A TEMPERATURE-INDUCED VARIATION IN THE INTRINSIC HYPERFINE SEPARATION OF A TIGHTLY BOUND NITROXIDE SPIN LABEL

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1. Introduction

Recently there has been increasing interest in studying the rotational motion of biological molecules by monitoring the electron paramagnetic resonance (EPR) spectra of spin labels which are tightly bound to the molecule of interest. Theoretical studies have shown that in the slow motion region ($\sim 5 \times 10^{-9}$ s to $\sim 5 \times 10^{-7}$ s) the correlation time may be determined by comparing the apparent hyperfine separation (HFS) in the presence of rotational motion with the rigid limit HFS in the absence of rotational motion [1,2]. Applications of this method include the measurement of rotational correlation times for spin-labelled oxyhemoglobin (HbO_2) [1] and α-chymotrypsin [3], and a prediction of the spin label orientation with respect to the α-chymotrypsin molecule [4].

The majority of work to date has assumed the tightly bound nitroxide label to act simply as a reporter group for molecular motion, exhibiting little or no intrinsic environmental or temperature sensitivity. However, we demonstrate here that the rigid limit EPR spectra exhibit a substantial intrinsic temperature dependence, with the rigid limit HFS of MAL-6-labelled HbCO decreasing by nearly 10 G over the temperature range −196°C to +45°C. The steepest temperature dependence is also found to occur over the 0–40°C temperature range where most biological measurements are made. This strong temperature dependence in the intrinsic HFS is shown to produce substantial errors in correlation time calculations if it is not explicitly recognized and appropriate corrections made. The detailed behavior of this intrinsic temperature dependence suggests that it is most probably produced by equilibrium hydrogen bonding between the nitroxide NO· group and an unidentified proton donor within the spin label binding site.

2. Materials and methods

Membrane-free carbonmonoxy Hb (HbCO) was prepared and labelled with 4-maleimido-2,2,6,6-tetramethylpiperidinooxyl (MAL-6) following the procedures in [1,5]. HbCO–sucrose solutions for variable viscosity studies and the frozen or ammonium sulfate-precipitated HbCO samples for direct immobilization studies were prepared by developed methods [6].

EPR spectra were measured on a Varian E-112 spectrometer following standard methods [6]. The field sweep width was calibrated using the residual Mn^{2+} in SrO as a standard [7]. Temperature was measured with a copper–constantin thermocouple and a potentiometer; accuracy of individual measurements is estimated to be ±1°C.

3. Results and discussion

Upon reaction with the Cys β93 residues of HbCO the MAL-6 label yields single component spectra characteristic of slow tumbling. Previous work has shown that the label nitroxide ring is quite strongly immobilized within its binding site [1,6], thus the spectra primarily reflect tumbling of the HbCO–MAL-6 complex as a whole. Since the spectra will

always reflect this rotational diffusion under normal solution conditions, it is not possible to directly observe the rigid limit HFS and any temperature dependence it may exhibit under such conditions. An indirect method for determining the rigid limit HFS is, however, available from [2], where it is shown that the correlation time, τ_R, can be calculated quite accurately from the expression:

$$\tau_R = a \cdot \left(1 - \frac{A^*_{zz}}{A^\circ_{zz}}\right)^{-b} \quad (1)$$

where a and b are parameters derived by spectral simulation, $2A^*_{zz}$ is the apparent HFS in the presence of motion, and $2A^\circ_{zz}$ is the rigid limit HFS. For rotational diffusion governed by the Stokes relation, $\tau_R = V\eta/kT$, where V is the effective HbCO molecular volume, η is the solvent viscosity, k is Boltzmann's constant, and T is the absolute temperature, eq. (1) can be rewritten in the form:

$$2A^*_{zz} = 2A^\circ_{zz} - a' \cdot \left(\frac{T}{\eta}\right)^{b'} \quad (2)$$

where $b' = 1/b$ and $a' = 2A^\circ_{zz} \cdot (ak/V)^{b'}$. Dissolving the labelled HbCO in sucrose solutions of varying viscosities and measuring $2A^*_{zz}$ at constant temperature for three temperatures, 4.5°C, 25°C and 45°C then generates the three curves shown in fig.1. Extrapolating this data to infinite viscosity ($T/\eta = 0$) yields the rigid limit HFS, $2A^\circ_{zz}$, as the zero intercept. The extrapolated values for $2A^\circ_{zz}$ are given in numerical form in table 1. From this table it can be seen that the rigid limit HFS changes by > 3 G over a 40°C temperature range under approximately physiological conditions. Thus it is clear that the intrinsic HFS of the tightly bound MAL-6 label is quite sensitive to temperature.

To determine whether this temperature dependence extends over a wider temperature range we have also examined the rigid limit HFS of MAL-6-labelled HbCO frozen in solution. From this data, shown as the solid circles in fig.2, it can be seen that the intrinsic HFS continues to increase steadily as temperature is decreased, until about −100°C where it begins to plateau, apparently going toward an asymptotic low temperature limit of about 77 G. The role of ionic strength (polarity) in this behavior was

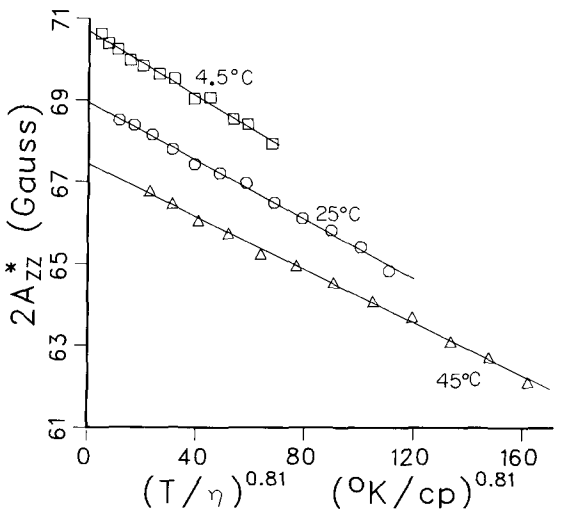

Fig.1. Apparent HFS as a function of viscosity and temperature. The symbols (□), (○) and (△), respectively, denote the values of the apparent HFS, $2A^*_{zz}$, as measured at 4.5°C, 25°C and 45°C. Measurement uncertainties for individual values are about ±0.3 G. The straight lines shown as a parameterization of eq. (2) in which nonlinear least square methods [8] were used to determine the best values for the exponent, b', the slopes, a', and the rigid limit HFS, $2A^\circ_{zz}$.

examined by comparing the behavior of the desalted HbCO system with that of ammonium sulfate precipitated HbCO, shown as the open squares in fig.2. From this data it can be seen that the high ionic strength system exhibits behavior qualitatively equivalent to that of the desalted system. The principal differences between the two systems are that the precipitated system exhibits a slightly lower rigid HFS at low temperature and a slightly higher one at high temperature. Increased polarity generally

Table 1
Temperature dependence of the infinite viscosity hyperfine separation

T (°C)	$2A^\circ_{zz}$ (G)[a]
4.5	70.64 ± 0.08
25.0	68.90 ± 0.09
45.0	67.34 ± 0.11

[a] Rigid limit HFS values are calculated by the least square methods noted in fig.1; quoted uncertainties are the standard errors of estimate

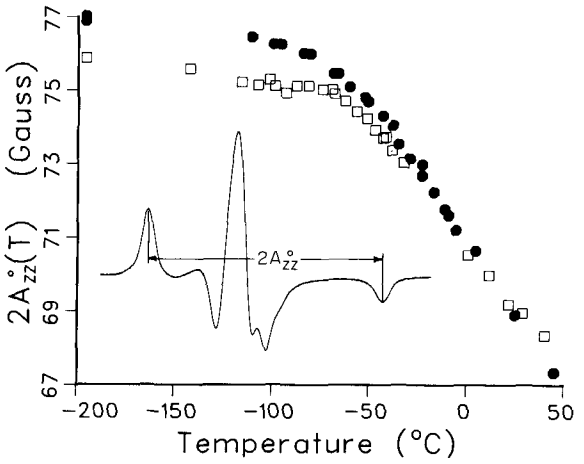

Fig.2. Temperature dependence of the rigid limit HFS. The symbols (•) and (□) are the values, respectively, observed for desalted and ammonium sulfate precipitated HbCO. Below 0°C the $2A^{\circ}_{zz}$ values for desalted HbCO were observed directly from the frozen solution; above 0°C the plotted values are taken from table 1. The precipitated HbCO is completely immobilized by the precipitation procedure, thus $2A^{\circ}_{zz}$ for that sample was directly observable over the full temperature range. Uncertainties for individual measurements of $2A^{\circ}_{zz}$ in the immobilized systems is about ±0.3 G; uncertainties of the viscosity extrapolated values for the desalted HbCO solutions are given in table 1. The spectrum inset into the lower left corner is from MAL-6-labelled HbCO frozen in solution at about −8°C. HFS values $2A_{zz}$ were measured as shown.

produces an increase in the hyperfine splitting constants [9], thus these differences also indicate that temperature-induced polarity changes are probably not the source of the observed HFS temperature dependence.

Another obvious mechanism which might produce shifts of the sort observed here would be a thermally-induced 'wobbling' or librational motion of the label within its binding site. Saturation transfer EPR measurements on the directly immobilized systems, however, have shown that the label is essentially completely immobilized within its binding site on time scales much longer than those to which normal absorption EPR methods are sensitive [6]. Thus the large temperature dependence observed here cannot be explained by such a mechanism.

The most probable source for this temperature dependence appears to be hydrogen bond formation between the nitroxide NO· group and some proton donor within the label binding site. A nitroxide radical trapped in an ethanol glass was shown [10] to exhibit an equilibrium between H-bonded and dissociated states, with the rigid limit HFS differing by about 4.5 G between the two states. Thus an H-bond which induces a slightly higher polarization of the free electron spin density could readily produce HFS changes of the magnitude observed here. The normal temperature dependence of the equilibrium constant between the associated and dissociated states would then produce the observed temperature dependent shifts in the HFS.

One of the most important consequences of this temperature behavior will be in the determination of correlation times using eq. (1). For example, if one were to take the −196°C value of $2A^{\circ}_{zz}$ as the rigid limit HFS, the rigid limit value at +45°C would then imply a correlation time somewhat less than 10^{-8} s, hardly corresponding to a rigidly immobilized label. Furthermore this temperature dependence appears to exhibit nearly its steepest slope over the approximate temperature range 0°–40°C. Thus accurate correlation time measurements using this method will require rather careful characterization of the intrinsic HFS temperature dependence in the system of interest. It is also likely that this strong spectral temperature dependence will be of importance in other biophysical spin label measurements.

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