

RAPID COMMUNICATION

Denaturing and refolding of protein molecules on surfaces

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Keeping protein molecules in the active state on a solid surface is essential to protein microarrays and other protein-based biosensors. Here, we show that the 2-D chemical environment controls the refolding of the denatured green fluorescent proteins tethered to solid surfaces. Refolding occurs readily on the repulsive PEG functionalized surface but is inhibited on the attractive $-NH_2$ functionalized surface. This result shows the critical importance of the 2-D chemical environment in the maintenance and revival of protein activity on surfaces and opens the door to designing 2-D molecular chaperones for protein folding.

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How a protein molecule folds into its native state is a major question in biological research. Protein folding *in vivo* occurs in a complex and dynamic environment crowded by other molecules. While small proteins with single domains may fold spontaneously by minimizing the exposure of hydrophobic residuals to the aqueous environment, the folding of larger protein molecules are often assisted by molecular chaperones [1, 2]. The molecular chaperones function by temporarily binding to the hydrophobic residuals/domains of nascent polypeptide chains to prevent aggregation and by providing confined environments to facilitate protein folding. Recently, there is growing interest in protein molecules confined to 2-D environments, *e.g.* solid surfaces. Examples include protein microarrays, biological sensors, and biocompatible materials. We ask the following questions: How does a protein molecule fold on a 2-D surface and what is the role of the local surface chemical environment on

the folding process? These questions are significant because keeping protein molecules in active states on surfaces is of paramount importance to the aforementioned technologies involving surface immobilized protein molecules.

For protein folding *in vivo*, there are at least two reasons for misfolding [1, 2]: (i) intermolecular interaction leading to peptide aggregates; and (ii) intramolecular interaction leading to the binding of domains that should be further apart in the native state. Indeed, some of the major functions of chaperones are to reduce or eliminate these two types of interactions. When a protein molecule is tethered to a solid surface, the first reason is no longer of concern because of the lack of mobility. Instead, it is replaced by a new type of interaction, *i.e.* protein–surface interaction, which may prevent the tethered protein molecule from folding into its native state. In fact, protein/peptide molecules are known to interact strongly with a wide variety of solid surfaces through hydrophobic, electrostatic, and hydrogen bond interactions. In the language of biomaterials, the tendency of a protein molecule to adsorb strongly on a solid surface is called “fouling” and the opposite is “nonfouling”. The presence of strong attractive interaction with the solid surface should introduce energetic barriers for a peptide chain to fold into its native state. Thus, we predict that a fouling surface prohibits the folding of a tethered peptide chain into its native state while a non-fouling surface does not. These issues have been addressed before by Möller and coworkers [3] based on fluorescence

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Abbreviations: **APS**, aminopropyltriethoxysilane; **GFP**, green fluorescent protein; **IDA**, iminodiacetic acid; **RMS**, root mean square

energy transfer in a dye tagged ribonuclease immobilized on nonfouling surfaces. In the present study, we probe denaturing and folding based on the activity of immobilized protein molecules.

We choose green fluorescent protein (GFP) [4] as the model system because it allows us to track unfolding and refolding by simply following its intrinsic fluorescence at 508 nm. In the native form, the chromophore of a GFP molecule is surrounded by a hydrophobic barrel which shields it from water molecules. Upon denaturing, the chromophore is exposed to the hydrophilic environment and fluorescence is quenched. Indeed, GFP has been one of the most popular model systems in previous studies on protein folding mechanisms [5, 6, 7]. We used three different types of surfaces, Fig. 1, to adsorb GFP with a 6 × histidine (6 × His) tag engineered to the N-terminus. The first surface (a) is a high-density PEG coated glass surface with Cu^{2+} ions chelated to iminodiacetic acid (IDA) groups that are covalently attached to the PEG coating. This surface is commercially available (MicroSurfaces, Minneapolis, USA) and is similar to that described in a previous publication [8]. This surface specifically binds 6 × His-GFP but resists the nonspecific adsorption of protein molecules without the poly-His tag. PEG is known to possess the best nonfouling property among a large number of surface functional groups studied [9]. The second surface (b) also consists of chelated surface Cu^{2+} ions to surface attached IDA groups but the underlying surface is 3-aminopropyltriethoxysilane (γ -APS) functionalized glass. While 6 × His-GFP can bind to surface chelated Cu^{2+} sites, they can also adsorb nonspecifically on the γ -APS coating due to hydrogen bonding and electrostatic interactions with $-\text{NH}_2$ groups. The third surface (c) is γ -APS coated glass and protein adsorption is only mediated by non-specific interaction with $-\text{NH}_2$ groups.

The Cu^{2+} /IDA/PEG, Cu^{2+} /IDA/ γ -APS, and γ -APS coated glass slides were from MicroSurfaces (<http://proteinslides.com>). These surfaces have root-mean-square (RMS) roughness of 1–2 nm, as determined by atomic force microscopy. The 6 × His-GFP samples were prepared as described previously [8]. All chemicals and solvents were

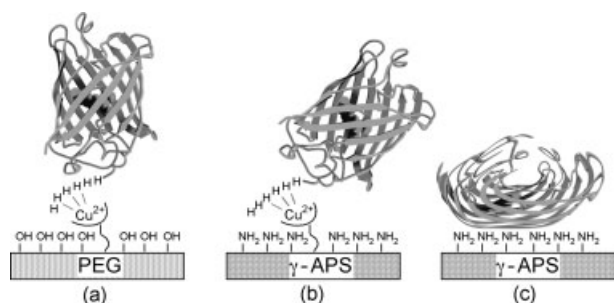


Figure 1. Schemes of the binding of 6 × His-GFP (a) to Cu^{2+} -IDA groups on high density PEG coated glass surface; (b) Cu^{2+} -IDA groups on γ -APS coated glass surface; and (c) nonspecifically to γ -APS coated glass surface. The H...H tail on each GFP represents the 6 × His tag.

purchased from Sigma/Aldrich (St. Louis, MO). Nanoliter droplets of 6 × His-GFP in a phosphate buffer (1 × PBS) containing 10% glycerol (pH = 7.5) were delivered onto a coated glass slide by a robotic spotter (Molecular Dynamics). The surfaces were incubated in a humidified chamber for 10 min at room temperature. After washing with 1 × PBS buffer twice (5 min each), the slide (kept hydrated all the time with 1 × PBS buffer) was examined under a fluorescent microscope (Nikon Eclipse 50i). We used acid-based [5] denaturing (De) and refolding (Re) [10, 11]. The slide with surface immobilized 6 × His-GFP was immersed in a denaturing buffer containing 50 mM Tris HCl (pH = 3.5) for 5 min at room temperature and examined under the microscope. Refolding involved incubating the surface with the renaturing buffer containing 1 × PBS, 20% sucrose, and 10% glycerol (pH = 8.1) at room temperature for 5 min. We found that both denaturing and refolding occurred on the time scale of ~ 1 min, faster than those observed in the solution phase [10, 11]. The 5 min incubation time ensured the completeness of denaturing and refolding. For immunostaining after denaturation, the slide containing denatured 6 × His-GFP was washed three times (5 min each) with 1 × PBS containing 0.05% Tween-20 and 20 mM imidazole (pH = 7.8) followed by incubation with anti-GFP antibody (Molecular Probes, OR) in a humidified chamber for 2 h at room temperature. The surface was subsequently washed extensively with the same washing buffer three times (15 min each), incubated with cy3-conjugated secondary antibody (Jackson Immuno Research Laboratory, PA) for 1 h at 25°C, washed with the same buffer three times (15 min each) and finally rinsed once with DI water. The slide was finally blown dry and examined under the fluorescence microscope.

The top panels (cycle 0) in Fig. 2 show fluorescence microscope images of 6 × His-GFP immobilized on the three surfaces before cycles of denaturing (De) and refolding (Re). The intrinsic fluorescence intensities are very different. The fluorescence intensity on Cu^{2+} /IDA/ γ -APS surface is 70% of that on the Cu^{2+} /IDA/PEG surface. For adsorption on the γ -APS surface, the fluorescence intensity is much lower, only less than 20% of that on Cu^{2+} /IDA/PEG. As shown below from immunostaining, there are similar amounts of GFP on the surface. Thus, the majority of GFP molecules on the γ -APS surface are denatured (unfolded) due to the strong nonspecific interaction. The lower fluorescence intensity on the Cu^{2+} /IDA/ γ -APS surface than that on Cu^{2+} /IDA/PEG can also be attributed to the portion of 6 × His-GFP molecules nonspecifically interacting with $-\text{NH}_2$ groups on the γ -APS coating.

While no fluorescence is detected after the denaturing step on all three surfaces, result of refolding is a strong function of the chemical nature of the surface. On the Cu^{2+} /IDA/PEG surface, we observe 40% recovery of fluorescence intensity even after three cycles of De/Re. In contrast, less than 10% of fluorescence intensity is left on the Cu^{2+} /IDA/ γ -APS surface and no fluorescence is observed

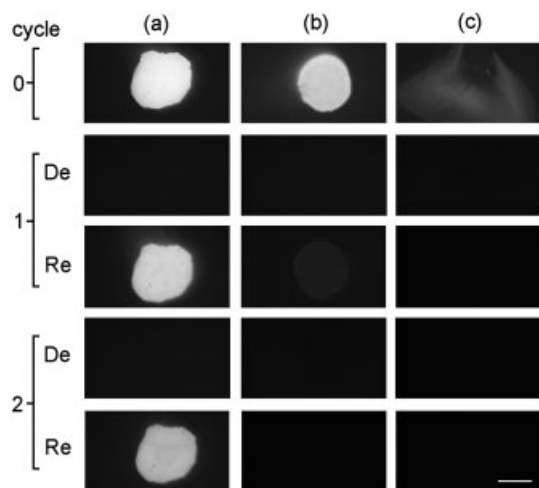


Figure 2. Fluorescence microscope images of $6 \times$ His-GFP before and after cycles of De/Re on three surfaces: (a) Cu^{2+} /IDA/PEG; (b) Cu^{2+} /IDA/ γ -APS; (c) γ -APS. Nanoliter droplets of crude lysate solution (4 mg/mL) containing $6 \times$ His-GFP and 10% glycerol were deposited onto the glass slide *via* a robotic spotter. Each slide was incubated at room temperature for 10 min, rinsed quickly with PBS buffer three times. The slide was covered with the buffer solution and imaged under the fluorescence microscope (excitation wavelength ~ 488 nm and detection wavelength ~ 508 nm). The images were taken before denaturing (cycle 0) and after two cycles (1 and 2) of acid denaturing (De) and refolding (Re). The scale bar (lower right) was 250 μm .

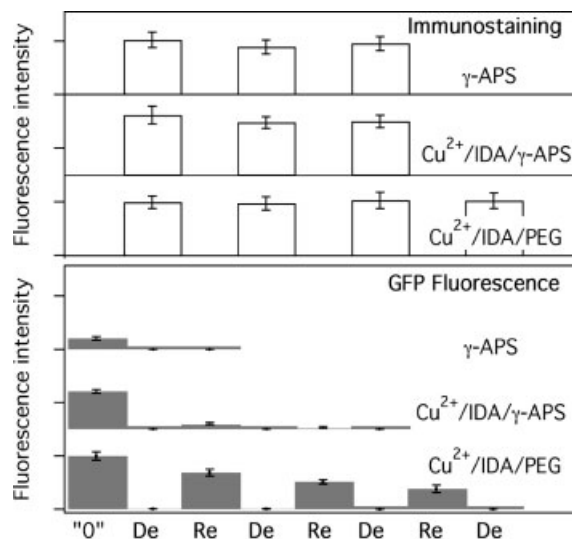


Figure 3. Lower panel: intensity of intrinsic fluorescence from $6 \times$ His-GFP as a function of cycles of denaturing (De) and refolding (Re) on the three surfaces: Cu^{2+} /IDA/PEG, Cu^{2+} /IDA/ γ -APS, and γ -APS. “0” represents the starting surface obtained from $6 \times$ His-GFP immobilization. Upper panel: Immunostaining of HGFP on the three surfaces. Slides with immobilized $6 \times$ His-GFP at each denaturing step are incubated with anti-GFP, washed extensively, incubated with cy3-conjugated secondary antibody, and washed again. Fluorescence intensities from the cy3-label are plotted as a function of the De/Re cycles.

on the γ -APS surface after only one cycle of De/Re. These results are summarized quantitatively in the lower panel in Fig. 3.

To verify that the loss of fluorescence intensity is not due to the desorption of $6 \times$ His-GFP molecules from the surface, we perform immunostaining to quantify the immobilized GFP molecules. We find that the primary antibody detects unfolded GFP more efficiently than it does against folded GFP. Thus, we carry out immunostaining after each denaturing step. The upper panel in Fig. 3 summarizes the immunostaining results as a function of the De/Re cycles. We arrive at two conclusions: the starting concentrations of $6 \times$ His-GFP are similar on the three surfaces and there is no desorption of $6 \times$ His-GFP molecules from the surface throughout the De/Re cycles.

Note that, even on the Cu^{2+} /IDA/PEG surface, refolding is not 100%. The fluorescence intensity is 70% after one De/Re cycle and 40% after three De/Re cycles. This could be an intrinsic property of the GFP molecule. Previous experiments carried out in the solution phase showed that 64–90% of denatured GFP molecules were renatured [10, 11]. Note also that we used the highest protein immobilization density (estimated to be of the order of 10^{13} molecules/ cm^2) [8] in the present study. At such a high surface density, intermolecular interaction becomes possible during denaturing and refolding. It is expected that denaturing and refolding should depend on the surface morphology and the 2-D density of

immobilized protein molecules. These issues are not addressed in the present report and are subjects of future studies.

Why does the immobilized $6 \times$ His-GFP molecule refold effectively on the Cu^{2+} /IDA/PEG surface, but not on the Cu^{2+} /IDA/ γ -APS or γ -APS surface? The answer lies in the protein–surface interaction discussed earlier. On the Cu^{2+} /IDA/PEG surface, each immobilized GFP molecule is linked only by the $6 \times$ His tag but otherwise prefers to stay away from the surface due to the repulsive nature of the PEG functionality. This repulsive or nonfouling nature of the surface ensures that the weak protein–surface interaction does not introduce additional barriers on the energy landscape for protein refolding. On the Cu^{2+} /IDA/ γ -APS surface, the active GFP molecules (possessing intrinsic fluorescence) may be held primarily by the $6 \times$ His tag, but there is also attractive and nonspecific interaction between tethered GFP and the “sticky” or fouling $-\text{NH}_2$ functional groups in the immediate surrounding. Upon denaturing, such nonspecific interaction with the sticky environment is expected to increase, thus effectively introducing insurmountable barriers on the energy landscape for protein refolding. On the γ -APS surface, the majority of immobilized $6 \times$ His-GFP is denatured to start with and there is no refolding on this sticky surface. We conclude that the 2-D chemical environment is of critical importance not only to the activity of immobilized protein molecules [12], but also to the maintenance and revival of

this activity. This study also raises the possibility of designing surface chemical environment as a 2-D model for the molecular chaperone effect.

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