

Heterogeneity of Protein Substates Visualized by Spin-label EPR

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ABSTRACT The energy landscape of proteins is characterized by a hierarchy of substates, which give rise to conformational heterogeneity at low temperatures. In multiply spin-labeled membranous Na,K-ATPase, this heterogeneous population of conformations is manifest by strong inhomogeneous broadening of the electron paramagnetic resonance (EPR) line shapes and nonexponential spin-echo decays, which undergo a transition to homogeneous broadening and exponential relaxation at higher temperatures (previous study). In this study, we apply these EPR methods to small water-soluble proteins, of the type for which the existence of conformational substates is well established. Both α -helical and β -sheet aqueous proteins that are spin-labeled on a single cysteine residue display spin-echo decays with a single phase-memory time T_{2M} and conventional EPR line shapes with predominantly homogeneous broadening, over a broad range of temperatures from 77 K to ~ 250 K or higher. Above ~ 200 K, the residual inhomogeneous broadening is reduced almost to zero. In contrast, both the proteins and the spin label alone, when in a glycerol-water mixture below the glass transition, display heterogeneity in spin-echo phase-memory time and a stronger inhomogeneous broadening of the conventional line shapes, similar to multiply spin-labeled membranous Na,K-ATPase below 200 K. Above 200 K (or the glass transition), a single phase-memory time and predominantly homogeneous broadening are found in both spin-label systems. The results are discussed in terms of solvent-mediated protein transitions, the ability of single spin-label sites to detect conformational heterogeneity, and the desirability of exploring multiple sites for proteins with the size and complexity of the Na,K-ATPase.

INTRODUCTION

The energy landscape of proteins is thought to be composed of conformational substates, which give rise to a heterogeneous protein population at low temperatures (1,2). In the conformational substates, individual protein molecules differ in the local arrangement of atoms or groups of atoms. Evidence for conformational substates comes from the rebinding kinetics of CO to myoglobin (3), Mössbauer experiments on haem proteins (4), and the temperature dependence of Debye-Waller factors in x-ray crystallography of myoglobin and ribonuclease (5,6). Conformational substates occur not only in haem metalloproteins; they have also been evidenced from rebinding kinetics of NO to the blue copper protein azurin (7). Moreover, for metalloproteins in the frozen state, *g*- and *A*-strain in electron paramagnetic resonance (EPR) spectra have been related to structural disorder and conformational substates (8–11). A correlation between protein dynamics and fluctuation between conformational substates has been suggested by using site-directed spin-labeling EPR spectroscopy (12,13).

In general, the energy landscape of proteins is modulated by temperature, pressure, and chemical perturbation of the solvent (i.e., pH, ionic strength, osmolyte concentration, etc.). A glass-like transition takes place at temperatures in the region of 200 K, above which the protein then fluctuates between the different conformational substates. Evidence

for a dynamical transition in this temperature range comes from neutron-scattering experiments, which show that the mean square fluctuation of the atomic positions in the protein increases more steeply with temperature above ~ 200 K (14,15). Mostly, work in this area has been performed with small water-soluble proteins. With the notable exception of bacteriorhodopsin (16), there has been little application to membrane proteins and none to large integral membrane proteins.

Recently, we showed that both phase-memory times and conventional EPR line shapes of spin-labeled membranous Na,K-ATPase provide evidence for a heterogeneous population of conformational substates at low temperatures (17). A glass-like transition to a uniform population was found to occur at higher temperatures. This transition coincided with the onset of rapid torsional librations on the nanosecond timescale and slower larger-scale motions on the microsecond timescale, both of which were detected by spin-echo EPR techniques. Partial unfolding by urea is found to increase the heterogeneity of conformational substates, as recorded by phase-memory times (18).

Na,K-ATPase is a large transmembrane protein that contains several sites for spin-labeling. It is therefore of interest to compare the spin-label results on Na,K-ATPase with similar EPR measurements on smaller soluble proteins, comparable with those with which evidence for conformational substates and glass-like transitions has been well established by other techniques such as x-ray diffraction and Mössbauer spectroscopy. Also of relevance is a comparison with the behavior of small spin labels in classical

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glass-forming media. In the present work, we determine spin-echo decay curves and analyze conventional EPR line shapes of two α -helical proteins (hemoglobin and serum albumin) and a β -sheet protein (β -lactoglobulin) that are spin-labeled on a single cysteine residue by using a maleimido nitroxide derivative. The proteins are examined in water, in the freeze-dried state, and in a glycerol-water mixture. Similar measurements are also made on spin labels alone in a glass-forming glycerol-water mixture. Comparison with previous results on Na,K-ATPase (17) then indicates, among other things, to what extent multiple labeling is necessary to sample the full conformational heterogeneity of large integral membrane proteins.

MATERIALS AND METHODS

Materials

Human hemoglobin (Hb), human serum albumin (HSA), bovine β -lactoglobulin (β -LG), spin-labeled maleimides (3-maleimido-1-oxyl-2,2,5,5-tetramethylpyrrolidine, 5-MSL; 4-maleimido-1-oxyl-2,2,6,6-tetramethylpiperidine, 6-MSL), and the TEMPONE spin label (4-oxo-1-oxyl-2,2,6,6-tetramethylpiperidine) were from Sigma/Aldrich (St. Louis, MO).

Spin-labeling

Hb (5 mM in 10 mM phosphate buffer, pH 7.0) was spin-labeled on Cys- β 93 by incubation with a 4:1 molar excess of 6-MSL for 3 d at 4°C. Unreacted spin label was removed by extensive dialysis against the same buffer. HSA in 10 mM phosphate buffer pH 7.2 was spin-labeled on Cys-34 with 5-MSL as described in a previous study (19). β -LG in 150 mM NaCl, 1 mM EDTA, pH 7, was spin-labeled on Cys-121 with 5-MSL, by reacting 1 ml of 40 mg/ml β -LG with 250 μ l of 2.5 mg/ml 5-MSL for 3 h at 37°C, followed by extensive dialysis and subsequent freeze drying.

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, Abingdon, UK). Samples were rapidly frozen in liquid nitrogen, and then introduced quickly into the pre-cooled cavity at 77 K. Data were recorded with increasing temperature from 77 K upward.

Two-pulse ($\pi/2$ - τ - π - τ -echo) spin-echo decays were obtained by integrating the echo and incrementing the pulse spacing, τ . The window for the integration was 160 ns. The microwave pulse widths were 32 and 64 ns, with the microwave power adjusted to provide $\pi/2$ and π -pulses, respectively. Use of soft pulses and integration of the echo largely suppresses the proton hyperfine modulations (see Fig. 1 later). The magnetic field was set to the EPR absorption maximum. The τ -dependence of the integrated echo intensity, $I(\tau)$, was fitted either with a single exponential or with a stretched exponential decay function as follows:

$$I(\tau) = I(0)\exp\left[-(2\tau/T_{2M,o})^\beta\right] \quad (1)$$

where the exponent $\beta = 1$ corresponds to a simple single exponential, and $T_{2M,o}$ is the unique phase-memory time in that case.

Conventional CW EPR spectra were recorded on an ESP-300 9-GHz spectrometer (Bruker, Karlsruhe, Germany) using 100-kHz field modulation; the spectrometer was equipped with an ER 4111VT temperature

controller. The low-field ($m_l = +1$) hyperfine extremum in the CW-EPR powder patterns was fitted by nonlinear least-squares minimization with a Voigt absorption line shape as follows:

$$v(H) = A \int_{-\infty}^{\infty} \frac{\exp(-(H' - H_o)^2/2\sigma_G^2)}{(\Delta H_L/2)^2 + (H - H')^2} dH' \quad (2)$$

where ΔH_L is the full width at half-height of each Lorentzian absorption component, σ_G is the standard deviation of the Gaussian distribution of Lorentzian components, and H_o is the center of the Gaussian distribution. The full width at half-height of the Gaussian distribution is given by $\Delta H_G = (2\sqrt{2 \ln 2})\sigma_G$. Note that the values of ΔH_L and ΔH_G that we report correspond with the data given in previous studies (17,18), where the definition was given incorrectly. The high-field wings of the low-field ($m_l = +1$) hyperfine extrema were omitted from the fitting procedure to eliminate any distortion by the powder pattern envelope.

RESULTS

We first present data on both spin-echo decays and conventional EPR line shapes for spin-labeled proteins in water and in the freeze-dried state. We then present comparable data on both proteins and small spin labels in glass-forming glycerol-water mixtures.

Echo decay curves and phase-memory time of spin-labeled proteins in water

Fig. 1 shows the temperature dependence of the spin-echo amplitudes as a function of echo delay time, τ , for spin-labeled β -lactoglobulin in aqueous buffer. The echo decays are reasonably well described by a single exponential function (dotted line), which specifies a unique phase-memory time, T_{2M} (see 20,21).

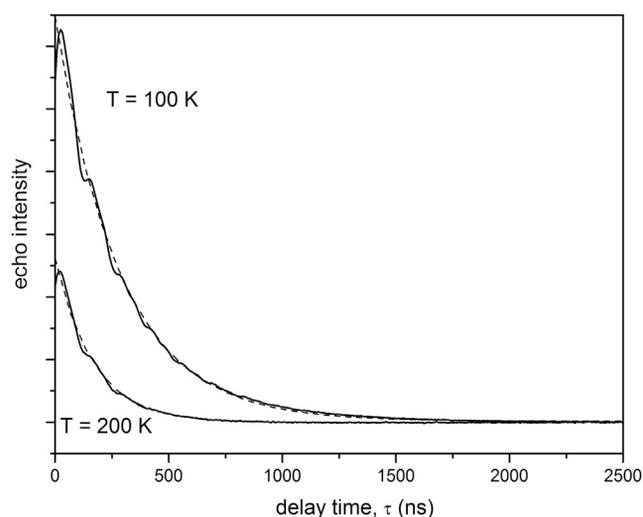


FIGURE 1 Spin-echo decay curves at the temperatures indicated for bovine β -lactoglobulin spin-labeled with 5-MSL and dissolved in water. Dashed lines are single exponentials. The vertical intensity axis is linear.

Figure S1 of the Supporting Material gives the temperature dependence of the phase-memory time for three different spin-labeled proteins, human hemoglobin, human serum albumin, and bovine β -lactoglobulin dissolved in aqueous buffer. Comparable data are given also for the lyophilized proteins. The phase-memory times were obtained by fitting the experimental echo decay curves with a single exponential (i.e., $\beta = 1$ in Eq. 1) in each case. Only for the proteins in glycerol-water mixtures below the glass temperature (see below) did we find that a single exponential function was inadequate, and the echo decays were fitted with a stretched exponential function appropriate to a distribution of phase-memory times.

Note that echo-detected spectra of spin-labeled hemoglobin (9) show that intermolecular dipole-dipole interactions are unlikely to contribute to the echo decay rates, because the center of the echo-detected spectrum is not suppressed relative to the wings, as is found for instantaneous diffusion induced by spin-spin interaction (see, e.g., 22,23).

Continuous wave (CW) EPR spectra of spin-labeled proteins in water

Fig. 2 shows the temperature dependence of the CW-EPR spectra from the spin-labeled proteins in water. The powder patterns obtained at low temperature have rather broad lines, which narrow progressively with increasing temperature. Diluting the spin-labeled protein by mixing with unlabeled protein has no effect on the line broadening at low temperatures. The crosses in Fig. 2 represent the results of fitting a Voigt absorption line shape (Eq. 2) to the low-field hyperfine extremum of each spectrum. This line shape corresponds to a Gaussian convolution of pure Lorentzian components.

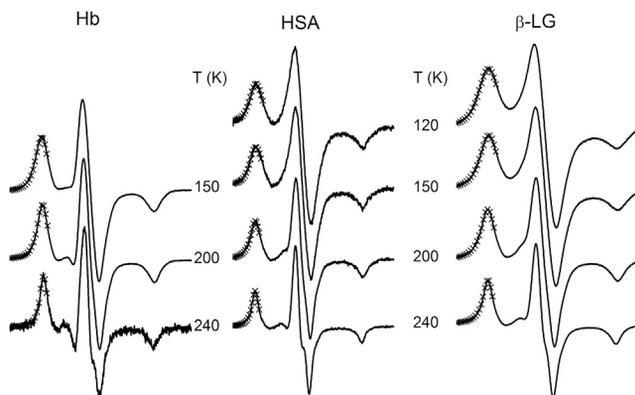


FIGURE 2 Temperature dependence of the CW-EPR spectra of 6-MSL-labeled human hemoglobin (Hb), 5-MSL-labeled human serum albumin (HSA), and 5-MSL-labeled β -lactoglobulin (β -LG) in water. Crosses represent fitting of the low-field ($m_l = +1$) hyperfine extremum with a Voigt line shape (Eq. 2). Total scan width = 12 mT for Hb and HSA, and 10 mT for β -LG.

Fig. 3 gives the temperature dependences of the widths of the Gaussian distribution (ΔH_G) and of the Lorentzian components (ΔH_L) that are obtained by fitting the CW-EPR spectra of each spin-labeled protein dissolved in water. For reference, data are also included for the freeze-dried proteins. At low temperatures, the Lorentzian line widths are considerably larger than the widths of the Gaussian distributions. In all cases, the Gaussian distribution widths decrease with increasing temperature, reaching zero or rather low values at temperatures above 260 K for both dissolved and lyophilized proteins. This corresponds to averaging of the inhomogeneous broadening by exchange processes at the higher temperatures (24). For the proteins dissolved in water, the Lorentzian line widths remain fairly constant up to 180–200 K, and then decrease gradually beyond this. The temperature dependence of the Lorentzian widths of the lyophilized proteins is much smaller. For

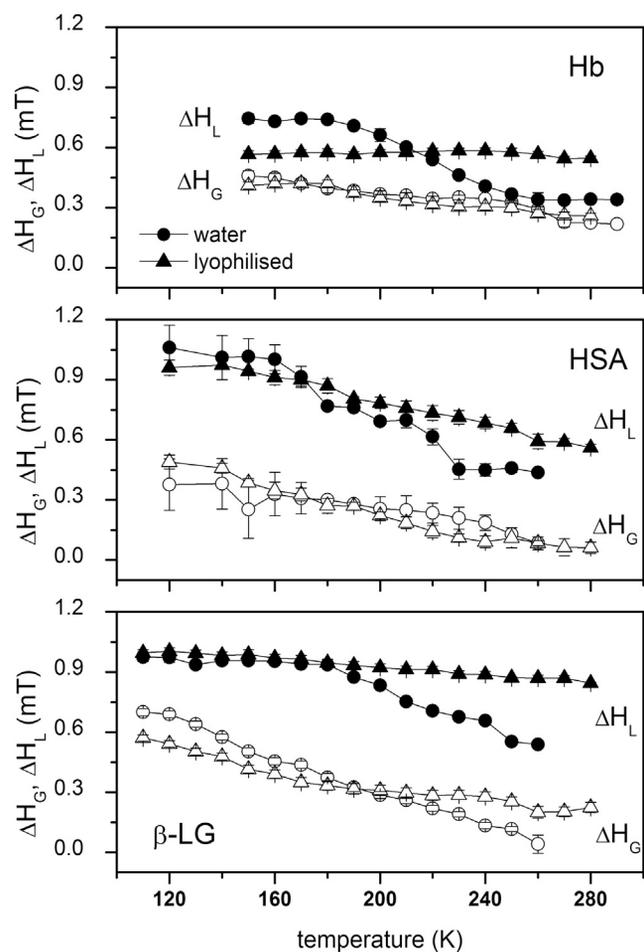


FIGURE 3 Temperature dependence of the deconvoluted Lorentzian (ΔH_L , solid symbols) and Gaussian (ΔH_G , open symbols) widths of the low-field ($m_l = +1$) hyperfine line obtained by fitting Voigt line shapes (Eq. 2) to the CW-EPR spectra of the spin-labeled proteins dissolved in water (circles) or lyophilized (triangles). Top panel: 6-MSL-labeled human hemoglobin (Hb); middle panel: 5-MSL-labeled human serum albumin (HSA); bottom panel: 5-MSL-labeled bovine β -lactoglobulin (β -LG).

lyophilized Hb and β -LG, the Lorentzian widths change hardly at all up to 280 K. On the other hand, the temperature dependence of the Gaussian broadening is rather similar for the hydrated and lyophilized proteins.

Echo decay curves and phase-memory time of spin-labeled proteins in glycerol

The upper panel of Fig. 4 shows the decays of the spin-echo amplitude as a function of echo delay time at 100 K, for β -LG and HSA spin-labeled with 5-MSL, and Hb spin-labeled with 6-MSL, in a 60% v/v glycerol-water mixture. At such low temperatures, the echo decays are not fit well by a single exponential function but can be described by a stretched exponential function (Eq. 1), which implies a distribution of phase-memory times. Above 200 K, the echo decays for β -LG and HSA are well described by a single exponential and the exponent β is close to unity. (For spin-labeled Hb at higher temperatures, the echo intensity is too low to distinguish possible heterogeneity.) Similar results are found with the 5-MSL or 6-MSL spin label alone, or with the small TEMPONE spin label, in 60% v/v glycerol-water mixtures, at temperatures below the glass transition. The echo decay curves for these spin labels at 100 K (shown in the lower panel of Fig. 4) also display heterogeneity of phase-memory times that is characteristic of the glassy media.

The upper pair of panels in Fig. S2 gives the temperature dependences of the parameters, $T_{2M,o}$ and β , which characterize the distribution of phase-memory times for β -LG, HSA, and Hb dissolved in a glycerol-water mixture that contains 60% v/v glycerol. These values were obtained by fitting the experimental echo decay curves with Eq. 1 for a stretched exponential. Below 200 K, a stretched-exponential fit is superior to a single exponential fit (i.e., the exponent, $\beta < 1$), indicating a heterogeneous distribution of phase-memory times for the spin-labeled proteins in glycerol-water mixtures, in this temperature regime. The exponent of the stretched exponential increases, and the phase-memory time decreases, with increasing temperature in this regime. Note that the range of uncertainty in the exponent β is less than the size of the symbols in Fig. S2, showing that the deviations corresponding to the less good fit for $\beta = 1$ (dashed line in Fig. 4) are significant. Above the glass transition temperature, the phase-memory time $T_{2M,o}$ increases to a local maximum in Fig. S2 and the stretched-exponential exponent increases to $\beta \approx 1$. At temperatures of 200 K and above, the echo decays become single exponential, corresponding to a single phase-memory time for all spin labels. A similar behavior is displayed by the small spin labels TEMPONE, and 5-MSL and 6-MSL alone, in the glass-forming 60% v/v glycerol-water mixture (see lower pair of panels in Fig. S2). The exponent of the stretched exponential reaches unity only at temperatures of 200 K and above.

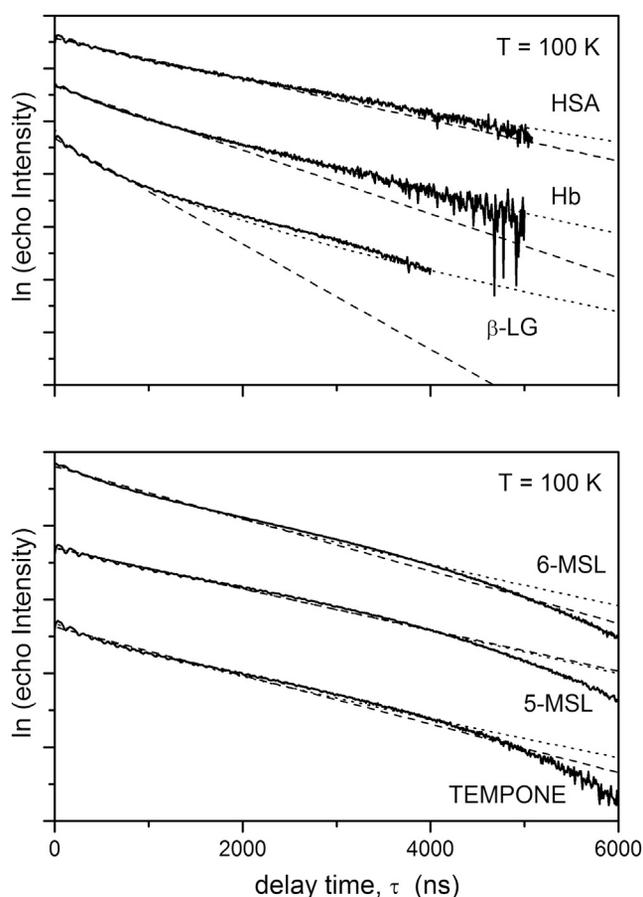


FIGURE 4 Spin-echo decay curves at 100 K for spin labels dissolved in 60% v/v glycerol-water. Upper panel: 6-MSL labeled human hemoglobin (Hb), and 5-MSL-labeled bovine β -lactoglobulin (β -LG), and human serum albumin (HSA). Lower panel: the 6-MSL and 5-MSL spin labels alone, and the small spin label TEMPONE. Dotted lines are stretched-exponential fits; dashed lines are single exponential fits. For clarity, the echo decays are shifted along the vertical axis, which is logarithmic; each minor increment corresponds to a change in intensity by a factor e .

Continuous wave (CW) EPR spectra of spin-labels in glycerol

Figure S3 shows the temperature dependence of the CW-EPR spectra from 5-MSL and from TEMPONE in 60% v/v glycerol-water. The powder patterns obtained at low temperature have extremely broad lines with inhomogeneously broadened line shapes of strong Gaussian character. These line shapes remain unchanged upon decreasing the spin label concentration by a factor of ten. With increasing temperature, the lines narrow progressively and the line shapes become more homogeneous until finally they become almost purely Lorentzian.

The lower panel in Fig. 5 gives the temperature dependences of the widths of the Gaussian distribution (ΔH_G) and of the Lorentzian components (ΔH_L) that are obtained by fitting the CW-EPR spectra of the small spin labels TEMPONE and 5-MSL alone in 60% v/v glycerol-water. Similar data for the spin-labeled proteins β -LG, HSA, and

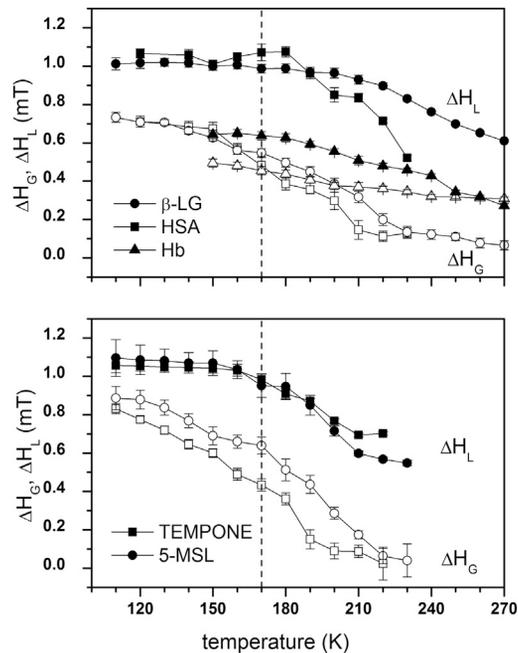


FIGURE 5 Temperature dependence of the deconvoluted Lorentzian (ΔH_L , solid symbols) and Gaussian (ΔH_G , open symbols) widths of the low-field ($m_l = +1$) hyperfine line obtained by fitting Voigt line shapes (Eq. 2) to the CW-EPR spectra of spin-labeled systems dissolved in 60% v/v glycerol-water. Upper panel: 5-MSL-labeled bovine β -lactoglobulin (circles) and human serum albumin (squares), and 6-MSL-labeled hemoglobin (triangles). Lower panel: 5-MSL alone (circles) and TEMPONE (squares). The vertical dashed line is the approximate position of the glass transition temperature for 60% v/v glycerol.

Hb in 60% v/v glycerol-water are given in the upper panel of Fig. 5. At low temperatures, the Gaussian distribution widths are comparable with the Lorentzian line widths but decrease steadily with increasing temperature. The Lorentzian line widths, on the other hand, remain approximately constant to between 160–180 K and then decrease gradually with increasing temperature. At the same time, the Gaussian distribution width decreases more rapidly, reaching zero or a rather small value at 220 K.

DISCUSSION

Energy landscapes of proteins are characterized by a hierarchy of conformational substates, transitions between which determine the various timescales in protein dynamics (25,26). These transitions are driven by librational fluctuations within the potential wells of the corresponding substates (see, e.g., 17,27). Such equilibrium protein fluctuations observed at lower temperatures can drive functionally important motions (i.e., on-pathway conformational transitions) at physiological temperatures, as embodied in the fluctuation-dissipation theorem (28,29). The conformational substates that are identified in this study by inhomogeneous broadening in the EPR spectra of small, singly labeled, soluble proteins at temperatures below 200 K are those that

have been studied classically by techniques such as x-ray diffraction (5,6,30), Mössbauer spectroscopy (4,31), and inelastic neutron scattering (14,15,32). Line-shape analysis in spin-label CW-EPR, therefore, provides a valuable addition to the armoury of biophysical methods available and can be further augmented by employing site-directed spin-labeling (12,13,33).

The small, singly labeled soluble proteins Hb, HSA, and β -LG show a behavior similar to that found previously for multiply labeled membranous Na,K-ATPase (17), as regards averaging of the inhomogeneous broadening in the conventional CW-EPR spectra at higher temperatures (Fig. 3). However, unlike Na,K-ATPase, they do not exhibit a clear heterogeneity in phase-memory times at low temperatures, when in aqueous solution (Figs. 1 and S1). Nevertheless, a pronounced heterogeneity in T_{2M} is found for the small, soluble proteins in the glassy state of glycerol-water mixtures (see Figs. 4 and S2). This coincides with a pronounced Gaussian broadening at low temperatures for both β -LG and HSA in glycerol-water mixtures (see Fig. 5), as occurs also with membranous Na,K-ATPase (17). This is to be expected because, as already mentioned, small soluble proteins display a heterogeneous population of substates at low temperatures (2). Presumably, in the case of phase-memory times, single labeling of the protein is not sensitive enough to detect heterogeneity of conformational substates via differences in T_{2M} , because this corresponds to limited and possibly highly localized conformational differences. Multiple labeling of the Na,K-ATPase is advantageous in this respect.

In glycerol-water mixtures, both the 5-MSL and TEMPONE spin labels, and the spin-labeled β -LG, HSA, and Hb proteins, are characterized by heterogeneity in phase-memory time (Figs. 4 and S2) and pronounced inhomogeneous broadening of the conventional CW-EPR spectra (Fig. 5), when at low temperatures below the glass transition. For temperatures above 200 K, spin-echo decays of the 5-MSL and TEMPONE spin labels alone display a single phase-memory time and the inhomogeneous (Gaussian) broadening of their CW-spectra becomes very small. This indicates heterogeneity in the spin label environment below the glass transition temperature of the glycerol-water mixture, which possibly is associated with different conformations, extents of hydrogen bonding and/or aggregation states of the spin labels alone. Most likely, these effects are also directly operative in the spin-labeled proteins, but additionally the glassy solvent promotes substate heterogeneity in the protein itself. Above the glass transition of the glycerol-water mixture, the spin label begins to sense a more uniform environment, as do also the protein-attached spin labels. The enhanced EPR signature of the small proteins in a glass-forming medium is highly significant, because bulk-solvent and/or hydration-shell fluctuations are thought to control protein motions and function (34). Also, the dynamic transition in proteins, which occurs somewhat above the glass transition (here at ~ 200 K),

depends directly on the solvent-protein interaction (14,35), as anticipated from Kramers theory for the coupling of dynamic processes to the environment (36). It is of further significance that different reaction steps of the Na,K-ATPase depend strongly on viscosity of the suspending medium (37).

The EPR-behavior of the spin labels and small proteins in glycerol-water mixtures is comparable with that found in a previous study on the spin-labeled membrane protein Na,K-ATPase dispersed in buffer (17). There are clear similarities in the temperature dependences, but also quantitative differences in the line broadening and phase-memory times. At low temperatures, the phase-memory times of both spin-labeled Na,K-ATPase and the spin-label systems in the glycerol-water glass are heterogeneous. With increasing temperature, the echo decays are characterized by a single, unique value of T_{2M} at 150 K and above for membranous Na,K-ATPase, and at 200 K and above for the proteins and spin labels in 60% v/v glycerol-water. The glass transition temperature for the latter mixture is at $T_g \sim 170$ K (38). The inhomogeneous broadening of both decreases with increasing temperature, being reduced to almost zero for 5-MSL, TEMPONE, β -LG, and HSA, above the glass transition temperature of 60% v/v glycerol-water, and to a low value for Na,K-ATPase, at comparably high temperature (17). Like the spin labels in a glycerol-water glass, the Gaussian broadening of spin-labeled Na,K-ATPase at low temperatures is smaller than that of the Lorentzian components. Similarly, the Gaussian line widths of membrane-bound Na,K-ATPase are greater than those for the smaller aqueous proteins that are given in Fig. 3. In part this may be attributed to the greater size of the Na,K-ATPase, but also multiple labeling of Na,K-ATPase allows to sample a spatially wider range of conformational substates than does the single labeling of the smaller proteins in Fig. 3. Note that the distinction between conformational substates may, in some cases, amount to rather small localized differences such as in the orientation of one particular amino acid residue. Exactly in this respect, future work with site-directed spin-labeling offers unique opportunities not available to the traditional methods of studying protein conformational substates.

SUPPORTING MATERIAL

Three figures are available at [http://www.biophysj.org/biophysj/supplemental/S0006-3495\(14\)00013-7](http://www.biophysj.org/biophysj/supplemental/S0006-3495(14)00013-7).

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SUPPORTING MATERIAL

Temperature dependence of T_{2M}

The temperature dependences of the phase memory times for proteins in water are shown in Fig. S.1, and of proteins or spin labels alone in aqueous glycerol are shown in Fig. S.2. The overall magnitudes of T_{2M} are similar to those found for non-clustered spin-labelled lipids in the low-temperature phases of bilayer membranes ref. 20; and cf. ref. 21). The temperature dependences for the spin-labelled proteins are complex and difficult to unravel completely. Below approximately 180 K for the proteins in water, or the glass transition temperature for samples in aqueous glycerol, the molecular motions are restricted to harmonic vibrations or librations without activation energy that are characteristic of the glassy state (35). Above this temperature, the librational motions become stochastic (i.e., diffusive) and are moderately activated ($E_a \sim 20 \text{ kJ mol}^{-1}$ for proteins in water; see ref. 35). From Figs. S.1 and S.2, it appears that the transition between harmonic and diffusive regimes is accompanied by a broad minimum in T_{2M} . Unfortunately, the temperature range over which the spin echo can be detected in the diffusive regime is rather limited.

Figures for Supporting Material to Guzzi et al. for BJ

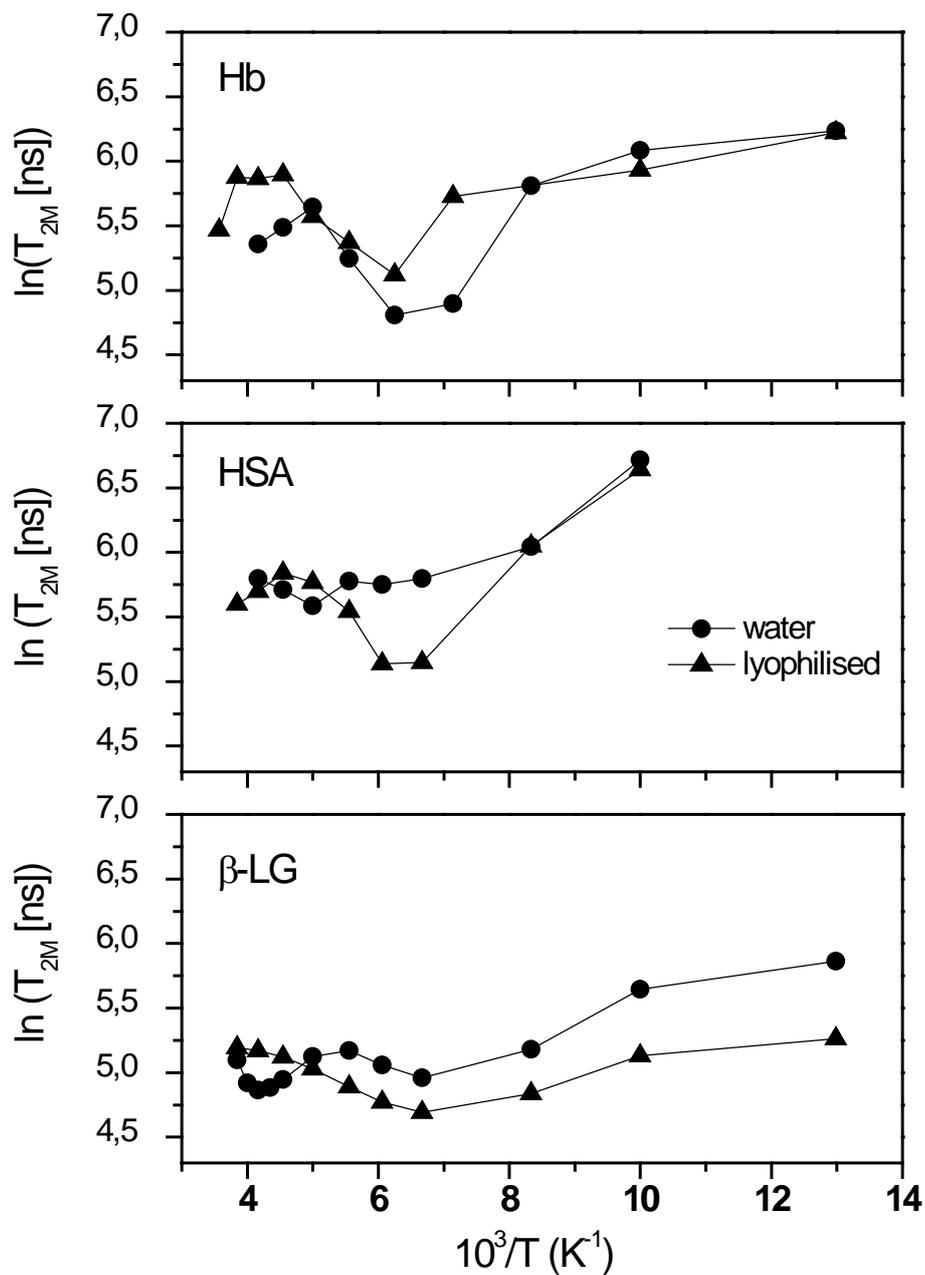


FIGURE S.1: Temperature dependence of the phase-memory time, T_{2M} , for spin-labelled proteins. *Top:* 6-MSL-labelled human haemoglobin; *middle:* 5-MSL-labelled human serum albumin; *bottom:* 5-MSL-labelled bovine β -lactoglobulin. The proteins are either lyophilised (triangles), or dissolved in water (circles).

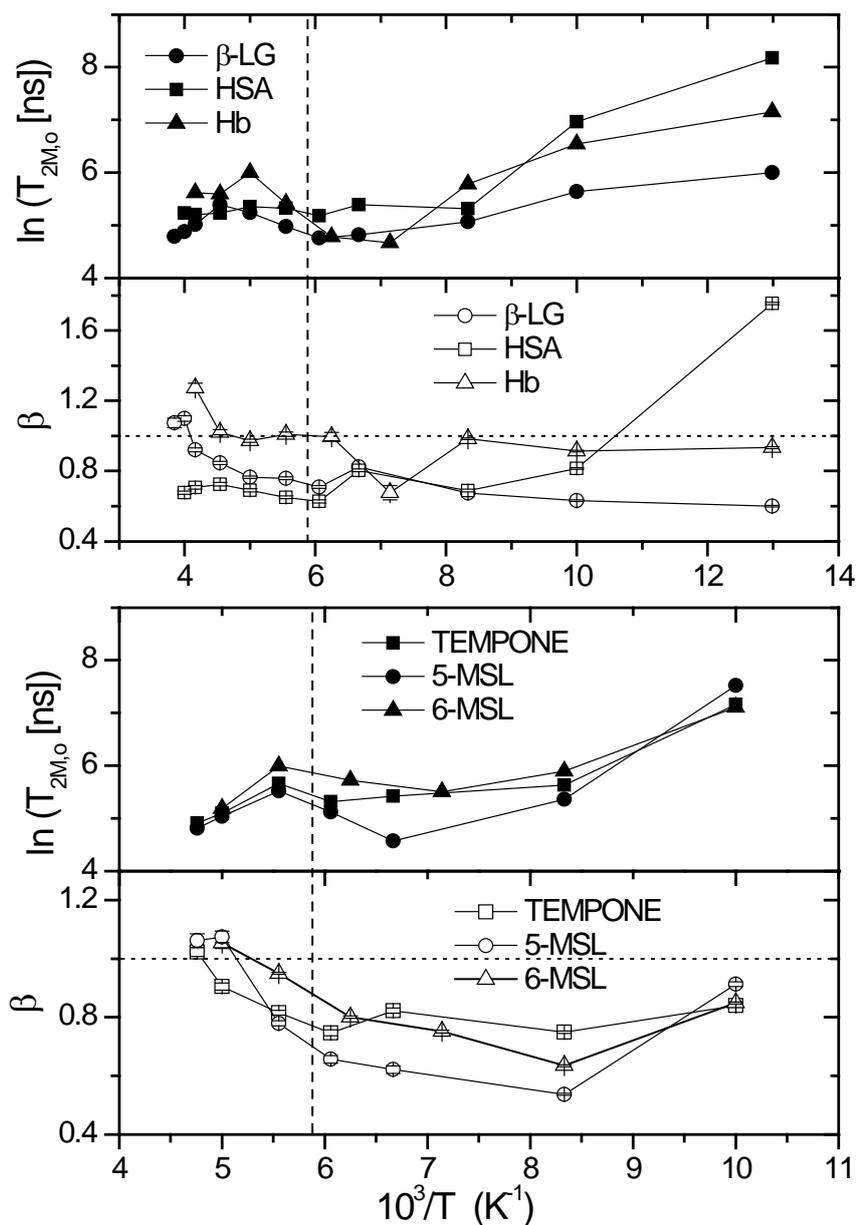


FIGURE S.2: Temperature dependence of the stretched exponential parameters, $T_{2M,o}$ (solid symbols) and β (open symbols), specifying the distribution of phase-memory times according to Eq. 1, for spin-labelled systems in 60% v/v glycerol-water. The upper of each pair of panels gives $T_{2M,o}$ and the lower of each pair gives the exponent β . *Top*: 5-MSL-labelled bovine β -lactoglobulin (β -LG, circles) and human serum albumin (HSA, squares), and 6-MSL-labelled haemoglobin (Hb, triangles). *Bottom*: TEMPONE (squares), and 5-MSL (circles) and 6-MSL (triangles) alone. The vertical dashed line is the approximate position of the glass transition temperature for 60% v/v glycerol.

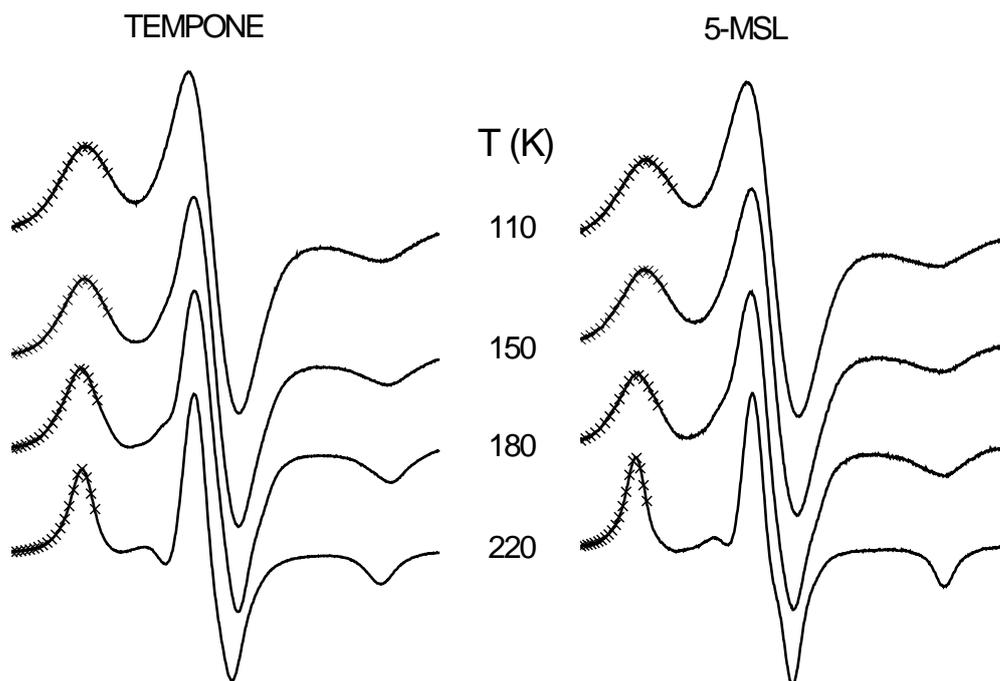


FIGURE S.3: Temperature dependence of the CW-EPR spectra of 5-MSL alone (*right*) and TEMPONE (*left*) in 60% v/v glycerol-water. Crosses represent fitting of the low-field ($m_I = +1$) hyperfine extremum with a Voigt lineshape (Eq. 2). Total scan width = 10 mT.