Motional properties of spin labels in proteins: Effects of hydration

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Conventional and saturation transfer (electron spin resonance) techniques are used to study the motional properties of several spin labels introduced in three proteins: lysozyme, sperm whale myoglobin and human hemoglobin. The mobilities of a maleimide spin label which binds covalently to the proteins, as well as of two small probes TEMPO and PD-TEMPOL were monitored in the temperature range from −10 to −150°C for samples in the dry and solution states. The three proteins show a similar temperature dependence as indicated by the parameters $2A_{zz}$ and ΔH. A small linear increase in $2A_{zz}$ with decrease in temperature is observed for the dry samples. For the proteins in solution, on the other hand, the $2A_{zz}$ temperature dependence shows a change of behaviour around −60°C that is related to the freezing of the water molecules in the hydration shell. The changes observed for the parameter ΔH are such that at temperatures below −60°C ΔH is greater for the solution sample, while at temperatures above −60°C ΔH is greater for the dry sample. Saturation transfer measurements show that the motion of the spin label is very restricted in all systems ($\tau_c > 10^{-5}$s) in the temperature range studied, so that the residual librational motion of the label is sensitive to the hydration, being responsible for the observed changes of the esr parameters with temperature.

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Faisant appel aux techniques de la rpe (résonance paramagnétique électronique) par transfert conventionnel et par transfert de saturation, on étudie le mouvement des marqueurs de spin introduits dans trois protéines, soit la lysozyme, la myoglobine du sperme de la baleine et l'hémoglobine humaine. Dans un intervalle de température allant de −10 à −150°C et opérant sur des échantillons secs ou en solution, on a mesuré les mobilités du marqueur de spin de la maléimide, qui se lie par covalence aux protéines, ainsi que les mobilités de deux petites sondes, TEMPO et PD-TEMPOL. En se basant sur les paramètres $2A_{zz}$ et sur le ΔH, on peut en déduire que les trois protéines subissent l'effet de la température de la même façon. Dans le cas des échantillons secs, on observe une faible augmentation linéaire du paramètre $2A_{zz}$ lorsque la température diminue. D'autre part, dans le cas des protéines en solution, l'effet de la température sur le paramètre $2A_{zz}$ change aux environs de −60°C et ceci qui correspond au point de congélation des molécules d'eau dans la sphère d'hydratation. Les changements observés sont tels qu'à des températures inférieures à −60°C, le ΔH de l'échantillon en solution est plus élevé tandis qu'au dessus de −60°C le ΔH de l'échantillon sec est plus élevé. Les mesures de rpe par transfert de saturation indiquent que dans l'intervalle de température étudié, le mouvement du marqueur de spin est très restreint dans tous les systèmes ($\tau_c > 10^{-5}$s). Par conséquent, le mouvement résiduel de libration du marqueur est sensible à l'hydratation et est responsable des changements des paramètres de la rpe en fonction de la température.

[Traduit par le journal]

Introduction

The hydration of biomolecules has been studied by several techniques such as calorimetry, absorption isotherms, infrared spectroscopy, nuclear magnetic relaxation (1,2), thermal stimulated depolarization (3), and computer simulation (4,5). Large discrepancies are observed when results obtained from different methods are compared. This is expected if one considers that different physical properties are probed in each case. Despite this difficulty in correlating new information with the known data, the hydration of macromolecules is an interesting subject for further studies.

The use of esr measurements to study the hydration of proteins was first proposed by Rupley *et al.* (6). They suggested that significant changes in the motional properties of a spin label (TEMPONE)[3] could be followed as a function of the sample hydration and interpreted as changes in the internal dynamics of the protein. The exclusion of possible structural changes induced in the protein by dehydration was crucial in the interpretation of these results. The absence of such structural changes was also suggested by Raman (7), infrared reflectance (8), and photoacoustic (9) spectroscopic studies.

The spin label motion as a function of temperature of hemoglobin–MAL-6 samples in the dry and solution states was studied by Johnson (10). For the lyophilized sample he observed spectral changes that were related to the librational motion of the spin label. A hydrogen bonding of the spin label to the protein or to a water molecule was the explanation given for the spectral changes observed for the solution sample.

These studies led us to consider the relevance of the spin label used and also the influence of the protein studied on the results obtained as a function of hydration. We propose to describe in this communication the esr studies in the dry and solution states of three different proteins: lysozyme, myoglobin, and hemoglobin using the spin labels: MAL-6, TEMPO, and PD-TEMPOL, as a function of temperature.

Experimental

Sample preparation

Hemoglobin solution was obtained from whole blood from healthy donors. The red blood cells were washed three times in phosphate buffered saline (135 m*M* NaCl, 5 m*M* phosphate, pH 7.4) and

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[3]Abbreviations: TEMPONE: 4-oxo-2,2,6,6-tetramethylpiperidine-1-oxyl; MAL-6: 4-maleimido-2,2,6,6-tetramethylpiperidine-1-oxyl; TEMPO: 2,2,6,6-tetramethylpiperidine-1-oxyl; PD-TEMPOL: 4-hydroxy-d_{17}-2,2,6,6-tetramethylpiperidine-1-oxyl; esr: electron spin resonance; st-esr: saturation transfer electron spin resonance.

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hemolysis was achieved by dilution of equal volumes of packed cells and distilled water. The hemolyzed cells were centrifuged for 40 min at 20 000 rpm (Sorvall RC-5B refrigerated centrifuge) at 4°C, and the supernatant hemoglobin solution was maintained, discarding the pelleted membranes. The hemolyzate was then filtered in a Sephadex G-25 column (11) avoiding substantial dilution. This hemoglobin solution was used in our studies. Solutions of sperm whale myoglobin and egg-white lysozyme were obtained by dissolving the lyophilized commercial material (Sigma Chemical Co.) in phosphate buffer 0.05 *M*, pH 7.0 so that the protein concentration was approximately 10%. After dilution a centrifugation step was used to remove unsoluble material from the solution.

Spin labeling of the proteins

Hemoglobin solution was spin labeled with 2,2,6,6-tetramethyl-piperidino-1-oxyl (TEMPO, Aldrich Chem. Co.) and 4-hydroxy-2,2,6,6-perdeuterotetramethylpiperidine-1-oxyl (PD-TEMPOL Merck Co.) in the following way: an aliquot of a stock solution of the label (approximately 10 m*M*) was introduced into the bottom of a test tube and dried under a N_2 flux. Then the hemoglobin solution was added on top in such volume as to make the final spin-label concentration 2×10^{-4} *M* for PD-TEMPOL and 5×10^{-4} *M* for TEMPO. In the case of the reaction of myoglobin and lysozyme with 4-maleimido-2,2,-6,6-tetramethylpiperidino-1-oxyl (MAL-6, Aldrich Chem. Co.) the procedure was similar: an aliquot of a stock solution of MAL-6 in acetone was introduced into the bottom of a test tube and dried under N_2. Then the protein solution was added in such a way as to make the following proportions: MAL-6/myoglobin 1:1 and MAL-6/lysozyme 1:1. The reaction mixture was left in the refrigerator at 4°C for 38 h. The excess spin label was removed by filtration of the reaction mixture in a small Sephadex G-25 column. All the labeled samples were lyophilized and dried over P_2O_5 in order to obtain the dry samples. In some experiments the sample was then exposed to a known water vapour pressure in a saturated salt solution container. The solution sample was used after the Sephadex G-25 filtration step.

Electron spin resonance measurements

Conventional esr and st-esr spectra were obtained using a Varian E-109 X-band spectrometer (9.14 GHz) equipped with a rectangular cavity E-248 and the temperature control accessory E-257. The temperature was monitored by a copper–constantan thermocouple attached to a Fluck potentiometer. The microwave power used was 2 mW and modulation amplitude 1.0 G. The st-esr spectra were obtained as the second harmonic out-of-phase signal. The self-null method was used in the adjustment of phase (12) with the sample in the cavity, modulation amplitude 5.0 G and 1-mW microwave power. After this adjustment the power was increased to 64 mW (measurements were also made at 30 mW at lower temperatures) and the second harmonic out-of-phase signal recorded. In the case of hemoglobin spin labeled with TEMPO or PD-TEMPOL, adjustments of phase were also made at the 0.1-mW power level, as at the lowest temperatures the sample is more readily saturated. The analysis of the st-esr spectra was made by comparison of the experimental spectra parameters L″/L and C′/C with calibrations obtained by Thomas (12).

Results and discussion

Covalent spin labels

In Fig. 1 the conventional esr spectra of spin labeled myoglobin are presented for different temperatures. In Fig. 1(a) the spectra were obtained for the solution sample while Fig. 1(b) is for the dry sample. The parameters used in our analysis are $2A_{zz}$, the splitting between the outer hyperfine lines, and ΔH, the linewidth at half height of the low field line. The spectra obtained with spin labeled lysozyme, in the temperature range -10 to -150°C, are similar to those of myoglobin, so that the spectra in Fig. 1 are characteristic of the two proteins. A careful inspection of the spectra shows clearly an increase in ΔH and $2A_{zz}$ as the temperature is lowered.

In Fig. 2(a) the parameter $2A_{zz}$ from the esr spectra is plotted

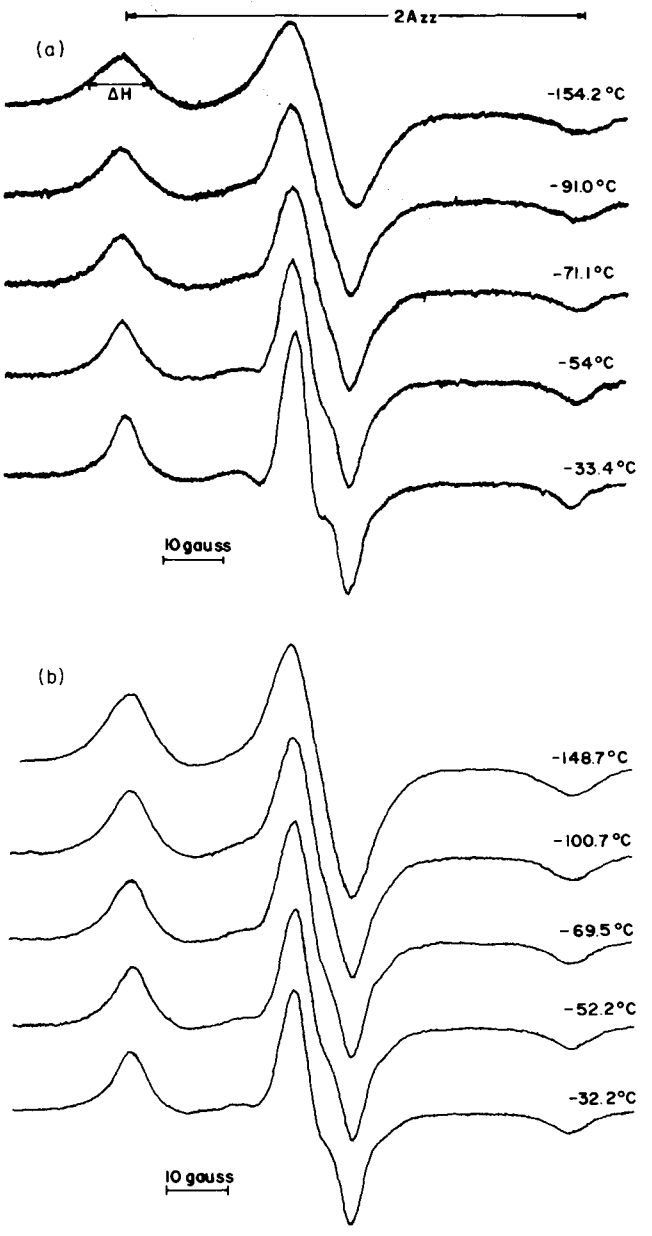

FIG. 1. (a) Conventional esr spectra of myoglobin–MAL-6 aqueous solution. The temperature is indicated in the spectrum as well as the esr parameters $2A_{zz}$ and ΔH. (b) Conventional esr spectra of myoglobin–MAL-6 dry powder sample. The temperature is indicated in the spectrum.

as a function of temperature for lyophilized and aqueous solution of myoglobin–MAL-6. For both samples a decrease is observed in $2A_{zz}$ with an increase in temperature, with the dry sample having a lower $2A_{zz}$ value over almost the whole temperature range studied. For the dry sample a single straight line could be fitted to the data as shown in Fig. 2(a). On the other hand, two straight lines were fitted to the data for the solution sample, one for temperatures below -60°C and another above this value. It is interesting to note that for the low temperature region the behavior of the two samples is similar, i.e., the straight lines fitted to the data are practically parallel, indicating that the rate of change of $2A_{zz}$ is the same. Above -60°C there is a faster decrease in $2A_{zz}$ for the solution sample, so that above ca. -10°C the value for the solution is smaller than the value for the dry sample.

In Fig. 2(b) the plot of the temperature dependence of the

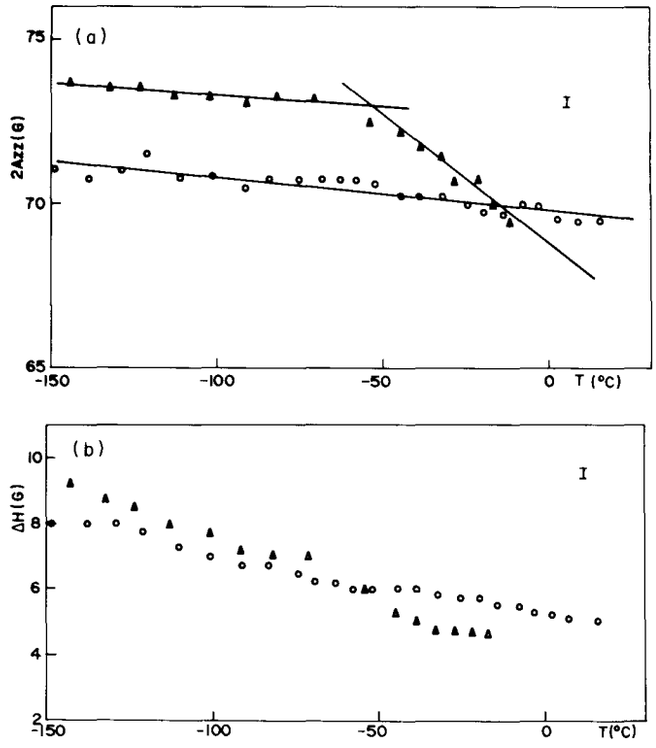

FIG. 2. (a) The parameter $2A_{zz}$ as a function of temperature for myoglobin–MAL-6 samples. The straight lines are least-square fits to the experimental points. Key: (Δ) aqueous solution; (o) lyophilized sample (water content: 0.12 g H_2O/g myoglobin). The error in the measurement above −100°C is 0.3 G; below −100°C it is increased to 0.5 G due to the broadening which introduces additional error especially in the high field. The bars in the figure correspond to the errors indicated above. (b) The linewidth of the low field line as a function of temperature for myoglobin–MAL-6 samples. Key: (Δ) aqueous solution; (o) lyophilized sample (water content: 0.12 g H_2O/g myoglobin). The error in ΔH is the same as in Fig. 2(a).

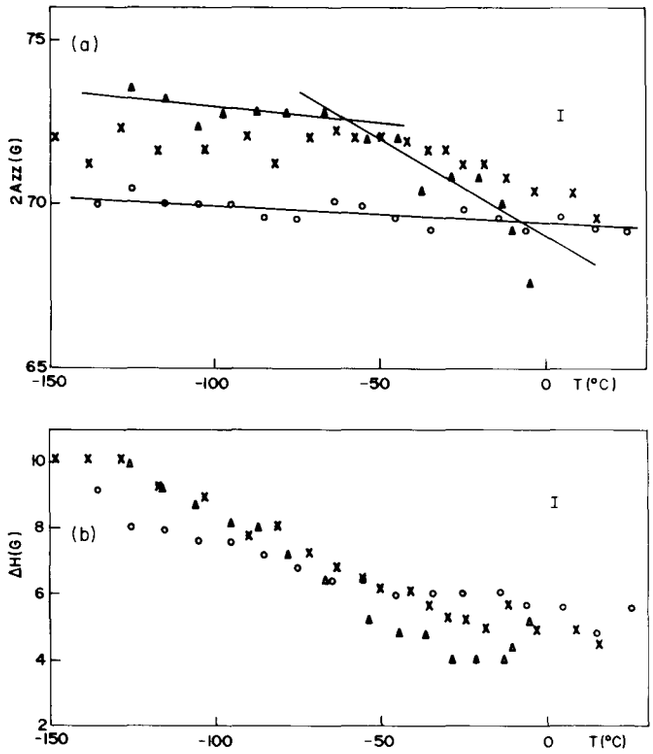

FIG. 3. (a) The parameter $2A_{zz}$ as a function of temperature for lysozyme–MAL-6 samples. The straight lines are least-square fits to the experimental points. Key: (Δ) aqueous solution; (o) lyophilized sample (water content: 0.07 g H_2O/g lysozyme); (x) intermediate water content sample (0.45 g H_2O/g lysozyme). The error is the same as in Fig. 2(a). (b) The linewidth of the low field line as a function of temperature for lysozyme–MAL-6 samples. Key: (Δ) aqueous solution; (o) lyophilized sample (water content: 0.07 g H_2O/g lysozyme); (x) intermediate water content (0.45 g H_2O/g lysozyme). The error is the same as in Fig. 2(a).

linewidth of the low field line is shown for the two samples of spin labeled myoglobin. A general decrease of ΔH is observed with an increase in temperature. In this case a different behavior can be noticed for the dry and solution samples below and above ca. −60°C. At lower temperatures (below −60°C) the linewidth for the dry sample is less than for the solution sample, while at temperatures above −60°C the linewidth for the solution sample is less than that for the dry sample. The variation of linewidth for the dry sample over the whole temperature range ($\Delta(\Delta H)$=3 G) is less than that for the solution sample ($\Delta(\Delta H)$ = 5 G). The error of the ΔH measurement is estimated to be 0.4 G.

In Figs. 3(a) and 3(b), respectively, the parameters $2A_{zz}$ and ΔH are presented as a function of temperature for lysozyme–MAL-6 with different water contents. The behavior of these parameters described for myoglobin is also valid in this case. The variation of $2A_{zz}$ for the dry sample is very small, while for the solution sample it is considerable. Again, for the dry sample a unique rate of decrease of $2A_{zz}$ with temperature is observed, while the solution sample shows this same rate below −60°C and a faster rate above −60°C. Over the whole temperature range, the variation of ΔH for the solution is greater than that for the dry sample. The two curves cross near −60°C, so that below this temperature the value for the solution is larger while above this temperature the value for the dry sample is larger. The experiment with lysozyme with an intermediate water content (0.45 g H_2O/g protein) resulted in a $2A_{zz}$ plot lying between the data for the dry and solution samples (Fig. 3(a)). Below ca. −45°C a straight line could be fitted to the data and it is parallel to the one fitted to the lyophilized sample data. Above ca. −45°C another straight line could be fitted to the data, with a slope between those obtained for the dry and solution samples. As shown in Fig. 3(b), −45°C is also the temperature at which the ΔH data obtained for this intermediate water content sample and for the lyophilized sample intersect.

The changes observed for the parameters $2A_{zz}$ and ΔH with temperature suggest that the spin label used is undergoing changes in motional properties (13,14). In order to verify this proposition the saturation transfer technique was used. In Fig. 4 the st-esr spectra obtained for myoglobin samples at different temperatures are shown. Fig. 4(a) is for the solution sample, while Fig. 4(b) is for the dry one. The parameters obtained from these spectra are L″/L and C′/C. They have different sensitivities to slow motion; L″/L is the most sensitive and for this reason was the parameter of our choice. The values of L″/L obtained for several samples studied in this work are presented in Table 1. Comparison of these values with the calibration curves of Thomas (16) shows that below −10°C the correlation times are greater than 10^{-5} s. The direct comparison of the lineshapes of our st-esr spectra with those reported in the literature (15,16) also shows that in our case the labels are very immobilized. In this way, st-esr indicates that the label is very

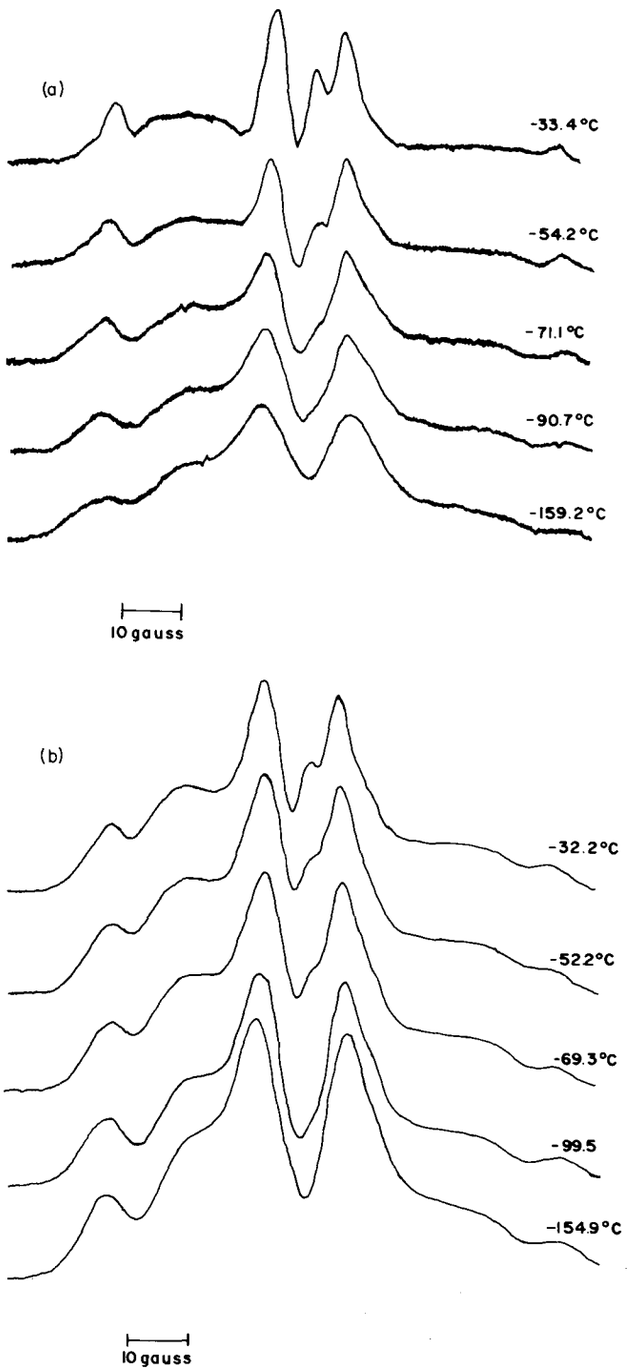

FIG. 4. (a) The st-esr spectra of myoglobin–MAL-6 aqueous solution. The temperature is indicated in the spectrum. The st-esr parameters measured are also indicated. (b) The st-esr spectra of myoglobin–MAL-6 dry powder sample at different temperatures.

immobilized so that the changes in $2A_{zz}$ and ΔH are not due to motional changes of the label.

The dependence of $2A_{zz}$ with temperature for the lyophilized samples can be interpreted in terms of the librational motion of the spin label, as proposed by Johnson (10). This model is based on the fluctuations of the $\diagdown$N—O$\diagup$ bond orientation with respect to the C—N—C plane of the nitroxide ring, which could produce torsional motion of the ring. This effect produces a temperature-dependent hyperfine separation of the form

$$2A_{zz}(T) = A^0_{zz}\,(1 + \cos\theta)$$

where $\theta = (RT/E_A)^{1/2}$, θ being the half angle of oscillation, E_A the torsional energy barrier, and $2A^0_{zz}$ the separation in absence of motion. For small oscillations it gives a linear dependence of $2A_{zz}$ with the temperature. This model was used to fit the experimental data for MAL-6 spin labeled lyophilized hemoglobin (10). For our lyophilized samples we observed this linear behavior over the whole temperature range studied. The solution samples at low temperatures also have this $2A_{zz}$ dependence, indicating that in the frozen solution the presence of the water does not affect the spin label motion. Its presence, however, increases the local polarity of the medium, causing an increase in the spin density on the nitrogen in the $\diagdown$N—O$\diagup$ group of the spin label, leading to an increase in $2A_{zz}$. The behavior of $2A_{zz}$ for the solution sample above -60°C can be explained as due to the thawing of the water of hydration, which will presumably facilitate the motion of the label. In the case of the lysozyme sample with intermediate hydration we found that the change in slope for the $2A_{zz}$ vs. T plot takes place at a higher temperature (-45°C) and the slope of this curve is less than that for the solution sample; in this way this temperature of slope change and the slope at this region are probably strongly dependent on the hydration of the protein. In the solution sample, as the temperature is raised above -10°C the free water also thaws and the spectra become typical of solutions. Therefore, below -10°C the same model, i.e., librational motion of the spin label, can be used for the dry and solution samples. Johnson (10) explained the dependence of $2A_{zz}$ upon temperature for hemoglobin–MAL solutions as due to the formation of a hydrogen bond between $\diagdown$N=O$\diagup$ group of the spin label and a proton that could be either in the protein (near the binding site of the label) or in a water molecule. The plots of the parameter $2A_{zz}$ as a function of temperature obtained for the proteins studied in the present work are similar to those described in ref. 10. The change of slope in the $2A_{zz}$ temperature dependence for the hemoglobin–MAL-6 solution sample in this reference also takes place around -60 to -70°C. This general behavior of $2A_{zz}$ suggests that the hydrogen bond is probably not due to the specific site of attachment of the label in the protein, as it is common to all three proteins, but that it involves a water molecule.

At a temperature near the transition between the first and second regions for $2A_{zz}$, considerable changes in ΔH are observed. Near -60°C the dependence of ΔH for the dry and solution samples cross one another so that at temperatures below -60°C ΔH is greater for the solution sample, while at temperatures above -60°C ΔH is greater for the dry sample. The increase in linewidth with a decrease in temperature comes from the inhomogeneous broadening due to the methyl protons of the spin label and the protons of water molecules near the label. Below -60°C the water in the solution sample is frozen and there is electron-nuclear interaction with the water protons and with the spin label methyl protons. For the dry sample, the interaction is mostly with the methyl protons since very few water molecules are present, hence the lower value of ΔH obtained. Above -60°C the water of hydration in the solution

TABLE 1. The st-esr parameter L″/L at different temperatures*

Temperature, °C	L″/L				
	Lyso–MAL-6 0.45 g H_2O/g lyso	Mb–MAL-6 solution	Mb–MAL-6 dry powder 0.12 g H_2O/g Hb	Hb–TEMPO dry powder 0.12 g H_2O/g Hb	Hb–PD-TEMPOL dry powder 0.12 g H_2O/ Hb
−156	—	—	1.29	—	1.64
−132	—	1.43	—	—	—
−114	—	1.31	—	—	1.60
−100	—	—	1.22	—	—
−90	2.05	1.31	—	—	—
−83	1.96	—	1.32	1.60	1.48
−70	—	1.10	1.45	—	—
−64	1.73	0.96	—	—	1.48
−52	1.64	0.93	1.45	1.57	—
−42	1.48	0.88	—	—	1.36
−33	1.30	0.79	1.35	—	—
−25	1.24	0.76	—	1.32	—
	The st-esr parameter C′/C in the temperature range −20 to −150°C				
	0.41–0.55	0.10–0.35	0.28–0.40	0.35–0.60	0.47–0.67

*From ref. 12 a value of L″/L of 0.98 corresponds to correlation times in the range 10^{-4} – 10^{-3} s; C′/C of 0.33 corresponds to correlation times in the range 10^{-5} – 10^{-4} s.

samples thaws, allowing the methyl groups and the water molecules to move more freely. This motion makes the electron-nuclear interaction less effective, and results in a smaller ΔH if compared to the value for the dry sample, where the methyl proton interaction is still effective. The temperature of −60°C, as noted above, also corresponds to the change in slope for $2A_{zz}$ vs. T plot. It is interesting that the linewidth changes described above can be observed directly from the esr spectra (Fig. 1): in the temperature range below −60°C, ΔH is increased in the solution sample to a greater extent than in the dry sample; the opposite occurs above −60°C. It is noticed that an improved resolution is obtained above −60°C for the solution sample in the central part of the esr spectra, consistent with the decrease in linewidth. Finally, it should be noted that the characteristic temperature for the lysozyme sample with intermediate hydration is the same both in the $2A_{zz}$ vs. T and ΔH vs. T plots (−45°C).

Noncovalent small spin probes

Since the results obtained with MAL-6 spin labeled samples suggest a general behavior of $2A_{zz}$ and ΔH for all proteins studied, an attempt was made to monitor the hydration of the samples with small noncovalent spin probes: human hemoglobin was labeled with two different small spin labels, TEMPO and PD-TEMPOL. In this second label the piperidine ring has deuterium instead of hydrogen, decreasing the inhomogeneuous broadening due to the electron-nuclear interaction. In Fig. 5(a) the parameter $2A_{zz}$ is presented for the spin labels TEMPO and PD-TEMPOL dissolved in hemoglobin solution and as dry powder samples. It can be seen that in the dry samples $2A_{zz}$ changes are quite small: a slight increase with lowering of the temperature is observed. The hemoglobin solution samples show a greater change of $2A_{zz}$ versus T: the slope of the curve becomes greater above ca. −60°C, and at low temperatures (below −60°C) the slope is similar to that in the dry sample. In fact, for PD-TEMPOL the slopes are the same. In this case, as the lines are relatively narrow and well resolved, $2A_{zz}$ is measured with good accuracy (0.2 G). In the case of TEMPO the lines are broader and the accuracy of measurement is not as good (around 0.4 G). For this reason the slopes are not exactly the same for this label. This behavior of $2A_{zz}$ vs. T is analogous to that obtained with the covalently labeled proteins. For the solution samples, again, a change of slope takes place around −60°C. On the other hand, the changes for the dry samples are quite small and linear over the whole temperature range. In Fig. 5(b) the linewidth ΔH for the samples described above is plotted as a function of temperature: it is clear that ΔH is much less for PD-TEMPOL than for TEMPO. As noted previously in the case of PD-TEMPOL, the inhomogenous broadening due to the protons in the piperidine ring is eliminated. This accounts for the difference of about 2 G in ΔH for TEMPO and PD-TEMPOL. The behavior of ΔH with temperature shows a very slight change over the whole temperature range. Furthermore, the differences in ΔH between the dry and solution samples are within experimental error. This result is different from that with the covalent spin label, where the changes in ΔH, although small, are significant. Perhaps this is related to the fact that the covalent label is tightly bound to the protein, while the small spin probes do not interact with the protein that remains dissolved in the solution (or powder). For this reason the linewidths for TEMPO and PD-TEMPOL represent a static distribution of orientations, which is the same both in the powder and in the frozen solution. The presence of water affects only the polarity of the label, making $2A_{zz}$ greater in the solution samples. In the case of the covalent label attached to the protein the linewidth is also affected by the restrictions imposed by the protein matrix. In this case the values of ΔH are not only given by a static distribution but also depend on the local state of the protein, which is a function of hydration. In this way the changes observed around −60°C can be correlated with the thawing of hydration water above this temperature: this will allow an increase in motion in the microenvironment of the spin label and a narrowing of the esr lines will take place for the solution sample (above −60°C, ΔH for the solution is smaller

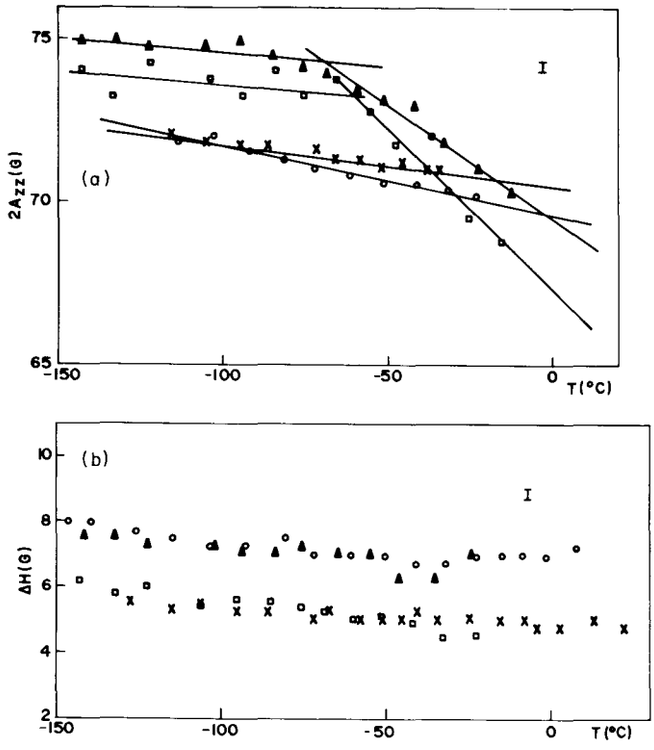

FIG. 5. (a) The parameter $2A_{zz}$ as a function of temperature for hemoglobin samples spin labeled with the small probes: TEMPO (Δ) aqueous solution and (o) dry powder sample (0.12 g H_2O/g hemoglobin), and PD-TEMPOL (o) aqueous solution and (x) dry powder sample (0.12 g H_2O/g hemoglobin). The error in the case of TEMPO is the same as in Fig. 2(a). For PD-TEMPOL it is reduced to 0.2 G due to the narrowing of the lines. (b) The linewidth of the low field as a function of temperature for hemoglobin samples. Key: (o) lyophilized hemoglobin–TEMPO (water content: 0.12 g H_2O/g hemoglobin; (Δ) hemoglobin–TEMPO aqueous solution; (x) lyophilized hemoglobin–PD-TEMPOL (water content: 0.12 g H_2O/g hemoglobin; (□) hemoglobin-PD-TEMPOL aqueous solution.

than ΔH for the dry sample). This would explain the ΔH changes for the covalent label. The $2A_{zz}$ changes are similar for all the labels used, as they reflect the polarity of the microenvironment. It is worth mentioning the results in refs. 17 and 18 on the dynamics of myoglobin at low temperature. These authors show that the mean-square displacements for most residues $\langle x^2 \rangle$ as a function of temperature show a discontinuity around 220 K (−53°C). It is suggested that these changes in the protein structure are correlated to the state of water in the protein. Studies of the absorption of microwaves in crystals of Met Mb also show a gradual thawing of water upon heating above −53°C (19). These results are in agreement with our data showing that a structural change takes place near −60°C.

Conclusions

Our conclusions can be summarized as follows.

(1) The behavior of the parameter $2A_{zz}$ with temperature is the same for the three proteins studied, hemoglobin, myoglobin, and lysozyme, as observed with the different spin labels, MAL-6, TEMPO, and PD-TEMPOL. This behavior must probably be common to all proteins and we believe reflects the sensitivity of the spin label to the polarity of its microenvironment.

(2) The lyophilized samples show a small linear increase in $2A_{zz}$ with decrease in temperature. Again, this is a feature common to proteins and spin labels used in this work. A restriction of the spin label librational motion could be responsible for this effect, as proposed by Johnson (10), although other possibilities are not excluded.

(3) The behavior of the protein in solution for temperatures below ca. −60°C is similar to the lyophilized protein ($2A_{zz}$ versus T). At this temperature the water present is already frozen and its presence does not affect the spin label motion. However, it changes the polarity of the medium and this explains the larger value of $2A_{zz}$ observed for the solution sample compared to the lyophilized sample. As the temperature is increased above −60°C the water of hydration starts to thaw. This facilitates the spin label motion and a larger rate of change of $2A_{zz}$ with temperature is observed. Around −10°C the thawing of the bulk water causes another change in the $2A_{zz}$ behavior.

(4) In the case of the parameter ΔH it is necessary to analyze separately the covalent spin label and the small probes. For the covalent maleimide label, the dependence of ΔH upon temperature also shows −60°C as a characteristic temperature. Below −60°C the sample in solution has a larger ΔH value than the lyophilized sample, while above −60°C the inverse is true. This can be interpreted as due to a motional narrowing effect in the solution sample due to the thawing of hydration water.

(5) The small probes TEMPO and PD-TEMPOL do not show differences in the behaviour of ΔH for solution and dry samples. This is explained as due to a similar static distribution of label orientations in both samples. As expected, the linewidth ΔH for the deuterated label PD-TEMPOL is 2 G less than ΔH for TEMPO. This is due to the elimination of inhomogenous broadening in PD-TEMPOL.

(6) The spectral changes observed as a function of temperature suggest that the parameter $2A_{zz}$ is quite sensitive to the hydration state of the sample. Since the sensitivity of ΔH depends on the spin label used and since its variation is small, it might be interesting to study in more detail the $2A_{zz}$ vs. T dependence as a function of protein hydration.

(7) Electron spin resonance measurements, as proposed by Rupley *et al.* (6) can be another method used to study protein hydration.

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