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ARTICLE *in* LANGMUIR · JANUARY 2008

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Investigating the Specific Interactions between Carbonic Anhydrase and a Sulfonamide Inhibitor by Single-Molecule Force Spectroscopy

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Received July 17, 2007. In Final Form: September 13, 2007

In this communication, we report on the interaction landscape of an active site-specific enzyme−inhibitor complex by single-molecule force spectroscopy. Electrostatic immobilization was employed to orient a carbonic anhydrase enzyme on a positively charged surface so its active site is pointing upward. This approach to immobilization effectively increases the number of specific interactions measured between the zinc ion of the active site on carbonic anhydrase and a sulfonamide inhibitor tethered to an atomic force microscope (AFM) probe. Further, it reduces the time required for data collection and thereby minimizes the possible mechanical damage to the probe and contamination of the enzyme surface. The rupture force measured at various loading rates is interpreted in terms of a single energy barrier for the carbonic anhydrase enzyme−sulfonamide inhibitor complex from which the kinetic and thermodynamic parameters were estimated on the basis of microscopic models and were compared to the Bell−Evans model. The dissociation rate for the enzyme−inhibitor complex was found to be significantly faster (∼35 times) than the natural spontaneous dissociation rate.

Introduction

Force spectroscopy has been used to examine the energy landscape of biomolecular interactions by probing single-molecule interaction forces between biorecognition molecules.[1] An insight into the dynamic environment in which these biomolecular complexes exist can be gained by examining the rupture forces of the recognition molecules under an external force. The rupture events of consequence are those arising from specific recognition interactions, and in force spectroscopy measurements, the reported binding probability of specific interactions is typically between 5% and 10%.[2] To improve this probability, and thereby reduce the data acquisition time and minimize the possible damage to the probe, it is important to control the orientation of the immobilized biomolecules. Here, we report a single molecule force spectroscopy study of an enzyme−inhibitor system that has an active site with high specificity and selectivity. There are several challenges to probing active enzymes immobilized on surfaces by force spectroscopy: first, the enzyme should retain its catalytic activity at all times; second, it should be oriented in a manner that its active site is easily accessible to inhibitor molecules. However, most immobilization techniques are based on covalent coupling methods that do not control the orientation of the enzymes, resulting in poor accessibility of the active site.[3] Recently, we demonstrated that an active enzyme, carbonic anhydrase (CA), can be controllably oriented on charged surfaces by electrostatic immobilization.[4] The enzyme was found to form a complete layer on both the positively (terminated with a pyridinium salt) and negatively (terminated with a carboxylic acid) charged surfaces with different orientations. On the positively charged surface, the enzyme is oriented such that the active site is predominantly pointed upward, while on the negatively charged surface the active site is pointed downward. Manipulation of enzyme orientation on charged surfaces makes it possible to probe the active site of the enzyme in a highly specific and selective manner.[5−8]

In this study, we examine the catalytically active site of CA using a tethered *p*-carboxybenzenesulfonamide (CBS) inhibitor (Figure 1, II). The enzyme was immobilized and oriented on an ultra-flat gold surface[9,10] via a self-assembled monolayer (SAM) of a pyridinium-terminated thiol (I) (Figure 1). The CBS inhibitor (II) has a tri(ethylene glycol) spacer to provide adequate length and flexibility. To perform the single-molecule study, a gold-coated AFM probe was functionalized with a SAM of II (surface concentration was diluted by a factor of 20 with 11-mercapto-1-undecanol (MCU)).

To estimate kinetic and thermodynamic parameters from the enzyme−inhibitor interaction, a loading rate dependence study was performed. The estimation of the kinetic parameters, the kinetic off-rate, and the barrier position can be obtained by the phenomenological model provided by Bell[11] and Evans.[12,13]

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10.1021/la702148v CCC: $37.00
Published on Web 11/01/2007

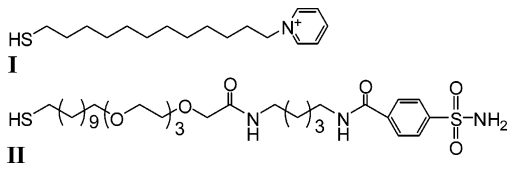

Figure 1. Molecular structures of linker (I) and inhibitor (II) molecules.

According to this model, under the application of an external force, the energy barrier of the potential energy surface is lowered, resulting in a faster barrier crossing rate. The rate of barrier crossing is expected to be exponentially dependent on the force. When analyzing the statistics of rupture force data, the Bell−Evans model is found to best fit rupture force data obtained at moderate pulling speeds, while the model deviates significantly from single-molecule rupture force data at low and high pulling speeds.[14] Since the off-rate for a rupture event is obtained at zero force by extrapolating the loading rate data, it is likely that the kinetic parameters predicted by the Bell−Evans model are grossly overestimated. Recently, Dudko et al. have demonstrated the successful use of microscopic theories to interpret single-molecule rupture force data and to estimate the off-rate, barrier position, and the activation energy barrier.[15,16] We use the analytical expressions from the microscopic theories developed by Dudko et al.[15,16] to estimate the kinetic and thermodynamic parameters for our enzyme−inhibitor system and make comparisons to the kinetic estimates obtained from the Bell−Evans model.

Experimental Section

Materials. Carbonic anhydrase II from bovine erythrocytes, tetrahydrofuran (THF) (HPLC grade), sodium acetate buffer (3 M, 0.2 μm filtered), 11-mercapto-1-undecanol (97%, MCU), acetazolamide (min. 99%, ACTZ), and 16-mercaptohexadecanoic acid (90%, MHDA) were purchased from Sigma-Aldrich (St. Louis, MO). Sodium acetate buffer was purchased at a 3 M concentration and adjusted to experimental concentrations (20 mM NaAc, 100 mM NaCl, pH 4.8) using NaCl (Mallinckrodt) and ultrapure water (18.2 MΩ-cm resistivity) obtained from a Barnstead water purifier (Fisher Scientific). This buffer was filtered (0.2 μm pore size) prior to use in force spectroscopy measurements and preparation of solutions. Ethanol (absolute, 200 proof) was purchased from AAPER Alcohol and Chemical Co (Shelbyville, KY) and filtered (0.2 μm pore size) prior to use. The tethered inhibitor molecule 4-[5-[2-(11-mercaptoundecyltri(ethylene glycol))acetylamido]petylamido]-phenylsufonamide was synthesized based on the *p*-carboxybenzenesulfonamide (CBS) moiety as previously reported.[4] The synthesis of *N*-(12-mercaptododecyl) pyridinium bromide (MDP.Br) is described by scheme S1 (see Supporting Information).

Substrate and Probe Preparation. Preparation of ultraflat template-stripped gold surfaces was performed as previously reported.[17] After stripping the mica layer, the gold surfaces were washed using several milliliters of THF followed by several milliliters of ultrapure water and dried in a stream of N_2 gas. The gold surface was immediately placed into a solution of 1.0 mM MDP.Br in filtered ethanol and allowed to incubate for 20 h prior to use. Standard Si_3N_4 cantilevers (MLCT, Veeco Probes) were modified by 10 nm of chromium evaporation as an adhesion layer followed by 30 nm of gold evaporation (at a rate of 1 Å/s). The tips were then placed immediately into a 1.0 mM total thiol solution made by combining 1.0 mM inhibitor with 1.0 mM MCU (both in ethanol) in a 1:20 ratio for all experiments except the competitive inhibition study, in which a 1:100 ratio of inhibitor to MCU was used. The cantilevers were incubated in this solution for 20 h prior to use. Before each force spectroscopy experiment, one of the surfaces was removed from the MDP.Br solution, rinsed with NaAc buffer (20 mM NaAc, 100 mM NaCl, pH 4.8) and ultrapure water, dried with N_2 gas, and placed in a 0.1 mg/mL solution of carbonic anhydrase in NaAc buffer for 1 h. For the control experiment in which the CA enzyme was immobilized on a negatively charged surface, the TSG surfaces were incubated in 1.0 mM MHDA in 2-butanol for 20 h, followed by washing and CA incubation as described above.

Instrumentation. All force spectroscopy experiments were performed using a PicoForce AFM (Veeco Metrology, California) equipped with a 40 μm scanner (20 μm in the *z* direction). Spring constants of gold-coated Si_3N_4 cantilevers were measured using the thermal fluctuation method.[18] Several different cantilevers were used, giving a range of spring constants from 42 to 48 pN/nm.

Force Spectroscopy Measurements. Force spectroscopy measurements were performed in a glass fluid cell (Digital Instruments) at room temperature (22 °C). Force measurements were conducted in sodium acetate buffer (20 mM NaAc, 100 mM NaCl, pH 4.8). Force measurements with the competitive inhibitor, acetazolamide (ACTZ), were carried out in 4 mM ACTZ in NaAc buffer using probes with a 1:20 and 1:100 ratio of inhibitor to MCU. For all force spectroscopy measurements, the ramp size was set to 250 nm and the surface delay to 1 s. A loading force study was performed by varying the loading force from 100 to 500 pN. All other experiments were conducted with a loading force of 100 pN. Loading rate dependence studies were carried out with apparent loading rates from 1370−87 000 pN/s. The apparent loading rate was obtained by taking the product of the slope just prior to the rupture point and the pulling speed.[19,20] Since the apparent loading rate varies slightly from one force curve to another, the average of 10 force curves was used as a reliable estimate of the apparent loading force. This method of averaging provides an apparent loading rate which is not too different from taking the maximum from a distribution of loading rates obtained from every single force−distance curve.[20] A fixed loading rate was used for loading force and competitive inhibition experiments. Force measurements were performed until ∼50−100 specific rupture force curves were collected over 1−2 h from ∼100−400 force−distance curves in at least 5−10 random spots on the surface. Special attention was paid to the shape of force curves both in data collection and analysis. Only force curves that indicated an extension of the force curve followed by an abrupt rupture of the retraction force curve were used in the analysis.

All force−distance curves were analyzed using the DI Nanoscope offline software provided by Veeco Metrology (version 6.13R1). Force data was analyzed using the freeware statistical package R (http://www.r-project.org/). Kernel density function plots were generated to examine the distribution of forces. All density curves reported were generated using 512 points and the Gaussian kernel function.

Results and Discussion

Orientation of the Enzyme. The orientation of the enzyme was experimentally determined by quantifying the specific interactions observed between the enzyme and its inhibitor. Not all contacts between the tip and surface led to a rupture event. While a majority of the observed force curves represented an adhesion force, others demonstrated zero adhesion. To identify specific interactions that led to rupture events, force curves were compared between a surface with a SAM of the positively charged pyridinium linker (MDP.Br) that had not been incubated in the CA solution and a surface that contained CA. In the absence of

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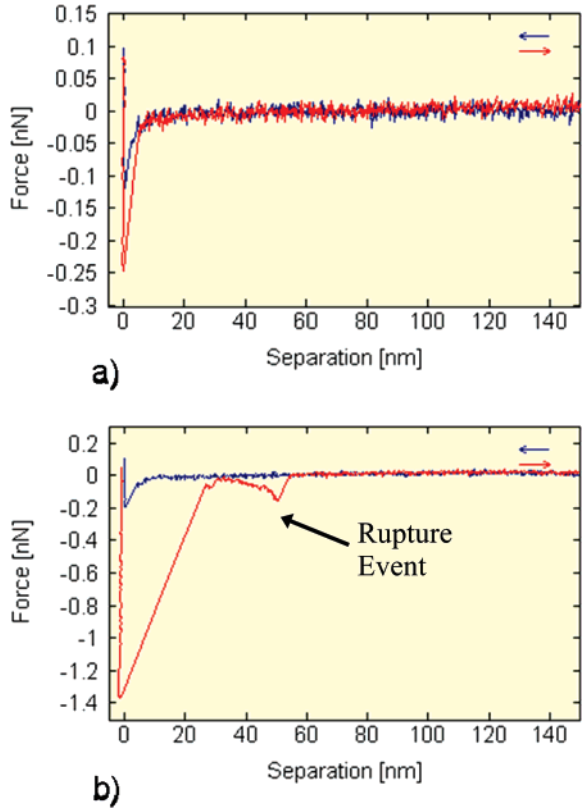

Figure 2. Comparison of force−distance plots obtained in the absence of CA (a) and in the presence of CA (b). The force extension curve followed by a rupture event seen in (b) indicates a specific interaction between the CA enzyme and inhibitor and is interpreted as the force required to rupture the CA enzyme−inhibitor complex.

the CA enzyme, the interactions between the pyridinium charged surface and the sulfonamide inhibitor are highlighted by a sample force curve displayed in Figure 2a. In the presence of immobilized CA, the force curve highlighted in Figure 2b shows an adhesion event followed by an extension of the force curve and a rupture. The appearance of a rupture event is indicative of a specific interaction between the enzyme and the tethered inhibitor.

On the positively charged pyridinium SAM surface, the number of specific interactions between the CA enzyme and the tethered CBS inhibitor was ~40%, while on a negatively charged MHDA SAM surface the number of specific interactions was ~1.5% of the total number of force curves. The observation of a very low number of specific interactions on a negatively charged surface is consistent with previous observations where the CA enzyme was covalently attached to a carboxylic acid terminated surface via amine coupling, resulting in random orientation of the enzyme.[3] The observation of a high percentage of rupture events observed from the CA immobilized on the positively charged surface is indicative of the active site of the enzyme being easily accessible to the tethered sulfonamide inhibitor.

Loading Force Measurements. To ensure that the measured rupture events were primarily occurring from single molecule interactions, the loading force was varied from 100 to 500 pN.[17] Rupture forces measured at loading forces of up to 500 pN at a fixed pulling rate are shown in Figure 3, with a mean rupture force of 52 ± 6 pN for the single-molecule interaction. Although at higher loading forces the number of multiple enzyme−inhibitor interactions increased, the single-molecule rupture events were still observed, albeit a smaller fraction. To minimize possible damage to the inhibitor and the enzyme molecules, all subsequent force spectroscopy studies were carried out at a loading force of 100 pN.

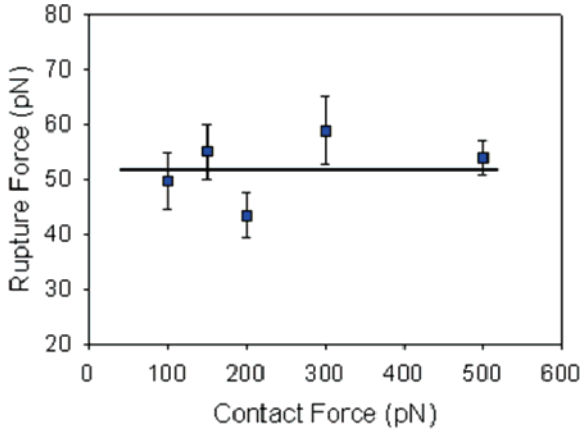

Figure 3. Loading force dependence. Measurement of single molecule rupture force between CA enzyme and sulfonamide inhibitor at varying loading forces (the solid line added to guide the eye).

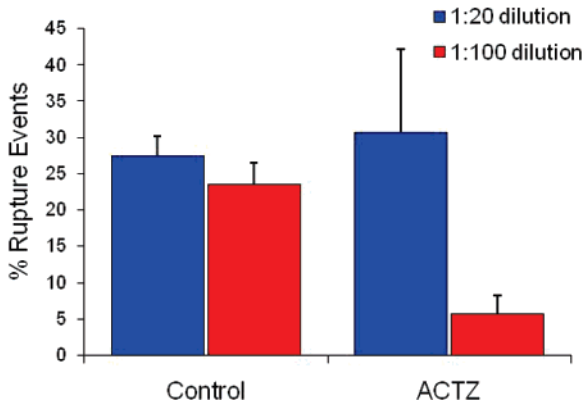

Figure 4. Percentage of rupture events observed between the CA enzyme and the tethered CBS inhibitor with and without the competitive inhibitor, acetazolamide (ACTZ). The blue bars highlight the percent of rupture events observed when the tip CBS molecules were diluted by MCU in a ratio of 1:20. The red bars highlight the percent of rupture events when the CBS molecules on the tip were diluted by MCU in a ratio of 1:100.

Competitive Inhibition. A competitive inhibitor, acetazolamide (4 mM, $K_i = 7$ nM), which binds reversibly to the zinc ion at the active site of CA,[21] was added into the solution after the measurement of forces as a control to discriminate between nonspecific interactions arising from the hydrophobic interactions and specific interactions arising from binding to the zinc ion. When using a CBS-tethered probe where the inhibitor molecules on the probe were diluted in a ratio of 1:20 (inhibitor/MCU), the number of rupture events observed between the CBS-tethered probe and CA enzyme appears to be indifferent in the presence or absence of the competitive inhibitor acetazolamide (Figure 4). However, when the inhibitor molecules on the probe surface were diluted at a ratio of 1:100 with MCU, a significant decrease in the number of rupture events was observed (Figure 4). Clearly the difference between these two probes is the number of inhibitor molecules available to interact with the enzymes on the surface. To observe a single-molecule rupture event, only one of the many inhibitor molecules (as many as 80 for a probe of 20 nm radius at 1:20 dilution) on the probe need to interact with one of the many immobilized enzymes (as many as 25) within the contact area (see scheme S2 in Supporting Information).[4] Since the acetazolamide can reversibly bind to the zinc ion at the enzyme active site, a single-molecule rupture event between the tethered

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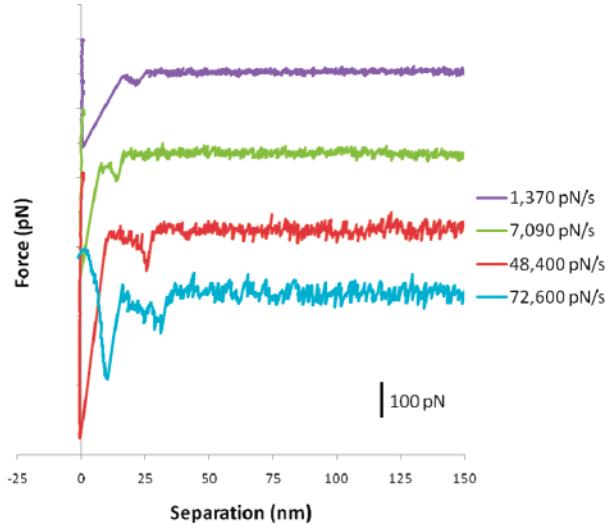

Figure 5. Sample force−distance curves obtained between the CA enzyme and the tethered sulfonamide inhibitor at various apparent loading rates.

CBS inhibitor and the CA enzyme is still possible given the large number of interactions taking place between the inhibitor molecules on the tip and the enzymes on the surface, in spite of a majority of the interactions resulting in the absence of a rupture event due to the active site being blocked by acetazolamide. When using the 1:100 probe, the number of inhibitor molecules available on the probe for interacting with enzymes on the surface is significantly smaller. Hence, the probability of observing a rupture event between the tethered CBS inhibitor and the CA enzyme in the presence of a competitive inhibitor should be smaller, as seen from the significant decrease in the number of rupture events observed when using the 1:100 probe in the presence of acetazolamide.

Kinetics and Thermodynamics of the CA−Inhibitor Complex. To examine features of the potential energy landscape and to extract kinetic and thermodynamic information for a single molecule interaction between the enzyme−inhibitor complex, a loading rate dependence study was performed. Rupture forces were measured at apparent loading rates from 1370 to 87 000 pN/s. Representative sample force curves at several different apparent loading rates are shown in Figure 5. At each apparent loading rate, force distribution plots were produced to obtain the most probable rupture force arising from a single-molecule rupture event, as shown in Figure 6 by fitting the force distribution data to a Gaussian. The peak value from the Gaussian fitting was considered as the most probable rupture force. A plot of the most probable rupture force obtained from similar Gaussian fits at various loading rates is shown in Figure 7 for a couple of different tip/sample combinations. The force dependence of the loading rate has been typically interpreted by the Bell−Evans model,[11−13] from which the barrier width (the location of the transition state) and the thermal off-rate (at zero force) is estimated. Recently, Dudko et al. have demonstrated that microscopic theories based on Kramer's theory of diffusive barrier crossing provides for better estimates of the kinetic parameters for single molecule force experiments.[15,16] On the basis of nanopore unzipping of DNA hairpins generated by an electric field, they were able to show the statistics of the rupture events deviated significantly from the Bell−Evans model. The kinetic off-rates predicted by the Bell−Evans model were nearly an order of magnitude off. Furthermore, the microscopic models were able to estimate the energy barrier of activation for DNA unzipping, which was not possible with the Bell−Evans model.

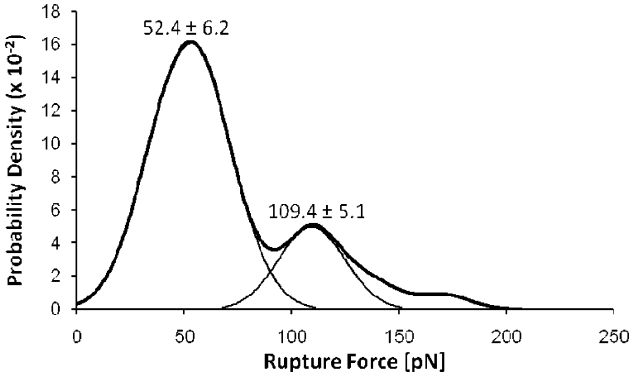

Figure 6. Typical density plot obtained from analysis of force−distance plots, showing the presence of single (52 ± 6 pN) and double (109 ± 5 pN) enzyme−inhibitor interactions. Only single interactions were reported, as they represent data obtained from single molecule interactions.

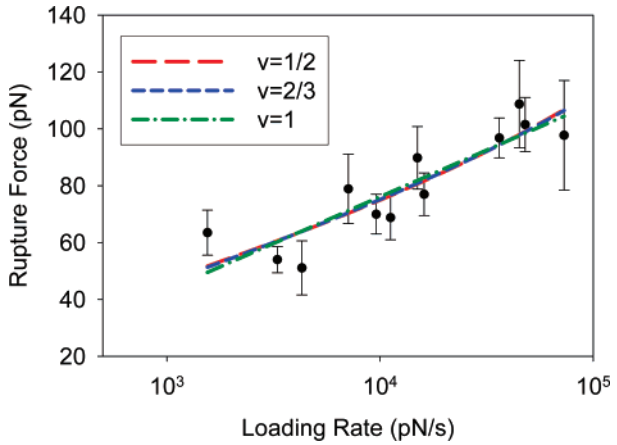

Figure 7. Dynamic force spectrum for the interaction between CA enzyme and the tethered CBS inhibitor. The rupture force is the peak rupture force obtained from a density function plot highlighting the most probable rupture event arising from a single-molecule interaction. The data includes rupture forces obtained at different apparent loading rates from two tip−sample combinations. The apparent loading rate is the product of the slope of the extension curve just before the rupture point and the pulling speed.

We have applied the microscopic models developed by Dudko et al.[15,16] to the loading rate dependence data displayed in Figure 7. The loading rate data were fitted to the analytical expression provided by eq 1, where the most probable rupture force, F, is plotted as a function of the loading rate, r.

$$F \cong \frac{\Delta G^{\#}}{\nu x^{\#}}\left\{1 - \left[\frac{1}{\beta \Delta G^{\#}} \ln \frac{k_0\, e^{\beta \Delta G^{\#}}}{\beta x^{\#} r}\right]^{\nu}\right\} \qquad (1)$$

where ν is a scaling factor, k_0 is the thermal off-rate, $\Delta G^{\#}$ is the activation free energy, $x^{\#}$ is the barrier width, and β is $(k_\beta T)^{-1}$. The loading rate dependence was examined for two microscopic models, the linear cubic theory ($\nu = 2/3$) and the cusp theory ($\nu = 1/2$) and compared to the Bell−Evans model ($\nu = 1$). The fits to the different models are displayed in Figure 7 from which estimates are obtained for k_0, $\Delta G^{\#}$, and $x^{\#}$. These estimates are tabulated for the two microscopic models ($\nu = 2/3$ and $1/2$) and compared with the Bell−Evans model ($\nu = 1$) in Table 1.

Since the variation in pulling speeds accessible by the AFM is not more than 1.5 orders of magnitude, a qualitative examination of the fits produced from the microscopic model and the phenomenological model does not appear to vary significantly. The estimates for the kinetic thermal off-rates obtained from the

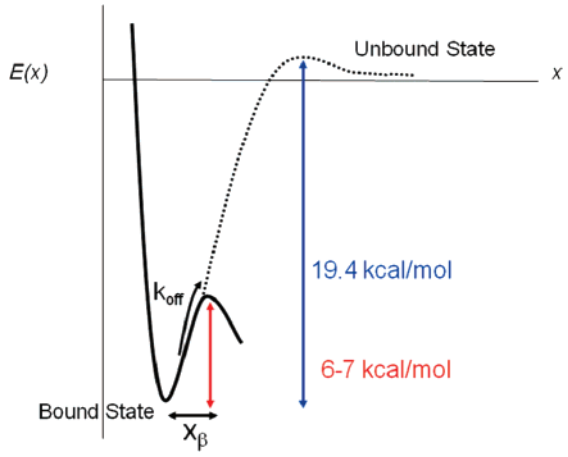

Figure 8. Schematic of the one-dimensional energy barrier for the CA enzyme−inhibitor complex. The dotted line represents the energy barrier for the CA enzyme−CBS inhibitor complex, where the CBS dissociation is under zero force. On the basis of SPR data,[22] the height of the energy barrier was calculated to be 19.4 kcal/mol. The solid line represents the lowering of the energy barrier using an external force. The height of the energy barrier calculated on the basis of the force spectrum data is 6−7 kcal/mol.

Table 1. Kinetic and Thermodynamic Parameter Estimates for CA Enzyme−Sulfonamide Inhibitor Interaction

ν	k_0 (s^{-1})	$x^{\#}$ (nm)	$\Delta G^{\#}$ (kcal/mol)
2/3	1.3 ± 3.7	0.43 ± 0.33	5.9 ± 2.5
1/2	0.82 ± 3.3	0.50 ± 0.51	6.7 ± 2.1
1	3.4 ± 2.8	0.29 ± 0.05	−

microscopic models are nearly a factor of 3 smaller than that predicted by the Bell−Evans model, while the barrier width is nearly 1.5 times greater. It is important to note that the errors associated with these parameters are still significantly large and are most likely due to the scatter present in the loading rate dependence data. When using different tip/sample combinations, even under identical pulling speeds, the shape of the force-distribution plot can be different, resulting in some uncertainty in predicting the most probable rupture force due to the presence of asymmetry in the rupture force distribution. This scatter is most noticeable when the pulling speed is only slightly varied. Nevertheless, the kinetic and thermodynamic parameters estimated from the single-molecule enzyme−inhibitor studies provide a crucial comparison to bulk measurements. The thermal off-rate measured from the free CBS in solution and CA II enzyme by surface plasmon resonance (SPR) is 0.037 s^{-1} with a complex lifetime of 27 s.[22] By using the Arrhenius equation,[23] the height of the energy barrier for the natural spontaneous dissociation was found to be 19.4 kcal/mol (Figure 8). On the basis of the microscopic models, the energy barrier is predicted to be ∼6−7 kcal/mol under the influence of an external force. The lowering of this energy barrier has been predicted by theory.[12,13] Further, the lowering of this energy barrier is also accompanied by faster escape rates, as shown by the thermal off-rates of ∼0.8 and 1.3 s^{-1} estimated from the microscopic models with a complex life time of ∼1 s for the enzyme−inhibitor single molecule study. However, there are some significant differences between the SPR study and the present study, most notably the different charged state of the enzyme and the inhibitor. In SPR, the carboxylic acid group on free CBS is deprotonated and thus negatively charged, while in our system the CBS moiety is covalently coupled to the linker and is uncharged. Furthermore, this study was carried out at pH 4.8, one unit below the pI of the enzyme (5.9), while in the SPR study the pH was 7.[22]

Conclusions

This study demonstrates for the first time the interaction landscape of an enzyme−inhibitor complex, where the enzyme was immobilized by electrostatic means and oriented to augment the specific interactions between the enzyme and inhibitor at the single molecule level. The use of an oriented enzyme not only increases the successful rate in measuring the single enzyme−inhibitor interaction but also reduces the chances of surface contamination and mechanical damage of the probe. Kinetic and thermodynamic parameters for the CA enzyme−sulfonamide inhibitor interaction were estimated on the basis of microscopic models and the phenomenological Bell−Evans model. The microscopic models provide kinetic parameter estimates in close proximity while significantly different from the Bell−Evans model. However, the loading rate dependence data were equally well fitted by the microscopic and the phenomenological models. In future studies, we plan to examine the role different lengths of tethers attached to the inhibitor molecule may affect the thermodynamics and kinetics parameters.

Acknowledgment. This work was supported by the National Institutes of Health (Grant No. 1R15GM073662-01). We wish to thank Dr. Olga Dudko and Dr. Gerhard Hummer of NIH for providing assistance in implementing their model to our enzyme−inhibitor system. R.B. acknowledges support of the Brummel Chair in organic chemistry at Calvin College.

Supporting Information Available: Synthesis of the molecules and AFM tip contact area calculations. This material is available free of charge via the Internet at http://pubs.acs.org.

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