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Self-Assembled Enzymatic Monolayer Directly Bound to a Gold Surface: Activity and Molecular Recognition Force Spectroscopy Studies

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Supporting Information

ABSTRACT: *Escherichia coli* dihydrofolate reductase (ecDHFR) has one surface cysteine, C152, located opposite and distal to the active site. Here, we show that the enzyme spontaneously assembles on an ultraflat gold surface as a homogeneous, covalently bound monolayer. Surprisingly, the activity of the gold-immobilized ecDHFR as measured by radiographic analysis was found to be similar to that of the free enzyme in solution. Molecular recognition force spectroscopy was used to study the dissociation forces involved in the rupture of AFM probe-tethered methotrexate (MTX, a tight-binding inhibitor of DHFR) from the gold-immobilized enzyme. Treatment of the ecDHFR monolayer with free MTX diminished the interaction of the functionalized tip with the surface, suggesting that the interaction was indeed active-site specific. These findings demonstrate the viability of a simple and direct enzymatic surface-functionalization without the use of spacers, thus, opening the door to further applications in the area of biomacromolecular force spectroscopy.

Molecular recognition force spectroscopy (MRFS) is a specific application of atomic force microscopy (AFM) measurements wherein the strength of ligand−receptor interactions, such as that between an enzyme and its inhibitor, is probed. In a typical experiment, the AFM probe and substrate are covalently modified to immobilize the ligands and receptors, respectively.[1,2] If the surface-bound receptor is a biomolecule such as a protein, a spacer is commonly used to ensure separation between the biomolecule and surface.[3−6] These studies not only require a complex design and assembly, but the flexibility and dynamics of the spacer often interfere with the AFM or molecular tweezers measurements.[3,6−8] A potentially simpler design, however, can be envisioned wherein the biomolecule is immobilized on a surface directly forming a self-assembled monolayer (SAM) through a direct interaction with the surface. Furthermore, close proximity of biomolecules may provide a unique stabilization that is absent in the solution, and may better resemble the crowdedness cytosolic enzymes face *in vivo*. If the ligand of interest is also bound to the AFM tip, the interaction between a ligand and the receptor can be studied using MRFS by pulling the tip away from the ligand−receptor complex until the applied force overcomes the interaction force and leads to dissociation of the complex. Such direct surface functionalization could potentially offer advantages in other experiments that require minimal distance between the surface and an enzyme active site (e.g., electrical conductivity[9] or catalyzed redox reactions using the surface as an electrode.[10,11])

Here, we present the use of MRFS to directly study the binding forces involved in the interaction of *Escherichia coli* dihydrofolate reductase (ecDHFR) with a tight-binding inhibitor, methotrexate (MTX). DHFR catalyzes the transfer of the pro-*R* hydride from C4 of reduced nicotinamide adenine dinucelotide phosphate (NADPH) to the *si*-face of 5,6-dihydrofolate (DHF), forming *S*-5,6,7,8,-tetrahydrofolate (THF) and $NADP^+$. This vital housekeeping enzyme is targeted by many chemotherapeutic and antibacterial agents. Because of its small size, lack of metals or S−S bonds, and simple catalyzed chemistry, ecDHFR has become a model system for studies of enzyme folding, activity, and dynamics, for both experimentalists and theoreticians.[12−19] Consequently, ecDHFR is a suitable and interesting candidate for the development of new MRFS studies.

The wild-type ecDHFR enzyme has a single cysteine (C152) on the outer surface opposite to and remote from the active site, and can, in principle, be used to bind the enzyme directly to a gold surface for use in MRFS experiments. The only other cysteine in the enzyme (C85) is located in an internal region of the protein and is unlikely to bind to the gold surface while the enzyme is folded in its globular conformation. Figure 1 presents the dimensions (nm) of ecDHFR as it would be bound to the gold surface through C152. In studies of surface-immobilized proteins, the use of linkers such as polyethylene glycol (PEG) or DNA to tether the biomolecules to surfaces is widespread.[3−5,20−22] However, the use of these linkers can be disadvantageous in studies of enzyme folding, dynamics, or function as the response becomes convoluted and in some cases even limited by the linker itself.[3,5−7] In particular, the polydispersity and nonlinear elasticity of the linker can significantly affect the dissociation rates and magnitudes of unbinding interactions.[1] The rupture forces measured without a spacer are typically larger,[2] hence, improving signal-to-noise ratio in MRFS experiments. Furthermore, the use of spacers has been shown to decrease lateral resolution, thus, diminishing the likelihood of single-molecule interactions.[23,24] The ability of ecDHFR to form a covalent bond between Cys152 and gold allows us to immobilize the enzyme onto the Au surface forming a SAM without the mediation of linkers or spacers. The SAM design results in closely spaced enzyme molecules, that are more crowded than diluted enzyme used in most *in vitro*

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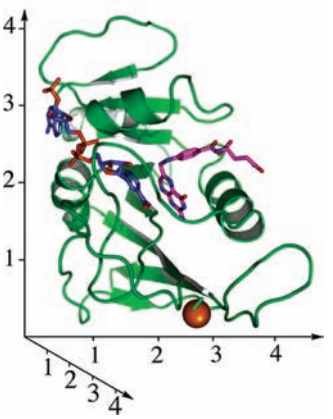

Figure 1. The dimensions in nanometers of ecDHFR in complex with $NADP^+$ (blue) and folate (magenta) (PDB ID 1RX2).[26] The thiol bound to the gold (C152) is highlighted as sphere.

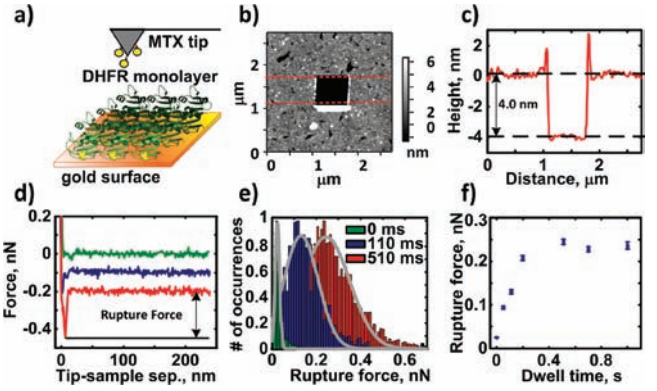

Figure 2. (a) Schematic cartoon of the experimental setup. (b) AFM height image of ecDHFR monolayer. The central region has been scratched to examine monolayer thickness. (c) Averaged cross section of 60 horizontal line scans between the red lines shown in panel b. (d) Representative force measurements (retract data only) of interactions at dwell times of 0 (green), 110 (blue), and 510 (red) ms. (e) Distributions of rupture forces at dwell times of 0, 110, and 510 ms. (f) Mean rupture forces versus dwell time at a velocity of 2 μm/s.

experiments. Nonetheless, it provides a closer approximation of crowded cell conditions.[25]

Proteins are conformationally labile molecules, and many studies indicate an important role of protein dynamics in the catalytic performance of enzymes.[27,28] AFM measurements on enzymatic SAMs can thus explore the effect of monolayer organization on enzymatic activity and conformational behavior.[29] Furthermore, the new method may provide insight into the effect of this unique environment (gold surface at the bottom, several neighboring proteins within the surface plane, and water molecules above) on the enzymatic function (i.e., enzyme catalysis and inhibition). To the best of our knowledge, direct demonstration of the catalytic activity in an enzymatic SAMs on gold surface has not been reported in the literature to date.

Figure 2a shows a schematic representation of the MRFS experimental setup utilized herein. For surface functionalization, ultraflat Au surfaces were immersed in a 10 μM ecDHFR solution in MTEN buffer (pH 7.5 at 25 °C), allowing the enzyme to self-assemble and covalently bind to the gold substrate (see Supporting Information (SI) for further details). Samples were incubated in that solution for 1 h and then rinsed several times in MTEN buffer to remove any physisorbed, noncovalently bound protein. The formation of a homogeneous monolayer was confirmed by AFM images (Figure 2b,c). Prior to and following all MRFS and activity studies, the ecDHFR-functionalized surfaces were scratched with the AFM tip to confirm the presence of an ecDHFR monolayer and to determine its thickness, as shown in the AFM height image in Figure 2b and the corresponding cross-section profile in Figure 2c. The scratch test involved the application of a force greater than 50 nN and imaging in contact mode in order to remove all material in a specified region.[30−32] By comparing height variations of the monolayer in the scratched region versus the surrounding unscratched region, we found a height variation of approximately 4 nm, consistent with the size of ecDHFR along the vertical dimension of Figure 1. The scratch test therefore supported a single uniform monolayer of ecDHFR molecules formed in all samples used in this study. Figures S2 and S3 show similar results for the scratch tests performed after the kinetic assay described below, both before and after treatment with MTX, respectively. These tests further confirmed that the monolayer was stable throughout the activity and AFM experiments.

Enzyme-immobilization may alter enzyme activity and behavior significantly from the solution behavior of the same protein.[33] Furthermore, one of the main reasons for the use of spacers in surface-immobilization of enzymes is to address the effect of the surface on enzyme folding, dynamics, and consequently function. In this case, the direct immobilization of the ecDHFR on the gold surface required careful examination of the catalytic activity prior to investigating MTX dissociation via MRFS. To conduct the activity assay of the ecDHFR-functionalized Au surface, we labeled NADPH with ^{14}C at the amide carbonyl to obtain [carbonyl-^{14}C] NADPH, and used this labeled substrate to follow ^{14}C-NADPH formation as a function of time, using HPLC and liquid scintillation analysis as described before.[34] Notably, conventional UV−vis activity measurements at 340 nm were not useful with the gold-immobilized enzyme due to the presence of the solid plate in the reaction mixture (i.e., heterogeneous, surface catalysis). A k_{cat} value of $8.8 \pm 1.2\ s^{-1}$ was determined using an ecDHFR concentration of 10 pM (see SI). Remarkably, this rate is about the same k_{cat} as that measured for ecDHFR under the same conditions in solution ($10-12\ s^{-1}$).[35] Since in solution the rate is determined by dissociation of the product tetrahydrofolate,[35] the k_{cat} of the Au-immobilized enzyme suggests that the dissociation was not altered and the overall functionality of the enzyme is similar to that in solution. This is especially important as the MRFS experiments presented below examined the dissociation of this product's analogue, MTX.

Once we had confirmed that the Au-bound ecDHFR monolayer was indeed active, we used MRFS to directly probe the interaction forces between ecDHFR and its pM inhibitor, MTX. Initially, three control measurements were conducted: (1) the interaction between a bare, unfunctionalized Si_3N_4 tip and gold-bound ecDHFR; (2) the same measurement for the MTX-functionalized tip; and (3) unfunctionalized Si_3N_4 tip and enzyme (see SI).[4,21,36] Then, the interaction between the tip-bound MTX and gold-bound ecDHFR was studied. Finally, the gold-bound ecDHFR was immersed in a 220 μM MTX solution in MTEN buffer and equilibrated for 20 min to saturate all active-sites with MTX, and then interaction forces between tip-bound MTX and the gold-bound ecDHFR−MTX complex were measured. The last experiment was critical to verify that the interaction between

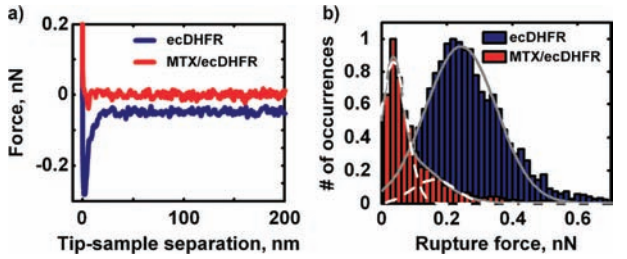

Figure 3. (a) Representative force measurement (retract data only) of interactions between the enzyme and MTX-functionalized tip (blue), and MTX-bound enzyme with MTX-functionalized tip (red). (b) Distributions of rupture forces for enzyme and MTX-functionalized tip (blue) and MTX-bound enzyme and MTX functionalized tip (red). All measurements collected at a dwell time of 510 ms and a velocity of 2 μm/s. For measurements with the gold-bound ecDHFR, 4995 measurements were performed on two independent samples, resulting in an average rupture force of 245 $\pm$ 110 pN (single Gaussian in gray). For control experiment with the MTX-blocked ecDHFR complex, 2304 measurements were performed on two independent samples, resulting in one major rupture force of 40 $\pm$ 20 pN and one minor rupture force of 200 $\pm$ 100 pN. Individual Gaussian fits are shown with dashed lines, while the combined sum by solid gray line.

the MTX-tip and functionalized gold surface is active-site specific.

For MRFS studies, multiple force measurements at various sample positions were collected (see SI for further details).[37] Upon retraction, the intermolecular contacts between gold-bound ecDHFR (or the DHFR−MTX complex) and the tip-bound MTX are ruptured as the probe moves away from the surface. The rupture force is defined as the maximum force required to remove the probe from the contact with the sample. To establish an optimal dwell time for the tip in the contact with the sample, force measurements were performed at several dwell times ranging between 0 and 1 s, while keeping the tip-velocity (2 μm/s) and maximum loading force (500 pN) constant. Figure 2d shows representative force-plots (retract data only) for measured interactions at three selected dwell times of 0, 110, and 510 ms; Figure 2e shows the corresponding distribution of rupture forces for these dwell times. Both figures clearly demonstrate the increase in rupture force with increasing dwell time. Figure 2f shows the mean rupture force as a function of dwell time. At dwell time of 0.51 s or greater, a maximum rupture force is reached for the interaction between ecDHFR and MTX. Hence, the dwell time of 0.51 s was selected as the optimal time for the MRFS experiments described below. We note the dependence of the rupture force as a function of the dwell time observed here is similar to that for vancomycin and D-Ala−D-Ala interactions, and can be used to estimate an apparent association rate constant.[38] Following similar approach, the interaction time for half-maximum probability of binding was estimated as 0.08 s, and the association constant k_{on} = 2.5 M^{-1} s^{-1}. The result is comparable with the rate constants measured for vancomycin and D-Ala−D-Ala interactions (5 M^{-1} s^{-1}).

Figure 3 shows representative force plots and the distribution of rupture forces measured for each experimental condition detailed above. The distributions are asymmetric, implying the occurrence of multiple binding events between MTX and ecDHFR.[5,39−41] Multiple-binding is probable since multiple MTX molecules are bound per tip and several enzyme molecules can be probed in the contact region between tip and substrate. Under the experimental conditions used herein, the estimated contact area (see SI for details) includes 6 ecDHFR molecules, assuming the enzyme is a sphere 4 nm in diameter (Figure 1). However, since the force-plot in Figure 3a suggests a single rupture in each force measurement, it is likely that a single interaction between MTX and ecDHFR is ruptured per force measurement. For the distribution of the interactions between DHFR and MTX, force measurements were fit to Gaussian distribution. The interaction between the MTX blocked enzyme surface and MTX tip show much weaker interaction but similar asymmetric nature, and there is an appearance of a second distribution at higher forces, possibly due to unoccupied active sites, so a double Gaussian fit was used.

As expected, the bare Si_3N_4 tip shows little interaction between the ecDHFR monolayer, with a mean rupture force of 30 $\pm$ 25 pN (Figure S4a), and similar small interactions are seen between an MTX-functionalized tip and the bare gold substrate (Figure S4b). However, the interaction between the ecDHFR monolayer and the tip-bound MTX is substantially higher (mean rupture force of 245 $\pm$ 110 pN), indicating that a binding event has taken place between a tip-bound MTX and substrate-bound ecDHFR (Figure 3b). A significantly reduced rupture force was measured between the tip-bound MTX and the gold-immobilized ecDHFR blocked by MTX, with an average value of 40 $\pm$ 20 pN with a second Gaussian distribution at 200 $\pm$ 100 pN. The second peak is similar to the specific MTX −DHFR interaction, suggesting that not all the active sites were blocked by MTX or, alternatively, the mechanical force induced by the AFM tip may remove MTX from the active site.[42] The significant difference in the rupture forces presented in Figure 3 indicates that the interaction of the MTX-functionalized tip was mostly active-site specific with regard to the gold-immobilized ecDHFR. Therefore, with the MRFS technique described herein, one can distinguish with pico-Newton accuracy between active and inhibitor-bound enzyme. ecDHFR−MTX complex rupture forces are within the range of values excepted for receptor−ligand complexes. For example, streptavidin−biotin complex has a mean rupture force of 340 pN.[32] Comparatively, vancomycin and D-Ala−D-Ala interaction strength is much weaker with a reported mean rupture force of 98 pN.[38]

In summary, we have demonstrated the formation of a stable monolayer of active ecDHFR directly bound to an ultraflat gold surface with limited disruption to the catalytic activity of the enzyme. Additionally, we have probed the rupture forces between the active-site of the immobilized enzyme and a tight-binding inhibitor, methotrexate. Our results indicate significant rupture forces (similar to streptavidin−biotin complex) upon dissociation of MTX from the enzyme's active site. Efforts are currently underway to extend these measurements to the natural substrates for ecDHFR (i.e., NADPH and dihydrofolate) to directly observe the forces involved with the dissociation of different enzymatic complexes. The SAM design described in this manuscript can be easily adapted for use with other enzymes provided a cysteine residue is introduced onto the protein surface, away from the active site, via site directed mutagenesis. Natural surface reduced-cysteines will also have to be modified but these are rare, not conserved, and commonly should not affect activity. Introduction of a surface cysteine located away from the active site in other enzymes will be used to evaluate the general applicability of the methodology developed here. If the ability of enzymes to form such functionalized monolayers is

found to be more general, enzymes catalyzing redox reactions will be tested.

■ ASSOCIATED CONTENT

Supporting Information. Details of the MRFS experimental setup, procedure, and data analysis. Procedure for radiographic ecDHFR activity measurement on surface-immobilized enzyme monolayer. This material is available free of charge via the Internet at http://pubs.acs.org.

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