Comment to the Editor

Comment on ‘‘Sub-Angstrom Conformational Changes of a Single Molecule Captured by AFM Variance Analysis’’

In their article, Walther et al. (1) have presented excellent data on the force-extension curve and on length and force fluctuations for a single Dextran molecule using the atomic force microscope (AFM). These measurements were done in the constant-velocity mode, in which the base of the cantilever is moved at a constant speed, so that its distance from the substrate (where the other end of the molecule is attached) is the macroscopically controlled variable, $D = vt$. They also operated the AFM in the force-ramp mode, where a feedback loop is used to ensure a linear rise in the average force between the cantilever tip and the molecule, $f = \bar{f} = \alpha t$. Based on previous work in our own group, the latter experiment was initially believed to correspond to the constant-force or Gibbs ensemble. However, the experimentally observed molecular fluctuations (Fig. 1 *B* in their article) showed quite clearly that this is not the case for the setup used in their experiment. Intrigued by the inadequacy of the Gibbs ensemble for such a force-ramp experiment, we have reinvestigated this situation and would like to add a theoretical clarification to the experimental findings.

In an AFM stretching experiment, the polymer is coupled to the AFM cantilever and from the deflection of the latter, one extracts both the length of the polymer and the applied force, as well as their fluctuations. Whereas the cantilever has a fixed force constant or stiffness (within its harmonic regime), the equivalent molecular elasticity $K = \partial f/\partial L|_T$ of a macromolecule varies continuously from zero, under zero force, to some rather large value just before its breaking point; here f is the applied force, L is the (average) length of the molecule, and T is the temperature. Thus, any analysis of data and any theory concerned with such experiments must be based on the coupled molecule-cantilever system (2), in which the position of the cantilever support is the macroscopically controlled variable. The idea behind an experimentally implemented force-ramp is to adjust this length so as to keep the overall force controlled, and thereby integrate out the effects of the cantilever. The result is supposed to be a system that is controlled by the force macroscopically and hence can be modeled in the (conceptually simpler) Gibbs ensemble.

Let us consider this force-ramp situation at some specific time t. The cantilever-substrate distance has a current value D and, at this instant in time, the molecule-cantilever system can access thermal fluctuations of width (2),

$$\langle \delta L^2 \rangle = k_B T/(k_c + \partial f/\partial L|_T), \tag{1}$$

where k_c is the cantilever's spring constant. The AFM now measures the force on the system and adjusts the substrate position to some value D' to keep the force on track with the linear force ramp. Again, the updated system can access thermal fluctuations of width given by Eq. 1, this time centered around position D'. If the cantilever spring constant k_c is comparable to or larger than the molecule stiffness $\partial f/\partial L|_T$, then we cannot simplify Eq. 1 by neglecting k_c. In this situation, it should be obvious that the cantilever remains the limiting factor regardless of the feedback, and that even the fastest feedback could not completely eliminate the effects of the cantilever, such as to have a fluctuation width $k_B T/\partial f/\partial L|_T$.

This could be shown rigorously by writing down the probability distribution of the molecule-cantilever system with an infinitely fast cantilever update. The spring constant k_c always enters such an expression, which precludes a general implementation of the Gibbs ensemble via a feedback loop in an AFM. However, the situation is quite different where the cantilever stiffness is much smaller than the molecular stiffness, or $k_c \ll \partial f/\partial L|_T$. We note that the last inequality is exactly what was originally proposed for systems in the Gibbs ensemble by Kreuzer et al. (2).

To substantiate this discussion we have presented in Fig. 1 the length fluctuations for a molecule with 170 monomers, calculated within the continuous two-state model for Dextran (3). Two calculations have been performed: one in the Gibbs ensemble for the isolated molecule, and one in the Helmholtz ensemble for the coupled molecule-cantilever system. The length fluctuations for a coupled molecule-cantilever system are in good agreement with the experimentally observed values shown in Fig. 1 *B* of Walther et al. (1). As expected, the length fluctuations for an isolated molecule are much larger, and hence do not agree with the experimentally observed values; this is in agreement with the statement of Walther et al. that a prediction in the Gibbs ensemble for an isolated molecule does not reproduce their data.

Note that the continuous two-state model employed here (3) is not equivalent to the one presented in Fig. 3 of Walther et al., which did not fully represent their own data over the transition region, but only worked in ranges where either chair or boat conformers alone were being stretched. Walther et al. do include the cantilever in the analysis presented in their Fig. 3 *B*; the ability or inability of a two-state model to

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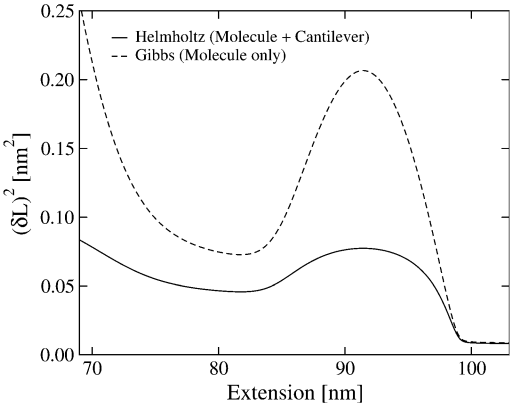

FIGURE 1 Length fluctuations for Dextran with 170 monomer units calculated with and without a cantilever (33 pN/nm); other parameters are as in Hanke and Kreuzer (3).

fit the transition in Dextran is thus related to the details of the model, and not to the analysis presented in this comment; see Hanke and Kreuzer (3) for further details.

It should also be noted that the cantilever spring constant for an experiment in this ensemble may be determined from the equation $\langle \delta f^2 \rangle = k_c^2 \langle \delta L^2 \rangle$; see Kreuzer et al. (2). If the force and length fluctuations are measured independently and the quotient of the two yields a constant equal to k_c^2, then one is definitely dealing with an experiment described by the Helmholtz ensemble, despite the force ramp.

In effect, our analysis shows that equilibrium in the Helmholtz ensemble for the coupled cantilever-molecule system should hold for both experiments performed by Walther et al. (1), regardless of the presence of a force-ramp mode. Thus the correct ensemble for the analysis of an AFM experiment is determined by the relative stiffnesses of the cantilever and the investigated molecule, and not on how the experiment is performed (force-ramp or constant-velocity). For a cantilever which is soft compared to the molecule ($k_c \ll \partial f / \partial L|_T$), one can make use of the Gibbs ensemble for the isolated molecule (2); the Helmholtz ensemble for the coupled molecule-cantilever system always applies and is the only ensemble appropriate for cantilevers of intermediate stiffness $k_c \sim \partial f / \partial L|_T$.

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