EPR AND ATR-FT-IR INVESTIGATION OF LYOPHILIZED CYTOCHROME c AT DIFFERENT pH

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Abstract. Conformational changes and interaction of spin label with cytochrome c protein at different pH values have been studied by FT-IR and EPR spectroscopy. Electron Paramagnetic Resonance (EPR) spectroscopy was used to investigate the mobility of Tempo spin label (3-carbamoyl-2,2,5,5-tetramethyl-3-pyrrolin-1-yloxy) in order to obtain useful information related to the interaction between the nitroxide group and the functional site of the lyophilized protein samples. A minimum mobility can be observed around the isoelectric point (pH_i = 9.8). The best information from infrared protein spectra is obtained in the amide I band which appears between 1700 and 1600 cm^{-1}. The results of qualitative and quantitative analysis by curve fitting to the inverted second derivative spectra of amide I features of lyophilized hemoglobin reveal a decrease in β-sheet content as the pH values increase and a decrease in α-helix content at lower or higher pH values as well as a decrease in turns content.

Key words: EPR, FT-IR, spin label, cytochrome c.

INTRODUCTION

Lyophilization or freeze-drying is the method most commonly used to prepare the protein for long-term storage. The acute freezing and drying stresses of lyophilization may irreversibly damage the protein. The damage is manifested as denaturation and aggregation when the protein sample is rehydrated. Denaturation is a process by which hydrogen bonds, hydrophobic interactions and salt linkages are broken and the protein is unfolded. The denaturation of secondary structure

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involves also changes in the ratio among the three common structures: α-helix, β-sheets and turns.

Therefore, preparation study of lyophilized (freeze-dried) proteins products is essential to obtain the requisite stability during months or even years at ambient temperature [3, 13].

In this paper, the non-covalent spin labeling EPR and ATR-FTIR techniques were used to study the interaction and vibrational change in lyophilized cytochrome c for different pH values. The techniques used here complement each other because Electron Paramagnetic Resonance (EPR) detects only the spin label in these experiments and has a time window that is optimal for protein chain dynamics, whereas Fourier transform infrared (FTIR) detects protein vibrations simultaneously on a fast time scale. Motional coupling between large, relatively immobile structure proteins and mobile residues results in motionally restricted protein-solvating first-shell of hydration [12]. By quantifying this degree of hydration, we have estimated changes in the size of protein assemblies of protein complexes. The partitioning of 2,2,6,6,-tetramethylpiperidine-1-oxyl (Tempo) between slow and fast motion depends on pH values of environments and can be measured with EPR [15, 16]

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 noncovalent spin labeling if the affinity of the protein for the label molecules is great enough to affect their motional freedom [17]. The rate of rotation (or tumbling) of the spin label influences the line shape of its EPR spectrum. Therefore, the EPR signal of a spin label covalently or noncovalently bonded to a bimolecular can yield a range of information about its structural environment (in conventional EPR) and allows full spectral coverage.

On the other hand, Infrared Spectroscopy is one of the most used techniques for studying stress-induced alterations in protein conformation and for quantifying the protein secondary structure [1, 7]. By establishing the frequencies of all IR-active amide modes of each element of the secondary structure together with their respective molar extinction coefficients, it is possible to determine the secondary structure of any given protein precisely from its vibrational spectrum [6, 8–10].

MATERIALS AND METHODS

The powder cytochrome c protein was obtained from Sigma Chemicals and used without further purification. The protein was rehydrated in phosphate buffer physiological saline at a final concentration of 10^{-3} mol/l. The pH range was adjusted at neutral values adding a small amount of NaOH. A small amount of 5 ml

from each sample was lyophilized for 30 hours at –5 ºC and then used as powder sample.

EPR SPECTROSCOPY

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://epr.niehs.nih.gov). 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* factor 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. 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 the 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]:

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

where A coefficient includes the 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 m-th line I_m [14, 16, 20]:

$$C = \frac{1}{2}\Delta H_0 \left(\sqrt{\frac{I_0}{I_1}} - \sqrt{\frac{I_0}{I_{-1}}} \right) \tag{2}$$

$$C = \frac{1}{2}\Delta H_0 \left(\sqrt{\frac{I_0}{I_1}} + \sqrt{\frac{I_0}{I_{-1}}} - 2 \right) \tag{3}$$

In the range from $5 \cdot 10^{-11}$ to 10^{-9} s (motion in the rapid tumbling limit) and the magnetic field above 3300 G, the correlation times τ_B and τ_C are directly related B and C coefficients by the following simple relations [20]:

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

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

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

$$\tau = \left(\tau_B \cdot \tau_C\right)^{\frac{1}{2}} \tag{8}$$

The slow motion of the spin probe leads 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. Another lineshape theory for slow isotropic Brownian rotational diffusion of spin-labeled proteins has been developed by J. Freed [19]. Thus, the correlation time can be evaluated from the ratio of the observed splitting between the derivate extrema a'_{zz} and principal value a_{zz}, determined from rigid matrix spectrum [20]:

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

The α and β parameters are empirical constants depending on the type of the diffusion process and are tabled in e.g. Poole and Farach, 1987 [17]. For small spin probe the intermediate jump diffusion is preferable [20].

ATR-FTIR SPECTROSCOPY

The FT-IR spectra of proteins were recorded in the region 4000–800 cm^{-1} by a Bruker EQUINOX 55 spectrometer, using an Attenuated Total Reflectance accessory with a scanning speed of 32 cm^{-1} min^{-1} with the spectral width 2.0 cm^{-1}. The internal reflection element was a ZnSe ATR plate (50 × 20 × 2 mm) with an aperture angle of 45°. A total of 128 scans were accumulated for each spectrum.

Spectra were recorded at a nominal resolution of 2 cm^{-1}. The resultant spectra were smoothed with a 9-point Savitzky-Golay smooth function to remove the white noise. The second derivative spectral analysis was applied to locate positions and assign them to different functional groups. Before starting the fitting procedure, the

obtained depths of the minima in the second derivative spectrum and, subsequently, the calculated maximum intensities were corrected for the interference of all neighbouring peaks. All second-derivative spectra, calculated with the derivative function of Origin7.0 software, were baseline-corrected, based on the method of Dong and Caughey [7] and area-normalized under the second derivative amide I region, 1700–1600 cm^{-1}.

The curve fitting is performed by stepwise iterative adjustment towards a minimum root-mean-square error of the different parameters determining the shape and position of the absorption peaks. The inverted second-derivative spectra were obtained by multiplying by (–1) the second-derivative spectra. Curve fitting was performed by setting the number of component bands found by second-derivative analysis with fixed bandwidth (12 cm^{-1}) and Gaussian profile. The best-average fit gave the intensity of each component band for each spectrum. The area under each peak was used to calculate the percentage of each component and finally used to analyze the percentage of secondary structure components. Thus, the areas corresponding to the different types of secondary structure are quantitatively and qualitatively evaluated by integration and curve fitting. The curve fitting is performed by stepwise iterative adjustment towards a minimum root-mean-square error of the different parameters determining the shape and position of the absorption peaks.

RESULTS

EPR SPECTROSCOPY

The characteristic EPR spectra of a Tempo spin label in lyophilized samples is due primarily to anisotropy in the nitrogen hyperfine coupling 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. The experimental and simulated spectra for Tempo spin label in lyophilized cytochrome c, at pH = 4.1, pH = 5.2, pH = 6.7, pH = 7.1, pH = 10.6 and pH = 12.0 are presented in Fig. 1. The main feature of the EPR spectra of Tempo spin label in lyophilized cytochrome c, is that it exhibits the characteristics to slow motion of spin label, but with a larger broadening of the lineshape [14, 15].

Simulation of experimental EPR spectra can be obtained by assuming the presence of two functional groups in heme proteins, associated with two nonequivalent paramagnetic species [18]. 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 the spin label molecules whereas the broadening of the Lorentzian lineshape is due to spin-spin interactions [4, 16].

From the computer analysis of spectra, we suggest that at low pH, the main contribution is due to species with Gaussian line shape (~80%). This contribution decreases to ~50% at high pH (pH = 12). The first species, with Gaussian lineshape and poor resolved hyperfine splitting, is not influenced by the presence of the heme iron and therefore we assume to be located far from the heme group.

The second species with Lorentzian lineshape and a well resolved hypefine structure is located, probably, near hem group giving rise to a spin-spin interaction between the nitroxide radical and the paramagnetic iron of the heme group. Our results are in accordance with covalently labelled other porphyrins in frozen samples under 50 K [2, 11, 19]. Due to increased spatial restrictions of protein structure in the vicinity of label, by lyophilization, the mobility of spin label is slow on the EPR time scale (~$5 \cdot 10^6$ 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 indicative of immobilization of the spin label, whereas sharpening of the peaks points to an increase in label mobility.

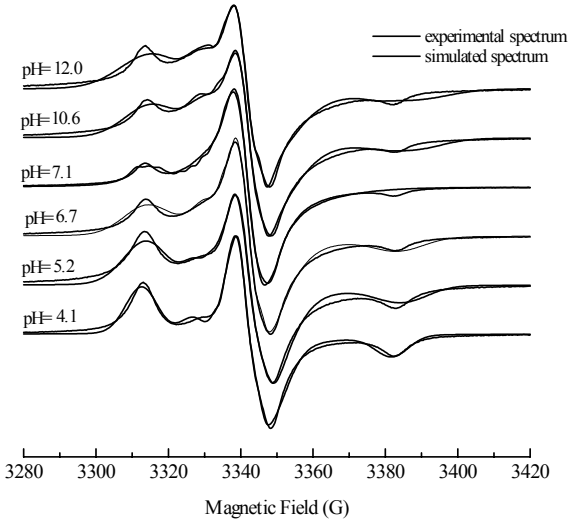

Fig. 1. Experimental and simulated spectrum of Tempo labelled cytochrome c.

By comparison of 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 influence of the 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 [20]. In our calculations, the intermediate jump diffusion model was considered, with coefficients values of $\alpha = 5.4 \cdot 10^{-10}$ s and $\beta = -1.36$ [20].

In Fig. 2 are plotted the averages of the correlation time for different values of the pH.

In acid 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 higher values in this range, followed by a significant decrease in the basic pH range, indicates a low mobility of spin label in acid environment while an increase of mobility can be noticed in basic pH range [4]. From the pH dependence of correlation time (involving the mobility of the label as well), we assume that in acid environment, the mobility of spin label molecules is reduced due to formation of the hydrogen bonds between NH_2 group of spin label and side chains of neighbouring amino acids. As shown in Fig. 2, the mobility of Tempo increases in acid environment, 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 with almost the same contribution in the broadening of the spectrum.

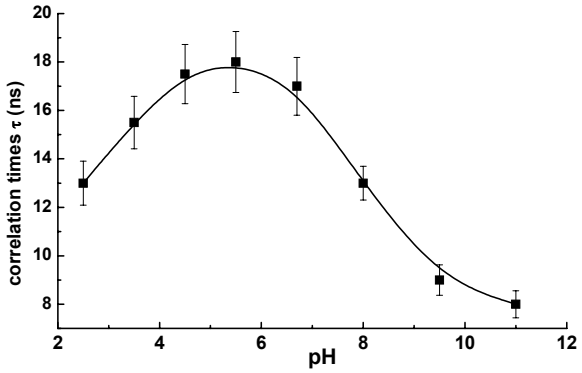

Fig. 2. Correlation times (τ) as a function of pH for Tempo spin label in lyophilized cytochrome c.

ATR FT-IR SPECTROSCOPY

FT-IR spectroscopy was used to monitor molecular conformational changes taking place during the lyophilization process. The amide I band is due to the in-plane C=O stretching vibration, weakly coupled with C–N stretching and in-plane N–H bending. Each type of secondary structure (i.e. α-helix, β-sheet, β-turn and unordered) gives rise to different C=O stretching frequencies, and hence, results in characteristic band positions. Band positions are used to determine the secondary structural types

present in each protein. The relative band areas (determined by curve fitting) can then be used to quantify the relative amount of each structural component. For instance, Figure 3 shows the inverted second derivative amide I of the cytochrome, lyophilized at pH = 6.7.

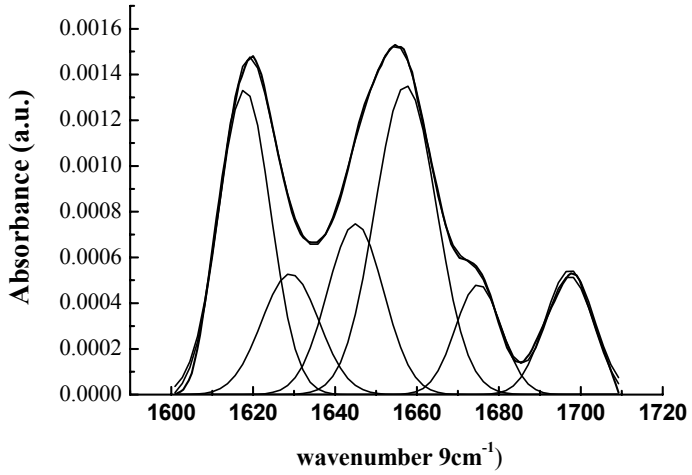

Fig. 3. The curve-fitted inverted second-derivative amide I spectrum of lyophilized cytochrome c at pH = 6.7. The inversion of second-derivative spectrum was done by factoring by −1. The curve-fitting was carried out as described under Materials and Methods.

Assignments of the bands were made on the basis of previously reported studies together with the quantitative analysis. Fourier deconvolution, used to determine the component band positions, reveals that both proteins exhibit a major band in range 1650–1653 cm^{-1} characteristic of α-helix structures. The bands in the region 1638–1640 cm^{-1} are expected to be characteristic for native β-sheet structures, while the bands in the ranges 1660–1667 cm^{-1} and 1682–1687 cm^{-1} may possibly be attributed to β-turns. On the basis of earlier IR studies, the bands from 1626–1628 cm^{-1} are indicative of intermolecular (antiparallel) β-sheet and, according to literature, are a common IR spectral features for both lyophilization and temperature-induced protein aggregation [9, 10]. The results in Table 1 show that the major change in cytochrome structure upon pH variation is the antiparallel β-sheet formation, and this transition has been associated with the intermolecular association of the unfolded protein molecules. A similar mechanism put forward for the heat-induced gelation of ovalbumin suggested that the aggregation was formed from partially unfolded protein molecules, through the cross-linking of intermolecular β-sheet structures, as a result of the exposure of hydrophobic residues [10].

Table 1

Relative areas and assignments of infrared second derivative amide I band of lyophilized cytochrome c at different pH

pH = 4.1			pH = 5.2			pH = 6.7		
ν (cm^{-1})	Areas (%)	Assign.	ν (cm^{-1})	Areas (%)	Assign.	ν (cm^{-1})	Areas (%)	Assign.
1693	4.73	aggreg.	1698	6.26	aggregates	1698	9.76	aggregates
1680	9.24	turns	1682	8.63	turns	1682	8.11	turns
1651	38.7	α-helix	1656	37.5	α-helix	1654	30.38	α-helix
1638	15.9	random	1640	18.76	random	1640	15.33	random
1625	22.6	β-sheets	1625	18.32	β-sheets	1626	11.12	β-sheets
1610	8.83	aggreg.	1620	10.53	aggregates	1620	25.29	aggregates

pH = 8.1			pH = 9.5			pH = 11.0		
ν (cm^{-1})	Areas (%)	Assign.	ν (cm^{-1})	Areas (%)	Assign.	ν (cm^{-1})	Areas (%)	Assign.
1697	10.8	aggregates	1691	7.73	aggregates	1687	8.1	aggregates
1675	9.6	turns	1676	7.1	turns	1677	8.22	turns
1656	32.6	α-helix	1656	36.77	α-helix	1656	41.1	α-helix
1645	18.8	random	1645	21.6	random	1645	23.0	random
1626	14.1	β-sheets	1629	17.8	β-sheets	1634	15.52	β-sheets
1616	13.8	aggregates	1618	8.8	aggregates	1625	3.11	aggregates

CONCLUSIONS

Noncovalent labeling of proteins can give valuable information on the magnetic interactions between the label molecule and the paramagnetic center of the proteins correlated with a chemical change in specific side chains in proteins. At the same time, the ATR-FTIR spectroscopic technique can be used to characterize the secondary structures of the proteins in the processes which involve their denaturation. Simultaneous qualitative and quantitative analysis of amide I ATR-FTIR spectra of cytochrome c by curve fitting of the inverted second derivative spectra reveals measurable changes in α-helix content of proteins.

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