PHYSICAL REVIEW E VOLUME 60, NUMBER 2 AUGUST 1999

Direct measurement of the viscoelasticity of adsorbed protein layers using atomic force microscopy

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(Received 23 December 1998)

Thick layers of the protein lysozyme have been deposited on mica, and their force-distance hysteresis measured using atomic force microscopy in the presence of different salts. Sodium thiocyanate, which is known to lower the melting temperature of proteins and increase their solubility, increases lysozyme deformability and lowers the viscosity of the protein layer, compared with sodium chloride. Sodium phosphate, known to raise the melting temperature and lower the solubility, decreases deformability and increases the viscosity. [S1063-651X(99)50208-0]

PACS number(s): 87.15.−v, 83.50.−v

I. INTRODUCTION

Many proteins are able to build up massed adsorpta (MA) at solid/liquid or liquid/air interfaces [1,2]. Such MA form the basement membranes ubiquitous in multicellular organisms; they constitute the scaffolding to which the cells are attached [3]. Up until now, attention has been primarily focused on the identification of the constituent molecules of such membranes [3]. Here we investigate the mechanical properties of MA.

When MA are subjected to an external force, either or both of two processes may conceivably occur: movement of the proteins with respect to one another and deformation of the individual molecules. Our strategy for investigating them is to compare responses to a probe penetrating the layer in the presence of different salts.

Many salts (and other small molecules) have been found to exert a definite influence on the stability of the native conformation of a protein, which can be quantified by the melting temperature T_m [4]:

$$T_m = T_0 + K_m c, \tag{1}$$

where T_0 is the melting temperature in the absence of salt, c is the salt concentration, and K_m is the corresponding coefficient (see Table I). Protein surface properties are also influenced, for which a measure is the protein solubility S, characterized by the salting-out coefficient K_s, defined by

$$\log_{10} S_0/S = K_s c, \tag{2}$$

where S_0 is the solubility in pure water. The constants K_m and K_s vary in the same sense over a range of salts; hence salts which decrease the solubility increase the stability of the native conformation. This has been rationalized by recognizing that the native conformation is the most compact state of the polypeptide chain and, as a consequence, all other conformations have a larger solvent/protein interfacial area, whereas precipitation (aggregation) diminishes it [6]. Salts arranged in order of their K_s or K_m follow the so-called Hofmeister series, named after one of its early observers and since corroborated by a huge body of data from many diverse fields [7,8]. It can be understood at least qualitatively on the basis of (i) specific salt-peptide interactions, opposing (ii) the general exclusion of ions from the vicinity of a low dielectric medium (the protein) due to repulsive image forces [8].

Here we report direct measurements on the nanomechanical properties of protein MA using the tip of an atomic force microscope as a probe, in the presence of salts from both extremes of the Hofmeister series and from its center. We find significant differences in the viscoelastic properties of the MA under these different conditions.

II. EXPERIMENT

A. Protein

Hen egg lysozyme (HEL) >99% pure was obtained from Worthington (Freehold, New Jersey). It was selected for the following reasons: under appropriate conditions (low to moderate ionic strength) it is known to form MA at surfaces,

TABLE I. Relevant anion attributes.

Anion	T_m/°C[a]	K_m/°C M^{-1}[b]	K_s/M^{-1}[c]	$\partial g_s/\partial g_p$[d]
PO_4^{3-}			0.36[e]	
Cl^-	28.0	−1.4	0.05	−0.0145
SCN^-	16.0	−10.0	−0.25	0.0071

[a]Melting temperature of 5% gelatin gel containing the sodium salt (1M) of the anion [5].
[b]For gelatin; potassium salts [4].
[c]For the oligopeptide ATGEE; sodium salts [6].
[d]Preferential interaction parameter, g_s, grams of salt; g_p grams of bovine serum albumin; lysozyme appears to behave similarly [13].
[e]Sodium dihydrogen phosphate.

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TABLE II. Aqueous buffer compositions and their designations.

Designation	Salt	c/M
P	phosphate[a]	0.2
W	(pure water)	
S	NaCl	0.2[b]
T	NaSCN	0.2[b]

[a]A mixture of Na_2HPO_4 and NaH_2PO_4 buffering the solution at *p*H 7.4.

[b]Containing additionally 5 mM *N*-2-hydroxypiperazine–*N'*-3-ethanesulfonic acid-NaOH (HEPES) in order to buffer the solution at *p*H 7.4.

due to intermolecular electrostatic interaction between its predominantly positively and negatively charged hemispheres [9]; it has a net positive charge at neutral *p*H [10], ensuring that it adheres to negatively charged mica; it has a known structure [11] that includes all of the usual protein structural features, such as α helices, β sheets, disulfide bonds, etc.; and it has a moderate compressibility [12]. It may thus be considered to be a good ''model'' protein, with the added advantage that high purity preparations are available commercially.

B. Deposition

HEL was deposited onto freshly cleaved mica from $0.2M$ of sodium phosphate solution buffered at *p*H 7.4 and containing 100 μg/cm^3 protein. After 40 min the sample was washed with a buffer solution selected from Table II to remove any protein not firmly adsorbed and exchange the phosphate ions. By this method a protein layer estimated at several hundred nanometers thick was produced.

C. Probe

An atomic force microscope (Explorer, TopoMetrix, Santa Clara, California) equipped with a 2.2-μm-liquid scanner was used. Experiments were carried out in a laboratory-built flow-through sample holder with a pyramidal Si_3N_4 tip (radius ca. 50 nm and height ca. 5 μm), mounted on a *v*-shaped cantilever of spring constant k_c. All of the experiments used to obtain the numerical results quoted in this paper were carried out with the same cantilever, which was 0.2 mm long. Some additional experiments used a cantilever 0.1-mm long, and 0.2-mm-long cantilevers with different tip shapes. Scans took place at room temperature in the presence of various salts, all at a concentration of $0.2M$: sodium chloride, considered to be ''Hofmeister neutral'' with K_s and K_m close to zero; sodium thiocyanate, a strongly salting-out/destabilizing salt (see Table I); and sodium phosphate, a strongly salting-in/stabilizing salt. Initially, the tip-sample distance *z* was automatically decreased to just bring the tip into contact with the sample, and the feedback setpoint was established to give minimal deflexion of the cantilever.

III. RESULTS

Figure 1 shows a typical force-displacement curve. At the extreme right there is a large tip-sample separation. Moving to the left, at a certain point *a* the detector response (force) moves from *a* to *a'*. This corresponds to movement of the monitoring beam reflected off the cantilever from an initial position near the perimeter of the four segment diode detector towards the center of the four segments, without the cantilever being deflected.

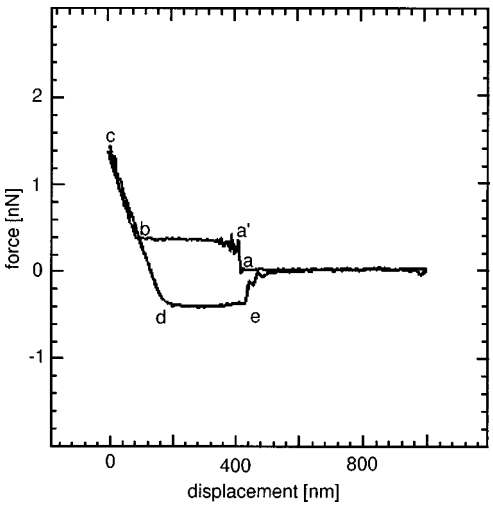

FIG. 1. Prototypical force-displacement cycle for tip movement velocities of ± 150 μm/s (see text). In the absence of protein the force $F=k_c d_c$, where the spring constant $k_c=32\pm 6$ pN/m (manufacturer's data), and the cantilever deflection $d_c=z$ (displacement) is obtained from the slope 0.19 ± 0.005 nA/nm of the measured sensor current-displacement plot.

During the displacement $a' \rightarrow b$ the beam is centered on the detector and a practically constant response, indicating a practically constant deflexion, i.e., a practically constant force, is observed. This appears to correspond to the tip moving in a viscous liquid (the protein layer), to which Stokes' law

$$F = f\eta v + F_0 \tag{3}$$

should apply, where F is the applied force, v is the velocity of tip movement, η is the viscosity of the liquid, and f is a characteristic dimension of the tip. F is plotted against v in Fig. 2. For each sample, a linear (Newtonian) region was observed (Fig. 3), which was severely truncated for the shorter cantilever. At higher v the cantilever oscillates with its resonant frequency and the average value of F no longer increases with v.

In the absence of protein, the slope $f\eta$ has a value of 3.17 ± 0.3 μN s m^{-1} in pure water. Since η has a known value of 0.894 mN s m^{-2}, we deduce $f=3.5$ mm, not an unreasonable value, considering that it is related to the dimensions of the perimeter of the cantilever.

In the presence of protein, the cantilever response depends on both the viscous drag of the entire cantilever together with that part of the tip not embedded in the protein moving in the aqueous solution, and the drag on the tip extremum embedded in the protein layer. Given that the total height of the tip is about 5 μm, of which a few hundred nm are embedded, we estimate the ratio of perimeters in contact with, respectively, the solution and the protein as 1000, with an uncertainty estimated at about 30%. Hence

$$F = [f\eta_{\text{water}} + (f/1000)\,\eta_{\text{protein}}]v. \tag{4}$$

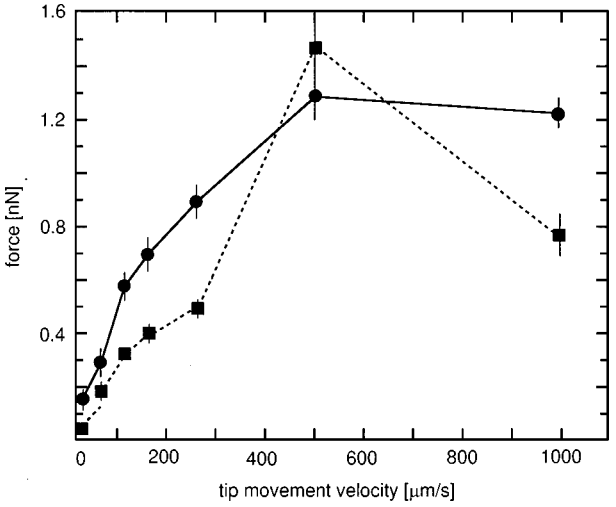

FIG. 2. F in the constant region ($a' \rightarrow b$) as a function of v. Protein layer in the presence of P, ● or T, ■. The lines are purely to guide the eye. The points show the averages of five to seven independent runs on different sites of the sample, and the error bars show the standard deviations. The slopes used to calculate η from Eq. (3) were determined from the portions of the curves lying between 80 and 260 μm/s (see Fig. 3).

The values of η_{protein} were calculated using Eq. (4) and the data from Fig. 3, and are given in Table III. The absolute values depend on the accuracy of the estimated ratio of perimeters, but since the same tip was used for all of the experiments contributing to Table III, the relative values are robust.

Moving the tip still further into the sample (Fig. 1, $b \rightarrow c$) causes a further increase in detector response. The slope dF/dz was always significantly lower in the presence of a protein layer than in its absence. The displacement z is the sum of the cantilever deflexion d_c and the protein layer deformation d_p, i.e.,

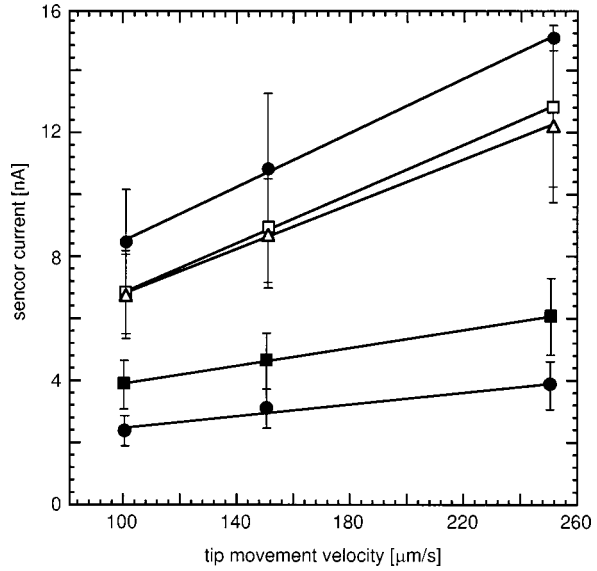

FIG. 3. Expanded plot of force vs tip movement velocity for protein layers in the presence of all the different liquids investigated (P, ●; W, □; S, △; T, ■; no protein, all solutions, ○). The straight lines are best fits to the data.

TABLE III. Viscosity [Eq. (4)] and deformability [Eq. (8)] parameters. Values are the averages of five to seven independent runs on different sites of the same sample, and the given uncertainty is their standard deviation.

Solution	η/mN s m^{-2}	k_p/mN m^{-1}	dA/dv[a]
P	1550±300	20±4	0.93±0.14
S	1050±210	15±3	0.98±0.15
T	810±160	6±0.1	0.31±0.05
W	1420±240	5±1	0.54±0.08

[a]Hysteresis is quantified by the slope dA/dv of plots of the force difference (see text) vs tip movement velocity. Since we do not offer any analysis of these results at present, they may be considered as relative values.

$$z = d_p + d_c, \tag{5}$$

and the response (i.e., F) can be written as

$$F = k_p d_p - k_c d_c, \tag{6}$$

assuming simple Hooke's law behavior. With the help of the further condition

$$k_p/k_c = d_p/d_c, \tag{7}$$

we obtain

$$F = z\frac{k_p^2 - k_c^2}{k_p + k_c}. \tag{8}$$

Since k_c is known (see the legend to Fig. 1), k_p can be determined from the slopes dF/dz and the resulting values are given in Table III.

Finally, the hysteresis was quantified as the force difference between the mean of $a' \rightarrow b$ and $d \rightarrow e$. It varied with v, and was smaller in the absence of protein. Figure 4 shows

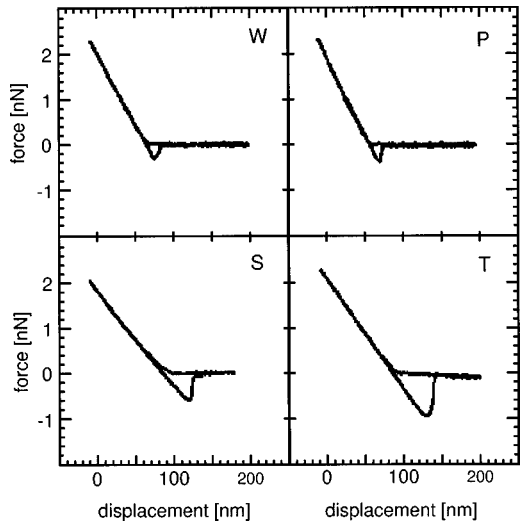

FIG. 4. Force-displacement cycles in the presence of protein showing the influence of anion type on the hysteresis. The tip velocity was ±1 μm/s. The difference in appearance between these curves and Fig. 1 is due to the difference in tip velocities.

graphically the striking differences between the protein layers in the presence of the different salts at low tip movement velocities.

IV. DISCUSSION

A. Viscosity

The highest viscosity occurs in the presence of phosphate. Collocating this observation with the fact that phosphate decreases the solubility of proteins (Table I), we may infer that if the main contribution to the viscosity is the friction caused by protein molecules rubbing against each other, then phosphate essentially roughens the surface. Conversely, the lowest viscosity occurs in the presence of thiocyanate, which must therefore lubricate the surface. Insight into the molecular origin of these effects comes from noting that the preferential ion interaction parameter (Table I) is positive for SCN^-, indicating enrichment of the salt at the protein surface [13]; since thiocyanate weakens the hydrogen bonding network of water molecules [8], a plausible consequence of SCN^- enrichment is enhanced lubrication. The Hofmeister-neutral chloride occupies an intermediate position.

B. Deformability of the protein molecules

k_p is lowest in the presence of thiocyanate, in accordance with its known destabilizing effect (Table I) and, conversely, highest in the presence of the stabilizing salt phosphate.

V. CONCLUSIONS

Detailed analysis of response-displacement curves obtained from atomic force microscopy yields estimates of the viscosity of a massed layer of adsorbed protein due to intermolecular friction, and of intramolecular deformability. The variations of these two parameters in the presence of selected salts from the Hofmeister series are well correlated with the effects of these salts on the solubility and conformational stability of the proteins.

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