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WATER DYNAMICS AND STABILITY OF MAJOR BLOOD PROTEINS AT PRE-DENATURATION STAGE

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ABSTRACT. We investigate the temperature effect on the size and stability of two major blood plasma proteins, human serum albumin and fibrinogen in aqueous NaCl solution. Dynamic Light Scattering measurements were carried out in the physiological temperature range up to 45°C. The analysis of the results provided the temperature dependences of the macromolecular hydrodynamic radius and the ζ-potential. For albumin the hydrodynamic radius remained unchanged, while the ζ-potential increased sharply at approximately 40°C. For fibrinogen the radius increased significantly above 45°C and the ζ-potential increased similar to albumin at slightly below 40°C. The dynamics of albumin macromolecule was simulated using classical Molecular Dynamics, which showed no change in the gyration radius, root mean square deviation, and the composition of disulfide and salt bridges, but substantial change in the secondary structure of the protein. We conclude that these changes in the structure and dynamics of the proteins are correlated with the qualitative change of water dynamics at 42°C in the hydration shell of the proteins.

1. Introduction

The stabilising effects of water on the protein structure have been discussed in (Takano *et al.* 2003; Park and Saven 2005). Single water molecules stabilise proteins by filling internal cavities. The backbone residues of loops and other polar atoms interact with the water molecules buried in the protein core. Buried water may also act as lubricant to favour loop dynamics. Various techniques have been used for investigations of the solvation properties of peptide and protein aqueous solutions (Abseher *et al.* 1996; Garcia and Hummer 2000; Makarov *et al.* 2000; Tarek and Tobias 2002; Pizzitutti *et al.* 2007; Frauenfelder *et al.* 2009; Sterpone *et al.* 2012; Rahaman *et al.* 2013) . The static and dynamic properties of the hydration water and their relation with the properties of the hydrated molecules have been investigated in recent experimental studies (Comez *et al.* 2016). Rotational and translational motion at the picosecond timescale, and the vibrational density of water molecules surrounding proteins and peptides were studied by quasi-elastic (QENS) and inelastic neutron scattering (INS) techniques (Russo *et al.* 2005; Frölich

et al. 2008; Khodadadi *et al.* 2010). Brillouin neutron scattering technique was used to investigate the fast collective dynamics in biomolecular solutions (Orecchini *et al.* 2009). The combination of QENS and Molecular Dynamics (MD) simulation has revealed spatial heterogeneity in the first hydration shell of the protein molecule. As it was shown the water translational and rotational dynamics within the critical hydration shell are slow in comparison with the bulk water and molecules of the first hydration layer form a barrier for the outer water molecules (Russo *et al.* 2004). The INS results (Orecchini *et al.* 2002; Paciaroni *et al.* 2008, 2009) indicate that the dynamics of the biomolecule is slaved by the surrounding water in pico- and nanosecond timescale reflecting the re-formation of the hydrogen bonds effect on the motion of the protein's surface. THz spectroscopy investigations (Born *et al.* 2009; Nibali and Havenith 2014) provided the possibility to probe the influence of the water structure on peptides and proteins and detect the radii of the hydration shells for such proteins as lysozyme, myoglobin, BSA (Bye *et al.* 2014) and (Sushko *et al.* 2015) and ubiquitin (Born *et al.* 2008). The values of the hydration shell thickness obtained in (Born *et al.* 2008) are substantiated by NMR studies (Mattea *et al.* 2008; Qvist *et al.* 2008), X-ray scattering (Svergun *et al.* 1998), and neutron scattering (Pertsemlidis *et al.* 1996).

Molecular dynamics studies have revealed that the perturbation of the inter-helical hydrogen bonds, which are important determinants of the local protein structure, can be coupled to the rapid changes in water dynamics (Del Val *et al.* 2014). It was shown in (Jiang *et al.* 2016) that water clusters are driven by the binding of various ions.

In our study we focused on the thermal stability of major blood plasma proteins (human serum albumin (HSA) and fibrinogen) and the dynamics of surrounding water at different temperatures. The region of the especial interest is the temperature point of 42°C , where in the vicinity of this temperature the spatial connectivity between H-bonded linear molecular chains is disrupted (Lokotosh *et al.* 2000; Bulavin *et al.* 2008; Fisenko *et al.* 2008; Fisenko and Malomuzh 2009; Lokotosh *et al.* 2010). Possibly this temperature can be considered as the threshold temperature of protein conformational stability and the dynamics of water can be related to the protein's conformational changes (Rezaei-Tavirani *et al.* 2006). As a subject of study we considered the temperature dependences of the hydrodynamic and the gyration radii and the ζ-potential, which can be used as an indicator of the stability and the degree of repulsion between adjacent protein molecules. Thus, we tried to correlate the changes of the macromolecular size and the ζ-potential with the thermal dynamic in water. We also have presented the results of MD simulation for HSA.

HSA and fibrinogen play important roles in functioning of living organisms and perform many different functions such as the transport of lipids, hormones, vitamins and metals in the circulatory system, the regulation of acellular activity and the immune system. HSA accounts for 55% of blood proteins, and it is a major contributor to maintaining the osmotic pressure of plasma to assist in the transport of lipids, steroid hormones, metabolites and binding of different ligands (Rothschild *et al.* 1988; Carter and Ho 1994; Kratz 2008; Rzga and Bal 2010). Fibrinogen comprises 7% of blood proteins. It is one of the key proteins in thrombosis and the conversion of fibrinogen to insoluble fibrin is essential for blood clotting.

2. Methodology, results, and discussion

2.1. Photon correlation spectroscopy. The photon correlation spectroscopy (PCS) (Cummins 1974) was actively used in the investigations of aqueous solutions of DNA, proteins, alcohols, and polymers (Magazù *et al.* 1989; Donato *et al.* 1996; Jannelli *et al.* 1996; Magazù *et al.* 1997; Branca *et al.* 1999; Faraone *et al.* 1999; Magazù *et al.* 1999a,b; Sidebottom 2007; Sidebottom and Tran 2010). Some studies based on the PCS showed that water furnished the temperature evolution of the hydration number, while incoherent quasi-elastic neutron scattering (IQENS) revealed the presence of entangled water and evidenced the effects of H-bond on the diffusive motions. The comparative studies of the rotational relaxation times obtained by IQENS and PCS for such systems as polymeric aqueous solutions, alcohols and homologous disaccharide aqueous solutions separated the self-particle contribution from the collective one (Magazù 1996; Magazù *et al.* 2007).

Hydrodynamic radius. At low concentrations of protein (Magazù 1996; Magazù *et al.* 2007) the macromolecular hydrodynamic radius is related to the diffusion coefficient by the Einstein-Stokes relation

$$R_H = kT/6\pi\eta D \tag{1}$$

where, T is temperature, k is the Boltzmann constant and η is the dispersant viscosity. The measurements were carried out in the range of temperatures 30-45°C, which corresponds to physiological temperature interval, using commercial spectrometer Zetasizer Nano ZS (ZEN3600).

ζ-potential (ZP).. We have measured ZP in the temperature interval 30-70°C in order to correlate the dynamic of water in the 'hydration shell' with conformational changes of a macromolecule. The ZP magnitude is one of key parameters which describes the surface charge of a protein and characterises its hydration layer (Sze *et al.* 2003; Maduar *et al.* 2015; Bhattacharjee 2016; Fischer and Schmidt 2016; Predota *et al.* 2016). The dynamics of surrounding ions is affected by the charge distribution at the macromolecule surface. We can separate two regions in the macromolecule environment: the so called Stern layer or an inner region where a macromolecule is tightly bound with the surrounding molecules and an outer region where the molecules are not strongly attached with the surface atoms of the protein. A protein macromolecule moves together with the surrounding molecules and the ions in the Stern layer, while in the outer region the drift of the macromolecule and the surrounding molecules is uncoupled. A boundary between the Stern and diffusive layers is the surface of the hydrodynamic shear or the so-called slipping plane and the potential existing at this boundary is the ζ-potential. ZP was defined using electrophoretic light scattering technique based on the measurement of electrophoretic mobility by the application of the Henry equation

$$\mu_r = \frac{2\varepsilon_r\varepsilon_0\zeta f(Ka)}{3\eta} \tag{2}$$

where, ε_r is the relative permittivity/dielectric constant, ε_0 is the permittivity of vacuum, ζ is the zeta potential, η is viscosity, $f(Ka)$ is the Henry's function, which generally accepts one of two values either 1.5 or 1.0. The electrophoretic mobility was defined with

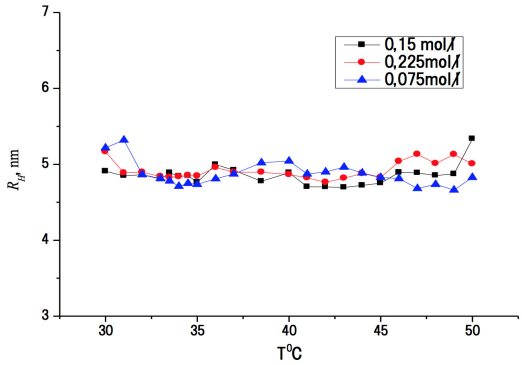

FIGURE 1. The hydrodynamic radius R_H of HSA in the temperature interval 30 – 50°C at HSA concentration 1mg/ml and NaCl concentrations: 0.075 mol/l, 0.15 mol/l, 0.225 mol/l.

the accuracy of $0.12 \cdot 10^{-8} m^2/V \cdot s$ for particle diameters 3.8nm – 100 microns. The error in calculation of the zeta potential did not exceed 5%.

Albumin. Such factors as pH and temperature affect the protein structure and dynamics (Brown 1977). HSA is a single chain protein with 585 aminoacid residues with a predominant α-helical heart-shaped structure (Carter *et al.* 1989; Carter and Ho 1994). For illustrative purpose we demonstrate here the measurements of HSA aqueous solutions at various NaCl concentrations, which have been already published in our previous paper (Atamas *et al.* 2017). One can see that the value of the hydrodynamic radius is practically constant in the temperature interval 30 – 50°C (Figure 1) and NaCl concentration does not cause a change in the hydrodynamic radius value. The temperature dependence of ZP in the temperature range 25-70°C is presented in Figure 2.

According to general principles (Hunter 1981; Sze *et al.* 2003) a colloid system usually loses stability when the ZP magnitude decreases to less than 25-30 mV (positive or negative). Thus, the vicinity with zero value of ZP (the isoelectric point, or IEP) is the region of instability where proteins may agglomerate in aqueous solutions or conformational changes may happen in the macromolecule's structure at the initial stage of denaturation process. In our case we observe the decrease of ZP magnitude starting from -27mV at temperature 35°C and pH 7.4 up to zero value around the temperature point 42°C, then the value of ZP varies from -2.5mV to 2.5mV up to the denaturation temperature.

Fibrinogen. Fibrinogen has been intensively studied by different methods, including transmission electron microscopy (TEM)(Hall and Slayter 1985; Weisel *et al.* 1985; Veklich *et al.* 1993), atomic force microscopy (AFM) (Agnihotri and Siedlecki 1999, 2004; Yermolenko *et al.* 2011; Zavyalova *et al.* 2011; Protopopova *et al.* 2015) and X-ray diffraction (Spraggon *et al.* 1997; Kollman *et al.* 2009; Protopopova *et al.* 2015). A fibrinogen macromolecule is a dimer, which comprises 2964 amino-acid and 4 carbohydrate residues. Each monomer unit consists of three non-identical polypeptide chains. The monomer units as well as

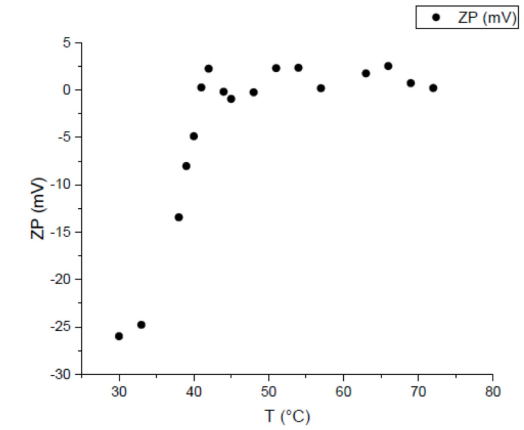

FIGURE 2. Temperature dependence of the zeta potential (ZP) for albumin aqueous solution (pH=7.4 at temperature 30°C) and NaCl concentration of 0.075 mol/l.

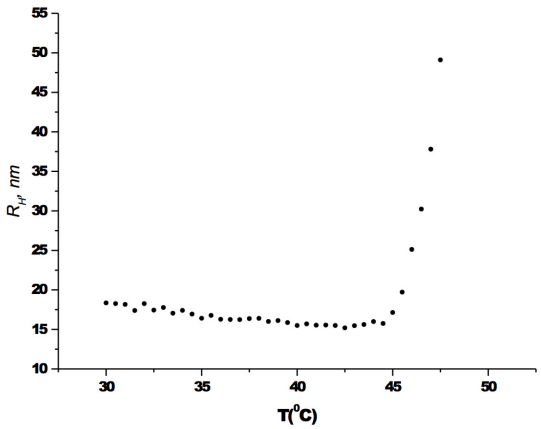

FIGURE 3. Temperature dependence of the fibrinogen macromolecular hydrodynamic radius R_H pH=7.4 and I=0.15 (NaCl concentration of 0.15 mol/l).

polypeptide chains are linked by the disulfide bonds. The measurements of R_H (Figure 3) and ZP (Figure 4) have been carried out under physiological condition pH=7.4 and ionic strength I=0.15 or NaCl concentration of 0.15 mol/l in the temperature interval 30–50°°C.

We can see insignificant changes of the hydrodynamic radius in the temperature interval 30-45°C where starting from 45°C the value of R_H increases significantly. Thus, in the case of fibrinogen we observe completely different behavior of the hydrodynamic radius

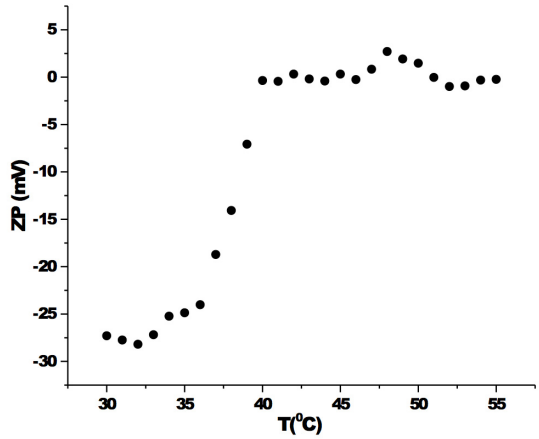

FIGURE 4. Temperature dependence of the zeta potential (ZP) for albumin aqueous solution (pH=7.4 at temperature 30°C) and NaCl concentration of 0.075 mol/l.

with temperature increase in contrast to albumin and substantially similar temperature dependence for ZP with the location of IEP in the vicinity of 42°C.

2.2. Simulation. In this section we demonstrate main results (Atamas *et al.* 2017).of the HSA macromolecule dynamics in aqueous solution at different temperatures obtained using the multifunctional package HARLEM, which combines the methods of molecular and Brownian dynamics. We have analysed the temperature effect on the gyration radius, the distances between the sulphur atoms (S_γ) in pairs of cysteines and COO-groups of aspartic or glutamic acids and NH3-groups of lysine or arginine (salt bridges). The values of the gyration radius in the temperature interval 25-45°C are presented in Fig.5. The gyration radius was calculated with using formula (3) and the crystallographic data (Sugio *et al.* 1999) for the coordinates of atoms, which change with temperature

$$R_g^2 = \sum m_i r_i^2 / \sum m_i \tag{3}$$

where, r_i is the distance from the centre of mass to the *i*-th atom, m_i is the mass of the *i*-th atom.

The gyration radius also remains unchanged in the temperature range 25-50°C. The values of the gyration radius differ from the hydrodynamic radius by approximately 1.5 nm, because the hydrodynamic radius indicates the *apparent* size of the solvated particle, while the gyration radius is calculated as the average distance from the centre of mass to the macromolecular surface (Kok and Rudin 1981). Interestingly, this value coincides with the values of the hydration shell (Bye *et al.* 2014; Sushko *et al.* 2015).

We present the results of simulation (Atamas *et al.* 2017) for the distances between S_γ (1) and salt bridges (2). The data for the dynamics of amino-acid residues studied by calculating the root-mean-square displacement (RMSD) are presented in Figure 6.

Distance between S_γ atoms, Å										
Disulfide bridges		**25°C**	**27°C**	**30°C**	**32°C**	**35°C**	**37°C**	**40°C**	**42°C**	**45°C**
Cys360	Cys315	2,079	1,936	2,085	2,079	2,079	2,067	2,09	2,054	2,032
Cys359	Cys368	2,099	2,009	2,109	2,033	1,962	2,085	2,025	2,107	2,038
Cys101	Cys90	2,054	2,064	2,061	2,038	2,043	2,042	2,061	1,993	2,083
Cys75	Cys91	2,029	1,965	2,01	1,969	2,01	2,066	2,017	2,011	1,979
Cys460	Cys476	2,046	2,002	2,062	2,044	2,016	2,027	2,042	2,049	2,068
Cys475	Cys486	2,114	2,07	2,111	2,09	2,091	2,054	2,117	2,021	2,119
Cys53	Cys62	2,043	2,009	2	1,978	2	2,056	1,949	2,085	2,048
Cys264	Cys278	2,105	1,984	1,961	2,076	2,081	2,009	1,965	2,046	2,138
Cys288	Cys277	2,053	2,091	2,059	2,05	2,011	2,078	2,01	2,032	2,064
Cys168	Cys123	2,036	2,051	2,038	2,051	2,053	2,103	2,03	2,033	2,001
Cys167	Cys176	2,062	2,108	2,036	2,077	2,079	2,077	2,083	2,025	2,034
Cys513	Cys558	1,992	2,054	1,989	2	2,023	2,124	2,022	2,047	2,028
Cys557	Cys566	1,981	2,049	1,981	2,001	1,97	1,996	2,067	2,041	1,996
Cys436	Cys447	1,977	2,021	1,982	1,998	2,021	1,972	2,046	1,993	1,979
Cys437	Cys391	2,072	1,996	2,036	2,046	2,054	2,029	2,009	1,951	2,081
Cys199	Cys245	2,069	2,039	2,09	2,04	2,051	1,928	2,111	2,025	2,082
Cys244	Cys252	2,029	2,054	2,078	2,025	2,019	2,049	1,938	2,102	2,052

TABLE 1. Distances between S_γ atoms in disulfide bridges of HSA (Atamas *et al.* 2017).

Amino-acid residues		\ Å	Amino-acid residues		\ Å	Amino-acid residues		\ Å
ASP72	ARG98	4,08	GLU424	ARG458	3,86	ASP255	LYS239	3,59
ASP108	ARG196	3,70	GLU441	ARG444	4,04	ASP313	LYS312	3,60
ASP236	LYS32	3,71	GLU449	ARG347	3,90	ASP517	LYS520	3,47
ASP254	ARG10	4,13	GLU449	ARG484	3,92	ASP254	LYS12	3,65
ASP450	ARG194	4,18	ASP13	LYS12	3,74	GLU6	LYS439	3,61
GLU6	ARG10	3,91	ASP108	ARG458	3,95	ASP86	ARG81	4,37
GLU16	LYS51	3,94	ASP108	ARG465	3,33	ASP311	LYS316	3,79
GLU73	LYS76	3,02	ASP311	LYS312	3,65	ASP258	LYS261	3,62
GLU140	ARG143	4,07	ASP313	LYS316	3,33	GLU332	ARG335	4,07
GLU152	ARG256	4,05	ASP450	LYS439	3,89	ASP323	LYS322	3,95
GLU186	ARG435	4,08	GLU152	LYS261	3,53	GLU424	ARG427	4,50
GLU243	LYS239	3,04	GLU530	LYS504	3,61			
GLU351	LYS375	3,41	GLU570	LYS556	3,19			
GLU357	LYS322	3,30	GLU570	LYS573	4,37			

TABLE 2. Lengths of salt bridges in HSA amino-acid residues (Atamas *et al.* 2017).

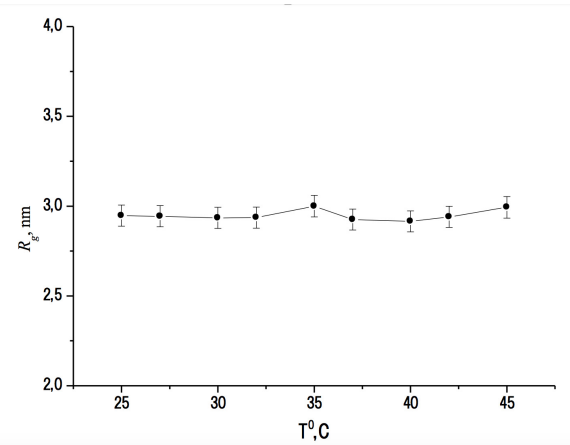

FIGURE 5. The gyration radius of HSA calculated from the simulation data (Atamas *et al.* 2017).

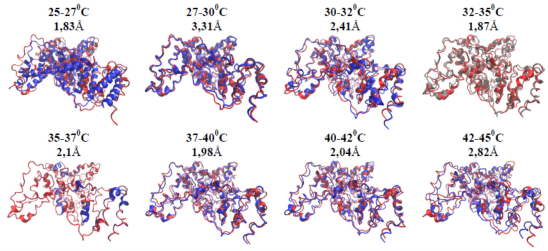

FIGURE 6. The amino-acids residue displacements of HSA and the RMSD values at corresponding temperature intervals. Blue and silver colours indicate the conformational state in the first temperature point, red indicates the conformational state in the second temperature point for every snapshot (Atamas *et al.* 2017)

We can see that the lengths of disulfide bridges and salt bridges are stable in the investigated temperature interval. The conformational changes of the structure by comparing the residue positions in the reference points were studied by the evaluation of RMSD values for close conformational states in certain temperature intervals with respect to the initial structure defined by PDB (Sugio *et al.* 1999).

We can conclude that the overall structure of the macromolecule does not undergo significant transformations with the temperature increase, only slight deflection of the RMSD values is observed with respect to the reference temperature in certain temperature interval.

3. Conclusions

The results presented here demonstrate specific correlation between the stability of two major blood plasma proteins, albumin and fibrinogen, and water dynamics in the

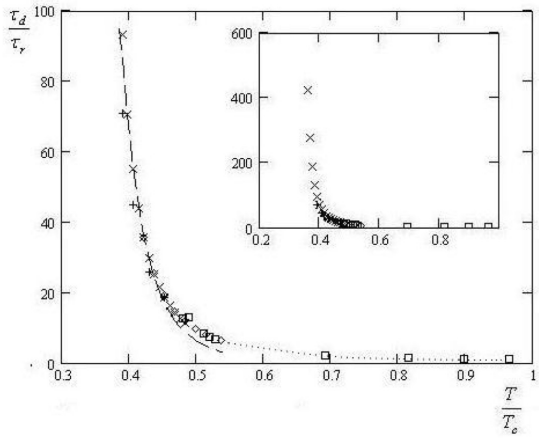

FIGURE 7. The behavior of $\tilde{\tau}_d(t)$ as a function of dimensionless temperature: $t = T/T_c$, where T_c is the critical temperature. Experimental data are taken from the works (Simpson and Carr 1958; Pruppacher 1972; Okada *et al.* 1999; Eisenberg and Kauzmann 2007). Points present interpolation values of τ_d , the dot line corresponds to exponential dependence.

physiological temperature range. For albumin the hydrodynamic radius and the simulated gyration radius, the stability of disulfide bonds and salt bridges support the fact of the macromolecule stability in the temperature range 25-50°C. The MD simulations provided the value of the gyration radius supported by the results (Bye *et al.* 2014; Sushko *et al.* 2015). Furthermore, the value 1.5 nm coincides with the thickness of the hydration layer obtained by THz spectroscopy (Bye *et al.* 2014). In the case of fibrinogen the R_H measurements indicate that the structure of the macromolecule undergoes significant transformations with the temperature increase and the temperature interval 42-45° can be considered as an initial stage of irreversible conformation changes in the macromolecule (denaturation). For both proteins the magnitude of the ζ-potential suggests instability in the protein structure and the surrounding. ZP behaviour may imply conformational changes in the macromolecule structure, surface modifications and also it indicates changes in water dynamics in the hydration layer close to the temperature 42°C. The concept of characteristic times (Bulavin *et al.* 2008; Fisenko *et al.* 2008; Fisenko and Malomuzh 2009; Lokotosh *et al.* 2010) gives deeper understanding of the water collective dynamics around the protein and the correlation with conformation changes. According to this concept the rotational motion of a molecule in the bulk is directly reflected in the dipole relaxation. The temperature dependence of the dipole relaxation time $\tau_d(t)$ is presented in Figure 7.

The value of dimensionless $\tilde{\tau}_d(t) = \tau_d(t)/\tau_r$ is close to unity in the temperature interval $0.6 < t < 1$ with the implication that the rotation of the molecules tends to be quasi-free. Here $\tau_r \sim 2\pi/\omega_T$ is the characteristic time of a molecule complete turn, $\omega_T \sim \sqrt{k_B T/I}$ is the characteristic value of the angular velocity, $I \sim m_H r_{OH}^2$ is the inertia moment of a water molecule (m_H is the mass of the hydrogen atom and is the distance between the hydrogen

and oxygen atoms). The essential deviation of $\tilde{\tau}_d(t)$ from unity is observed in the region $t < 0.5$ and especially in the super cooled region $t < 0.42$.

For these temperatures the behaviour of $\tilde{\tau}_d(t)$ is approximated by the exponential function: $\tilde{\tau}_d = \tilde{\tau}_d{}^{(0)} exp(\varepsilon_H/t)$, where $\tilde{\tau}_d{}^{(0)} = 5.1 \cdot 10^{-4}$ and $\varepsilon_H = E_H/k_B T_c = 4.71$ is the characteristic activation energy. The estimated value of takes the same magnitude as the H-bond. Thus one can suggest that at temperatures the rotation of the molecules has a discontinuous character: every rotation on a small angle is realised after the destruction of the H-bond. It is clear that the character of the molecular rotation reflects the stability of the H-bond network in the bulk water. The stability of the H-bond network is significantly changed in the close proximity of 42°C or the dimensionless value 0.5.

This fact should be manifested in all phenomena whose specificity is caused by the H-bonds. Similar behaviour of the H-bond network is appropriate for proteins aqueous solutions due to the fact that the H-bonds are formed between: 1) water-water molecules, 2) water molecules and fragments of protein macromolecules as well as 3) various fragments in protein macromolecules. Therefore the essential changes in the temperature behaviour of the ζ-potential are well substantiated. In other words, the destruction of the H-bond networks in the blood plasma plays determinative role in the death of living organisms starting from the temperature of 42°C.

Acknowledgments

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