Steered molecular dynamics and mechanical functions of proteins

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Atomic force microscopy of single molecules, steered molecular dynamics and the theory of stochastic processes have established a new field that investigates mechanical functions of proteins, such as ligand–receptor binding/unbinding and elasticity of muscle proteins during stretching. The combination of these methods yields information on the energy landscape that controls mechanical function and on the force-bearing components of proteins, as well as on the underlying physical mechanisms.

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Abbreviations
AFM atomic force microscopy
DFS dynamic force spectroscopy
LOT laser optical tweezers
SMD steered molecular dynamics

Introduction

Numerous functions of cells involve mechanical properties of biopolymers. Recent studies of single-molecule measurement techniques, such as atomic force microscopy (AFM) [1], laser optical tweezers (LOT) [2] and biomembrane force probe [3] experiments, have greatly advanced our knowledge of these functions. Steered molecular dynamics (SMD) seeks to complement these observations and provide atomic level descriptions of the underlying events. SMD applies external forces to manipulate biomolecules in order to probe mechanical functions, as well as to accelerate processes that are otherwise too slow to model. Here, we review recent publications that have established SMD and force spectroscopy.

A sample process investigated by these new techniques is ligand binding. AFM and molecular dynamics simulations have revealed that proteins have evolved optimal pathways that guide ligands into binding sites. The investigations have established the interactions that steer ligands and have elucidated the energy landscape that controls the kinetics of the binding and unbinding processes.

Other studies focused on proteins that can be stretched to many times their original size, while keeping the ability to contract back to their original length. Such proteins exist in muscle tissue, the extracellular matrix and the cell nucleus. The extracellular matrix proteins can signal, via stretch intermediates, the mechanical state of the surroundings to cell receptors such as integrins. The adhesion forces between cell-surface receptors and their ligands are another focus of recent studies.

In the following, we will first introduce the new fields of force spectroscopy and SMD through examples. We will then describe the various simulation and analysis techniques that have been developed. Finally, we will review applications of these techniques in the light of experimental observations.

Unbinding biotin from avidin

Force probe spectroscopy and SMD are fields with experimental, as well as theoretical, roots. The fields were initiated with the 1994 AFM experiments by Gaub and coworkers [4] on biotin–streptavidin that reported the first measurement of the unbinding force of individual ligand–receptor pairs. Streptavidin and the related avidin are tetrameric proteins that bind four molecules of biotin with strong affinity. Florin *et al.* [4] derivatized both the tip of an AFM and a substrate with biotin, added avidin (or streptavidin), which binds to the substrate, then upon lowering the AFM tip observed binding to avidin and upon raising the tip observed rupture. They were able to work under conditions in which a single biotin was pulled out of its avidin binding site, revealing the properties of a single ligand–receptor (biotin–avidin) pair. The force needed to rupture the ligand–receptor bond was found to be 160 pN. To appreciate the magnitude of this force, we note that pulling a ligand with a constant force of 41 pN over 1 Å corresponds to an energy of 1 k_BT. Hence, pulling biotin with the constant force of 160 pN out of a 10 Å binding pocket would generate 23 kcal/mol, a value that compares well with the binding free energy of biotin of 22 kcal/mol.

The AFM measurements also provide information on the position of the tip, such that one can correlate applied force with relative position. The correlation can require interpretation in terms of a polymer model [5•] that accounts for the elastic behavior of the molecular link to the AFM tip, for example, the worm-like-chain model.

One must also realize that results of AFM experiments are sensitive to the timescale of the experiment, typically in the microsecond to second range. Biotin is so strongly bound to avidin that spontaneous unbinding via thermally activated events happens on timescales of weeks; if experimentalists were to carry out observations on such slow timescales, they would measure rupture at very small or even vanishing forces. But even on a microsecond scale, rupture is dominated by thermal events that exhibit a probabilistic nature. As a result, observations that sample

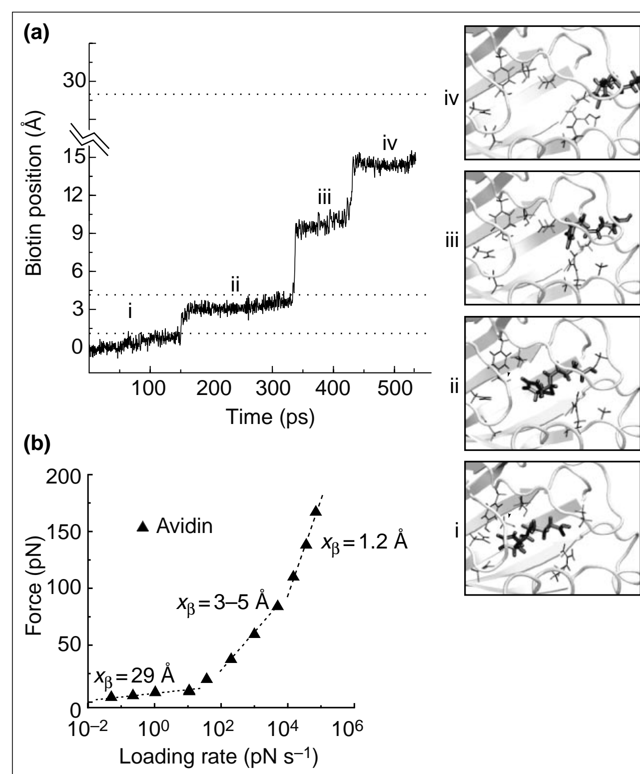
over multiple rupture events actually reveal a distribution of forces.

The observations in [4] have been extended by several researchers. Recent (strept)avidin–biotin unbinding studies involve mutagenesis [6], surface adhesion [7] and so-called dynamic force spectroscopy (DFS) observations [8••]. The unique information provided by AFM observations on force distributions and, to a more limited degree, on the relative motion characterized by a one-dimensional extension of the AFM tip provides a fascinating glimpse into ligand–receptor binding. One wishes, however, to relate this information to the underlying molecular structure and energy landscape. For this purpose, one needs to complement AFM observations with molecular dynamics simulations and nonequilibrium statistical mechanics analysis. It should be noted here that the relationship of unbinding times and applied force was previously described by Bell in 1978 [9].

Molecular dynamics simulations of biotin–streptavidin and biotin–avidin were reported in [10] and [11], respectively. Figure 1a presents the results from [11], showing the stepwise extraction of biotin as it slips out of the binding pocket in discrete steps that correspond to a series of quasi-stable intermediates; the figure shows which ligand–receptor interactions correspond to the intermediates. The simulations in [10] described a solvated monomer of the protein and reported quantitative agreement with observed AFM rupture forces. Such agreement could not be reproduced with a full tetramer, albeit unsolvated [11]. Izrailev *et al.* [11] provided a comprehensive analysis of the unbinding of the ligand–receptor pair on the basis of a description using a one-dimensional Smoluchowski equation that accounts for diffusive molecular motion in the strong friction limit. It was shown that simulation and AFM observation operate in different regimes, the drift regime and the thermally activated regime, respectively, such that agreement between simulation and AFM results cannot be expected. However, once a potential of mean force describing the energy landscape of ligand binding is constructed, for example, from a simulation, the AFM results can be reproduced using the theory of first passage times. This approach also permits one to evaluate the distribution of rupture forces measured in AFM experiments [11].

DFS [8••] samples many unbinding events, varying force-loading rates over several orders of magnitude (see Figure 1b), and seeks to determine the positions of unbinding barriers. The results agree well with SMD data, for example, in the case of avidin–biotin unbinding [8••,11]. Evans and colleagues [5•,8••,12] developed the necessary stochastic theory of bond breaking under varying force-loading rates to reveal the locations of barriers along an unbinding path. DFS has been successfully applied to explain the conflicts among different force techniques for unfolding immunoglobulin domains in terms of loading rates [5•]; for connecting biomembrane force probe

Figure 1



Transition events in avidin–biotin unbinding in SMD simulations and DFS experiments. **(a)** Unbinding transitions in stiffening-spring SMD simulations [11]. The solid line shows the position of biotin during the SMD simulation. Quasi-stationary intermediates i–iv correspond to snapshots from the simulation shown on the right. Three dotted horizontal lines are drawn at the three x_β values shown in (b). **(b)** DFS of avidin–biotin unbinding, as presented by Merkel *et al.* [8••]. Each data point represents a DFS experiment at a given loading rate; the three slopes fitted to the data points correspond to three distinct positions (x_β) along the unbinding pathway (projected along the direction of applied force) at which activation barriers arise.

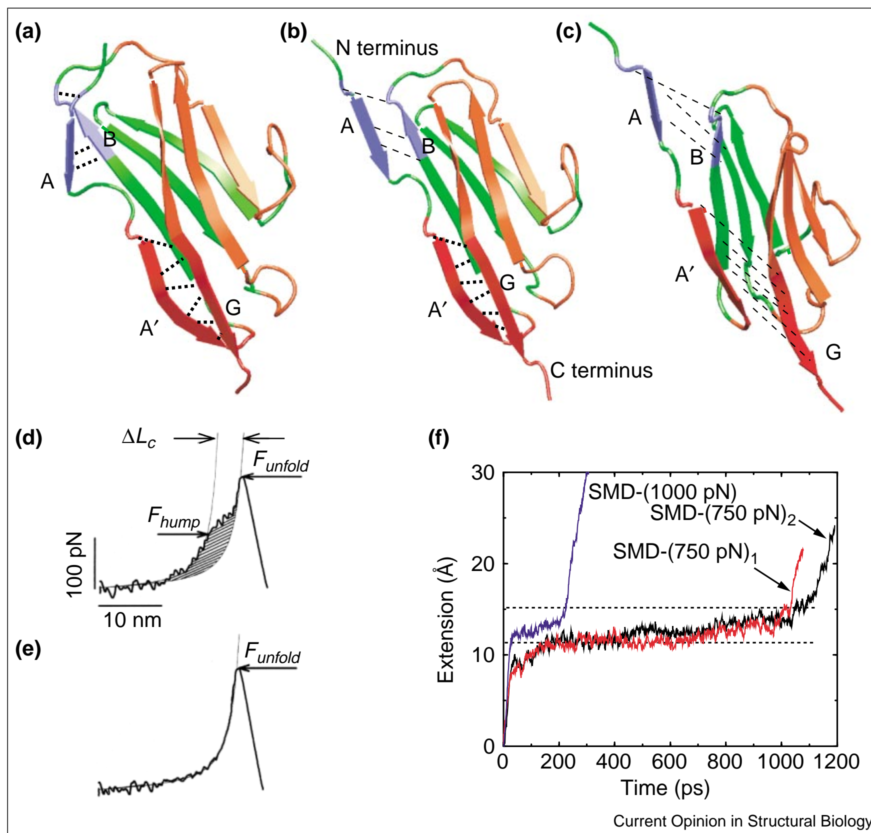
experiments to SMD simulations in locating the positions of energy barriers of the biotin–avidin system [8••]; for examining forces in fibrinogen–silica unbinding [13]; and for demonstrating a transition, as the loading rate is increased, between spontaneous unbinding and force-induced unbinding [14]. Recently, the theory of DFS was further refined [15•] to account for the shape of barriers, as well as for both stiff-spring and soft-spring experiments.

DFS experiments on avidin–biotin [8••] identified three unbinding barriers (Figure 1b), the first two of which match the positions of plateaus i and ii shown in Figure 1a. Plateaus iii and iv are not reproduced because, in the SMD simulation underlying the data shown in Figure 1a [11], a loop at the pocket entrance was moved out of the exit path.

Stretching of immunoglobulin and fibronectin domains

A second major application of AFM was in the investigation of the muscle protein titin. The native protein

Figure 2



Forced unfolding of titin immunoglobulin domain I27 by SMD simulations and AFM experiments. (a–c) Representative snapshots of the three-phase unfolding scenario of I27 observed in SMD simulations [50]. **(a)** Native structure of I27. **(b)** An unfolding intermediate of I27 at extension of 10 Å. Hydrogen bonds between β-strands A and B (colored blue) are broken (thin lines), but those between β-strands A′ and G (colored red) are intact (thick lines). **(c)** At 25 Å extension, a force extends the A′–G patch. All hydrogen bonds between β-strands A and B and between β-strands A′ and G are broken in this snapshot (thin lines). The first peak in the force/extension curve **(d)** for an I27 octamer and **(e)** for the Lys6→Pro mutant from AFM experiments (adapted from [21••]). In (d), one can recognize a hump corresponding to a force F_{hump} smaller than the complete unfolding peak force F_{unfold}. The change in contour length ΔL_c before and after the hump, determined by fitting a worm-like-chain model to the AFM data (thin lines), indicates an extension of 6.6 Å per module (shaded area indicates the work needed for the event). SMD simulations (b) suggest that the extension results from the breaking of hydrogen bonds between β-strands A and B. The elimination of the deviation from the constant contour length worm-like-chain model by a point mutation replacing Lys6 with a proline (e) further confirmed the suggestion. **(f)** Extension profiles from SMD simulations for constant force protocols. Three phases (preburst, burst, postburst) can be recognized in each of three SMD simulations, two applying a force of 750 pN and one applying a force of 1000 pN (adapted from [50] with permission).

contains a string of about 300 individually folded domains and a central region that acts as an entropic spring. Upon stretching, titin straightens, the domains stretch out by about 6 Å each and, upon further stretching, the domains unfold one-by-one. The unfolding events were demonstrated with AFM observations initiated in the Gaub group [16,17] and with LOT measurements by Kellermayer *et al.* [18]. These observations were later extended in a series of ground-breaking publications from the Fernandez group [19,20,21••,22•] (reviewed in [23•]). The observations relied on genetically engineering strings of protein domains, focusing mainly on polyproteins made of the immunoglobulin-like titin domain I27, for which a structure had been obtained earlier [24,25]. The AFM observations revealed a series of force peaks as the tip is pulled away from the substrate, each peak corresponding to the unfolding of a domain; the last peak corresponds to the rupture of the tip–protein connection. The overall process can be modeled by means of the worm-like-chain model as a series of polymer stretching events, with a stepwise increase of the polymer length by 28–29 nm, that is, the length of a completely unfolded and stretched I27 domain. Analysis of the observations revealed that the stretching of domains occurs in two steps: forces above 50 pN extend the domains by about 6 Å, forces above 150–200 pN extend the domains by a further 10 Å, leading to a complete unfolding of the domains. The observations also produced distributions of stretching forces that agreed qualitatively with the predictions derived in [11]. A comparison of solvent denaturation experiments revealed that spontaneous unfolding and stretched unfolding are governed by the same effective energy barrier [20].

These observations called for an interpretation in terms of the architecture of the I27 domain, a double β-sheet structure shown in Figure 2, in order to reveal the mechanism that determines the forces required to extend and unfold the units. In a series of SMD simulations [21••,26,27•,28•] that reproduced the stretching events for individual solvated I27 domains, a clear interpretation emerged. The simulations identified the force-bearing structural components, the β-strands directly connected to the C and N termini of the domain. These β-strands are connected to the other β-strands of the protein by two sets of three and six hydrogen bonds. The first set (three bonds) breaks at weak forces and results in a 6 Å extension of the domain;

the second set (six bonds) breaks at strong forces and thereby initiates complete unfolding. This identification inspired experiments that confirmed the emerging picture. A mutant that severed the first set of hydrogen bonds was found to abolish the stretching intermediate (see Figure 2d,e) [21••].

The simulations in [26] were criticized by Paci and Karplus [29,30•] on the basis of results from implicit solvent model simulations. The authors claimed that the faster pulling speeds applied in the simulated systems overestimated the strength of interstrand hydrogen bonds as, on the nanosecond timescale of the simulations, solvent is essentially frozen around these bonds. However, an analysis of the role of water during simulated stretching showed clearly that, over the time period of a nanosecond, solvent (water) repeatedly attacks the interstrand hydrogen bonds and is, in fact, the rate-determining element for the initiation of unfolding [28•]. Moreover, when a constant force of, say, 500 pN is applied, the protein enters an intermediate state in which it resides until unfolding occurs. One needs to note in this regard that the stability of interstrand bonds does not depend solely on the actual hydrogen-bonding interactions, but also depends on interactions connected with the packing of the side groups involved in the bonding, as well as on interactions between polar elements of the respective side groups.

The stretching scenario of I27 domains under simulation conditions is governed by statistical events. This has been demonstrated in [27•], in which the dwell time in the stretching intermediate was computed for a random ensemble of domains subjected to 750 pN stretching forces. The simulations exhibited a distribution of dwell times. This distribution could be reproduced through a simple energy landscape involving a linear barrier separating the folded state from the unfolded state. A peak position with a height of the barrier that compared well with the denaturation and AFM observations was yielded. The simulations also determined dwell times for a series of different forces and could match the resulting dwell times well to a first passage time expression that assumed the same barrier characteristics.

Besides immunoglobulin-like domains, titin also contains fibronectin-like domains, which have a double β-sheet architecture that is similar to the structure of immunoglobulin domains. Fibronectin is a key constituent of the extracellular matrix, where it connects cells to substrates and links to the integrin receptors at the cell surface. A series of four consecutive fibronectin domains has been structurally resolved for fibronectin type III_{7-10}. The proteins are less amenable than titin to genetic expression and AFM observations [17]. Molecular dynamics simulations have been carried out nevertheless [29,31], showing a behavior under stretching that is reminiscent of that of the I27 domain, but more complex. The SMD simulations in [31] also reproduced a dominant force peak that can be attributed to the breaking of interstrand hydrogen bonds involving the strands that merge directly into the C and N termini. The simulations for fibronectin module III_{10} revealed a decrease in the curvature of the so-called RGD loop in these domains, named after a segment with neighboring arginine, glycine and aspartic acid side groups, that binds to the integrin receptors. The affinity of the loop for the receptor decreases significantly with the flattening of the loop, suggesting that a cell receives information about the mechanical strain affecting the extracellular matrix through integrins.

Variations of steering forces

The force applied in SMD simulations was initially inspired by AFM extension experiments. Accordingly, molecular systems were tethered to a spring that was pulled at its free end. The simulations explored various types of pulling speeds, mainly to induce a desired event in the longest time that could be covered in simulations, typically a few nanoseconds. Naturally, the simulations at higher pulling speeds required stronger forces than those observed, for example, for the biotin–avidin system. The simulations therefore diverged from the observations, such as by using a spring constant increasing in time and by applying constant forces to a system, for example, to the stretching of I27 domains. The constant force method resulted in new scenarios that did not alter the qualitative pathways of stretching and unfolding, but that exhibited intermediates more clearly as they survived longer. A second advantage of constant force pulling is a simpler analysis of data, from either observation or simulation. For example, the powerful theory of mean first passage time can be applied in a straightforward way, although generalizations of the theory for time-dependent force have been worked out [32] that can, in principle, be applied.

Molecular dynamics simulations permit one to apply forces that are more complex than can be realized in an AFM experiment. For example, simulations explored the binding/unbinding pathway of retinal in bacteriorhodopsin. In this case, the prosthetic group can be extracted, but for this purpose the applied forces need to change directions during the extraction process. The simulations [33] thereby showed that retinal should enter the membrane protein through the lipid phase, rather than the aqueous phase, as had been suggested earlier.

Before the advent of AFM observations, Schlitter and colleagues [34,35] had already suggested that conformational transformations or ligand unbinding could be studied through applied forces. They suggested a method, termed targeted molecular dynamics, in which forces are defined such that they reduce the distance $d(t)$ between an initial state and a target state defined in a multidimensional conformation space. The corresponding forces act simultaneously on many coordinates and directions, but are one-dimensional in that they act along $d=d(\vec{r}_1,\ldots,\vec{r}_N)$, where $\vec{r}_j$ denotes atomic positions. This

choice makes the forces too limited to produce the desired results, as barriers cannot be circumnavigated. Steered and targeted molecular dynamics were jointly applied to a stretched prostaglandin H_2 synthase-1 [36].

Paci and Karplus [29,30•] employed a potential that corresponds to a spring that can only push particles, that is, it only exerts forces if a particle moves opposite to the specified direction. A disadvantage of such forces is that resulting data cannot readily be analyzed, as succinct descriptions for stochastic processes with such forces are not available. Wade *et al.* [37] added random forces that permitted a ligand to find an unbinding route in the case of cytochrome P450 enzymes. Such forces indeed appear to be suitable for cases in which a hypothesis for unbinding routes does not exist and a user wishes to rely on random search. An alternative approach based on an *a priori* hypothesis of binding/unbinding pathways has been applied to suggest pathways in a nuclear hormone receptor [38].

A simplified description of protein unfolding simulations has been suggested by Klimov and Thirumalai, who employed a lattice model in [39] and an off-lattice model in [40•]. The coarse-grained continuum representation adopted in [40•] makes it possible to conduct many runs of simulations for pulling speeds four orders of magnitude slower than SMD simulations [26,41]. The simulations, which the authors call steered Langevin dynamics simulations, apply nearly the same forces as SMD simulations, but damp system movements via coupling to a heat bath. Combined with Evans and Ritchie's DFS theory [5•,12], the simulation results are able to correctly predict rupture forces in AFM experiments on I27 and spectrin. The unfolding pathway revealed in their simulations of I27 is in excellent agreement with SMD simulations, although the break-up of hydrogen bonds cannot be represented because of the simplified model employed.

SMD simulations can adapt applied forces to nonlinear coordinates that may capture the nature of conformational transitions. The need for this arose in a study of the motion of a protein domain in a membrane protein complex, the cytochrome bc_1 complex. It was suggested on the basis of crystallographic observations that the iron–sulfur domain of this system undergoes a rotation around a hinge provided by a long helical segment anchored in the transmembrane part. An SMD simulation applied a torque to the domain and demonstrated that it could readily be rotated as suggested [42]. Another interesting case using a generalized force arose in a recent study of the mechanosensitive channel MscL of *Escherichia coli*. The channel exists in a closed form and in a wide-open form, with a channel diameter of about 30 Å. Patch clamp measurements [43] had shown that MscL opens spontaneously when the protein together with its membrane surroundings is sucked into the apparatus, inducing a bulge in the membrane, the radius of which can be quantitatively related to lateral tension in the membrane. Accordingly, simulations applied anisotropic pressure to the isolated protein and to the protein embedded in an artificial membrane, and could thereby induce opening of the channel [44]. The underlying motion involved an increase in the tilt of transmembrane helices, conforming to observations in which the opening of the channel is induced by placing the channel into membranes with shorter lipids that can accommodate the protein only when helices tilt down [44].

Analysis of steered molecular dynamics simulations

SMD simulations, like umbrella sampling, superimpose a time-dependent force on simulated biopolymers that pulls the systems along certain degrees of freedom. SMD simulations are equivalent to umbrella sampling when applied forces are weak, change very slowly in time and induce minor overall changes. Of interest here is the opposite limit: when major changes are induced (e.g. a ligand is extracted from an enzyme or a protein's termini are stretched to initiate unfolding); when superimposed forces change rapidly in time; and when significant deviations from equilibrium arise. This is the limit that one is supposed to avoid in umbrella sampling, free energy perturbation theory and weighted histogram analysis methods, but that one seeks to exploit in SMD, expecting of course certain limitations. There are several reasons why this seems possible and, indeed, wise.

One can assume from the outset in the analysis of simulation results that systems described are not at equilibrium and employ nonequilibrium descriptions for the analysis. That this is possible, in principle, has been shown in the work of Jarzynski [45,46], who demonstrated that free energy differences can be obtained through exponential averages of irreversible work.

Evans and Ritchie [12] have employed the simulation data of [11] and constructed a potential of mean force through low pass filtering of the data. Balsera *et al.* [47] demonstrated likewise that one can construct potentials of mean force from SMD trajectories and derived an error bound for this construction that is proportional to the irreversible work done in the simulated system. Gullingsrud *et al.* [48•] carried this construction further, introducing a time series analysis method. The authors suggest modeling the trajectory from a simulation as the path of a particle moving in a one-dimensional potential $W(z)$ with a Langevin equation of the form:

$$\gamma\dot{z} = -dW(z)/dz + F(z,t) + \sigma\xi(t).$$

Here, γ is the viscous friction coefficient and $\sigma\xi(t)$ represents a stochastic force due to thermal fluctuations in the channel environment. The potential $W(z)$ determines the dynamics of the ligand and reconstructing this term is the goal of the analysis. The method needs to assume knowledge of γ that discounts the irreversible work or needs to determine an appropriate γ, possibly dependent on location z. The authors

compared a weighted histogram analysis treatment that assumes quasi-equilibrium with methods in which such an assumption is not made. The most promising method suggested is based on the Onsager–Machlup function for the Smoluchowski equation connected with the above stochastic differential equation. This approach permits a sampling of multiple paths and was shown, for generic models, to reproduce $W(z)$ potentials well when γ is known. The methods have not yet been applied successfully to real simulation data.

Conclusions

We have reviewed the field of force spectroscopy and, in particular, the role of SMD simulations in this field. The advance of computer power will lend an exciting new dimension to force spectroscopy. Routine simulations will become fast enough to complete, in a few seconds, typical conformational relaxation processes that arise after mechanical perturbations of biopolymers. This will permit a user to monitor an ongoing simulation through molecular graphics and to interfere with the simulation by exerting external forces to manipulate the simulated system. In this way, one can speed up thermally activated processes and explore the mechanisms underlying protein function. For example, a researcher can pull an ion through a membrane channel and, through an analysis of the applied forces and of the system response, can determine conductivities or filter mechanisms. The feasibility of this vision has already been demonstrated in [49], in which a molecular dynamics program on a large-scale parallel computer, a molecular graphics program and a force feedback input device were combined to manipulate various biomolecular systems.

Acknowledgements

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