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ELECTRON PARAMAGNETIC RESONANCE STUDY OF THE COMPLEX DYNAMICS OF THE SPIN LABEL ATTACHED TO CYTOCHROME C

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Measurements of an EPR signal of a nitroxide spin label attached to a protein allow for exploring the motion and structural changes of the macromolecule at the level of the backbone fold. X-band EPR spectra of the spin label attached to the iso-1-cytochrome *c* at position 102 (recorded for temperatures between 4–40°C) contain two components with different mobility. They were analyzed using "the microscopic order –macroscopic disorder" model (MOMD). The spectra of the spin labeled protein, either dissolved in a sucrose solution or bound to the CM Cellulose gel, have been compared. Experimental lineshapes have been successfully fitted to the theoretical model. Two components of the spectra are characterized by different values of rotational diffusion rates and ordering potential coefficients. Their values increase with temperature, but the relative fraction of either component in the spectrum is independent of temperature. The mean correlation times characterizing the faster component resolved in the EPR spectrum are different for these two kinds of samples. Analysis of the prototype system: "cytochrome c electrostatically bound to the gel" prepares the way for study the interaction of this protein with cytochrome bc_1, one of the three major respiratory enzyme complexes.

INTRODUCTION

Electron Paramagnetic Resonance Spectroscopy represents a very useful technique for studying the protein dynamics. The information about the motion of the whole molecule, or its domain or even backbone is retrieved form the EPR spectrum of a spin label, attached to the protein (Columbus & Hubbell, 2002). Advances in „site-directed spin labeling" (Hubbell, Cafiso & Altenbach, 2000) enable introduction of a spin label, usually MTSSL [(1-oxyl-2,2,5,5-tetramethylpyrroline-3-methyl) methanethiosulfonate], at a chosen site of the protein chain. The resulting motion determines the complex EPR spectrum of the spin label, and is the sum of the macromolecule tumbling, subdomain movement or backbone fluctuations and internal dynamics of the nitroxide chain. The consequence of the interaction between the spin label and its local neighborhood is the restricted, anisotropic rotational diffusion of the spin probe. Commonly, the observed complex EPR spectrum of the spin label attached to the protein is interpreted within MOMD model (Meirovitch, Nayeem & Freed, 1984).

Recent investigations on structure and dynamics of cytochrome c, with application of site directed spin labeling and EPR spectroscopy (Pyka, Osyczka, Turyna, Blicharski & Froncisz, 1999, 2001), revealed the existence of two different dynamic modes of the spin label motion. In that topography the nitroxide is partially exposed to the solution. The existing model "X_4/X_5" (Langen, Oh, Cascio & Hubbell, 2000) of the MTSL motion, well working for labels placed at solvent-exposed helical sites, does not seem to be relevant in this specific case. Below is presented a detailed analysis of the EPR spectra recorded for the MTSL attached to the yeast cytochrome *c* at naturally occurring cysteine 102. The measurements were taken for a range of temperatures. In order to focus on the spin label motion itself, the unwanted rotary diffusion of the macromolecule was reduced either by increasing solution viscosity or binding the protein to the gel. Rotational motions of the nitroxide within the few nanoseconds range are expected, thus the slow motional theory of EPR spectrum must be applied to interpret the results.

MATERIALS AND METHODS

The spin labeling of iso-1-cytochrome *c* from yeast was performed as described in (Pyka *et al*. 1999).

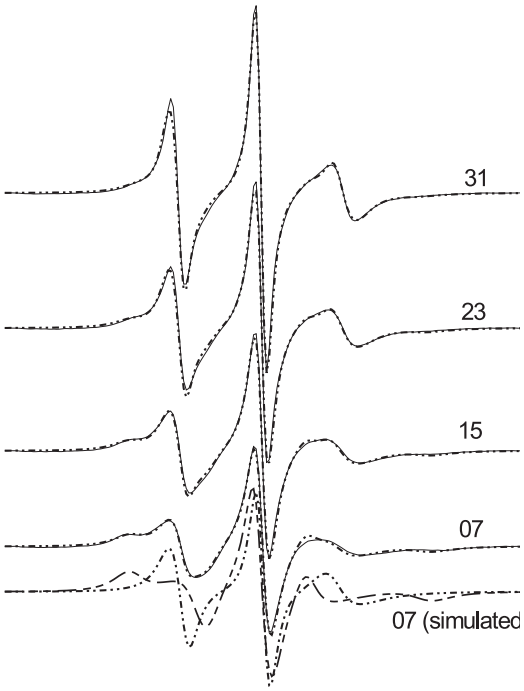

Fig.1. X-band EPR spectra of MTSL+iso-cytochrome *c* (spin label attached at C102) in a 32% sucrose solution recorded at +7, 15, 23 and 31°C. The experimental (solid lines) and simulated (dashed) to MOMD model traces are shown. All fits (performed with NLSL program) resolved the presence of 2 components with different mobility. The lowest trace presents these individual components of the simulated EPR spectrum (at +7°C) as an example.

The samples were prepared as follows: 68 mg of 0.2 mM solution of spin-labeled cytochrome *c* in 50 mM sodium phosphate buffer (pH 7.4) was mixed with 32 mg of sucrose (to obtain 32% w/w sucrose). Alternatively, stronger immobilization was achieved by bounding the labeled cytochrome *c* to CM Cellulose: approx. 1 mL gel solution was mixed with 0.2mL of 0.1 mM cytochrome c., in 12.5 mM sodium phosphate buffer, pH 7.4. Samples were loaded into glass tubes. Measurements were performed in air atmosphere.

The temperature dependent EPR spectra of the labeled protein in sucrose were recorded on a Bruker ELEXSYS spectrometer equipped with ER4122SHQ cavity and nitrogen flow temperature-controller (at National Biomedical ESR Center in Milwaukee, USA). The spectra of spin-labeled cytochrome *c* bounded to CM Cellulose were recorded on a locally constructed X-band spectrometer equipped with loop-gap LG-PRO resonator (Ilinicki, Kozioł, Oleś, Kostrzewa, Galiński, Gurbiel & Froncisz, 1995, Piasecki, Froncisz & Hubbell, 1998).

Analysis of experimental X-band EPR spectra was carried out employing simulation of the EPR spectrum (based on the stochastic Liouville equation) and least- squares fitting procedure. Program NLSL (Budil, Lee, Saxena & Freed, 1996) was used. Dynamics of the spin label was modeled as a constrained diffusion around the internal rotation axis bound to the protein molecule, and assuming an isotropic distribution of these axes' orientations ("microscopic ordering with macroscopic disordering"-MOMD model). The values of the **A** and **g** tensor components for MTSL attached to the cytochrome *c* at C102 were taken from (Qu, Vaughn, Sienkiewicz, Scholes & Fetrow, 1997). The parameters varied in simulations were: diffusion tensor elements (axial symmetry assumed); coefficients of ordering potentials; the tilt angle orienting diffusion tensor with respect to the molecular frame of the nitroxide.

RESULTS AND DISCUSSION

A minimum least-squares fit to experimental data has provided optimum values for rotational diffusion rates and ordering potential coefficients for a whole range of temperatures. Two kinds of samples (differing by the method of reduction of protein rotary diffusion) were analyzed. The presence of two dynamic components has been resolved in each spectrum of MTSL-protein molecule in viscous aqueous sucrose solution and in case of MTSL-protein molecules bound to gel. For the whole series of experimental spectra the unique set of structural parameters – coefficients defining the restoring potential and the tilt angle β_D - has been established. The main difference between spectra, as temperature is increased, is accounted for the change of the average rotational diffusion rate $\overline{R}$. Two components, designated by I and II, contributing to the overall EPR spectrum are characterized by different mobility ($\overline{R} = \overline{R}(T)$ and $\overline{R}_1 < \overline{R}_2$) and distinct coefficients of ordering potential found as follows:

$$c_{20}(1) = 2.2,\ c_{22}(1) = 0.54,\ c_{20}(2) = 0.4,\ c_{22}(2) = 0\,.$$

The tilt angle was found to be equal 17°. The series of the recorded spectra and theirs satisfactory fits are presented in Fig.1. Fraction of the slower component was estimated as 70% (at lowest temperature +4°C) decreasing to 50% (above 30 °C) for labeled protein in sucrose; and as 60% for protein bound to gel. The greater value of ordering potential coefficient $c_{20}(1)$ indicates the more con-

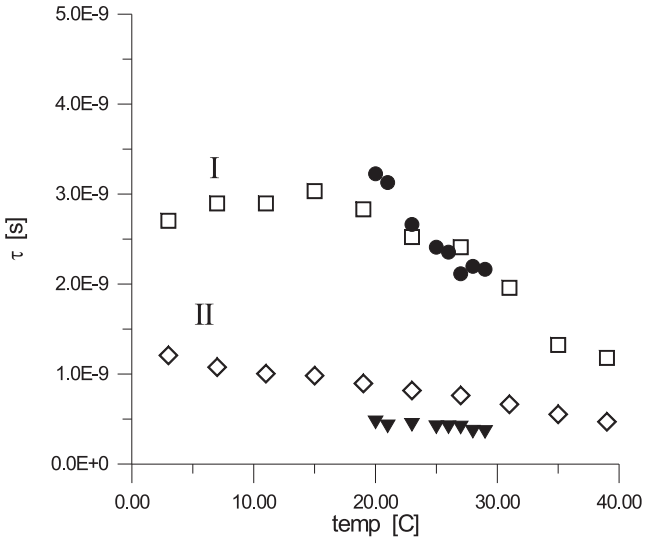

Fig.2. The whole EPR spectrum of the MTSL+cyochrome *c* complex is the sum of two components characterized by the mean correlation time τ. The values of τ for the component I and II are calculated from „the best-fit" parameters generated during simulation and they vary with temperature. The „open" symbols represent data obtained for the labeled protein in a sucrose solution. Values obtained for the protein immobilized on gel are marked with the 'solid" symbols.

strained motion of the population giving rise to I (slower) component in the EPR spectrum. Additionally, nonzero value of $c_{22}(1)$ points to the orienting potential asymmetry about the internal ordering axis. In contrast, the faster component is characterized by low order parameter and $c_{22}(2)=0$.

The mean correlation times (calculated from the formula: $\tau = 1/(6\overline{R})$) for both components are compared in Fig.2. Within the error, data for slower component yield the same rates of motion for both kinds of samples. The values characterizing the faster motion, extracted from the spectra of immobilized macromolecules, are systematically lower than data obtained for protein in sucrose. This discrepancy can be explained by the insufficient reduction of cytochrome *c* (rather small protein) tumbling in sucrose solution. It results in observing the intermediate motional rate, with contribution from nitroxide's internal rotation and slow tumbling. Effective τ got from MOMD model is longer than the true value characterizing fast motion of the spin label "alone". Elucidation of the origin of two different "dynamic populations" of spin label placed at C102 of cytochrome *c* based on Molecular Dynamics simulation (Murzyn, Róg, Blicharski, Dutka, Pyka, Szytuła & Froncisz, 2004) concerns the effect of conformation of the torsion angle about S-S bond in the nitroxide side chain. Consistent results obtained for the MTSL-protein molecule bound to the gel can be very helpful in studies of interaction between the cytochrome c and complex bc_1. The proposed "prototype" system corresponds to the situation which occurs when the cytochrome c_2 binds to the bc_1 complex. The mobility of the c_2 slows down because of binding to a relatively bigger molecule of the bc_1 complex. For such a situation a new model for further CW EPR spectra simulation describing probed system was successfully designed. The investigations of that interaction using SDSL and EPR spectroscopy are planned in our further studies.

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