On the dynamic behaviour of the forced dissociation of ligand–receptor pairs

Philip M. Williams,* Adam Moore, Molly M. Stevens, Stephanie Allen, Martyn C. Davies, Clive J. Roberts and Saul J. B. Tendler

Laboratory of Biophysics and Surface Analysis, School of Pharmaceutical Sciences, The University of Nottingham, Nottingham, UK NG7 2RD

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Measurement of the forced rupture of biotin from streptavidin, using the force microscope, revealed a logarithmic dependence on the rate of loading. Transition state theory predicts that the rupture force is dependent on the rate of force loading and the dissociation rate constant of the interaction. Analysis of the dependence of the rupture force with loading rate reveals that the barrier to unbinding under the loading rates employed here is situated between 0.12 and 0.18 nm away from the bound state. The position of the barrier determined is similar in value to the results obtained using complementary force techniques, and is matched by calculations from computational simulation. Thus, force rupture measurements may be used to profile the internal energy pathway of molecular dissociation events. The results suggest, however, that the loading rates employed here, using cantilever retract velocities as low as 1 nm s^{-1}, are still too high to explore the whole of the unbinding energy landscape, and suggests further avenues for instrumental and experimental development.

Introduction

Dynamic interactions between molecules engender biological functionality and a greater understanding of such interactions is key to advancement in many avenues of research. New analytical techniques have emerged which can directly measure interaction forces on a molecular scale, including the surface force apparatus,[1–3] optical tweezers,[4,5] and micro-pipette suction.[6,7] In particular, the forced rupture experiment afforded by the atomic force microscope (AFM) has proven to be a powerful technique for measuring the strength of the interaction between individual molecules over a large dynamic range. The AFM has seen application in the analysis of protein–ligand,[8–11] antibody–antigen,[12–16] and base pair and DNA strand interactions.[17–19] The streptavidin–biotin system has been used as a model system on which the development of the AFM-based forced rupture experiment has been made, due primarily to the high affinity of the complex at 10^{15} M^{-1}. Many reports of AFM investigations on this system exist in the literature, with average rupture forces determined between 200 to 409 pN.[20,21] The ability to reduce the measured rupture force to zero by the addition of excess ligand suggests that the force measured is indeed related to the rupture of the specific interaction. Questions remain, however, as to the origin of the wide distribution of forces reported for this system, and the relationship between the thermodynamic properties of the interaction and the measured rupture force.

The relationship between thermodynamics and forced rupture measurements by AFM has been explored through site-directed mutagenesis of the streptavidin system,[22] indicating a relationship between the rupture force and the enthalpic barrier to dissociation. These findings supported previous experimental results[23] and have been confirmed through computational modelling.[24,25] However, attempts at the simulation of the ligand rupture experiment by molecular dynamics procedures have suffered problems due to the rate at which the ligand is removed from the binding pocket.[26]

An interaction is expected to have a finite lifetime, and thus would break spontaneously if left alone for a suitable period of time. There exists, therefore, a dependence on the amount of work required to break the interaction and the time over which the interaction is forced to rupture. Bell showed that the lifetime of an interaction, τ_W, can be related to the dissociation energy barrier, E_b, and the work done, W, in removing the ligand.[27]

$$\tau_W \propto \exp\left(\frac{E_b - W}{k_B T}\right) \quad (1)$$

In order to achieve rupture within the time available through traditional computational methods, where τ_W is in the order of 1 ns, the rate at which the ligand is removed from the binding pocket in the simulation is several orders of magnitude greater than that of the experiment (τ_W of the order of 1 ms) thus more energy has to be given. Simulations of streptavidin–biotin undocking at various rates showed a linear relationship of the forces, attributed to the dominance of molecular friction at high rates.[26,28] Linear extrapolation of the forces from the high rates of simulation to those near zero of the experiment reveal that the predicted rupture force is of correct order (250 pN for the streptavidin–biotin system),[26] but there is no evidence from the simulation that this extrapolation to low rates should be linear; indeed the linear relationship indicates that the simulation was operating in an (ultra-fast) friction-domination regime. With the rupture forces dominated by molecular friction such simulations reveal little of the thermal barriers to dissociation.

The force required to break a molecular interaction depends on the rate at which it is forced to rupture. However, this dependence is only observed experimentally when the natural life time of the interaction is comparable to the length over which the observation is made (seconds to hours). Here, therefore, our discussion centres upon ligand–receptor interactions such as those between binding proteins and their substrates. When subjected to low loading rates such an interaction will yield, whereas at high stresses the system will offer resistance and withstand large forces. Such phenomena are shown by leukocytes which roll over endothelial cell walls under the stress of low hydrodynamic flow but withstand detachment from these surfaces at high flow rates.[29] The forced rupture experiment afforded by the AFM and other methods outlined above represent, therefore, a single snap-shot of a dynamic process.

The theory of the dynamic strength of molecular interactions under stress has been explored and developed by several workers, and the work of Evans gives particular insight into the effect of loading rate on rupture force.[6,7,30–32] Here, we reiterate the pertinent theory in order to interpret our AFM measurements of the streptavidin–biotin system.

In thermodynamic equilibrium, the off rate of an interaction, v_0, can be related to the energy barrier to dissociation through the Arrhenius equation,[33] giving eqn. (2) where t_D is the

$$v_0 = \frac{1}{t_D} \exp\left(\frac{-E_b}{k_B T}\right) \quad (2)$$

diffusive relaxation time of the ligand in the binding pocket. By performing work through loading, the AFM reduces the energy barrier and thus decreases the lifetime of the interaction. The lifetime of an interaction, $\tau = 1/v$, can be shortened by adding energy through work and lowering the barrier to dissociation (see eqn. (1)).[27]

The streptavidin–biotin complex has a barrier to dissociation of 1.7×10^{-19} J (24.4 kcal mol^{-1}) and a dissociation rate constant, measured by label exchange experiments, between 1×10^{-5} and 8.7×10^{-7} s^{-1} depending on pH.[22,34] The t_D prefactor in eqn. (2), the relaxation time, is calculated to be of the order 1×10^{-9} s.[30] Notably, this is three to four orders larger than the gas value (Erying equation) often used in calorimetry ($k_B T/h \approx 10^{-13}$ s, itself similar to the period of a simple harmonic oscillator (mass $m = 243$ Daltons) in a potential well of $E = 1.7 \times 10^{-19}$ J of $x = 1$ nm width of $\pi x(m/2E)^{1/2} \approx 10^{-12}$ s), suggesting the barrier heights determined from solution studied off rates using the Eyring equation are incorrect.

Substitution for t_D from eqn. (2) into eqn. (1) reveals the dependence eqn. (3) from which the relationship between the

$$v_W = \frac{1}{\tau_W} = v_0 \exp\left(\frac{W}{k_B T}\right) \quad (3)$$

amount of work done and the rate at which the ligand dissociates can be seen. The AFM does work in pulling the ligand by retracting the piezo on which the cantilever is attached. If the ligand was attached to an infinitely stiff cantilever, restricting all thermal motion, then the force that it experiences at each position along an undocking pathway would be equal to the local change in the energy potential. The force required to remove the ligand would be equal to the maximum in this energy gradient, and invariant to loading rate. However, experimentally the rate at which the system is loaded allows the ligand to remain in the thermally active regime. Attached through alkyl or polymer linkers to cantilevers of typical stiffness ($K \approx 0.01$ N m^{-1}), a biotin ligand is expected to be able to fluctuate several angstroms within the binding pocket and can follow the same undocking pathway regardless of the speed of retraction.[28] Biotin, therefore, experiences all the force throughout its life within streptavidin binding pocket under these experimental loading rates. This would not be true, however, for more conformationally flexible systems and with interactions that occur over long distances, such as those in oligonucleotide duplexes.

Substitution for work, W, in eqn. (3), as the product of the rupture force F_R and the barrier position x, gives eqn. (4).

$$F_R = \frac{k_B T}{x} (\ln(\tau_0) - \ln(\tau_W)) \quad (4)$$

Evans showed that the lifetime of the interaction can be related to the rate of loading, and a plot of rupture force F_R against the logarithm of the loading rate ($r = \Delta F/\Delta t = s_R k$) has a slope of $k_B T/x$, from which x may be estimated.[30] The logarithmic intercept r_0 of the force line with the rate axis permits the barrier height to be determined,[32] as eqn. (5).

$$E_b = k_B T \left[\ln\left(\frac{k_B T}{t_D x}\right) - \ln(r_0)\right] \quad (5)$$

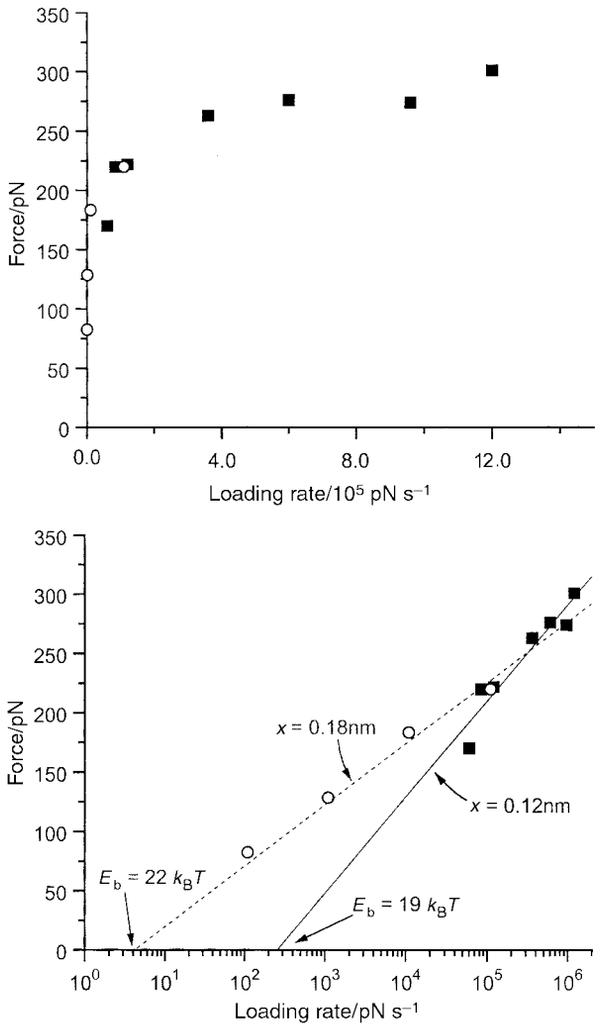

Fig. 1 The rupture force/rate dependence measured by the AFM. (a) Plot of rupture force *versus* loading rate for the streptavidin–biotin system measured using cantilevers of stiffness 0.12 N m^{-1}. (b) Data as (a) but plotted on a logarithmic scale of loading rate. Linear regression reveals a force scale of 23.5 pN indicating a barrier to dissociation 0.18 nm from the bound state.

Here, we show through experimental investigations the dependence of the force required to rupture the streptavidin–biotin interaction on the rate at which the complex is forced to rupture. The results go some way to explain the range of rupture forces reported for this single system and, importantly, show that both thermodynamic properties of single molecular interactions and features of the energy landscape of forced undocking can be measured using force techniques. The results also reveal experimental considerations for force measurements by the AFM and suggest that instrumental and experimental developments are required to allow the full exploration of unbinding energy potentials.

Results and discussion

Fig. 1a shows a plot of the rupture force for varying retract velocities of the piezo in the AFM for the streptavidin–biotin interaction for two different experiments. The open circles represent data with poor statistics, where the rupture force from less than 20 measurements at each rate has been deter-

mined. Values derived including these data are included below in brackets. The *x*-axis is the apparent loading rate calculated as the product of the retract velocity and the cantilever spring constant of 0.12 N m^{-1}. The forces range from 83 pN at a retract velocity of 1 nm s^{-1} (0.12 nN s^{-1}) to 301 pN at 10 000 nm s^{-1} (1200 nN s^{-1}). The relationship between the measured force and the speed at which the cantilever is retracted is clearly not linear in this regime. When plotted on a log scale (Fig. 1b) the data appear to follow a logarithmic trend. The widths of the distributions for each force are comparable to the forces themselves, as expected,[32] necessitating a large number of measurements to describe each distribution.

It is possible to relate the rupture force to the work required to break the bond at a given rate and hence determine the length *x* over which the work is done.[30] The force-scale of the data in Fig. 1b is 35 pN (23.5 pN). Since this scale equates to k_BT/x the barrier is calculated to be 0.12 nm (0.18 nm) from the bound position. A similar position of the barrier was found by Merkel *et al.* using the biomembrane force probe (BFP) technique.[30] For the streptavidin–biotin system they found a force-scale of 34 pN indicating a barrier at 0.12 nm. Using the BFP technique and its ability to probe lower loading rates, however, Merkel *et al.* were able to show the existence of barriers further away from the bound state; specifically at 0.5 nm for streptavidin and, for the structurally similar avidin, a barrier as far away as 3 nm. This suggests that the loading rates employed here with the AFM are still too high to adequately map the unbinding potential surface; it is worth noting that the lowest retraction velocity employed here was 1 nm s^{-1}, which is probably close to the limit of current instrument design. To lower the loading rate further requires either the use of even slower retract velocities, in which the measurements become increasingly subject to mechanical noise, or the use of cantilevers of lower spring constants, which would prohibit the exploration of high loading rates. One may foresee the use of 'smart' cantilevers, the attachment of ligands *via* stimuli-responsive polymers or molecules for example, that would permit the spring constant, and hence the loading rate, to be changed *in situ* during an investigation to explore more of the unbinding potential surface.

Previous adiabatic mapping[24] of the energy landscape on undocking shows maxima in the energy profile at 0.22, 0.95, 1.33 and possibly 0.58 nm. The experimental data above suggest that it is the first energy barrier, near 0.22 nm, which dictates the rupture force at the AFM loading rates employed here. The logarithmic intercept of the rate dependence data with the loading rate axis permits the magnitude of the energy barrier to be calculated (eqn. (5)) and using a relaxation time t_D of 1×10^{-9} s^{-1} the experimental data predict the barrier height to be 19 k_BT (23 k_BT) (1 $k_BT = 0.6$ kcal $mol^{-1} = 2.5$ kJ mol^{-1}). This compares favourably with the energy profile determined by Klaus Shulten and co-workers where molecular dynamics simulations predicted the barrier to be in the order of 25 k_BT at this position.[28]

Since the rate of loading affects the rupture force measured, it is necessary to know this rate in order to interpret experimental results. Although piezo displacements may be calibrated to sub-angstrom accuracy, calibration of the cantilever spring constant is more difficult, and determination is usually made to within ±10%. Fortunately, forces measured are proportional to the logarithm of the loading rate. The value determined for *r* is insensitive to the scale of loading rate since it is derived from the rate of change of force with the rate logarithm (the slope of the force rate logarithm as k_BT/x) and is therefore not affected by mis-calibration of the instrument.

Instrumental noise and experimental errors in the measurement of the rupture force limit the force-scales (slope of the force *versus* log(rate) data) that may be confidently measured. Fig. 2 shows how, over 4 orders of rate, the force-scale may be over or under read by linear regression assuming a 10 pN error

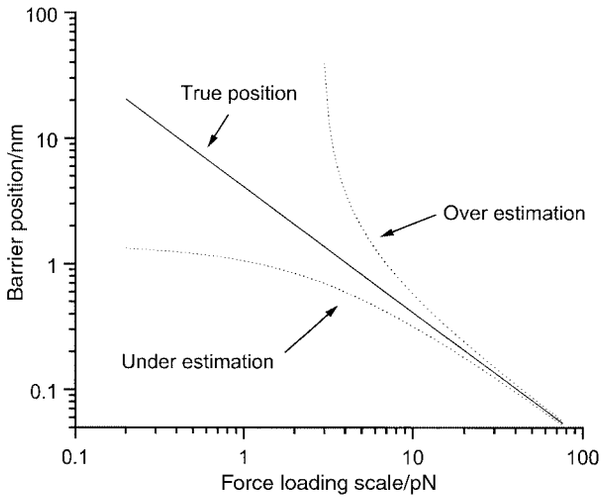

Fig. 2 Effect of measurement error on the determination of barrier position. Plot of the effect of measure error (±10 pN) on the determination of true barrier position using the scale for rupture force/rate data over 4 orders of rate.

in each measurement. With such an error in force determination, the smallest force-scale that can be determined is 3 pN equating to a barrier distance of 1.4 nm. Here, the barrier may be overestimated as far as 39 nm away. The graph shows this over or underestimation in barrier distance as a function of the true distance, revealing that the determination is more accurate when the energy barrier is close to the bound state, such as those probed by the AFM at high loading rates. The errors in determining the position of barriers distal to the bound state are compounded by the fact that they are only profiled experimentally at low loading rates, and thus the data may span one order of rate at best.

The analysis presented here of the effect of loading rate on the rupture force measured by the AFM neglects the effects of friction and damping of the cantilever movement with velocity. Consideration should be made of the viscous damping of the cantilever in the surrounding liquid, since all force rupture measurements are performed in an aqueous environment. The forces due to the viscous drag of the cantilever can be approximated[33] to eqn. (6) where η is the viscosity of water at

$$F_D = \frac{4\pi\eta L s_R}{(\frac{1}{2} - \lambda_E - \log(s_R R/4\nu))} \qquad (6)$$

1.2×10^{-3} Ns m^{-2}, *L* is the cantilever beam length of 2×10^{-4} m, λ_E is Euler's constant of 0.577, *R* is the effective radius of 1×10^{-5} m and ν is the kinematic viscosity of the liquid at 1.5×10^{-6} m^2 s^{-1}. Viscous drag at the highest retraction speed used here, at $s_R = 1 \times 10^{-5}$ m s^{-1}, would produce a maximum force on the lever of the order of 6 pN. Therefore, although this drag will obviously add to the forces measured, it has little significance on the results obtained and lies within the errors for each force determination.

Conclusions

The AFM reveals a non-linear dependence between the rupture force and the rate at which the system is loaded with force. This dependence is explained by transition state theory that suggests that the rupture force measured is dependent on the dissociation rate constant of the interaction, and the loading rate employed.

Since it is the rate of loading which affects the measured rupture force, specification of the rupture force alone is not enough to describe the strength of the interaction. Rupture values can only be interpreted by consideration of the rate of loading, which for AFM investigations is a function of the

cantilever spring constant, piezo retract speed and the vertical calibration of the piezo actuator. These findings go some way to suggest why the reported values of rupture force of identical systems vary considerably, and confirm the importance of both accurate assessment of the system calibration and citing all of the relevant parameters when publishing results.

The loading rate dependence of the rupture forces has unveiled a new experimental technique for the elucidation of the thermodynamic properties of a molecular complex and exploration of the energy landscape of forced unbinding. The large dynamic range of loading rates afforded by the AFM makes it ideally suited to these investigations of the energy barrier deep within the binding pocket, and allows the exploration of thermodynamic properties of single interacting molecular systems under stress.

Experimental

Rupture force measurements were made between the streptavidin-coated silicon surfaces and biotin-functionalised AFM probes. Silicon nitride cantilevers (Nanosensors, CA) and silicon wafers (Micro-Image Technology, Derbyshire, UK) were functionalised with biotinylated bovine serum albumin (BBSA) (100 μg ml^{-1} in 100 mM potassium phosphate buffer, pH 7) using a method described previously.[14] Before use, probes and surfaces were rinsed in deionised water to remove loosely bound material. To confirm functionalisation with protein force measurements were first recorded between the BBSA tips and surfaces without the presence of streptavidin. Such measurements typically displayed little or no adhesion. The BBSA coated silicon surfaces were then incubated in streptavidin (100 μg ml^{-1} in phosphate buffer, 1 hour) and force measurements recorded at piezo retract speeds between 1 nm s^{-1} and 10 μm s^{-1}.

All force measurements were made on an instrument constructed in the laboratory. Designed for adhesion mapping over large (25 mm by 25 mm) areas, this instrument exhibits good thermal and mechanical stability. The sample is mounted on a 2-axis positional stage, controlled by piezo drives (Inchworm, Burleigh) under a 12 μm *z*-axis piezo. The instrument is controlled using commercial electronics and software (Thermo-Microscopes Inc., Sunnyvale CA, USA).

The rupture forces were determined from the force-distance data obtained using software developed in the laboratory running off a Hewlett Packard J-210 workstation. Raw data (sensor response *versus* piezo displacement) were converted to force-probe displacement using the methods presented previously.[14] The software verifies the nature of each force measurement by a number of objective assessments. The intercept between the contact region and non-contact region of the approach curve is used to determine the point of contact, and along with a measure of the significance of the adhesion with respect to the noise, used to accept or reject the data. Rupture forces were determined as the position of the first mode in the frequency distribution of acceptable forces measured.

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