Plastic Deformation of Protein Monolayers

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ABSTRACT Globular proteins are peculiar solids that display both local stability of their conformation and the ability to undergo large cooperative changes of shape (conformational changes). If one forces a large deformation of the molecule, such that the structure is necessarily changed, it is not obvious whether the deformed globule can still remain a solid or whether it will melt. Is it possible to plastically deform a protein? Here we investigate this question with a micro-mechanical experiment on a small region (~10 molecules) of a protein monolayer adsorbed on a rigid surface. For the two proteins studied, albumin and myoglobin, we observed that the molecules can be substantially deformed (~1–2 nm deformation) by comparatively small stresses applied for sufficiently long times. The deformation is irreversible, and the protein remains in the solid state (i.e., displays a nonzero shear modulus). The dynamics of the deformation is approximately logarithmic in time, similar to creep in solids. These results show that globular proteins adsorbed on a surface can be plastically deformed.

INTRODUCTION

The native state of proteins is to a first approximation a solid, with most atoms in the molecule occupying fixed average relative positions. This phase is separated by first-order transitions from the unfolded state, which could be described as the gaseous phase of the system, or from the molten globule state, with characteristics similar to a liquid phase. The question arises whether the solid phase is realized by only one conformation (the native state) or many. Although misfolded proteins cannot be readily crystallized, we know that enzymes, for example, undergo substantial conformational changes during catalysis, and in many cases the structure of two distinct conformations has been solved. Also, for a polypeptide chain of several hundred amino acids, which can interact through hydrogen bonds, van der Waals forces, the hydrophobic effect, and electrostatically, one expects a complex energy landscape characterized by many nearly isoenergetic minima (Ansari, 1985). Thus the protein has some of the characteristics of a glass (Frauenfelder et al., 1991). This view is supported by experimental observations and computer simulations (Austin et al., 1974; Elber and Karplus, 1985). Whether these states correspond to a solid or not is then a question of how deep the minima are with respect to the thermal energy kT. However, the energy levels themselves depend on the temperature, as the strength of the interactions between residues, most notably the hydrophobic effect, are temperature dependent.

If one takes the point of view that it is sensible to talk about the material properties of proteins (Howard, 2001), one possible approach to these questions is to examine the response to large mechanical deformations; a solid must have by definition a nonzero shear modulus. The question we have in mind is the following. Suppose we subject a protein to a large deformation, such that the structure is necessarily changed (one can think, for example, of elements of the secondary structure slipping with respect to each other during the deformation, so that the tertiary structure is substantially altered); can the deformed globule still be a solid, or will it, on the contrary, melt into a collapsed polymer state with mechanical properties similar to a viscous liquid? Is there such a property as plasticity for proteins?

Experiments in which a large multidomain protein was mechanically unfolded by pulling on the polypeptide chain demonstrated the sequential unfolding of domains (Kellermayer et al., 1997; Tskhovrebova et al., 1997; Rief et al., 1997); however, once a domain starts to unfold, there does not seem to be a shear modulus.

Here we perform experiments in which we push on proteins instead of pulling. However, our sample is not a single molecule but consists instead of a small number of molecules that are part of a protein monolayer adsorbed on a hard surface. We apply a load force to a small area of the layer composed of an estimated 15 molecules and follow the dynamics of the ensuing deformation. The striking observation is that the molecules undergo large deformations (several nanometers), while retaining a nonzero shear modulus. That is, the protein remains in the solid state when deformed against a rigid surface. The dynamics of the deformation is approximately logarithmic in time. This suggests that there is no characteristic time scale for the process, reminiscent of the dynamics of plastic flow in solids, which is termed creep. The deformation is irreversible: if the surfaces are separated and then pressed together again, the dynamics is completely different; i.e., the system exhibits hysteresis. We further investigate how the dynamics is affected if we chemically alter the stability of the folded state. We present measurements on two different proteins: albumin (bovine serum albumin, BSA) and myoglobin (horse heart myoglobin, Mb).

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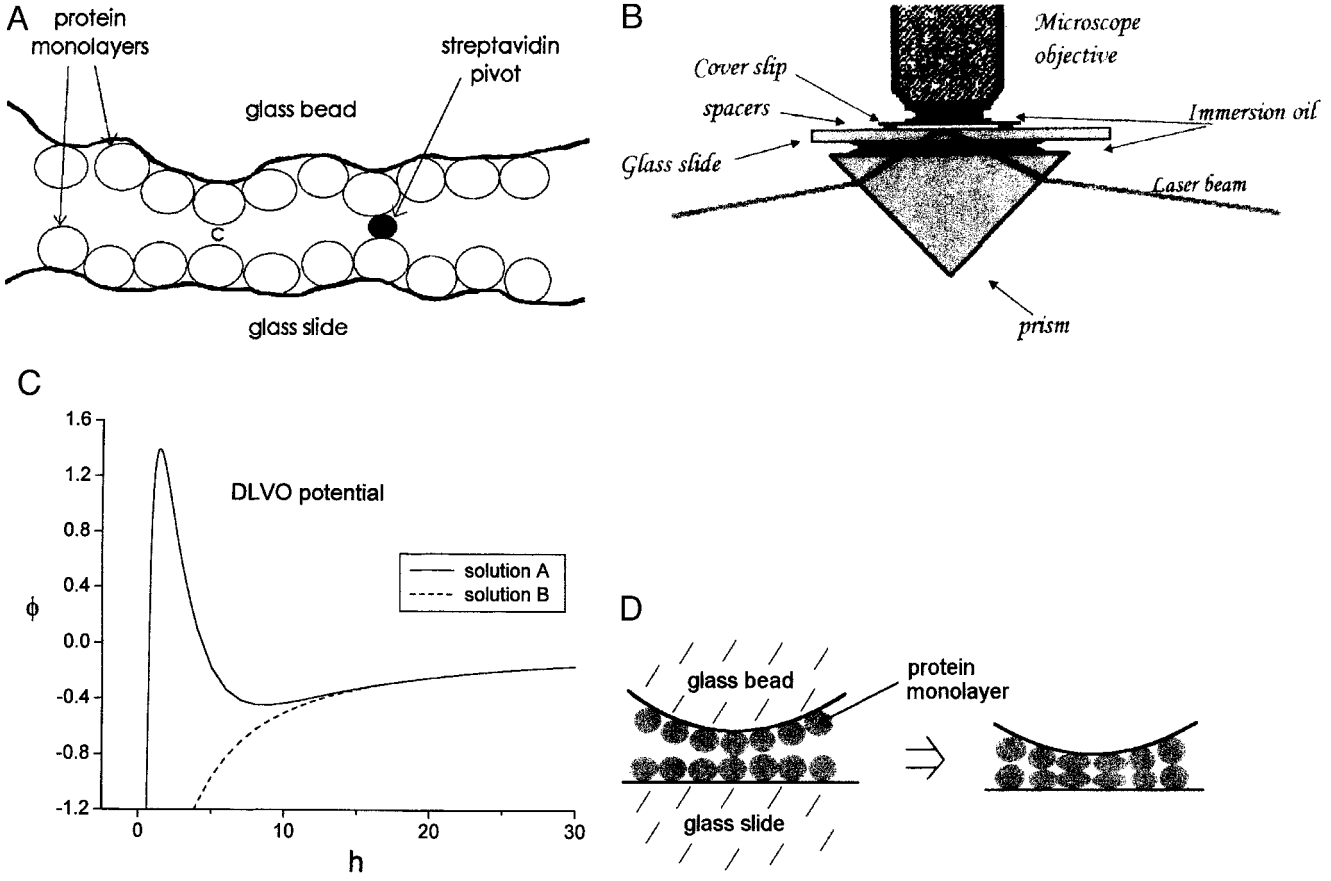

FIGURE 1 Sketch of the experimental configuration. (*A*) A 6-μm-diameter glass bead is tethered to the surface of a microscope slide by a single biotin-avidin-biotin contact, and the glass surfaces are covered with a monolayer of either BSA or Mb. In the initial state, the bead can pivot about the contact point; the surfaces are kept apart by an electrostatic repulsive barrier (see *C*). When this barrier is removed, by changing to a high ionic strength solution, the two surfaces jump into contact, e.g., at *c*. The contact region is compressed by the action of the attractive Van der Waals force (see *D*). (*B*) Sketch of the optical setup. The flow chamber is optically coupled to a prism that guides a laser beam to create an evanescent wave at the slide's upper surface. Light scattered by a single bead is collected through a microscope objective. (*C*) Sketch of the interaction potential between the bead and the slide, in the initial state (solution A, ——), and the final state (solution B, — — —). In the initial state, the bead is trapped in the secondary minimum. (*D*) Sketch of the deformation process. The protein monolayers are squeezed in the contact region.

MATERIALS AND METHODS

A micron-size glass bead is tethered to a glass slide through a single-point molecular attachment (a biotin-streptavidin bond), as previously described (Singh-Zocchi, 1999; Zocchi, 2001) (see Fig. 1 *A*). The glass slide forms the bottom of a flow cell of dimensions 2 cm $\times$ 2 cm $\times$ 100 μm. Both glass surfaces (the bead and the slide) are covered with a monolayer of the protein under study. The bead can still pivot around the fixed attachment point, whose function is to prevent it from being swept away when we change solution in the cell. The distance of closest approach h between the two surfaces is monitored with sub-nanometer resolution using a near-field optical technique that we have described in detail elsewhere (Jensenius and Zocchi, 1997; Zocchi, 1997). The slide is coupled through immersion oil to a Dove prism, and a parallel laser beam (20 mW He-Ne) is steered through the prism to be reflected at the upper surface of the slide (the slide-solution interface) at an angle beyond the critical angle for total internal reflection (Fig. 1 *B*). The intensity of the evanescent wave created at the bottom of the flow cell decays exponentially with the distance from the slide's surface. The bead scatters some of this light, and the scattered intensity I is a measure of the distance h between the bead and the slide, according to $I \propto \exp(-h/\Delta)$; here Δ is the penetration depth of the evanescent wave, which in our setup is $\Delta = 86$ nm. We see from the formula above that a 1-nm change in h produces a change in I of more than 1%, which is easily measured. The scattered light is collected through a microscope objective ($\times$53; NA 0.90) and focused on a photodiode; the voltage from the amplifier is measured by a lock-in amplifier connected to a computer for data acquisition. The resolution of the measurement is limited by the Brownian motion of the bead, but if the bead is pressed against the plate, as we show later, fluctuations can be reduced below 1 nm. We obtained an independent calibration of the relative distance measurements by observing the Brownian motion of a free (nontethered) bead and constructing the potential energy of the bead, which at large separations is gravitational and can be checked against the known weight of the bead (Prieve and Frej, 1990). Furthermore, it has been shown both experimentally and theoretically that the exponential law for the scattered intensity remains valid down to contact (Prieve and Walz, 1993).

The monolayers and attachment points were prepared as follows. For BSA (Sigma Chemical Co., St. Louis, MO), the protein was adsorbed for 30 min from a 1 mg/ml solution in PBS at pH 6.0; for both the slides and the beads, a small amount (in the molar ratio $1{:}10^3$) of biotinylated BSA (Sigma) was also present. After washing, the slides were incubated with streptavidin (0.5 pmol/μl for 30 min) to form isolated binding sites for the beads. For Mb (horse heart; Sigma), the protein was adsorbed overnight from a 10 mg/ml solution in PBS pH 7.4, with biotinylated BSA present in

the molar ratio $1{:}10^4$ for both surfaces; subsequent steps were the same as for the BSA preparation.

This configuration (Fig. 1 *A*) can be used as a mechanical probe down to the single-molecule scale because the surfaces are rough at the nanometer scale, so that when the bead is in contact with the slide, the contact region is formed, in most cases, by one, two, or three asperities of the surfaces, which typically have a radius of curvature much smaller than the radius of the bead. We have shown this to be the case by demonstrating single molecular attachment of beads to the slide and by measuring the extent to which such beads can pivot around the attachment point (Zocchi, 2001). This method represents an alternative to other techniques currently used for mechanical studies at the molecular scale, notably the atomic force microscope (AFM), the surface force apparatus, and the micropipette-supported biomembrane technique. For example, we have shown (Zocchi, 2001) that one can reproduce measurements of single molecular bond rupture forces obtained with the micropipette technique (Merkel et al., 1999) and with the AFM in (Florin et al., 1994; Wong et al., 1999). The main difference with the AFM is that the probe is not held by a mechanical arm. This makes it easier to measure displacements, particularly over long times, and harder to measure forces. This technique may generally be of interest to study the mechanical properties of polymer films, thus complementing other optical tools such as reflection interference contrast microscopy and ellipsometry (Elender et al., 1996; Kuhner and Sackmann, 1996).

The interaction between the bead and the slide consists of a van der Waals attraction, $\propto 1/h$ in this geometry, and an electrostatic repulsion (the surfaces are charged) which decays exponentially with distance because of the screening effect of ions in solution. The resulting Derjaguin–Landau–Verwey–Overbeek (DLVO) potential (Israelachvili et al., 1991) has a secondary minimum at a certain distance from contact and a primary minimum at contact ($h = 0$; see Fig. 1 *C*), and the particles that fall into this minimum do stick. In our experiment, both surfaces (the slide and the bead) are prepared with a protein monolayer adsorbed. Our initial conditions (solution A) are such that the bead is sitting in the secondary minimum of the DLVO potential, at $h \approx 10$ nm. Namely, the monolayers are negatively charged at this pH and provide an electrostatic barrier that prevents the bead from sticking. The basic idea of the experiment is to suddenly neutralize the layer, so that the attractive van der Waals force will press the bead against the slide. It is easy to modify the DLVO potential, because we can change the screening length, by changing ionic strength of the solution, and we can change the surface charge, even reverse its sign, by moving the pH around the isoelectric point of the protein (which for BSA is $pI \approx 5.5$ and for Mb is $pI \approx 9.0$). The experiment consists in suddenly removing the barrier that separates the secondary from the primary minimum of the interaction potential by changing to a high ionic strength solution at pH close to the isoelectric point (solution B; see Fig. 1 *C*). The bead falls into the primary minimum (i.e., sticks), and from then on we observe a slow process in which the protein monolayers are progressively squeezed between the bead and the slide (see Fig. 1 *D*). The driving force for the deformation originates from the attractive van der Waals interaction between the surfaces; thus we can also describe this as surface-induced denaturation.

Solution A was phosphate-buffered saline (PBS) at an ionic strength $[Na^+] = 25$ mM, pH 7.4. Solution B was, for the BSA experiments, PBS with $[NaCl] = 0.55$ M, $[MgCl_2] = 0.1$ M, pH 5.0. Solution B was the same for the Mb experiments but at pH 7.9. The time scale for exchanging solutions in our system is ~10 s. Diffusion of the new solution over the relevant length scales is even faster (~1-ms diffusion time over a 1-μm distance). These time scales are fast compared with the dynamics of the deformation process studied.

RESULTS

In Fig. 2 we show the deformation process for BSA monolayers, observed over a time span of 30 min; $h = 0$ is the reference height corresponding to the two BSA monolayers

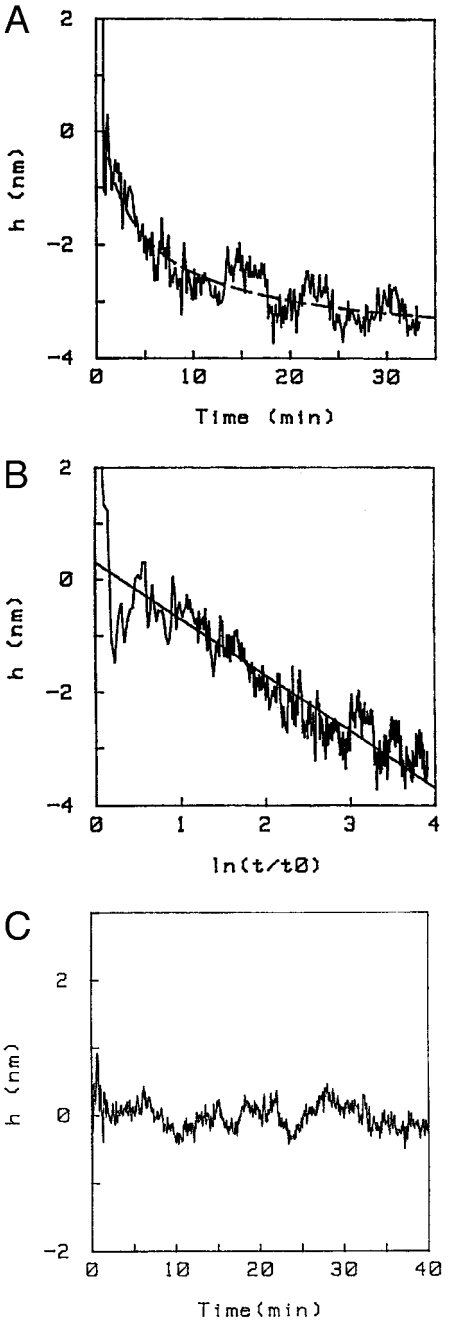

FIGURE 2 (*A*) Slow dynamics in the mechanical compression of albumin monolayers. The figure shows the time course of the separation between the bead and the slide. The vertical scale is in nanometers, and $h = 0$ corresponds to the albumin layers coming into contact (see Fig. 1 *D*, left). The initial condition is with the sphere in the secondary minimum of the DLVO potential, at $h \approx 10$ nm (not visible in the figure; see Fig. 3). At $t \approx 40$ s, solution A is exchanged with solution B, and the sphere falls into the primary minimum (sharp vertical line close to $t = 0$). From then on a slow deformation sets in, with a total amplitude of ~3 nm over the 30 min of the experiment. The dashed line is a power law fit: $h = \lambda\{1/(1 + \gamma\,[(t - t_0)^n] - 1\}$ with $n = 0.8$. (*B*) The same data as in *A* plotted versus ln (*t*). The solid line is drawn to show that the plot is roughly linear. (*C*) Control experiment with no protein layers on the solid surfaces. After exchanging solutions (A → B), the position of the bead remains stable within ±0.5 nm.

coming into contact. After contact, the total compression in the course of the experiment is, in this case, ~3 nm, or ~1.5 nm per monolayer. This is a considerable deformation: albumin in the unperturbed, native state is a globule of dimensions 8 × 8 × 4 nm (He and Carter, 1992).

It is known that albumin adsorbs on glass as a monolayer, not a multilayer (Brynda and Houska, 1996); if the molecule is adsorbed from a solution at a relatively low concentration (<1 mg/ml), the preferred orientation is with the minor axis perpendicular to the surface (i.e., a monolayer thickness of ~4 nm). Thus a 1.5-nm deformation represents a substantial strain: $s = 1.5/4 = 0.37$, presumably beyond the elastic regime. When the monolayers are formed by adsorbing from a high-concentration solution (10 mg/ml), then the preferred orientation is with the minor axis parallel to the surface (monolayer thickness, ~8 nm); consistent with this, for this system, we obtain, under the same conditions, larger deformations (~3 nm per monolayer).

In Fig. 2 *B* we present the same data plotted versus the log of time, to point out that in this representation the curve is roughly linear. Similar dynamics is observed in the plastic deformation of solids, in particular glasses (Phillips, 1905; Cottrell, 1953). In Fig. 2 *A* we also show a power law fit. The physical significance of these forms is that there appears to be no characteristic time scale for this process.

Fig. 2 *C* is a control with no proteins on the surfaces. Going through the same sequence of steps (solution A → solution B) the position of the bead remains stable within ±0.5 nm. Thus the mechanical stability of the experiment allows us to detect deformations of ~1 nm over times of ~1 *h* for a sample of ~10 molecules, as we discuss below.

When solution A is changed to solution B, the two monolayers on the bead and slide surfaces come into contact. This is evidenced by the fact that the center of mass of the bead abruptly drops toward the slide by typically ~10 nm; at the same time, vertical Brownian motion is drastically reduced, from ~5-nm amplitude in solution A (this corresponds to the rocking motion of the bead around its single attachment point) to below measurable (<1-nm amplitude) in solution B (Fig. 3). Because there is a clear separation of time scales for the processes of dropping into contact (~10-s time scale, set by the speed with which the solutions are exchanged) and for the subsequent squeezing of the monolayers (~20-min time scale), we can determine by inspection the zero in time for the process of deforming the monolayers (see Fig. 3) and therefore a reference intensity of the scattered light that corresponds to contact between the monolayers ($h = 0$ in the figures). A more objective determination of contact ($h = 0$) is obtained by plotting h vs log t and seeing where the straight-line fit to the slow deformation departs from the data.

The contact interface between proteins on opposite monolayers may include a hydration layer. However, the observed ~3-nm slow approach of the rigid surfaces cannot be attributed only to the removal of water layers, because 3 nm is already 12 water layers, which would behave like bulk liquid water, and the time scale τ for viscous flow of this water out of a gap between surfaces of curvature $1/R$, $\tau \approx \eta R^2/F$ where η is the viscosity and F the force on the surfaces ($F > 100$ pN for this experiment; see below) is at least six orders of magnitude faster (<0.1 ms) than the time scales we observe. Moreover, when we chemically soften the proteins (as explained later), we observe even larger (~7nm) deformations, which would correspond to removing an implausible ~25 water layers.

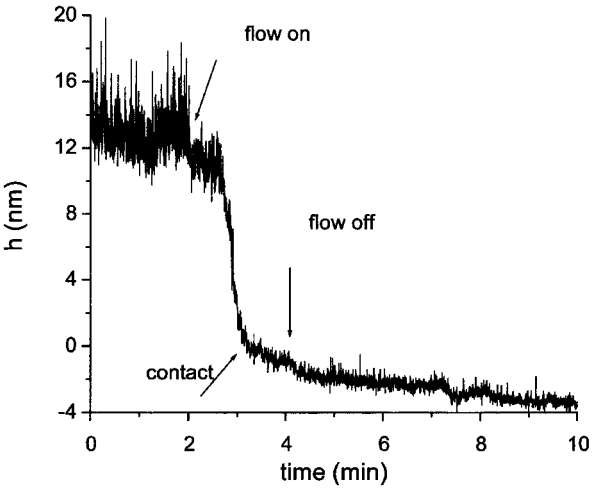

FIGURE 3 This figure shows the process of bringing the monolayers into contact. Initially ($t < 2$ min), the tethered bead, in solution A, is in the secondary minimum of the DLVO potential; fluctuations are large (~5 nm), because the bead is pivoting around the single tether point (note that these fluctuations do not represent a compression of the molecules). When a flow is switched on to exchange solutions in the chamber, the bead is pushed down slightly by the drag force; fluctuations are reduced. When the front of solution B reaches the bead, the repulsive electrostatic barrier is removed and the bead's center of mass drops abruptly by ~10 nm, bringing the monolayers into contact. Fluctuations are reduced to below measurable (<1 nm). The slow squeezing process sets in.

To quantify the reproducibility of the experiments, we took five different curves from independent runs under the same conditions as in Fig. 2 *A* and fitted the curves with the form $h = -\Delta\ln(t/t_0)$ for $t_0 < t < t_{end}$, where $\Delta\ln(t_{end}/t_0)$ represents the total deformation, and t_0 and t_{end} are the times at which the deformation starts and stops, respectively; we obtained the values $\Delta\ln(t_{end}/t_0) = (3.3 \pm 0.7)$ nm and $(t_{end} - t_0) = (27 \pm 12)$ min, where the error is the root mean square of the data. The plot in Fig. 4 was obtained by averaging these five experimental curves.

The magnitude of the observed deformations (~1.6-nm compression for a 4-nm-thick monolayer and, in the presence of denaturants, ~3.5-nm compression) indicates that the monolayers are squeezed beyond the elastic response regime; thus one expects the deformations to be irreversible. Indeed, it seems implausible that a globular protein, once subjected to a strain of $1.6/4 = 0.4$ or more against a solid

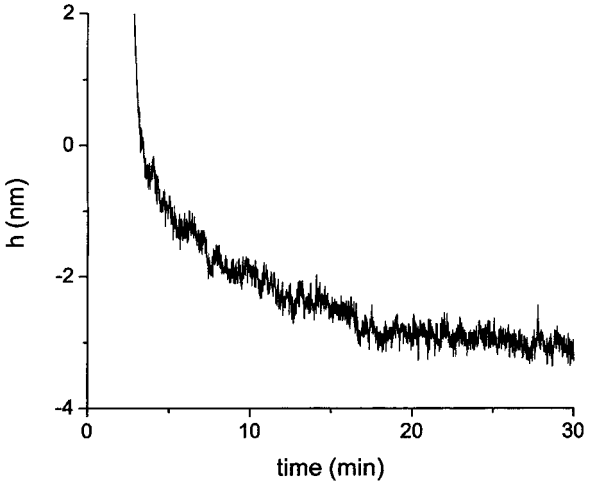

FIGURE 4 Average of five experimental curves obtained with BSA monolayers.

surface, with which it presumably forms additional hydrophobic contacts in the course of the deformation, would reversibly come back to its original state on the surface if the stress were released. We tested the irreversibility by cycling the experiment a second time. After the initial compression (Fig. 5 *A*), the bead and slide are pulled apart again through the electrostatic repulsion obtained by changing to a low ionic strength solution away from the isoelectric point (solution C: ionic strengths of 25–10 mM and pH 9–10); then solution B is reintroduced. The surfaces again drop into contact, but essentially in the same position as was the final state of the previous cycle, and no slow compression is visible (Fig. 5 *B*). The hysteresis manifest in Fig. 3 indicates that the monolayers are permanently deformed. We checked that solution C does not by itself cause the proteins to flatten against the glass, by first introducing solution C and then performing the first squeezing cycle; in this case we observe the usual ~3-nm deformation.

Our next step was to investigate the effect of chemically modifying the stability of the protein. To this end, disulfide bonds of the protein (BSA has 17 disulfide bridges) were reduced by placing the system in 0.1 M dithiothreitol (DTT). The same experiment as before was then performed; i.e., solution A (plus DTT) was exchanged with solution B (plus DTT). A typical curve obtained in this way is shown in Fig. 6, plotted versus log *t*. The overall effect over the 30 min of the experiment is now approximately two times bigger (~7 nm; Fig. 6), and there is an initial fast 2-nm jump after the albumin layers come into contact. However, the slow part of the deformation is still logarithmic in time. We also explored an alternative way of reducing the stability of the protein, by placing the system in a mildly denaturing solution (0.5 M urea). The resulting phenomenology is similar to what we observe in the presence of DTT, i.e., a bigger (~7-nm) deformation. Thus if we chemically reduce the stability of the folded state, we observe in the experiment that the proteins become mechanically softer, which further establishes that the compression observed in the experiments corresponds indeed to a deformation of the molecules.

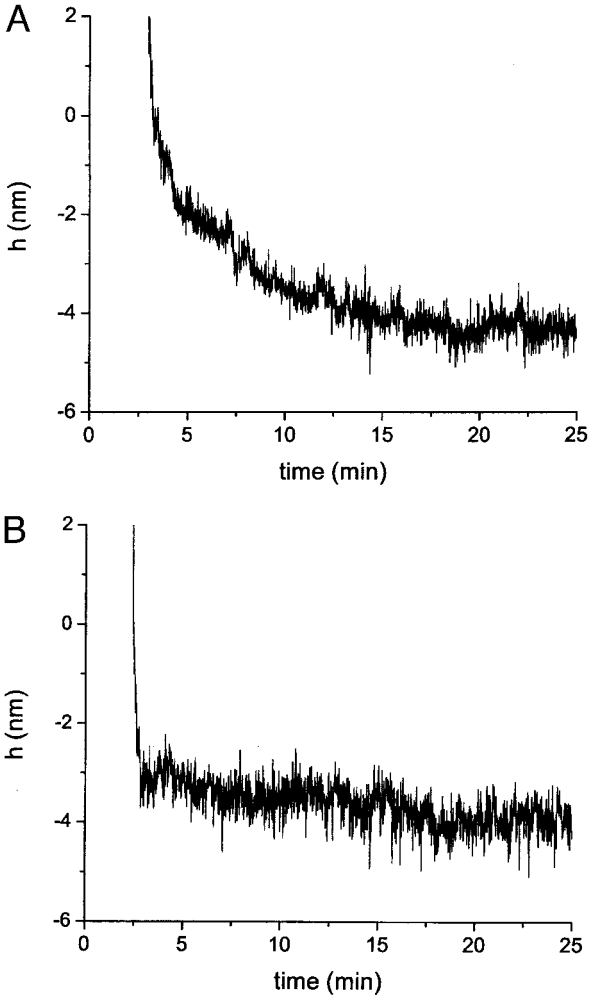

FIGURE 5 This figure shows that the deformation of the (BSA) monolayers is permanent. (*A*) After a first squeezing cycle, the monolayers are pulled apart again by increasing the electrostatic repulsion; (*B*) Then solution B is introduced again. The monolayers now come into contact at a bead-slide separation corresponding to the final state of the first squeezing cycle, and there is no additional deformation.

To test a second system, we performed similar experiments on Mb, a 17-kD globular protein that is particularly well studied from the point of view of its thermodynamic stability (Privalov, 1992) and energy landscape (Ansari et al., 1985). The dimensions of the molecule are ~5 × 4 ×3 nm (see, e.g., Protein Data Bank structure 1AZ1), the structure consisting predominantly of α-helices. Fig. 7 shows the results obtained with this system. The dynamics of the deformation is very similar to the albumin case; one difference is that with myoglobin the deformation process consistently stops after 20 min. This feature of the Mb experiments appears clearly as a break in the slope of the log plots

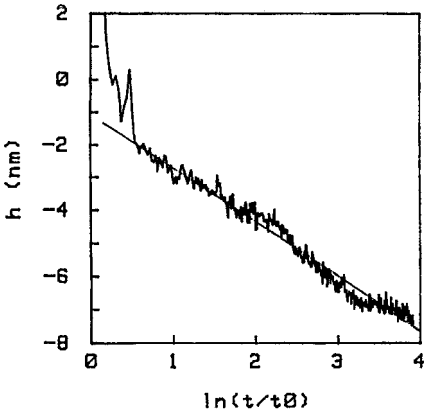

FIGURE 6 Squeezing of BSA monolayers with DTT present in the chamber to reduce the disulfide bonds of the albumin. In this case, after the layers come into contact ($h = 0$), we reproducibly observe a fast deformation of amplitude ~2 nm before a slow process sets in similar to the one in Fig. 2. The total deformation, including the initial 2-nm jump, is now ~7 nm, much bigger than in Fig. 2. This log plot shows that the slow part of the deformation is again roughly linear in this representation.

(Fig. 7) for $t \approx 10^3$ s. Fig. 7 *B* further shows that in this case also the dynamics is approximately logarithmic in time.

DISCUSSION

This study addresses the following question. Does a monolayer of globular proteins subjected to large mechanical deformations behave like a solid, in the sense that it can be plastically deformed, while retaining a finite shear modulus, into a permanently altered shape, or does it on the contrary behave like a viscous liquid, which can be made to flow reversibly from one shape into another. We find solid-like behavior, characterized by a finite shear modulus, and permanent deformations. In addition, the dynamics of the deformation is logarithmic in time: (Goldman et al., 1967) $h = \Delta \ln(t/t_0)$ for $t_0 < t < t_{end}$, where t_0 and t_{end} are the times at which the deformation starts and stops, respectively; this is reminiscent of creep in solids.

To clarify the regime under which the experiment is performed, we now discuss the load force and contact area, i.e., the stress on the molecules. We estimate the force from the Hamaker constant and the contact area from measurements of the flow velocity necessary to detach adhering beads. The load on the molecules is given by the adhesion force that originates from the van der Waals attraction between the layers. This adhesion force increases in the course of the experiment, as the contact area increases (see Fig. 1 *C*). However, we expect the force per unit contact area, i.e., the stress on the molecules, to be essentially constant, independent of contact area. This stress is on the order of γ/D_0, where γ is the surface energy (characteristic of the protein layers), and D_0 a microscopic scale on the order of the interatomic distances ($D_0 \approx 0.2$ nm). Namely,

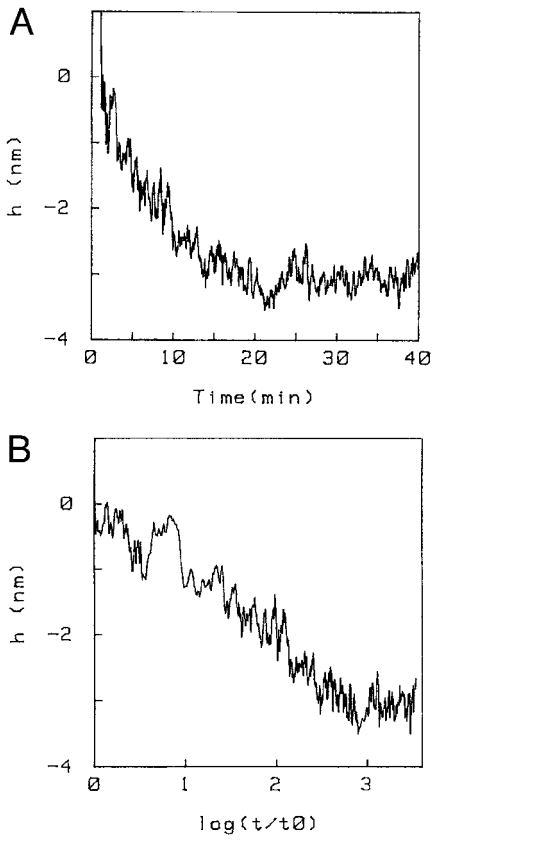

FIGURE 7 (*A*) Deformation of a Mb layer. The phenomenon is qualitatively the same as with albumin. Under these conditions, the deformation reproducibly stops after ~20 min. (*B*) The same data as in *A* plotted on a log scale show that the dynamics is roughly logarithmic, as is characteristic of creep in solids.

the work W required to separate two surfaces adhering through a contact area S_c is $W/S_c = 2\gamma = A/(12\pi D_0^2)$, where A is the Hamaker constant and $D_0 \approx 0.2$ nm (Israelachvili et al., 1991); the adhesion force is $F_{ad}/S_c = 4\gamma/D_0$. We measured the Hamaker constant A for our system, by fitting a DLVO form to the interaction potential measured for the nonsticking sphere, as detailed previously (Singh-Zocchi et al., 1999). Our measured value is $A \approx (1.0 \pm 0.3) \times 10^{-14}$ ergs (the known value for the glass-water-glass system, taking into account ionic screening, is $\sim 3 \times 10^{-14}$ ergs, but our value is different because of the protein monolayers on the glass). With this value for A we obtain an adhesion force per unit contact area $F_{ad}/S_{contact} \approx 6$ pN/(nm)^2. Taking a contact area per protein molecule of ~20 nm^2 gives then a force per molecule of ~100 pN, independent of the number of molecules making contact.

We obtain an estimate of the adhesion force at contact using a flow to detach the spheres. As detailed previously (Zocchi, 2001), the horizontal drag force on the bead results in a largely vertical force, and we obtain typical adhesion forces $F_{ad} \approx 2 \times 10^3$ pN. From the two estimates, $F_{ad}/S_{contact} \approx 6$ pN/(nm)^2 and $F_{ad} \approx 2 \times 10^3$ pN we obtain, for

the surface of contact: $S_{contact} \approx 300$ nm^2, or $S_{contact} \approx 15$ molecules. If the solid surfaces were smooth at the molecular scale, the number of contact molecules would be much larger: for a sphere of radius $R = 3$ μm and a deformation $\delta = 1$ nm, the contact area is $S_{contact} \approx 2\pi R\delta \approx 2 \times 10^4$ nm^2, or $\sim 10^3$ molecules. As estimated above, the actual contact area in the experiment is much smaller, because the surfaces are rough at the nanometer scale. Indeed, from our previous detailed work on single molecular attachments with this system (Zocchi, 2001), and from observing that the center of mass of a bead attached at a single point drops by typically $\sim$10 nm when the bead is collapsed on the slide (see Fig. 3), we know that there are asperities protruding by several nanometers from the average surface. Consistent with this, surface profiles of microscope slides show an average feature height of $\sim$2 nm over a 1 μm $\times$ 1 μm surface (Tristram-Nagle et al., 1998). Given the relatively small contact area, the experiment is performed in a regime that is in between single-molecule measurements and mesoscopic surface layer measurements.

We now examine how our observations compare with what is known about the forces needed to disrupt the structure of globular proteins. The force required to break a ligand-receptor bond (Bell, 78; Florin et al., 1994; Hinterdorfer et al., 1996; Allen et al., 1996; Shao and Hochmuth, 1999) depends on the time over which it is applied: it increases with the logarithm of the pulling rate (Evans and Ritchie, 1997; Merkel et al., 1999). Experiments in which single molecules of the multidomain protein titin were unfolded by mechanically pulling on the polypeptide chain with an AFM (Rief et al., 1997; Tskhovrebova et al., 1997) or optically trapped bead (Kellermayer et al., 1997) showed the sequential, cooperative unfolding of individual domains; here also the force necessary to unfold a domain increases with the logarithm of the pulling rate. The measurements on titin are well represented by a typical survival time τ for the folded domain on order of $\tau \approx \tau_0 \exp(-F\Delta x/kT)$, where $\tau_0 \approx 3 \times 10^4$ s is the survival time at zero force, F is the applied force, and $\Delta x \approx 0.3$ nm represents a barrier width (Rief et al., 1997). This gives a survival time $\tau \approx 20$ s for $F = 100$ pN and $\tau \approx 800$ s for $F = 50$ pN. In our experiment, the time over which we obtain a large deformation of the proteins is on the order of $\sim$10 min. This is essentially consistent with the titin results, given that 1) titin has been selected to resist stress (more typical proteins may unfold at smaller forces (Best et al., 2001)), 2) our geometry is different (squeezing versus pulling), and 3) we could be overestimating our load force F (a factor 2 difference would bring our time scales to coincide with that extrapolated from the titin experiments).

In the present experiments, as in the titin experiments, the structure of the molecule is disrupted, i.e., we are far from the regime of elastic deformations. The Young modulus for bulk proteins, as measured, e.g., through viscoelasticity (Rosser et al., 1977) or the speed of sound in the solution (Tamura et al., 1993), is on the order of $Y \approx 10^9$ N/m$^2 = 10^3$ pN/nm^2; however, the stresses applied in our experiment are on the order of $\sigma \approx 100$ pN/20 nm$^2 = 5$ pN/nm^2, and with the Young modulus above, the corresponding elastic strain $s = \sigma/Y$ would then be $s \approx 5 \times 10^{-3}$, two orders of magnitude smaller than the deformation observed in the experiments. Yield stresses have been measured for some of the structural proteins (Howard, 2001) and vary in the range 10^8–10^6 N/m^2. However, one has to keep in mind that these quantities are frequency dependent: one can substantially deform a protein, even cause it to unfold, by applying comparatively small forces for sufficiently long times. This is demonstrated by a number of studies, including the titin experiments and the present study; the physical reason for this is thermally assisted barrier hopping.

We also note that in the present experiment the work done by the load force on the single protein, $W \approx 100$ pN $\times$ 1.5 nm $\sim$ 40 kT (T is room temperature), is comparable to the stability of the protein (free energy difference between folded and unfolded state), which is on the order of $\sim$20 kT/molecule for a small single-domain protein (Creighton, 1990).

A number of control experiments were performed to confirm that the proteins are actually deformed in the experiments and that the dynamics is set by this deformation process.

First, a second compression cycle reveals hysteresis. Hence the monolayers in the contact region were permanently deformed by the first compression.

Second, chemical softening of the molecules (by addition of small amounts of denaturants) results in larger observed deformations for the same load force. Thus the experiment probes the mechanical strength of the molecules.

Third, we cross-linked the proteins within the monolayers (both on the slide and the bead) using gluteraldehyde. Thus the molecules cannot escape from the contact region. We still observe the same phenomena, although the total amplitude of the deformation is reduced (for albumin, $\Delta h \approx$ 1.5 nm typically compared with $\Delta h \approx 3$ nm typically without gluteraldehyde; however, this can be attributed to a stiffening of the molecules caused by intramolecular cross-linking). This indicates that the slow approach of the bead and slide does not merely correspond to displacing the molecules away from the contact region.

Fourth, we doubled the viscosity of the solvent, using a water-glycerol mixture, and the dynamics of compression was not affected. This shows that the flow of solvent out of the gap does not determine the dynamics.

Other facts that are consistent with our interpretation of the experiments are that the observed deformation is always smaller than the combined thickness of the two monolayers (and comes close to this limit when denaturants are used) and that if the surface coverage by the proteins is small (our initial Mb preparations had small surface coverage, because it turns out that Mb adsorption is slower than BSA adsorption), then no slow compression is observed, because the contact region is then most likely glass-glass.

Because the molecules being deformed are part of a larger monolayer, a second issue is whether the observed creep dynamics is a single-molecule or a monolayer phenomenon, i.e., whether the observed dynamics of deformation is affected, or even controlled, by interactions between nearby molecules. This question cannot be resolved by the present experiment, but noting that in the monolayer the protein still has room to expand laterally, we propose that the creep may be characteristic of the single molecule interacting with the surface.

In conclusion, we have shown on two examples that a monolayer of globular proteins adsorbed on a rigid surface and subjected to load forces on the order of ~100 pN/molecule (stresses $\sigma \approx 5$ pN/nm^2 $= 5 \times 10^6$ N/m^2) can be deformed substantially (strain $s > 0.4$) if the stress is applied for a sufficiently long time (~20 min in this case). Throughout the deformation the protein remains solid. The process is reminiscent of plastic deformations in solids: it is irreversible, and the dynamics is logarithmic in time. Presumably this plasticity is conferred upon the proteins by the presence of the rigid surface against which they are squeezed.

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