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Protein Unfolding with a Steric Trap

Tracy M. Blois, Heedeok Hong, Tae H. Kim, and James U. Bowie*

Department of Chemistry and Biochemistry, UCLA-DOE Institute for Genomics and Proteomics, Molecular Biology Institute, University of California, Los Angeles 90095

Abstract

The study of protein folding requires a method to drive unfolding, which is typically accomplished by altering solution conditions to favor the denatured state. This has the undesirable consequence that the molecular forces responsible for configuring the polypeptide chain are also changed. It would therefore be useful to develop methods that can drive unfolding without the need for destabilizing solvent conditions. Here we introduce a new method to accomplish this goal, which we call steric trapping. In the steric trap method, the target protein is labeled with two biotin tags placed close in space so that both biotin tags can only be bound by streptavidin when the protein unfolds. Thus, binding of the second streptavidin is energetically coupled to unfolding of the target protein. Testing the method on a model protein, dihydrofolate reductase (DHFR), we find that streptavidin binding can drive unfolding and that the apparent binding affinity reports on changes in DHFR stability. Finally, by employing the slow off-rate of wild-type streptavidin, we find that DHFR can be locked in the unfolded state. The steric trap method provides a simple method for studying aspects of protein folding and stability in native solvent conditions, could be used to specifically unfold selected domains, and could be applicable to membrane proteins.

Examination of protein folding requires a method to control the folding-unfolding equilibrium, which is generally accomplished by altering solution conditions with chemical denaturants, temperature, or pressure to destabilize native interactions^{1, 2}. These approaches have the disadvantage that they alter the molecular forces that drive folding and the properties of the

bowie@mbi.ucla.edu.

Supporting Information Available: Materials and methods, additional figures.

denatured state³. Moreover, solvent perturbation is global and does not permit the specific manipulation of individual domains. When folding is used to screen for ligand binding⁴⁻⁷, it would be preferable to maintain strong interactions. Single molecule pulling experiments can be used to manipulate folding under native solvent conditions^{8,9}, but the methods are not generally accessible or high throughput, and it is impossible to characterize the nature of the stretched state in detail using techniques such as NMR or hydrogen exchange that require large populations of molecules⁴. Loh and coworkers developed an elegant method for unfolding without the need for solvent perturbation, called mutually exclusive folding, in which two proteins are fused so that folding of one precludes folding of the other¹¹⁻¹³. Here we describe a new, more-flexible method to drive unfolding under non-denaturing conditions. The approach allows the characterization of unfolding thermodynamics in native solvent conditions, the generation of large quantities of the unfolded state for detailed study, the selective unfolding of specific domains, and the screening for compounds that bind to the folded state without the addition of perturbing denaturants. The method could also be applicable to membrane proteins in lipid bilayers.

In our method we sterically trap a target protein in an unfolded state using a second binding protein, illustrated in Figure 1, thereby coupling unfolding to a measurable binding event. We introduce two biotin tags on a target protein that are close in space and employ monovalent streptavidin (mSA)⁵ as our steric trap. A single mSA can bind without steric hindrance to the folded protein, but a second mSA can only bind when the protein unfolds, or partially unfolds, due to steric overlap in the native conformation. Thus, the apparent binding affinity of the second mSA is coupled to the unfolding free energy. If $\Delta G_{\text{bind}} = \Delta G'_{\text{bind}}$ (the mSA molecules do not interact), the difference in binding affinities gives ΔG_u . When comparing the stabilities of two variants, however, it is not necessary to make this assumption.

We tested the steric trap on a well characterized protein, mouse dihydrofolate reductase (DHFR)^{6, 7}. To allow site-specific biotin labeling, a cysteine-free construct, C7A, was used for all experiments described here. To set the steric trap, Arg29 and Lys64 in DHFR were replaced with cysteine and labeled with a thiol-reactive biotin tag, N-(biotinoyl)-N'-(iodoacetyl) ethylenediamine (BE), either singly (BE-DHFR-R29C and BE-DHFR-K64C) or in combination (BE₂-DHFR). These sites were chosen for their close proximity to one another, their location on structured α -helices, and their high solvent accessibility (Figure 2a). We utilized two forms of mSA, one with a single wild-type subunit and one with a single S45A mutant subunit (mSA_{S45A}) that has ~ 1000 -fold reduced biotin binding affinity (3.6×10^{-11} M) and an accelerated off rate^{8, 9}. Binding of mSA is essentially irreversible over many hours^{5, 10} while binding of the mSA_{S45A} variant can be rapidly reversed upon the addition of free biotin.

If BE₂-DHFR can be sterically trapped in the unfolded state, we expect a loss of activity with the addition of excess streptavidin. As shown in Figure 2b, the activity of BE₂-DHFR is lost with increasing molar ratios of mSA_{S45A} to BE₂-DHFR. The loss of activity was completely reversible, as indicated by the restoration of activity upon the addition of free biotin competitor. Maximum activity loss occurs when mSA_{S45A} is in a 2-fold or greater molar excess, suggesting that inactivation requires double streptavidin binding (Figure 2c). Moreover, the singly labeled mutants, BE-DHFR-R29C and BE-DHFR-K64C, were not inactivated by mSA_{S45A} (Supporting Information, Figure S1). In fact, BE-DHFR-K64C was activated ~ 3 -fold by streptavidin binding. Activation could be due to slight increases in conformational flexibility upon binding, as DHFR is known to be activated by dilute denaturants¹¹. As a further indication that two mSAs could bind simultaneously, we observed fluorescence resonance energy transfer between two labeled mSA proteins in the presence of BE₂-DHFR (Supporting Information, Figure S2).

To further investigate whether the two bound mSAs drive protein denaturation, we employed limited proteolysis to detect unfolding¹². BE₂-DHFR samples were incubated in the absence and presence of mSA, followed by exposure to chymotrypsin. The reactions were then quenched and analyzed by SDS-PAGE. As shown in Figure 2c, BE₂-DHFR becomes protease sensitive in the presence of mSA, consistent with protein unfolding.

If the reaction scheme presented in Figure 1 is correct, the binding affinity of the second streptavidin should be indicative of protein stability. Anything that increases protein stability, such as compounds that bind to the folded state, will decrease the apparent affinity of streptavidin. To test this prediction we observed the effects of increasing concentrations of the DHFR cofactor NADPH on the binding of the second mSA_{S45A} (Figure 2d). As expected, the binding affinity of mSA_{S45A} decreased with increasing concentrations of NADPH. If mSA_{S45A} binds exclusively to the unfolded state, NADPH binds exclusively to the folded state, and the unfolding equilibrium constant is very small¹⁴, then the apparent mSA_{S45A} dissociation constant, $K_d^{mSA}(\text{app})$, should be given by:

$$K_d^{mSA}(\text{app}) = K_d^0 (1 + [\text{NADPH}] / K_d^N),$$

where K_d^0 is the dissociation constant observed in the absence of NADPH, reflecting the intrinsic affinity of mSA_{S45A} for biotin and the unfolding equilibrium, and K_d^N is the dissociation constant for NADPH binding to DHFR. Fitting the data in Figure 2d with our measured K_d^N of 120 nM (Supporting Information, Figure S3), yields a K_d^0 of 0.08 ± 0.01 μM . Based on these parameters, at 0.6 μM NADPH we expect a $K_d^{mSA}(\text{app})$ of 0.38 μM and at 12 μM , we expect a $K_d^{mSA}(\text{app})$ of 7.6 μM . The measured values were 0.50 ± 0.07 μM and 6.6 ± 0.9 μM , respectively. If we make the assumption that $\Delta G_{\text{bind}} = \Delta G'_{\text{bind}}$, the observed K_d^0 corresponds to a ΔG_u of 4.5 kcal/mol, which compares favorably to the ΔG_u of 3.9 ± 0.6 kcal/mol we measured by urea denaturation (Supporting Information, Figure S5). These results indicate that the steric trapping method can both detect and faithfully measure changes in protein stability.

The results reported above indicate that steric trapping is reversible, but another potential advantage of the steric trapping method is that the protein can be essentially locked in the denatured state by employing the slow off rate of wild type streptavidin. As shown in Figure S4, when wild-type mSA is employed to unfold BE₂-DHFR, the addition of a tight binding DHFR inhibitor, methotrexate, does not protect the protein from proteolysis even at concentrations of 70 μM , which is thousands fold higher than its K_d of 1.2 nM¹⁵. Thus, the bound mSA effectively blocks refolding.

Steric trapping provides a convenient and versatile means for driving unfolding under native solvent conditions. Our steric trapping method can take advantage of the many tools developed around the streptavidin-biotin interaction including streptavidin mutants with a range of affinities, numerous biotin labels, and diverse assay methods.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.
The steric trap method. The biotin tag, B, is represented by the orange circles. The active subunit of mSA is shown in teal.

Figure 2.

The steric trap method applied to mDHFR. Error bars are from triplicate experiments. (a) Crystal structure of mDHFR (PDB Code 1U72)¹³, shown with the sites of the engineered cysteine mutations, K64C and R29C. The native Cys7 (yellow) was mutated to alanine. (b) Activity assays of BE₂-DHFR in the presence of mSA_{S45A} (black) or after addition of 5 mM free biotin (grey). The control sample indicates a 3-fold molar excess of mSA, preincubated with biotin, was used. (c) Limited proteolysis of BE₂-DHFR. DHFR with and without mSA was incubated with and without protease as indicated. (d) BE₂-DHFR unfolding curves in the presence of 0, 0.6 or 12 μM NADPH.