Research Article

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Low-temperature librations and dynamical transition in proteins at differing hydration levels

<https://doi.org/10.1515/bmc-2022-0007>

received January 27, 2022; accepted February 14, 2022

Abstract: Hydration of water affects the dynamics and in turn the activity of biomacromolecules. We investigated the dependence of the librational oscillations and the dynamical transition on the hydrating conditions of two globular proteins with different structure and size, namely β -lactoglobulin (β LG) and human serum albumin (HSA), by spin-label electron paramagnetic resonance (EPR) in the temperature range of 120–270 K. The proteins were spin-labeled with 5-maleimide spin-label on free cysteins and prepared in the lyophilized state, at low ($h = 0.12$) and full ($h = 2$) hydration levels in buffer. The angular amplitudes of librations are small and almost temperature independent for both lyophilized proteins. Therefore, in these samples, the librational dynamics is restricted and the dynamical transition is absent. In the small and compact beta-structured β LG, the angular librational amplitudes increase with temperature and hydrating conditions, whereas hydration-independent librational oscillations whose amplitudes rise with temperature are recorded in the large and flexible alpha-structured HSA. Both β LG and HSA at low and fully hydration levels undergo the dynamical transition at about 230 K. The overall results indicate that protein librational dynamics is activated at the low hydration level $h = 0.12$ and highlight biophysical properties that are common to other biosamples at cryogenic temperatures.

Introduction

Since the seminal work of Frauenfelder and coworkers, it is well accepted that biomacromolecule dynamics is affected by the hydration level of the medium, and this plays a key role in regulating several molecular aspects of the structure/function relationship in biosystems [1–4]. A molecular motion whose characteristics depend on the water content is the librational motion, consisting of rapid vibrations of small amplitude [5,6]. Librational oscillations are readily detected at low, cryogenic temperatures, such as those reached with liquid nitrogen down to 77 K or with helium below 77 K, when large-amplitude motions are suppressed. They are present also at higher physiological temperatures and are relevant for functionally important processes in biosamples. Indeed, librations can be involved in the transitions among conformational substates in proteins, in ions conduction, and ligand migration within macromolecules; also, librations in the lipid environment can be coupled to solvent-mediated transitions in membrane proteins [3,4,7–10]. Rapid librations are also important for the cryopreservation of biological tissues and genetic materials [11].

Librational motion has been found to occur in several proteins, including soluble globular proteins, membrane proteins, and intrinsically disordered proteins (IDP) [4,12–21], nucleic acids [22,23], lipid bilayers [24–30], and natural membranes [7,8,10]. The low-temperature librational dynamics in biosystems is properly studied by using X-ray diffraction and neutron scattering techniques [1,3,4,6,17,20,23,28] and methods of electron paramagnetic resonance (EPR) spectroscopy [19,21,24,25,29]. Other spectroscopic techniques, such as Raman [25,31], Mossbauer [3,4], dielectric relaxation [4,23,32], and nuclear magnetic resonance [33,34] as well as differential scanning calorimetry [32] and computational investigations (e.g., [7,20,22,35]) have provided findings on low-temperature dynamics at different time, length, and energy scales.

Interesting insights into the librational motion of various hydrated supramolecular complexes and non-biological

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samples are obtained from neutron and EPR techniques. Indeed, the temperature dependence of the mean-square atomic displacement $\langle r^2 \rangle$ in scattering measurements [3,4,6,20,28,35] is quite similar to that of the mean-square angular amplitude $\langle \alpha^2 \rangle$ measured by EPR [18,19,21,25,27,29]. Both $\langle r^2 \rangle$ and $\langle \alpha^2 \rangle$ are first weakly and linearly dependent on temperature and then increase sharply at a temperature T_d called dynamical transition temperature. The nature of the dynamical transition is a discussed topic in the biophysics literature [3,18,20]. It has been associated with the transition from harmonic vibrations to anharmonic large-amplitude motions [e.g., ref. 20], and depending on the sample type and experimental conditions, T_d lies in the 190–240 K interval.

Quite generally, in biosamples, the librational dynamics is restricted and the dynamical transition is absent in the dry state, whereas the librations and the transition are activated by the presence of water molecules [4,12–15,17,20,21,28,30]. Within this contest, in the literature, there are data which link the characteristics of the librational motion to the hydrating conditions of biosystems having different structure and function. The findings vary widely. Indeed, significant differences have been found in the dynamics of proteins [4,14,17] and in that of proteins and nucleic acids [22,23] as a function of water content. Moreover, at the same level of hydration, small differences are observed in the mean square displacements of diverse sized globular proteins, whereas a greater motional flexibility is detected in the IDP tau compared to the globular folded maltose binding protein [16]. Also, Dzuba and coworkers reported that the temperature differently affected the dynamics of lysozyme and that of IDP casein both in the dehydrated and hydrated state [21].

In this work, we focus on the effects of the hydration level on the librational dynamics of two model globular soluble proteins, namely β -lactoglobulin (β LG) and human serum albumin (HSA). β LG is the most abundant protein in the milk of different mammals, not including humans, and it is involved in the binding and transport of hydrophobic and amphiphilic molecules [36]. HSA is the principal extracellular protein with a high concentration in blood plasma where it performs several functions, and in particular binds, stores, and delivers to target sites a wide variety of exogenous and endogenous molecules, drugs, and metals [37]. The two proteins have different structures, mainly α -helical for HSA and β -sheets for β LG, and size (162 residues, 18.4 kDa for β LG and 585 residues, 66 kDa for HSA) (Figure 1).

We aim at investigating the angular librations and detecting the dynamical transition in the proteins in the lyophilized state, at low ($h = 0.12$) and full ($h = 2$) hydration levels in aqueous buffer, where the degree of hydration, h , is defined as the weight ratio of water to protein. The study

Figure 1: Crystal structure of β LG (PDB ID 3NPO; [38]) and HSA (PDB ID 1AO6; [39]). Cysteins in the proteins are indicated with the ball-and-stick model. The structure of 5-MSL is also given.

has been carried out in the temperature range of 120–270 K by using spin-label EPR on proteins covalently spin-labeled with the maleimide nitroxide derivative 5-MSL on the single free cysteine residue located at the protein–water interfaces: Cys121 in β LG and Cys34 in HSA.

The EPR results show similarities and differences in the low-temperature librational dynamics of β LG and HSA under different hydrating conditions.

Materials and methods

Materials

Fatty acid-free and globulin-free albumin from human serum (HSA, type A-3782, purity approximately 99%), beta lactoglobulin from bovine milk (β LG, type L3908, purity > 90%), the nitroxide derivative of maleimide, and 3-maleimido-2,2,5,5-tetramethyl-1-pyrrolidinyloxy (5-MSL) were from Sigma-Aldrich (St Louis, MO). Dulbecco's phosphate-buffered saline (DPBS) solution (10 mM and pH 7.4), also from Sigma-Aldrich, was used throughout to prepare all the protein solutions.

Sample preparation

Proteins were spin-labeled covalently on the unique free sulfhydryl group by reaction with the spin-label 5-MSL as reported elsewhere [10,40]. Briefly, 5-MSL dried down from ethanol was mixed with the proteins in solution (protein concentration = 5 mM) at a five-fold molar excess. The mixtures were incubated for 24 h at 4°C with gentle stirring. Excess free spin label was then separated from the spin-labeled proteins by extensive dialysis at 4°C against the same buffer until no free spin-label was detected in

the EPR spectra of the dialysis medium. After covalent labeling, proteins were prepared in the freeze-dried lyophilized state, at low ($h = 0.12$) and full ($h = 2$) hydration levels in DPBS, where the hydration level $h = g_{\text{water}}/g_{\text{protein}}$ is expressed as gram of water per gram of protein.

EPR

EPR measurements were carried out on an ESP-300 9 GHz spectrometer (Bruker, Karlsruhe, Germany) equipped with a ER 4111VT temperature controller and with a ER4201 TE₁₀₂ rectangular cavity (both from Bruker). Standard 3 mm EPR quartz sample tubes were centered in the cavity and spectra were recorded on heating from 120 to 270 K by evaporating liquid nitrogen. The following spectrometer settings were used: 100 kHz field modulation frequency, 2–3 G_{p-p} peak-to-peak modulation amplitude, 10 mW microwave power, 332.5 mT central magnetic field, 11 mT in 3 min sweep

width rate, and 82 ms time constant. Spectra were accumulated several times to improve signal-to-noise ratio.

Data reproducibility were assessed by repeating the experiments two or three times.

Results and discussion

EPR spectra of spin-labeled β LG and HSA: temperature and hydrating conditions affect the spectral anisotropy

EPR spectra, at selected temperatures, of spin-labeled β LG/5-MSL and HSA/5-MSL in the lyophilized, low and fully hydrated states are reported in Figure 2.

On the whole, the spectra are anisotropic powder patterns typical of immobilized nitroxides and indicative of covalent spin-label binding to the proteins. Moreover,

Figure 2: EPR spectra at selected temperatures of β LG/5-MSL and HSA/5-MSL at differing hydration states: lyophilized and low hydration and full hydration levels.

the spectral lineshapes change only a little with temperature and hydrating conditions. Specifically, at the lowest temperatures, the spectra of both proteins are characterized by large spectral widths and broad lines with the spectral anisotropy changing in the order: lyophilized < low hydration < full hydration. For lyophilized proteins, the spectral anisotropy moderately reduces and the linewidths narrow only at the highest temperatures. For hydrated proteins, from $T > 200$ K, the spectra progressively narrow and achieve sharper lineshapes at the highest temperatures, more in the fully hydrated conditions than in the low hydrated conditions.

Hydration influences the $2\langle A_{zz} \rangle$ temperature dependence for β LG/5-MSL and HSA/5-MSL

A quantitative evaluation of the influence of hydration on the spectral anisotropy can be obtained from the temperature dependence of the motionally averaged ^{14}N -hyperfine splitting, $2\langle A_{zz} \rangle$, that is the separation between the outer resonances in the EPR spectra (Figure 2). This spectral parameter reflects the amplitude and the rate of motion in all motional regimes of the EPR timescale [41]. It correlates with the spectral anisotropy: the larger the anisotropy, the greater the $2\langle A_{zz} \rangle$; it also relates to the mean-square angular amplitude of librational motion (equation (1) given in Section Hydration level impacts on the $\langle \alpha^2 \rangle$ temperature dependence for β LG/5-MSL and HSA/5-MSL): a decrease in $2\langle A_{zz} \rangle$ corresponds to an increase in the librational amplitudes [5,42].

By comparing the temperature dependence of $2\langle A_{zz} \rangle$ in the two panels in Figure 3, it can be seen that the hyperfine separation has an almost constant value of about 6.90 mT in both lyophilized proteins, although it slightly decreases in β LG on increasing the temperature.

Moreover, the $2\langle A_{zz} \rangle$ temperature dependence in the proteins at low and full hydration levels is not monotonic: for $T < 200$ K, the $2\langle A_{zz} \rangle$ values increase with temperature, more rapidly in HSA than in β LG; for $T \geq 200$ K, the $2\langle A_{zz} \rangle$ values decrease with temperature as a result of progressive motional narrowing by librations [42]. The rate of this decrease is similar for HSA at low and full hydration whereas it increases with hydration for β LG; the two fully hydrated proteins show the same temperature decrease in the $2\langle A_{zz} \rangle$ values.

The almost temperature independence of $2\langle A_{zz} \rangle$ in the lyophilized proteins indicates that the mobility is restricted in the dry state; in contrast, the mobility intensifies (i.e., $2\langle A_{zz} \rangle$ decreases) in the high temperature regime for both hydrated proteins, the extent being hydration independent for HSA, whereas it increases with hydration

Figure 3: Temperature dependence of the outermost hyperfine separation, $2\langle A_{zz} \rangle$, for β LG/5-MSL and HSA/5-MSL at differing hydration conditions: lyophilized (triangles), low hydration (circles), and full hydration (squares).

for β LG. These findings clearly indicate that protein dynamics is activated in the presence of a low number of water molecules.

The increase in $2\langle A_{zz} \rangle$ in the low-temperature interval for the two spin-labeled proteins at low and full hydration levels is anomalous. Most probably it is due to the contribution to the outer hyperfine splitting from the environmental polarity [41,43] and the temperature dependence of the hydrogen-bonded water [44]. A similar behavior has been reported for fully hydrated HSA complexed with stearic acids [45] or prepared in different amount of hydration water molecules [46], and on lipid membranes spin-labeled in the upper hydrocarbon region [24].

Hydration level impacts on the $\langle \alpha^2 \rangle$ temperature dependence for β LG/5-MSL and HSA/5-MSL

From the motionally averaged hyperfine splittings, $2\langle A_{zz} \rangle$, can be derived the mean-square angular amplitude of

librations, $\langle \alpha^2 \rangle$, for the spin labeled proteins according to the following equation [5,42]:

$$A_{zz} = A_{zz} - (A_{zz} - A_{xx})\alpha^2, \quad (1)$$

where A_{xx} and A_{zz} are the principal values of the hyperfine interaction tensor. A_{zz} is obtained from the highest value of the motionally averaged hyperfine splitting in Figure 3 (i.e., A_{zz} at 190–210 K) and $A_{xx} = 0.59$ mT is taken from ref. [41].

The temperature dependence of $\langle \alpha^2 \rangle$ for the β LG/5-MSL and HSA/5-MSL samples are reported in Figure 4.

The plots of $\langle \alpha^2 \rangle$ give results consistent with those of $2\langle A_{zz} \rangle$. Indeed, from Figure 4 it can be seen that in the lyophilized proteins, the angular amplitude maintains small and constant values of about 6–7° in β LG and about 3° in HSA in the whole temperature range. In the hydrated proteins, after an initial constant and low $\langle \alpha^2 \rangle$ values, the

angular librational amplitude starts to rise rapidly at the same temperature of about 230 K in both HSA and β LG. The temperature behavior of $\langle \alpha^2 \rangle$ in both proteins at $h = 2$ is similar to that recorded for spin-labeled stearic acids associated with HSA [45] and β LG [47]. The different temperature dependence of the mean-square librational amplitude between lyophilized and hydrated proteins (i.e., restricted vs activated) is in accordance with previous results obtained with EPR [15,19,21]. It is also in agreement with neutron scattering data of the mean-square atomic displacement [3,14,16,17,20].

From the EPR results in Figure 4, another feature can be pointed out for β LG: at the lowest temperatures, the librational amplitudes increase with hydration level in the order: low hydration $\approx$ full hydration $<$ lyophilized state, whereas at the highest temperatures they become larger in the order: lyophilized $<$ low hydration $<$ full hydration. In other words, hydration water hinders β LG dynamics at low temperatures and favors it at high temperatures. A similar behavior is reported for the barrel-like green fluorescent protein [17] and, to a minor extent, for other proteins such as lysozyme [14] and myoglobin [3], as well as for lipid membrane model systems [30], where the most dehydrated sample has higher dynamics at low temperatures than the hydrated sample and *vice-versa*.

The effect of the solvent on the librational amplitudes can be better seen from the plots of the difference of the mean-square angular amplitude, $\Delta\langle \alpha^2 \rangle$, between the hydrated (fully and low) and the lyophilized proteins as a function of temperature reported in the insets of Figure 4. These plots show more clearly how the presence of water molecules affects the protein librational dynamics: the rise in $\langle \alpha^2 \rangle$ vs temperature is hydration-dependent in β LG, whereas it occurs at the same rate in the low and fully hydrated HSA. Our results on HSA are in reasonably good agreement with previous findings obtained by EPR and saturation transfer EPR at room temperature, which reported hydration-independent outer hyperfine splitting and rotational correlation time for the spin-labeled protein at $h > 0.2$ –0.5 [46]. For comparison, it is worth to mention other studies. A neutron study on model systems formed by various homomeric polypeptides showed that i) the dynamics is restricted in the dry samples; ii) in some hydrated samples, the motional contribution does not depend on the hydration at $h \geq 0.2$, whereas in some other cases, the librational fluctuations are enhanced with temperature by increasing the water content [48]. Moreover, similarities in the temperature dependence of the librational oscillations have been reported for three proteins of diverse size, namely RNase (124 residues, ≈ 14 kDa), myoglobin (153 residues, ≈ 17 kDa), and maltose binding

Figure 4: Temperature dependence of the mean-square librational amplitude, $\langle \alpha^2 \rangle$, for β LG/5-MSL and HSA/5-MSL at differing hydration conditions: lyophilized (triangles), low hydration (circles), and full hydration (squares). Inset: Difference, $\Delta\langle \alpha^2 \rangle = \langle \alpha^2 \rangle$ (hydrated) – $\langle \alpha^2 \rangle$ (lyophilized), between $\langle \alpha^2 \rangle$ values for the hydrated (fully and low) and lyophilized proteins.

protein (387 residues, ≈ 43 kDa), at the same water content [16]. In contrast, significant differences have been found in the dynamics of lysozyme, t-RNA, and DNA [22,23] or of green fluorescent protein and lysozyme [17] at different degrees of hydration. Also, the presence of water molecules affects the dynamics of folded proteins (such as myoglobin and lysozyme) more than that of IDPs (such as tau and casein) [16,21].

Dynamical transition in hydrated proteins

As already mentioned in the previous paragraph, at a variance with lyophilized proteins that do not undergo the dynamical transition, both β LG and HSA at low and fully hydration levels show a rapid increase in the mean-square angular amplitude at the same $T_d \approx 230$ K. T_d is therefore independent from protein type and hydration level. While in literature there is a general consensus with the fact that the dynamics is restricted to harmonic oscillations and the dynamical transition is absent in the dry state (e.g., [20] and references therein), there are however cases in which T_d varies as a function of sample type and it is either dependent or independent on the amount of hydration water molecules. For example, fully hydrated bilayers with interdigitated chains (unsaturated lipids) manifest the dynamical transition at higher (lower) temperature than ester-linked, saturated symmetrical chain phosphatidylcholines [26,29,49], whereas hydrated lysozyme undergoes

the dynamical transition at lower T_d than the IDP casein [21]. Also, the dynamical transition occurs at the same temperature in the three differently sized proteins, RNase, myoglobin, and maltose binding protein, at the same water content [16]. A clear hydration dependent decrease in T_d is observed for tRNA [22], whereas T_d is almost unchanged in lysozyme [14] and in lipid bilayers [30] in buffer at various levels of hydration.

For $T > T_d$, the data in Figure 4 are described by an Arrhenius law of the form $\langle \alpha^2 \rangle \propto \exp(-E_a/RT)$ [3]. The activation energy values, E_a , for the onset of diffusive motions can be evaluated from the linear regression of the $\ln \langle \alpha^2 \rangle$ vs T^{-1} data which are reported in Figure 5. They are: $E_a = 16.44 \pm 0.72$ kJ/mol and $E_a = 21.04 \pm 2.10$ kJ/mol for β LG and $E_a = 18.27 \pm 0.84$ kJ/mol and $E_a = 21.53 \pm 0.53$ kJ/mol for HSA in the low and fully hydrated states, respectively.

The E_a values are similar for low and fully hydrated HSA, increase with hydration level in β LG (i.e., E_a [low hydrated β LG] < E_a [fully hydrated β LG]), and are similar for the two fully hydrated proteins. In any sample, in close agreement with literature [18,33,34], at the dynamical transition, β LG and HSA cross the low-energy barriers in the range of 15–30 kJ/mol.

Conclusion

In this study we have addressed the effect of hydration water on the librational dynamics and the dynamical transition of surface labeled β LG and HSA by using EPR in the temperature range of 120–270 K. In spite of the different structures, shapes, and sizes, the two proteins share same aspects that characterize their low-temperature librations. For the proteins in the lyophilized state, the librational oscillations are small and temperature-independent, and the dynamical transition is absent. For proteins, both in the low and in the fully hydrated conditions, the angular librational amplitudes increase rapidly at the same dynamical transition temperature of about 230 K where they cross low energy barriers of about 20 kJ/mol. For the different structure flexibility, the proteins are differently affected by the hydrating water level at temperatures higher than T_d : the compact β -structured β LG show librational amplitudes which became progressively larger with hydration, whereas the loosened packed α -structured HSA shows hydration-independent librational oscillations.

The spin-label EPR results obtained at cryogenic temperatures deepen the biophysical characterization of soluble proteins that are normally studied at higher

Figure 5: Arrhenius plots characterizing the temperature dependence of the librational amplitude, $\langle \alpha^2 \rangle$, in the high temperature regime for β LG and HSA at different hydrating conditions. Dashed lines are linear regressions of the data.

temperatures. They indicate that protein dynamics is activated in the presence of a low number of hydrating water molecules. Some properties evidenced in this study in β LG and HSA are common to a variety of frozen hydrated samples, including lipid membranes and non-biological polymers, and would imply that they are typical of the low-temperature behavior of samples around 200 K independently from their structure and functioning.

Acknowledgements: This work was financially supported by University of Calabria.

Conflict of interest: Authors declare no conflict of interest.

Data availability statement: The datasets generated and analyzed during the current study are available on request.

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