Abstract

Interactions of spin label with 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. In the present work, noncovalent spin labelled hemoglobin with tempyo spin label was investigated in the pH range 2.5-11 in order to obtain useful informations related to the interaction between the nitroxide group and the active site (hem group) of hemoglobin. Complementary, Raman and SERRS investigations were performed in order to emphasize the characteristic spectrum of hemoglobin with the three proeminent bands, called “markers” of the hemic group, packed in the polypeptidic chain. These bands are assigned to “in plane” vibration of the porphyrinic ring. It was concluded that these techniques are invaluable tools for probing microscopic molecular motions in biomolecules and, in the same time emphasize the selective character of SERRS techniques, using the adsorption of hemoglobin on silver colloidal soil.
Introduction

Electronic spectra and magnetic properties of iron are sensitive reporters of the iron environment. The wide range of oxidation states, spin states and geometries already observed for iron in biological systems necessitates probing the iron with the most sensitive techniques available. One example is the use of two paramagnetic species, which permits study of their interactions with each other in addition to their interaction with other aspects of their environment. 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 [1-4]. EPR has been an invaluable tool for probing microscopic molecular motions in a variety of systems, including isotropic solvents [5,6], liquid crystals [7,8], model membranes and bimolecules [9,10]. In the same time, in spite of the existence of a large amount of data in the field of biological application of Raman spectroscopy, and, in particular, of resonance Raman applied to hemic proteins, it is very difficult to outline a complete vibrational characterisation of them [11]. The current traditional methods of analysis of proteins are time consuming and expensive and therefore more efficient techniques are desirable. Raman spectroscopy is an important tool for the determination of the biomolecules structure and extensive research has been undertaken [12-14]. In the present work, noncovalent spin labeled bovine hemoglobin (BH) with Tempyo spin label (3-carbamoyl-2,2,5,5-tetramethyl-3-pyrrolin-1-yloxy) was 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
active site of the protein. Interactions of spin label with hemic or nonhemic 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. In this pH range, we followed the effect of protein conformational changes on the interactions between the nitroxide and the active site of proteins and also the pH influence on molecular motion emphasized by the EPR spectra of the spin label. Also, in this paper we present vibrational Raman and SERS investigations on hemoglobin. The lower sensitivity and effects of fluorescence on the normal Raman spectra of this biomolecule have lead to the extension of research in the direction of the surface enhanced Raman scattering (SERS) [15].

**Materials and Methods**

Powder hemoglobin (>95% methemoglobin) from SIGMA Chemicals was used without further purification. The protein was 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 was added to the liquid samples 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÷12. The amount of 5 ml from each sample was lyophilized for 30 hours at $-5\degree C$ and used for the EPR measurement, at room temperature.

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 20mW. 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). Colloidal silver substrate for SERRS measurement was prepared according to the Lee-Meisel procedure [12]. The maximum absorption of the freshly prepared colloid was centered at 423 nm. The protein was rehydrated in buffer solutions for each pH value. A small amount of about 10 µl 10^{-2} mol/l protein solution was added to 2 ml colloidal silver, resulting a final sample concentration of 5 x 10^{-5} mol/l.

A micro-Raman setup was employed in order to record the Raman spectra of lyophilized powder samples. The 514.5 nm line of an argon ion laser (Spectra Physics, Model 166) was applied for excitation. The scattered light was collected in back-scattering geometry by focusing a x50 objective (Olympus ULWD MSPlan50) on the entrance slit of a spectrometer LabRam, Dilor with 1800 grooves/mm diffractive grating. The detection system consisted of a charge–coupled multichannel detector (CCD, SDS 9000 Photometrics).

Results and discussions

a) EPR investigations

EPR spectra of liquid samples are typical for fast motion nitroxide radicals in liquid environment, being similar for both proteins, at all pH
values (data not shown). The characteristic powder EPR spectrum of a nitroxyl radical at X band is due primarily to anisotropy in the nitrogen hyperfine coupling.

Fig.1 and table1 show the spectra of tempyo labelled hemoglobin and the magnetic parameters, respectively. In perpendicular plane, the nitrogen hyperfine splittings are $A_{xx} = 6.8 \text{ G}$ and $A_{yy} = 7.5 \text{ G}$, on the average. Along the z axis, $A_{zz} = 35 \text{ G}$ on the average, the values being similar to those calculated for covalently labelled methemoglobin and other porphyrins in frozen samples under 50 K [16,17].

![EPR spectra of noncovalent tempyo-labelled hemoglobin at various pH values.](image)

In these previous studies, spectra of covalently labelled methemoglobin were analysed by using perturbation calculations in order to estimate the iron to nitroxy distances and it was suggested that plausible distances are in the range of 14.5÷17.5 Å.
Table 1. EPR parameters for noncovalent tempyo labeled hemoglobin, at various pH values.

<table>
<thead>
<tr>
<th>pH</th>
<th>$g_{xx}$</th>
<th>$g_{yy}$</th>
<th>$g_{zz}$</th>
<th>$\Lambda_{xx}$ (G)</th>
<th>$\Lambda_{yy}$ (G)</th>
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(L) - Lorentzian line shape  
(G) - Gaussian line shape

In the present study of noncovalent labelled hemoglobin, the best fit of the experimental spectra can be obtained by assuming the presence of two sites in hemoglobin, associated with two nonequivalent paramagnetic species [10]. Computer simulations indicate a weighted sum of Gaussian lineshapes (static case) and Lorentzian lineshapes (dynamic case).
The first species, with Gaussian lineshape and well resolved hyperfine splitting, is not influenced by the presence of the hemic iron. The correlation time versus pH (fig.2) reveals that in acid pH range the mobility of tempyo decreased and has a minimum value near the isoelectric pH of hemoglobin (pH$_i$=6.8). By comparing with BSA case [18], we can 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 pH.

The second species in hemoglobin, with Lorentzian lineshape, is obviously influenced by the presence of the hemic iron. The lack of hyperfine structure of this specia is due to the dipolar and spin-spin interaction between the nitroxide radical and the paramagnetic iron of the hem group.

As shown in fig.2 the mobility of this species increases in acid 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 preferential manifested. However, the latter are less intense than the former, as results from fig.2.
Fig. 2. Correlation times ($\tau$) as a function of pH for Tempyo labeled hemoglobin.

According with [19-21], when rotational motion is slow enough that the spectra approach the powder spectrum limit, the rotational correlation time ($\tau$) can be evaluated using the relation: $\tau = \alpha (1 - A_{zz}/A_{zz'})^\beta$, where $\alpha = \ldots$
2.25 \cdot 10^{-9}, \beta = -0.615 \text{ and } A_{zz'}/A_{zz} \text{ is the ratio of the observed splitting between the derivative extreme } 2A_{zz'} \text{ and the principal value of } A_{zz} \text{ determined from the powder spectrum. The results are consistent with the “moderate jump diffusion” model for rotational diffusion [20-22] in which the label molecule has a fixed orientation for some average residence time } \tau \text{ and then “jumps” through an average angle of } (6D\tau)^{1/2} \text{ radians, where } D \text{ is the diffusion coefficient.}

b) Raman and SERRS investigations

In hemoglobin case, it was recently point out (1999-2000) the identification of a single molecule adsorbed on nanometric silver colloidal particles[23].

According with these studies, the excitation with 514,5 nm, lead to an absorption spectrum characterised by three prominent bands: 1375, 1586, 1640 cm\(^{-1}\).

These bands are called “markers” of the haemic group, packed in the polypeptidic chain, and are assigned to in plane vibration of the porphyrinic ring.

In the present study, due to the preresonant excitation with the green light, the Raman response of hemoglobin is covered by a fluorescence background.

Figure 3 present the Raman spectrum of pure hemoglobin compared with the SERRS spectra of increased concentration of hemoglobin.
In these spectra, the main bands are located at 1610, 1586, 1568 cm$^{-1}$ ($\nu_{C=C}$ and $\nu_{C=N}$), 1375 cm$^{-1}$ ($\nu_{C=N}$), 1167 cm$^{-1}$ ($\delta_{C-H}$ bending vibration), 957, 761, 674 cm$^{-1}$ ($\pi$-ring vibration out of plane). One can observed a good reproducibility of the SERRS spectra corresponding to all hemoglobin concentrations. By increasing concentration we can notice a little modification in relative intensity of 1375 cm$^{-1}$ band with respect to 1586-1610 cm$^{-1}$ range (meaning the amplification of $\nu_{C=N}$ detrimental to $\nu_{C=C}$ and $\nu_{C=N}$ vibrations). This observation suggest an electronic delocalisation of chromophor group. In the same time, by comparison with an other hemic protein, the cytochrome c [24], the enhancement of the bands assigned to
the porphyrin macrocycle stretching modes allowed the supposition of the N-adsorption from porphyrin ring to the colloidal surface.

In the present case, the SERRS signal represent a double resonant contribution:
1) plasmonic resonance of colloidal particles excited with green light, representing the electromagnetic amplification in SERRS effect, 2) Raman resonant contribution of hemoglobin excited with green light. Hence, the final amplification is greater than the usual SERS amplification due to the well-known mechanisms: electromagnetic enhancement and charge transfer.

Our results are in agreement with that reported in the case of a single molecule of hemoglobin adsorbed on silver nanoparticle, isolated [23]. SERRS spectra of labelled hemoglobin do not reveal a scattering contribution in the protein–tempyo complex, due to the folding complexity of the protein which limits the scattering effect of the small tempyo label. We suppose that the label is bound within a distance from the heme group in the basic pH range from 6.7 to 11. Also we can notice that the SERRS signal intensity do not change with the increasing concentration of hemoglobin, indicating that this signal arise only from the particles adsorbed on the first monolayer.

Conclusions

EPR spectroscopy is very useful to study the mobility of nitrooxide radicals with respect to hemic or nonhemic proteins in different environmental conditions. In the framework of the “moderate jump diffusion” model for rotational diffusion, the rotational correlation time is
strongly influenced by pH, due to the electrostatic interactions and hydrogen bonding.

From the SERRS study of the tempyo labeled cytochrome c and hemoglobin on the silver surface, a chemical contribution to the total enhancement was concluded. The enhancement of the bands assigned to the porphyrin macrocycle stretching modes allowed the supposition of the N-adsorption from porphyrin ring to the colloidal surface. The adsorption on the Ag surface under resonance condition is independent on the pH (in the range from 6.7 to 11), concentration, or the presence of the tempyo spin label.

References


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