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Ordering effect of protein surfaces on water dynamics: NMR relaxation study

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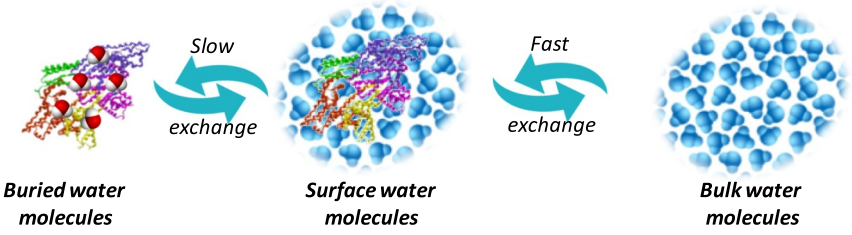
HIGHLIGHTS

- An NMR relaxometry method was developed.
- Water molecules ordering contribution is a function of protein size and conformation.
- Average correlation time of water molecules in protein hydration layers was studied.

GRAPHICAL ABSTRACT



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ABSTRACT

Proteins in solution affect the structural and dynamic properties of the bulk water at the protein-water interface, resulting in a contribution to the order of the hydration water. Theoretical and experimental NMR relaxation methods were developed to study the dynamic properties of water molecules in the protein hydration shell. Water non-selective and selective relaxation rates, were shown to be sensitive to contributions from ordered solvent molecules at protein surface. The average rotational correlation time of water molecules in the protein hydration shell was determined for three protein systems of different size: ribonuclease A, human serum albumin and fibrinogen. The knowledge of these properties is an important step toward the determination of the size of the water ordering contributions originate in proteins systems.

1. Introduction

Water molecules surrounding a protein system are highly organized, giving rise to structures of different complexity and creating a long range ordering effect to a distance of several Angstroms [1,2]. These water molecules show restricted dynamics compared to the free tumbling motion of bulk water molecules [3–5]. Water solvation of biomolecules [6,7] and metal ions [8–12] is an essential process in chemical and biochemical functioning [13–15]. Moreover, protein hydration leads to an increased flexibility, an important property for protein functional roles [16–22]. In the past, theoretical and experimental studies have highlighted the role of water and specifically of hydrogen bonds formation in the protein-protein interactions [23–26], as well as in the study of metal-macromolecules recognition processes [27–31].

In the present study, we investigate the dynamical state of water molecules interacting with the protein molecules. Three proteins with different conformation and molecular weight were studied to highlight the water behavior in specific surrounding environments. In particular, the ribonuclease A (RNase), human serum albumin (HSA) and fibrinogen were used. RNase is a small (~13.7 kDa) and stable enzyme [32]; HSA is a globular, water-soluble, non-glycosylated polypeptide that contains 585 amino acids with a molecular weight by ~66.2 kDa [33] and fibrinogen is a ~340 kDa homodimeric glycoprotein consisting of 2Aα, 2Bβ, and 2γ polypeptide chains linked by 29 disulfide

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bridges [34]. Proteins in solution can define three water environments, *i.e.*, the buried water molecules (which are integral part of the protein structure, often present in protein crystals [35,36]), the surface water which is present in layers around the macromolecules, and the bulk water. Different dynamical properties are typical of each environment [37–39]. NMR relaxometry can be used to characterize protein dynamics and to determine the rotational correlation time of the buried water molecules [40–43]. Water proton spin-lattice relaxation rates, obtained in selective and non-selective modes were used to determine the rotational correlation time of the water molecules in the layers around a protein surface [44].

Bulk water dynamics were previously characterized, revealing orientation relaxation of the order of picoseconds [45–47]. The results indicate that the macromolecules induce an ordering effect on the water molecules present on the protein surface. This study reports an NMR relaxometry method aimed to quantify the effect of water in protein systems, highlighting that the ordering contribution on water molecules is a function of both protein size and conformation. This will indicate that the protein conformational change, induced by protein-ligand interaction, modifies the protein ordering effect of the water molecules in the protein surface layers.

2. Materials and methods

2.1. Experimental

Ribonuclease A, human serum albumin and fibrinogen were purchased from Sigma Chemical Co. and used without any further purification.

The solutions for the NMR experiments were obtained by dissolving the appropriate amounts of protein in D_2O. In all the experiments protein concentration was 2×10^{-2} mol/L.

^{1}H NMR spectra were obtained on a Bruker AMX 400 and a Bruker AC 200 spectrometer, operating at 400.00 MHz and 200.13 MHz respectively. The spin-lattice relaxation rates were measured using the $(180^\circ\text{-}\tau\text{-}90^\circ\text{-}t)_n$ sequence. The τ values used for the selective and non-selective experiments were: 0.01, 0.02, 0.04, 0.06, 0.08, 0.1, 0.2, 0.4, 0.8, 1, 1.5, 2, 3, 4, 5, 7, 20 s, respectively, and the delay time (t) was 20 s. The 180° selective inversion of the proton spin population was obtained by a selective soft perturbation pulse, generated by the decoupler channel. All the selective and non-selective spin-lattice relaxation rates refer to the water protons. The maximum experimental error in the relaxation rate measurements was 5%.

All the spectra were processed using the Bruker Software XWINNMR, version 2.5 on Silicon Graphics O2 equipped with RISC R5000 processor, working under the IRIX 6.3 operating system.

NMRD profiles were measured on a Stelar Spinmaster FFC field cycling spectrometer (FFC2000, Stelar, Mede, Italy), operating in the field range from 2.4×10^{-4} to 8.2×10^{-1} T (corresponding to proton Larmor frequencies from 0.01 to 35 MHz). NMRD profiles were acquired from samples of the proteins in water solution. The field-switching time was 3 ms, the polarization field was 30 MHz, the free induction decays were recorded after a single 90° excitation pulse applied at 14.8 MHz by using a receiver-delay time by 10 μs.

2.2. Theory

In a proteins system, we assume a distribution of water molecules in three different environments accordingly to their dynamically properties.

Using this model, the system contains:

(i). bulk water with a rotational correlation time typical of the order of picoseconds [44–47].

(ii). water present in the hydration layers around the protein surface which exhibits a restricted re-orientational motion. The dynamical properties of water molecules in this environment it is assumed be represented by a distribution of correlation time values. The limit values are the protein rotational motion (which include the buried water molecules), and the free tumbling bulk water. A fast chemical exchange conditions between the water molecules present in each existing micro-environments of the protein hydration layers and the bulk water is assumed.

(iii). buried water molecules. These long lived water molecules show dynamics which are determined by the slow rotational motion of the protein with correlation times values (τ_c), typically of the order of tens nanoseconds. These molecules are in slow chemical exchange (on the NMR time scale) with the water molecules present in the layers around the macromolecular surface.

The magnetic relaxation dispersion studies [6,43], give information about the rotational mobility of a protein and the buried waters, as well as on the number of buried water molecules that are associated with the protein. The number of buried water molecules is usually a very small fraction of the total number of water molecules in contact with the protein [6]. Nevertheless, relaxometric investigation cannot be used to monitoring the dynamics of water molecules present in the hydration layers around the protein which are characterized by shorter rotational lifetimes, in the limits from nanoseconds to few picoseconds.

Water proton spin-lattice relaxation rates obtained in either selective or non-selective mode allows to compute the protein contributions to the water relaxation for those molecules present in the hydration layers. The fast chemical exchange between the bulk (A) and the water molecules in the layers around a protein surface (B), can be described by:

$$M(H_2O)_n^B + n(H_2O)^A \rightleftharpoons M(H_2O)_n^A + n(H_2O)^B \quad (1)$$

where M is the protein.

On the basis of the previous equilibrium both R_1^{NS} and R_1^{SE} can be described as:

$$wR_{1\,\mathrm{exp}} = \chi_b R_{1b} + \chi_f R_{1f} \quad (2)$$

where wR_{1exp} is the experimental relaxation rate of water in the presence of the protein, R_{1b} and R_{1f} the water relaxation rates of the pure bound and free environments respectively. The values of χ_b and χ_f are the molar fractions of water in bound and bulk conditions and χ_b can be defined as [48,49]:

$$\chi_b = \frac{n[M(H_2O)_n^A]}{[H_2O)] + [M(H_2O)_n^A]} \approx \frac{n[M(H_2O)_n^A]}{[M(H_2O]} \quad (3)$$

where χ_f is the free water molar fraction: $\chi_f = 1 - \chi_b \approx 1$, considering that $[H_2O] > > [M(H_2O)_n{}^A]$.

Dipolar non-selective R_1^{NS} and selective R_1^{SE} spin-lattice relaxation rates can be described as [50,51]:

$$R_1^{NS} = \sum \rho_{ij} + \sum \sigma_{ij} \quad (4)$$

$$R_1^{SE} = \sum \rho_{ij} \quad (5)$$

where ρ_{ij} and σ_{ij} are the direct and the cross-relaxation contributions to proton spin-relaxation, respectively.

For any (i,j) dipolar coupling, R_1^{NS} and R_1^{SE} assume the explicit form:

$$R_1^{NS} = \frac{3}{10}\frac{\gamma_H^4 \hbar^2}{r_{ij}^6}\left[\frac{4\tau_c}{1+4\omega_H^2\tau_c^2} + \frac{\tau_c}{1+\omega_H^2\tau_c^2}\right] \quad (6)$$

$$R_1^{SE} = \frac{1}{10}\frac{\gamma_H^4 \hbar^2}{r_{ij}^6}\left[\frac{3\tau_c}{1+\omega_H^2\tau_c^2} + \frac{6\tau_c}{1+4\omega_H^2\tau_c^2} + \tau_c\right] \quad (7)$$

where τ_c is the rotational correlation time modulating the internuclear interactions, r_{ij} is the internuclear distance between i and j atoms, ω and γ are the proton Larmor frequency and the proton magnetogyric ratio,

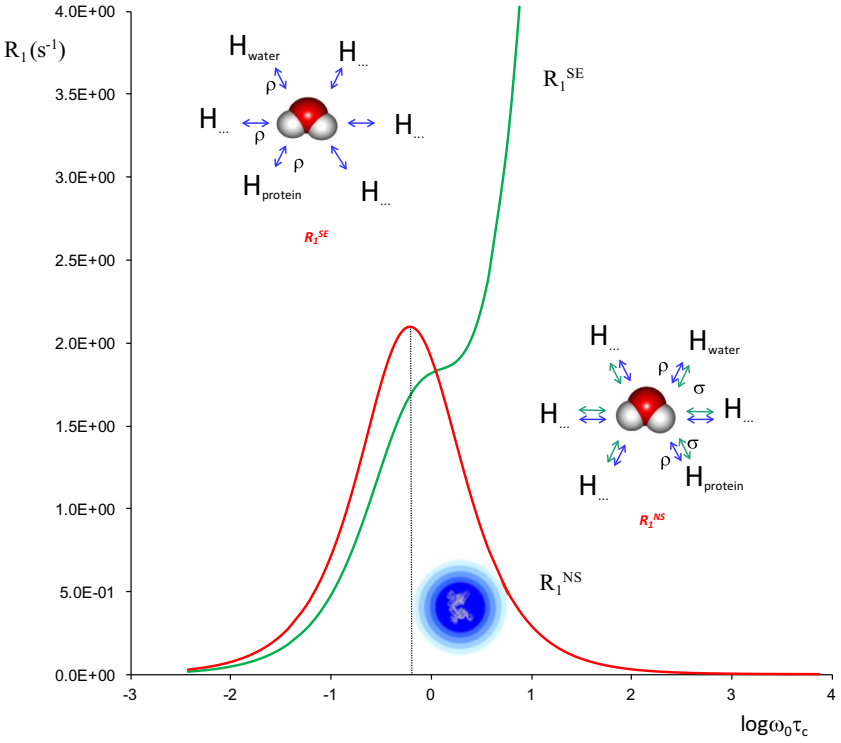

Fig. 1. Water proton R_1^{NS} and R_1^{SE} dependence on τ_c.

respectively and $\hbar$ is the Plack's constant. The water proton R_1^{NS} and R_1^{SE} dependence on τ_c are shown in Fig. 1 [52].

The cross-relaxation term σ_{ij} is described by:

$$\sigma_{ij} = \frac{1}{10}\frac{\gamma_H^4 \hbar^2}{r_{ij}^6}\left[\frac{6\tau_c}{1+4\omega_H^2\tau_c^2} - \tau_c\right] \quad (8)$$

In pure water both water non-selective and selective spin-lattice relaxation rates, wR_1^{NS} and wR_1^{SE} respectively, assume the same values as the water cross-relaxation term (σ_{ww}), affects the selective and non-selective measurements to the same extent. Being the water proton spin population affected in similar way by the 180° pulse of the inversion recovery pulse sequence used for the experimental measurements of the relaxation rates.

In a water protein system a negative protein-water cross-relaxation contribution to the non-selective spin-lattice relaxation rates is expected. The negative cross-relaxation contributions originate from water molecules in close contact with the protein whose motions are outside the fast motion limits: $\omega_H\tau_c << 1$ (see Eq. (8)). As a consequence $R_1^{SE} > R_1^{NS}$ is observed.

In protein solution both the non-selective and selective water relaxation rates can be described as a sum of the following terms:

$$wR_{1exp}^{NS} = \sum \rho_{ww}^f + \sum \sigma_{ww}^f + \chi_b \sum \rho_{ww}^b + \chi_b \sum \sigma_{ww}^b + \chi_b \sum \rho_{wp}^b + \chi_b \sum \sigma_{wp}^b \quad (9)$$

$$wR_{1\,exp}^{SE} = \sum \rho_{ww}^f + \sum \sigma_{ww}^f + \chi_b \sum \rho_{ww}^b + \chi_b \sum \sigma_{ww}^b + \chi_b \sum \rho_{wp}^b \quad (10)$$

where ρ_{ww}^f and σ_{ww}^f, are direct and cross-relaxation terms for bulk water, ρ_{ww}^b and σ_{ww}^b the intra- and inter-molecular water interactions in the bound conditions and ρ_{wp} and σ_{wp} are the water-protein direct and cross-relaxation terms respectively (the indexes *f* and *b* indicate the free and bound water environments). The difference between the non-selective and selective measurements is due to the $\chi_b \sum \sigma_{wp}^b$ term, which contains all the negative cross-relaxation contributions arising from protein-water interactions. The $\chi_b \sum \sigma_{wp}^b$ term is responsible of: $wR_{1exp}^{SE} > wR_{1exp}^{NS}$.

In D_2O solutions, both Eq. (9) and Eq. (10) are simplified as:

$$wR_{1\,exp}^{NS} = \sum \rho_{ww}^f + \sum \sigma_{ww}^f + \chi_b \sum \rho_{wp}^b + \chi_b \sum \sigma_{wp}^b \quad (11)$$

$$wR_{1\,exp}^{SE} = \sum \rho_{ww}^f + \sum \sigma_{ww}^f + \chi_b \sum \rho_{wp}^b \quad (12)$$

Combining Eqs. (4) and (5) and Eqs. (11) and (12), the following expressions can be obtained:

$$wR_{1\,exp}^{NS} = wR_1^{NS} + \chi_b\left(\sum \rho_{wp} + \sum \sigma_{wp}\right) \quad (13)$$

$$wR_{1\,exp}^{SE} = wR_1^{SE} + \chi_b\left(\sum \rho_{wp}\right) \quad (14)$$

from which the protein contribution to the water relaxation rates, ΔR_1, can be calculated as:

$$\Delta R_1^{NS} = wR_{1\,exp}^{NS} - wR_1^{NS} = \chi_b\left(\sum \rho_{wp} + \sum \sigma_{wp}\right) = \chi_b R_{1b}^{NS} \quad (15)$$

$$\Delta R_1^{SE} = wR_{1\,exp}^{SE} - wR_1^{SE} = \chi_b\left(\sum \rho_{wp}\right) = \chi_b R_{1b}^{SE} \quad (16)$$

where R_{1b}^{NS} and wR_{1b}^{SE} are the non-selective and selective relaxation rates of the water molecules present in the bound conditions.

If **W**, is defined as the $\Delta R_1^{NS}/\Delta R_1^{SE}$ ratio, then the average $\tau_{r(ave)}$ value of water molecules present in the protein hydration layers, can be determined. This value represents the average rotational correlation time among all the contributions arising from the water molecules present in the hydration layers around the protein:

$$\boldsymbol{W} = \Delta R_1^{NS}/\Delta R_1^{SE} = \chi_b R_{1b}^{NS}/\chi_b R_{1b}^{SE} = R_{1b}^{NS}/R_{1b}^{SE} = \frac{\frac{12\tau_{c1}}{1+4\omega_H^2\tau_{c1}^2} + \frac{3\tau_{c1}}{1+\omega_H^2\tau_{c1}^2}}{\frac{6\tau_{c1}}{1+4\omega_H^2\tau_{c1}^2} + \frac{3\tau_{c1}}{1+\omega_H^2\tau_{c1}^2} + \tau_{c1}} \quad (17)$$

The $\tau_{r(ave)}$ value is expected to be in between the protein rotational motions ($10^{-7}/10^{-8}$ s) and the water free tumbling reorientation (picoseconds).

In Fig. 2 the frequency dependence of **W**, ($\mathbf{W} = \Delta R_1^{NS} / \Delta R_1^{SE}$) on the basis of Eq. (17), is reported. Three values of the proton Larmor frequencies were considered: 200, 300 and 400 MHz. Three values of the proton Larmor frequencies were considered: 200, 300 and 400 MHz.

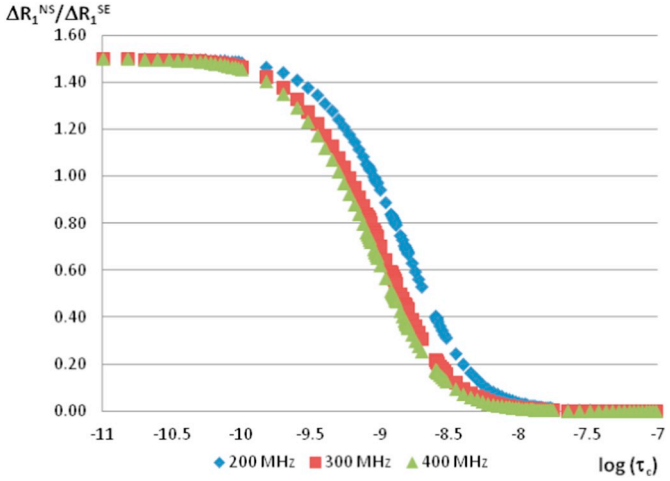

Fig. 2. Frequency dependence of **W**, (**W** = $\Delta R_1^{NS}/\Delta R_1^{SE}$) on the basis of Eq. (17).

3. Results and discussion

Three systems containing proteins of different size and conformation were investigated: ribonuclease A, human serum albumin and fibrinogen [32–34,53,54]. The water selective and non-selective spin-lattice relaxation times at 400 MHz were determined as a function of ribonuclease A, human serum albumin (HSA) and fibrinogen concentrations. The experimental results are reported in Fig. 3 and showed that the water selective spin-lattice relaxation rates for protein systems assume a larger value with respect to the water non-selective spin-lattice relaxation rates. This is consistent with the presence of a negative protein-water cross-relaxation contributions to R_1^{NS}. Both ΔR_1^{SE} and ΔR_1^{NS} *vs* protein concentration showed a linear behavior. The values of the W ratios were calculated for ribonuclease, human serum albumin and fibrinogen systems, and reported in Table 1.

Similarly, selective and non-selective spin-lattice relaxation measurements were performed on both, human serum albumin and human serum albumin-Lamotrigine systems using a spectrometer operating at a proton frequency of 200 MHz. These measurements were performed to verify the validity of the proposed approach using a different operating proton frequency. Lamotrigine an anticonvulsant drug is known to have a specific affinity toward human serum albumin. As reported in literature, the interaction between HSA and lamotrigine was studied by spectroscopic techniques and molecular modeling to highlight the aminoacids involved in the binding site. Experimental data reported by Poureshgh et al. 2017 suggest that subdomain IB of HAS was involved in the interaction process and that Van der Waals interactions and hydrogen bonds were the dominant forces [55–57]. This system has been studied to analyze the contribution of protein conformational change on protein ordering effects on the surface water molecules. The plots of ΔR_1^{SE} and ΔR_1^{NS} *vs* protein concentration for HSA and HSA-Lamotrigine systems, are reported in Fig. 4 and calculated **W** ratios for these two systems are also reported in Table 1.

On the basis of the computed **W** ratios and using Eq. (14) the average rotational correlation time values, $\tau_{r(ave)}$, of water molecules present in the protein hydration layer was calculated.

The rotational correlation time of the protein buried water, the average correlation time of the water molecules in the protein hydration layers, and the tumbling rotational correlation time of the bulk water molecules, together with the protein molecular weight, are reported in Table 1.

The rotational correlation time of the buried water molecules were obtained from relaxometric measurements and then compared with the previously obtained measurements [6,43]. Fig. 5 reports the "fast field cycling", FFC-^{1}H relaxation dispersion curves of ribonuclease A, human serum albumin and fibrinogen systems. The results indicate that the dispersion region was not detected in the range of frequencies used. The reason of such behavior is probably due to the fact that fibrinogen is not a globular protein. The fibrinogen sizes are: longitudinal axis 434 Å and transversal axis of 50 Å. The protein may contains also few buried water molecules no detectable by a relaxometric study.

In protein systems, water protons spin-lattice relaxation rates contain information on both bulk and protein surface water environments. Protein presence induces an ordering effect, which depends on the size of the protein and on its conformation. Fig. 6 shows a model of a HSA

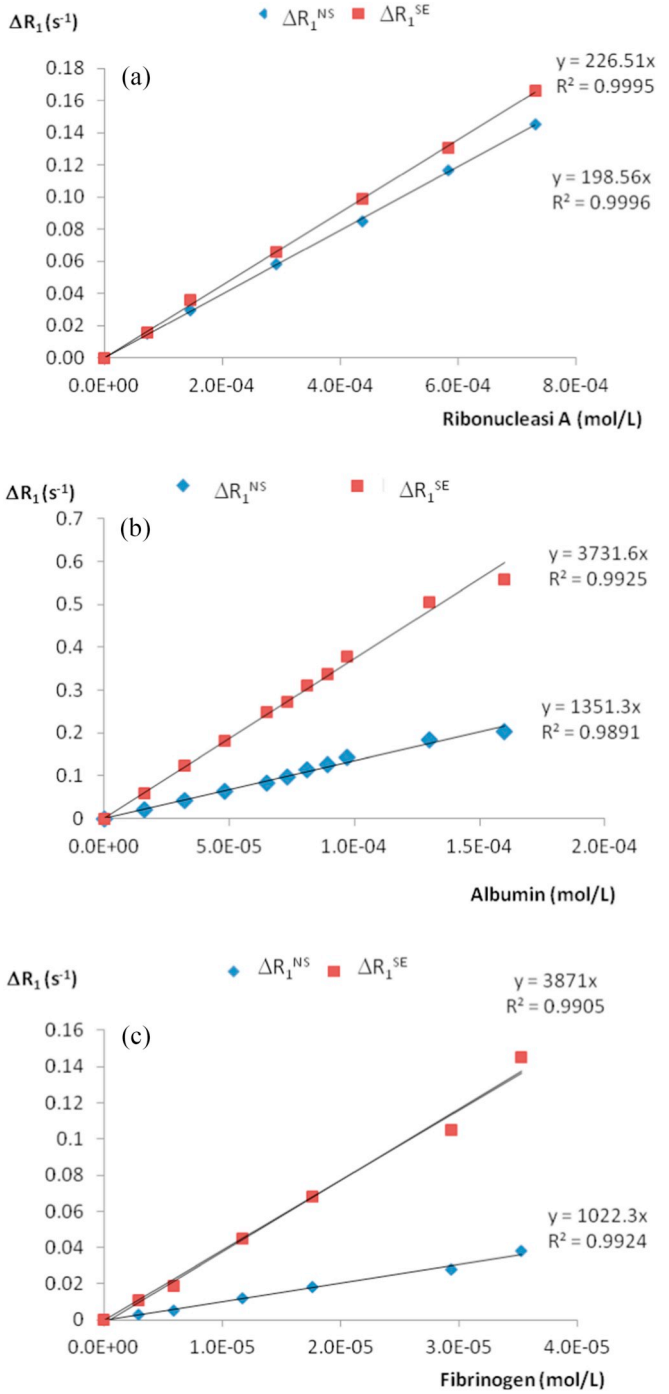

Fig. 3. ΔR_1^{SE} and ΔR_1^{NS} water selective and non-selective spin-lattice relaxation rates dependence as a function of protein concentrations: (a) ribonuclease A, (b) human serum albumin, (c) fibrinogen systems at a Larmor frequency of 400 MHz.

Table 1
W, τ_c, $\tau_{r(ave)}$ and τ_w correlation times, of water molecules in different environments, protein buried water, protein hydration shell and bulk water. Values obtained for different proteins systems, at 400 MHz and 200 MHz, and at 298 K.

	Mw (kDa)	W	τ_c (ns) Buried water	$\tau_{r(ave)}$ (ns) Surface water	$\tau_{w(ave)}$ (ns) Bulk water
ω_o (400 MHz)					
Ribonuclease A	13.7	0.88	6.6	0.60	2.5×10^{-3}
HSA	66.2	0.36	48	1.50	2.5×10^{-3}
Fibrinogen	340	0.26	–	1.70	2.5×10^{-3}
ω_o (200 MHz)					
HSA	66.2	0.71	48	1.50	2.5×10^{-3}
HSA-Lamotrigine	66.2	0.54	48	2.00	2.5×10^{-3}

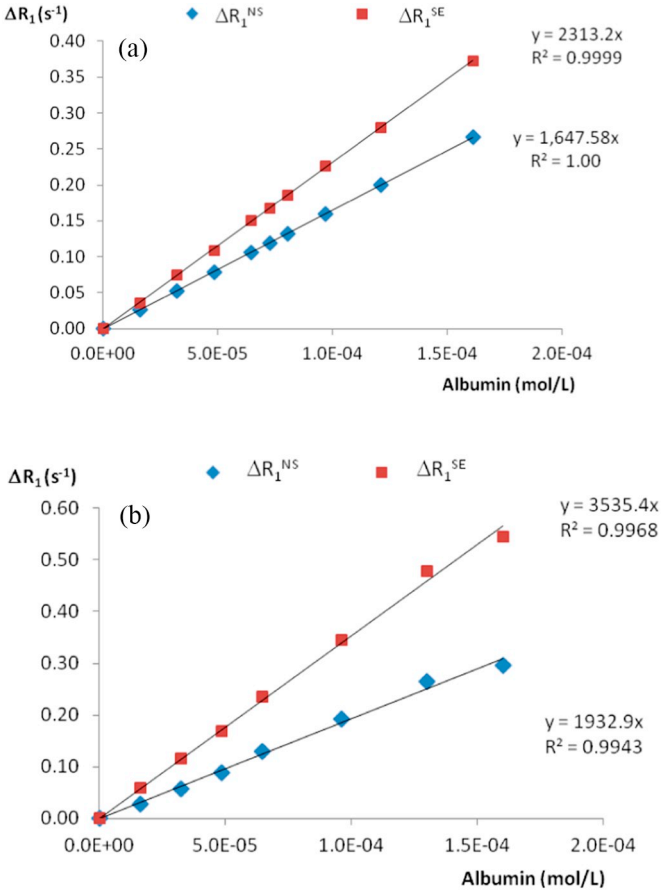

Fig. 4. ΔR_1^{SE} and ΔR_1^{NS} water selective and non-selective spin-lattice relaxation rates dependence as a function of human serum albumin concentrations at a Larmor frequency of 200 MHz: (a), pure human serum albumin and (b) human serum albumin-Lamotrigine system. Lamotrigine concentration 1×10^{-2} mol.dm^{-3}.

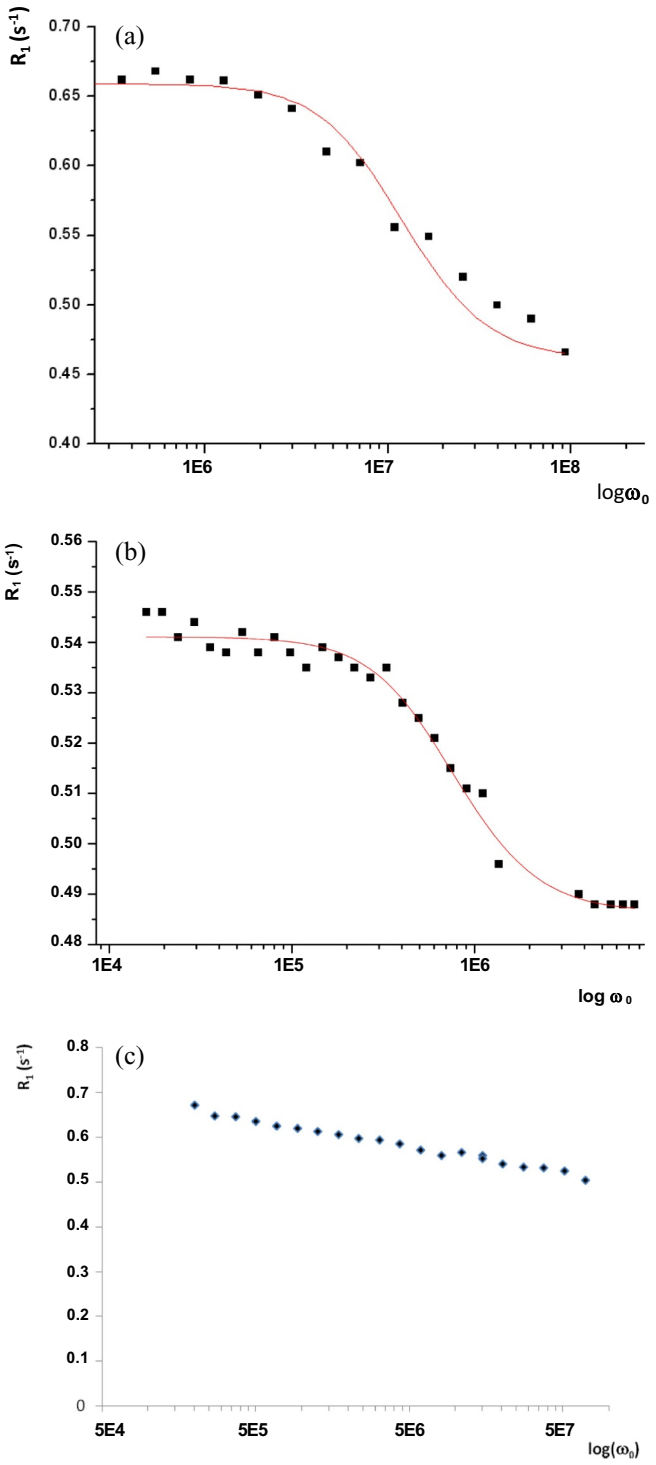

Fig. 5. FFC ^{1}H relaxation dispersion curves of (a) ribonuclease A, (b) human serum albumin, and (c) fibrinogen systems.

protein with ordered and bulk water, as obtained by molecular modeling simulation starting from the HSA structure (pdb code 1AO6) in a water cap (Amber 16.0, tLEaP suite) [58,59]. Three environments with different dynamics, each characterized by a different profile of the normalized Spectral Density function J(ω) (Fig. 7, for HSA protein system), can be defined on the basis of Eq. (18):

$$J(\omega) = 2\tau_c/[1 + (\omega^2\tau_c^2)] \quad (18)$$

4. Conclusion

The protein ordering contribution on water molecules was quantified using the average correlation time of the water molecules present in the protein hydration layers. This result shows the importance of quantifying the size of the protein ordering contribution on the water molecules. This study confirms the theoretical and experimental approaches developed for the calculation of the average correlation times of water molecules in the protein hydration layers. These systems are very important in both theoretical and experimental studies, allowing for the simultaneous identification of dynamical properties of each of three different water environments.

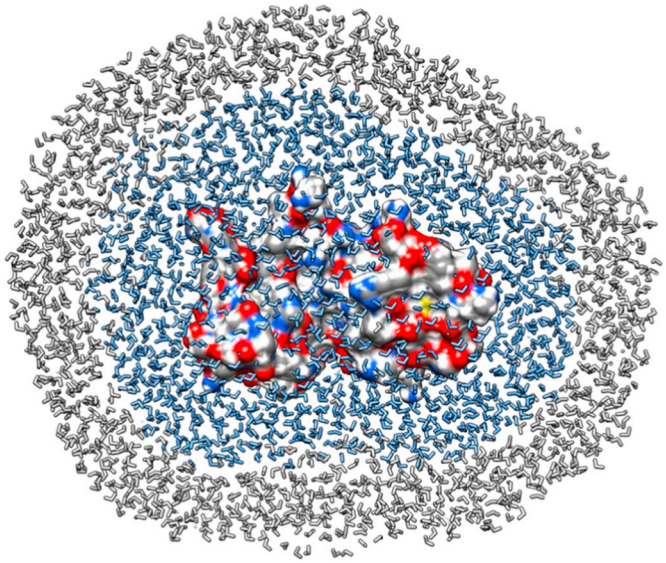

Fig. 6. Schematic model of HSA protein in which ordered and bulk water are shown.

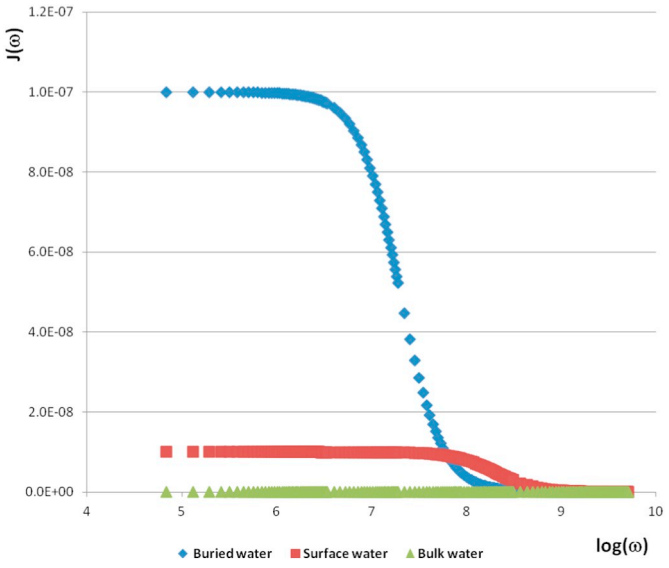

Fig. 7. Normalized spectral density function profile for water molecules in the three different environments of a protein system *i.e.* buried, surface and bulk water.

Conflicts of interest

The authors declare no conflict of interest.

Acknowledgments

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