Kinetic Monte Carlo simulation of titin unfolding

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Recently, it has become possible to unfold a single protein molecule titin, by pulling it with an atomic-force-microscope tip. In this paper, we propose and study a stochastic kinetic model of this unfolding process. Our model assumes that each immunoglobulin domain of titin is held together by six hydrogen bonds. The external force pulls on these bonds and lowers the energy barrier that prevents the hydrogen bond from breaking; this increases the rate of bond breaking and decreases the rate of bond healing. When all six bonds are broken, the domain unfolds. Since the experiment controls the pulling rate, not the force, the latter is calculated from a wormlike chain model for the protein. In the limit of high pulling rate, this kinetic model is solved by a novel simulation method. In the limit of low pulling rate, we develop a quasiequilibrium rate theory, which is tested by simulations. The results are in agreement with the experiments: the distribution of the unfolding force and the dependence of the mean unfolding force on the pulling rate are similar to those measured. The simulations also explain why the work of the force to break bonds is less than the bond energy and why the breaking-force distribution varies from sample to sample. We suggest that one can synthesize polymers that are well described by our model and that they may have unusual mechanical properties. © *2001 American Institute of Physics.* [DOI: 10.1063/1.1369622]

I. INTRODUCTION

Many proteins and other large molecules make chemical bonds with a variety of surfaces. An adsorbed protein touched by the tip of an atomic force microscope (AFM) will sometimes bind to the tip. If this happens, the molecule can be stretched by moving the surface away from the tip. Pulled by the receding molecule, the AFM cantilever (on which the tip is located) bends (see Fig. 1). From this bending, one can calculate the pulling force (the force constant of the cantilever is known). Such experiments[1–11] determine the elongation of the molecule as a function of the applied force, for various pulling rates. One makes thus, on one molecule, the kind of measurements used to determine the plasticity of macroscopic wires.

If the molecule being pulled is the protein titin,[3,5,6,9,10] the plot of the force versus molecular elongation looks like the one shown in Fig. 2. If the load is removed the molecule folds back; pulling again generates a new saw-tooth pattern that is very similar to the initial one. This process can be repeated hundreds of times. We will argue shortly that the sudden drop in the force corresponds to the unfolding of a domain in the protein. There are many saw-teeth because the protein has many domains. We will call the maximum force in a tooth the unfolding force; the time when the drop is seen is called the unfolding time.

The saw-tooth graphs obtained by repeating the experiment are not identical: the unfolding times and the force at which unfolding take place are stochastic variables; they vary with each repetition of the experiment. The probability distribution of the unfolding force has been measured,[3] that of the unfolding time was not.

For the purpose of the present paper, titin consists of folded immunoglobulin (Ig) domains located along a string[12] (see Fig. 1). The drop of the force in the saw-tooth graph takes place when one of the Ig domains unfolds. The increase of force, following its collapse, occurs because the unfolded domains are being stretched by the force.

This interpretation is supported by several observations. The measured distance between peaks is[7] on the order of 25 to 28 nm, while the maximum length gained by unraveling an Ig domain is expected to be 31 nm.[13] An additional argument in favor of this interpretation was provided by Rief, Gautel, Oesterhelt, Fernandez, and Gaub[7] who performed this kind of experiment with recombinant titin molecules ''engineered'' to have either four or eight Ig units. In these experiments, they never observed more than four (or eight) teeth in the saw-tooth pattern, although pulling and refolding were repeated many times. In some experiments, they saw less than four (or less than eight) teeth because, presumably, the tip did not bind to the end of the molecule.

The titin unfolding experiments revealed a number of intriguing facts that a model should explain. The magnitude of the unfolding force and the work expended to induce unfolding (since one measures the force and the displacement, one can calculate the work) changes with the pulling rate. If

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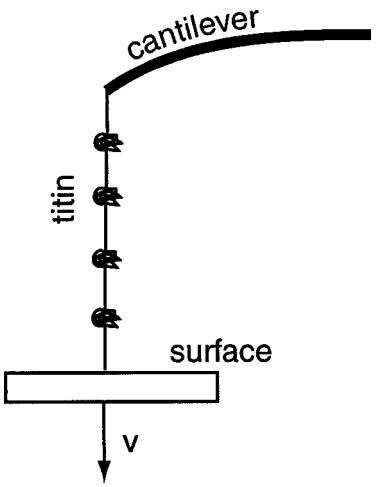

FIG. 1. A schematic representation of the protein unfolding experiment.

the unfolding of the domain takes place by breaking weak chemical bonds, one would naively expect that the work of the pulling force should be equal to the energy of the bonds being broken. The fact that the work depends on the pulling rate, and is smaller than the energy of a hydrogen bond, is a puzzle that requires an explanation.

In the experiment, the force F at which the unfolding takes place increases with the pulling rate v. The empirical relation $F=a+b\log v$ (a and b are constants) fits the experimental data. A model of the process must explain, or at least reproduce this dependence.

The unfolding forces of the Ig domains in titin, measured by different groups, are different.[6,10] Specifically, the unfolding forces measured by Rief *et al.*[6] are larger than those observed by Viani *et al.*,[10] for experiments using the same pulling rate. These experiments differ only in the number of domains in the titin molecule. Rief *et al.* studied genetically engineered titin with four or eight Ig domains, while Viani *et al.* were working with native titin, having substantially more domains. It is surprising that the force required to unfold a single Ig domain depends on the number of such domains present in the molecule. A model of the process must explain this observation also.

Important insights regarding the unfolding mechanism have been obtained from the molecular dynamics simulations performed in Schulten's group,[3,14,15] who studied the behavior of an Ig domain pulled by an externally applied force. They concluded that unfolding takes place if six hydrogen bonds, holding the protein folded, are broken. The calculations also point out that two other hydrogen bonds break prior to unfolding. Those are believed[14,15] to have a minor effect on unfolding and are neglected in the present work.

FIG. 2. A typical dependence of the stretching force on the extension of the protein titin.

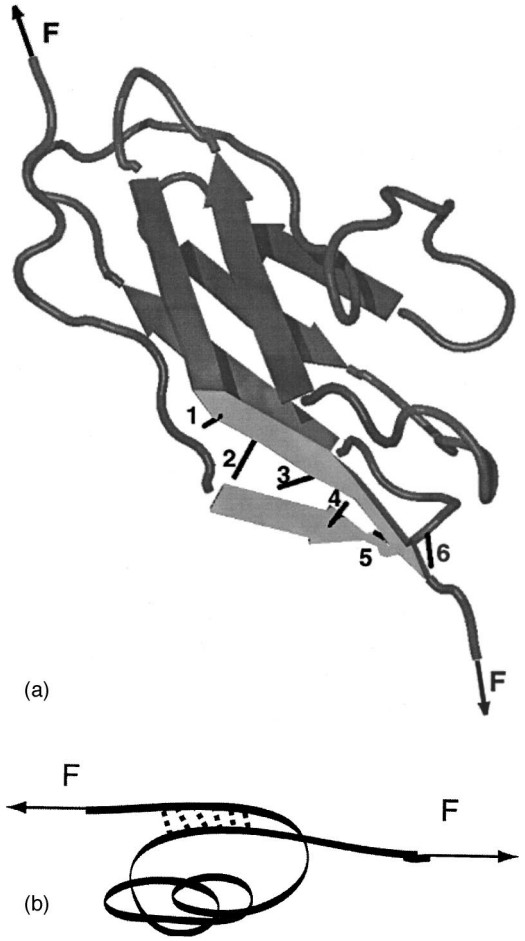

FIG. 3. (a) The structure of the Ig27 domain. Six hydrogen bonds resist sliding of two β-strands (shown in the lighter shade) with respect to one another. (b) A schematic representation of the folded Ig domain assumed in our model.

Figure 3(a) shows the experimental structure of the Ig27 domain[16] and the location of the six hydrogen bonds that resist sliding of one β-strand with respect to another and are believed to be crucial to unfolding.[14] Other hydrogen bonds are not shown. Figure 3(a) was generated by the VMD program that was developed by the Theoretical Biophysics Group in the Beckman Institute for Advanced Science and Technology at the University of Illinois at Urbana–Champaign.[17] A complete description of the hydrogen-bonding pattern of the domain can be found in Ref. 14. It is shown there that the other hydrogen bonds between various β-strands break rapidly once the bonds indicated in Fig. 3(a) are broken.

It is often pointed out that the pulling rate in molecular dynamics simulations is six to eight orders of magnitude larger than the one achieved in the laboratory. It is likely that this affects the unfolding process. However, it is improbable that the conclusion that unfolding is caused by breaking hydrogen bonds is an artifact due to the large pulling rate. For this reason, we include this feature in our kinetic model of the unfolding process.

The simulations also provide information about the energies controlling the unfolding of the molecule under load. We are not using this information here for two reasons. First,

we are not sure that the potential energy used in the simulation has sufficient accuracy. Second, as pointed by Paci and Karplus,[18] Schulten's simulations use a large number of water molecules which form a ''droplet'' around the protein. When the Ig domains unfold this droplet changes shape and this costs a certain amount of free energy. In the simulation this is part of the unfolding energy. In the experiments, the protein is surrounded by bulk water and this effect is absent.

Before moving on, we point out that not all proteins unfold because hydrogen bonds are broken. Paci and Karplus[18] find that the plastic response of fibronectin type III domains is controlled by van der Waals interactions.

We also mention that molecular dynamics simulations have been performed to examine unfolding of other proteins. Bryant, Pande, and Rokhsar,[19] for example, studied unfolding of a β-hairpin forming polypeptide. The requirement that the simulation time should be short forces one to perform the simulation at temperatures or pulling forces that are much higher than the ones used in experiment. The kinetic Monte Carlo approach allows one to access longer time scales. In the context of protein unfolding, it has so far been only applied to a lattice model.[20]

In this paper we propose and examine a kinetic model of titin unfolding under the influence of an external force. We assume that the folded Ig domain [Fig. 3(a)] can be schematically represented as shown in Fig. 3(b), where the polypeptide chain is held together by six hydrogen bonds. The potential energy along the reaction coordinate for each individual hydrogen bond is represented by a double well. The external force, pulling along the reaction coordinate, distorts the double well potential and affects the barriers to bond breaking and reforming; the rate of bond breaking is enhanced and that of bond forming is diminished. Thus pulling enhances the net rate of bond breaking. When all the bonds are broken the domain unfolds. To simulate the experimental observations we need to examine the unfolding of many Ig domains, taking place independently of each other.

Since we are interested in kinetic events taking place in a single molecule, we cannot use the standard kinetic equations, which deal with the evolution of the concentration of an ensemble of molecules. A probabilistic treatment is needed which calculates the probability that a domain unfolds at a given time, when the pulling force reaches a certain value. The outcome of the calculation should be the probability distribution of the unfolding times and that of the unfolding force.

The model uses rate equations for the probability that a bond is broken or is in place. In principle one could simulate unfolding by solving these differential equations and using the resulting probabilities in a Monte Carlo program that makes or breaks bonds. This procedure would require an excessive amount of computer time because the time step used in solving the differential equations is at least eight orders of magnitude smaller than the unfolding time. For this reason we had to develop new simulation methods that are described in Sec. III. Even though these methods are very efficient, they still cannot cope with the case when the pulling rate is very low. For this situation we develop a quasi-equilibrium approximation, which is described in Sec. V.

We find that the model reproduces all experimental observations and provides insights into the unfolding kinetics. Unfortunately, this does not mean that the mechanism proposed here must be valid. Its validation can only come from detailed, reliable quantum mechanical calculations and from further experiments. It is however possible to synthesize linear polymers that have a few side groups that can bind to each other. It is likely that when the polymer is left alone the side groups will accidentally come in contact and form bonds. If these bonds are weaker than those holding the chain together, the folded polymer will come apart when pulled by an external force. The behavior of this unfolding will be described by the model studied here. Such materials would have interesting mechanical properties.

II. THE MODEL

A. Bond breaking and recombination

In our model, we assume that Ig-like domains are attached to (and connected by) soft springs that represent the rest of the titin molecule including the already unfolded domains. A folded Ig domain is held in place by $N=6$ hydrogen bonds. For lack of more detailed information we assume that all these bonds are identical. In reality, the force that stretches each bond will depend on the angle it forms relative to the pulling force and the structure of the protein backbone. These angles are themselves dependent on the pulling force as the protein is deformed under the load. In our simplified model, we assume that each bond is subject to a force

$$f=F/n, \tag{1}$$

where F is the total pulling force and n is the number of bonds that have not been broken. The force per bond increases every time a bond breaks. At present, we do not have enough microscopic information about the Ig domains to assess the validity of these assumptions. We therefore use the simplest possible model Eq. (1). However, by experimenting with bead and spring models we found that the force on the surviving bonds grows substantially whenever one of the bonds is broken. If detailed molecular simulations will provide better information about the forces acting on each bond and the rate of each bond breaking, they can be easily included in the model.

The potential for each bond is a double well modified by the applied force:

$$V(r)=D_1\{1-\exp[-a_1(r-r_1)]\}^2 + D_2\{1-\exp[a_2(r-r_2)]\}^2-fr. \tag{2}$$

Here r is the ''reaction coordinate'' along which the bond breaks, $D_1=0.35\,\mathrm{eV}$, $D_2=0.19\,\mathrm{eV}$, $a_1=a_2=1.5\,\mathrm{bohr}^{-1}$, $r_1=0$, $r_2=4$ bohr. The term $f\,r$ describes the effect of the stretching force f. This potential is shown in Fig. 4, in the absence (solid line) and the presence (dashed line) of the force. The right well corresponds to a broken hydrogen bond, and the left well to an intact bond. As the fragments produced by breaking a bond are held close to one another by the remaining bonds, there is a possibility that a broken bond reforms. This event is represented here by a transition from the right well to the left well. At zero force, the state in

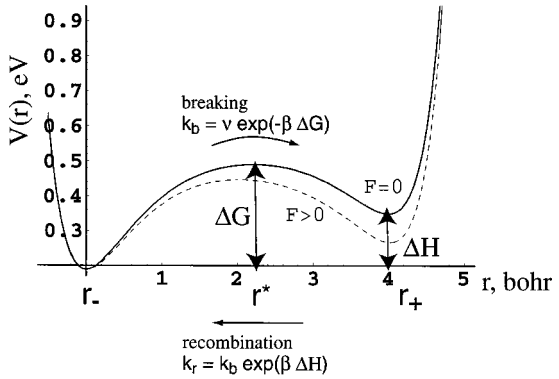

FIG. 4. The potential for a single hydrogen bond [Eq. (2)] in the absence of the force (solid line) and in the presence of a stretching force (dashed line). It is assumed in Eq. (2) that the component of the stretching force that acts along the hydrogen bond is the same as *f*.

which the bond is broken has a higher energy than the bonded state. Stretching the bond lowers the energy barrier for bond breaking and increases the stability of the broken-bond state. As the force is increased the broken-bond state will eventually become energetically more favorable and the energy barrier preventing bond breaking will eventually disappear. This does not mean that the bond breaks when the external force eliminates the barrier. Thermal fluctuations can cause bond rupture before this situation is reached. The effect of these fluctuations is important and is included in the stochastic kinetic model developed here.

The rate constant for the bond breaking, i.e., a transition from left to right in Fig. 4, is given by the Arrhenius expression:

$$k_b(f) = \nu \exp[-\Delta G(f)/k_B T]. \tag{3}$$

The recombination rate k_r is related to k_b via detailed balance

$$k_r(f) = k_b \exp[\Delta H(f)/k_B T], \tag{4}$$

where $\Delta G(f) = V(r^*) - V(r_-)$, and $\Delta H(f) = V(r_+) - V(r_-)$ (see Fig. 4). Here r_- and r_+ are, respectively, the coordinates of the left and right minima, and r^* is the coordinate of the maximum of $V(r)$. In our simulations we choose the value $\nu = 10^{12}\,\mathrm{s}^{-1}$ for the pre-exponential in Eq. (3). This is a typical value for unimolecular reactions.

A single Ig domain unfolds when all N bonds are broken. At zero force, this is a very unlikely process because breaking a bond is energetically unfavorable and any broken bond will be likely to recombine before the other $N-1$ links get a chance to break. This is why the spontaneous unfolding of a domain does not occur on the experimental time scale despite the fact that, according to Eq. (3), it takes less than a microsecond to break one bond.

B. The time dependence of the force $F(t)$

Because the molecule is stretched by moving the surface, with uniform speed, away from the cantilever (see Fig. 1), the force per bond $f = F(t)/n(t)$ is time dependent. Here $n(t)$ is the number of bonds in the domain at time t and $F(t)$ is the total stretching force at the same time. To simulate the breaking of the Ig domains we need to know the time dependence of $F(t)$. In the experiment this is being measured. In our simulation we follow Rief *et al.*[6,21] and use the wormlike chain model,[22–24] which gives the force $g(x_p)$ needed for stretching the chain a length x_p:

$$g(x_p) = (k_B T/p)[0.25(1 - x_p/L)^{-2} - 0.25 + x_p/L]. \tag{5}$$

Here p is the persistence length, chosen to be[21] $p = 4$ Å. The polymer's length is $L = 58$ nm (Ref. 21) when all Ig domains are folded. In our model, this is incremented by $\Delta L = 28$ nm every time one of the domains unfolds. We emphasize that the purpose of our model is to generate the correct breaking time and breaking force distribution not the raising part of the saw-tooth pattern, which is represented by Eq. (5). We could have used a fit to the measured force-displacement curve, instead of Eq. (5). However, since Rief *et al.* found that Eq. (5), with the parameters indicated above, provides a good fit, we decided to use this expression. It is not clear that the agreement between Eq. (5) and measurements is physically meaningful. The experiments stretch the whole molecule, consisting of folded domains and the chains connecting them (which includes the unfolded domains). One could imagine that the folded domains are rigid and that the chains connecting them behave like wormlike chains. There is however no hard evidence that this interpretation is correct. Perhaps a more realistic representation of the force is provided by a recent model proposed by Erickson[25] which accounts for the fact that different parts of the molecule have different elastic properties. However, we have not studied this model here. We feel that since the present model fits the data well, not much would be gained by introducing features with more parameters, unless new experimental details become available.

We encounter here a slight complication. The experiment does not measure the amount $x_p(t)$ by which the polymer is stretched at time t. This has to be calculated from the equation

$$x_p = vt - x_c. \tag{6}$$

Here v is the velocity of the surface (moving away from the cantilever) and x_c is the displacement of the cantilever. The latter can be calculated from

$$x_c(t) = F(t)/k_c, \tag{7}$$

where $k_c = 0.06$ N/m is the cantilever force constant. Combining (6) and (7) we can express x_p as a function of $F(t)$. Inserting this expression in Eq. (5) gives an implicit equation for the force:

$$F(t) = (k_B T/p)[0.25(1 - (vt - F(t)/k_c)/L)^{-2} - 0.25 + (vt - F(t)/k_c)/L]. \tag{8}$$

This expression is used to calculate the force per bond [Eq. (1)], which is then used in Eq. (2) to calculate the barriers to bond breaking and bond forming. These in turn go in Eqs. (3) and (4) to give the rate constants for bond breaking and bond forming. These rate constants depend on time through $F(t)$ and the number $n(t)$ of bonds in place at time t. We

have therefore all the information needed to simulate the stochastic single molecule kinetics for the unfolding caused by surface displacement.

III. MONTE CARLO SIMULATION OF UNFOLDING

A. A bootstrapping approach to simulate unfolding of many Ig domains

A brute-force simulation of a protein with many domains would simulate, in parallel, the state of all the bonds in the protein. However, there is every reason to believe that the domains evolve independently of each other. Therefore, if we know the statistical properties of one domain unfolding, we could use them to simulate the unfolding of M domains. Let $S(0,t)$ be the survival probability for one domain, that is, the probability that the domain has not unfolded by the time t, in an experiment in which pulling started at time 0. The survival probability for M domains $\sigma_M(0,t)$ (that is, the probability than none of them has unfolded by the time t) is a product of the survival probabilities for each domain, i.e.,

$$\sigma_M(0,t)=\{S(0,t)\}^M. \tag{9}$$

The probability that one of the domains will unfold at a time between t and $t+dt$ is given by

$$\begin{aligned}\pi_M(t)dt&=-(d\sigma_M(0,t)/dt)dt\\&=-M\{S(0,t)\}^{M-1}(dS(0,t)/dt)dt.\end{aligned} \tag{10}$$

The quantity $\pi_M(t)$ is the unfolding time distribution for a system of M Ig domains. Given $\sigma_M(0,t)$, we can generate a time t_1 at which one of the domains unfolds[26] by solving the equation:

$$\sigma_M(0,t_1)=\xi. \tag{11}$$

Here ξ is a uniform random number between 0 and 1. It does not matter which of the domains unfolds, since all of them are equivalent. At the time t_1 immediately after unfolding takes place, the length of the protein is increased and becomes $L+\Delta L$, which means that the force (8) drops. We now have $M-1$ domains and so we compute the survival probability $\sigma_{M-1}(t_1,t_2)=\{S(t_1,t_2)\}^{M-1}$ that no unfolding event takes place between t_1 and t_2. We then generate the unfolding time t_2 according to this probability and so on. This procedure is repeated until all M domains are unfolded. It is clear from this discussion that all we need to know to simulate the unfolding of many Ig domains is the probability $S(t_1,t_2)$ for one domain to survive by the time t_2, if pulling started at time t_1.

B. Unfolding a single Ig domain by a Monte Carlo method

For the purpose of the present simulations the state of a domain at time t is specified by the number of bonds $n(t)$ that are intact at that time. Since the total number of bonds in a domain is N, the number of broken bonds is $N-n$. The kinetics of unfolding is described by the function $n(t)$; unfolding occurs at the first time at which $n(t)=0$. Since we are dealing with a single molecule, the kinetic equations provide us with probabilities: for a given value of n there is a certain probability that a bond reforms and n goes into $n+1$, and a given probability that the bond breaks and n goes to $n-1$. Therefore, the unfolding kinetics for a single molecule can be described as a random walk of the variable $n(t)$. In what follows we describe the properties of this random walk and arrive at an efficient Monte Carlo algorithm for simulating it.

Suppose that at time t the Ig domain contains n bonds. The probability that one bond is broken between t and $t+dt$ is $p_b(t)dt=nk_b[F(t)/n]dt$. In this formula, $k_b[F(t)/n]$ is the rate constant for bond breaking; the notation indicates that this ''constant'' depends on time through the force per bond $F(t)/n(t)$. The probability that one of the broken bonds will reform is $p_r(t)dt=(N-n)k_r[F(t)/n]dt$. If at time t the domain has n bonds then at the time $t+dt$ the number of bonds will be $n-1$ [with the probability $p_b(t)dt$], $n+1$ [with the probability $p_r(t)dt$], or n [with the probability $1-p_b(t)dt-p_r(t)dt$].

These three probabilities are sufficient for generating the random walk of the variable n. The conventional procedure would be to start at time $t=0$ with the domain in the state n. We will discuss later how the initial state is chosen. We increase the time to dt and calculate the rate constants k_r and k_b at that time (using the value of the force at that time). Then we imagine laying down the three probabilities $p_b(t)dt$, $p_r(t)dt$, and $1-p_b(t)dt-p_r(t)dt$ as segments on a line of length one. Then we draw a uniform random number ξ between zero and one. If ξ falls on the segment $p_b(t)dt$ we increase $n(t)$ by one; if it lands on the segment $p_r(t)dt$ we decrease $n(t)$ by one; if ξ falls on the segment $1-p_b(t)dt-p_r(t)dt$ we leave $n(t)$ unchanged. If n is not changed we increase the time by dt, calculate the rate constants k_r and k_b at time $2\,dt$, and repeat the procedure. If n has been changed we change the force per bond ($f=F/n$) and recalculate k_r and k_b for the new force. After this we use the three probabilities to generate another event: n increased or n decreased, or n unchanged. This is repeated until $n=0$ for the first time.

This ''conventional'' algorithm forces us to use small time steps and is inefficient. The inefficiency appears because for a large number of time steps n will not change and we are wasting computer time testing whether a change should be performed or not. It would be much better if we could directly generate the time when n changes, together with an indication of the type of change. This can be done as follows. Suppose that at time $t=0$ we start the simulation with n bonds. We refer to either bond breaking or recombination as an event. The time t_1 when the first event occurs can be determined[26] by solving the equation

$$A(0,t_1)=\xi, \tag{12}$$

where ξ is a uniform random number between 0 and 1, and $A(0,t)$ is the survival probability of the state n, i.e., the probability that no event has occurred between 0 and t. This quantity should not be confused with $S(0,t)$, the survival probability of the domain.

To calculate $A(0,t)$, we note that the probability that the first event (after the simulation has started) will happen between t and $t+dt$ is equal to the probability $A(0,t)$ that no

event took place before t, times the probability that an event happens between t and dt. The latter is equal to

$$p_b(t)dt+p_r(t)dt=nk_b[F(t)/n]dt + (N-n)k_r[F(t)/n]dt.$$

The survival probability therefore satisfies the equation

$$dA(0,t)=-A(0,t)\{nk_b[F(t)/n]dt + (N-n)k_r[F(t)/n]\}dt. \quad (13a)$$

This gives

$$A(0,t)=\exp\left\{-\int_0^t dt'[nk_b(F(t')/n) + (N-n)k_r(F(t')/n)]\right\}. \quad (13b)$$

At first glance, using Eq. (13b) to compute the time of the next event has no advantage over the ''conventional'' algorithm. The transition rates are time dependent and the integral in Eq. (13b) is evaluated numerically. When the integral is calculated the time interval $(0,t)$ is divided into small time steps. Denote by h_1 the time step in the conventional algorithm and by h_2 the time step required by the integration routine in Eq. (13b). The conventional algorithm is accurate if h_1 satisfies the inequalities $k_b h_1 \ll 1$ and $k_r h_1 \ll 1$. The integration in Eq. (13b) is accurate if the time step h_2 is small compared to the time during which the rate constants change significantly. That is, h_2 is set by the time scale on which the time-dependent force $F(t)$ changes, rather than the time scale of bond breaking/recombination. Roughly speaking, h_2 is much smaller than the unfolding time for the entire Ig domain and much larger than $\max\{1/k_b,1/k_r\}$, which is the lifetime of a state n of the domain. For the parameters adopted here, $\max\{1/k_b,1/k_r\}$ is on the order of microseconds or shorter, while the time scale of unfolding in a typical experiment ranges from milliseconds to seconds. Therefore, h_2 is at least three orders of magnitude larger than h_1. We find that it takes more than 10^7 bond breaking, bond forming events before the Ig domain unfolds; the time it takes to break one bond is often so much shorter than the unfolding time, that one can get away with only one time step in evaluating Eq. (13b) [i.e., one can take $F(t)$ to be a constant].

We summarize now the more efficient algorithm used here.

(1) Generate a random number ξ and solve Eq. (12), with $A(0,t)$ given by Eq. (13b). This gives the time t_1 when an event takes place.
(2) Generate a new uniform random number $0<\zeta<1$. If $\zeta < nk_b(t_1)/\{nk_b(t_1)+(N-n)k_r(t_1)\}$ then remove one bond; otherwise increment n by one. Thus, we decide whether the event at t_1 is a bond breaking or a bond formation, with probabilities proportional to $n(t_1)k_b(t_1)$ and $(N-n(t_1))k_r(t_1)$, respectively.
(3) Since n has changed at t_1, recalculate the force per bond, and the rate constants $k_r[F(t_1)/n(t_1)]$ and $k_b[F(t_1)/n(t_1)]$.
(4) Generate a new random number ξ and solve $A(t_1,t_2)=\xi$ to find the time t_2 when the next event takes place. $A(t_1,t_2)$ is given by Eq. (13b) with the integration from t_1 to t_2.

Repeat steps (2), etc. This iteration goes on until $n=0$ (the domain has unfolded).

C. The initial condition

We need now to explain how we chose the initial state of the domain. At $t=0$, when no force acts on the protein, we assume that the Ig domain is in thermal equilibrium. The probability p_n of having n bonds at $t=0$ is then determined by using the detailed balance equations:

$$p_n nk_b(0)=p_{n-1}(N-n+1)k_r(0), \quad n=2,...,N. \quad (14)$$

The left-hand side of Eq. (14) is the probability, per unit time, of breaking of one of the n existing bonds, thereby reducing n by 1. The right-hand side is the probability of adding one more bond to the existing $n-1$ bonds. Now we assume that the time it takes the domain to equilibrate is much shorter than the time it takes to unfold. Therefore we can neglect the (small) probability of unfolding so that the probabilities in Eq. (14) satisfy the normalization condition $p_1+p_2+\ldots+p_N=1$. Equations (14) together with the normalization condition can be solved to obtain the equilibrium probabilities p_n. In each Monte Carlo run we select the initial number of bonds, n, with the probability p_n.

D. The simulation of many domains revisited

According to the arguments given in Sec. III A, the time t_1 when the first domain unfolds is a solution to Eqs. (9) and (11). This means that we need the single-domain survival probability $S(0,t)$. The straightforward procedure for calculating $S(0,t)$ would be to simulate unfolding of a single domain in a large number of Monte Carlo runs and make a histogram of the unfolding times $\{t_j\}$. The resulting data will be noisy so that the histogram of the unfolding times would have to be fitted by some smooth function to obtain $S(0,t)$. Such a procedure would be inconvenient and cumbersome. It would be desirable to pick the unfolding times of the domain from the array $\{t_j\}$, with probabilities satisfying Eq. (11), without having to fit the distribution of $\{t_j\}$ by any smooth function. The probability distribution of $\{t_j\}$ is, by definition, $p_t(t)=-dS(0,t)/dt$. However, we want to pick times from the array $\{t_j\}$ but in such a way that they have the distribution given by Eq. (10). This means that some elements of the array $\{t_j\}$ would be more likely to be selected and some less. Here is how this can be done. Denote by $\nu(t)$ the number of elements in the list $\{t_j\}$ that are larger than t. If N_t is the number of elements in the list $\{t_j\}$, we have

$$S(0,t)\approx \nu(t)/N_t. \quad (15)$$

When N_t is very large this equation gives a very good approximation to the survival probability $S(0,t)$ of a domain.

The time when a domain unfolds can be calculated from Eq. (11). When we use Eq. (15) this becomes

$$\nu(t)/N_t=\xi^{1/M}, \quad (16)$$

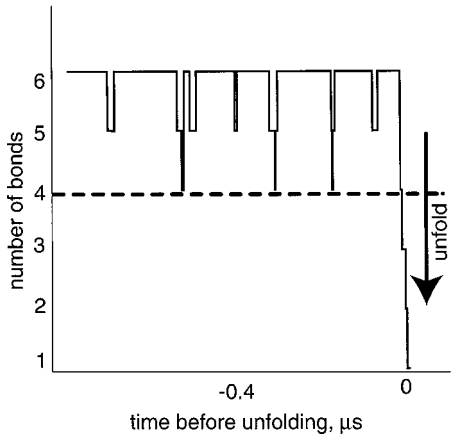

FIG. 5. The number of bonds in the domain as a function of time generated in one of the Monte Carlo runs for the last few microseconds before the domain unfolds. The pulling rate is 1 μm/s.

where ξ is a uniform random number between zero and one and M is the number of Ig domains in the protein. This equation is very easy to solve. If we sort the array $\{t_j\}$ in order of decreasing times, then the time satisfying Eq. (16) is the $N_t(\xi)^{1/M}$-th element in the sorted list. This gives the time t_1 when the first domain unfolds.

To generate the time t_2 when the second domain unfolds we increase the contour length L in Eq. (8) by $\Delta L = 28$ nm, the length of the unfolded segment.[6] We then proceed to generate a list of unfolding events for a single domain using the new force-extension curve, Eq. (8), and generate a new unfolding time as described above. This is repeated until all domains unfold.

We have described above an efficient procedure for performing Monte Carlo simulations of the kinetics of unfolding. Unfortunately, in spite of all the improvements made here the method is not sufficiently efficient to perform simulations in the case when the pulling rate is very slow. To deal with this regime we develop an approximate quasiequilibrium theory, which is accurate at low pulling rates. This is described in Sec. V.

IV. SIMULATION RESULTS

In Fig. 5, we show the time evolution of the number of bonds $n(t)$ in a single domain, for the last few microseconds before the domain unfolds. We note here an important effect, which is used in developing the quasiequilibrium theory of force induced unfolding (Sec. V). In this particular run the number of bonds fluctuates but never goes below four. As soon as n becomes less than four, the domain unfolds almost instantaneously. We have observed this in all simulations: when the number of bonds drops below a critical number the folded domain is no longer stable and unravels very rapidly. We will use this feature to develop a quasiequilibrium model in Sec. V.

By repeating such a simulation 5000 times and recording the time at which the last bond breaks we obtain a histogram that gives us the probability distribution of the domain unfolding time, $p_t(t)$. The latter is related to the domain survival probability, $S(0,t)$, via $p_t(t) = -dS(0,t)/dt$. In Fig. 6(a) we plot $p_t(t)$ for a pulling rate of 1 μm/s.

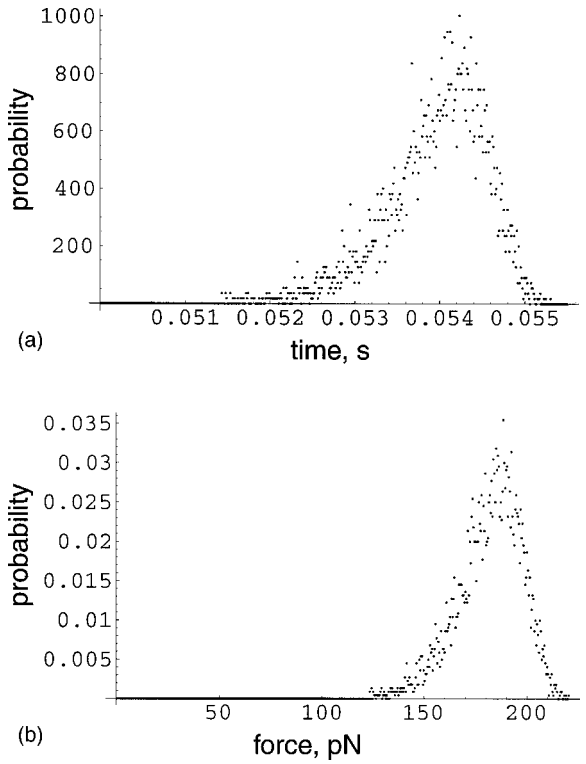

FIG. 6. The unfolding (a) time and (b) force distribution for a pulling rate of $v = 1$ μm/s.

Figure 6(b) shows the histogram of the unfolding force, which is the unfolding force distribution $p_F(F)$. It is interesting to note that the unfolding force can vary in a relatively broad range, from 140 pN to 220 pN, while the unfolding time distribution $p_t(t)$ in Fig. 6(a) has a narrow maximum at $t = 54$ ms with a width of about 2 ms. Since the force is a function of time [through the Eq. (8)] the two distributions are related through

$$p_F(F) = p_t(t)/|F'(t)|. \tag{17}$$

It so happens that the breaking time in these calculations is such that for a small change in time, Eq. (8) gives a large change in the force. This means that small fluctuations around the mean value of breaking time lead to large fluctuations in the breaking force around its mean value. This is the reason why the distribution $p_F(F)$ is broader than $p_t(t)$.

Figure 7 shows the dependence of the mean unfolding force on the pulling rate. The filled circles are the data of Rief *et al.*[6] and the empty squares are the results of Viani *et al.*[10] Both experiments were performed with titin. Viani *et al.* worked with a native titin while Rief *et al.* used a genetically engineered titin molecule that contained a small number (4 or 8) of Ig domains. We will assume in what follows that the properties of a single domain, and therefore the domain survival probability $S(0,t)$, were the same in both experiments. Therefore, the difference in the distributions of the unfolding force in the two experiments must be due to the difference in the number of domains. To verify this conclusion we have performed simulations for a mol-

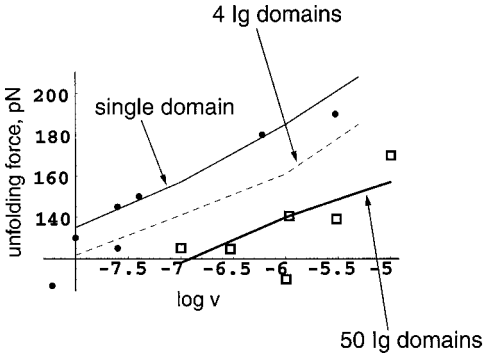

FIG. 7. Mean unfolding force as a function of the pulling rate for different values of the Ig domains in the chain. Also shown are the experimental data. Filled circles: Ref. 6. Empty squares: Ref. 10.

ecule that contains $M=1$, 4, and 50 Ig domains. Figure 8 shows a typical force-extension curve generated in the course of a simulation of the unfolding of four Ig domains. This is very similar to the experimentally observed dependences. For low pulling rates ($v<0.1\ \mu$m/s), the Monte Carlo simulation becomes slow so we used the quasiequilibrium unfolding theory of Sec. V instead. The results are also plotted in Fig. 7. The exact number of Ig domains in the experiments of Viani *et al.*[10] is not known (and it may vary from sample to sample) because the AFM tip does not necessarily bind to the end of the titin molecule. In our simulations we chose $M=50$. Since the dependence of the results on M is not strong, the uncertainty in M does not affect the conclusions that follow. It is seen from Fig. 7 that the effect of the number of domains M on the mean unfolding force is consistent with the trend that is seen experimentally. While the agreement between simulation and experiments is not perfect, the trend is clearly displayed.

It is easy to understand how the number of domains affects the mean unfolding force. Because the domains unfold independently of each other, the first unfolding in a molecule with many domains will take place earlier than in a molecule with few domains. If the unfolding time is earlier, the unfolding force given by Eq. (8) will be smaller.

V. QUASIEQUILIBRIUM THEORY

A. General theory

If the pulling rate is low, it takes a long time to develop a sizeable pulling force and the time required to unfold a domain becomes very long. During this long period the bonds in the system break and reform many times. We find that when the pulling rate is less than 0.1 μm/s, the computer time needed by the Monte Carlo simulation for generating so many bond breaking or forming events becomes prohibitive. Unfortunately, these are the pulling rates used currently by experiments. Here we propose a quasiequilibrium theory that provides an ''analytical'' solution for the case when the pulling rate is low.

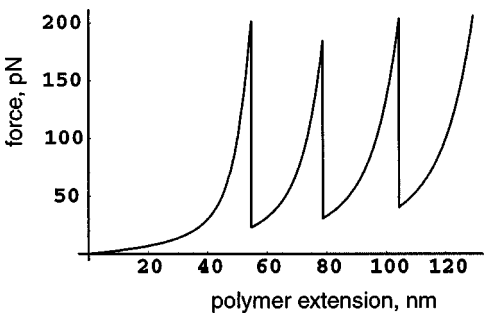

FIG. 8. A typical force-extension curve generated for $M=4$ in one of the Monte Carlo runs.

Besides providing a very efficient algorithm the quasiequilibrium theory is conceptually important. Rief *et al.*[21] have shown that their experimental data could be fitted by a phenomenological, two-well model, in which force-induced unfolding is characterized by a time-dependent rate. This suggests that, for the pulling forces used by these experiments, our model should reduce to an effective two-well model. The quasiequilibrium theory shows that this reduction does take place and allows us to interpret the effective rate constant in the two-well model in terms of the bond breaking and bond forming rates used in the present work.

To explain the quasiequilibrium model we start by mentioning three properties of the random walk of the variable $n(t)$, established by our simulations.

(1) The random walk of the variable $n(t)$ ''forgets'' the initial number of bonds, $n(0)$, on a time scale much shorter than the unfolding time. The survival probability $S(0,t)$ is independent of the initial value of n, as long as we do not start with $n=1$. If we start with $n=1$, the simulation generates a large spike in $p_t(t)$, at t close to 0, because there is a nonzero probability that the domain unfolds immediately after starting the simulation, by breaking its last bond.

(2) The unfolding of a domain (i.e., the breaking of the last bond) is a rare event; a huge number of changes in $n(t)$ take place before the domain unfolds.

(3) By examining the last few microseconds prior to the time when the domain unfolds (such as in Fig. 5) we find that there is a critical number $n^{\#}$ of bonds with the following property: as soon as $n(t)<n^{\#}$, the domain will unfold practically instantaneously. An example of such a rapid unfolding is shown in Fig. 5, where $n^{\#}$ is equal to 4. Such spontaneous unfolding is not hard to understand qualitatively: each time one of the n bonds is broken, the force per bond increases from F/n to $F/(n-1)$. At some point this has an ''autocatalytic'' effect and the bonds are broken ''explosively.''

Properties (1) and (2) suggest that the dynamics of the domain can be described in terms of a probability $p_n(t)$ to have n bonds at time t. Probabilities $p_n(t)$ are time dependent because the transition rates from n to $n\pm1$ change in time. If the force were time independent, $p_n(t)$ would be constant and equal to the equilibrium probability p_n that is obtained by solving Eq. (14). If the pulling rate is small, the force changes slowly in time and we can assume that $p_n(t)$ is in equilibrium with the value of the force at time t. For the calculation of rate, we can use equilibrium populations, which we calculate from the detailed balance

$$p_n(t)nk_b[F(t)/n(t)]=p_{n-1}(t)(N-n+1)k_r[F(t)/(n-1)],\quad n=2,...,N \tag{18}$$

together with the normalization condition.

Property (3) suggests that, in the spirit of transition state theory, the unfolding rate is

$$k_{\mathrm{eff}}(t)=p_{n^{\#}(t)}k_b[F(t)/n^{\#}(t)]n^{\#}. \tag{19}$$

To unfold, $n(t)$ needs to reach $n^{\#}$ (the probability of having $n^{\#}$ bonds is $p_{n^{\#}(t)}$) and break one of the $n^{\#}$ bonds (the rate for this is the rate constant $k_b[F(t)/n^{\#}(t)]$ times the number of bonds).

To complete the theory we must provide a recipe for calculating $n^{\#}$. If we knew it, the effective rate would be given by Eq. (19). If we substitute $n^{\#}=1,2,...,6$ into Eq. (19) we can calculate six effective rate constants k_{eff}, one for each value of $n^{\#}$. We can use now the fact that the rate constant calculated by assuming that if $n<n^{\#}(t)$ the domain unfolds, is an upper bound for the exact rate constant. This means that, like in the variational transition state theory, the effective rate constant is the smallest value among the six rate constants:

$$k_{\mathrm{eff}}(t)=\min_n[p_n(t)k_b[F(t)/n]n]. \tag{20}$$

Given the effective rate $k_{\mathrm{eff}}(t)$ it is easy to determine the probability distribution of the unfolding time: the survival probability of the domain is given by

$$S(0,t)=\exp\left[-\int_0^t dt'\,k_{\mathrm{eff}}(t')\right]. \tag{21}$$

The probability distribution of the unfolding time is

$$p_t(t)=-dS(0,t)/dt=k_{\mathrm{eff}}(t)\exp\left[-\int_0^t dt'\,k_{\mathrm{eff}}(t')\right]. \tag{22}$$

We plot this distribution (solid line), along with the results of a full Monte Carlo simulation, in Fig. 9(a), for a pulling rate of $v=5\ \mu$m/s. In Fig. 9(b), the unfolding force distribution predicted by Eqs. (20), (22), and (17) is compared to that generated by a full Monte Carlo simulation. The agreement between the quasiequilibrium theory and the simulations is excellent. We have tested the quasiequilibrium theory for pulling rates in the range $10\ \mu\mathrm{m/s}\geqslant v\geqslant 0.1\ \mu$m/s and found good agreement in all cases.

B. The critical number of bonds $n^{\#}$ as a function of force F

The critical number of bonds $n^{\#}$, for which the minimum in Eq. (20) is achieved, is also a function of the force $F(t)$ (and therefore a function of time). In Fig. 10 we plot $n^{\#}$ as a function of the force F. These values have been calculated by the procedure described above for calculating the effective rate constant in the quasiequilibrium theory. They are valid only if the pulling rate is small enough for the quasiequilibrium theory to be valid. Our simulations indicate however that the trend is general: as the force increases $n^{\#}$ becomes larger.

This sheds some light on the results of Lu and Schulten.[15] Since they use extremely large pulling rates, the pulling force becomes large before the protein has a chance to unfold. Our calculations suggests that in their experiments

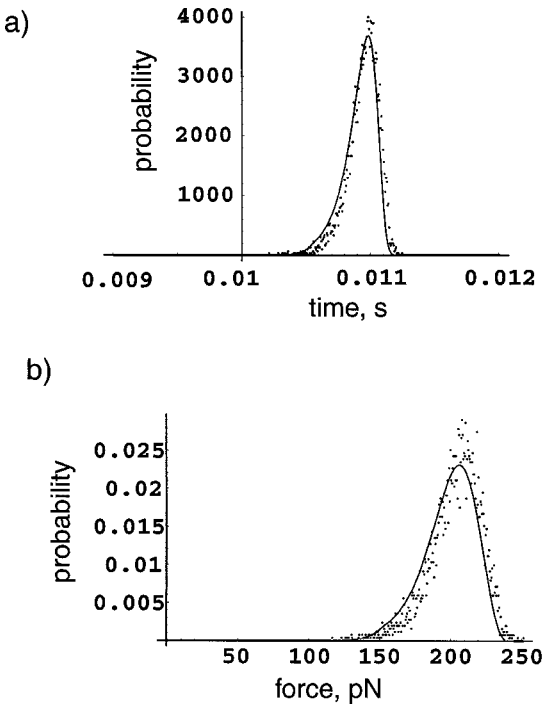

FIG. 9. The unfolding (a) time and (b) force distribution for a pulling rate of 5 μm/s. The solid lines are obtained using the quasiequilibrium theory [Eqs. (20) and (22)].

$n^{\#}$ is likely to be equal to 6. This means that all the hydrogen bonds will break simultaneously, which is what they observe in the simulations. Our model also implies that this would not be the case had the pulling rate been smaller.

C. The case of a low pulling rate (effective two-well model)

When the force is low enough the critical number of bonds is $n^{\#}=1$. This means that all bonds have to be broken, in a stepwise fashion and the ''catastrophic'' breaking of several bonds in a very short time will not be observed. This is probably what happens at the low pulling rates used by Reif *et al.*[21] If this is the case, the unfolding rate is the probability of having one bond left multiplied by the rate of breaking it:

$$k_{\mathrm{eff}}(t)=p_1(t)k_b(F(t)). \tag{23}$$

If we solve Eq. (18) and use Eqs. (3) and (4), we get

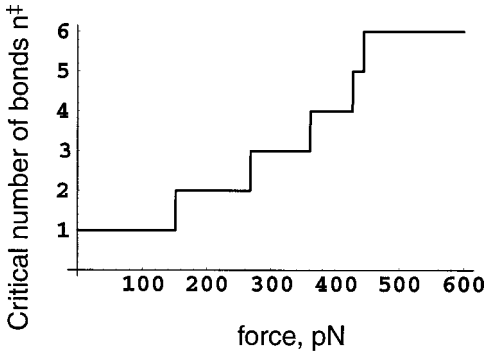

FIG. 10. The critical number of bonds in the domain as a function of the applied force.

$$p_1(t) = N p_N(t)[k_b(F(t)/N)/k_r(F(t))] \times \exp[-\beta \Delta H(F(t)/(N-1))] \times \exp[-\beta \Delta H(F(t)/(N-2))] \cdots \exp[-\beta \Delta H(F(t)/2)], \quad (24)$$

where $\beta = 1/k_B T$. Substituting Eq. (24) into Eq. (23) and using Eq. (3), we get

$$k_{\text{eff}}(t) = N p_N(t) \nu \exp(-\beta E_a(t)) \quad (25)$$

with

$$E_a(t) = \Delta G(F(t)/N) + \Delta H(F(t)/(N-1)) + \cdots + \Delta H(F(t)). \quad (26)$$

The probability $p_N(t)$ can be obtained by solving Eq. (18), and it is

$$p_N(t) = 1/(1 + \alpha_N + \alpha_N \alpha_{N-1} + \alpha_N \alpha_{N-1} \cdots \alpha_2), \quad (27a)$$

where

$$\alpha_n = k_b[F(t)/n] n/((N-n+1) \times k_r[F(t)/(n-1)]). \quad (27b)$$

If the breaking rate is much smaller than the recombination rate, then $\alpha_n \ll 1$ and $p_N(t) \sim 1$. This is the case in our model when the force F is low enough. In this case $E_a(t)$ can be interpreted as a time-dependent effective activation energy.

Equation (25) has the appearance of the rate of surmounting a single time-dependent barrier $E_a(t)$ with an attempt frequency on the order of $N\nu$. The real mechanism, however, consists of successive breaking of N bonds. If the experiments are performed at low pulling rates, it is not possible to use them to distinguish between a two-well and a many-bonds mechanism. Evans and Ritchie[27,28] proposed and studied a phenomenological model, in which unfolding involves surmounting a single energy barrier. Assuming a linear dependence of the barrier on the force, they obtained a linear dependence of the unfolding force on the pulling rate, similar to the one in Fig. 7. It is seen from our discussion that in the limit of low pulling rates our model gives the same result as that of Refs. 27 and 28, if one assumes that the barrier in the Evans–Ritchie model depends on the force in a nontrivial manner, as given by Eq. (26).

VI. DISCUSSION

We have proposed a model for titin unfolding when it is pulled with a time-dependent force. We have assumed that each Ig domain is held folded by six hydrogen bonds, as suggested by molecular dynamics simulations.[15] The external force increases the rate of breaking these bonds. The evolution of the probability that a bond is in place is described by the equations of phenomenological kinetics, with time-dependent rate constants.

The state of a domain under stress is described by the variable $n(t)$, the number of hydrogen bonds in the domain at time t. This quantity undergoes a random walk in which bond breaking decreases $n(t)$ by 1 and bond forming increases $n(t)$ by 1. Our simulation generates the times when these events occur. The domain unfolds when $n(t) = 0$ for the first time. By repeating such a simulation many times we generate a list of unfolding times. This list is used to perform a simulation of the unfolding of many domains that does not require any further calculations except for sorting the list.

This procedure allows us to simulate unfolding under stress when the pulling rate is high. Unfortunately, it requires too much computer time when the pulling rate is low. In this regime, we had to develop a new method. We do this by assuming that during a slow pull, the variable $n(t)$ is in equilibrium with the force $F(t)/n(t)$. This allows us to calculate the probabilities $p_n(t)$ that a domain has n bonds at time t. In simulations we have observed the existence of a critical value $n^{\#}$ of $n(t)$: if $n(t)$ drops below this value, the domain unfolds very rapidly. The rate of unfolding is the rate for $n(t)$ to drop below the critical value. The critical value plays a role similar to the transition state in the transition state theory. We determine it by using a variational method similar to the variational transition state theory. This quasiequlibrium model has been validated by simulations.

The model describes well the experimental observations even though we did not try to vary its parameters to fit the data. Here is a summary of the more important results. The distribution of the force at which the domains unfold resembles that obtained experimentally. The distribution of the unfolding times generated by simulations is very narrow, in accord with the experimental observations. Because we use the wormlike chain model to connect the extension of the polymer to the force acting on it, we can connect the width of the unfolding time distribution to that of the distribution of the unfolding force. The latter happens to be broad because the mean breaking time happens to be such that $|F'(t)|$ is very large; small fluctuations in time lead to large fluctuations in force.

The dependence of the mean unfolding force $\langle F \rangle$ on pulling rate v is similar to that seen in experiment, which is close to the form $\langle F \rangle = a + b \ln v$.

The model explains why the work of the force is less than the energy required to break the hydrogen bonds. Except for the case of an extremely high pulling rate, the bonds are always broken by thermal fluctuations that move the system over the barrier. By lowering the barrier, the external force makes these fluctuations more efficient. The difference between the energy needed to break the bond and the work of the force is taken from the medium.

The simulations and the quasiequilibrium theory led us to the concept of critical number of bonds $n^{\#}$. This depends on the pulling force, and its value is 6 at the largest pulling force and 1 at the smallest one. This means that, when the pulling rate is very high, if one bond is broken the others follow in very rapid succession. This has been observed in molecular dynamics simulations,[15] which are forced to use an extremely high pulling rate.

The quasiequilibrium theory allows us to show that the unfolding of a domain can be represented by an effective two-well system. Our theory gives an equation for the effective rate constant as a function of the rate constant of the elementary processes in our model.

Finally, we note that the success of the model does not

prove its correctness. As is well known in chemical kinetics of complex reactions, several mutually exclusive mechanisms can fit the data equally well. Nor do we want to suggest that the model used here, postulating that folding and unfolding of proteins is a matter of forming or breaking several weak chemical bonds, is a general one. Other proteins may behave differently.

It is possible to create synthetic polymers that have small number of side groups that can bind to each other. When these bonds are formed the chain is folded. If the fastening bonds are the weakest in the system, then these chains will unfold, when pulled by a force, in a manner described by our model. This may lead to materials with unusual mechanical properties: they can be stretched for a very large distance and will recover their folded configuration when the stress is removed. The behavior of these polymers will be similar to that of titin.

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