Prediction of the Translocation Kinetics of a Protein from Its Mechanical Properties

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ABSTRACT Proteins are actively unfolded to pass through narrow channels in macromolecular complexes that catalyze protein translocation and degradation. Catalyzed unfolding shares many features that characterize the mechanical unfolding of proteins using the atomic force microscope (AFM). However, simulations of unfolding induced by the AFM and when a protein is translocated through a pore suggest that each process occurs by distinct pathways. The link, if any, between each type of unfolding, therefore, is not known. We show that the mechanical unfolding energy landscape of a protein, obtained using an atomistic molecular model, can be used to predict both the relative mechanical strength of proteins when unfolded using the AFM and when unfolded by translocation into a pore. We thus link the two processes and show that the import rate through a pore not only depends on the location of the initiation tag but also on the mechanical properties of the protein when averaged over all the possible geometries that are relevant for a given translocation initiation site.

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When proteins are actively translocated across the mitochondrial membrane or degraded by chambered proteases (such as the proteasome or the Clp proteases), the protein substrate must first be unfolded as the entry pores of these large macromolecular complexes have a diameter that is much smaller than the size of the folded protein (1). It is thought that these complexes catalyze unfolding by the application of a mechanical force onto the protein, thereby decreasing the unfolding barrier and exponentially increasing the unfolding rate constant. Proteins targeted for either translocation or degradation by a suitable polypeptide tag are unfolded significantly faster than in solution. The precise mechanism, however, by which these ''unfoldases'' are able to denature proteins, remains unresolved. In the last 10 years, many proteins have now been mechanically unfolded using the AFM—a process that shares many features of active unfolding catalyzed by the macromolecular complexes described above. For both types of unfolding it has been shown that i), the unfolding pathway is distinct to the intrinsic unfolding pathway that is probed by chemical denaturation experiments (2–4); ii), the unfolding rates of substrate proteins do not correlate with their global thermodynamic or kinetic stability (3,5,6); and iii), the mechanical strength is determined by the local structure of the polypeptide chain relative to the initiation point of import or degradation (2,3,7,8). Although these results suggest a common mechanism, direct comparison of mechanical strength measured by the atomic force microscope (AFM) and import rates (the mitochondrial TOM and TIM complexes (9,10)) or degradation rates (ClpXP (11)) have proved inconclusive. Furthermore, simulation studies that allow the unfolding pathways of proteins to be observed at the molecular level suggest that the unfolding pathways of barnase, when dragged into a very simple geometrical model of a pore, are different to the pathway observed when the two ends of the protein are pulled apart (12). An analogous result was also found for ubiquitin (13) using an even simpler model of a pore and a coarse-grained model of the protein. Although the mechanisms between pore translocation and AFM pulling were found to be different, both sets of simulations showed that the local stability of the part of the protein that enters the pore first determines its overall resistance, in qualitative agreement with experimental observations.

What then is the relationship between catalyzed mechanical unfolding and unfolding a protein using the AFM? In AFM, unfolding force is applied locally to two distinct locations within the protein (Fig. 1 *a*), and in this case the mechanical strength of the protein is thought to be dominated by the stability of the contacts between these regions (e.g., the hydrogen bond mechanical clamp in I27 (4)). When translocated through a pore, however, force is applied at a precise location, but the areas of the protein resisting the extension are diffuse (those touching the rim of the pore, Fig. 1 *b*). Therefore, although force is acting as a highly localized peturbant in both processes, the mechanical stability measured by the unfolding force (in AFM experiments) or unfolding rates (translocation experiments) will depend in some manner on the strength of all of the contacts being perturbed, and these differ in each process. If these processes are both accelerated by force, information about both pathways should be contained in the mechanical unfolding landscape of the protein. We have previously used a C_α native-centric or Gō-like model to create such a mechanical landscape for a variety of proteins (14) in which the mechanical strength of a protein is simulated when extended by

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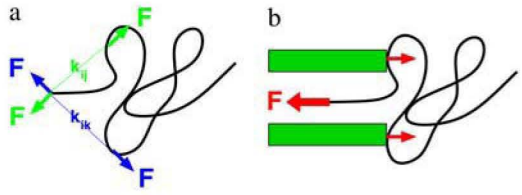

FIGURE 1 ***(a)*** **AFM single molecule experiments can pull pairs of atoms apart under a constant loading force *F*. By repeating the same experiment a number of times, one may determine the unfolding rate for a given residue pair and force. (*b*) A simple steric model of a pore. Assuming that the protein unravels as a consequence of being dragged through the pore, a special role will be played by the residues that contact the rim of the pore. Their identity depends not only on the site of the translocation initiation site but also on a possible partial unraveling of the protein in the region of the initiation site.**

pulling every pair of C_α atoms. We showed that these ''mechanical unfolding landscapes'' capture the anisotropy of the response of a protein to mechanical peturbation: a protein can either resist a mechanical force or yield easily to it (*yellow* and *blue* areas in Fig. 2) by altering the *pulling geometry*.

To investigate the relationship between the mechanical unfolding landscape of a protein and its rate of translocation through a pore, it was first necessary to simulate the latter process. A simple model of a pore was designed using a previously proposed model (13) of an infinitely long pore interacting repulsively with the protein. The chosen pore diameter was 12 Å, comparable to the diameter of narrowest constriction in the degradation channel of the proteosome (1), which ensures that translocation could only occur with the complete unfolding of the domain. We then performed

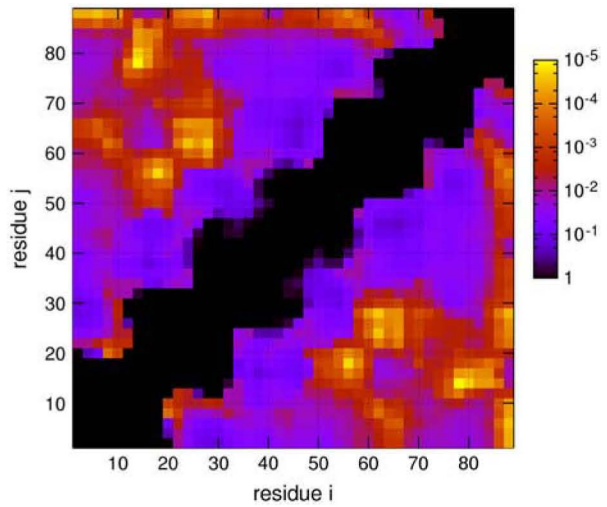

FIGURE 2 Mechanical unfolding landscape of I27 generated by estimating unfolding rates pulling apart all pairs of residues at a constant force of 150 pN, $T = 300$ K. Yellow to blue colors denote high to weak mechanical resistance; black denotes pairs not pulled. Scale is in ps^{-1}.

simulations where a selected residue was centered on the pore, the protein oriented so that the vector joining the selected residue and the center of geometry is parallel to the pore axis and a constant force oriented along this axis applied to the selected residue. The resulting translocation rates are shown in Fig. 3 *(black line)*.

Interestingly, the rate of translocation changes by more than two orders of magnitude when the force is applied to different residues. Slowest translocation occurs when the initiation site is close to residues 11, 23, 59, 69, and 83, and I27 is translocated relatively rapidly when dragged through the pore from the N-terminus. This is particularly surprising, since it is has been shown by experiment and simulation that I27 is able to withstand large forces when pulled between the N- and C termini using the AFM. This also contrasts with the conclusion of Wilcox et al. (9) that the unfolding force when pulling the N- and C termini correlates in general with the translocation rate when the targeting sequence is attached to the N-terminus.

To predict the translocation rates through a pore from a knowledge of the mechanical properties of the protein, a rough approximation consists in assuming that all possible pulling geometries between that residue and other residues contribute equally; i.e., that all mechanical pathways contribute in parallel, which gives the rate

$$k_i^{\text{force}} = \sum_{j \neq i} k_{ij}^{\text{force}}, \tag{1}$$

where k_{ij}^{force} is the unfolding rate constant for a residue pair i,j. The resulting k_i^{force} is shown as a blue line in Fig. 3. Although some features are shared between these data and the pulling through a pore simulation, the correlation between $\log k_i^{\text{force}}$ and $\log k_i^{\text{pore}}$ is only 0.48. The primary reason for this moderate correlation comes from the fact that if a single

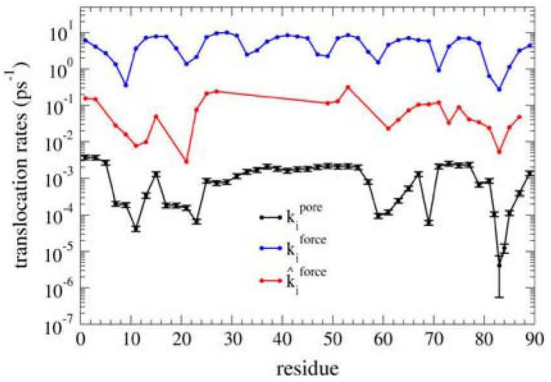

FIGURE 3 Unfolding rates measured when pulling I27 by a single residue through a pore (*black line*). The translocation rate through the pore is related to the mechanical landscape when averaged over suitable geometries. The blue line is the unfolding time averaged over all possible residue pairs; the red from averaging over all pairs with c_{ij} determined as described in the text.

unfolding rate k_{ij} is much larger than all the others, it will dominate the sum in Eq. 1. A better approximation consists in considering only those residues that are in contact with the rim of the pore when the force is applied to residue *i*. Their identity cannot be exactly determined, as the conformation which corresponds to the rate limiting step in the unfolding process (the transition state) is not necessarily the native one (although for mechanically robust proteins we do not expect it to be very topologically different from the native state). If one assumes that the mechanical unfolding transition state is close to the native state, then a reasonable assumption is to consider residues which are close in space to the initiation site and not buried inside the protein. An effective rate is then obtained by summing only over the relevant pairs of residues:

$$\hat{k}_i^{\text{force}} = \sum_{j \neq i} c_{ij} k_{ij}^{\text{force}}, \qquad (2)$$

where $c_{ij} = 1$ if residue *j* is at most 9 Å apart from residue *i* (slightly larger than the pore radius) and at least 40% of its surface is solvent-accessible in the native state (since buried residues are unlikely to get stuck on the outer rim of the pore) and that are at least 10 residues apart along the polypeptide chain (it makes little sense to define an unfolding rate between residues close in the chain as their extensions might not lead to the unfolding of the protein). The resulting unfolding rate constant ($\hat{k}_i^{\text{force}}$) profile calculated in this manner (*red line*, Fig. 3) agrees more closely with k_i^{pore} (the correlation increases to 0.68).

One obvious difference between the rate constant profiles between simulated translocation through a pore and that obtained from the mechanical energy landscape is that the latter are about one order of magnitude larger than the former. This is simply due to the fact that the effective constant force F acting on each of the M residues, which are stuck on the rim (see Fig. 1 *a*), is $\sim 1/M$ the force acting on each pair when the same constant force F is used to determine k_{ij}^{force}.

Although the translocation rate profile predicted from the mechanical map and directly estimated from the pore model is similar in the case of I27, this method may not be reliable for all proteins. We performed analogous estimations for E2Lip3 and barnase (both at a constant force of 100 pN) and the best correlation between $\hat{k}_i^{\text{force}}$ and k_i^{pore}, which could be found was 0.53 and 0.48, respectively. In particular, for proteins whose mechanical transition state is far from the native state and may have possible unfolding intermediates, this approximation is less accurate. In this case, any partial unraveling of the protein into the pore will make the prediction less accurate. One other factor that might hinder a reliable prediction of the translocation rate from mechanical properties alone is the fact that the geometry of the protein relative to the pore might differ from that probed by the analogous AFM experiments on the various pairs of atoms that might be involved.

Can we predict the translocation rates through a pore from the knowledge of the mechanical properties of the protein? The answer is yes, but it depends on the full mechanical properties of the protein and not only on those probed by extending the protein from two points as measured in AFM experiments. The results presented here could be experimentally tested by measuring the rate at which the ClpXP protease degrades I27 variants in which an ssrA degradation peptide tag is attached to the side chain of unique cysteine residues inserted at specific points (15).

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