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ORIGINAL PAPER

Solvent effect on librational dynamics of spin-labelled haemoglobin by ED- and CW-EPR

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Abstract Two-pulse, echo-detected electron paramagnetic resonance (ED-EPR) spectra and continuous-wave EPR (CW-EPR) spectra were used to investigate the solvent effect on the librational motion of human haemoglobin spin-labelled on cysteine β93 with the nitroxide derivative of maleimide, 6-MSL. Protein samples fully hydrated in phosphate buffer solution (PBS), in a 60% v/v glycerol/water mixture and in the lyophilized form were measured at cryogenic temperature in the frozen state. The protein librational motion was characterized by the amplitude–correlation time product, $\langle\alpha^2\rangle\tau_c$, deduced from the ED-EPR spectra. The librational amplitude, $\langle\alpha^2\rangle$, was determined independently, from the motionally averaged hyperfine splitting in the CW-EPR spectra, and the librational correlation time, τ_c, was derived from the combination of the pulsed and conventional EPR data. Rapid librational motion of small amplitude was detected in all samples. In each case, the librational dynamics was restricted up to 180 K, beyond which it increased steeply for the hydrated protein in PBS and in the presence of glycerol. In contrast, in the dehydrated protein, the librational dynamics was hindered and less dependent on temperature up to $\sim$240 K. In all samples, $\langle\alpha^2\rangle$ deviated from small values only for $T > 200$ K, where a rapid increase of $\langle\alpha^2\rangle$ was evident for the hydrated samples, whereas limited temperature variation was shown in the lyophilized samples. The librational correlation time was in the subnanosecond regime and weakly dependent on temperature. The results evidence that solvent favours protein dynamics.

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Introduction

It is well recognized that protein dynamics plays a key role in regulating molecular aspects of biosystem functions. However, global and local motions in proteins are strongly affected by the physico-chemical properties of the solvent. Indeed, it has been evidenced that the solvent controls protein dynamics and affects macromolecule activity (Rupley and Careri 1991; Fenimore et al. 2004; Frauenfelder et al. 2009).

Biophysical studies on proteins are nowadays routinely carried out at low, cryogenic temperatures. These studies allow the separation of details of the molecular motion that are inevitably present as part of the more complex protein dynamical modes found at higher, physiological temperatures. It is also worth noting that low-temperature studies of biomaterials are the subject of intensive research not only in basic but also in applied science because of their relevance to (cryo)preservation and bioprotection of cells, tissues, therapeutic proteins and food.

A number of experimental methods and theoretical approaches, sampling different timescales, have been used to investigate protein samples in different environments and under different conditions (Frauenfelder et al. 1979; Rasmussen et al. 1992; Steinbach and Brooks 1996; Gabel et al. 2002; Poluetkov et al. 2003; Parak et al. 2006; Klare and Steinhoff 2009; Bridges et al. 2010; Doster 2010; Khodadadi et al. 2010a, b).

In particular, for studies at low temperature, electron spin-echo (ESE) methods of pulsed electron paramagnetic resonance (FT-EPR) have proved to be a powerful means

to define details of the librational motions in a variety of biosystems. Using ESE techniques, librations are detected, although with different spatio-temporal characteristics, not only in small magnetic probes dispersed in glass-forming liquids (Dzuba 1996; Dzuba 2000; Kirilina et al. 2001) but also in model phospholipid membranes (Bartucci et al. 2003; Erilov et al. 2004a; Erilov et al. 2004b; Bartucci et al. 2006; Isaev and Dzuba 2008), in mixed lipid–peptide dispersions (Bartucci et al. 2008) and in Na,K-ATPase enzymes (Guzzi et al. 2009).

In the present work, human haemoglobin (Hb) was spin-labelled with the cysteine-specific nitroxide derivative of maleimide, 6-MSL. Haemoglobin is a hetero-oligomeric protein composed of two α- and two β-subunits arranged in a quaternary structure in the form $\alpha_2\beta_2$. The label covalently binds to the cysteines at position β93. When frozen, it is buried in a protein pocket and reports on overall protein dynamics (Banham et al. 2007). The librational dynamics of 6-MSL bound to Hb (SL-Hb) was characterized in different environments, namely in aqueous buffer dispersions, in a glass-forming glycerol/water mixture and in the lyophilized state. The study was carried out at low, cryogenic temperature by two-pulse ESE spectroscopy combined with CW-EPR measurements. Primary, two-pulse ED-EPR spectra of SL-Hb as a function of interpulse delay time yielded values for the librational amplitude–correlation time product, $\langle\alpha^2\rangle\tau_c$. Conventional EPR spectra allowed independent determination of the mean-square torsional amplitude, $\langle\alpha^2\rangle$, and combining data from ED and CW spectra, the librational correlation time, τ_c, was obtained. How the solvent affects the librational parameters of SL-Hb is shown and discussed.

Materials and methods

Materials

Human haemoglobin, the spin-labelled maleimide 4-maleimido-1-oxyl-2,2,6,6-tetramethylpiperidine (6-MSL) and pure glycerol were obtained from Sigma-Aldrich (St. Louis, MO). The reagent-grade salts for the 10 mM phosphate buffer solution (PBS) at pH 7.2 were from Merck (Darmstadt, Germany). All materials were used as purchased with no further purification. Bidistilled water was used throughout.

Sample preparation

Hb (5 mM in 10 mM PBS, pH 7.2) was spin-labelled on Cys-β93 by incubation with 4:1 M excess of 6-MSL for 3 days at 4°C with periodic gentle vortexing. Excess free spin label was then separated from the spin-labelled protein by extensive dialysis against the same buffer. After covalent labelling, protein dispersions were prepared in PBS at pH 7.2, in a 60% v/v glycerol/water mixture and in the freeze-dried state.

EPR spectroscopy

Pulsed EPR data were collected on an ELEXSYS E580 9-GHz Fourier-transform FT-EPR spectrometer (Bruker, Karlsruhe, Germany) equipped with a MD5 dielectric resonator and a CF 935P cryostat (Oxford Instruments, UK). The protein samples in the quartz tubes were rapidly frozen in liquid nitrogen and then accommodated in the cavity pre-cooled at 77 K; the measurements were carried out on increasing the temperature.

Primary, two-pulse ($\pi/2$–τ–π–τ–echo) echo-detected EPR spectra were obtained by recording the integrated spin-echo signal at fixed interpulse delay τ, whilst sweeping the magnetic field. The microwave pulse widths were 32 ns and 64 ns, with the microwave power adjusted to provide $\pi/2$ and π pulses, respectively. The integration window was 160 ns.

The original ED spectra, $\mathrm{ED}_T(2\tau,H)$, were corrected for instantaneous spin diffusion arising from static spin–spin interaction, by using spectra recorded at 77 K, where motional contributions are negligible. The corrected spectra, $\mathrm{ED}_T^{\mathrm{corr}}(2\tau,H)$ recorded at temperature T are plotted as a function of magnetic field, H, according to Erilov et al. (2004a):

$$\mathrm{ED}_T^{\mathrm{corr}}(2\tau,H) = \mathrm{ED}_T(2\tau,H)\frac{\mathrm{ED}_{77\mathrm{K}}(2\tau_0,H)}{\mathrm{ED}_{77\mathrm{K}}(2\tau,H)}, \quad (1)$$

where τ_0 is the smallest value of τ for which ED spectra were obtained.

Relaxation rates, $W(H,\tau_1,\tau_2)$, were determined from the ratio of corrected ED spectra recorded at two different values, τ_1 and τ_2, of the interpulse delay by using the following relation (Erilov et al. 2004b):

$$W(H,\tau_1,\tau_2) = \ln\left[\frac{\mathrm{ED}(2\tau_1,H)}{\mathrm{ED}(2\tau_2,H)}\right]\cdot\frac{1}{2(\tau_2-\tau_1)}, \quad (2)$$

where $\mathrm{ED}(2\tau,H)$ is the ED spectral line height at field position H.

Conventional CW-EPR spectra were recorded on an ESP-300 9-GHz spectrometer (Bruker, Karlsruhe, Germany) equipped with a ER 4111VT temperature controller and with a ER4201 TE_{102} standard rectangular cavity (both from Bruker). The spectrometer settings were: 100 kHz field modulation frequency, 3 $\mathrm{G}_{\mathrm{p-p}}$ peak-to-peak modulation amplitude and 10 mW microwave power, well below saturation.

Results and discussion

Echo-detected EPR spectra of spin-labelled haemoglobin fully hydrated in PBS, dispersed in a 60% v/v glycerol/water mixture and in the lyophilized form were acquired at different cryogenic temperatures.

Figure 1 shows ED-EPR spectra of SL-Hb in the three different environments recorded at 200 K and for various values of the echo delay time, τ.

All ED-EPR spectra were corrected for instantaneous spin diffusion according to Eq. 1 by using spectra recorded at 77 K, where little molecular motion is expected (Erilov et al. 2004a). Moreover, they have been normalized to the central line to display only the field-dependent part of the relaxation.

In each set of spectra, the dependence of the ED line shapes on the τ interpulse separation reveals preferential relaxation in the intermediate spectral regions at low and high field, which is characteristic of rapid, small-amplitude torsional libration (Dzuba et al. 1992; Dzuba 1996).

To characterize the librational motion of the spin-labelled protein, the anisotropic part of the relaxation rate, W, was calculated from the ED-EPR spectra according to Eq. 2.

As an example, Fig. 2b presents W relaxation rates evaluated for different pairs of τ_1 and τ_2 of the ED-EPR spectra of SL-Hb hydrated in the glycerol/water dispersion at 200 K as reported in Fig. 2a.

The W curves coincide within the noise level, showing that the relaxation is close to exponential. This is consistent with the so-called "isotropic" model for librational dynamics, in which uncorrelated librational motions take place simultaneously about the nitroxide x-, y- and z-axes (Erilov et al. 2004a; b). The relaxation rates are characterized by the maximum values, W_L and W_H, determined in the low- and high-field regions, respectively, of the ED spectra (Fig. 2b).

Figure 3a shows plots of both the W_L and the W_H values of SL-Hb in PBS, in glycerol/water and in the lyophilized form as a function of temperature.

The temperature profiles of W_L and W_H for the same sample are similar, showing consistency between the relaxation effects on the low- and high-field regions of the

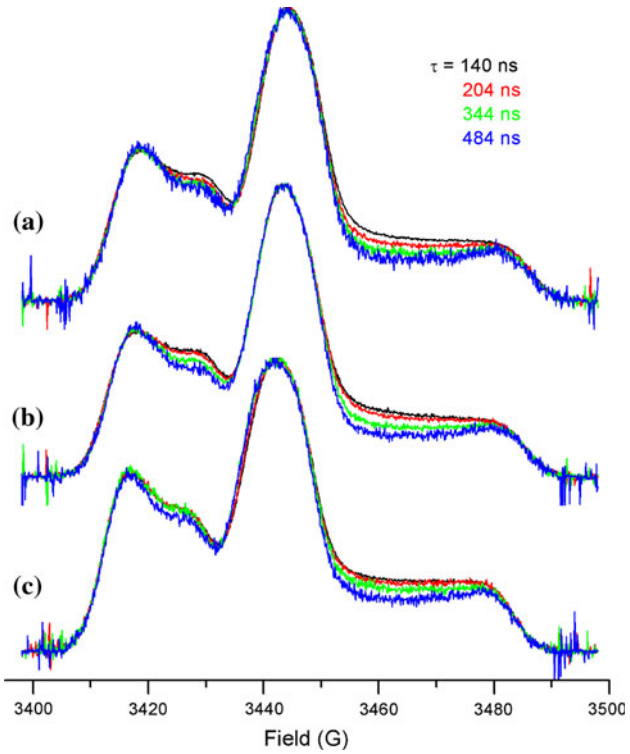

Fig. 1 Echo-detected EPR spectra at 200 K of SL-Hb that is either dispersed in aqueous buffer (**a**), or dispersed in 60% v/v glycerol/water mixture (**b**), or lyophilized (**c**). The ED spectra are recorded for interpulse spacings of (*top* to *bottom*) τ = 140, 204, 344 and 484 ns. Spectra are corrected for instantaneous diffusion according to Eq. 1, and are normalized to the maximum line height

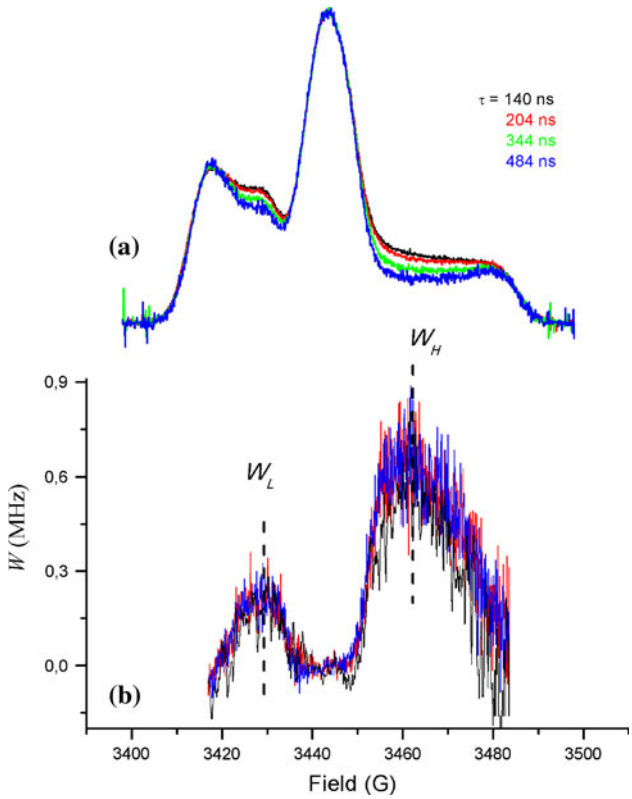

Fig. 2 **a** Corrected ED-EPR spectra at 200 K of SL-Hb dispersed in a 60% v/v glycerol/water mixture. The ED spectra are recorded for interpulse spacings of (*top* to *bottom*) τ = 140, 204, 344 and 484 ns. **b** Anisotropic part of the relaxation rate, W, obtained according to Eq. 2 from pairs of spectra in (**a**) with interpulse separations of $\tau_1 = 140$ and $\tau_2 = 204$ ns, or $\tau_1 = 140$ and $\tau_2 = 344$ ns, or $\tau_1 = 140$ and $\tau_2 = 484$ ns

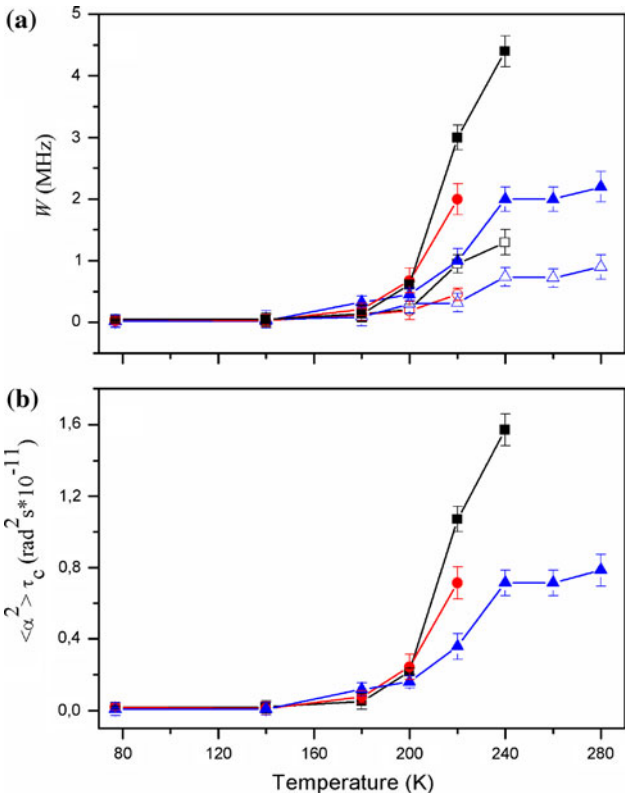

Fig. 3 Temperature dependence of the (**a**) W_L (*open symbols*) and W_H (*solid symbols*) relaxation rate parameters and of the (**b**) $\langle\alpha^2\rangle\tau_c$ amplitude–correlation time product of SL-Hb that is either dispersed in aqueous buffer (*red circles*), or dispersed in 60% v/v glycerol/water mixture (*black squares*), or lyophilized (*blue triangles*). The number of points is not the same for all samples, because primary echo vanishes at different temperatures

ED-EPR spectra. The different absolute values of W_L and W_H arise simply because of the different inherent sensitivities of the low- and high-field spectral regions to librational motion (Erilov et al. 2004a, b). In each sample, the librational motion is suppressed up to about 180 K. Above this temperature, in the hydrated samples, i.e. SL-Hb in PBS and in glycerol/water, the librational intensity increases with temperature more rapidly for Hb in glycerol/water mixture than in aqueous buffer (compare circle and square symbols in Fig. 3a). In the lyophilized protein, instead, the librational intensity increases moderately with temperature and reaches a plateau value at 240 K (triangles in Fig. 3a).

Figure 3b shows the temperature dependence of the amplitude–correlation time product, $\langle\alpha^2\rangle\tau_c$, of the librational motion of SL-Hb dispersed in aqueous buffer, in the glycerol/water mixture and lyophilized. The $\langle\alpha^2\rangle\tau_c$ values are obtained from the measurements of W_L presented in Fig. 3a by using a conversion factor relating $\langle\alpha^2\rangle\tau_c$ to W_L of 1.05×10^{17} rad^{-2} s^{-2}, as established previously both for phospholipids spin-labelled in the lipid chain and for alamethicin spin-labelled with TOAC, by using spectral simulations with the "isotropic" librational model (Erilov et al. 2004b; Bartucci et al. 2008) and used by De Simone et al. (2007) and Guzzi et al. (2009).

For all samples, $\langle\alpha^2\rangle\tau_c$ remains very low from 77 to 180 K and then increases more steeply for the hydrated protein, whereas it increases more gradually and up to 240 K for the lyophilized protein (Fig. 3b).

To determine the mean-square amplitude, $\langle\alpha^2\rangle$, of the librational motion, conventional CW-EPR spectra of the three spin-labelled protein preparations were also recorded as a function of temperature.

Figure 4 shows typical first-derivative CW-EPR absorption spectra of SL-Hb at full hydration recorded in buffer at different temperatures.

Powder patterns with broad lines are obtained at low temperature. From 200 K onwards, they progressively narrow and achieve almost sharp line shapes at the highest temperature.

The solvent effect on the CW-EPR spectral line shapes can be seen from the temperature dependence of the motionally averaged hyperfine splitting, $2\langle A_{zz}\rangle$, which is shown in Fig. 5a.

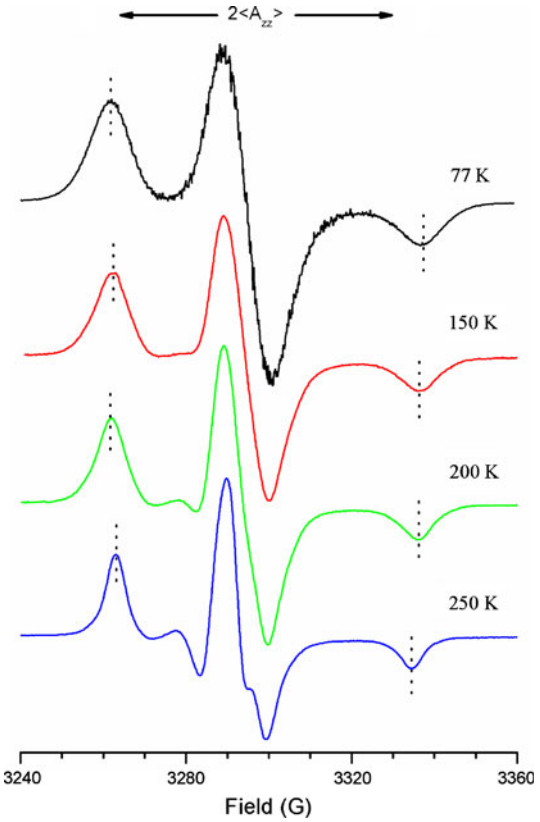

Fig. 4 Conventional CW-EPR spectra at the indicated temperatures of SL-Hb dispersed in aqueous buffer

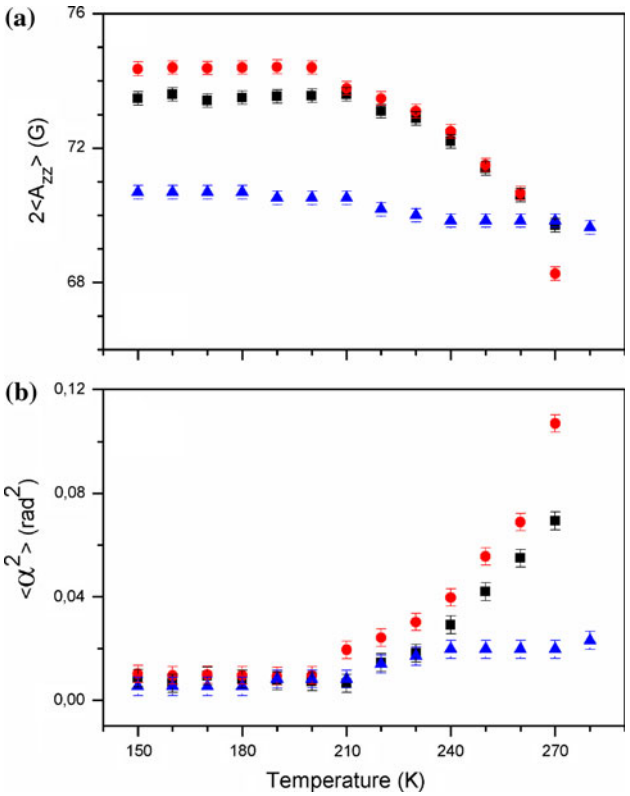

Fig. 5 Temperature dependence of the (**a**) outer hyperfine splitting $2\langle A_{zz}\rangle$ and of the (**b**) librational amplitude $\langle\alpha^2\rangle$ of SL-Hb that is either dispersed in aqueous buffer (*red circles*), or dispersed in 60% v/v glycerol/water mixture (*black squares*), or lyophilized (*blue triangles*)

For SL-Hb fully hydrated in PBS, initially (at low temperature), the hyperfine splitting is approximately constant. This represents the temperature range over which the hyperfine splitting corresponds to its rigid-limit value, $2A_{zz}$, which is dependent mainly on the polarity of the spin-label environment (see, e.g., Marsh 2001; Kurad et al. 2003). Above 200 K, the values of $2\langle A_{zz}\rangle$ decrease with increasing temperature as a result of progressive motional narrowing by librations (Van et al. 1974). Another contribution to the decrease in hyperfine splitting is unfreezing of the rotation of label methyl groups, a mechanism which leads to averaging of the anisotropic proton hyperfine couplings in nitroxides (Kulik et al. 2005).

The temperature dependence of $2\langle A_{zz}\rangle$ for SL-Hb in glycerol/water dispersions is rather similar to that seen for the protein in buffer. Indeed, at low temperature, constant values of the hyperfine splitting are obtained, followed by a progressive decrease starting above 210 K. In glycerol-containing sample, the lower $2\langle A_{zz}\rangle$ values in the low-temperature range result from the lower solvent polarity of the glycerol/water mixture.

Figure 5a shows that the departure from the linear dependence of $2\langle A_{zz}\rangle$ occurs at about 210 K for the protein in PBS, and at about 220 K when glycerol is present in solution. This corresponds to the onset of a dynamical regime in the hydrated proteins which shows up at temperatures higher than the glass-transition temperature of the solvent matrices (Angell 2008; Li et al. 2008).

The absence of water molecules in the dehydrated SL-Hb leads to much smaller hyperfine splitting that keeps an almost constant value over the whole temperature range investigated (see blue triangles in Fig. 5a). This finding indicates restricted dynamics in the lyophilized protein also at high temperature.

It is worth noting that similar behaviour with regard to hydration conditions has been observed in the temperature dependence of the outer hyperfine splitting for myoglobin and lysozyme labelled with 6-MSL (Ruggiero et al. 1985) and of the order parameter for 5-MSL/Hb (Steinhoff et al. 1989). In fact, the hydrated samples show a discontinuity in the above-mentioned parameters around 200–220 K, whereas it is absent in the freeze-dried samples.

The mean-square amplitude, $\langle\alpha^2\rangle$, values of the librational motion of SL-Hb are given in Fig. 5b as a function of temperature. For small-amplitude, fast libration, they are derived from $\langle A_{zz}\rangle$ according to the following equation (Van et al. 1974):

$$\langle A_{zz}\rangle = A_{zz} - (A_{zz} - A_{xx})\langle\alpha^2\rangle. \quad (3)$$

The rigid-limit hyperfine splitting A_{zz} was derived by linear extrapolation of the data in Fig. 5a to zero temperature, and A_{xx} was taken from literature (see, e.g., Berliner 1976; Marsh 1981).

Up to 200 K, very small $\langle\alpha^2\rangle$ values are obtained for the three spin-labelled protein samples. Thereafter, the $\langle\alpha^2\rangle$ values increase progressively for hydrated SL-Hb, reaching higher values for SL-Hb dispersions in PBS than in glycerol/water mixture. In contrast, the increase of $\langle\alpha^2\rangle$ is very limited for the protein in the freeze-dried state, and a plateau level is observed from 240 K onwards. The temperature dependence of $\langle\alpha^2\rangle$ in the hydrated protein samples is similar to that observed previously by CW-EPR and pulsed EPR for small spin probe in glass-forming solvents (Paschenko et al. 1999; Dzuba 2000; Kirilina et al. 2001), for spin-labelled frozen lipid dispersions (Erilov et al. 2004b; Isaev and Dzuba 2008), for chain-labelled stearic acids inserted in the binding sites of human serum albumin (De Simone et al. 2007), for spin-labelled Na,K-ATPase (Guzzi et al. 2009) and for photosynthetic bacterial reaction centre (Poluetkov et al. 2003). It is also interesting to note that the behaviour of $\langle\alpha^2\rangle$ as a function of temperature is analogous to that of the mean-square atomic displacement,

$\langle r^2 \rangle$, determined by neutron scattering (Doster 2010) and Mossbauer spectroscopy (Parak et al. 2006).

In all samples, below 210 K, the amplitude of the librational motion is about 5 ± 1° and then increases with the temperature. However, in the lyophilized SL-Hb sample, α has lower variation and reaches 8 ± 1° at the highest temperatures, whereas α is 12 ± 1° and 10 ± 1° at 240 K for SL-Hb hydrated in buffer and in the presence of glycerol, respectively.

For the hydrated protein samples, the observed differences in α values can be related to the solvent intermolecular bonds. In fact, glycerol is known to form a strong glass, which is due to the formation of a network of hydrogen bonds. In glycerol/water mixture the hydrogen bonds are strengthened compared with those formed in PBS. Accordingly, the variation of α with temperature is smaller in the presence of glycerol than PBS. Another contribution to this difference is the higher viscosity of the glycerol/water mixture. The lyophilized spin-labelled protein has small variation of the librational angle in the temperature range investigated, indicating that the presence of the solvent is important to activate the protein dynamics. Indeed, water acts as a plasticizer that favours protein dynamics (Doster 2010). Likely, the limited increase in the mean-square librational amplitude could be due to the presence of residual water molecules entrapped in the protein structure. A smooth increase in $\langle r^2 \rangle$ with temperature is also observed in dry lysozyme and in tRNA (Roh et al. 2009).

Finally, combining $\langle \alpha^2 \rangle \tau_c$ and $\langle \alpha^2 \rangle$ values, the librational correlation time τ_c of the spin-labelled protein in the different solvents can be determined. Evaluations were made only at temperatures above 200 K, for which $\langle \alpha^2 \rangle$ is different from quasi-zero. The correlation time for all samples is in the sub-nanosecond range and depends weakly on temperature. Moreover, τ_c results 0.30 ± 0.06 ns for SL-Hb in PBS at 220 K, 0.54 ± 0.06 ns for SL-Hb in glycerol/water mixture and 0.36 ± 0.06 ns for the lyophilized SL-Hb at 240 K.

It is interesting to compare the characteristics of the librational motion obtained with EPR methods in other fully hydrated biosystems. Similarly to spin-labelled stearic acids in human serum albumin (De Simone et al. 2007), to TOAC spin-labelled alamethicin in lipid membranes (Bartucci et al. 2008) and to maleimide spin-labelled pig and shark Na,K-ATPase (Guzzi et al. 2009), the librational dynamics in SL-Hb is activated at temperature around 180–200 K. This is in contrast to the results in model membranes, in which chain-labelled lipids both at the first segments of the alkyl chain and at the terminal methyl end show a remarkable intensity of librations already at 120 and 80 K, respectively (Erilov et al. 2004b; Isaev and Dzuba 2008). At 240 K, the librational angular amplitude is much greater for SL-Hb relative to the above-mentioned biosystems and comparable to that in chain-labelled membranes (Erilov et al. 2004b). This comparison indicates that SL-Hb possesses significant librational dynamics in the frozen state. Finally, the librational correlation time of SL-Hb, which is on average 0.4 ns, has the same magnitude as that found in the other frozen spin-labelled biosystems, but is longer than that in the spin-labelled peptide backbone in phospholipid membranes (0.7–0.8 ns) (Bartucci et al. 2008). Solvent effect and hydration in maleimide spin-labelled Hb have been studied by linear CW-EPR (Johnson 1978; Ruggiero et al. 1985; Steinhoff et al. 1989) and non-linear, saturation transfer (ST)-EPR (Johnson 1978). The spectral changes observed in a wide temperature range were interpreted in terms of librational model of the spin-labelled protein.

The nanosecond vibrational dynamics of different biosystems (DNA, RNA, proteins, lipid membranes) can also be conveniently studied by neutron scattering (Fitter et al. 1999; Roh et al. 2009; Khodadadi et al. 2010a, b; Doster 2010, and references therein). In particular, for lysozyme, both the quasi-elastic neutron scattering intensity and the mean-square atomic displacement increase from the dry to the hydrated state. The increase is significant with hydration for $T > 200$ K (Roh et al. 2009), as observed in our work on haemoglobin.

In conclusion, combining data from two-pulse ED spectra of electron spin-echo experiments and from the hyperfine splitting of CW-EPR spectra, the librational motion of spin-labelled haemoglobin has been characterized in the low cryogenic temperature regime for samples hydrated in PBS, in a 60% v/v glycerol/water mixture and in the lyophilized form. Fast stochastic librational motion of limited amplitude was detected for all protein preparations. The librational motion is suppressed up to ca. 200 K and then increases progressively in the hydrated protein, whereas it is more restricted and reaches a plateau level at 240 K in the dry protein. The same trend is observed in the temperature dependence of the librational amplitude, whereas the correlation time of the librations is less dependent on temperature and on solution conditions and lies in the sub-nanosecond regime. On the whole, the results indicate that the presence of solvent favours protein dynamics and that addition of glycerol has a slight influence on the characteristics of the librational oscillations in haemoglobin.

High-frequency librational motions of small angular amplitude are inevitably present also at higher, physiological temperature, where slower, large-scale rotations make it difficult to resolve the rapid librational modes explicitly. Such rapid librational oscillations of small angular amplitude are generally important for glass-like state of proteins and could be relevant in Hb function to

permit oxygen accessibility to the iron atoms of the embedded haem groups and, possibly, to favour oxygen transport.

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