CHEMPHYSCHEM

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Properties of Hydration Shells of Protein Molecules at their Pressure- and Temperature-Induced Native-Denatured Transition

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Properties of water at the surface of biomolecules are important for their conformational stability. The behaviour of hydrating water at protein transition (t) pressures P_t and temperatures T_t, with the points (P_t,T_t) lying in the Native–Denatured (N-D) transition line, is studied. Hydration shells at the hydrophilic regions of protein molecules with surface charge density σ are investigated with the help of the equation of state of water in an open system. The local values of σ rather close to each other ($\sigma_D \approx 0.3\ C\,m^{-2}$) are found for six different experimental lines of the N-D transition found in the literature. The values σ_D correspond to the crossings of the total pressure ($P_t + \Pi$) vs σ isotherms at different T_t (Π−electrostriction pressure). The pressures P_t and temperatures T_t appear to be related with some selected sites at the surfaces of the protein molecules.

1. Introduction

Thermodynamics of pressure-temperature denaturation of proteins was described in the early 1970s (refs. [1,2] and references therein) with the assumption that the transition involves two conformation states: native (N) and denatured (D). Recently, the thermodynamics of pressure-temperature phase diagrams of proteins, and the phase equilibrium between the native and the denatured state N-D as controlled by the difference in the chemical potentials $\Delta\zeta = \zeta_D - \zeta_N$, has been reviewed[3]. The pressure-temperature phase diagrams have been plotted[1—4] for N-D equilibrium, that is, for conditions in which the chemical potential ζ_N of the native state is equal to the chemical potential ζ_D of the denatured state as given by Equation (1):

$$\zeta_N = \zeta_D \qquad (1)$$

It is generally known from experiment[3,5] that proteins undergo denaturation on heating and sometimes also on cooling. Proteins also undergo denaturation under the effect of pressure. It is worth recalling that already in 1914, Bridgman incidentally discovered that the white of an egg subjected to hydrostatic pressure at room temperature becomes coagulated, presenting an appearance much like that of a hard boiled egg.[6] Also, protein denaturation results from addition of denaturants and too low or too high pH.

The authors of refs. [1,2] have shown that the pressure-temperature phase diagram can be fitted to a relatively simple equation which elicits approximately elliptic contours. More recently, a number of pressure-temperature phase diagrams that can be approximated by elliptic contours have been found for various biomolecules[3—5,7]. For the pressure-temperature-pH phase diagram of myoglobin, a similar behaviour has also been observed, but no attempt to fit the phase boundaries using ellipses has been undertaken.[3] The protein structure and dynamics at high pressure are reviewed by Heremans and Smeller.[8]

It has been shown beyond doubt that water has a stabilizing effect on the native protein structure; "by binding strongly to its outer surface, water reinforces the structure of a protein, keeping the hydrophilic regions firm on the outer side of the protein"[9] (for the role played by hydrophilic regions see also ref. [10]). Hence it is important to look at the properties of the hydration shell as the properties of water in close vicinity to the polar and charged groups on the protein surface are significantly different from that in the bulk.

The surfaces of the protein molecules attain local (at about 40% of its solvent-accessible surface[11] but in the particular case of lysozyme estimated[12] to about 75%) electric charges of surface density σ that give rise to the electric fields. These local fields induce inhomogeneity in the hydration shell through:

1. Reorientation of the H_2O dipoles.[13,14] The strength of the local field is $\approx 10^9\ V\,m^{-1}$ and for such field values the water permittivity is $\varepsilon < 35$.[15,16]

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2. A denser packing of the H_2O dipoles. The hydration shells around the protein molecules are denser than bulk water, as found experimentally by Svergun et al.[17] The electric charges at the surface immersed in water exert a pull on the water dipoles into the region of the action of field *E*. Consequently, water within reach of the field is denser. This is due to the pressure Π, termed electrostriction pressure,[18] which adds to the external pressure *P* in the open thermodynamic system. If one knows the total local pressure $P+\Pi$, one can determine the local mass density[19], the local coefficient of thermal expansion, the local heat capacity[20] and local compressibility of hydration water which are dependant on the charge density σ at the adjacent charged or polar surface. Herein, the values of these local quantities are the same as they would be for bulk water under pressure $P+\Pi$. One may remark that since the hydration water is denser, it freezes at a higher or lower temperature than bulk water, depending on the pressure $P+\Pi$ (see ref. [21] and references therein). The "antifreeze" action of protein on water may be of biological relevance since "many organisms are able to survive subzero temperatures at which bodily fluids would normally be expected to freeze".[22]

The equilibrium state between the hydration shell in the high field and the bulk water outside the reach of the field is defined by the equality of the chemical potentials given by Equation (2):

$$\zeta^{i} = \zeta^{o} \qquad (2)$$

where the superscripts *i* and *o* denote the characteristics of water inside and outside the field, respectively. In this way, the description of water in terms of thermodynamic quantities is kept at the same level of theory as that of the N-D transitions known from the literature. The equilibrium condition concerning water in the field[19] [Eq. (2)], as well as the condition concerning the equilibrium N-D of the protein in solution[3] [Eq. (1)], follow from the second principle of thermodynamics. Equation (2) represents the concise form of the thermodynamic equation of state for H_2O in an open system, with one subsystem situated within the electric field.[18,19] Equation (4) shows a more explicit form of the equation; the solution of which leads to a relation between the mass density within the hydration shell and the surface charge density σ. Knowing these values, one establishes a link between hydration shell characteristics [mass density (higher than that of the bulk water)] and protein properties (surface charge density).[14]

We shall look for possible correlations between the properties of hydration water and the equilibrium line of proteins at the N-D transition (at temperature T_t and external pressure P_t, where *t* stands for transition). What happens to water in such conditions? Anticipating the unexpected result, we can say that water at P_t and T_t is univocally correlated with a specific value of the surface charge density σ (within a narrow range). Such a value is encountered at some selected hydrophilic regions which might be termed "sensitive". This intriguing result opens a way for speculating the possible role that such regions, with a specific value of σ, play in the N-D transitions. Since the result is unexpected, how credible can it be? We can only say that it is based on a statistical and thermodynamic approach to open systems comprising water in a high electric field that previously succeeded to account, mostly quantitatively for (despite no adjustable parameters involved) X-ray and neutron scattering, dielectric, picosecond photothermal/photoacoustic, thermal and electrochemical measurements.[23] The importance of the presence of water is clearly proven by the fact that the elliptic (or distorted) diagram can only be observed for protein solutions, and not for the dry solute.

It has been generally conceived up to now that the hydration shells surrounding protein molecules reside at the same temperature and pressure as the protein itself, and that the protein molecules together with their hydration shells are immersed in bulk water. Does this mean that the hydration layer and the protein are under the same conditions? Some of the water in the hydration layer is subjected to high local electric fields due to the charges at the surface of the protein molecule. Hence, the answer to the above question is no, since part of the hydration layer immersed in bulk water is subjected to an additional local electrostriction pressure Π, which is not the case for the protein molecule itself. The electrostriction pressure (making the hydration water more dense) is calculated from the equation of state of H_2O [Eq. (4)].

Let us note that the N-D (folding/unfolding) transition may proceed over several steps and can be metastable or out of equilibrium. As discussed in detail below, our approach cannot be applied to such situations.

2. Rigorous Equation of State of H_2O Expressed in Terms of T, P, σ

The rigorous equation of state of H_2O appearing in previous work[19,24] makes basis for a discussion on the pressure and thermal behaviour of H_2O in high electric fields. It is briefly recalled herein for the sake of completeness. It stems from the equilibrium condition shown in Equation (2). The electric field strength *E* is due to a charged or polar surface (say, of an electrode, an ion, a micelle, or a protein molecule) immersed in bulk water. *E* diminishes with distance of the surface and practically vanishes at larger distances. At these distances one encounters water outside the field. There is no wall between water in the field (subsystem *i*) and water in no field (subsystem *o*): the subsystem *i* represents a thermodynamic open system. 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. To attain a thermodynamic equilibrium, the chemical potential gradient created induces the pull of the dipoles into the field. We are interested in the new equilibrium state reached as a result of this process, characterized by a water density increase in the high field (say, in the hydration shell of the protein). The work *L* related to water compression in the field—the electrostriction work—enhances the chemical potential of water by ζ_L. After attaining the equilibrium, the latter compensates the negative increment ζ_W and Equation (2)

takes the form of Equation (3):

$$-\zeta_W = \zeta_L \tag{3}$$

From Equation (3), in a way described earlier,[24] one obtains the equation of state of H_2O expressed in the variables T,P,σ as [Eq. (4)]:

$$\frac{V}{\varepsilon_o}\left\{\frac{\partial}{\partial\varepsilon}\int_{a_l}^{b_l}\frac{\sigma}{\varepsilon}d\left[\sigma\left(1-\frac{1}{\varepsilon}\right)\right]\right\}\left(\frac{\partial\varepsilon}{\partial N}\right)_{T,V,\sigma}=\int_{a_r}^{b_r}\frac{\partial V}{\partial N}dP \tag{4}$$

where d denotes the differential, N is the number of water molecules in the volume V and ε is the permittivity of water. The l.h.s. represents ζ_W and the r.h.s. ζ_L, respectively. The lower limit of the integral on the l.h.s of Equation (4) is zero ($a_l=0$) and its upper limit represents the value $b_l=\sigma(1-1/\varepsilon)$. The lower limit of the integral on the r.h.s. is equal to the value of the external pressure ($a_r=P$) and the upper limit is $b_r=(P+\Pi)$, where Π denotes the local electrostriction pressure.[18] The latter represents the only unknown quantity in Equation (4). The permittivity $\varepsilon=\varepsilon(\sigma,P,T)$ has been taken from refs. [15, 16, 25]. Since the actual dependence of the volume $V=V(\Pi)$ on the local electrostriction pressure Π is not available, the isotherms $V=V(P)$ of H_2O under external pressure P in the absence of a field[26] are applied instead.

3. Results

3.1 What Happens to the Hydration Shells of Proteins in Conditions Specific to the N-D Transitions?

Figure 1 shows six lines representing the N-D conformation transitions of proteins taken from the literature.[4,7,27] The lines of transitions have been chosen for which the ranges of pressures (P_t) and temperatures (T_t) are markedly distinct. In each line, several points (P_{t1},T_{t1}), (P_{t2},T_{t2}) etc. are marked with symbols. The solutions of the rigorous Equation of state (4) of H_2O depending on external pressure P as well as temperature T are considered. On the basis of Equation (4) we have found the values of pressure $P+\Pi$ as a function of σ for those external pressures and temperatures (P_t,T_t) marked with symbols in Figure 1. Let us consider a general example of a solution of Equation (4), shown in Figure 2, not limited to the protein hydration problem.

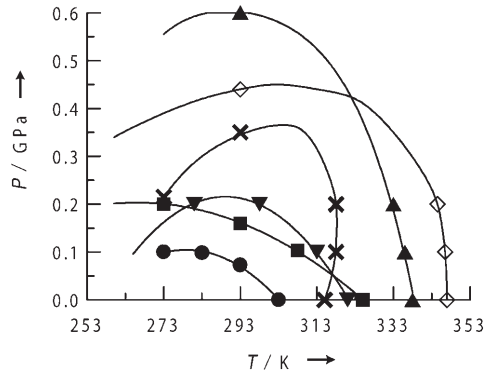

Figure 1. Pressure-temperature (P–T) plot of the N-D transitions of proteins. ●—ribonuclease,[4] ■—human interferon γ,[27] ▼—staphylococcal nuclease (SNase) (at pH 5.5),[7] x—chymotrypsinogen (at pH 2.07),[4] ▲—ribonuclease A (RNase A),[7] ◇—lysozyme (at pH 4.0).[27] The coordinates of the points marked with symbols are chosen for further calculations.

Characteristic features of the plots in Figures 2, as well as 3 and 4 are the crossing points of the isotherms ($P+\Pi$) vs σ.

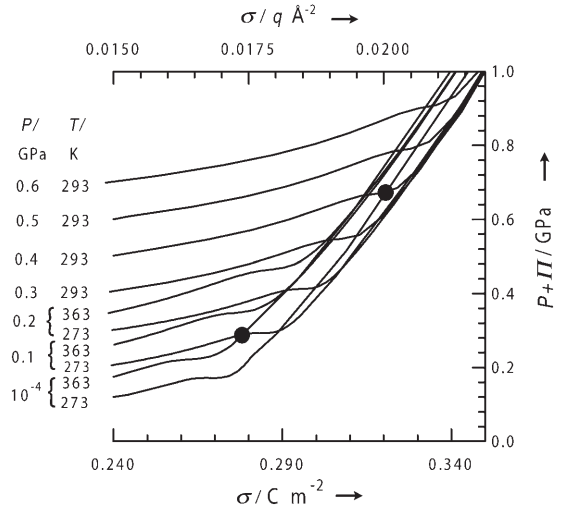

Figure 2. Pressure $P+\Pi$ acting on water in the field, plotted as a function of the surface charge density σ [C m^{-2}] at an adjacent surface. The additional abscissa axis defines σ in units of the elementary charge q per Å^2. The numbers at the left-hand side of the plot mark different values of the external pressure P and temperature T of individual lines. ● marks the exemplary crossing points of isotherms.

In Figure 2, two full circles mark the exemplary crossing points. The isotherm of water at $T=363$ K and at atmospheric pressure ($P=10^{-4}$ GPa) crosses at the point marked with the lower circle with the $T=273$ K isotherm at a higher external pressure of $P=0.1$ GPa. The isotherm at 273 K and 10^{-4} GPa crosses another one at 293 K and 0.4 GPa at the point marked with the upper circle.

Two pairs of isotherms are calculated and plotted in Figure 3 for chosen values of P,T equal to the coordinates (P_t,T_t) of the points marked with symbols in Figure 1. For the human interferon γ, the isotherm of hydration water at $T_{t1}=323$ K at atmospheric pressure ($P_{t1}=10^{-4}$ GPa) crosses at the point $\sigma_D=0.2877$ C m^{-2} and $(P+\Pi)_D=0.3150$ GPa (■) with the $T_{t2}=308$ K isotherm at external pressure of $P_{t2}=0.1$ GPa. For ribonuclease A (RNase A), the isotherm of hydration water at $T_{t1}=333$ K and external pressure $P_{t1}=0.2$ GPa crosses at the point with coordinates $\sigma_D=0.34$ C m^{-2} and $(P+\Pi)_D=0.92$ GPa (▲) with the $T_{t2}=293$ K isotherm at $P_{t2}=0.6$ GPa. For a given line and all possible pairs of points (P_t,T_t) in it marked with symbols in Figure 1, the relations of $P+\Pi$ as a function of σ have been calculated and plotted in a way described above. This procedure has been repeated for each of the six lines. The coordinates of the cross-points $\sigma_D,(P+\Pi)_D$ found in this way are collected in Table 1.

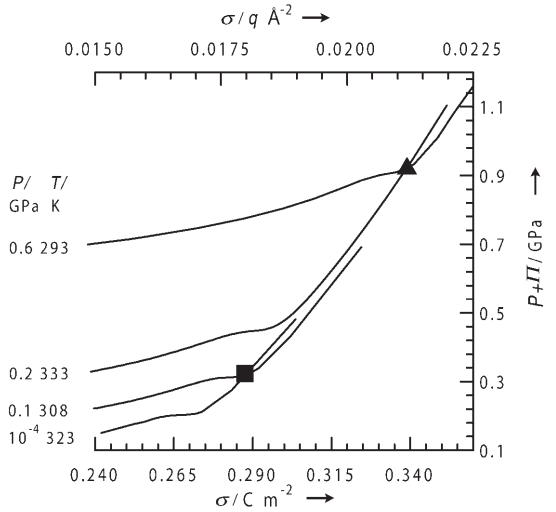

Figure 3. Pairs of isotherms of water in the shells of two exemplary proteins. The isotherm at $T_{t1}=323$ K at atmospheric pressure ($P_{t1}=10^{-4}$ GPa) crosses at the point $\sigma_D=0.2877$ C m^{-2} and $(P+\Pi)_D=0.3150$ GPa (■) with the $T_{t2}=308$ K isotherm at external pressure of $P_{t2}=0.1$ GPa for human interferon γ. The isotherm at $T_{t1}=333$ K and external pressure $P_{t1}=0.2$ GPa crosses at the point with coordinates $\sigma_D=0.34$ C m^{-2} and $(P+\Pi)_D=0.92$ GPa (▲) with the $T_{t2}=293$ K isotherm at $P_{t2}=0.6$ GPa for RNase A. The numbers at the left-hand side of the plot mark different values of the external pressure *P* and temperature *T* of individual lines.

The exemplary Figure 4 shows the $P+\Pi$ isotherms for staphylococcal nuclease (SNase) as a function of σ. Let us look for a correlation between the properties of the hydration shells and the lines of the N-D conformation transition of proteins. Surface charge density σ is distributed at the surface adjacent to the layer of water under consideration in an inhomogeneous way. Water remains at the pressure $(P+\Pi)_D$ when adjacent to the surface with the local surface charge density value σ_D. It is a local portion of the hydration shell adjacent to the surface just at the site where σ takes this specific value σ_D. The fact that the isotherms cross each other at the point corresponding to this value of σ_D means that the same pressure $(P+\Pi)_D$ acting on this portion of water can be attained for different temperatures by a well-chosen simultaneous change in the values of the external pressure *P*. Each isotherm corresponds to well-defined points (P_t,T_t) at the N-D transition line. Their crossing defines a **single** common value $\sigma_D(P_{t1},T_{t1},P_{t2},T_{t2})$ of the surface charge density and a single value of $(P+\Pi)_D$ related to **two** different points at this transition line. The surface charge density σ_D gives rise to the field locally acting on water and $(P+\Pi)_D$ gives rise to the local increase in mass density which induces the compression of water. If more than one crossing of various isotherms is considered for each transition line, the σ_D values are scattered within a relatively narrow range. The cross-points fall within the regions, one of which has been marked by a dashed-line rectangle in the exemplary Figure 4. The lengths of the edges of such rectangles parallel to the abscissa shall be denoted by $\Delta\sigma_d$ and their midpoints by σ_d. The values σ_d of the midpoints of the dashed-line rectangles and their half-widths $\frac{1}{2}\Delta\sigma_d$ are collected in Table 2.

Table 1. Coordinates σ_D and $(P+\Pi)_D$ of the crossing point of isotherms $(P+\Pi)$ vs σ corresponding to the symbols in the six lines of the N-D transitions in Figure 1, external pressure (P_t) and temperature (T_t) of the transition, permittivity [$\varepsilon(\sigma_D,P_t,T_t)$] and energy ($\mu E_{On}N^o$) of H_2O dipoles in the field.

σ_D [C m^{-2}]	$(P+\Pi)_D$ [GPa]	P_t [GPa]	T_t [K]	$\varepsilon(\sigma_D,P_t,T_t)$	$\mu E_{On}N^o$ [kJ mol^{-1}]
Ribonuclease					
0.2855	0.275	10^{-4}	303	15	14
		0.073	293	21.5	10
0.2872	0.295	10^{-4}	303	14.5	14.5
		0.1	273	27	8
0.2881	0.305	10^{-4}	303	14	15
		0.1	283	25	8.7
0.2915	0.310	0.073	293	18.5	11.7
		0.1	273	22.7	9.6
Human interferon γ					
0.2877	0.315	10^{-4}	323	15	14
		0.1	308	23	9.4
0.2957	0.415	0.15	293	24	9.3
		0.2	273	28	8
0.3010	0.423	0.1	308	15	14.75
		0.2	273	26.2	8.7
Chymotrypsinogen					
0.2895	0.337	10^{-4}	315	14	15
		0.1	318	20	11
0.2970	0.410	10^{-4}	315	11	19.5
		0.2	273	30.5	7.4
0.3070	0.510	0.1	318	16	14
		0.2	318	17	13
0.316	0.625	0.2	318	17	13
		0.35	293	17.5	14
Lysozyme					
0.2880	0.360	10^{-4}	347	16	13.4
		0.1	346	35	6.3
0.3180	0.665	0.1	346	14	16
		0.2	345	22.5	10
0.3230	0.730	10^{-4}	347	7	32
		0.1	346	29.5	8.3
		0.2	345	49	5
		0.45	293	30	8
Staphylococcal nuclease (SNase)					
0.2890	0.329	10^{-4}	320	16	13
		0.1	313	21	10
0.2980	0.420	10^{-4}	320	13	17
		0.2	283	28.5	8
0.3005	0.430	0.1	313	15	14.7
		0.2	283	25.9	9
0.3000	0.440	10^{-4}	320	13	17
		0.2	298	18.5	12
0.3050	0.476	0.1	313	14	16
		0.2	298	19	12
Ribonuclease A (RNase A)					
0.2910	0.365	10^{-4}	338	11	19
		0.1	335	17.5	12
0.3050	0.510	10^{-4}	338	10	22
		0.1	335	13	17
		0.2	333	17	13
0.3400	0.920	0.1	335	8	30
		0.2	333	9	27
		0.6	293	25	10

One feature should be noted: the exemplary crossing points lie not far from the region where the slope of the isotherms shows an abrupt change to higher values (Figure 2, 3, 4). As discussed in more detail elsewhere,[25] in this region small varia-

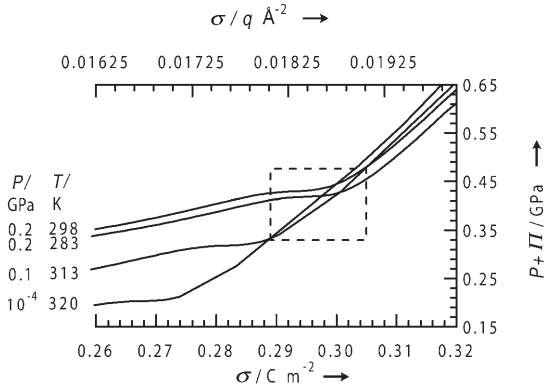

Figure 4. Four ($P+\Pi$) vs σ isotherms of water in the field plotted for the temperatures and external pressures taken in the N-D transition line of SNase (from ▼ marked in Figure 1). The isotherms cross each other within a dashed-box comprised within the limits: $\sigma = 0.289$–$0.305\ C\,m^{-2}$, $(P_t+\Pi) = 0.329$–0.476 GPa. The numbers at the left-hand side of the plot mark different values of the external pressure P and temperature T of individual lines.

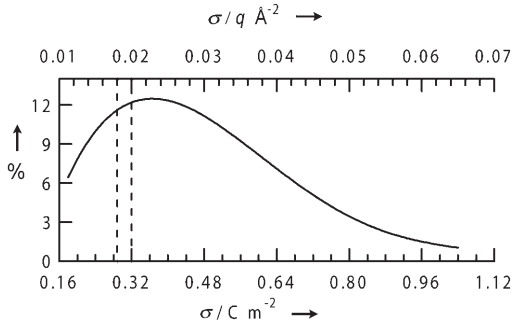

Figure 5. Statistical distribution (%) of the population of surface charge densities σ, at the surface of lysozyme.[13] The additional abscissa axis defines σ in units of the elementary charge q per Å^2. The vertical dashed lines added at $\sigma = 0.0181$ and $\sigma = 0.0203\ q\,Å^{-2}$ represent the values of σ for which the crossing of the isotherms occur ("sensitive" range for lysozyme, cf. Table 2).

Table 2. The widths of the regions ($\Delta\sigma_d$) within which crossings occur of the isotherms (see dashed-box in Figure 4) and the midpoints of these regions (σ_d).

	$\sigma_d \pm \frac{1}{2}\Delta\sigma_d$ [C m^{-2}]	$\sigma_d \pm \frac{1}{2}\Delta\sigma_d$ [q Å^{-2}]
Ribonuclease	0.289 ± 0.003	0.0180 ± 0.0002
Human interferon γ	0.294 ± 0.007	0.0184 ± 0.0004
Chymotrypsinogen	0.303 ± 0.013	0.0189 ± 0.0008
Lysozyme	0.306 ± 0.017	0.0192 ± 0.0011
SNase	0.297 ± 0.008	0.0186 ± 0.0005
RNase A	0.316 ± 0.025	0.0197 ± 0.0016

tions of σ correspond to large variations in $P+\Pi$ and, consequently, large variations in water mass density.

Let us compare the value of $\sigma_d \pm \frac{1}{2}\Delta\sigma_d$ characterizing the "sensitive" regions with the statistical distribution of the population of surface charge densities σ at the surface of lysozyme shown in Figure 5. The vertical dashed lines at $\sigma_d - \frac{1}{2}\Delta\sigma_d = 0.0181\ q\,Å^{-2}$ and $\sigma_d + \frac{1}{2}\Delta\sigma_d = 0.0203\ q\,Å^{-2}$ (cf. Table 2 for lysozyme) bound from the bottom and from the top the values of σ for which the crossing of the isotherms can occur. It is apparent that the range found is situated close to the maximum of the population of the surface charge densities σ at the surface of lysozyme,[13] but is narrow with respect to the width of the whole distribution.

3.2 Energy of the Dipole Moment of the H_2O Molecule in the Field and the H-bond Energy

In the preceding subsection, on the basis of the solutions of the Equation of state (4), it was pointed out that at the surface of the protein molecule there are distinct sites, characterized by local values σ_D of the surface charge density, which have been termed "sensitive". Herein, the question shall be considered which electric fields are encountered at these "sensitive" sites and how do they affect the H_2O molecules? The value of energy of the dipole moment μ of the H_2O molecule in the field due to the charge of surface density σ_D shall be calculated. Electric field of the strength E_{On} (cf. ref. [15], see equation (7) therein) orients the dipole moment μ of the H_2O molecule [Eq. (5)]:

$$E_{On} = \frac{\sigma(n^2+2)}{\varepsilon_o(2\varepsilon+n^2)} \tag{5}$$

where On stands for Onsager and n is the refractive index. The value of the dipole moment of the H_2O molecule is $\mu = 6.03 \times 10^{-30}$ C m; its energy in the electric field E_{On} amounts to μE_{On} and the energy of one mole of dipoles in the field E_{On} equals to $\mu E_{On} N^o$, where N^o is the Avogadro number. Energy values of H_2O dipoles μ found at $\sigma_D(P_t,T_t)$ at several external pressures P_t and temperatures T_t (points marked by symbols in Figure 1) for the six transition lines under consideration are collected in Table 1. These values of $\mu E_{On} N^o$ (Table 1) are very roughly comparable with literature hydrogen bond (HB) energy data[28,29,30] for different donors and acceptors of protons comprised in the limits specified in Table 3. In a rough approximation, the energies $\mu E_{On} N^o$ of the dipole moments of H_2O molecules in the field at $\sigma \approx \sigma_D$ at the external pressures P_t and temperatures T_t corresponding to the N-D transitions, are sufficient to disrupt the hydrogen bonds inhibiting orientation of the H_2O molecules along the field.

The above correlates with a very recent finding by Merzel and Smith. Using the MD method, they found that the number

Table 3. H-bond energies at ambient conditions (from ref. [28], p. 141–142).

H-bond type	Binding energy [kJ mol^{-1}]
OH···O	12–67
OH···N	9–43
NH···O	5–25
CH···O	10–19

of hydrogen bonds between water molecules per water molecule as a function of the separation from the surface of lysozyme decreases with decreasing separation from this surface (cf. ref. [31], figure (7) therein). It cannot be excluded that the H_2O molecules closest to the protein molecule's surface, having on average fewer HBs between themselves, could create HBs with donors or acceptors of protons belonging to the protein. If so, just the latter HBs might be able to be disrupted at sites with $\sigma \approx \sigma_D$. This interpretation eventually might lead to explaining the role of these sites in the N-D transitions, that is, their relations to the (P_t,T_t)—points marked by symbols in Figure 1.

4. Why Is the Thermodynamic Approach Valid?

A question may arise concerning the validity of the thermodynamic description of the hydration shell around the protein molecule; particularly at specific chosen parts of the shell. Thus, we shall discuss the statistical calculation of average quantities applied to the case of water at specific chosen parts of the shells of large protein molecules. Strong electric fields generated by the charges at the protein surface decay rapidly with the distance into water due to the screening by ions present in water or aqueous electrolyte. As a consequence, the water molecules within the first hydration shells at the sites neighboring high surface charge densities are in fields of considerably higher strength than those outside them. In this way, the first hydration shells around the protein molecule are distinguished from the whole electrolyte. They still can be subdivided according to the higher or lower field values conforming to the surface charge density distribution around the surface of a protein molecule. Taken together, all the portions of the first shells residing at the protein boundaries with the same surface charge density form a subsystem of water molecules in the same physical conditions, although they do not have a common macroscopic boundary. The chemical potentials of various parts of the system in equilibrium, including the set of these portions of the first hydration shells, the sets of the further more or less well defined shells, and the rest in the zero field, are equal. The subsystem of these portions of the first shells of protein molecules forms a macroscopically large ensemble of molecules in the same physical conditions and thus can be subjected to the procedure of statistical averaging leading to values of their thermodynamic parameters. Although dispersed in space, the set of these portions of hydration shells residing at the protein boundary with the same surface charge density can be treated in much the same way as a layer of molecular thickness in the theory of electrolytes at an electrode[19,32]. Thus, one can derive thermodynamic quantities concerning the macroscopic portions of hydration shells residing at the boundary of biomolecules with the same surface charge density by statistical methods, as done throughout this paper.

The phase diagrams like those shown in Figure 1 concern only the reversible, two-state N-D (folding/unfolding) transitions[33] and do not take into account the possibility of existence of intermediate (partially unfolded) or metastable states. Also, such a diagram "suggests that the unfolded state ensemble has the same properties throughout the pressure-temperature plane",[33] which may not be the case since pressure, cold and heat unfolded states can be conformationally different.[34] For metastable states the curve of phase equilibrium is usually not unique, and can depend on, for example, the velocity of the change of state process.[35] Our approach does not apply to such changes of state: we cannot define the properties of H_2O at the transition line between metastable states since such a line consisting of definite (T_t,P_t) points is not defined.

5. Summary and Discussion

There is some literature[14,31,36] which is concerned with the problem of water properties in the hydration shell of lysozyme, following the seminal paper by Svergun et al.[17]. The question of how these properties are related to the conditions at the N-D equilibrium, in these papers has not been addressed. This problem has been undertaken in the current paper. Herein the question is asked what happens to hydrating water at protein surfaces at temperatures and pressures corresponding to the N-D transitions? We have correlated the properties of the hydration shells at external pressures P_t and temperatures T_t in the electric field originating in the charged and polar (hydrophilic) regions of the surface of the protein with the N-D transitions at the same external pressures P_t and temperatures T_t. We have arrived at an unexpected result that the N-D transitions for different proteins are related with the local surface charge density not far from the value $\sigma \approx 0.3\ C\,m^{-2}$ ($\sigma \approx 0.019\ q\,Å^{-2}$). In addition, it has been noticed that the energy μE_{On} of orienting the dipole of a H_2O molecule along the related field is very roughly comparable with the HB energy (compare Tables 1 and 3). The picture that emerges from the results of the current work is that there exists some "sensitive" regions at the surface of protein characterized by the surface charge density σ_d, that are correlated with the whole N-D transition line. The values of σ_d are close to each other for various proteins (Table 2).

One should carefully distinguish between the results of our calculations concerning water in high electric fields at various temperatures T and external pressures P, and the related considerations concerning proteins. The results are:

1. The occurrence of crossings of the total pressure $P+\Pi$ isotherms (as a function of the field E or surface charge density σ) of water in the field at various T and P values.
2. The crossings occur close to the region where the slopes of the isotherms suffer an abrupt change. As known from another work, water in this region is characterized by high electrostriction.
3. Given the limits for T ($273 < T < 373$ K) and P (< 0.6 GPa), the crossings occur in a relatively narrow range of field (or surface charge density) values, irrespective of its source (charged metallic electrode, biomolecule etc.).
4. At regions of the surfaces of the protein molecules with surface charge densities comprised in a relatively narrow

range close to σ_d water reveals properties described in the preceding points. In particular, this occurs at the values of (P_t, T_t) corresponding to the N-D equilibria of proteins.

Let us discuss briefly the solvent effect. In general, protein molecules are not immersed in pure water, but rather in aqueous solutions of salts and/or organic molecules, which are often polar (see ref. [37] and references therein). The closest water molecules of ions as well as dipolar molecules are situated in their corresponding local fields E and form their own hydration shells. As a consequence, the state of hydration water of the protein molecule does not depend solely on the charge density σ at its surface. This more complex situation is not considered herein. In particular, in the limiting case, a very large concentration of ions and/or dipolar molecules in the solution can result in a lack of H_2O molecules outside the field (lack of bulk water) and can change or destroy the mechanism of the pull of the dipoles into the field acting on the hydration shell of the protein molecule.

Let us stress that the current work does not belong to the wealth of papers dealing with the N-D transitions of proteins with the role of hydration water taken into account. Rather, the main concern herein is the hydration water itself. A similar point of view has been adopted in a series of papers devoted to the possible percolation transition in the HB network in the hydration water covering a biomolecule in a dilute aqueous solution (refs. [12, 38, 39] and references therein) studied by molecular dynamics (MD). The authors of refs. [12, 38, 39] were able to correlate the temperature of the breaking (percolation transition) of the HB network spanning the biomolecule into nonspanning water clusters with the protein thermal unfolding temperature. It is remarkable that the "non-spanning largest water cluster is usually attached to some strongly hydrophilic part of lysozyme"[12] which, as their definition of HB binding refers to the closest H_2O molecules, it seems to correspond to regions of enhanced mass density in accordance with our approach. However, the question of a more detailed comparison between these two approaches is deferred until the MD approach is extended to find the effect of pressure on the percolation.[38]

It is worth noting that our calculations do not involve any adjustable parameters whatsoever and the value of σ_d in the "sensitive" region stems directly from the calculation. It could a priori occur that the value of σ_d in question is zero. This would infer regions of protein surface with no charge or polarization (hydrophobic), which is not the case. Note also that our conclusions predict the behaviour of hydration water accompanying the N-D transition of proteins and are only weakly dependent on the particular protein, at least among those considered. This is in agreement with our knowledge on the essential role of water in the protein transition. However, it relates the transition not with the total energy balance of the whole protein molecule surface, but rather with a small selected part of it. Since the general balance certainly cannot be neglected, one is tempted to speculate that the "sensitive" regions selected by their value of σ_d might be, in some way, active in initiating a transition governed thereafter by the total balance.

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