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Protein Unfolding with a Steric Trap

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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] These approaches have the disadvantage that they alter the molecular forces that drive folding and the properties of the denatured state.[2] Morever, solvent perturbation is global and does not permit the specific manipulation of individual domains. When folding is used to screen for ligand binding,[3] it would be preferable to maintain strong interactions. Single molecule pulling experiments can be used to manipulate folding under native solvent conditions,[4] 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 molecules.[5] Loh and co-workers 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 other.[6] Here we describe a new, more-flexible method to drive unfolding under nondenaturing 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

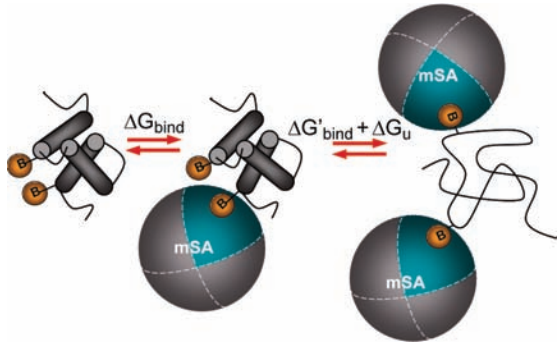

Figure 1. Steric trap method. The biotin tag, B, is represented by the orange circles. The active subunit of mSA is shown in teal.

monovalent streptavidin (mSA)[7] 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 $\Delta G_{bind} = \Delta G'_{bind}$ (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).[8] 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 (BE_2-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.[9] Binding of mSA is essentially irreversible over many hours,[7,10] while binding of the mSA_{S45A} variant can be rapidly reversed upon the addition of free biotin.

If BE_2-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 BE_2-DHFR is lost with increasing molar ratios of mSA_{S45A} to BE_2-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 denaturants.[11] 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 BE_2-DHFR (Supporting Information, Figure S2).

To further investigate whether the two bound mSAs drive protein denaturation, we employed limited proteolysis to detect unfolding.[12] BE_2-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, BE_2-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

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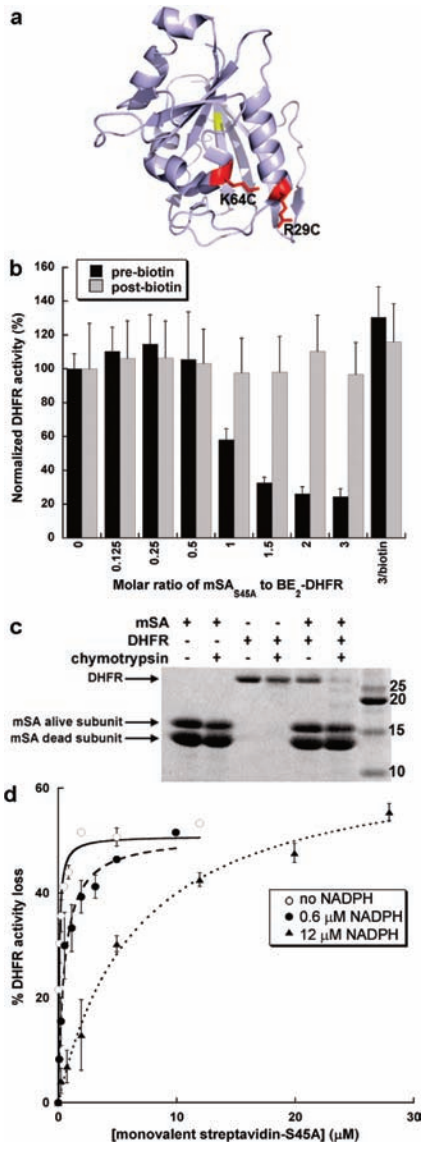

Figure 2. Steric trap method applied to mDHFR. Error bars are from triplicate experiments. (a) Crystal structure of mDHFR (PDB Code 1U72),[13] shown with the sites of the engineered cysteine mutations, K64C and R29C. The native Cys7 (yellow) was mutated to alanine. (b) Activity assays of BE_2-DHFR in the presence of mSA_{S45A} (black) or after addition of 5 mM free biotin (gray). The control sample indicating a 3-fold molar excess of mSA, preincubated with biotin, was used. (c) Limited proteolysis of BE_2-DHFR. DHFR with and without mSA was incubated with and without protease as indicated. (d) BE_2-DHFR unfolding curves in the presence of 0, 0.6, or 12 μM 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 small,[14] then the apparent mSA_{S45A} dissociation constant, K_d^{mSA}(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 $\pm$ 0.01 μM. Based on these parameters, we expect a K_d^{mSA}(app) of 0.38 μM at 0.6 μM NADPH and a K_d^{mSA}(app) of 7.6 μM at 12 μM. The measured values were 0.50 $\pm$ 0.07 and 6.6 $\pm$ 0.9 μM, respectively. If we make the assumption that $\Delta G_{bind} = \Delta G'_{bind}$, 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 $\pm$ 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 BE_2-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 >10 000-fold higher than its K_d of 1.2 nM.[15] 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.

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Supporting Information Available: Materials and methods, additional figures. This material is available free of charge via the Internet at http://pubs.acs.org.

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