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EPR study of non-covalent spin labeled serum albumin and hemoglobin

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Abstract

Electron Paramagnetic Resonance (EPR) was used to investigate the Tempyo spin label (3-carbamoyl-2,2,5,5-tetramethyl-3-pyrrolin-1-yloxy) as a report group for the interactions and the conformational changes of lyophilized bovine serum albumin (BSA) and bovine hemoglobin (BH), as function of pH values in the range 2.5–11. The EPR spectra are similar with those of other non-covalently spin label porphyrins in frozen solution at very low temperatures. This behavior indicated a possible spin–spin interaction between the hemic iron and the nitroxide group. The changes in the EPR spectra as function of the pH are discussed in terms of conformational changes of the proteins. Spectral simulations and magnetic EPR parameters reveal the following: (i) one single paramagnetic species, with Gaussian line shape, was used for the best fits of experimental spectra in the case of serum albumin samples; and (ii) a weighted sum of Lorentzian and Gaussian line shape in the case of hemoglobin samples. The representation of correlation time vs. pH, reveals a dependence of degree of immobilization of spin label on the conformational changes of proteins in acidic and basic environment.

Keywords: EPR; Spin label; Serum albumin; Haemoglobin

1. Introduction

Proteins are polymeric chains that are built from monomers called amino acids. All structural and functional properties of proteins derive from the chemical properties of the polypeptide side-chain. The ionizable groups of the side-chains of charged amino acids are often involved in biochemical transactions (binding, catalysis). Therefore, pH usually has rather dramatic effects on the function of proteins. Polar residues are both buried as well as on the surface of the protein. They either form hydrogen bonds with other polar residues in the protein or with water. Non-polar residues do not interact favorably with water. However, a significant number of non-polar residues are also found on the surface of the protein. Therefore, it is important to study the interactions of proteins with well-defined molecules that will confer the total

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molecules sufficient affinity for the binding site of interest on protein [1,2]

The successful application of spin labeling to protein structure investigations is limited by the possibility to chemically change specific side chains in proteins. However, useful information on protein properties can be obtained by non-covalent spin labeling if the affinity of the protein for the label molecules is great enough to affect their motional freedom [3–6]. The rate of rotation (or tumbling) of the spin label influences the lineshape of its EPR spectrum. Therefore, the EPR signal of a spin label covalently or non-covalently bonded to a biomolecule can yield a range of information about its structural environment in conventional ESR and allows for full spectral coverage [7,8].

In the same time, EPR has been an invaluable tool for probing microscopic molecular motions in a variety of systems, including isotropic solvents [9], liquid crystals [10], model membranes and biomolecules [11–13].

In the present work, non-covalent spin labeled bovine serum albumin (BSA) and bovine hemoglobin (BH) with Tempyo spin label (3-carbamoyl-2,2,5,5-tetramethyl-3-pyrrolin-1-yloxy) were investigated both in liquid and lyophilized samples, in the pH range 2.5–11, in order to obtain useful information related to the interaction between the nitroxide group and the functional site of the proteins.

Interactions of spin label with hemic or non-hemic proteins might affect the spin label spectra and in the same time it is well known that the pH strongly influences the conformation of proteins leading to significant changes in the type and degree of these interactions [14]. In this pH range, we followed the effect of protein conformational changes on the interactions between the nitroxide and the functional groups of proteins and also the pH influence on molecular motion emphasized by the EPR spectra of the spin label.

2. Materials and methods

Powdered bovine serum albumin and hemoglobin (>95% methemoglobin) from Sigma Chemicals, were used without further purification. Proteins were hydrated in phosphate buffer physiological saline at a final concentration of 10^{-3} M. Tempyo spin-label (3-carbamoyl-2,2,5,5-tetramethyl-3-pyrrolin-1-yloxy), from Sigma Chemicals (Fig. 1), was added to the liquid samples of each protein in a final concentration of 10^{-3} M (protein/spin label molar ratio 1:1) and the pH values were adjusted to the desired value in the range 2.5–11. An amount of 5 ml from each sample was lyophilized for 30 h at −5 °C and used for the EPR measurement, at room temperature.

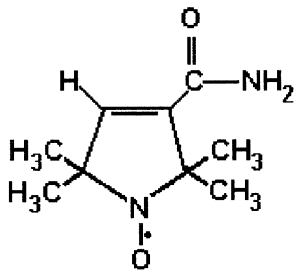

Fig. 1. The chemical structure of Tempyo spin label.

EPR spectra for both liquid and lyophilized samples, were recorded at room temperature with a JEOL-JES-3B spectrometer, operating in X-band (9.5 GHz), equipped with a computer acquisition system. Samples were placed in quartz capillary tubes. The spectrometer settings were: modulation frequency 100 KHz, field modulation 1 G, microwave power 20 mW. The computer simulation analysis of spectra, for obtaining the magnetic characteristic parameters, was made by using a program that is available to the public through the Internet (http://alfred.niehs.nih/LMB).

The lineshape of an EPR spectrum depends, among other factors, on the orientation of the paramagnetic center with respect to the applied magnetic field. In a powder, or a frozen aqueous solution, the paramagnetic centers will be fixed with a random distribution of orientations, and in the case of anisotropic g- and hyperfine interactions this will lead to a broadened EPR spectrum, since all orientations contribute equally. In the liquid state, however, the paramagnetic centers are not fixed but undergo rotational fluctuation. In the case of fast rotation, the anisotropic interactions are thereby averaged to zero, giving rise to sharp EPR lines. If the velocity of the rotational motion

decreases, the EPR spectrum will approach that of the powder spectrum. Therefore, a rotational correlation time for a paramagnetic molecule can also be determined by EPR using semiempirical formula [15].

For isotropic motion in the rapid tumbling limit, the spectra will be isotropic with the averages of the principal components of the g-values and hyperfine splitting factor, a^N. The rate of the isotropic motion determines the relative widths of resonances and the width, ΔH_m, of an individual (hyperfine) line, in the first approximation can be written as a function of the z component of the nitrogen nuclear spin number ($m=-1,\ 0,\ 1$) [16,17]:

$$\Delta H_m = A + B\cdot m + C\cdot m^2 \tag{1}$$

where the A coefficient includes other contributions than motion. The terms B and C are functions related to the rotational correlational time (τ) and can be defined as a function of peak-to-peak line width of the central line, ΔH_0 [G], and the amplitudes of the mth line I_m [14]:

$$B = \frac{1}{2}\Delta H_0\left(\sqrt{\frac{I_0}{I_1}} - \sqrt{\frac{I_0}{I_{-1}}}\right) = 0.103\omega_e[\Delta g\Delta a^N + 3(\delta g)(\delta a^N)]\tau_B\left[1+\frac{3}{4}(1+\omega_e^2\tau_B^2)^{-1}\right] \tag{2}$$

$$C = \frac{1}{2}\Delta H_0\left(\sqrt{\frac{I_0}{I_1}} + \sqrt{\frac{I_0}{I_{-1}}-2}\right) = 1.181\cdot 10^6\left[(\Delta a^N)^2 + 3(\delta a^N)^2\right]\tau_c\left[1-\frac{3}{8}\times(1+\omega_c^2\tau_c^2)^{-1} - \frac{1}{8}(1+\omega_e^2\tau_c^2)^{-1}\right] \tag{3}$$

in which

$$\Delta a^N = a_{zz}^N - \frac{1}{2}(a_{xx}^N + a_{yy}^N),\ \ \delta a^N \frac{1}{2}(a_{xx}^N - a_{yy}^N) \tag{4}$$

$$\Delta g = g_{zz} - \frac{1}{2}(g_{xx} - g_{yy}),\ \ \delta g = \frac{1}{2}(g_{xx} - g_{yy}) \tag{5}$$

and $\omega_N = 8.8\times 10^{-6}\ \langle a^N\rangle$, a^N is the isotropic hyperfine splitting and ω_e the ESR spectrometer frequency in angular units.

In the range from 5×10^{-11} to 10^{-9} s (motion in the rapid tumbling limit) and magnetic field above 3300 G, Δg and Δa^N vanish, and the correlation times τ_B and τ_C are directly related to the B and C coefficients by the following simple relations [5]:

$$\tau_B = \tau_z = K_1\cdot B \tag{6}$$

$$\tau_C = \tau_{x,y} = K_2\cdot C \tag{7}$$

where $K_1 = 1.27\times 10^{-9}$ and $K_2 = 1.19\times 10^{-9}$. The average correlation times is:

$$\tau = (\tau_B\cdot\tau_c)^{1/2} \tag{8}$$

The slow motion of spin probe, lead to a broadening of the EPR lines. In this case, the rotational correlation time, τ, is larger than 10^{-9} s, and thus, the relation (8) is not applicable.

The isotropic nitrogen hyperfine splitting changes to a powder like spectrum, with the peak-to-peak distance between the external peaks of the spectrum [$2\cdot a'_{zz}$ (N)] depending on the magnitude of the rotational correlation time, τ [18,19]. Another lineshape theory for slow isotropic Brownian rotational diffusion of spin-labeled proteins has been developed by Freed [9]. Thus, the correlation time can be evaluated from the ratio of the observed splitting between the derivate extreme a'_{zz} and principal value a_{zz}, determined from rigid matrix spectrum [5,7]:

$$\tau = \alpha\left(1 - \frac{a'_{zz}}{a_{zz}}\right)^{\beta} \tag{9}$$

The α and β parameters depend on the kind of the diffusion process, where a and b are empirical constants, which are tabulated in e.g. Poole and Farach (1987) [15]. For small spin probe, the intermediate jump diffusion is preferable [15].

3. Results and discussion

In the low concentration liquid state BSA and BH solution, the Tempyo spin label which is a relatively small molecule, gives rise to a spectrum with narrow lines and constant hyperfine splitting, typical for fast isotropic rotational motion (Fig. 2) with a very low rate of migration between protein molecule and water. For this kind of rotation, the

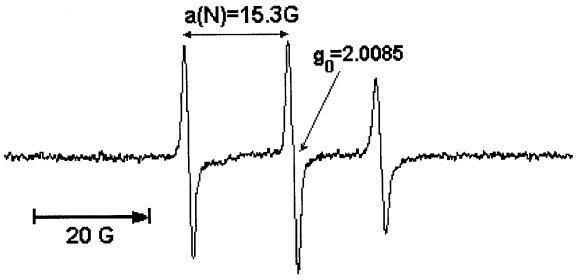

Fig. 2. EPR spectrum of the Tempyo spin label at low pH.

rotational correlation time can be estimated from the intensity ratio of the low-field and high-field N-lines using a semi-empirical formula (8). For the Tempyo spin label in BSA and BH aqueous solution, the calculated rotational correlation times was 2×10^{-10} s and 2.5×10^{-10}, respectively, which is consistent with fast rotation as expected for a small molecule.

No detectable changes were observed in EPR spectra of aqueous solution of Tempyo spin label without proteins, at different pH values.

The characteristic EPR spectra of a Tempyo spin label in lyophilized BSA is due primarily to anisotropy in the nitrogen hyperfine coupling which is typical for slow rotation. For slow rotations, the EPR spectrum of spin labels depends, in a much more complicated fashion, on the combined influences of molecular motion and magnetic interactions. Fig. 3 displays the experimental and simulated spectra for Tempyo spin label in lyophilized BSA at different pH values. In order to find the magnetic parameters, the experimental EPR spectra were simulated. The best fit of experimental EPR spectra of Tempyo spin label in lyophilized BSA, was obtained assuming a single paramagnetic species with magnetic parameters listed in Table 1, and a Gaussian lineshape corresponding to static dipolar interactions.

The main feature of the EPR spectra of Tempyo spin label in lyophilized haemoglobin (Fig. 4), like the Tempyo spin label in lyophilized BSA, exhibit the characteristics to slow motion of spin label but with more broadening of the lineshape. The best fit of the simulated experimental EPR spectra can be obtained by assuming the presence of two functional groups in hemoglobin associated

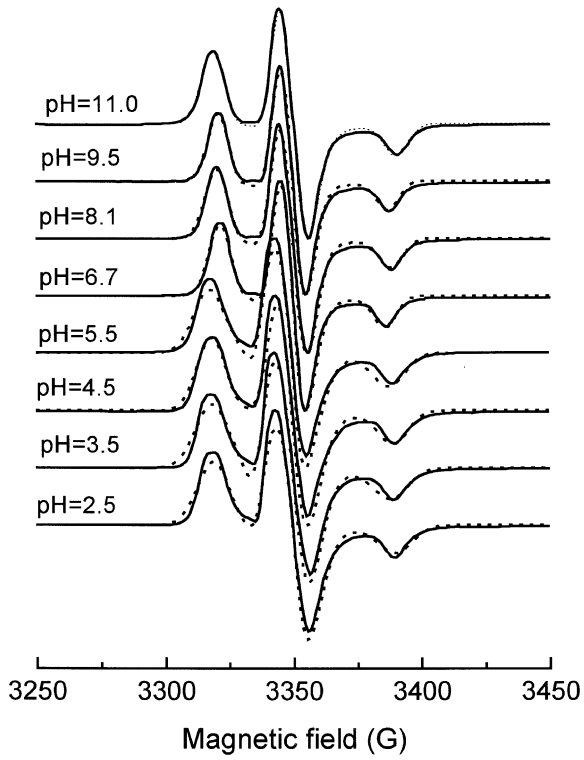

Fig. 3. Experimental (——) and simulated (……) EPR spectra of Tempyo spin label in lyophilized bovine serum albumin at various pH values.

with two non-equivalent paramagnetic species. Computer simulations indicate a weighted sum of mixed Gaussian lineshapes (static dipolar interactions) and Lorentzian lineshapes (spin–spin interactions). Generally, the broadening of the Gaussian lineshape are due to static dipolar interactions of

Table 1
Magnetic parameters values of Tempyo spin label in lyophilized bovine serum albumin at various pH values

pH	g_{xx}	g_{yy}	g_{zz}	A_{xx} (G)	A_{yy} (G)	A_{zz} (G)
11	2.01028	2.00841	2.00441	4.72	8.53	35.48
9.5	2.00113	2.00762	2.00484	6.16	5.91	33.96
8.1	2.01128	2.00781	2.00461	6.41	5.69	32.98
6.7	2.00113	2.00742	2.00500	6.15	6.55	32.20
5.5	2.01147	2.00809	2.00505	6.03	7.83	35.45
4.5	2.01259	2.00672	2.00588	7.79	6.98	35.52
3.5	2.01243	2.00660	2.00538	8.28	5.34	35.46
2.5	2.01269	2.00662	2.00459	6.38	5.66	35.91

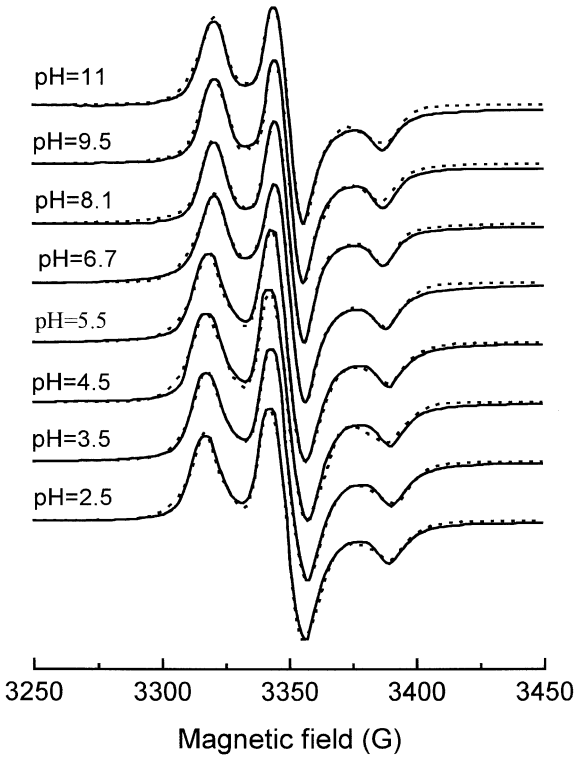

Fig. 4. Experimental (——) and simulated (……) EPR spectra of Tempyo spin label in lyophilized bovine hemoglobin at various pH values.

the spin label molecules whereas the broadening of the Lorentzian lineshape is due to to spin–spin interactions [20]. Figs. 5 and 6 present the EPR spectra of Tempyo spin label in the lyophilized BH at low and high pH, in which the contribution of each species is displayed. At low pH (pH 2.5), the main contributions are due to species with Gaussian line shape ($\sim$80%). This contribution decreases to $\sim$50% at high pH (pH 11). The first species with Gaussian lineshape and poor resolved hyperfine splitting is not influenced by the presence of the hemic iron and, therefore, we assume to be located far from the hemic group. The second species with Lorentzian lineshape and a well resolved hyperfine structure is located, probably, near the hem group, giving rise to a spin–spin interaction between the nitroxide radical and the paramagnetic iron of the hem group. Our results are in accordance with covalently labeled methemoglobin and other porphyrins in frozen samples under 50 K [21,22]. In these previous studies, spectra of covalently labeled methemoglobin were analyzed by using perturbation calculations in order to estimate the iron to nitroxyl distances and it was suggested that plausible distances are in the range of 14.5–17.5 Å. The g tensor and A tensor components used in the simulation for the best fit values of the simulation of the effective powder spectrum, are presented in Table 2.

Due to increased spatial restrictions of protein structure in the vicinity of the label, by lyophilization, the mobility of spin label is slow on the EPR time scale ($\sim 5\times 10^6$ s^{-1}), leading to a broadening of the EPR lines, with the peak-to-peak distance between the external peaks of the spectrum [$2\cdot a'_{zz}$ (N)] depending on the magnitude of the rotational correlation time, τ. Generally, broadening of the peaks in an EPR spectrum is

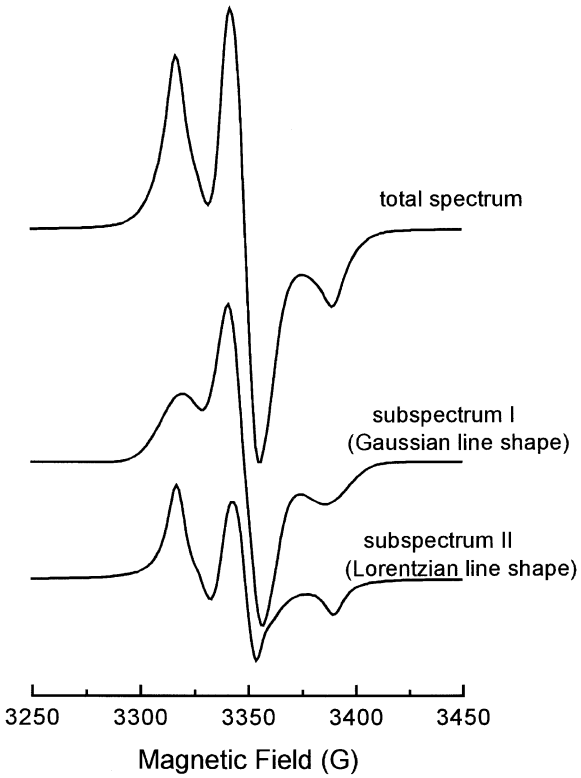

Fig. 5. The experimental EPR spectrum and its subspectra of Tempyo spin label in lyophilized hemoglobin at pH 2.5

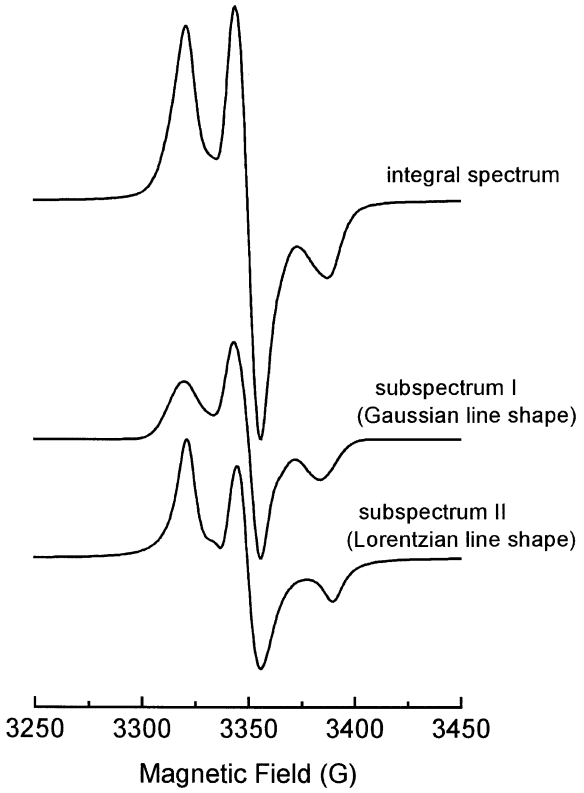

Fig. 6. The experimental EPR spectrum and its subspectra of Tempyo spin label in lyophilized hemoglobin at pH 11.

indicative of immobilization of the spin label, whereas sharpening of the peaks is indicative of an increase in label mobility. By comparing the apparent nitrogen hyperfine splitting [termed a'_{zz} (N)] with the nitrogen hyperfine splitting obtained from their rigid limit values [a_{zz} (N)], the rotational correlation times can be calculated using formula 9. The values of α and β coefficients depend on the motional model. The study of the influence of different diffusional models on the spectral line shape in the regime of slow motional spin label by high EPR fields has shown that jump diffusion mainly affects the line widths at the same motional rates [23]. In our calculations, the intermediate jump diffusion model was considerate, with coefficients values of $\alpha = 5.4 \times 10^{-10}$ s and $\beta = -1.36$ [15]. The average correlation time for different values of pH are plotted in Fig. 7. As shown in figure, the pH influences the rotational correlation

Table 2
Magnetic parameters values of Tempyo spin label in lyophilized bovine hemoglobin at various pH values

pH	g_{xx}	g_{yy}	g_{zz}	A_{xx} (G)	A_{yy} (G)	A_{zz} (G)
11	2.01242	2.00771	2.00372	4.81	8.70	34.01$^{(L)}$
	2.01117	2.00497	2.00610	6.42	7.76	33.16$^{(G)}$
9.5	2.00935	2.00839	2.00478	6.19	13.89	34.82$^{(L)}$
	2.01079	2.00695	2.00465	6.11	7.29	33.06$^{(G)}$
8.1	2.01133	2.00702	2.00464	8.38	6.68	33.87$^{(L)}$
	2.01500	2.00428	2.00592	8.64	6.50	32.60$^{(G)}$
6.7	2.00115	2.00773	2.00425	3.27	8.20	33.73$^{(L)}$
	2.01213	2.00476	2.00556	7.43	8.88	35.84$^{(G)}$
5.5	2.012442	2.00821	2.00471	9.52	7.32	34.62$^{(L)}$
	2.001050	2.00592	2.00541	5.86	6.12	35.45$^{(G)}$
4.5	2.01609	2.00768	2.00522	9.20	7.66	36.03$^{(L)}$
	2.01198	2.00416	2.00523	7.43	8.88	35.15$^{(G)}$
3.5	2.01265	2.00816	2.00413	4.14	5.39	36.59$^{(L)}$
	2.01169	2.00287	2.00506	7.06	9.36	35.46$^{(G)}$
2.5	2.01344	2.00844	2.00506	9.83	10.38	36.26$^{(L)}$
	2.01270	2.00471	2.00548	4.67	5.80	35.78$^{(G)}$

(L), Lorentzian lineshape; (G), Gaussian lineshape.

time. In the acidic pH range the NH_2 groups of the label molecule as well as those of the amino acids residues are protonated. The fact that τ shows greater values in this range followed by a significant decrease in the basic pH range, indicate a low mobility of spin label in acidic environment while an increasing of mobility can be noticed in the basic pH range. A minimum mobility can be observed around the isoelectric point of both proteins (for BSA $pH_i = 4.8$, and for BH, $pH_i = 6.8$). From the pH dependence of correlation time (involving the mobility of the label as well), we assume that in an acidic environment the mobility of spin label molecules are reduced due to the

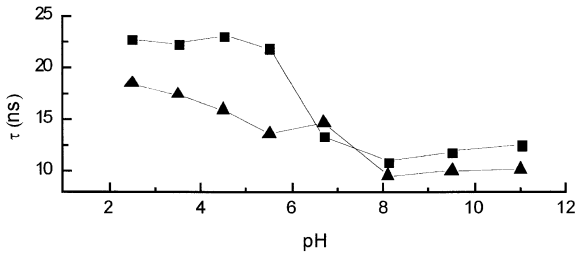

Fig. 7. Correlation times (τ) as a function of pH for Tempyo spin label in lyophilized bovine serum albumin (■) and bovine hemoglobin (▲).

formation of hydrogen bonds between the NH_2 group of spin label and side chains of neighboring amino acids. One can correlate this observation with the fact that serum albumin undergoes reversible isomerization in the pH range 2.7–7 from the expanded form characterized by 35% α-helix content, to normal form characterized by 55% α-helix content accompanied by a decrease in β-sheet [24,25]. It is well known that β-sheet conformation favors the formation of hydrogen bonding.

By comparing with the BSA case, we notice that the mobility of Tempyo is greater with respect to hemoglobin, which is not surprising if we take into account that hydrogen bonding opportunities depends on the β-sheet content: in hemoglobin the β-sheets represent 50% while in BSA the percentage varies from 70 to 45%, depending on the pH.

As shown in Fig. 7 the mobility of this species increases in an acidic environment, reaching a maximum value corresponding to pH_i 6.8 followed by a slow decrease. We suggest that in basic pH range, where the label is not subject to strong electrostatic interactions, dipolar and spin–spin interactions are manifested almost with the same contribution in the broadening of the spectrum.

4. Conclusions

EPR spectroscopy is very useful to study the mobility of nitroxide radicals with respect to hemic or non-hemic proteins in different environmental conditions. Non-covalent labeling of proteins can give valuable information on the magnetic interactions between the label molecule and the paramagnetic center of the proteins. The relevance of this interaction can be obtained from lineshape analysis: computer simulations for non-hemic protein assume a Gaussian lineshape, while for hemic protein a weighted sum of Lorentzian and Gaussian components is assumed. The contribution of the each line shape to experimental spectrum depends on the pH.

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