Tight knots in proteins: can they block the mitochondrial pores?

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Abstract

Proteins need to be unfolded when translocated through the pores in mitochondrial and other cellular membranes. Knotted proteins, however, might get stuck during this process since the diameter of the pore is smaller than the size of maximally tightened knot. In the present article, I briefly review the experimental and numerical studies of tight knots in proteins, with a particular emphasis on the estimates of the size of these knots. Next, I discuss the process of protein translocation through the mitochondrial pores and report the results of molecular dynamics simulations of knotted protein translocation, which show how the knot can indeed block the pore.

Introduction

In recent years, there has been a surge of interest in the properties and functions of knotted proteins. As more and more knotted structures are deposited in the PDB, it becomes increasingly important to understand how, if at all, non-trivial topology affects the protein's function in the cell [1–7]. In particular, it has been hypothesized that the presence of a knot in the polypeptide backbone may affect the ability of knotted proteins to be degraded in the proteasome or translocated through the intercellular membranes, e.g. during import into mitochondria [8,9]. The smallest constrictions in the mitochondrial pores or proteasome opening are 12–14 Å (1 Å = 0.1 nm) in diameter [10,11], too narrow to accommodate folded structures, thus translocation must be coupled to protein unfolding. An exact mechanism by which the mitochondrial translocases unfold the proteins is, however, still elusive. In particular, it is debatable whether the import motors actively unfold the proteins by generating a pulling force (a so-called 'power-stroke' mechanism) or merely act as Brownian ratchets, trapping the fluctuations and preventing the retrograde movement [12,13]. However, no matter which mechanism is at work, the tension induced in the protein backbone during unfolding might lead to the tightening of the knot. In the present article, I briefly review a number of recent studies, both experimental and theoretical, exploring the process of knot tightening in the proteins. Next, I analyse the implications of these results for a translocation process and present the results of molecular dynamics simulations of knotted protein translocation process using a coarse-grained model of both the pore and the protein.

Knot tightening in stretched proteins

The process of knot tightening in proteins has been analysed in three previous studies [9,14,15], two of them numerical and one experimental. In the paper by Sułkowska et al. [14], a coarse-grained Go-type model was used to analyse knot tightening in 20 different proteins, 18 of them with 3_1 knots and two with 5_2 knots. As the chain was stretched and the protein unfolded, the authors tracked the position of the knotted core, i.e. the minimal segment of the peptide chain that can be identified as a knot. In that way, the trajectories of knot's ends in the sequential space were obtained, analogous to those shown in Figure 1. The striking result of [14] was that the motion of the knot's ends was not diffusive, as reported in the case of polymeric structures such as DNA [16], but instead took place through a series of jumps to well-defined positions along the protein backbone, associated with sharp turns, usually involving proline or glycine. After several of such jumps, the knot finally gets to a tightened conformation, in which the knotted core reduces to 12–14 amino acids (for a trefoil knot) and the radius of gyration is of the order 7–8 Å. Interestingly, it was also found that, after the tension is released, the knotted protein often does not come back to the initial conformation. This is reminiscent of the behaviour of knots in polymeric chains [16–19]. As already noted by de Gennes [17], when a tight knot is made, it becomes extremely difficult to untie it even after the tension is switched off. A bottleneck in this process is the initial loosening of the knot, i.e. the insertion of some stored length into the knotted core associated with the distortion of a number of molecular bonds. On the effective level, this can be described in terms of the friction between the two segments of the protein chain. A simple estimate by Kirchner and Neukirch [19] leads then to the following relation between the external tension (t) applied to the chain and the radius a knot (R):

$$\left|\frac{tr^2}{\kappa} - \frac{1}{2}\left(\frac{r}{R}\right)^2\right| \sim \frac{1}{2}\mu\left(\frac{r}{R}\right)^{3/2} \quad (1)$$

Here, κ is the bending stiffness of the chain, μ is the effective friction coefficient, and r is the radius of the filament. In particular, eqn (1) predicts that, after the knot is tightened and subsequently released (by putting $t = 0$), it will not go

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Abbreviation used: Hsp70, heat-shock protein 70.
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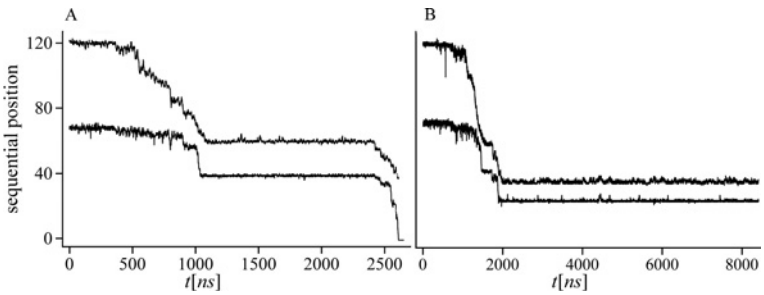
Figure 1 | **Sequential movement of a knot's ends during the translocation process of *E. coli* methyltransferase (PDB code 1NS5)**
The knot either slides off the chain (**A**) or is tightened (**B**).



back to its original configuration but become locked in a conformation with $R \sim r/\mu^2$.

The above effective model has only a limited applicability to protein molecules. In the latter, the presence of a complex network of bonds between amino acids gives rise to a highly non-uniform effective friction along the chain. This heterogeneity is most dramatically manifested in the fact that, as mentioned above, the knot's ends are stopped at the sharp kinks in the backbone, whereas they slide easily over other fragments of the chain. Nevertheless, on a qualitative level, the effective friction model reproduces the observed behaviour of the knots as the force is switched off; initially they swell slightly, increasing their length by one or two amino acids. Next, the knot enters a relatively long-lived metastable state which lasts until a large thermal fluctuation partially breaks its structure, allowing for the insertion of some stored length into the knotted core. After that, the knotted core expands relatively quickly, the knot begins to move back across the chain, and the protein can refold.

As mentioned above, in the study by Sułkowska et al. [14], a coarse-grained model of a protein was used, which allowed the authors to obtain statistically meaningful results for a relatively large number of proteins. Nevertheless, the lack of atomic details in these models can potentially result in an inaccurate description of the knot-stabilizing interactions, in particular for tight structures, in which the packing of side chains becomes important. It is thus important to validate the results of the coarse-grained models against fully atomistic models, such as those of Dzubiella [15] who has performed atomistic simulations of the knot-tightening process in polypeptide chains using Amber ff03 forcefield with an explicit solvent. He has analysed the sizes and radii of gyration of tight protein knots, finding their lengths to be $\Delta l \approx 47 \pm 4$ Å (involving 12–14 amino acids) for a trefoil and $\Delta l \approx 69 \pm 4$ Å (involving 18–20 amino acids) for a figure-of-eight knot. The length of the tight knot is defined here as $\Delta l = l_c - l$, where l_c is the contour length of the peptide chain, and l is the end-to-end distance of the protein in the tightened configuration. The corresponding radii of gyration of the tightened knots reported in [15] are $R_g \approx 7.2 \pm 0.2$ Å and 7.8 ± 0.2 Å for a trefoil and figure-of-eight knot respectively. Importantly, there is only a weak dependence of the knot size on the pulling force applied to tightened the knot, which seems to suggest a very tight peptide packing leading to strong steric hindrance effects [9].

Interestingly, as remarked in [15], a very reasonable estimate of the size of tight protein knots can be obtained by relating to the ideal tight knots in perfect ropes [20]. These are uniquely characterized by their length/diameter ratio:

$$\Lambda = \frac{\Delta l}{D} \tag{2}$$

with $\Lambda = 10.1$ and 13.7 for 3_1 and 4_1 knots respectively. Assuming the diameter of the polypeptide chain (as given, e.g., by tuning the thick-polymer model to its stretching response [21]) to be of the order 5 Å, we get the estimate of the length of the tight protein knots as $\Delta l \approx 50$ Å for a trefoil and 69 Å for a figure-of-eight knot, in good agreement with both the atomistic simulations and the results of the coarse-grained models. However, Dzubiella [15] also found some novel atomic-scale effects in the tight knot structures, which are not observable using coarse-grained models. These include strong hydrogen-bonding propensities and water-trapping effects in the tight knotted cores, which lead to the increased stability of the tight peptide knots.

Finally, let us mention a unique single protein experimental study by Bornschlögl et al. [9], in which the figure-of-eight knot in the phytochrome was tightened using an atomic force microscope. By analysing the force–extension curves the authors of [9] were able to estimate the apparent shortening of the polypeptide chain due to the presence of the knot and hence obtain the size of the knotted core of the 4_1 knot in the phytochrome of the order $\Delta l \approx 62 \pm 10$ Å, corresponding to 17 ± 3 amino acids, again consistent with both the numerical studies and the simple estimate of eqn (2).

Implications for protein translocation

The above-discussed studies give a consistent estimate of the minimal size of tight protein knots, with the radius of gyration in the range 7–8 Å. This brings up the question of whether a tight knot of this size can get stuck during the translocation through a mitochondrial pore or proteasome opening. In fact, there are two problems to be considered: (i) could the knot

tighten during the translocation process, as the protein is being unfolded, and (ii), if so, will the tightened knot block the pore, thus preventing the further translocation of the chain?

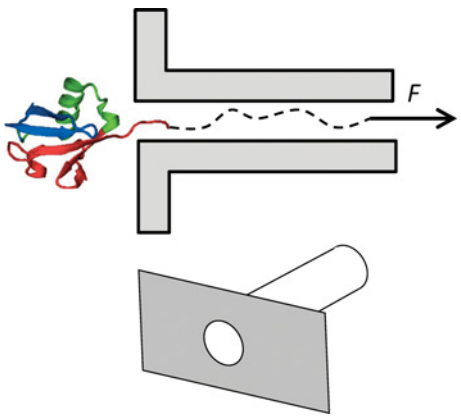
The second problem is relatively straightforward, as it involves the comparison of geometric characteristics of tight knots and the mitochondrial pore openings. Since the smallest constrictions of the mitochondrial pores are approximately 13 Å in diameter [11], the knots will probably not be able to pass through. On the other hand, the answer to the first problem depends on the actual mechanism by which the protein is translocated. This is still under debate, with three competing models. In one, a so-called 'lever-arm' or 'power-stroke' model [22,23], it is assumed that the motor proteins tethered to the outlet of the import channel undergo a conformational change as a result of ATP binding and hydrolysis. This conformational change is then translated into a mechanical force imposed on the associated translocating protein chain, which drives unfolding of the domain. In the second ('Brownian ratchet') model [24,25], a central role is played by the thermal fluctuations that trigger spontaneous unfolding of the parts of the translocating protein. In this model, the import motor acts as a Brownian ratchet, preventing the retrograde movement of the chain and thus trapping the partially unfolded intermediates. Finally, the third mechanism, so-called 'entropic pulling model' was proposed [26], in which the binding of the chaperone protein Hsp70 (heat-shock protein 70) to the polypeptide emerging from the pore on the inner side of the mitochondrial membrane decreases the freedom of Brownian movements due to the large volume of Hsp70 involved. As a result, the chain tends to diffuse away from the channel outlet to increase its configurational entropy, which results in a force of entropic origin that can unfold the protein domain on the cytosolic side and pull it further inside the pore. In the context of the present article, the important question is whether the unfolding and translocation of a protein driven by each of these mechanisms can be accompanied by the tightening of the knot. Below, I consider the power-stroke model only, leaving the two other mechanisms as the subject of further work.

The most important difference between the force-induced translocation and the AFM (atomic force microscopy) pulling considered in the previous section is the fact that in the former case one of the ends of the protein is free, hence the knot can, in principle, slide off the chain. To test whether this is indeed the case, we have performed coarse-grained simulations of the translocation process of several proteins in a simplified model, in which the protein is described by a structure-based Go-type analogous to that in [14], whereas the pore is represented as a rigid cylinder, mostly to take into account the steric constraints present during the translocation process.

A schematic illustration of the simulation set-up is presented in Figure 2. At the beginning of the simulations, the protein in its native conformation is placed at the cytosolic side of the membrane near the pore entrance. In the cell, the transport of proteins into mitochondria is usually mediated by a loosely folded presequence, which is modelled here as a loose piece of a peptide chain (ten amino acids long). One end of the presequence is attached to the N-terminus, whereas the other end is pulled with a constant force F. Analogously to [14], we record the positions of the knot's ends in the sequential space, thus tracking the movement of the knotted core along the chain. The example of such a trajectory is shown in Figure 1. Ultimately, there are two scenarios possible: the knot either slides off the chain, as shown in Figure 1(A) or it is tightened, as in Figure 1(B). Note that this is in contrast with the study of Huang and Makarov [27], where the parameters of the pore allowed the knotted chain to enter it.

Figure 2 | A schematic view of a simulation set-up

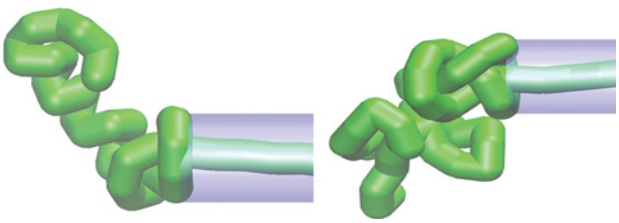
Upper panel: cross-section showing the protein, presequence and the pore. Lower panel: side view of the membrane with the pore.



Example tightened conformations are presented in Figure 3: the tightened configurations of a trefoil will usually involve a fastened loop around the entrance of the pore, whereas the 4_1 knot will make a tight figure-of-eight with the long axis oriented parallel to the pore. The final fate of the knot is determined stochastically, with a tightening probability strongly increasing with the pulling force. This behaviour is similar to that reported by Rosa et al. [28] for force-induced translocation of knotted polyelectrolyte chain, such as ssDNA (single-stranded DNA). On the other hand, no jamming was observed in the passive ejection of DNA out of a spherical cavity [29,30]. However, as mentioned above, there are also important differences between the motion of the knots in proteins and in DNA, which manifest itself during the translocation process. In particular, similarly to [14], we find that both the intermediate and final position of the knot's ends are almost always associated with sharp turns in the protein backbone (involving mostly proline and glycine, but also occasionally serine). If such locations are absent from the stretch of the protein backbone between the initial position of the knotted core and the free end of the protein, then the knot slides off easily and never tightens. Conversely, if the knot is deep and there are many potential trapping sites, the tightening probability is high and the knot survives thus blocking the pore. Note, however, that the forces used in the simulations (70–400 pN) are larger than

Figure 3 | Example tight-knot configurations stuck at the entrance to the pore

Left: trefoil in *E. coli* methyltransferase (PDB code 1NS5). Right: figure-of-eight in FLIN2 chimaeric protein (PDB code 1J20 [34]).



the estimates of the characteristic forces that can be exerted by protein translocases, which are approximately 30 pN [31]. Unfortunately, smaller force regimes are computationally inaccessible because of the large translocation times involved. Finally, it is important to mention that in the simulations we have used the simplest protocol, in which a constant force was acting on the translocating protein. We did not take into account Brownian ratchet effects nor did we account for the fact that the real biological forces produced by the motors are always cyclic in character, since the motors transform chemical energy into directed motions via nucleotide-hydrolysis-mediated conformational changes. These effects can significantly affect the unfolding pathways [32,33]. Whether this can prevent the knot from tightening is the subject of further study.

Conclusions

In summary, it was shown that the translocation of the knotted peptide may lead to the tightening of the knot and subsequent blocking of a mitochondrial pore. Several limitations of this study need to be acknowledged, including a crude model of a translocation process (constant pulling force, cylindrical pore and lack of ratcheting). Clearly, further study is needed regarding the influence of these factors on the knot dynamics.

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