, disembodied side-chains in the tunnel¹³. They find significant energy barriers for tryptophan binding all along the tunnel ($\Delta G = 3-7k_B T$) compared to those for alanine. Only at regions approximated to be 45 Å and 68–70 Å from the PTC is there little difference in energy barriers for the two side-chains. These data are not comparable to our experimental findings and may result from the many differences in our two systems of study: we have

studied a mammalian tunnel hosting a peptide, which includes the possibility for allosteric communication, vis-à-vis an archebacterial tunnel devoid of peptide in which disembodied side-chains were simulated one at a time. Both results may be reconciled if an allosteric model with cooperative interactions within the nascent chain were included in the simulations of energy barriers. We suggest that the conformational rearrangements underlying the detected increased accessibility in the region Δ PTC 18–22 might reduce the energy cost of a tryptophan being at this location. A second MD simulation of tunnel-peptide interactions investigated TnaC, the leader peptide of the tryptophanase operon²⁵. W12 of TnaC peptide resides at the constriction site of the tunnel, at the convergence of L4 and L22, at the same location as tryptophan in our Δ PTC 13 tape measure construct. Schulten and co-workers demonstrate a cation- π interaction with an arginine or lysine donated from L4 and L22 (L17), with interaction energies as much as -13.5 kcal/mol²⁵. It is possible that this cation- π interaction lowers the energy barrier for a tryptophan at this position and obviates a Trp-induced rearrangement. This is consistent with our findings that at this location, there is no apparent conformational change when tryptophan replaces alanine (Figure 4).

Regional discrimination, or lack thereof, may reflect specialized function. The middle of the tunnel apparently can sense steric differences in the peptide whereas the constriction and the vestibule do not. The mid-region of the tunnel is permissive for peptide rearrangement. As the peptide is confined in the constriction, or forms compact secondary or tertiary hairpin structures in the folding vestibule, the peptide segment in the mid-gut may consequently need to reorient. The altered accessibilities along the tunnel, dependent on primary sequence, suggest that the nascent peptide may twist and turn along the tunnel during elongation. Such gyrations may influence translational arrest and even chaperone interaction, folding, and rates of elongation. It is possible that both backbone and side-chains allosterically dictate the location of segments of the nascent chain²⁶ and hence the functional consequences for chain elongation and folding. There is some structural evidence that a specific sequence of the nascent peptide may influence the conformation of an adjacent region of the peptide chain². Moreover, gaps in the cryo-EM structures of nascent peptide suggest that the nascent peptide does not adopt a single conformation in some locations in the tunnel¹, perhaps indicating the potential for a multiplicity of conformations or trajectories for the nascent chain. This may be side-chain dependent and consistent with our findings. Moreover, long range allosteric changes in a nascent peptide have been implicated in relocation of the nascent chain in the translocon²⁷.

Are there other interpretations of our results? If we introduce a large side-chain adjacent to the cysteine and assume nothing in the tunnel moves, then a reagent molecule will have a smaller environment to maneuver in and this would predict a slower modification rate due to the restricted degrees of freedom in the vicinity of the cysteine. This is contrary to our data. Nor can we reasonably attribute the cause of increased modification rate with increased volume of the adjacent side-chain to a change in dielectric constant or water (see above). The most consistent and parsimonious explanation is that sterics is the major determinant for the side-chain dependence of modification rate. All of these considerations support a rearrangement of the peptide relative to the tunnel that scales with sterics of side-chains and suggests the primary sequences of nascent peptides influence the dynamics of the tunnel-peptide interactions.

A final consideration is that a modest change of diameter of the tunnel from $10\text{\AA}$ to $13\text{\AA}$ can alter the volume for an accommodated cargo, approximated as a sphere, by 3-fold ($510\text{\AA}^3$ to $1150\text{\AA}^3$). Thus, peptide-tunnel rearrangements that are on the order of a couple of angstroms can have dramatic effects on the water accessible volume and hence on the trajectory and rate of progression of a peptide through the tunnel, the mechanism of

allosteric communication related to translational arrest, and perhaps protein folding and chaperone interactions.

MATERIALS AND METHODS

Constructs and *in vitro* translation

Standard methods of bacterial transformation, plasmid DNA preparation and restriction enzyme analysis were used. The nucleotide sequences of all mutants were confirmed by automated-cycle sequencing performed by the DNA Sequencing Facility at the University of Pennsylvania School of Medicine on an ABI 377 sequencer using Big dye terminator chemistry (ABI). The tape measure DNAs were sequenced throughout the entire coding region. Engineered cysteines were introduced into pSP64/Kv1.3/cysteine-free¹⁷ using Stratagene's QuikChange site-directed mutagenesis kit.

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-extended⁵. The cysteine engineered for all experiments is 62C in the native Kv1.3. To introduce different size side-chains, 63F was replaced either by A, S, Q, R, or W using Stratagene's QuikChange site-directed mutagenesis kit. To move 62C closer to the PTC site, residues were deleted from the C-terminus of the 62CΔ34 tape measure constructs using PCR methods to generate 62C63A(W)Δ6, 62C63A(W)Δ10, 62C63A(W)Δ13, 62C63A(W)Δ18, 62C63A(S,Q,F,R,W)Δ20, 62C63A(W)Δ22, 62C63A(W)Δ24, and 62C63A(W)Δ27, as described previously¹⁴. For the first three pairs of constructs, 62C63A(W)Δ6, 62C63A(W)Δ10 and 62C63A(W)Δ13, the methionine codon close to the C-terminus was deleted, hence we re-introduced it in the N-terminus. Capped complementary RNA was synthesized *in vitro* from linearized templates or PCR fragments using Sp6 RNA polymerase (Promega). Linearized templates for Kv1.3 biogenic intermediates were generated using a native *NcoI* site, which produced a longer nascent peptide and positioned 62C at 74 amino acids away from the PTC. Proteins were translated *in vitro* with [³⁵S]methionine Express (2 μl per 25 μl translation mixture; ~10 μCi μl⁻¹; Amersham) for 1 h at 22 °C in rabbit reticulocyte lysate according to the Promega Protocol and Application Guide.

Synthesis of maleimide probes

The synthesis of TMA is as described previously¹⁴. The other maleimido-quaternary ammoniums (TEA, TPA and TBA) were synthesized by mixing the corresponding trialkylamine (20 mmol) with N-(2-Bromoethyl)phthalimide (4 mmol) in 20 mL acetonitrile and heating to reflux for 2 – 4 days. The reaction mixture was concentrated to dryness and partitioned into equal volumes of ethyl acetate and water. The aqueous layer was washed with ethyl acetate and then concentrated to a brownish residuum. The crude material was dissolved in concentrated hydrogen bromide (48%) and heated to reflux for 8–16 hr to remove the phthalimide protecting group. The excess hydrogen bromide was removed by repeated evaporations from water and the crude amino ammoniums (TMA was from Fluka) were basified with hydroxide exchange resin (Dowex 1×8 200–400) to pH=13. After filtration of the resin, the solvent was evaporated to dryness. Crude aminoethyltrialkylamines were dissolved in a saturated sodium bicarbonate solution to a final concentration of 0.15 M and then 1.2 molar equivalents of N-methoxycarbonylmaleimide were added to the reaction. After 1 hr, the reaction was quenched with trifluoroacetic acid (TFA); longer reaction times resulted in lower yields due to competing maleimide hydrolysis. The crude maleimido-quaternary ammoniums were purified by HPLC on a C18 reverse phase column eluting with various gradients of A: 0.1% TFA in water; B: acetonitrile. Approximate %B at elution: TMA: 0%; TEA: 5%; TPA: 17%;

TBA: 55%. All maleimido-quaternary ammoniums were characterized by proton and carbon NMR, and mass spectrometry. TEA (TFA⁻): ¹H NMR (400 MHz, D₂O): δ 1.18 (t, 9 H, J=7.2), 3.23 (m, 8 H), 3.78 (m, 2 H), 6.77 (s, 2 H); ¹³C NMR (100MHz, D₂O): δ 6.720, 30.325, 52.421, 53.344, 134.876, 172.049; ESI-MS: m/z ([M⁺], calculated: 225.19, found: 225.2). TPA (TFA⁻): ¹H NMR (400 MHz, D₂O): δ 0.93 (t, 9 H, J=7.2), 1.72 (m, 6 H), 3.24 (m, 6 H), 3.38 (m, 2 H), 3.90 (m, 2 H), 6.88(s, 2 H); ¹³C NMR (100MHz, D₂O): δ 9.804, 14.947, 30.442, 54.146, 60.486, 134.854, 171.990; ESI-MS: m/z ([M⁺], calculated: 267.21, found: 267.3). TBA (TFA⁻): ¹H NMR (400 MHz, D₂O): δ 0.80 (t, 9 H, J=7.2), 1.22 (m, 6 H), 1.53 (m, 6 H), 3.13 (m, 6 H), 3.25 (m, 2 H), 3.74 (m, 2 H), 6.74 (s, 2 H); ¹³C NMR (100MHz, D₂O): δ 12.911, 19.175, 23.226, 30.530, 54.120, 58.867, 134.883, 172.056; ESI-MS: m/z ([M⁺], calculated: 309.25, found: 309.3).

Kinetics of cysteine modification with maleimides

As described previously⁵, 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 (critical requirement) 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).

The reaction was started by adding 1/10 dilution of the MAL reagent to the peptide-containing buffer. The reaction mixture was incubated on ice for times indicated in the time course experiments, quenched by addition of 2 mM DTT, and centrifuged through a sucrose cushion (120 μl; 0.5 M sucrose, 100 mM KCl, 5 mM MgCl₂, 50 mM HEPES, 1 mM DTT (pH 7.3)). The pellet was resuspended by pipetting in 25 μl buffer containing 20 mM HEPES, 1% (w/v) SDS, 10 μg ml⁻¹ of RNase, 100 mM NaCl, and 50 μM DTT. Pegylation was started by adding a final concentration of 2 mM PEG-MAL and continued for 3 hours at 4 ~ 8 °C in a refrigerator. The reaction was then terminated by adding 50 mM DTT and vortexing.

Data Analysis

The following analysis is taken from Elinder et al.¹⁹ The modification rate, ρ , of a given cysteine in the nascent peptide is equal to

$$\rho = k_{\text{MAL}} A_{\text{cys}} P_{\text{S}^-} [\text{MAL}] \quad \text{eqn. 1}$$

where k_{MAL} is the intrinsic reaction rate of a cysteine for a specific reagent, A_{cys} is the accessibility of the thiol group, P_{S^-} is the probability of the thiol group being in its unprotonated thiolate state, and $[\text{MAL}]$ is the local concentration of TMA in the vicinity of the cysteine. P_{S^-} depends on the local electrostatic potential (ψ). The rate ρ was measured experimentally from the time course of pegylation, which was exponential and of the form $y(t) = ae^{-\rho t} + c$. The quality of the exponential fit was assessed from the coefficient of determination, R^2 , which was always ≥ 0.98 . The values of k_{mod} presented in the Results are equal to the product ($k_{\text{MAL}} A_{\text{cys}} P_{\text{S}^-}$), and were calculated using the equation $k_{\text{mod}} = \rho / [\text{MAL}]$. A negative ψ attracts protons to the thiol, a positive ψ repels protons (i.e., effectively raises the local pH). This is quantitatively expressed as

$$P_{\text{S}^-} = 1 / (1 + 10^{(\text{pK}_a - \text{pH})} \exp(-\psi F / RT)), \quad \text{eqn. 2}$$

where pKa is the pH at which 50% of the cysteines are ionized (8.5 in physiological salt solution) and pH is the negative logarithm of the $[H^+]$ in the bulk solution. Because the surface potential inside the tunnel is negative¹⁴, then $pH_{local} \ll pKa$ and eqn. 3 reduces to

$$P_{s-} = 1 / (10^{(pKa-pH)} \exp(-\psi F/RT)) \quad \text{eqn. 3}$$

As with protons, the local concentration of charged maleimide is also exponentially dependent on the local electrostatic potential:

$$[MAL] = [MAL]_{bulk} \exp(-z\psi F/RT) \quad \text{eqn. 4}$$

where z is the valence of the charged MAL reagent. Substituting eqns. 3 and 4 into eqn. 1, and making a ratio of the modification rates for a given cysteine measured in two different constructs (ρ_1/ρ_2 , e.g., same cysteine inside and outside the tunnel), the following relationship is derived.

$$\rho_1/\rho_2 = (k_{MAL} A_{cys,1} 10^{(pKa-pH)} \exp(-\psi_2 F/RT) [MAL]_{bulk} \exp(-z\psi_1 F/RT)) / (k_{MAL} A_{cys,2} 10^{(pKa-pH)} \exp(-\psi_1 F/RT) [MAL]_{bulk} \exp(-z\psi_2 F/RT)) \quad \text{eqn. 5}$$

If the charge on the maleimide is +1 (e.g., TMA, TEA, TPA, TBA) and the contribution of the P_{S-} term is less than the A_{cys} term, as indicated by the data in Figures 3A, B, D, and 5B (see Results and Discussion), then eqn. 5 simplifies to

$$\rho_1/\rho_2 = (A_{cys,1}/A_{cys,2}) \quad \text{eqn. 6}$$

indicating that the relative rates of modification of the cysteine by the positively charged reagent equals the fold increase in accessibility for the cysteine in nascent peptide 1 versus nascent peptide 2. Note that this formalization ignores the possibility that pKa might not be equal in different tunnel locations. Our data, however, suggest that sterics play a significantly larger role than pKa changes.

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% or 12% gels and Mes 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Experimental Approaches

A. The Tape Measure in the Tunnel. A schematic is shown of the ribosomal tunnel (black brackets) and an all-extended nascent peptide (red), the tape measure, containing an engineered cysteine (yellow circle). The peptidyl transferase center (PTC) and exit port (EXIT) are shown at the ends of the tunnel. **B.** Single Cysteine Tape Measures. Successive shortening of the chain length produced the different length nascent peptides shown with their primary single-letter amino acid sequence. For each construct, the number of residues from (and including) the cysteine (red) to the PTC is indicated as ΔPTC and written to the left of each construct. **C.** Maleimide Probes. The structures of a series of maleimides of increasing size: trimethyl (TMA), triethyl (TEA), tripropyl (TPA), and tributyl (TBA) ammonium ethylmaleimide are displayed using DS Viewer Pro (<http://www.accelrys.com>). Volumes are indicated to the right of each structure. For comparison, the structure of tryptophan, the largest amino acid side-chain is also shown (beta-carbon included). All structures were optimized with Jaguar (www.schrodinger.com) using a Hartree-Fock (HF) model with the STO3* basis set. The volume calculations were done with the program Gaussian03, and used the HF model with a 6-31++G* basis set. The solute-enclosed volume calculation derives from a static isodensity surface-polarized continuum model (IPCM) ²⁸.

Figure 2. Kinetics of Cysteine Modification

A. Time course of cysteine modification by TMA for a cysteine located 74 (top) or 20 (bottom) residues from the PTC. After treatment with TMA (3 and 20 μM , respectively), the reaction was quenched and the peptide pegylated and fractionated on gels as described in the Materials and Methods. Numbers to the left of the gels represent molecular mass standards (kD); numbers to the right indicate unpegylated (0) and singly pegylated (1) protein. The plot to the right of each gel is the fraction of individual cysteine residues pegylated, which is inversely correlated with the fraction of individual cysteine residues modified by the TMA. The fraction pegylated is calculated as radioactivity (cpm) in band 1 divided by the sum of radioactivity in bands 1 and 2. A single-exponential function was fit to the data to give modification rate constants of 3172 and 287 $\text{M}^{-1}\text{s}^{-1}$, respectively, for ΔPTC 74 and 20. **B.** Time course of cysteine modification by TBA for a cysteine located 74 (top) or 20 (bottom) residues from the PTC. After treatment with TBA (3 and 20 μM , respectively), the reaction was quenched and the peptide pegylated and fractionated on gels as described in the Materials and Methods. Gels and plot of fraction pegylated are as described in A.

Figure 3. Dependence of k_{mod} on Tunnel Location

A. The rate constant, k_{mod} , determined at several locations for a cysteine with a C-terminally adjacent alanine, Cys(A). Locations probed are 74, 27, 24, 22, 20, 18, 13, 10 and 6 amino acids from the PTC. The cysteine was modified by either TMA (filled circles) or TBA (open triangles). Data, obtained from experiments similar to those shown in Figure 2, are means $\pm$ SEM for triplicate experiments plotted on a semi-log scale (y-axis). **B.** The rate constant, k_{mod} , determined at several locations for a cysteine with a C-terminally adjacent tryptophan, Cys (W). Locations and reagent probes are as described in A. Data are means $\pm$ SEM for triplicate experiments. **C.** Tunnel discrimination between TMA and TBA. For the data shown in A. and B., the ratio of rate constants for TMA to TBA, at each location, is plotted for alanine (black bars) and tryptophan (gray bars). A ratio of one indicates no difference in reactivity for TMA versus TBA, which only occurs far outside the tunnel at location ΔPTC 74. Data are means $\pm$ SEM as propagated errors²⁹. **D.** Rate constants for each probe, determined at location ΔPTC 20 in the tunnel, as described above in Figure 2. The amino acid C-terminally adjacent to the cysteine is the native residue (F) in the tape measure. Data are means $\pm$ SEM for triplicate measurements.

Figure 4. Regions of Discrimination in the Tunnel

A. Relative accessibility to TMA for tryptophan compared to alanine. Rate constants for tryptophan at each location were normalized to the value obtained outside the tunnel and divided by the similarly normalized rate constant for alanine. Data are shown for triplicate determinations as means $\pm$ SEMs calculated as propagated errors²⁹. **B.** Relative accessibility to TBA for tryptophan compared to alanine, as described in A.

Figure 5. Effect of Side-Chain Volume on Relative Cysteine Reactivity

A. Schematic of the nascent tape measure peptide (red line) in the tunnel (delineated by brackets) containing an engineered cysteine (yellow circle) and one of the indicated amino acids (A, S, Q, F, R, W, green circle) at the C-terminally adjacent position between the PTC and the cysteine. The cysteine, located at a ΔPTC of 20 amino acids ($\sim 60 \text{ \AA}$) from the PTC, is modified by a maleimide probe (red circle), either TMA or TBA. **B.** Modification rate constants for constructs described in A. Rates are measured as described in the Materials and Methods and Figure 2. For each side-chain at the C-terminal position to the cysteine (i.e., Cys(X)), data are normalized to the rate constant for a cysteine with an alanine at the C-terminal adjacent position (i.e., normalized to Cys(A)). This ratio, k_X/k_A , is plotted versus the side-chain volume ($\text{\AA}^3$) of each C-terminally adjacent side-chain (beta-carbon included), which is marked along the x-axis with a single-letter amino acid code (red). The volume calculations were done with the program Gaussian03, and used the HF model with a 6-31++G* basis set. The solute-enclosed volume calculation derives from a static isodensity surface-polarized continuum model (IPCM)²⁸.