An enthalpic approach to the analysis of the scanning force ligand rupture experiment

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The scanning force microscope has developed into a technique for the examination of key events involved in molecular interactions. Here we present a novel method to analyse the new family of force data obtained from this biophysical tool. Adiabatic mapping of the streptavidin–biotin interaction reveals a range of ligand rupture forces from 253 to 393 pN, which are in close agreement with experimental data. Analysis of the undocking process shows the importance of hydrogen bonding in this interaction. The potential of this combined approach as a method of studying rupture force data is highlighted.

Introduction

The scanning force microscope (SFM) has attracted considerable interest as a tool for studying molecular interactions. In comparison with other techniques, such as optical tweezers and pipette suction,[1,2] the SFM has high force sensitivity over a large dynamic range.[3,4] Using the SFM intermolecular forces as low as 10 pN, corresponding to individual hydrogen bonds, have been resolved[5] and numerous systems have been investigated including avidin–biotin,[6,7] cellular adhesion proteoglycans,[8] antibody–antigen[9,10] and hydrogen bonding between nucleotide bases.[11] In this emergent field of rupture force measurement it is important to validate and understand the data obtained.

The SFM was conceived as an instrument to image the surface structure of insulating materials.[12] This is achieved by scanning a probe attached to a cantilever over the sample and monitoring the deflection of the lever. By using a very sharp probe on a flexible lever, atomic resolution of surface structure is possible.[13] The force sensitivity of the SFM also allows the spatial measurement of material properties, such as hardness, plasticity and friction and it is an extension of these property measurements which is used to measure molecular interactions. In a typical ligand rupture experiment the receptor is bound to a substrate and the ligand bound to the SFM probe surface. The ligand covered probe is brought into contact with the substrate and binding occurs. The probe is then withdrawn from the surface, pulling the ligand out of its binding pocket. The deflection of the lever, and therefore the force exerted on the probe by the ligand–receptor interaction, is recorded. Two recordings of the lever deflection are made: on approach to, and retraction from the substrate. The difference between these two traces occurs through adhesion between the probe and surface and is attributed to the ligand–host interaction. The maximum adhesive force measured is quoted as the ligand rupture force.

We have used SFM extensively to investigate several biomolecular forces of interaction, including the widely studied streptavidin–biotin systems.[14] A typical rupture measurement is shown in Fig. 1. We found the ligand rupture force for streptavidin–biotin to be 409 ± 166 pN, a value similar to others quoted in the literature (340 pN Lee,[6] 257 pN Moy[7]). Measured on an uneven, industrial substrate the large value of 409 pN and the range may suggest that double interactions occurred, each *ca*. 270 pN. The similarity of these data obtained through independent research suggests that the forces are representative of the specific ligand interaction. However, questions arise as to how this force relates to the systems's free energy, affinity constant and the strength of the association. To further our understanding of the ligand rupture data we studied the thermodynamic and reaction kinetics of the streptavidin–biotin system which led to the development of computer simulations for the modelling of the experiment. In this paper, we review this particular interaction, outline our motivation for the simulations and discuss the results in the light of recent publications in this field. A thermodynamic consideration of the streptavidin–biotin interaction is detailed in the Experimental section.

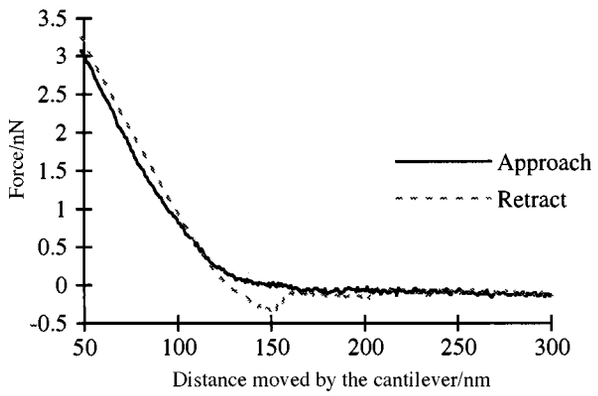

Fig. 1 A typical force measurement of ligand dissociation. The data are plots of the cantilever deflection *vs*. the vertical distance moved by the sample. Two plots are shown; the deflection on approach to, and retract from, probe–sample contact. The difference in deflection between the retract curve and the approach curve at contact reflects the adhesion between the probe and sample. The adhesion is related to the force required to remove the ligand from its receptor.

Molecular dynamics has been applied to profile the energy of forced ligand undocking and determine the rupture force. In these simulations the calculated forces were, unsurprisingly, higher than those determined experimentally. The energy barrier calculated by Izrailev using molecular dynamics for the avidin–biotin system was *ca*. 30 kcal mol^{-1} over 1.3 nm, and exhibits an intermediate maximum of 24 kcal mol^{-1} at 0.6 nm

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along the reaction coordinate.[15] The barrier is significantly higher than that found experimentally, with ΔH° −21.5 kcal mol^{-1} and ΔG° −20.4 kcal mol^{-1}. Such a barrier would increase the predicted thermally-driven dissociation time [eqn. (4)] from *ca*. 200 days to 2×10^6 years. In addition, the 400 pN force required to undock the ligand within the timescale of the simulation (up to 500 ps) is far in excess of those experimentally measured (*ca*. 160 pN). Similar discrepancies were reported by Grubmuller *et al*.,[16] whose simulations of the streptavidin–biotin system (using a streptavidin monomer with a fully hydrated binding pocket) predicted forces ranging from 700 pN down to 300 pN as the speed of rupture was decreased to a lower value of 1.5 nm ns^{-1}.

Although the experimental measurement of molecular interactions is possible with the SFM, the magnitude of the rupture force lies between those predicted by a simple thermodynamic analysis and those indicated through molecular simulation. In order to interpret the SFM ligand rupture data it is therefore necessary to translate from the results of molecular dynamics and thermodynamics calculations to the experimental environment. Recently, Balsera showed that it is possible to predict the unbinding potential using molecular dynamics with an uncertainty linearly proportional to the magnitude of the force required to dissociate the complex.[17] This, however, indicates that it is non-trivial to predict the results of a rupture experiment since the force required to undock a ligand within the nanosecond, and therefore the error, is large. A different approach to the prediction of the experimental data was indicated by Moy *et al*. who in measurements of avidin and streptavidin systems showed a correlation between the measured rupture force and the change in enthalpy on dissociation.[7] This correlation with ΔH° and not the total free energy change of the system, ΔG°, is explained thus: whilst the ligand remains in the binding pocket, entropic contributions to the energy are negligible and ΔH and ΔG follow a similar path. As the ligand leaves the pocket, entropy will have an increasingly significant contribution to ΔG and the free energy will decrease from that of ΔH. The correlation between the force of rupture and ΔH indicates that the measured force is a consequence of the energy change of the system whilst the ligand is within the pocket, at the position along the reaction coordinate before ΔG and ΔH differ.

A correlation between ΔH and the rupture force opens up an efficient method to predict and analyse experimental measurements since enthalpy can be calculated without consideration of the thermal fluctuations and dynamic properties of the system. We therefore studied the streptavidin–biotin ligand rupture experiment from an enthalpic perspective by performing an adiabatic mapping of the ligand dissociation.[18] The energy of the system during ligand undocking was analysed, forces required for ligand removal calculated and the sources of the interactions assessed.

Results and discussion

The adiabatic mapping took a total of 316 201 iterations, with a maximum of 3543 iterations for one step and a mean of 1054 per step. The run was completed in just under 900 h of CPU time on one processor of the J-210 using unoptimized code.

The energy of the system during the mapping is shown in Fig. 2(*a*), which is a plot of energy *vs*. distance undocked. The energy of the system increased from −1537 kcal mol^{-1} to a maximum value of −1488 kcal mol^{-1}, at 1.33 nm separation increase, and then fell through a 5 kcal mol^{-1} trough to an undocked value of −1506 kcal mol^{-1}. The calculated enthalpy difference was −32 kcal mol^{-1} which is significantly larger than that reported by Chilkoti *et al*.,[19] at −24.5 kcal mol^{-1}, but identical to that determined by Weber *et al*.[20] The large calculated energy barrier to dissociation, at 49 kcal mol^{-1} compared to the 32 kcal mol^{-1} of Chilkoti, reflects the methods adapted to simulate the ligand rupture experiment. A natural barrier of such magnitude would prohibit the unforced dissociation of the complex.

In order to simulate the experiment we chose to constrain the separation between two atoms; an α carbon of the streptavidin distal from the binding pocket and a carboxyl oxygen of the biotin. This distance constraint was increased to induce undocking. However, the streptavidin and biotin will experience the undocking force along their lengths and consequently distort in geometry. The energy measured in the mapping will, therefore, contain contributions from distortion in the internal structures of the protein and ligand away from the sites of interaction. This is evident from the position of the energy maximum, at a separation of 1.33 nm, which is larger than expected. Despite the difference in the barrier height the energy profile is similar to that measured by Izrailev for the avidin–biotin system, with an intermediate maximum 70% along the coordinate 7 kcal mol^{-1} below ΔH°.[15] The internal energies of the streptavidin and biotin during the undocking are plotted in Fig. 3(*a*) and 3(*b*) respectively, and Fig. 3(*c*) is a plot of the total system energy minus the sum of the internal energies of the streptavidin and biotin. This plot represents the interaction enthalpy between the receptor and ligand. From this analysis the enthalpy change of interaction, which should be unaffected, is only slightly lower at −31 kcal mol^{-1}, and the enthalpy barrier to dissociation has been reduced to 35 kcal mol^{-1}. The results demonstrate that within the COSMIC(90) force-field[21] it has been possible to obtain realistic enthalpies of interaction between this protein and ligand and thus may permit a quantitative interpretation of the ligand rupture data.

As detailed in the introduction, it is possible to determine the force required to reduce the energy barrier to zero and undock a ligand. The critical force, f_c, is the maximum of $E(r)/r$, where $E(r)$ is the energy of the system along the reaction coordinate at position r. In other words, $E(r) - f_c r \leq 0$ for all values of r. For the enthalpy profile of Fig. 2(*a*), the critical force is 306 pN at 0.9 nm whilst for the interaction enthalpy of Fig. 3(*c*), f_c is 257 pN at 0.6 nm. Both these values of r_0 are predicted to lie within the binding pocket and we may reasonably neglect entropy in our calculations. The critical force is not expected to match that measured by the SFM since in the experiment the force applied to the ligand is not constant and the rate at which energy is added to the system varies.

A simple method of estimating the force of rupture is to determine the maximum of the energy gradient. Gradients are, however, susceptible to convergence errors in the minimization, particularly with the small increment of 0.01 nm between calculations. There is a need to either filter the data or find a best-fit polynomial. Since we are concerned with the energy profile from the bound state up to the maximum we chose to fit the enthalpy profile using eqn. (1),[17] where L is the position of

$$H = \Delta H^{\ddagger}\left[\left(\frac{r}{L}\right)^4 - 2\left(\frac{r}{L}\right)^2\right] \qquad (1)$$

the maximum in enthalpy along the reaction coordinate from the bound state. A fit is found with $\Delta H^{\ddagger}$ at −43.7 kcal mol^{-1} and L at 1.19 nm, giving a maximum gradient equal to 393 pN which is higher than the both critical force and most of the experimental data.

The forces obtained in the adiabatic analyses above are similar in value and range to the experimental data reported in the literature. This enforces the assumption that, in this system, the force of ligand rupture which is responsible for the measured SFM value is a property of the interaction of the biotin ligand within the streptavidin binding pocket and that the change of entropy is small during the undocking process. Determination of the exact relationship between the interaction energy and the experimental force plot can only by approached by consideration of the thermal and chaotic effects and the

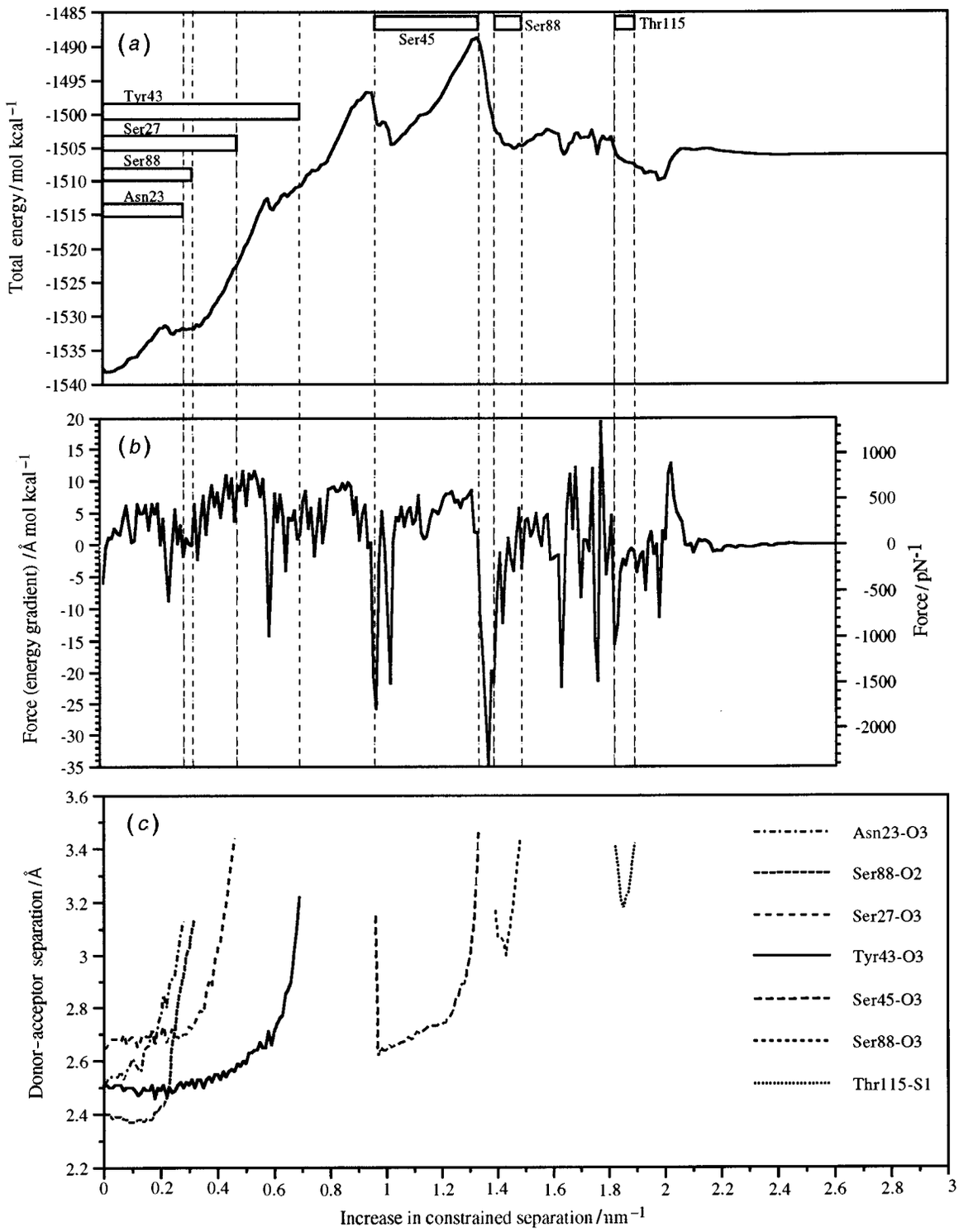

Fig. 2 Results from the adiabatic mapping experiments: (*a*) the energetic profile of the undocking, (*b*) energy derivative (force), (*c*) the hydrogen bonding patterns calculated. The hydrogen bonding domains of (*c*) are overlaid on (*a*) and (*b*).

mechanical behaviour of the lever detection system over experimental timescales.

The value of such experimental simulation lies in the ability to determine the origin of the forces and interactions involved, giving possible pointers to further experimental investigation. The hydrogen bonding donor–acceptor distances for biotin–amino acid interactions are shown in Fig. 2(*c*) and summarized in Table 1. The initial hydrogen bonding pattern differs slightly from that of the crystal structure[22] in that there is no bond detected with Asp[128]. Initially biotin forms a network of hydrogen bonds between the carbonyl oxygen of the biotin ureido head group (O3) and the amino acids Asn[23], Ser[27] and Tyr[43]. The carboxyl tail is stabilised by a hydrogen bond between the carbonyl oxygen (O2) and Ser[88]. We also observe a biotin carboxyl atom(O1)–water–Asn[49] hydrogen bonding network (not shown). As undocking progresses the initial hydrogen bonds are broken, in the order Asn[23], Ser[88], Ser[27] and Tyr[43]. The breaking of the Ser[27] bond coincides with the steepest energy gradient, with the Tyr[43] interaction having the longest duration (0.69 nm). There is a short period without detectable direct receptor–ligand hydrogen bonding, until the formation of the bond to Ser[45] by O3. This is then followed by a short interaction with Ser[88] and then a long period without hydrogen bonding. There is a weak hydrogen bond formed to Thr[115] by the sulfur of the biotin (S1) before unbinding finally occurs.

It is clear that this simulation method will be a useful tool for

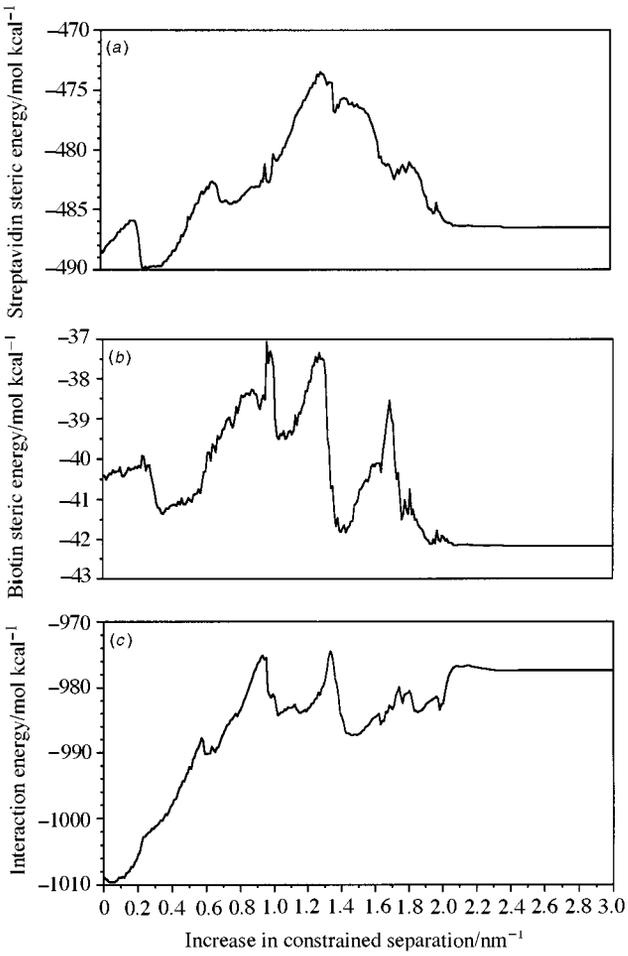

Fig. 3 Determination of the interaction energy between biotin and streptavidin. The steric energy of (*a*) the streptavidin and (*b*) the biotin is subtracted from the total system energy [Fig. 2(*a*)] to produce (*c*) the energy of interaction.

Table 1 Receptor–ligand hydrogen bonding during unbinding, showing the recorded spring force and separation increase as each bond is made and broken

Hydrogen bonds		Bond formation		Bond breaking	
Donor	Acceptor	Force	Separation	Force	Separation
Asn^{23}	Biotin–O3	0	0	1.08	2.8
Ser^{27}	Biotin–O3	0	0	1.75	4.6
Ser^{88}	Biotin–O2	0	0	0.92	3.2
Tyr^{43}	Biotin–O3	0	0	1.37	6.9
Ser^{45}	Biotin–O3	0.91	9.6	0.72	13.4
Ser^{88}	Biotin–O3	0.59	13.9	0.51	14.8
Thr^{115}	Biotin–S1	0.18	18.2	0.14	18.9

examining the mechanisms underlying SFM force–distance experiments. Our results show a series of force peaks, which can be related to both polar and nearest neighbour contacts made by the biotin as it traverses the unbinding pathway. It may be observed that most of the contacts are made to O3, suggesting a key role for this substituent in the interaction of host and ligand. Superposition of the hydrogen bonding distances and force curve shows a strong correlation between the two, suggesting that the biotin feels a strong force as the hydrogen bond is formed or broken. Examination of the energy curve suggests a stabilising role for the Ser^{45}–O3 interaction at the transition state, the two main peaks in the energy graph being either side of the hydrogen bond lifetime.

Comparison of the interactions detected with Grubmüller's reported on the same system[16] show some difference in the amino acids that interact with biotin. In the initial binding pocket they show the Asp^{128} interaction present, and the carboxyl tail stabilized by Asn^{49}, with subsequent interactions on the unbinding pathway between an NH substituent of the ureido ring and Val^{47}, followed by O3 with Arg^{84}. These results are part of a larger set of experimental data obtained, and it is possible that the sequence of interactions reported here are seen in some of their runs. The general shape of force curve, with hydrogen bond breaking and formation reflected by peaks is similar to our results, and they also attribute the highest force to the breaking of the Asn^{23} interaction, although the decay of the Tyr^{43} bond is seen close to this event. The presence of many water molecules in the binding pocket during the simulation may be responsible for the low forces calculated, as many water-mediated hydrogen bond networks are reported, allowing for much more flexible associations. This seems unlikely to be the case in the biological system, where the X-ray structure shows no solvent molecules in the binding pocket, which has little solvent accessibility. We only use the water molecules present in the X-ray structure, and see no water-mediated hydrogen bonding, except for the Asn^{49}–O1 interaction.

In contrast, Izrailev's work with a structurally similar system, the avidin tetramer,[15] shows markedly different results. Their simulations display three key regions of interaction, with the biotin jumping sharply from one region to the next in a slip-stick fashion, rather than following a smooth path. This jumping from pocket to pocket is a more realistic model for the forced undocking events since the spring constant used in these simulations was much less than ours and Grubmüller's, permitting the ligand to drift within the pocket.[23] It may be possible to use our system to investigate this behaviour by having lower energetic penalties for deviation from the set position of the constrained atoms, allowing the ligand to dwell in energetically favourable positions, but the value of such modelling is doubtful since we still neglect thermal motion of the ligand and receptor. Nevertheless, a slip-stick process is evident in the force plot shown in Fig. 2(*b*).

Finally, the speed of our calculations compares favourably with those of the other groups, both of whom required multiple runs over many thousands of hours of CPU time. It can be seen from the above results that energy-minimized simulations of receptor–ligand undocking have particular differences to those utilising molecular dynamics, and produce further valuable data with which to explore the origins of the ligand rupture force.

Conclusions

From an adiabatic mapping of the streptavidin–biotin forced undocking within a general force-field, it has been possible to determine the enthalpy of dissociation reasonably accurately and show that the position of the energy barrier lies within the binding pocket. The force experienced by the ligand during an undocking experiment was calculated at between 253 and 393 pN, which is in agreement in both value and range to those values measured by the SFM and extrapolated from molecular dynamics calculations.

These results demonstrate the potential of adiabatic mapping as a technique to simulate the underlying events with ligand rupture experiments using the SFM. Unlike the SFM experiments, the simulation allows a detailed examination of the whole sequence of events occurring during an interaction. This can allow an investigation of the bonds that are formed, and broken, between the ligand and the amino acids present in a binding pocket. Specifically, we have identified interactions in the streptavidin–biotin system, and have been able to relate these to the force on the ligand as it is undocked.

Molecular dynamics may be the obvious approach for the simulation of biomolecules but the current limits of processing power do not allow the extension of the technique to

examine ligand rupture events within the experimental milieu. The method employed here is relatively fast to compute, given the strictness of the convergence criteria involved, and can certainly be speeded up by the careful selection of scaling rates for stringency. This simulation approach can be a useful aid to suggesting further avenues of experimental investigation and in the interpretation of the results.

Experimental

Adiabatic mapping

Adiabatic mapping was performed using the Program for the Undocking of Ligands *via* Minimisation (PULMIN). This is a novel adaption of the minimization algorithms contained within DMC—the dynamic Monte Carlo software[24,25]—and utilises the COSMIC(90)[21] force field and the Genesis Graphics System.[26] Code development was on a Hewlett-Packard 735 UNIX workstation (HP-UX 9.03) using standard FORTRAN-77. Input consists of a ligand–receptor structure and constraint file. For the input structure partial atomic charges were generated using an adaptation of the Charge2 program,[27–29] which allows for the extensive π-bonding systems found in biomolecules. The constraint file details atoms which are considered anchored to the substrate and the SFM probe, typically a carbon atom on the protein backbone of the receptor and a suitable site on the ligand. The constrained atom–atom separation is increased along the undocking vector by a specified distance, the ligand is translated by that distance and the structure minimized. The new separations of the atoms are preserved by the imposition of high energetic penalties for deviation from the set value, of the form given by eqn. (2), where d is the

$$E_c = F_s|(d - d_s)| \tag{2}$$

constrained atom separation, d_s is their required separation and F_s a scaling factor. The undocking vector is equal to that which the ligand followed between the previous two iterations. This increase–minimize loop is performed until a defined increase in separation is achieved. The minimizing algorithm used is the standard Fletcher–Reeves conjugate gradients procedure.[30] In order to obtain convergence of the minimizer at the strictest possible level, whilst still maintaining a reasonable speed for the program, the convergence criteria, D, which is the maximum first derivative allowed in a fully minimized conformer, is linearly increased over each minimization step, such that [eqn. (3)]:

$$D = \begin{cases} 0.1D_{max} & \text{for } I \geq 0.1I_{max} \\ D_{max}\left(\dfrac{I}{I_{max}}\right) & \text{for } I > 0.1I_{max} \end{cases} \tag{3}$$

Here, D is the current convergence criteria, D_{max} is the target maximum criteria, I is the iteration number and I_{max} is a target limit of iterations permitted. D_{max} and I_{max} are defined at the beginning of the run. This ensures that the run does not end before convergence and limits any discontinuities between iterations.

X-Ray structural data of the streptavidin monomer and biotin complex[20,22] were used as the starting structures for simulation. Charges were assigned using the modified Charge2 algorithm and the complex minimized within the COSMIC(90) force-field using 2673 iterations of a conjugate gradients minimizer. The α-carbon atom of Ala[100] of the streptavidin, which is near its surface distal to the binding pocket, and the carboxyl oxygen of the biotin were chosen as the constrained atoms. These had an initial separation of 3.37 nm. Adiabatic mapping using the PULMIN algorithm was performed in 0.01 nm steps over 3 nm with D_{max} and I_{max} set to 5.0 and 500 respectively. The mapping was performed on a Hewlett-Packard J-210 workstation (HP-UX 10.20).

Hydrogen bonding between ligand and receptor was monitored using the HBPLUS algorithm of McDonald and Thornton.[31]

Kinetic analysis

It is possible to estimate the force required to undock a ligand without resorting to atomistic simulation of the experiment by a thermodynamic consideration of the dissociation. The streptavidin–biotin interaction is highly specific and has a dissociation constant of $K_d \approx 10^{-15}$. The complex is stable over a wide range of pH and temperatures, with a dissociation time, τ, of several days.[32] From Arrhenius,[33] the lifetime of a system in a potential energy well can be estimated from the size of the energy barrier to dissociation, E_0, using eqn. (4).

$$\tau = \tau_0 \exp\left[E_0/k_B T\right] \tag{4}$$

Immediately, the exponential relationship between τ and E_0 highlights a significant problem for the accurate simulation of thermally-dissociated ligand interactions since an overestimation of the energy barrier is compounded exponentially in the lifetime of the interaction.

The application of force to break an interaction quicker than that through thermal dissociation adds energy to the system, since work is done, and lowers this energy barrier. A critical force, f_c, may be calculated as that which reduces the barrier to zero, or at least less than $k_B T$. It is interesting to note that when power (rate at which the work is done) is constant, the position where the energy barrier is greatest, r_0, reduces and there is a foreshortening of the measured binding potential. Bell extended the Arrhenius equation to apply this reduction in the barrier to the lifetime of the bond,[34] through eqn. (5).

$$\tau = \tau_0 \exp\left[(E_0 - \gamma f)/k_B T\right] \tag{5}$$

The force required to rupture a bond within a given period can be estimated. In contrast to the exponential relationship between E_0 and τ, there exists a linear relationship between E_0 and f for a given τ. Thus, it should be possible to simulate the experiment and calculate a rupture force with a similar error as in the determination of the energy barrier.

For the streptavidin–biotin system the free energy, $\Delta G°$, has been determined[19] at -18.3 kcal mol^{-1}, an energy barrier to dissociation, $-\Delta G^{‡}$, of 24.4 kcal mol^{-1} and the half-life of the interaction varies with pH from 7×10^4 to 8×10^5 s.[32] Since the timescale of the ligand rupture measurement, at $\tau \approx 10^{-3}$ s, is eight orders of magnitude smaller than this, the absolute determination of τ for zero force is not necessary in order to estimate the pre-factor τ_0, at *ca.* 1.3×10^{-12} s. This value is similar to that calculated for a simple harmonic oscillator, with a period τ_h given by eqn. (6),

$$\tau_h = 2\pi r_0 \sqrt{\frac{m}{2E_0}} \tag{6}$$

where m is the mass of the oscillator. Biotin, with a mass of 243 D, has a simple harmonic period $\tau_h \approx 7 \times 10^{-12}$ s for a 1 nm binding pocket. For a typical SFM ligand rupture experiment the interaction is broken within 1 ms, indicating $\gamma f \approx 12.2$ kcal mol^{-1}. Assuming a value of γ equal to r_0 in the order of 1 nm, the critical force would be $f \approx 85$ pN. The predicted rupture force is one third of that measured. Izrailev extended this further by applying Kramers' relationship of reaction kinetics, which accounts for the shape of the potential well and the diffusivity of the ligand, and showed that the barrier to unbinding, including the applied force, is *ca.* 9 kcal mol^{-1} for a 1 ms measurement.[15] This value is similar to the 12 kcal mol^{-1} obtained from the simple consideration above.

The discrepancy between the rupture force predicted by a

kinetic analysis and that measured experimentally is, in part, due to the assumption that the force applied to the system is constant throughout the unbinding. In the experiment, the force applied to the ligand is related to the rate of change of the potential at its current position along the reaction coordinate. The force, therefore, will vary as the ligand is withdrawn since the change in free energy is not linear. The velocity of the ligand and the force applied can vary, as long as the total time taken to undock is constant. Such an analysis, however, requires knowledge of the energy profile, and such data is difficult to ascertain. Izrailev showed that if the force on the ligand is gradually increased during the experiment, the maximum force required for rupture increases.[15] This is expected since the total work done in both regimes is similar.

The time over which the bond is broken leads to a logarithmic relationship between speed of rupture and the predicted force. These ideas were expanded further by Evans and Ritchie[23] and Izrailev *et al*.[15] who show a biphasic relationship with a transition between friction and thermal limited dissociation, the Smoluchowski limit.[33] The rate at which the rupture is performed, and the difficulty in predicting the point of this transition, complicates the simulation of the ligand rupture experiment. With molecular dynamics, the maximum period over which a simulation can be made is in the order of 10^{-8} s. Here, neglecting friction, $\gamma f \approx 19$ kcal mol^{-1}. If r_0 remains at 1 nm, f increases from 85 to 133 pN, and in addition r_0 is likely to shorten. A molecular dynamics simulation will indicate a rupture force in excess of that measured in the experiment through the disparity in timescales of the measurements. When molecular friction is considered, the simulated force will deviate further and, moreover, the extent of deviation is very difficult to predict.

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