

Complex Stability of Single Proteins Explored by Forced Unfolding Experiments

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ABSTRACT In the last decade atomic force microscopy has been used to measure the mechanical stability of single proteins. These force spectroscopy experiments have shown that many water-soluble and membrane proteins unfold via one or more intermediates. Recently, Li and co-workers found a linear correlation between the unfolding force of the native state and the intermediate in fibronectin, which they suggested indicated the presence of a molecular memory or multiple unfolding pathways (1). Here, we apply two independent methods in combination with Monte Carlo simulations to analyze the unfolding of α -helices E and D of bacteriorhodopsin (BR). We show that correlation analysis of unfolding forces is very sensitive to errors in force calibration of the instrument. In contrast, a comparison of relative forces provides a robust measure for the stability of unfolding intermediates. The proposed approach detects three energetically different states of α -helices E and D in trimeric BR. These states are not observed for monomeric BR and indicate that substantial information is hidden in forced unfolding experiments of single proteins.

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The past few years have seen a dramatic increase in our understanding of the processes that stabilize single proteins (2). Atomic force microscopy (AFM) unfolding experiments, where an external pulling force plays the role of the denaturant, revealed the unfolding pathways and kinetics of individual water-soluble (1,3) and membrane proteins (4,5). It was found that many water-soluble and membrane proteins unfold in a well-defined sequence of one or several partly unfolded intermediates (1,4,5). Until very recently it was assumed that unfolding transitions from the native state to the intermediate(s) and from the intermediate(s) to the fully unfolded state occur independently from each other. Interestingly, Li and co-workers lately reported a linear correlation between the unfolding force of the native state and the intermediate in fibronectin (Fn) (1). This correlation suggests either the presence of multiple hidden unfolding pathways or a molecular memory in Fn.

As proposed by Li and co-workers (1), we have used linear regression analysis to look for a correlation between the unfolding forces of α -helices E and D of bacteriorhodopsin (BR) (Fig. 1), which represent stable mechanical unfolding intermediates of the membrane protein (6). On plotting the unfolding force of α -helix D against that of α -helix E for each single molecule (Fig. 1 A), we found linear correlation coefficients (R -values) between 0.043 and 0.636 depending on the pulling speed (Fig. 1 B). R -values as large as 0.636 could indicate a significant linear correlation for unfolding of the two α -helices. However, we observed that the R -values are scattered heavily and show no clear tendency with pulling speed. Because one would expect constant (or increasing) correlation with pulling speed the

scattering suggests a different, other than molecular, origin for the observed correlation between α -helix E and D.

Measuring forces with AFM requires precise knowledge of the spring constant of the micromachined cantilevers (7). The most commonly applied method to determine spring constants, thermal fluctuation analysis, is known to be associated with an error of at least 10% (8). Due to this systematic error, unfolding forces measured in the same experiment will have either a bit smaller or a bit larger values than an ideally calibrated reference. Therefore, these data sets tend to cluster in a plot such as shown in Fig. 1 A. Consequently, if data recorded in different experiments are pooled, this clustering might result in a (linear) correlation. To estimate the influence of error in force calibration on linear correlation analysis we performed Monte Carlo (MC) simulations for unfolding α -helices E and D (9,10). MC simulations are well suited as a reference because consecutive elements unfold independently (no memory) and a single unfolding pathway exists (1,9). At all pulling speeds, we observed R -values near zero suggesting no correlation of the simulated unfolding forces (Fig. 1 B, *open symbols*). Fig. 1 C shows the result of a typical simulation where 1000 force pairs have been computed (*red symbols*). To simulate a typical experimental situation (five experiments, $\pm 10\%$ error in spring constant determination), we added an error of 10%, 5%, 0, -5% , and -10% each to a fifth of these force pairs. Remarkably, linear correlation analysis now yields an R -value of 0.391 (Fig. 1 C, *blue dots*) showing that

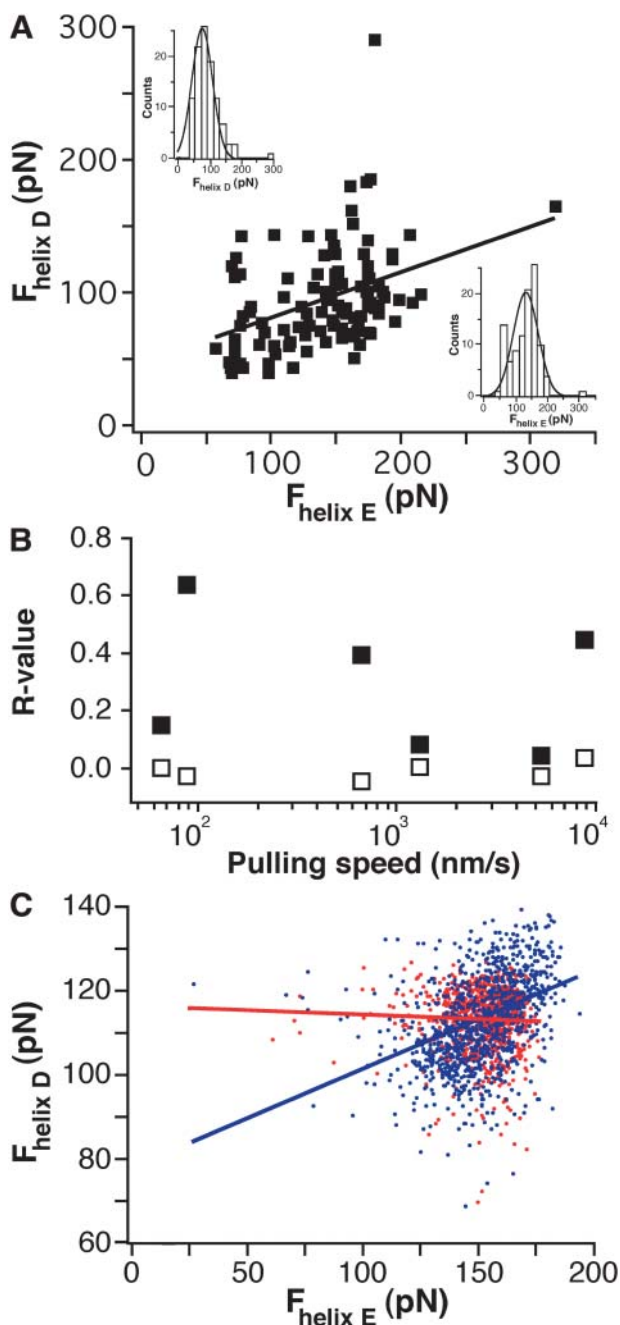


FIGURE 1 Linear correlation analysis of unfolding BR α -helices E and D. (A) Analysis of experimental unfolding forces ($v = 654$ nm/s, R -value = 0.392, $n = 104$). (Insets) Normally distributed unfolding force of each helix. (B) R -values observed at different pulling speeds (solid symbols, experimental data; open symbols, Monte Carlo simulations). (C) Analysis of simulated unfolding forces (red dots, perfect calibration, R -value = 0.043; blue dots, calibration error, R -value = 0.391; $v = 654$ nm/s, $n = 1000$).

uncertainties in spring constant calibration could be responsible for the correlations observed in Fig. 1 B. This effect might also have implications for the recent observation in Fn (1).

Because a comparison of absolute forces is sensitive to inevitable errors in force calibration, we propose an alternative analysis method based on force ratios. Here, we define force ratio as the normalized unfolding force of α -helix D obtained by dividing it by the unfolding force of α -helix E for each protein. This approach yields a population of force ratios near 1 and is insensitive to errors in force calibration. An easy yet powerful way for analyzing this population is to compile it as a histogram because this allows immediate access to the distribution of the force ratios. Fig. 2 A shows the force ratio histogram compiled from unfolding data determined on native (trimeric) BR. Three peaks are visible in the histogram that can be well described with three Gaussian fits. This indicates that, in the trimeric BR, α -helices E and D coexist in three distinct states, which differ in the relative strength of the α -helices. Surprisingly this is not observed for the simulated data (Fig. 2 A, inset) or monomeric BR (T. K. Sapra, H. Besir, D. Oesterhelt, and D. J. Müller, unpublished data) (Fig. 2 B). In latter cases, α -helices E and D unfold at a fixed force ratio as indicated by a single peak in the histograms. Detecting an increased energetic complexity of α -helices E and D in trimeric BR may not be a surprising finding if one considers that these

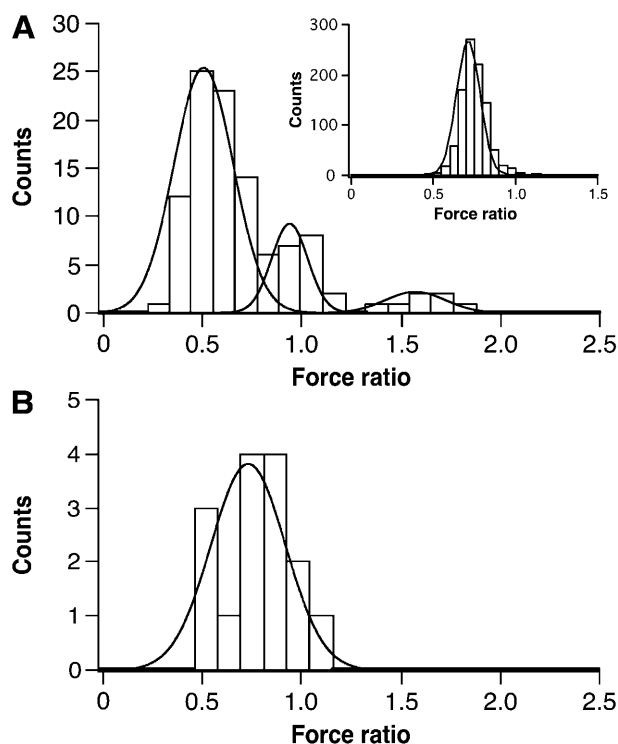


FIGURE 2 Force ratio histograms for α -helices E and D. (A) Trimeric BR shows a complex distribution well described by three Gaussian fits centered at 0.51 ± 0.02 , 0.94 ± 0.03 , and 1.57 ± 0.02 ($n = 104$; $v = 654$ nm/s). (Inset) Force ratio histogram of the MC simulation with a single peak centered at 0.72 ± 0.01 ($n = 1000$). (B) Data from monomeric BR show single peak centered at 0.74 ± 0.02 ($n = 16$).

α -helices, in combination with certain lipid molecules, are responsible for intermonomer contacts (11).

Our results indicate the presence of substantial hidden information in forced unfolding experiments of single proteins. For BR, more detailed studies using different types of molecular assemblies and proteins with point mutations are required to determine the origin of the molecular interactions stabilizing the observed coexisting states. Unraveling such hidden information of protein stability will build one important step toward understanding the complex energetic properties of single proteins.

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