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PAPER

Role of electromechanical and mechanoelectric effects in protein hydration under hydrostatic pressure

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Recent measurements of lysozyme hydration water density under non-denaturing pressure show that it is higher than that of bulk water in the same conditions. High protein hydration layer density has earlier been observed at ambient conditions and ascribed to electrostriction. We calculate the pressure-induced protein mean surface charge density increment $\Delta\sigma$. Within the hydration layer, the higher fields due to $\Delta\sigma$ lead to an additional water compression *via* electrostriction. The increment $\Delta\sigma$ is considered as due to a mechanoelectric effect in protein molecules. The mean value of the effective mechanoelectric coefficient d is calculated and compared with piezoelectric coefficients of amino acids and their compounds.

I. Introduction

It is widely accepted that characterization of protein hydration is essential for understanding the protein structure, folding and stability as well as biological functions.[1–3] It is known that the properties of hydration water differ from those of bulk water away from the protein's surface (ref. 4 and 5 and references therein). In particular, recent small-angle neutron (SANS),[6] small-angle X-ray (SAXS)[6,7] and wide-angle X-ray (WAXS)[8] scattering measurements have provided density values of the first water layer at the surfaces of some proteins higher than those of bulk water at the same temperature and pressure conditions. These proteins were: chicken egg white lysozyme,[6–8] thioredoxine reductase from *Escherichia coli*[6] and ribonucleotide reductase protein R1 from *E. coli*.[6] The higher values of hydration water density at protein molecules at ambient pressure P_{atm} have been explained as due to the effect of electrostriction in the electric field generated by protein surface charges.[9,10] Moreover, under high hydrostatic pressure the related density enhancement[7] is quantitatively different from that observed at P_{atm} and depends on the pressure applied. Herein, we provide an explanation of the latter effect. We refer to our earlier result that the calculation of the surface charge density of proteins leads to a reasonable value of the mean electric field within its hydration layer.[9] The same approach provides also a satisfactory description of the hydration enthalpy of proteins.[10]

The investigations of the coupling between the electric and mechanical properties of biological systems start with Galvani's late 18th century experiments on electrically induced mechanical response in muscle tissue. Since the 1960s it was realized that piezoelectricity is a fundamental property of biological materials, in particular that of the protein amino acids (see, *e.g.*, Vasilescu *et al.*[11]) and its investigation was recently the subject of numerous studies (see Lemanov[12] and Gruverman *et al.*[13] for recent reviews). At present, the interest in piezoelectricity and related effects in biomaterials is motivated in part by their possible applications as non-linear optical materials[14] or acoustic transducers, sensors/actuators and other electromechanical systems destined to work in a biological environment.[15] It is remarkable and essential that piezoelectric phenomena in biomaterials can be observed at a nanometre scale.[15–17] This is the scale of dimensions of the bio-macromolecules, in particular proteins. We argue that the mean density of hydration water of lysozyme observed by Ortore *et al.*[7] can be explained as due to the electric charge density inherent to the hydrophilic sites of the protein molecules as well as to the additional surface charge density appearing under hydrostatic pressure. The latter one is attributed herein to a local mechanoelectric effect in protein, possibly in its building blocks, *e.g.*, amino acid groups. Differences are emphasized between the invoked local mechanoelectric phenomena at specific sites at the surfaces of protein molecules in aqueous environment and the global ones observed in systems showing spatial periodicity (crystals, fibrils).

Recent molecular dynamics simulations of a globular protein HP-36 suggest that "high density of water close to the protein surface is more distinct for the native state of the protein" in contrast to water around the protein in an unfolded state that has a mass density close to that of bulk water.[18] If it were also applicable to lysozyme, in a regime in which the unfolding process has already begun, hydration

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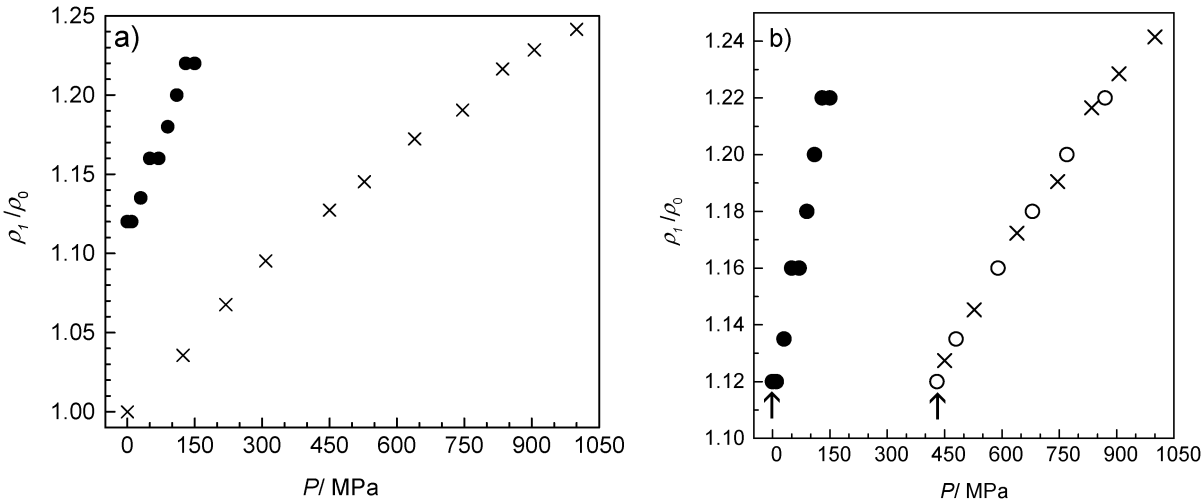

Fig. 1 Relative mass density of water ρ_1/ρ_0 as a function of pressure P. Crosses (×) mark bulk water density under pressure P from ref. 38 (read from Fig. 3 therein), ○ lie on the line (not shown) interpolating between × (*cf.* eqn (A.1)), full circles (•) mark densities of lysozyme hydration water under pressures $P = P^{h}$ from SAXS measurements (ref. 7, read from Fig. 4 therein). (a) Density (•) of hydration water under pressure $P = P^{h}$ between 0.1–150 MPa is higher than that (×) of bulk water under the same pressure and increases steeper with pressure than that of the latter. (b) Open circles (○) mark bulk water densities equal to those of the corresponding (•), but under pressures $P = P^{b}$ between 430–870 MPa. The distance between the arrows pointing to the lowest •–○ pair represents the electrostriction pressure $\Pi \cong 430$ MPa acting on hydration water at ambient conditions, other horizontal •–○ distances provide Π values at the corresponding density levels, *cf.* Table 2.

water density should *decrease* on approaching the completely unfolded protein state with increasing pressure, contrary to the *increase* in density with increasing pressure observed by Ortore *et al.*[7] (*cf.* Fig. 1). Indeed, unfolded protein makes a higher number of apolar (hydrophobic) groups accessible to the solvent than the folded one, wherein a majority of hydrophobic groups is buried (*cf.*, *e.g.*, ref. 19). Now, the hydrophobic groups are in contact with less dense water than that at the hydrophilic ones, where water is electrostricted.[10] This is corroborated by recent model calculations indicating that "maximum density in the pRDFs [proximal radial distribution functions] for hydrogens [of the hydration layer] attached to the charged/polar atoms... exceed[s] those for hydrogens attached to the more hydrophobic... atoms".[20] In this context, it remains a matter of speculation if the "development of low-density water from the protein surface to the bulk"[21] deduced from intramolecular O–H stretching bands under pressures exceeding by far our range (0.1–200 MPa) of interest could be considered as a manifestation of less dense hydration water at the surface of unfolded lysozyme.

It follows that a beginning of denaturation seems to be difficult to reconcile with the results of Ortore *et al.*[7] However, evidence can be found that there are no traces of denaturation in the range of pressures (0.1–150 MPa) applied by them.[7] It concerns the following experimental methods applied to lysozyme under pressure:

nuclear magnetic resonance (NMR),[22,23]
dynamic light scattering (DLS),[24]
Fourier transform infrared (FTIR)[25–27] spectroscopy,
Raman spectroscopy,[21]
circular dichroism (CD),[28]
ultraviolet (UV) fluorescence,[29]
X-Ray diffraction[30] and small angle X-ray scattering (SAXS).[7,27]

The papers listed above confirm that at ambient temperature there is no apparent unfolding of lysozyme structures from atmospheric pressure up to at least 200 MPa and that the minor observed deformations have an elastic character. Note that according to Kundrot and Richards[30] and Refaee *et al.*,[22] the contractions are non-uniformly distributed: the beta-sheet and one of the helices under pressure of 200 MPa are deformed less than the other helices.

Thus, it seems to be no incentive to consider unfolding processes in the context of the properties of hydration water of lysozyme observed in Ortore *et al.* experiments[7] and we turn to the explanation related to electrostatic properties of the protein surface.[9,31] The elastic character of the lysozyme globule deformations under pressures below 200 MPa can lead to changes in the polarity of particular chemical groups of lysozyme. This opens the way to the considerations on elasto-electric phenomena.

The paper is organized as follows. Firstly, a thermodynamic equation of state of an open system consisting of water in high electric field in contact with water in no field[32,33] is recalled. Hydration water at hydrophilic places of protein is considered as residing in an electric field and bulk water—as being outside the field. The equation describes the relation between the mechanical quantities (specific volume, pressure) and the electric ones (permittivity, surface charge density). Hydration water (h) is compressed by the sum P^{b} of the applied pressure P^{h} and the electrostriction pressure[34] Π due to the field. Secondly, with the knowledge of Π one calculates the mean surface charge density σ at the surface of the protein molecules with the help of the thermodynamic equation of state. The unexpected result is that, in the pressure range of the experiments,[7] the mean surface charge density σ and the mean field E acting on hydration water increase nearly linearly with increasing applied pressure. This is attributed to a mechanoelectric

effect akin to piezoelectricity. The value of the mechano-electric coefficient d—the quotient of $\Delta\sigma$ and ΔP^{h}—is calculated and compared with those of the bulk amino acid compounds. Finally, yet another experiment is proposed that could confirm the mechanoelectric properties of lysozyme in solution.

II. Thermodynamic equation of state

Our calculations are based on the thermodynamic equation of state of water in high electric field introduced earlier.[32,33]

The chemical potential of a water molecule, situated in a high electric field at the expense of the work W needed for reorienting it, is reduced by ζ_W with respect to that of a molecule outside the field. The chemical potential gradient induces the pull of the dipoles into the field. A resulting equilibrium state is characterized by a water density increase in the high field. The work L related to water compression in the field—the electrostriction work—enhances the chemical potential of water by ζ_L. At equilibrium, the latter compensates the negative increment ζ_W:

$$-\zeta_W = \zeta_L. \tag{1}$$

From eqn (1), one obtains[32] the equation of state of H_2O expressed in the variables T, P and σ as:

$$-\frac{V}{\varepsilon_0}\left\{\frac{\partial}{\partial\varepsilon}\int_0^y \frac{\sigma}{\varepsilon}\,\mathrm{d}y\right\}\left(\frac{\partial\varepsilon}{\partial N}\right)_{T,V,\sigma} = \int_P^{P+\Pi}\frac{\partial V}{\partial N}\,\mathrm{d}P, \tag{2}$$

where d denotes the differential, N is the number of water molecules in the volume V, σ is the surface charge density, ε is the permittivity of water and ε_0 is the permittivity of vacuum. $T = 293$ K was assumed everywhere. On the l.h.s. of eqn (2), $y = \sigma(1 - 1/\varepsilon)$, while $V\mathrm{d}y$ is the electric polarization increment. The integral on the r.h.s. goes from the applied pressure P to $P + \Pi$. The permittivity $\varepsilon = \varepsilon(\sigma, P, T)$ has been taken from ref. 35–37. Since the actual dependence of the volume $V = V(\Pi)$ on the electrostriction pressure Π is not available, the isotherms $V = V(P)$ of H_2O under external pressure P in the absence of field[38] are applied instead. It represents a good approximation.[32] In eqn (2) there appears only the square of the σ value or, equivalently, the square of the *value* of the electric field strength E, since

$$E = \frac{\sigma}{\varepsilon\varepsilon_0}. \tag{3}$$

These squared values are *scalar quantities*. Hence, our results are completely invariant under the direction reversal of the field or the sign reversal of the charges giving rise to them.

III. Mechanical and electric properties of the system protein–water

The relative mass densities ρ_1/ρ_0 (ρ_1 is the hydration water density, ρ_0 is the bulk water density at ambient conditions) of bulk and hydration water under pressure are compared in Fig. 1. It illustrates the fact that there are two ways of obtaining water of the same density higher than unity: either by applying the hydrostatic pressure to bulk water in no field, or by applying the electric field of suitable strength giving rise to an electrostriction pressure Π high enough to compress, jointly with a lower hydrostatic pressure, hydration water to the same density. One finds the electrostriction pressure Π immediately from experiments as the difference $\Pi = P^{b} - P^{h}$ between pressures $P = P^{b}$ acting on bulk water (○) and pressures $P = P^{h}$ acting on protein hydration water with the same density (●). Since the slope of the set of full circles (●) is higher than that of the open circles (○), Π increases with increasing P^{h}, *cf.* Fig. 1a.

The value $\rho_1/\rho_0 = 1.12$ of the mean relative density of hydration water of lysozyme at ambient conditions[7] is comparable to the earlier results by Svergun *et al.*[6] and Koizumi *et al.*[8] (Table 1). In particular, the WAXS values "are comparable to the reported value of 1.07 (Svergun *et al.*[6])".[8] The values of $\rho_1/\rho_0 > 1$ of hydration water mass density have been interpreted as due to the compression under the atmospheric P_{atm} and electrostriction Π pressures acting on hydration water in the electric field E of the atom charges of protein.[9] In Fig. 1b, the full circle with an arrow marks the hydration water density ρ_1/ρ_0 at P_{atm}. The open circle with an arrow at the same water density is situated at $P^{b} = P_{atm} + \Pi$. Their distance Π in the pressure scale amounts to $P^{b} - P_{atm} \cong 430$ MPa.

The numerical solution of the equation of state eqn (2) for $P = P^{h} = P_{atm}$ provides the corresponding mean surface charge density of the lysozyme molecule amounting to $\sigma = 0.303$ C m^{-2} (see Fig. 2, lower horizontal segment).

A. Results of the calculations of the surface charge density

We start with considering the mean relative mass density ρ_1/ρ_0 of its hydration layer as due to the action of the sum $P^{h} + \Pi = P^{b}$ (Table 2).

The lines a, b, c, and d plotted in Fig. 2 represent the solutions of eqn (2) for the values 0.1, 60, 100 and 200 MPa of the pressure P^{h}, respectively. The pressures P^{h} applied in ref. 7 fall within the range 0.1–200 MPa (Table 2). In the range 430–870 MPa of pressures $P^{b} = P^{h} + \Pi$ given in Table 2, all the lines a, b, c, and d can be approximately drawn as a common line (Fig. 2). Mean surface charge density σ and electrostriction pressure Π increase with increasing applied pressure P^{h} (Table 2).

B. Why does the surface charge density on the hydrated lysozyme molecule increase with pressure?

Could the enhanced surface charge density be due to the change in the net charge $Z(e)$ on the lysozyme molecule under

Table 1 Mean relative density ρ_1/ρ_0 of hydration water of lysozyme found by different scattering methods at ambient pressure P_{atm}. ρ_1 is the hydration water density, and ρ_0 is the bulk water density at ambient conditions

ρ_1/ρ_0	Method	Ref.
1.07	SAXS	6
~1.07	WAXS	8
1.11	SANS	6
1.12[a]	SAXS	7

[a] Read by this author from Fig. 4 in ref. 7.

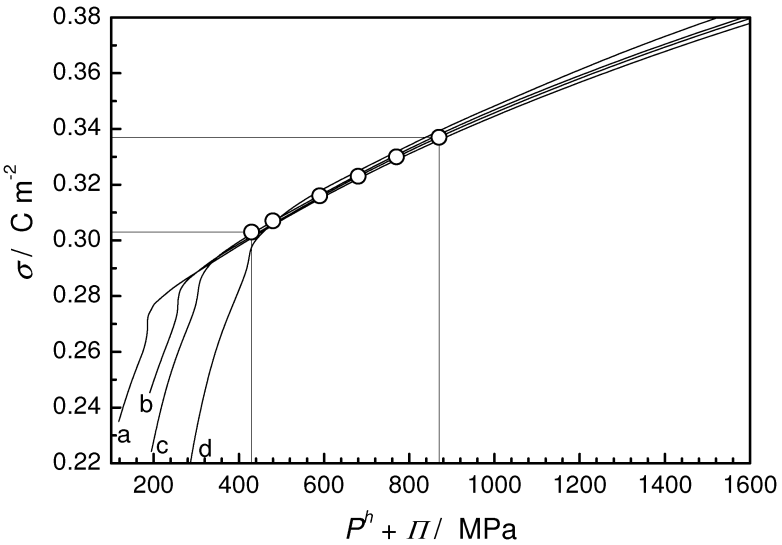

Fig. 2 Mean surface charge density σ as a function of the sum $P^{b} = P^{h} + \Pi$ of the hydrostatic pressure P^{h} and electrostriction pressure Π, calculated for P^{h} equal to $P_{atm} = 0.1$ MPa (line marked a), 60 MPa (line b), 100 MPa (line c) and 200 MPa (line d). The vertical segments mark the extreme values of $P^{b} = P^{h} + \Pi$ given in Table 2. The horizontal segments mark the corresponding extreme values of σ. The points $(P^{h} + \Pi, \sigma)$ corresponding to the relative density ρ_1/ρ_0 data of lysozyme hydration water are marked by open circles (○).

Table 2 Mean relative density ρ_1/ρ_0 of lysozyme hydration layer,[7] measured under pressure P^{h}, the same density ρ_1/ρ_0 values of bulk water under pressure P^{b} ((eqn (A.1), fitted to the data × in Fig. 1a), electrostriction pressure $\Pi = P^{b} - P^{h}$ and the corresponding calculated mean surface charge density σ values

P^{h}/MPa	ρ_1/ρ_0	P^{b}/MPa	Π/MPa	σ/C m^{-2}
0.1	1.12	430	~430	0.303
10	1.12	430	420	0.303
30	1.135	480	450	0.307
50	1.16	590	540	0.316
70	1.16	590	520	0.316
90	1.18	680	590	0.323
110	1.20	770	660	0.330
130	1.22	870	740	0.337
150	1.22	870	720	0.337

pressure? The answer is no, since $Z(e)$ of 8 electrons per lysozyme molecule noted by Ortore *et al.* is almost pressure-independent within the accuracy of about 12% (ref. 7, Fig. 4 therein). Its nearly constant value corresponds to the mean surface charge density $\sigma \cong 0.04$ C m^{-2}. The latter value is considerably lower than $\sigma \cong 0.3$ C m^{-2} calculated herein. Hence, the variation in the net charge on the globule cannot make the main contribution to the effect observed: its origin does not lie in the change in the net charge, but in the changes in polarity.

In the current work we suggest that additional local surface charge densities appearing at various places of the protein molecule surface come from the local polarizations due to local mechanoelectric effects in some strained chemical groups, *e.g.*, the amino acid ones. Thereby, a specific physical mechanism leading to the observed phenomenon is invoked and its quantification made possible. Let us mention that Ortore *et al.*[7] ascertain that "pressure induces changes in protein hydration properties"' and that "hydration modifications probably affect ...amino acid charge on the protein surface".

In general, the local surface charge density σ is dependent on the electric field originating from all charged or polar groups within the large and chemically heterogeneous protein molecule (*cf.* ref. 39). Yet varying pressure-induced local mechanical strains accompanied by a non-uniform deformation[30] likely affect polarizations of specific chemical groups only. The local polarizations of such groups could combine to lead to a global polarization characteristic for a piezoelectric on conditions of forming periodic structures (crystals, fibers). As well known, the uniform polarization of a piezoelectric is accompanied by a uniform electric field outside and a homogeneous surface charge density increment $\Delta\sigma$ on its boundary. Yet the non-uniform local polarizations inside protein molecules give rise to inhomogeneous distributions of the increment $\Delta\sigma$ on their boundaries and, consequently, non-uniform distributions of fields of different values and signs on the outside. While the piezoelectric properties can easily be observed on the macroscopic scale, the local mechanoelectric properties of protein molecules can be observed intermediately *via* their effect on the density of hydration water, as discussed in the current work. This is possible because, as already mentioned, our results are invariant *vs.* the sign reversal of the field and chemical groups with opposite polarizations have the same, and not opposite, effect on hydration water density. Kalinin *et al.*[16] describe essentially the same effect slightly differently, relating the mechanoelectric properties of the tobacco mosaic virus with the "surface piezoelectricity due to the presence of carboxyl groups on the outside and amino groups inside the [virus] shell".

C. Mechanoelectric coefficient of lysozyme in water solution

For the pressures applied by Ortore *et al.*[7] and surface charge densities σ presented in Table 2, one can find the functional dependence of σ on the applied pressure P^{h}. We admit that the strain induced within the protein molecule by the applied

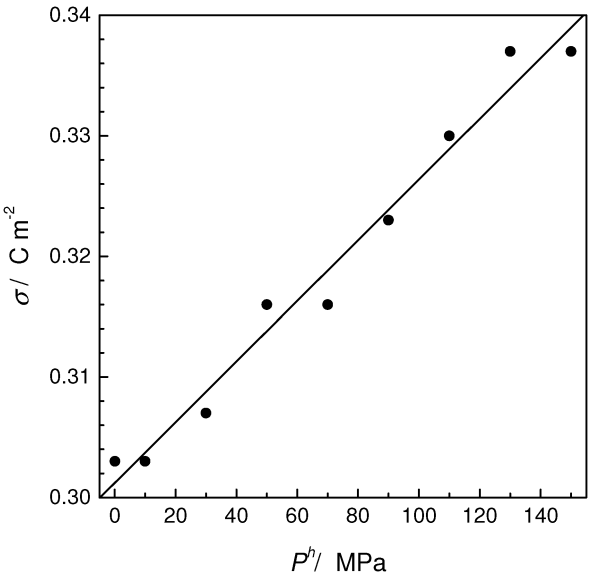

Fig. 3 Mean surface charge density σ as a function of the hydrostatic pressure P^h applied in the experiment.[7] Straight line represents a fit to the data.

pressure leads to variations in the *local* polarizations. This is akin to the variation in the *global* electric polarization involved in the piezoelectric effect in massive media. (Yet another possible mechanoelectric effect is the flexoelectric effect observed, *e.g.,* in liquid crystals). If such a local mechanoelectric effect does exist in protein molecules, the dependence of $\sigma(P^h)$ on the applied pressure P^h deduced from hydration water density *could be linear* in analogy to the piezoelectric phenomena or nonlinear in the case of analogy to the flexoelectric effect (strain proportional to the field gradient). A look at Fig. 3 leads to a rather unexpected observation that within the range of pressures applied by Ortore *et al.*[7] the calculated σ varies, to a good approximation, linearly with P^h. This observation speaks in favor of the idea that the mean surface charge density increment of a protein molecule is due to the mechanoelectric effect. Note that the nearly linear behavior observed in the range ($0.303 < \sigma < 0.337$ C m^{-2}) corresponds to a rather wide range of total pressures (applied P^h plus the electrostriction Π one, see Fig. 2). On the other hand, this range of σ corresponds to the whole range of lysozyme hydration water densities (see Table 2) investigated up to now.[7]

Let us define the slope d of the line in Fig. 3 for lysozyme in aqueous environment as:

$$d = \frac{\Delta\sigma}{\Delta P^h}, \qquad (4)$$

where $\Delta\sigma$ is the surface charge density increment corresponding to the pressure increment ΔP^h. In the range $0.303 < \sigma < 0.337$ C m^{-2}, one obtains the value $d = 2.5 \times 10^{-10}$ C m^{-2} of the mean effective mechanoelectric coefficient.

Since the linear mechanical strain-electric field dependence is characteristic for the piezoelectric effect, it makes sense to compare the results of our calculations for lysozyme in aqueous environment with the data on piezoelectricity in other biomaterials.

IV. Selected examples of mechanoelectric effects in proteins and related substances

Piezoelectricity is observed if global electric polarization either appears or varies under the influence of strain. Since many amino acids bear electric charges or dipoles, the universal presence of piezoelectricity in biopolymers[40] is not surprising. Since the size of the lysozyme globule is of about 4 nm, it is natural to look for available data on the nanometric scale. Recently, it became possible to study piezoelectric properties of biological systems (tobacco mosaic virus of radius of $\sim$9 nm) by piezoresponse force microscopy (PFM).[16] According to Kalinin *et al.*,[16] one can encounter "intrinsic piezoelectric properties of proteins" as well as "surface piezoelectricity" in the tobacco mosaic virus. Yet another example of the piezoelectric effect on the nanometre scale, apart of the mechanoelectric one discussed in the current work, is encountered in the 100 nm long bioactive peptide nanotubes made of diphenylalanine peptide monomers.[15]

How does the mean effective mechanoelectric coefficient $d = 2.5 \times 10^{-10}$ C m^{-2} of lysozyme molecules compare with the piezoelectric ones of *bulk* amino acids and their compounds? Piezoelectricity is frequent in aminoacids and has been observed in bulk DL-Ala, L-Val, L-Glu, L-Ser, and their compounds by Nuclear Quadrupole Resonance.[41] Lemanov[12] finds that L-alanine, L-valine, L-glutamic acid and DL-tyrosine show a piezolectric effect weaker than that of quartz crystals (with only $d_{11} = 2.2 \times 10^{-12}$ C m^{-2}), but the piezoelectric effect of bulk DL-alanine crystals has been found as comparable to, or even stronger than that. However, no numerical data were provided.[12,41] On the other hand, such compounds as L-histidine hydrochloride monohydrate amino acid[42] (with $d_{36} = 2.3 \times 10^{-10}$ C m^{-2}) and L-arginine hydrochloride monohydrate amino acid[14] (with $d_{16} = 2.3 \times 10^{-9}$ C m^{-2} and $d_{36} = 2.2 \times 10^{-9}$ C m^{-2}) show d coefficients of the same order of magnitude as, or higher than, that of lysozyme globules found herein.

The enumerated examples make it likely that the value of the effective mechanoelectric coefficient d of lysozyme molecules, deduced herein on the basis of the data presented by Ortore *et al.*,[7] is reasonable.

V. Discussion

We considered a system of two media in contact. The first medium, protein, becomes strained under applied pressure and admittedly shows a *mechanoelectric* effect, giving rise to an enhanced mean surface charge density. The contacting medium, hydration water, is affected by the electric field originating from the protein and compressed due to electrostriction—an *electromechanical* effect. The high hydration water density due to that compression was observed by Ortore *et al.*[7]

Since an individual lysozyme globule shows no crystalline structure, one must take care to call the effect under consideration as a mechanoelectric rather than a piezoelectric one. In the case considered, one has to do with the pressure-dependent changes in local polarizations and the corresponding changes in the local values of the surface charge density on the lysozyme molecule. It follows that the pressure applied

enhances the hydrophilicity of the specific chemical groups at the protein surface.

Since the putative mechanoelectric effect in lysozyme in water solution was characterized as a localized one, a suitable probe verifying its occurrence should be localized as well. One of the well-known methods of this kind is NMR. One could apply the nuclear acoustic resonance (NAR) in aqueous solution of lysozyme, analogous to that of NAR in a dispersion (colloid) of piezoelectric and ferroelectric $Pb(Ti,Zr)O_3$ (PZT) nanoparticles in water solution.[43] The sizes of the nanoparticles are about 100 nm for a PZT grain[43] and about 4 nm for a lysozyme globule. However, PZT nanoparticles have the mean surface charge density σ and piezoelectric coefficient d comparable to those of lysozyme globules, thus the check appears feasible.

An important question arises whether one can expect similar behavior of other proteins apart from lysozyme. Since the hydration mechanism is similar in various proteins (see, *e.g.*, ref. 10) and the presence of piezoelectricity in biopolymers is by no means exceptional,[40] answering this question can represent an interesting subject of investigation.

VI. Conclusion

Pressure dependence of the density of hydration water of lysozyme in solution found in a SAXS experiment[7] is explained in terms of electrostriction of water in the fields originating from the charges due to the mechanoelectric effect in lysozyme globules. Our conclusion relies on the finding that the calculated mean surface charge density (reflecting the strain-induced variations in the local polarizations of protein globules) depends nearly linearly on the pressure applied, in analogy to the piezoelectric effect. It can be attributed to the local mechanoelectric effect in the chemical components contained in the lysozyme molecule, including the amino acid groups. The calculated value of the effective mechanoelectric coefficient d of protein globules does not differ much from those of the piezoelectric coefficients d of some other compounds containing amino acid groups.

It is suggested that Nuclear Acoustic Resonance experiments could provide additional evidence on mechanoelectric properties of lysozyme (and other proteins) in water solutions.

Appendix A

Second-order interpolation polynomial in ρ_1/ρ_0 fitted to the bulk water data marked by $\times$ in Fig. 1a by the program MICROCAL™ ORIGIN5.0™ is:

$$P/\mathrm{MPa} = 2298.14057 - 7404.9665\rho_1/\rho_0 + 5112.17389(\rho_1/\rho_0)^2. \quad (A.1)$$

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