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What is the biological relevance of the specific bond properties revealed by single-molecule studies ?

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During the last decade, many authors took advantage of new methodologies based on atomic force microscopy, biomembrane force probes, laminar flow chambers or optical traps to study at the single molecule level the formation and dissociation of bonds between receptors and ligands attached to surfaces. Experiments provided a wealth of data revealing the complexity of bond response to mechanical forces and the dependence of bond rupture on bond history. These results supported the existence of multiple binding states and/or reaction pathways. Also, single bond studies allowed us to monitor attachments mediated by a few bonds. The aim of this review is to discuss the impact of this new information on our understanding of biological molecules and phenomena. The following points are discussed: i) which parameters do we need to know in order to predict the behaviour of an encounter between receptors and ligands, ii) which information is actually yielded by single-molecule studies and iii) is it possible to relate this information to molecular structure ?

Keywords: **laminar flow chamber, atomic force microscopy, biomembrane force probe, optical traps, surface-attached molecules, binding strength, unbinding force, off-rate, Bell's law**

1 - INTRODUCTION

During the last decade, simultaneous development of several methods allowing to monitor bond formation and dissociation at the single molecule level yielded a new kind of information on molecular behaviour. The aim of this review is to discuss the input of this advance to current biological wisdom. For the sake of clarity and homogeneity, we shall focus on proteins.

Importance of molecular interactions.

Life is based on molecular interactions. Indeed, considering proteins, enzymes need to bind specifically to substrates they will maintain in close proximity with adequate reagents. Scaffolding proteins bind to each other in order to maintain the cell architecture. Signalling is based on the formation of multimolecular complexes that will be generated through dedicated binding sites frequently borne by widely spread structures such as SH2, SH3 or plecskstrin homology domains. Membrane receptors bind to specific ligands. Membrane adhesion receptors are a prominent example: nearly all steps of cell life including survival, proliferation, differentiation, migration or activation are heavily dependent on adhesive interactions. It is therefore not surprising that between 25 % and 50 % of cell membrane molecules may well be adhesion receptors (Barclay, 1998). Also, it was stated in standard treatises that "the biological functions of proteins almost invariably depend on their direct physical interaction with other proteins" (Creighton, 1993).

Thus, it is not surprising that most biologists are deeply interested in molecular recognition and biomolecule interaction. However, while these phenomena are taught in every textbook and may be felt as well understood, several lines of research that steadily increased during the last decade

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brought dramatic changes to our understanding of molecular interactions.

Recent progress in studying molecular interactions.

At least five major advances led to dramatic conceptual advances.

i) extensive use of structural studies based on crystallography or nuclear magnetic resonance yielded an increasing number of available molecular complexes known with angström resolution. Thus, we probably know fairly well what protein-protein binding interfaces look like (Lo Conte et al., 1999). It seems a reasonable order of magnitude to assume that the association of two protein molecules will make several hundreds of squared angströms inaccessible to water, with several tens of close interatomic contacts.

ii) In addition to descriptive information, systematic use of site-directed mutagenesis following the pioneering study of Cunningham et al. (1989) opened the possibility to assess the quantitative importance of the interactions that were "seen". The basic principle consisted of systematically replacing individual aminoacids constituting the binding surface with alanine and measuring the influence of this change on affinity. This approach yielded information on the influence on affinity of individual hydrogen bonds or ionic interactions.

iii) Following the pioneering work of Tha et al. (1986) and Evans et al. (1989), several innovative methods were systematically used to monitor individual bond formation and dissociation between surface-attached biomolecules subjected to controlled force in the piconewton range. These include laminar flow chambers (Kaplanski et al., 1993 ; Alon et al., 1995 ; Pierres et al., 1996), atomic force microscopy (Florin et al., 1994 ;Lee et al., 1994 ; Hinterdorfer et al., 1996), optical tweezers (Miyata et al., 1996 ; Rinko et al., 2004 ; Litvinov et al., 2005) biomembrane force probes (Evans et al., 1994 ; Merkel et al., 1999). In addition to unprecedented accuracy, these methods yielded qualitatively new information such as bond response to mechanical forces.

iv) The need to interpret the new kind of information yielded by aforementioned methods was an incentive to reexamine the physical basis of molecular interaction. Thus, pioneering work initiated by Eyring (1935) and Kramers (1940) to account for the rate of molecular reactions and basic analysis of the effect of force and attachment to surfaces (Bell, 1978) was revived (Evans and Ritchie, 1997). Older reports formed the basis of a recent surge of theoretical analyses that are intended to process and interpret currently available experimental data.

v) Simultaneously, computer simulation evolved as a new approach for understanding as well as predicting the behaviour of biomolecules. While thirty years ago, it appeared as a remarkable feat to simulate the behaviour of a few tens of water molecules in a box (Dashevsky and Sarkisov, 1974), it becomes feasible to simulate interactions between proteins comprising thousands of atoms (Schueler-Furman et al., 2005 ; Gray, 2006 ; Rueda et al., 2007) and mimic force-induced bond rupture as studied experimentally on models such as streptavidin-biotin interaction (Zhou et al., 2006).

Thus, it seems reasonable to examine the impact of aformentioned lines of research on the conceptual framework we should use to consider biomolecule interactions.

An agenda for studying molecular interactions.

A biologist willing to explore and understand the molecular interactions underlying cell function will need the following three kinds of information.

i) A first requirement consists of defining a ***set of quantitative parameters*** that are both necessary and sufficient to account for molecular behaviour when they have been measured. As an example, it has long been considered that the affinity constant might account for most aspects of biomolecule association.

ii) Once useful parameters have been defined, it is necessary to elaborate efficient methods for measuring them. In fact, the development of powerful ways of studying individual bonds was certainly an incentive to look for more accurate ways of describing and understanding these bonds.

iii) Finally, it is certainly useful to obtain quantitative links between molecular structure and interaction properties. First, this would allow us to predict interaction parameters

between known molecules without a need to perform lengthy and delicate experiments. Second, this would markedly improve our understanding of biological phenomena. Thus, an important effort was done in building so-called docking algorithms intended to predict the interaction between two molecules or to devise a synthetic molecules with a capacity to bind to a biologically important mediator. This may be particularly useful for drug design (Brooijmans and Kuntz, 2003). Also, this is certainly useful to ***summarize*** data.

The aim of the present review is to provide a brief description of recent progress along these three lines of research, with a special emphasis on methodological points. As mentioned above, we shall focus on protein interactions. Even with this restriction, it is not feasible to do justice to all advances performed during the past years and we apologize for the omission of important work.

2 - LOOKING FOR A SUITABLE SET OF PARAMETERS TO ACCOUNT FOR MOLECULAR INTERACTIONS

The affinity constant.

The law of mass action is a basic rule that defines the amount of moles of complex formed between two reactants, say A and B, in equilibrium:

$$A + B \rightleftharpoons AB \qquad (1)$$

A common way of expressing the law of mass action is:

$$[A_{eq}]\,[B_{eq}]/[AB_{eq}] = K_d = 1/K_a \qquad (2)$$

Where $_{eq}$ stands for the equilibrium conditions, and K_d and K_a are the dissociation constant and the affinity constant, that are expressed in mole and mole^{-1} respectively (see Bongrand, 1999, for some additional remarks).

As was emphasized by Alan Williams (1991) in the early nineties "The concept of affinity dominated most thinking about complex biological reactions for many years". This means that about 15 years ago biologists were entitled to think that the affinity constant accounted for most important properties of any intermolecular reaction. However, at this moment, several important experimental findings suggested that other parameters had to be considered. Here are two important examples.

A prominent step in the development of immune responses is the interaction of T lymphocytes and antigen presenting cells exposing on their membranes cognate complexes formed between molecules encoded in the major histocompatibility complex and antigen-derived oligopeptides of about 10 aminoacid residues (pMHC). Indeed, a major task of T lymphocytes is to scan cells composing a living organism to detect possible abnormalities, essentially the expression of foreign structures likely to be encoded by potentially harmful microorganisms (see e.g. Bongrand and Malissen, 1998, for more details on this task and involved challenge). Now, an important feature of this interaction is that a given T lymphocyte may display different responses depending on the interaction properties of its membrane receptor (T cell receptor, abbreviated as TCR) and pMHC. While immunologists had long tried to relate the outcome of interaction with affinity (Ashwell, 2004), it appeared in the early nineties that **different** peptide ligands recognized by a TCR with **similar** affinity could induce widely **different** responses (Matsui et al., 1994). What then might be the interaction parameter determining the T cell response ?

An other example is integrin regulation. Integrins are important cell membrane receptors that mediate a number of key interactions, such as adhesion to extracellular matrices or lymphocyte adhesion to other cells. An important property of integrins is that their binding efficiency is highly regulated, in order that cells may bind to appropriate surfaces (see Vitte et al., 2005 for a review). Now, while recent studies clearly established the integrin capacity to display major conformational changes with concomitant affinity modification (e.g. Shimaoka et al., 2001), intriguing experiments showed that affinity was not the only parameter involved in integrin regulation: indeed, Cai and Wright (1995) showed that a monoclonal antibody could induce a high affinity conformation in CR3, an integrin complement receptor of phagocytic cells, but this antibody **displayed similar binding affinity** to both active and inactive forms of CR3. Thus, rigorous thermodynamic reasoning showed that activity regulation performed by

this antibody was not based on affinity. What then might be the basis of increased integrin efficiency ?

Kinetic constants: the on-rate and off-rate. Several reports suggested that kinetic reasoning might provide an answer to aforementioned questions. This consisted of replacing equation (2) with (3) for describing a bimolecular reactions:

$$d\,[AB]/dt = k_{on}\,[A]\,[B] - k_{off}\,[AB] \qquad (3)$$

This kinetic view was related to the thermodynamic description through the simple equation:

$$K_a = k_{on}/k_{off} \qquad (4)$$

An important consequence of (4) is that two reactions of similar affinity might display widely different timescales. Thus, some receptors might form rapidly fairly transient bonds, while others with similar affinity might require higher amounts of time to form durable bonds. Several examples supported the interest of this concept.

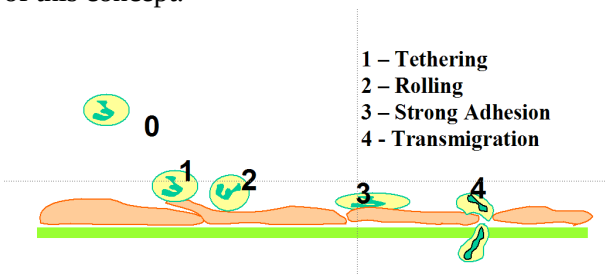

Figure 1: Capture of flowing leukocytes by activated endothelial cells. The arrest of flowing leukocytes on inflamed blood vessels involves several sequential steps including tethering, induction of rolling (often mediated by selectins) and firm arrest (requiring integrins).

First, in their aforementioned paper, Matsui et al. (1994) provided experimental support to the hypothesis that the outcome of interaction between T cell receptor and pMHC ligand was determined by the lifetime of interaction rather than the affinity. A widely quoted model suggested to explain experimental data (McKeithan, 1995) relied on the involvement of biochemical reactions requiring a minimal occupation time to trigger cell a signalling cascade in T lymphocytes.

A second example is the so-called maturation of the antibody response: when an organism is repeatedly stimulated with a given antigen, the "efficiency" of the antibodies produced to get rid of the antigen will progressively increase. While it was first thought that this improvement was essentially due to an increase of affinity, Foote and Milstein (1991) reported that in addition to the affinity increase, there was a shift towards an antibody family with an extremely high association rate (k_{on}), suggesting a selection mechanism "with a premium of binding target antigens rapidly".

A third example, that raised a major interest of the biological community in biophysical aspects of cell adhesion is the interaction of blood leukocytes with the blood vessels leading to adhesion and subsequent transmigration to peripheral tissues. This phenomenon is a key step of inflammation, which plays an essential role in the organism defence against aggression (Harlan and Liu, 1992). It has long been found, using intravital microscopy, that local activation of endothelial cells lining the blood vessels allowed them to capture flowing white blood cells in remarkable way involving at last two steps (Figure 1). First, cells undergo a nearly hundredfold velocity decrease and display a jerky displacement in contact with the vessel walls. Their velocity is then reduced to a few micrometers per second. This is called rolling.

Cells then stop completely, which is a prerequisite for the transmigration step. The molecular basis of this phenomenon was demonstrated by Lawrence and Springer (1991) who used a laminar flow chamber to demonstrate that rolling could be initiated and maintained by an interaction between endothelial receptors belonging to the selectin family and ligands on the leukocyte surface. Complete stop could be achieved through an interaction between a leukocyte integrin (such as LFA-1, also called CD11aCD18) and its ligand on endothelial cells (e.g. ICAM-1, also denominated as CD54). While selectins could tether leukocytes to endothelial cells, they were unable to stop them completely. Conversely, integrins could stop leukocytes only if their motion had been slowed down by selectins. A reasonable interpretation of these findings was that selectins had high association and dissociation rate, while integrins might display comparable affinity and lower kinetic constants.

The reports from Foote and Milstein (1991) and Lawrence and Springer (1991) indeed arose the interest of the biological community in kinetic aspects of intermolecular reactions (Williams, 1991). Interestingly, available methodologies based on surface plasmon resonance provided a suitable way of measuring these constants when receptor and ligand molecules were available (Schuck, 1997 ; Rich and Myszka, 2006).

Interaction between bound molecules: there is a need for two functions rather than two constant parameters to account for molecular behaviour.

Since the late seventies, it became more and more apparent that the conventional kinetic description of molecular interactions as described in standard textbooks of physical chemistry was insufficient to deal with important biological processes. Indeed, biologically relevant intermolecular reactions often involve bound molecules. This is obvious when we consider cell adhesion, but intracellular processes also frequently involve multimolecular assemblies. Thus, molecular interactions often involve molecules bound to large size complexes.

A first important consequence is that bonds formed between attached molecules are frequently subjected to mechanical forces that are likely to alter their lifetime. Thus, several experimental data suggested that adhesive strength might behave as a physiologically relevant parameter in the triggering of defence processes such as phagocytosis (Capo et al., 1978) or T-lymphocyte-mediated cytotoxicity (Bongrand et al., 1983).

A second point is that the rate of bond formation between attached molecules cannot be derived from properties measured in solution. The most obvious illustration of this fact may be that 2-dimensional (2D) and 3-dimensional (3D) on-rates have different dimensions (Dustin et al., 1996 ; Pierres et al., 2001). Also, association between bound molecules may be heavily dependent on properties such as binding length or flexibility that are not important in solution (see Pierres et al., 1998a, for additional remarks and Jeppesen et al., 2001, for a spectacular illustration of the importance of the link between molecules and surfaces).

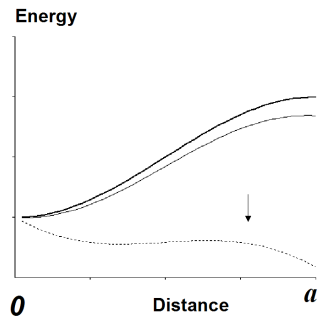

Figure 2: Effect of force on the energy landscape. The figure shows ***i)*** *bold line*: a simple energy well with a barrier (abscissa a). ***ii)*** *thin line*: the alteration induced by an external force equal to 10 % of the maximal slope of the native curve. The barrier position is not markedly changed, and Kramers's theory may be used, leading to Bell's law. ***iii)*** *broken line*: the alteration induced by an external force equal to about 90% of the maximal slope of the native curve. The barrier is very low and approximations used to derive Bell's law are no longer valid.

These points were indeed discussed in an insightful and premonitory theoretical paper by George Bell (1978): he clearly emphasized at least two important points: i) the kinetics of bond association and dissociation may be widely different when molecules are freely moving (3D conditions) and when they are bound to surfaces (2D interactions). ii) the effect of forces on intermolecular bonds should be dependent on specific structural parameters (e.g. the location of energy barriers on energy/distance curves) that might vary independently of affinity. Indeed, Bell proposed a simple exponential dependence of the off-rate on disrupting force following a formula that is now currently denominated as Bell's law:

$$k_{off}(F) = k_{off}(0)\exp(F/F°) = k_{off}(0)\exp(Fa/k_BT) \quad (5)$$

where k_B is Boltzmann's constant, T is the absolute temperature, and *a* is the distance between the energy minimum and the maximum of the energy barrier on the force distance curve (Figure 2). An intuitive explanation for this law was that the rate of bond rupture might be viewed as the product of

"attempt frequency" to escape and the probability of success for a given attempt. A force F should decrease the activation barrier (Figure 2) by F*a*, thus increasing by $\exp(Fa/k_BT)$, according to Boltzmann's law, the probability that a complex had sufficient energy to break the bond. Note that in contrast with Bell, several authors chose to make us of parameter F° (i.e. k_BT/a) instead of *a*. Indeed, F° may be used as a phenomenological parameter without any reference to the energy landscape structure.

Thus, predicting the formation and dissociation of bonds between attached molecules should require **at least** a knowledge of two functions rather than two parameters (Pierres et al., 1996), i.e. the probability $\mathbf{k_{on}(d)}$ of bond formation as a function of the distance between the anchoring points of the molecule, and the frequency $\mathbf{k_{off}(F)}$ or rupture of a bond subjected to a force F. Note that even this set of parameters may prove insufficient in the future. Thus, the force-mediated rupture of a bond may be dependent on the force orientation in addition to its intensity (Astroff et al., 2006 ; Ke et al., 2007).

There is some experimental support to the view that this information is indeed relevant to biological situations. Thus, elegant studies by Patel et al. (1995) strongly suggested that the selectin capacity to tether leukocytes to endothelial cells under flow was at least partly due to the length of these molecules, which allowed them to interact with their ligand at sufficient distance to avoid excessive repulsion by the pericellular matrix of interacting cells. This concept is fully consistent with another report by VonAndrian et al. (1995) who showed that selectins needed to be located on the tip of cell membrane protrusions to mediate rolling, thus emphasizing the importance of interaction range.

An other point that was less frequently considered is the relationship between cell rheological properties and mechanical properties of the bonds formed by cytoskeletal constituents, such as actin and actin binding proteins. Indeed, cell deformation under force is tightly related to the kinetics of attachment to and detachment from actin microfilaments of molecules such as alpha actinin (Wachsstock et al., 1994). Also, cell capacity to generate forces is related to the mechanisms of microfilament elongation (Kovar et al., 2004).

There is a need for information allowing us to derive the properties of multivalent interactions from single bond parameters.

It is striking that the efficiency of many bond species was found to be related to their capacity to form dimers or multimers. Here are some examples.

The aforementioned ligand of LFA-1, ICAM-1/CD54, an ubiquitous molecule, was reported to occur as a dimer on cell membranes, and this dimerization seemed important for adhesive activity (Miller et al., 1995).

Cadherins are a prominent family of homotypic calcium-dependent adhesion molecules that play a key role in maintaining tissue integrity (Wheelock et al., 2003). In a very elegant study, Yap et al. (1997) made cells express modified cadherin with an intracytoplasmic moiety that allows the induction of lateral clustering at will by mere addition of a membrane-permeable drug. This clustering was concluded to be required for cadherins to exert efficiently their adhesive function.

Antibody molecules have long been shown to be multivalent. It has also been recognized that the potential of an antibody to capture its cognate antigen was not always entirely accounted for by **affinity**, which led immunologists to use the empirical concept of **avidity** which seemed related to the capacity of an antibody sample to form multivalent bonds.

Integrins are a prominent example that has been already mentioned. CR3/CD11bCD18 is an integrin receptor for complement components that is found on neutrophils and macrophages. It has long been known that this receptor was fairly inefficient on resting cells and it required some kind of activation. As described above, activation was sometimes found to be related to a suitable conformational change resulting in increased affinity or association rate. However, another hypothesis was supported by other experiments. Indeed, Detmers et al. (1987) used colloidal gold labelling and electron microscopy to report that treatment of neutrophils with PMA, a well known protein kinase C activator, induced both CR3 activation and clustering of these receptors into

aggregates of a few (two to six) integrins. Further experiments showed that the mere clustering of receptors on a membrane could be sufficient to activate adhesion (Hermanowski-Vosatka et al., 1988). Note that the importance of receptor clustering in integrin activation was supported by a number of experimental reports (Yauch et al., 1997 ; Hato et al., 1998)

As a consequence, it is important to provide links between multivalent attachment and the properties of single bonds. However, this raises major difficulties:

i) linking the rupture of multivalent attachments to the behaviour of individual bond requires assumptions relative to the sharing of forces between bonds and the possibility of rebinding. As discussed in theoretical studies (Seifert, 2000 ; Erdmann and Schwarz, 2004), these properties may dramatically influence bond rupture. Indeed, the lifetime of a divalent attachment may be close to the lifetime of a single bond in the absence of rebinding, whereas active rebinding may increase this lifetime by several orders of magnitude. This possibility is also exemplified by a recent report by Gopalakrishnan et al. (2005) who used computer simulation to show that ligand dissociation from a surface might be strongly influenced by receptor clustering.

ii) when multiple bonds are allowed to form between surfaces, the rate of bond formation is usually dependent on the number and position of already existing bonds as well as molecular length and flexibility (see e.g. Pierres et al., 1998a, for a simple treatment of a representative example).

Thus, the only rigorous way of extracting single bond properties from experimental studies is to study attachments mediated by **single bonds**. This explains the outstanding impact of single bond studies on our understanding of molecular interactions.

A point of caution: importance of defining intrinsic association parameters.

It is important to emphasize a major difficulty underlying the experimental study of bond formation between surface-attached molecules. When molecular associations are studied in solution, measured thermodynamic and kinetic constants may be considered as fairly intrinsic to the studied binding sites: when molecular species A and B have been defined, the equilibrium constant in a standard medium (of known composition, temperature and pressure) may be considered as fully defined. On the contrary, aforementioned parameters concerning surface-attached molecules are dependent on many extrinsic factors. If these are not adequately controlled, experimental data obtained with a given experimental setup may not be relevant to other situations. Here are a few examples.

The effect of a force applied to a bond may depend on the stiffness of linker molecules. Thus, when Evans et al. (1999) subjected molecular bonds to a disruptive force increasing linearly with respect to time, the dependence of the unbinding force (i.e. the force at the moment of rupture) on the rate of force increase might depend quantitatively and even qualitatively on the nature of the linker. Also, when Chen et al. (2000) studied the force required to separate cells from concanavalin-A-coated atomic force microscopy tip, increasing cell rigidity with glutaraldehyde resulted in twofold increase of the separation force. This finding may be highly relevant to the physiology of cell adhesion, since older investigations showed that cell detachment from a surface might follow a decrease of membrane stiffness, with subsequent decrease of adhesive strength (Rees et al., 1977 ; Badley et al., 1980). Clearly, linker mechanical properties may be easily overlooked when forces parameters are measured on bonds involving cell membrane molecules, since results are dependent on cell mechanical properties which are fairly complex.

The significance of association rates is probably even more dependent on environmental parameters than that of unbinding forces. Indeed, experimental association rates are not only dependent on the length and flexibility of interacting molecules but also on their mobility and on the topography of surrounding surfaces (Huang et al., 2004).

In summary, bond formation and dissociation between biomolecules is often dependent on parameters that are not accounted by standard 3D association and dissociation rates. It is necessary to determine **force-dependent dissociation rate** and **distance-dependent association rates**. These quantities are highly

complex since i) functions are obviously more complex than constant numbers, ii) they are dependent on molecular environment, and iii) as will be shown below, a third cause of complexity is the multiplicity of binding states, which was clearly revealed by single molecule studies.

3 - WHICH PARAMETERS CAN BE OBTAINED WITH SINGLE MOLECULE STUDIES?

The aim of the present section is to provide a brief description of currently available methods of studying single bond formation and dissociation in order to discuss the reliability and significance of experimental data. At least four approaches were repeatedly used during the last decade: flow chambers, atomic force microscopy (AFM), biomembrane force probes (BFP) and optical tweezers. Although much interesting information was obtained with the latter approach (Miyata et al., 1996 ; Rinko et al., 2004 ; Litvinov et al, 2005), we shall consider only on the one hand flow chambers, and on the other hand AFM and BFP, since these methods were probably used most often and they yield fairly complementary information, making comparison fruitful.

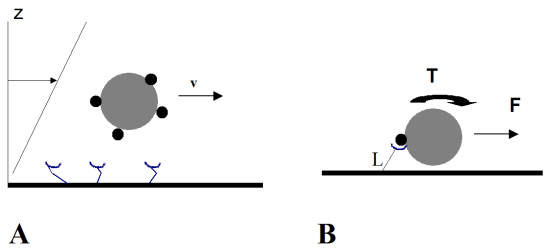

Figure 3: Studying ligand-receptor interaction in the flow chamber. **A**: a receptor-coated particle is driven along a ligand-coated surface by a hydrodynamic flow. The unperturbed flow velocity at distance z from the surface is Gz, where G is the wall shear rate. **B**: a particle maintained at rest by a bond is subjected to a hydrodynamic force F and torque T. The force on the bond is dependent on the shape of the particle surface.

Studying single bonds with flow chambers. We shall only present major conclusions. The reader is referred to a recent review for more details (Pierres et al., 2006).

Basic principles. As shown on Figure 3, the principle consists of monitoring the motion of a receptor-bearing cell or particle along a ligand-coated surface in presence of a laminar shear flow. The velocity of a particle of radius a close to the surface is **on the order** of aG, where G is the wall shear rate (in second^{-1}). The hydrodynamic force on a sphere attached to the surface is $\approx 32\ \mu\ a^2\ G$, where μ is the medium viscosity. When the sphere is maintained at rest by a single bond, the force on the bond may be 5-10 fold higher at most, depending on the length of the lever arm (see Figure 3B).

In many laboratories, flow chambers were used with a shear rate on the order of 100 s^{-1} or more, to mimic physiological conditions in blood vessels. In this case, the Reynold's number is low enough to warrant complete neglect of inertial effects. The velocity of a particle of 5 µm radius is on the order of 500 µm/s, allowing a contact duration of about 0.1 millisecond between receptor and ligands of total length of 50 nm. If a cell is stopped by a bond, it may be subjected to a force of order of the 100 pN, and the bond may experience a rupture force of several hundreds of pN, unless cells undergo extensive deformation such a extension of long membrane tethers (Schmidtke et al., 2000). These conditions were mostly used to study interactions between selectin molecules and their ligands.

We found it more rewarding to operate flow chambers at a much lower shear rate ranging between about 1-10 s^{-1} and replacing cells with microspheres of about 1.4 µm radius. In this case, the intermolecular contact time for bond formation might be higher than 1 millisecond, and the force on a bond maintaining cells at rest was less that about 5 pN. Under these conditions, it appeared possible to monitor single bond formation and dissociation between involving most adhesion receptors, including integrins, cadherins, collagen, fibronectin, members of the immunoglobulin superfamily, and antibodies, as well as selectins. Another advantage of this setup is that freely moving spheres are easily followed, and their position could be easily determined with an accuracy of about 50 nm with a simple image processing system. The

moment of bond formation and dissociation could thus be followed with high accuracy.

Results obtained with flow chambers.

Bond rupture: Bell's law first seemed to account for a number of reported results.

An experiment easily yields hundreds of particle trajectories (Figure 4A) that may be used to detect a number of arrests of widely varying duration. Values of arrest durations can be used to obtain unbinding curves by plotting the probability P for particles to remain at rest at time t after initial stop (Fig 4b) . Assuming simple dissociation kinetics, P is expected to be equal to exp(- k_{off} t), and the dissociation rate is simply the slope of the curve on a semi-log plot. If the experiment is repeated with different values of the shear rate, the dependence of k_{off} on the force can be determined provided the force on the bond is determined with simple reasoning (Pierres et al., 1995 ; Alon et al., 1995). A simple way of varying specifically the force consisted of changing the medium viscosity (Mège et al., 1986 ; Chen et al., 2001). The force might thus be increased without changing the kinetics.

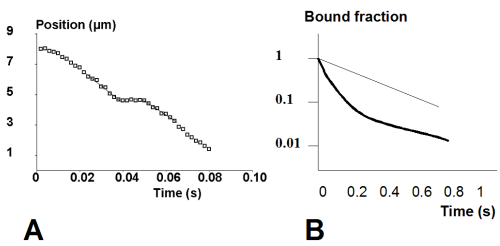

Figure 4: Unbinding curves may be obtained with a flow chamber. **A**: a typical trajectory revealing a binding event. **B**: the distribution of binding events durations is used to build an unbinding plot. A straight line (bold curve) is suggestive of a monophasic reaction. Curvature (thin line) is indicative of bond strengthening that may be due to delayed formation of additional bonds or strengthening of a single bond.

Many experimental data were consistent with Bell's law (Alon et al., 1995 ; Pierres et al., 1996a ; Alon et al., 1997 ; Chen et al., 2001) with a force constant varying on a nearly 100 fold range since reported F° was 2.4 pN for outer fragments of E-cadherins (Perret et al., 2002) and about 218 pN for L-selectin (Alon et al., 1997). The spontaneous dissociation rate also varied on a large range, as exemplified by CD2-CD48 (k_{off} = 7.8 s^{-1}, Pierres et coll., 1996a) and $\alpha_2\beta_1$ integrin - collagen (k_{off} = 0.01 s^{-1} after integrin activation ; Masson-Gadais et al., 1999).

Bond rupture: Multiplicity of binding states.

Soon after the first determination of bond lifetime with a flow chamber (Kaplanski et al., 1993), it appeared that unbinding plots did not always follow monophasic kinetics (Pierres et al., 1995) in accordance with previous studies made with conventional methods of chemical kinetics (Beeson et al., 1994 ; Foote et al., 1994). Thus, it was shown at the single bond level that ligand-receptor couples such as antigen-antibody (Pierres et al., 1995), Streptavidin-biotin (Pierres et al., 2002), integrin-fibronectin (Vitte et al., 2004a) or C-cadherin/C-cadherin (Pierres et al., 2007) could display a series of binding states with different lifetimes. As will be discussed below, this situation accounts for a variety of results that have been formulated in different ways such as dependence of rupture on bond history (Pincet et al., 2005 ; Marshall et al., 2005) or bond heterogeneity (Raible et al., 2006). Also, this imposes a tremendous complexity on the concept and experimental determination of association rates.

Bond rupture: catch bonds behave at variance with Bell's law.

The concept of catch-bonds was put forward nearly twenty years ago by Dembo et al. (1988). The basic idea was that while a disruptive force should decrease bond affinity, as shown with thermodynamic reasoning, it was in principle conceivable that it might **increase** bond lifetime. A few years later, when in vitro systems were used to study the rolling phenomenon, it was reported that a minimal shear force was required to allow rolling (Finger et al., 1996), but it was only recently that flow chambers allowed clearcut demonstration that catch bonds actually existed, as exemplified with bacterial receptors (Thomas et al., 2002) or P-selectin (Marshall et al., 2003).

Bond formation: measuring association rates with the flow chamber.

Flow chambers are in principle well suited to measure association rates provided these are not too high. This may be explained as follows: consider a molecule of binding range r passing near a ligand with a velocity v. As a rule of thumb, the contact time will be of order of r/v and the local concentration during passage will be about $1/r^3$ (in molecule per unit of volume). if we express r in nanometer and v in µm/s, the probability p that a contact might result in binding might be of order of

$$p = k_{on} * (r/1000v) * (10/6r^3) \approx k_{on} /(600\ v\ r^2) \quad (6)$$

where k_{on} is expressed in $Mole^{-1}s^{-1}$ and the number of molecules per mole is written as $6\ 10^{23}$.

Taking the binding range as about 20 nm, which matches the length of many adhesion molecules, and using velocities of order of 10 µm/s, it is concluded that the association rate may be a limiting parameter for binding if it is lower than about $2.4\ 10^6\ M^{-1}s^{-1}$. Note that this is probably an underestimate since the mobility of surface-attached binding sites is likely to be lower than in free molecules (however, the difference may be moderate: Pierres et al., 1998a). Note also that if k_{on} is higher than the limit we calculated, the binding frequencies measured with the flow chamber only reflect the number of ligand-receptor encounters, i.e. binding site densities on interacting surfaces.

However, the experimental determination of association rates with flow chambers is made difficult due to the following reason: a high shear rate is required to make encounter time short enough to comply with aforementioned condition. However, a high shear rate generates a high disruptive force which makes break nascent bonds.

A possible way of overcoming this limitation might be to use particles of smaller radius than cells. Indeed, this will increase the ratio between the particle velocity and the hydrodynamic force. However, a new difficulty is the importance of Brownian motion that makes particle motion very complex. However, this may be used to obtain an estimate of the binding range. Computer simulation may then be required to process experimental data. This approach was used to study the rate of bond formation between surfaces decorated with outer domains of cadherin 11 (Pierres et al., 1998b). The binding range was estimated at 10 nm and the binding frequency between molecules maintained at binding distance was estimated at $0.0012\ s^{-1}$.

A major difficulty in studying association rates is that the complexity of energy landscapes makes their definition quite arbitrary: indeed, defining a molecule as bound when a threshold force F_t during a threshold period of time t_t, the bound state depends on these parameters (Figure 5). Also, depending on the shape of the energy landscape, the probability that a molecular encounter of duration dt might result in binding may vary as a power of dt higher than 1. This adds to the definition of k_{on} a complexity that has not been thoroughly discussed, to the best of our knowledge (see e.g. Pierres et al., 2007, for an experimental study illustrating this point.

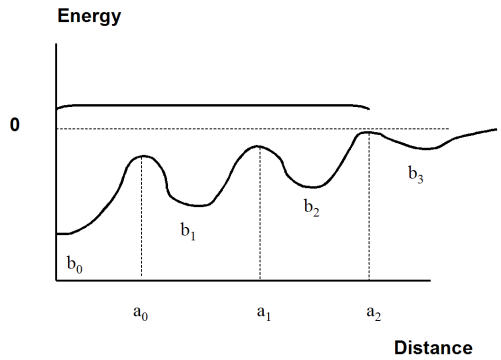

Figure 5: ligand-receptor interactions are often multiphasic. The curve shows a sequence of bound states of decreasing stability. The horizontal bracket shows a group of minima representing what is called a bound state. The proportion of complexes occupying b_0, b_1, b_2 depends on bond history. State b_3 may be too weak to be detected, but it may influence binding kinetics. Thus, if transition from b_3 to b_2 is slow enough, the attachment probability may vary as a power of contact duration.

Studying multivalent attachments with a flow chamber.

As emphasized, unravelling biologically relevant phenomena requires not only a quantitative understanding of single bond behaviour but also a good knowledge of combinations rules. As an example, the integrin-mediated attachment of monocytic cells to fibronectin-coated surfaces was studied under flow (Vitte et al., 2004a): unexpectedly, when cell surface integrins were clustered with antibodies, a simultaneous increase of binding duration and binding frequency was found. Since it was checked that cross-linking

antibodies did not change integrin conformation, the simplest interpretation of these experiments was that monovalent encounters between integrins and fibronectin molecules resulted in a majority of transient associations that were too short to be detected, while receptor clustering might result in strong potentialization of interactions, thus markedly raising the proportion of detectable interactions.

Studying single bonds with atomic force microscopy or biomembrane force probes.
While selectins were the first molecules studied at the single bond level with flow chambers (Kaplanski et al., 1993 ; Alon et al., 1995), avidin-biotin interaction was first chosen for AFM studies in view of its exceptionnally high affinity, on the order of 10^{15} M^{-1} (Florin e al., 1994 ; Lee et al., 1994).

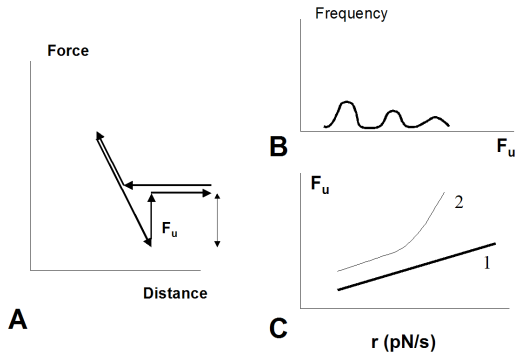

Figure 6: Information yielded by AFM and BFP. **A** : An unbinding force (F_u) can be measured . **B**: information can be extracted from the frequency distribution of F_u. **C**: a plot of F_u (either mean or peak) versus the rate of force increase gives some information on the energy landscape (Figure 5). However, several assumptions may be required to extract this information. Also, objective separation of the curve into straight segment lines may not be straightforward.

Basic principle.
The basic principle consisted of coating an AFM tip with a low density of receptor molecules and repeating cycles of approach and retraction at constant speed on a fixed point of a ligand-coated surface. As shown on Figure , bonds formed between the tip and the surface generated a pulling force increasing linearly with time during the tip retraction. For a value of the pulling force, the bond was broken, resulting in a sharp discontinuity of the recorded curve (Figure 6A). The measured quantity was called the unbinding force (F_u). After performing hundreds of cycles, it was possible to obtain the frequency distribution of unbinding forces (Figure 6B). In the pioneering study made on avidin-biotin couple (Florin et al., 1994), the hypothesis that single bonds were observed was based on the finding that the unbinding force appeared as an integer multiple of a constant value of 160 pN. In many studies, the surface density of binding molecules was made low enough that only a small fraction of contact/retraction cycles revealed binding events. Thus, if binding was a rare events, the probability of simultaneous occurrence of multiple bonds was deemed negligible based on Poisson law.

Although the cycle and contact duration were subject to wide variations between different reports, typical values for the contact time were on the order of 50-100 ms, cantilever velocity was on the order of 100-1000 nm/s, and cantilever stiffness on the order of 0.1 N/m. The impingement force during contact might be on the order of several hundreds of pN (Hinterdorfer et al., 1996 ; Ros et al., 1998 ; Fritz et al., 1998).

While only "unbinding forces", i.e. the maximum force supported by a bond, were first reported, it was soon recognized that bond rupture should be considered as a stochastic event. It was first indicated by Evans et al. (1997) that the **most probable force** at the moment of rupture should be proportional to the loading rate r (i.e. the rate of force increase per unit of time). However, the relationship between the unbinding force distribution and the loading rate is fairly complex and depends on the precise form of function $k_{off}(F)$. Using Bell's law as the standard model, the following equation if often used (Merkel et al., 1999 ; Yuan et al., 2000 ; Perret et al., 2004 ; Rinko et al., 2004)

$$F^* = F° \ln (r / k_{off}(0) F°) \qquad (7)$$

Note that the **average unbinding force** $\langle F_u \rangle$ is not equal to F* and Bell's parameters were sometimes derived from rupture force distribution with numerical fit (e.g. Fritz et al., 1998).

Results obtained with AFM and BFP.
Studying bond rupture with AFM and BFP.
Interestingly, many ligand-receptor models were studied with both flow chambers and

AFM or BFP. Indeed, the latter approach was applied to streptavidin-biotin (Florin et al., 1994 ; Merkel et al., 1999 ; Yuan et al., 2000), selectin-ligand couples, and particularly P-selectin/PSGL1 (Fritz et al., 1998 ; Hanley et al., 2003 ; Evans et al., 2004), Integrins (Zhang et al., 2002 ; Li et al., 2003) or cadherins (Baumgartner et al. 2000 ; Perret et al., 2002 ; Panorchan et al., 2006).

Merkel et al. (1999) soon showed that Equation (7) applied only over a limited range of loading rates, and plots of F* versus r appeared as a sequence of line segments, which was interpreted as revealing a succession of energy barriers that had to be overcome for complex dissociation. The distance between each barrier and the energy minimum was calculated as $k_BT/F°$, according to equation (5). This first report was rapidly confirmed by other investigators who reported multiple segments in F*/r plots revealing a growing complexity.

As an example, while a study of the homotypic interaction between the two distal domains of E cadherin (EC12) yielded a dissociation rate k_{off} and a force constant F° equal to 0.45 s^{-1} and 2.4 pN respectively with a flow chamber (Perret et al., 2002), Panorchan et al. (2006) reported values of 1.09 s^{-1} /13 pN, and 4 s^{-1} /41 pN with an atomic force microscope operated with a loading rate of 1,000 and 10,000 pN/s respectively. Finally, using a biomembrane force probe, Perret et al., disclosed a hierarchy of four substates displayed by complexes formed with EC12 or the entire five-domain extracellular region of E cadherin (EC15): parameters were respectively about 10 s^{-1}/6pN, 0.7 s^{-1}/6 pN, 0.01 s^{-1}/5 pN and 10^{-5}-10^{-6} s^{-1}/3-4pN. In order to reveal these widely different binding states, it was necessary to improve the usual procedure involving constant loading rate. The authors took advantage of a special "jump ramp" procedure to detect very transient complexes.

This multiplicity of binding states was not unique to E-cadherin. Thus, Li et al. (2003) studied the interaction between a fibronectin fragment and $\alpha_5\beta$integrin : Bell parameters were respectively 0.13 s^{-1}/10 pN and 33.5 s^{-1}/48 pN when the loading rates fell in the 20-10,000 and 10,000-50,000 pN/s range.

Thus, AFM and BFP forcefully demonstrated that Bells' law held only on restricted domains of loading rates.

Catch bonds were also evidenced with AFM and BFP.

While catch bonds were demonstrated by Thomas et al. (2002) with a flow chamber, Marshall et al. (2003) found with both flow experiments and atomic force microscopy that the P-selectin/PSGL-1 couple displayed a catch bond behaviour. This conclusion was confirmed by a collaboration between E. Evans and C. Zhu (Evans et al., 2004) with the biomembrane force probe. This was interpreted as a consequence of the occurrence of two dissociation pathways for the P-selectin/PSGL-1 complex.

Studying the rate of bond formation with AFM and BFP.

Several authors attempted to estimate association rates at the single molecule level with AFM or BFP. The first quantitative report was probably authored by Hinterdorfer et al. (1996) who studied the interaction between an albumin-coated surface and an AFM tip coated with specific antibodies. They estimated the range of the interaction by subjecting the AFM tip to rapid vertical movement and slow lateral displacement, and determining the binding probability as a function of position near a surface coated with albumin at low density. Estimating that binding could occur when the tip was less than 6 nm from the surface, and assuming that the binding frequency was constant when the distance was lower than the threshold value, they could derive a tentative association constant from the binding probability. They obtained an estimate of 5 10^4 M^{-1} s^{-1} that they considered as satisfactory on the basis of reported values obtained on soluble molecules with conventional methods (although much higher values were often reported).

In their study of VE-cadherin, Baumgartner et al. (2000) estimated at 10^3-10^4 $M^{-1}s^{-1}$ the association rate of these molecules. This falls within the range reported with measurements made on cadherins with surface plasmon resonance (Syed et al., 2002).

In addition to these attempts at estimating association rates, several authors reported that the binding probability increased as a function of the duration of contact during

receptor and ligand-coated surfaces (Fritz et al., 1998).

Reliability and significance of measured parameters.

Before concluding this experimental section, we shall consider four pending problems.

Can we really measure and even define association rates ?

An association rate k_{on} may be defined by assuming that the probability that an encounter of duration dt between two molecules will result in bond formation is k_{on}.dt. There are two problems with this definition: i) since the energy landscape of the molecular complex probably involves a huge number of minima (see e.g. Ansari et al., 1985) there is no objective way of defining the region we shall call "bound". Thus, as often discussed in the description of flow chamber experiments, a particle is defined as arrested when its displacement during a time interval τ is less than a threshold distance ξ. This requires an arbitrary choice. ii) simple kinetic reasoning shows that the probability that a sufficient number of energy barriers be overcome during a molecular encounter, the probability of bond formation during a period of time dt may be proportional to a power of dt, which makes "apparent" association rates time dependent. Thus, "apparent" association rates might increase together with encounter time. This might provide an explanation for the low value of the association rate we obtained with cadherin moieties (Pierres et al, 1998b). The existence of ultrashort undetectable binding states was indeed demonstrated in recent studies (Vitte et al., 2004a ; Pierres et al., 2007).

How can we prove that we are actually measuring single bonds ?

As previously argued (Zhu et al., 2002), It is difficult to prove formally that single bonds are actually detected for several reasons:

- An argument used in many AFM studies is that when a low proportion of cycles reveal detectable binding events, only a low proportion of these events should involve multiple bonds according to Poisson's law. However, it must be emphasized that this argument holds only if it has been proved that single bonds are actually detectable.
- A more complete argument consists of comparing results obtained with serial dilutions of binding sites: if the frequency of binding events decreases as a linear function of surface receptor density, but event duration or strength remain constant, it seems reasonable to assume that single bonds are detected. Although this conclusion seems reasonable, it may be pointed out that there remain a possibility of an error: indeed, suppose only divalent attachments are detectable, and receptor solution contains a proportion of molecular aggregates (which is quite common: Vitte et al., 2004). In this case, it is possible to observe linearly decreasing frequency of identical events.
- A definitive proof would thus require to check the precise density and distribution of binding sites on surfaces (imaging mode AFM might be useful for this purpose) in order to rule out the requirement for aggregates in view of the frequency of binding events. This is by no means easy.
- A reasonable argument might be that the properties (strength or duration) of observed binding events match the expected order of magnitude on the basis of other published reports. However, while this argument also seems reasonable, the multiplicity of binding states and possibility that a combination of incomplete bonds might resemble a single full attachment makes also this argument questionable.

Thus, it must be accepted that despite adequate controls the conclusion that single bonds are studied may often be considered as reasonable, although not formally proven.

How can we relate the properties of attachments mediated by a few bonds to single-bond parameters?

As emphasized in the first section of this review, many important biological interactions are mediated by parallel bonds. An important example is antigen binding by antibodies. Indeed, antibodies are multivalent and it has long been known that the efficiency (often called "avidity") of antibody samples is often related to their capacity to form multivalent bonds. It is therefore of interest to mention recent experimental studies using dynamic force spectroscopy to analyze parallel bonds between antibodies and antigens such as mucin (Sulchek et al., 2005) or chelated uranyl (Odorico et al., 2007). Results clearly suggest

that multivalency can result in dramatic enhancement of receptor efficiency.

Is Bell's description convenient, and is it sufficiently accurate to summarize experimental data?

At the present time, Bell's law remains the standard way of **describing** bond rupture and extracting simple parameters from experimental data, as acknowledged in both experimental (Chen et al., 2001 ; Rinko et al., 2004 ; Perret et al., 2004) and theoretical (Pereverzev et al., 2005 ; Dudko et al., 2006 ; Lou et al., 2007) reports. However, it seems now well established that we must abandon the earlier view that a single pair of parameters $k_{off}(0)$ and F° can fit experimental observations in all accessible situations (Alon et al., 1995 ; Fritz et al., 1998). This is exemplified by a comparison of several parameters reported for the same molecular couple, i.e. P-selectin/PSGL1 (Table 1). The main reason for this situation stems from the multiplicity of binding states that is usually observed in studied ligand-receptor couples. There are two consequences for this situation:

- Different transitions may be observed under different situations. Indeed, it was suggested that AFM or BFP often probed transitions from more stable binding states than flow chambers (Pierres et al., 2002 & 2007). Thus, in a study of streptavidin-biotin interaction, the distance between energy barriers estimated with Bell's law on the basis of AFM or BFP data (Merkel et al., 1999 ; Yuan et al., 2000) ranged between 0.05 an 1 nm whereas the flow chamber revealed two barriers 1.3 nm apart, at some distance from the minimum energy state. Also, in a study made on C-cadherin homotypic interaction (Pierres et al., 2007), Results were consistent with the existence of two barriers 2.15 nm apart.
- Since what is called a "bound state" is in fact a mixture of different molecular states, the proportion of these isoforms may be dependent on the **history** of complex preparation in absence of equilibrium, as was demonstrated directly (Pincet et al., 2005).

Thus, describing bond rupture with Bell's law and a single set of parameters is only feasible in a limited range of experimental conditions (i.e. loading rate in AFM experiments, and shear rate in flow chamber experiments).

Table 1. Analysis of P-Selectin/PSGL1 interaction at the single molecule level

Method /Loading rate	Bell parameters		Reference
	$k_{off}(0)$ $second^{-1}$	F° pN	
Flow chamber	0.95	82	Alon et al., 1995
AFM/10^4 - 10^5 pN/s	0.022	16.6	Fritz et al., 1998
Flow chamber	2.4	104	Smith et al., 1999
AFM/$5x10^3$-$6x10^5$pN/s	0.22	30	Hanley et al., 2004
BFP/300-$3x10^4$ pN/s	0.37	18	Evans et al., 2004

This table displays estimates of Bell's parameters for PSGL1-P-Selectin interaction reported by several investigators using AFM, BFP or flow chambers.

Conclusion.

The aim of this section was to review the type of information that was obtained during the last decade when flow chambers, atomic force microscopes or biomembrane force probes were used to study bond formation and dissociation at the single molecule level.

Bond rupture. At the present time, Bell's law remains the standard way of **describing** bond rupture. However, this is more and more difficult to use since a given set of parameters (k_{off} and F°) can only be used in a limited number of conditions. Therefore, there is probably a need to determine which physiological situation is relevant to a given set of Bell's parameters. Thus, adhesion in a flow chamber operated under a wall shear rate of 100-200 s^{-1} is probably well representative of leukocyte-endothelium adhesion in blood vessels. The disruptive forces experiences by bonds in a flow chamber operated at lower shear rate may be comparable to the force required to extend microvilli, i.e. several tens of piconewtons (Shao et al., 1998). It would certainly be useful to determine which loading rate is most representative of physiological situations.

Bond formation. The most common way of **describing** bond formation between **individual** attached molecules is based on two parameters, i.e. binding range and binding frequency for two molecules maintained within

binding range (Hinterdorfer et al., 1996 ; Fritz et al., 1998 ; Pierres et al., 1998b). As previously emphasized, these parameters are likely to be highly dependent on surface structure and environmental conditions. It must be emphasized that a different approach should be used to study the rate of bond formation between surfaces that have been **aligned** by a sufficient number of bonds. In this case, the relevant formalism that has been developed for many years is the use of 2D affinity constant, and association and dissociation rates (see e.g. Zhu et al., 2007).

4 - RELATIONSHIP BETWEEN BINDING PARAMETERS AND STRUCTURE OF BINDING SITES

While a quantitative description of molecular interactions is *per se* of utmost interest to understand and model cell processes such as signalling or adhesion (Bell et al., 1984), it would be of prominent interest to relate these parameters to molecular structure. Indeed, in addition to its intellectual appeal, a reliable method of deriving interaction parameters from molecular structure would allow us to take advantage of all structural information available in data banks and avoid lengthy and delicate experiments.

There are currently to ways of relating cell interaction parameters to their structure: while a purely theoretical approach is conceivable, computer simulations are more and more widely used to illuminate the behaviour of biomolecules.

While a thorough review of both approaches would not fall into the scope of this review, it is certainly warranted to give a brief sketch of recent attempts relevant to the phenomena we studied.

Theoretical approach.

A theoretical basis for Bell's law.

The structure of a complex formed by two **rigid** macromolecules may be defined with five independent parameters (e.g. a distance and four angles). The energy landscape of the complex may thus be viewed as a hypersurface in a 6-dimensional space. The interaction between molecules may be viewed as a motion of a point defining the complex on this hypersurface. Since proteins are quite flexible (Rueda et al., 2007), the number of dimensions of the relevant space may be strongly increased.

A simplification that was considered as acceptable until a few years ago was suggested by H. Eyring (1935) who elaborated the transition state theory of reaction rates. The main assumption was that there was a *single preferred path* for a bimolecular reaction. This path was supposed to follow the bottom of a valley in the energy landscape. The reaction could thus be viewed as a 1-dimensional displacement along the energy/distance curve (Figure 3). The dissociation rate could then be calculated by using the Boltzmann velocity distribution to determine the particle flux across the energy barrier opposing the exit.

A following step was achieved by Kramers (1940), who took care of viscosity by studying the escape rate of a particle experiencing a random Langevin force as well as viscous drage in a well.

More recently, following a seminal intuition by Bell (1978), Evans and Ritchie (1997) built on these early reports to achieve a quantitative analysis of the effect of forces on particle escape (or bond rupture). Following Bell's remark that the energy U(x) becomes U - Fx in presence of force, the diffusion current J(x) may be written as:

$$J(x) = - D\, \partial c/\partial x - (Dc/k_BT)\, (dU/dx - F) \qquad (8)$$

where D is the diffusion coefficient and c(x,t) the particle concentration. Assuming a stationary state (J is constant, c only depends on x), and writing that the total number of particle (i.e. $\int c(x)\, dx$) is one, we can calculate the escape frequency 1/J. Now, assuming that **F is low enough** not to alter drastically the shape of the potential, the escape rate may be viewed as a product of four terms (Evans and Ritchie, 1997 ; Lin et al., 2007):

$$k_{off} \approx (D/l_0 l_a)\, \exp\, [(- E°_a + a\, F)/k_BT] \qquad (9)$$

D represents the particle diffusion coefficient. This is likely to be much lower than in free state due to different phenomena including hydrodynamic effects (Evans and Ritchie, 1997) and the microstructure of the energy curve (Zwanzig, 1988 ; Hyeon and Thirumalai, 2003)

l_0 represents the width (or the reciprocal of the curvature) of the energy well, and l_a represents the width of the energy barrier opposing the particle escape. Finally, the exponential term represents the escape probability that may be approximated as the sum of the zero force activation energy and the product of the force and the distance of the barrier to the minimum energy. This approximation is acceptable if the barrier is fairly steep.

Extracting barrier position from force spectroscopy data

Assuming the existence of a series of sharp barriers, Evans et al. predicted that the plot of most probable unbinding force versus the logarithm of the loading rate should appear as a sequence of linear segments with ascending slope (Evans et al., 1997 ; Merkel et al., 1999) and they concluded that the time required to cross a series of barriers was the sum of times required to pass each barrier.

$$1/k = \sum t_{off}(n) \exp(- F/F°(n)) \qquad (10)$$

Where n refers to the nth barrier. Note that this result was based on several assumptions concerning particle equilibration between barriers.

However, this simple interpretation was recently questioned for several reasons.

Even if Bell's equation is valid for the passage of a single barrier, it may be difficult to relate dissociation kinetics to the shape of the energy/distance curve. Indeed, Strunz et al. (2000) showed that even with a single intermediate barrier, different dissociation kinetics could be observed when forces were varied. Also, Derényi et al. (2004) demonstrated that when the curve contained a number N of barriers, plots of most probable unbinding force might display up to N(N+1)/2 segments.

Single path approximation.

Bartolo et al. (2002) questioned the single path assumption in a theoretical study. Interestingly, the interest of the point raised by these authors was soon supported by the experimental demonstration of the reality of catch bonds (Thomas et al., 2002 ; Marshall et al., 2003) whose rupture behaviour was found consistent with the hypothesis that there indeed were two dissociation pathways with different sensitivity to external forces (Evans et al., 2004 ; Pereverzev et al., 2005). Note that the reality of multiple pathways is also supported by molecular dynamics simulation (Martinez et al., 2005).

Reexamination of Kramers theory.

The increasing flow of experimental studies on single bond rupture was an incentive to reexamine the basis of Kramers theory, and it was an incentive to study theoretical relationships between experimental results and free energy profiles (Hummer and Szabo, 2001 & 2003 ; Dudko et al., 2003). As clearly explained in recent reports (Dudko et al., 2006 ; Lin et al., 2007), it seems now possible to state that Bell's law is an acceptable approximation when the pulling speed is low enough. However, at high pulling speed, it may happen that the force experienced by the bond is close to the maximum slope of the binding potential along the reaction coordinate. In this case, there is no longer any sharp barrier on the free energy profile and the unbinding force may be proportional to $r^{2/3}$ rather than r (r is the loading rate), following an insightful analysis from Garg (1995). Further, some important points were added.

- First, it was emphasized that the statistical distribution of unbinding forces conveyed useful information. A useful guideline (Dudko et al., 2006) is that Bell's law may no longer be applicable when the variance of the unbinding force is dependent on the loading rate. This is important since this may indicate that curvature of the F_u versus r plot may not be indicative of an actual energy barrier.
- Also, it was noticed that additional information could be obtained by varying the way of exposing bond to forces. Thus, Lin et al. (2007) concluded that a constant force experiment was better suited to obtain the activation energy of the bond, and a constant loading rate might be useful to extract the critical force corresponding to the maximal slope of the energy/distance curve. Also, Braun et al. (2005) pointed out the potential interest of periodic loading.

Conclusion.

While Bell's law remains the standard way of **describing** experimental data, much theoretical work is currently devoted to the analysis of quantitative relationship between these data and energy landscapes. The interest

of this current line of research is increased by present attempts made at taking advantage of computer simulation to predict fine aspects of molecular behaviour more and more closely related to measurable properties.

Computer simulation.

Molecular dynamics was applied for more than two decades to simulate protein behaviour, and there is much interest in the development of algorithms for predicting the capacity of synthetic molecules to interact with biologically important targets (Brooijmans and Kuntz, 2003). While the reliability of available methods has long been felt insufficient, continuous progress makes it more and more warranted to use simulations as an additional tool both to help understand and predict experimental data. Systematic ways of testing new methods such as CAPRI (critical assessment of protein interactions) markedly increased the credibility of current approaches (Schueler-Furman et al., 1995).

Thus, while Evans et al. (Evans and Ritchie, 1997 ; Merkel et al., 1999) were able to compare their experimental results on the rupture of spectravidin-biotin bonds with earlier simulations (Tavan et al., 1996 ; Izrailev et al., 97), there was a gap of several orders of magnitude between the pulling duration in simulated and actuel experiments. Recently, this difference was overcome by Zhou et al. (2006) with a so-called "hybrid simulation technique" consisting of decoupling the motion of the tip and molecular equilibration. It was thus possible to simulate realistic loading rates. Experiments suggested the occurrence of three energy barriers in accordance with the experimental report from Merkel et al. (1999). Also, the authors were able to link energy barriers to the disruption of well identified hydrogen bonds.

Thus, it is likely that computer experiment will complement more and more efficiently single molecule experiments to yield an accurate view of protein dynamics.

5 - SUMMARY

During the last decade, numerous investigators used new approaches based on atomic force microscopy, biomembrane force probes, laminar flow chamber or optical tweezers to study bond formation and dissociation between ligand and receptor molecules at the single cell level. Most studies were devoted to the monitoring of the rupture of bond subjected to a constant or steadily growing external force. The standard way of expressing results is based on Bell's hypothesis that the dissociation rate of a bond subjected to an external force F increases exponentially with respect to F.

It was initially considered that bond rupture might be viewed as the passage of a single energy barrier in a single reaction path, following a scheme elaborated by Eyring and Kramers during the first half of the twentieth century. In this case, it was felt relatively easy to extract from experimental data the spontaneous dissociation rate of a bond, as well as the height of the energy barrier and its distance to the energy minimum.

During the last years, it became more and more obvious that this standard framework was unable to account for all the complexity of actual bonds. It is likely that bond formation and dissociation may follow more than one path in the complex energy landscape, and each path involves several energy barriers. As a consequence, more work is required to draw full benefit of recent experimental advances:

i) Firstly, it would be useful to define criteria to assess the biological relevance of the multiple reaction paths that are disclosed by ultrasensitive experimental devices. This requires a quantitative analysis of the conditions of encounter between biomolecules composing living cells.

ii) Secondly, in order to rule out any ambiguity in the extraction of structural information on molecular complexes from dissociation experiments, it is necessary to improve the procedure of single bond analysis by using more appropriate and/or systematic ways of varying the force cycles.

iii) Thirdly, much effort must be done to rule out possible causes of interpretation errors in order to rule out misinterpretations due to the unexpected occurrence of multivalent attachment, or to inexact estimate of the forces actually experienced by tested bonds.

iv) Fourthly, measuring association rates probably requires extensive information on energy landscapes (as obtained by studying bond rupture), topographic distribution and environment of binding molecules as well as geometrical and mechanical properties of linkers.

It may be hoped that the improvement of computer simulation methods makes them useful to progress in the understanding of molecular interactions.

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