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Protein–protein unbinding induced by force: single-molecule studies

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Experiments in which two specifically interacting protein molecules are dissociated by external force have yielded new insights into mechanisms involved in cell adhesion, leukocyte rolling, regulation of integrin activity, antigen–antibody interactions and other protein-mediated reactions contingent upon molecular recognition. Another important aspect of force-induced protein–protein unbinding studies is the new information being gleaned about the thermodynamics and kinetics of bond rupture.

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Abbreviations

AFM	atomic force microscopy
BFP	biomembrane force probe
ConA	concanavalin A
ICAM-1	intercellular adhesion molecule-1
LFA-1	leukocyte function-associated antigen-1
LT	laser tweezers
PSGL-1	P-selectin glycoprotein ligand-1
SGCT	sodium glucose co-transporter
vWF	von Willebrand factor
WASP	Wiscott–Aldrich syndrome protein

Introduction

Protein–protein unbinding studies are a part of the major field of investigation termed receptor–ligand interactions or molecular recognition. During the past decade, important new information about receptor–ligand interactions at the single-molecule level has complemented conclusions based on conventional methods, which measure the properties of large ensembles of molecules or observe the behavior of whole cells. The main advantages of studying individual receptor–ligand pairs are: minimized cooperative and/or clustering effects; the possibility of probing conformational transitions of individual molecules, such as activation/inactivation; revealing the structural and functional heterogeneity of seemingly identical molecules; knowing the number of molecules involved in reactions; quantifying directly the magnitudes and working distances of forces in ligand–receptor interactions to elucidate the relationships between molecular structure and the thermodynamics of bond dissociation. Watching individual events and distributions rather than observing average values may reveal rare but physiologically important functional fluctuations [1•,2].

The study of protein–protein interactions has been dominated by a static viewpoint, such that the emphasis is on molecules in solution under equilibrium conditions, whereas their real-life biological interactions generally occur on surfaces under nonequilibrium conditions; the latter is the focus of the papers summarized in this review. The study of the mechanics of protein interactions is necessary to understand the many cellular functions and properties, such as rolling, motility, adhesion, deformability and so on, that are mediated by specific receptor and ligand molecules, and controlled by mechanical forces produced by either external (shear flow) or internal (cytoskeletal rearrangement, motor proteins) sources. In addition, the study of protein–protein unbinding by an applied force has turned out to be a precise and unique tool for analyzing protein structure and function, as well as mechanisms of their regulatory changes [3].

Theory

The results of mechanical rupture (pulling) experiments have been analyzed using two distinct theoretical methods [4]. The first, called the energy landscape model, is based on Kramers' rate theory and leads to general predictions about the distribution of rupture forces [5•]. In this model, it is assumed that an applied load changes the energy of the transition state as well as the equilibrium of the bound and unbound states, thereby altering the kinetics of association and dissociation [6]. An important conclusion from the model is that the distribution of rupture forces depends on the loading rate (i.e. the rate that the applied force is increased) and that this dependence provides information not obtained from equilibrium experiments [7•]. Pulling experiments in which the loading rate is changed over many orders of magnitude are called dynamic force spectroscopy. One important open question is whether these force spectra can be used to uniquely identify the dissociation mechanism [8•]. A recent advance in nonequilibrium thermodynamics [9•], based on an insight from Jarzynski [10], further suggests that the complete free energy diagram in the direction of the applied load can be inferred by correctly averaging repeated traces of the force versus

time (trajectories) acquired at constant pulling velocity. Although the method has not yet been applied to protein–protein interactions, it was verified for the mechanical unfolding of RNA [11••].

The second method uses molecular dynamics simulations to investigate the disruption of specific pairs of molecules in atomic detail [12]. Ideally, this type of model provides new insights into the mechanisms of protein–ligand interactions [13•,14,15]. However, meaningful simulations require complete X-ray crystal structures of the proteins and huge computational resources. Even with the most powerful current computers, the simulations only last ~10 ns, roughly three orders of magnitude less than typical pulling experiments. One of Jarzynski's predictions is that his equality holds even very far from equilibrium or at very high loading rates. Recent computational and theoretical advances allow us to be more confident that dynamic force spectroscopy may provide important information on how proteins bind and unbind.

Principles and methodology

Force-induced receptor–ligand unbinding studies are always performed at an interface. Molecular binding and rupture result from controlled touching and separation of two surfaces, one bearing receptors and another coated with ligand. The different techniques used to perform these kinds of experiments differ from each other mainly by the surfaces to which the proteins are bound, as well as by the methods of generating, sensing and measuring mechanical forces. The techniques used during the past several years for studies of single-protein molecular mechanics are: atomic force microscopy (AFM), also termed scanning force microscopy (SFM) [16]; laser tweezers (LT) [17]; biomembrane force probe (BFP) with pipette suction [18]; and hydrodynamic methods [19•]. The forces generated and sensed during single-molecule experiments are in the range of several piconewtons ($1\ \mathrm{pN} = 10^{-12}\ \mathrm{N}$) to about one nanonewton ($1\ \mathrm{nN} = 10^{-9}\ \mathrm{N}$), although no single instrument can adequately cover the entire range of forces. The magnitudes of these forces correspond to the range of noncovalent interactions, but they are insufficient to break a covalent bond [20].

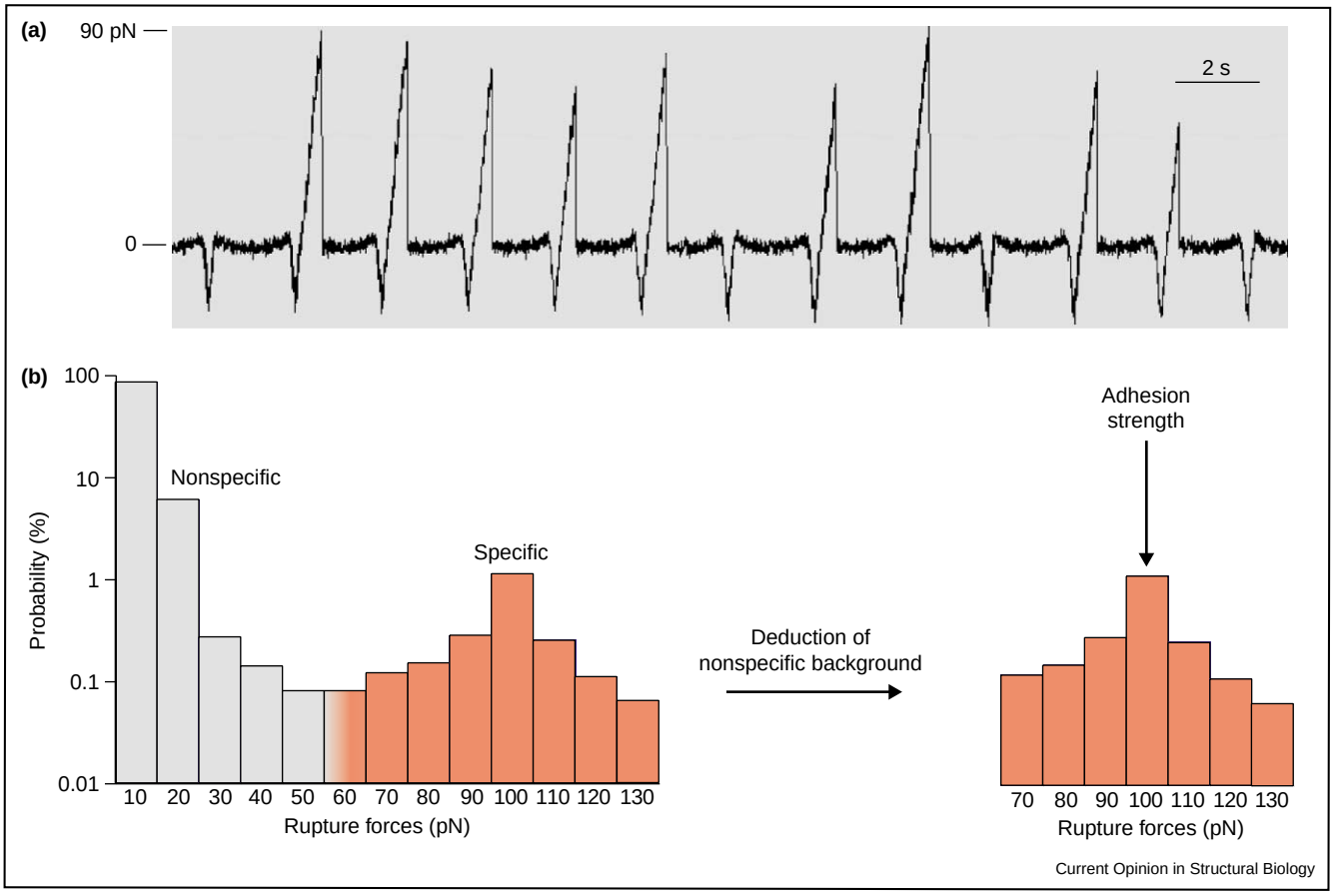
The interacting receptor and ligand molecules both have to be firmly bound to their underlying surfaces to ensure that the measured forces reflect protein–protein unbinding rather than the detachment of molecules from the surface [21]. When experiments are performed with purified proteins, they should be coupled to surfaces either covalently or at least via biotin–(strept)avidin interactions, which are stronger than any known noncovalent protein–protein adhesion. Covalent linkages are made either directly using bifunctional reagents [22••,23] or via spacer molecules [24•,25], which is seemingly preferable because of the higher flexibility and better spatial orientation of surface-bound proteins. However, stretching of polymeric linkers changes the unbinding profile by giving rise to delayed nonlinear receptor–ligand rupture force signals [24•].

As in ensemble experiments, discrimination between specific and nonspecific interactions is a major concern, requiring carefully chosen control experiments. Two important control experiments are replacing one of the reacting partners with an inert protein (e.g. albumin) or applying specific inhibitors that abolish the interaction in a dose-dependent manner. Irrespective of the origin of nonspecific surface-to-surface attraction, several studies have shown that nonspecific protein–protein adhesion strength may reach the value of several tens of piconewtons, partly overlapping the forces produced by specific receptor–ligand interactions (Figure 1) [26,27]. These weaker nonspecific protein–protein interactions may have physiological significance in cell adhesion in cooperation with the specific interactions [22••].

An important question that is difficult to answer unequivocally is whether one or multiple pairs of molecules interact during pulling experiments. Two pieces of evidence, both based on statistics, are commonly used to determine whether single-molecule interactions are detected. The first requires that only a limited fraction of touching cycles between the surfaces result in binding [19•,27]. For example, when only ten IgG molecules were bound per bead and the protein A surface density was also very low, the probability of formation of more than one IgG–protein A pair at a particular scanning velocity was calculated to be at most 9% [27]. Secondly, histograms of the distribution of rupture forces can show a series of quantized peaks that are multiples of a single value [23,28]. Although there are other important indirect criteria supporting individual molecule interactions [29•], reasonable conclusions must be substantiated by combinations of data obtained independently.

Protein–protein unbinding studies performed on live cells with exposed receptor molecules have several limitations and uncertainties. One of them is the chance of uprooting receptor molecules from the membrane, which depends on pulling time and force as well as on membrane tension [30,31]. A related problem is mechanical compliance and membrane tethering, combined with interactions of receptors with the cytoskeleton [30,32]. Receptor clustering also can potentially affect quantification of single-molecule receptor–ligand interactions, because it was shown that chemical cross-linking of receptors led to increased ligand binding strength as a result of receptor cooperativity [33,34•]. Similarly, self-association of selectins led to shear resistance and changed unbinding parameters for P-selectin and its ligand, P-selectin glycoprotein ligand-1 (PSGL-1) [35]. Nevertheless, properly performed receptor–ligand binding studies with proteins naturally residing

Figure 1



Raw data trace and force histogram for integrin–fibrinogen interactions as measured using LT [22••]. **(a)** A series of attachment events illustrating the variability of the unbinding forces produced by the same individual integrin–fibrinogen pair. In each touching/separation cycle, the forces are displayed as two peaks: a negative, compressive force between a receptor-coated surface and a ligand-coupled latex bead, and a positive, unbinding force that increases linearly with time until the receptor–ligand bond is ruptured, after which the force rapidly returns to zero. If attachment does not occur, the positive rupture force is absent. **(b)** Rupture forces following detachment of surfaces are collected and displayed as normalized force distribution histograms. The results of many experiments under similar conditions are summed so that each histogram includes the many contacts needed for representative statistics. Nonspecific protein–protein interactions may partly overlap the specific interactions.

on metabolically active cells are physiologically more relevant and most promising.

Some recent results of enforced single-molecule protein–protein unbinding, along with a partial description of the experimental conditions, are summarized in Table 1. It can be seen that the majority of peak rupture forces measured over a wide range of loading rates are in the interval from several tens up to ~150 pN. The forces measured at similar loading rates appear to be independent of underlying surfaces, coating chemistry and measuring techniques. However, there is an obvious difference in the rupture forces for different protein–protein pairs. These results imply that the different rupture forces reflect multiple mechanisms of receptor–ligand dissociation, rather than diversity of experimental conditions.

Applications

The strength of cell attachment to substrata and/or to another cell is a good example of how the mechanical characteristics of single molecules determine cell function. That is why integrins, selectins and cadherins, which mediate cellular interactions, were among the first proteins studied at the single-molecule level using force-induced unbinding methodology. Cell adhesion and aggregation are strongly influenced by the mechanical plasticity of cells, by the direction and rate of applied external forces, and by the mutual accessibility of the receptor and ligand molecules [4,36,37].

The regulation of binding-site exposure was directly demonstrated in a study in which the interactions of fibrinogen with the integrin $\alpha_{IIb}\beta_3$ were studied on live platelets with different degrees of activation [22••]. Using

Table 1

Values of rupture forces required to separate individual protein molecules.

Receptor* and receptor-bearing surface	Ligand* and ligand-bearing surface	Measuring technique	Reported loading or shear rate/stress	Rupture forces (pN)	References
Adhesion molecules					
GPIIb-IIIa ($\alpha_{IIb}\beta_3$) Covalently bound to latex beads	Fibrinogen Free in solution	Hydrodynamic flow	0.6–2.9 N/m^2 shear stress	70–150 (15.6%) 150–230 (16%) 230–310 (17%)	[19•]
$\alpha_{IIb}\beta_3$ On native adherent platelets	GSSSGRGDSPA Covalently bound to tips via glutaraldehyde	AFM	12 nN/s	~93	[28]
$\alpha_{IIb}\beta_3$ On resting or activated native adherent platelets Covalently bound to modified silica beads via glutaraldehyde	Fibrinogen Covalently bound to latex beads via carbodiimide	LT	20 nN/s	60–150, with a peak at 80–100	[22••]
$\alpha_V\beta_3$ $\alpha_5\beta_1$ $\alpha_V\beta_3$ $\alpha_V\beta_3$ All on adherent osteoclasts partly fixed with paraformaldehyde	GRGDSP GRGDSP Osteopontin Echistatin All adsorbed on tips via noncovalently bound PEG	AFM	30 nN/s†	42 ± 4 32 ± 2 50 ± 2 97 ± 15	[50]
GP Ib-IX ($\alpha\beta$IX) $\alpha\beta$IX $\alpha\beta$IX On native transfected CHO cells	von Willebrand factor (vWF) Ultralarge vWF A1 domain of vWF Adsorbed on latex beads	LT	Not reported	6.5 ± 0.8 8.8 ± 0.3 11.4 ± 2.1 11.5	[51]
P-selectin Bound to silanized glass cover slips via biotin–avidin	PSGL-1 Bound to silanized tips via biotin–avidin	AFM	168 nN/s†	159 ± 30	[52]
P-selectin Bound to cantilever as Fc-chimera via anti-Fc-Ab	PSGL-1 On intact neutrophils	AFM	250 nN/s†	175	[44•]
PSGL-1 and other selectins' ligands On neutrophils	P-, E-, L-selectins or peripheral node addressin Adsorbed on a plastic surface	Hydrodynamic flow	0.5–5.0 dyn/cm^2 shear stress	37–250	[42]
L-selectin Covalently bound to glass spheres via PEG	PSGL-1 Covalently bound to glass spheres via PEG	BFP	0.01–100 nN/s	5–200	[39••]
LFA-1 (αLβ1) On murine T-cell hybridoma cells (3A9) coupled to cantilever via ConA	ICAM-1 Expressed by fibroblasts FT 16.11 or adsorbed in soluble form	AFM	0.02–50 nN/s	20–320	[40••]
β1-integrins On native mouse 3T3 fibroblasts	Fibronectin Adsorbed on glass	LT	0.005–0.1 nN/s	13–28	[53]
$\alpha_5\beta_1$ On K562 cells activated with mAb TS2/16 and attached to tips via ConA	Fibronectin fragment 7-10 Adsorbed on tissue culture dishes	AFM	1.8–2.0 nN/s	69 ± 15 93 ± 1.5	[41••]
VE-cadherin Covalently bound to SiOH plates via PEG	VE-cadherin Covalently bound to tips via PEG	AFM	6 nN/s† 24 nN/s† 120 nN/s†	33 40 54	[24•]
Glycoprotein csA On *Dictyostelium discoideum* AX20214 strain cells attached to tips via lectin	csA On *Dictyostelium discoideum* AX20214 strain cells spread on polystyrene	AFM	12 nN/s†	23 ± 8	[54]

Table 1 Continued

Receptor* and receptor-bearing surface	Ligand* and ligand-bearing surface	Measuring technique	Reported loading or shear rate/stress	Rupture forces (pN)	References
Mucin Covalently bound to tips via 11-C spacer molecules	Mucin Mucin gel deposited on mica	AFM	1–132 nN/s†	100–4000	[55]
Immunoglobulins					
Anti-ferritin mouse mAb Covalently bound to silicon wafers via glutaraldehyde	Ferritin Covalently bound to tips via glutaraldehyde	AFM	Not reported	49 ± 10	[56]
Polyclonal anti-HSA Ab Covalently bound to tips via PEG	Human serum albumin (HSA) Covalently bound to mica via PEG	AFM	54 nN/s†	240 ± 48	[57]
Anti ICAM-1 mouse mAb F10.2 Covalently linked to tips via PEG	ICAM-1 Adsorbed on mica in a soluble truncated form (sICAM-1)	AFM	54–191 nN/s†	100 ± 50	[26]
Ab against γ-glutamyl-transpeptidase (γ-GT) Ab PAN3 against a segment of SGCT Both covalently bound to tips via PEG	γ-GT SGCT Both on brush border membrane vesicles adsorbed on gold surfaces	AFM	6 nN/s†	131 ± 44 100 ± 47	[45•]
Polyclonal antibodies #8 against ryanodine receptor 1 (RYR 1) Covalently linked to tips via PEG	RYR 1 Adsorbed on mica	AFM	2 nN/s 6 nN/s 9 nN/s	42 ± 6 66 ± 11 73 ± 17	[58]
Mouse antibody against β subunit of human chorionic gonadotropin (βhCG) Covalently bound to silicon wafers via glutaraldehyde	βhCG Covalently bound to tips via glutaraldehyde	AFM	43–140 nN/s†	132 ± 16	[23]
Anti-β3 mAb (F11) Adsorbed on tips via noncovalently bound PEG	$\alpha_V\beta_3$ On adherent osteoclasts partly fixed with paraformaldehyde	AFM	30–1500 nN/s†	127 ± 16	[50]
Rabbit anti-mouse IgG Covalently linked to synthetic resin beads via dextran	Staphylococcal protein A Covalently co-linked along with lectin to synthetic resin beads via dextran	BFP	50–60 pN/s	19 ± 1	[25]
Rabbit, mouse, bovine or goat IgG Covalently linked to latex beads	Protein A Covalently linked to a glass surface	LT	0.4–5.3 nN/s	25–44	[27]
Plant lectin from *Ricinus commutis* Plant lectin from *Viscum album* Bovine galectin-1 IgG All covalently linked either to agarose beads directly or to tips via amylose	Asialofetuin Covalently linked either to agarose beads directly or to tips via amylose	AFM	3 nN/s	37 ± 3 43 ± 5 65 ± 9 45 ± 6	[59]
Cytoskeletal and motor proteins					
Actin from rabbit skeletal white muscle Attached to gelsolin-coated latex beads	Heavy meromyosin prepared from rabbit skeletal white muscle myosin Adsorbed on glass	LT	12 pN/s	9.2 ± 4.4	[46•]
Bundles of actin from rabbit skeletal muscle Adsorbed on positively charged polyacrylamide beads	Heavy meromyosin prepared from rabbit skeletal muscle myosin Attached to polyacrylamide beads via biotin–avidin	AFM	≈140 pN/s†	14.8 ± 4.0	[60]

Table 1 Continued

Receptor* and receptor-bearing surface	Ligand* and ligand-bearing surface	Measuring technique	Reported loading or shear rate/stress	Rupture forces (pN)	References
Microtubules Adsorbed to glass	Two- or one-headed kinesin Covalently linked to latex beads	LT	5.0 ± 1.68 pN/s	3.3–10	[49••]
Actin from rabbit muscle Attached to gelsolin-coated latex beads	Bovine Arp2/3 complex with VCA Arp2/3 adsorbed on a VCA-coated surface	LT	50.5 ± 17.3 pN/s	6–7	[48•]
Various proteins					
Bovine insulin Covalently linked to aminosilanized mica using photoreactive azido groups	Bovine insulin Covalently linked to aminosilanized tips using photoreactive azido groups	AFM	7.4 nN/s†	1340–1350	[61]
Chaperonin GroEL from *E. coli*	Mutated (Gly→Ala) citrate synthase from yeast	AFM	≈6–60 nN/s†	420 ± 100	[62]
	Double mutated RTEM (Cys→Ala) β-lactamase			240 ± 70	
	Horseradish peroxidase			130 ± 30	
	Bovine serum albumin (BSA)			570 ± 60	
Adsorbed on mica	All covalently linked to tips via glutaraldehyde				

*The term 'receptor' is relevant by definition only to integrins and selectins, whereas other interacting proteins were named 'receptor' or 'ligand' arbitrarily. †Calculated from reported values of a transducer spring constant and retraction velocity.

receptor–ligand adhesion strength as a measure of single receptor activity, it has been shown that $\alpha_{IIb}\beta_3$ activation is an all-or-none phenomenon, that is, each integrin molecule resides on the platelet in an either completely on or off conformation [22••]. The strength of fibrinogen–$\alpha_{IIb}\beta_3$ binding was shown to be dependent on the 'age' of bonds; 'young' complexes formed within minutes were capable of dissociation under hydrodynamic force, whereas 'older' complexes could not be ruptured [19•]. Probing interactions of individual integrins on the surface of live cells with ligands containing the RGD (Arg-Gly-Asp) binding motif revealed a considerable extension of the flexible membrane surface under load [28], as well as a paradoxical increase in the number of cells that remain adherent when larger pulling forces were applied [34•].

The interaction of the platelet integrin GPIbα with von Willebrand factor (vWF) was shown to have kinetic characteristics similar to those of binding reactions of selectins [38•]. Dynamic force spectroscopy studies revealed that the rupture forces of both integrins and selectins with their protein ligands depend on applied force loading rates. When the range of applied force loading rates was wide enough and reached 10^4 pN/s or more, two regimes were observed as different slopes in the peak force versus logarithm of loading rate curves. They were interpreted as reflecting two sequential energy barriers along the unbinding pathway. Changing experimental conditions revealed that these barriers had different susceptibility to divalent cations [39••] and may have different physiological significance [40••,41••]. The first, inner barrier for the $\alpha_5\beta_1$–fibronectin pair, observed at high forces, was sensitive to RGD deletion, but not to deletion in the synergy site of fibronectin. The second, outer barrier, operating at lower, physiologically relevant forces, was affected by both RGD and synergy binding regions, and was characteristic of the site of integrin activation [41••]. In a study of leukocyte function-associated antigen-1 (LFA-1) and intercellular adhesion molecule-1 (ICAM-1), it was shown that, in the slow loading regime, Mg^{2+}, known to stabilize the LFA-1–ICAM-1 interaction, increased the unbinding forces, whereas the fast loading regime was susceptible to an inhibitory effect of EDTA. Hence, the outer activation barrier was attributed to an equilibrium dissociation constant, whereas the inner barrier was thought to determine resistance to pulling forces [40••]. These thermodynamic characteristics, along with kinetic parameters and bond elasticity, fully control the dynamic process of cell adhesion and leukocyte rolling [13•,42,43•], and may account for their impairment in pathologically altered cells [44•].

The binding strength between antigen and antibody has been used as a tool to probe conformational states of a single protein molecule in several studies. AFM tips functionalized with antibodies were used to produce two images, the first, an adhesion image of individual ICAM-1 molecules, compared to the second, topographical one [26]. In another AFM study, antibodies were used to confirm proper membrane orientation in cell-derived vesicles and to probe the conformational changes of an

antibody-binding epitope of sodium glucose co-transporter (SGCT) during D-glucose transport [45•].

Direct measurement of the strength of rigor bonds between actin and myosin [46•,47], studying detachment of the actin–Arp2/3 complex from the VCA domain of N-WASP (Wiscott–Aldrich syndrome protein) [48•] and using binding strength in the molecular analysis of kinesin motility ([49••]; see also Update) are additional but not the last examples (see Table 1) of diverse and important biological applications of force-induced individual protein–protein unbinding studies.

Conclusions and perspectives

Most of the results of those experiments listed in Table 1 that were carefully designed and executed show that rupture forces for adhesion proteins are characteristic of each ligand–receptor pair. The usual range for typical proteins appears to be ~100 pN +/− 50 pN, but there may be exceptions. Although loading rates under most physiological conditions have not been determined, it is likely that these forces were measured with loading rates that cells might really experience, for example in flowing blood. It should be noted that the experiments described in some papers listed here are incomplete, lacking important controls or have problems with methodology or interpretation, reflecting the difficulty of these studies. The results of all single-molecule protein–protein unbinding induced by force experiments still need to be interpreted with caution. Future research in this field should take into account the cautions presented in this review.

Although studies of protein–protein unbinding induced by force are still in their infancy, there have been important theoretical and experimental breakthroughs, but much remains unknown. The theory behind nonequilibrium events needs to be further tested and related to equilibrium processes. More studies will explore the detailed events during unbinding and binding via simulation of experimental data by molecular dynamics calculations, which need to be extended in time. The current literature on prototypical purified protein–protein systems will be expanded to include many other biologically important molecules, especially adhesive proteins, but much of the excitement in this field is likely to lie with studies involving live cells. Specific mutations in functionally important sites can be designed to elucidate molecular mechanisms of ligand–receptor interactions. More complex systems should be investigated, including clustering of receptors in the membrane, interactions between two different receptors and cytoskeletal interactions. Studies in the future may involve the simultaneous measurement of forces and other functional parameters, such as phosphorylation or other biochemical activity, distribution of GFP-labeled proteins, channel movement or the behavior of other stress sensors. Single-molecule studies of protein–protein unbinding address fundamental questions on the origins of the specificity and dynamics of cellular interactions. In the future, it may be possible to relate single-molecule studies to whole cell interactions and the changing protein–protein interactions that occur during the development of the organism, and during cellular physiological and pathological processes.

Update

Recently, Kawaguchi *et al.* [63••] have used unbinding force distribution studies to reveal more mechanistic details of kinesin motility.

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