Elasticity of Globular Proteins Measured from the ac Susceptibility

Yong Wang and Giovanni Zocchi*

Department of Physics and Astronomy, University of California Los Angeles, Los Angeles, California 90095-1547, USA

(Received 9 August 2010; published 3 December 2010)

We introduce a new method to measure the elastic constants of globular proteins. Gold nanoparticles, tethered to a gold surface by the protein, are driven by an ac electric field while their displacement is synchronously detected by evanescent wave scattering, yielding the mechanical response function of the macromolecular sample in the frequency domain. We apply the method to measure the stiffening of an enzyme upon binding its substrate.

DOI: 10.1103/PhysRevLett.105.238104 PACS numbers: 87.15.−v, 83.60.Bc, 87.85.G−

Introduction.—Biological macromolecules are structurally ordered but ''soft.'' The mechanical properties of such a structure are necessarily peculiar and form an interesting object of study in condensed matter. Moreover, the mechanics of these molecules is tightly coupled to their function, as conformational change (static or dynamic) [1,2] is ubiquitous in molecular recognition [3,4], catalysis, and regulation [5]. We are specifically interested in the mechanical properties of globular proteins and short DNA molecules because we use the latter as ''molecular springs'' to perturb the conformation of the former (reviewed in Ref. [6]). Here we present an experimental method to investigate the elasticity of nm size biological molecules such as globular proteins. The method is based on measuring the mechanical response function of the sample in the frequency domain, or what we might call nanorheology [7]. Different from spectroscopic techniques [8–10] which rely on thermal fluctuations, here the sample is mechanically forced. In contrast to AFM-based force spectroscopy techniques [11–13], the method can preserve the native conformation of the protein, with deformations of only a few fractions of one angstrom.

The molecule under study is used to tether gold nanoparticles to the surface of a microscope slide coated with a thin (30 nm) layer of gold; the purpose of this layer is to obtain a conducting electrode, and also take advantage of the affinity of thiol groups (which can be introduced into proteins and DNA) for gold surfaces [14,15]. The layer is thin in order to allow optical measurements. The experiment consists in mechanically driving the gold nanoparticles using an ac electric field and detecting their motion transverse to the slide by evanescent wave scattering, in a phase locked loop. The amplitude and phase of the particles' motion, averaged over many particles, are recorded in the frequency range 10 Hz–10 kHz; these curves contain the information about the (visco)elastic properties of the system. In the present setup, both the actual force on the gold particles and the average number of molecular tethers per particle are uncalibrated (although these quantities can, of course, be estimated), so the method is not suitable to extract the absolute value of the elastic constants of the tether; however, it provides a relatively simple and robust way to measure changes in the elastic parameters, such as may be caused by ligand binding, temperature changes, etc. First we demonstrate the method using the change in stiffness of DNA oligomers upon hybridization, then we apply it to detect the change in stiffness, along a specified direction, of the protein Guanylate Kinase (GK), upon binding of the substrate guanosine monophosphate (GMP).

Materials and sample preparation.—Gold nanoparticles (GNPs, 20 nm diameter) were from Nanocs (New York, NY); unmodified and thiol-modified DNA oligonucleotides from Integrated DNA Technologies (Coralville, IA); other chemicals from Sigma-Aldrich. Experiments were performed in saline-sodium citrate buffer (SSC; Invitrogen) diluted with deionized water to a final concentration of 50 mM sodium chloride and 5 mM trisodium citrate, pH 7.0 (SSC/3). Guanylate Kinase (GK) was prepared by mutagenesis with the internal Cys changed to Ser and Cys substituted at positions 171 and 75, as described in Ref. [16], for coupling to the gold surfaces. Glass slides and cover slips were thoroughly cleaned before evaporating a 3 nm layer of Cr followed by 30 nm of gold, using an e-Beam vacuum evaporation system. To couple the thiol-modified DNA, the Au slides were immersed in a solution containing the DNA (1 μM DNA in 1M KH_2PO_4, pH 4.0) overnight. To remove nonspecifically bound DNA the slide was then immersed in 1.0 mM 6-Mercapto-1-hexanol (MCH) for 1 h and finally rinsed with deionized water and dried by a nitrogen flow. DNA-GNP conjugates were prepared essentially as described in Ref. [17]. The coverage of DNA on the GNPs or gold films was quantified by a βME displacement assay [14] and found to be $\sim$300 molecules per particle or 4×10^{12} molecules/cm^2 on the gold films. The DNA-GNP conjugates were coupled to the DNA-modified slide through a DNA linker [Fig. 1(b)]. This proess was monitored by measuring the light intensity scattered in the evanescent wave apparatus.

To couple the protein, GK was prepared at 1 μM concentration in 1M KH_2PO_4. The pH was optimized to minimize nonspecific binding of the protein to the gold (Fig. 4). The Au slides were immersed in the GK solution

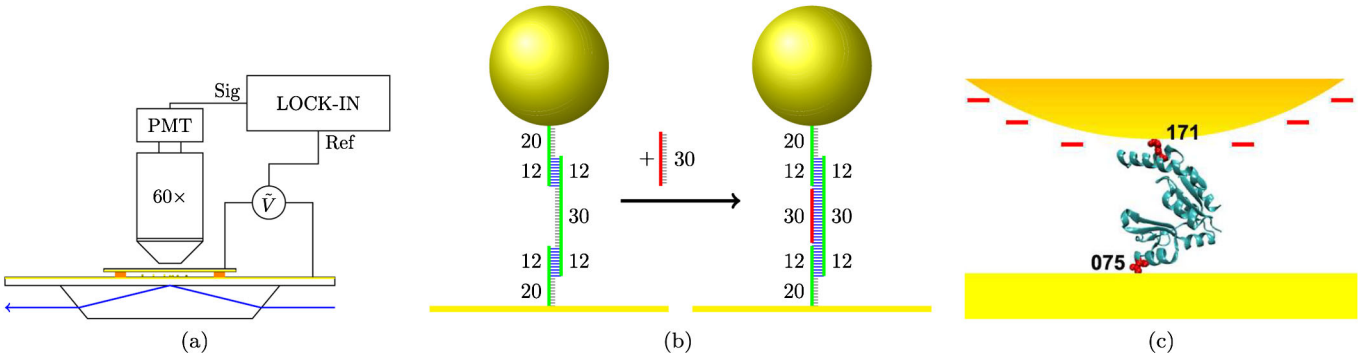

FIG. 1 (color online). Schematics of the experimental method. (a) The flow chamber, electric excitation, and optical readout. (b) The DNA construction used to test the method. (c) Geometry of the protein attached by the 171 and 75 sites. The protein and the radius of the GNP are drawn to scale.

overnight and then washed with deionized water. The GNPs were then introduced and incubated at room temperature for 3 hours, followed by washing with water. The slide was then immersed in a solution containing thiol-modified DNA (1 μM DNA 32-mers in 1M KH_2PO_4 at pH 4.0) overnight to increase the net charge on the GNPs. The chamber was then washed with water, and the buffer changed to SSC/3 for the measurements.

Experimental method.—The molecules under study tether the gold nanoparticles (GNP; 20 nm diameter) to the gold film on the microscope slide [Figs. 1(b) and 1(c)] which forms the bottom of a flow chamber [Fig. 1(a)]. The chamber is constructed with the slide and a cover slip (also coated with a gold thin film) separated by 200 μm spacers, resulting in a volume of $\sim$20 μl in a parallel plates capacitor configuration. The GNPs are negatively charged (through surface modifications: see sample preparation) and can thus be driven by the electrophoretic force arising from the electric field established by applying a potential difference between the gold films.

The motion of the GNPs transverse to the gold surface is monitored by evanescent wave scattering. The optical setup is similar to the one described in Ref. [18] in the context of single-molecule measurements. The flow chamber is optically coupled to a prism through immersion oil. The beam from a 100 mW argon laser (488 nm) is steered through the prism to create an evanescent wave at the bottom of the flow chamber. Light scattered by the GNPs is collected through a microscope objective (60 $\times$, NA = 0.80) and focused on a photomultiplier (Hamamatsu H6780). The intensity I of the light scattered by a GNP varies exponentially with the distance h of the particle transverse to the surface, $I = I_0 e^{-h/\delta}$, where δ is the penetration depth ($\delta \approx 64$ nm in our setup). Thus for small displacements $z = \Delta h = \delta \Delta I/I$ where ΔI is the change in scattered light intensity. The field of view of the microscope objective is 0.35×0.35 mm^2, corresponding to approximately 10^7 GNPs; we detect the average displacement of this collection of GNPs. In the experiments, an ac voltage at frequency f ($\sim$ 1 V amplitude) is applied to the gold films, and the scattered light signal is measured in a phase locked loop, using a lock-in amplifier [Fig. 1(a)]. At each frequency, the amplitude of the sine wave which drives the system is adjusted so that the voltage applied to the gold films is the same (as the impedance of the flow chamber varies with frequency). The driving frequencies are a geometric series of ratio 2, from 10 to 10240 Hz. The response (amplitude and phase) at each frequency is averaged over 50 seconds. This averaged response contains the information on the viscoelastic properties of the sample. Because the measurement is averaged over many GNPs, it is insensitive to thermal fluctuations even for small driving forces; in fact we can easily measure displacement amplitudes of the GNPs of 0.1 Å. The temperature of the flow chamber, monitored by a bolometer, is constant during the measurements.

Results.—We first demonstrate the method using the well-known change in stiffness of DNA upon hybridization [5]. The GNPs are tethered by a DNA oligomer construction consisting partially of ds and partially of ss DNA (Fig. 2). Figure 2 shows the measured (normalized) amplitude of the response in the frequency range 10 Hz–10 kHz, above which frequency the signal goes into the noise (the electronic frequency cutoff with the present apparatus is just above 10 kHz and does not significantly affect the curves of Fig. 2). The amplitude of the low frequency, flat part of the curves corresponds to approximately 5 Å displacement of the GNPs in the unhybridized case (calculated from the measured signal $\Delta I = 0.401 \pm 0.002$ mV divided by the measured scattered light intensity $I = 49.9 \pm 0.2$ mV). The equation of motion for a GNP is

$$m\ddot{z}(t) + \gamma \dot{z}(t) + \kappa z(t) = F(t) \qquad (1)$$

where m is the mass of the particle, $1/\gamma$ is a mobility, F is the force due to the applied electric field, and κ is a spring constant representing the elastic properties of the sample for small deformations z. There is no Brownian motion term because the measurement method averages it out. Depending on the nature of the sample, γ can be dominated by the Stokes drag on the GNP, by the internal

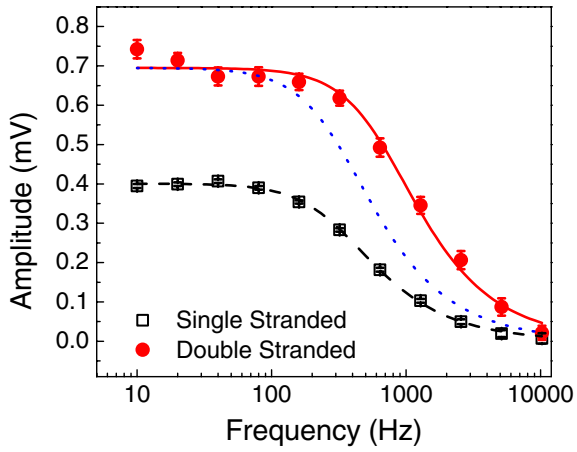

FIG. 2 (color online). Amplitude of the response function of the DNA sample before (squares) and after (circles) hybridization. The solid and dashed lines are fits with Eq. (4), yielding $f_c(ss) = 328 \pm 6$ Hz and $f_c(ds) = 675 \pm 39$ Hz. We have also plotted (dotted line), for comparison, the function Eq. (4) for the hybridized case, using the corresponding amplitude $z_0(ds)$ but the cutoff frequency of the unhybridized case $f_c(ss)$.

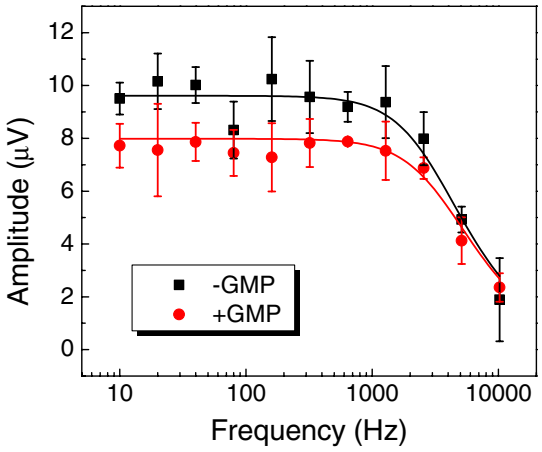

FIG. 3 (color online). Amplitude of the frequency response of the GK sample in the absence (squares) and presence (circles) of the substrate GMP. Lines are fits with Eq. (4), showing the protein's stiffness increases by 20% upon binding GMP.

friction of the polymers, or be a combination of effects. In any case $\gamma \geq 6\pi\eta R$ where η is the viscosity of the solution and R is the radius of the GNP. In the experiments, the driving frequency is therefore "small" ($\omega \ll 6\pi\eta R/m$), and the inertial term can be neglected. Thus,

$$\gamma \dot{z}(t) + \kappa z(t) = F(t) \tag{2}$$

and we arrive at

$$z(t) = \frac{F_0}{\kappa\sqrt{1 + \left(\frac{\gamma\omega}{\kappa}\right)^2}} e^{i[\omega t + \phi(\omega)]} \tag{3}$$

where F_0 and ω are the amplitude and (angular) frequency of the applied force. Thus the amplitude and phase of the response are

$$z_0(\omega) = \frac{F_0}{\kappa\sqrt{1 + \left(\frac{\gamma\omega}{\kappa}\right)^2}} = \frac{z_0(0)}{\sqrt{1 + \left(\frac{f}{f_c}\right)^2}} \tag{4}$$

$$\phi(\omega) = -\arctan\left(\frac{\gamma\omega}{\kappa}\right) \tag{5}$$

where $z_0(0) = F_0/\kappa$ and $f_c = \kappa/2\pi\gamma$, i.e., from the zero frequency amplitude $z_0(0)$ and the cutoff frequency f_c one obtains the spring constant κ and the friction coefficient γ. The lines in Fig. 2 are fits using Eq. (4), from which we obtain $f_c(ss) = 328 \pm 6$ Hz, $f_c(ds) = 675 \pm 39$ Hz, indicating a stiffening of the molecular tether by a factor 2.1 upon hybridization. A simple estimate based on the entropic elasticity of the random coil (ss) parts of the tether [Fig. 1(b)] is consistent with this result.

We now apply the method to measure the change in stiffness of the enzyme GK along the direction 171-75 (the numbers refer to the amino acid sequence, see Fig. 1(c)) upon binding of its substrate GMP. Binding of GMP induces a conformational change which closes the cleft between the two lobes of the molecule [19], presumably stiffening the structure along the 171-75 direction [20]. Indeed, Fig. 3 shows this effect in the response function measured in the absence (squares) and presence (circles) of 1 mM GMP. This concentration of GMP is much higher than the binding constant ($K \approx 200$ μM [21]). The introduction of GMP has negligible effect on the ionic strength since the buffer used in the measurements is SSC/3 which contains 55 mM Na^+. The net charge of the GNPs also remains the same and thus the driving force does not change, which allows us to compare amplitudes z_0 at zero frequency, $z_0(0) = F_0/\kappa \propto 1/\kappa$. By fitting the data with Eq. (4) we find the zero frequency amplitudes $V_0^+ = 8.0 \pm 0.1$ μV, $V_0^- = 9.6 \pm 0.2$ μV and the cutoff frequencies $f_c^+ = 3.7 \pm 0.3$ kHz and $f_c^- = 3.1 \pm 0.2$ kHz for the two cases with and without GMP. Thus the protein is 20% stiffer ($\kappa_+/\kappa_- = f_c^+/f_c^- = 1.2$) with GMP bound, while $(z_0^+ f_c^+)/(z_0^- f_c^-) = (F_0^+\gamma^-)/(F_0^-\gamma^+) = 1.0$ represents an internal consistency check that the amplitude F_0 and the friction coefficient γ remain the same. A control experiment with 1 mM CMP (not a substrate) instead of GMP showed no change in stiffness within experimental resolution. On the other hand, the true increase in stiffness with GMP bound is probably larger than 20%, because the molecules are only imperfectly oriented with respect to the gold surface. Figure 4 shows that the ratio of specific (through Cys residues) to nonspecific binding of GK to the gold was, under the best conditions we found, approximately 1.4. Assuming there is no significant change in stiffness of the molecule along the directions orthogonal to 171-75 (which could be checked in future

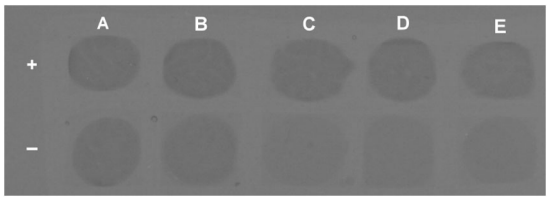

FIG. 4. Specific vs nonspecific binding of GK to the gold surfaces, under various conditions. The *p*H of the buffer increases left to right from 4.0 (A) to 10.0 (E). Upper row (+): GNPs are tethered by the GK mutant with Cys at 171 and 75. Lower row (−): control with a mutant without Cys. The ratio of specific (thiol-gold) to nonspecific binding reaches a maximum 1.4 at *p*H 7.0 (D).

experiments), the actual change in stiffness is probably close to a factor 1.34.

Discussion.—We present a relatively simple and robust method to measure the elastic constants of globular proteins. For the present measurements, the largest deformation amplitude is ~0.3 Å, the size of the protein is 4 nm, so the maximum strain is ~1%. This is apparently within the linear elasticity regime, since Eq. (2) describes the data well. However, the measurements can probably be extended into the nonlinear (visco)elastic regime, though care will be required about heating the sample at higher driving amplitudes. In order to place oneself in the interesting regime where the dissipation is dominated by the internal rheology of the protein, one would like to reduce the size of the GNP as much as possible. The limitations are the scattered light intensity and the driving force on the GNP: both drop with decreasing size. Probably measurements at different temperatures (the protein ''softens'' at higher temperature) will help both access the nonlinear elasticity regime and distinguish between hydrodynamic and internal friction as the origin of dissipation. In conclusion, we present a method to explore the internal rheology of soft, nm size objects. We apply it to measure the change in stiffness of an enzyme upon binding its substrate. Measurements of the stiffness along a specific direction are possible, with perturbation amplitudes such that the sample remains in the linear elasticity regime. It remains to be seen whether the nonlinear viscoelastic regime is accessible also. Finally, the method may possibly form the basis of an electronic screening device for small molecules binding at the active site of enzymes.

*zocchi@physics.ucla.edu

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