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Nitroxides in the solution of some problems of chemical biophysics

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Abstract - The efficiency of the nitroxide radicals (NR) unravelle biophysical problem increases markedly by use of combine approach including: 1. Measuring of relaxation characteristics of the ESR signals of NR; 2. Study of NR effect on exited triplet stated of chromophores; 3. High resolution (2 mm band) ESR technique; 4. Use of methods fluorescent, triplet, mossbauer and radicals pairs labeling.

Results of study of molecular dynamics in wide range of correlation time values, protein molecules conductivity for spin exchange exxects, long-distance electron transfer, structures of a number complex enzymatic systems (nitrogenase, reaction center of photosynthetic bacteria and cytochrome P-450, etc.) have been reported.

Some new conceptions which have been introduced in connection with these results, namely, multi-electron mechanisms of redox processes, chemical mechanism of energetic coupling, cascade scheme of photoseparation at photosynthesis, role molecular dynamics, exchange phenomena and complexation in electron transfer have been discussed.

INTRODUCTION

The many structural and dynamical problems in molecular biology and biophysics can be solved by using methods based on the introduction of various labels into biological systems. The approach is widely used in many biophysical and biochemical laboratories. One of the most employed methods appeares to be the spin-labeling technique. Nitroxide radicals (NR) as spin-labels present a number of unique features which enable them to serve as probes for molecular dynamics, microstructure and micropolarity of surroundings, as indicators of spin exchange and dipole interaction, and as redox reagents. The achievments and limitations in this field in 1960^{th}–1970^{th} were generalized in a number monographs and reviews /1-10/.

The traditional versions of the spin-label method have a number of inherent limitations. For instance these method can record only comparatively fast diffusion process with characteristic times 10^{-7}–10^{-8}s and distaces between the paramagnetic centers no more then 3,0 nm. In some cases large concentrations of labels (up to 10^{-2}–10^{-3}M) have to be introduced into the systems under study, which can violate their structural and functional integrity. In the last few years several approaches have been proposed and developed, and the efficiency of the NR application as spin labels has been increased /5-7/. Some of these methods utilize phenomena with large charac-

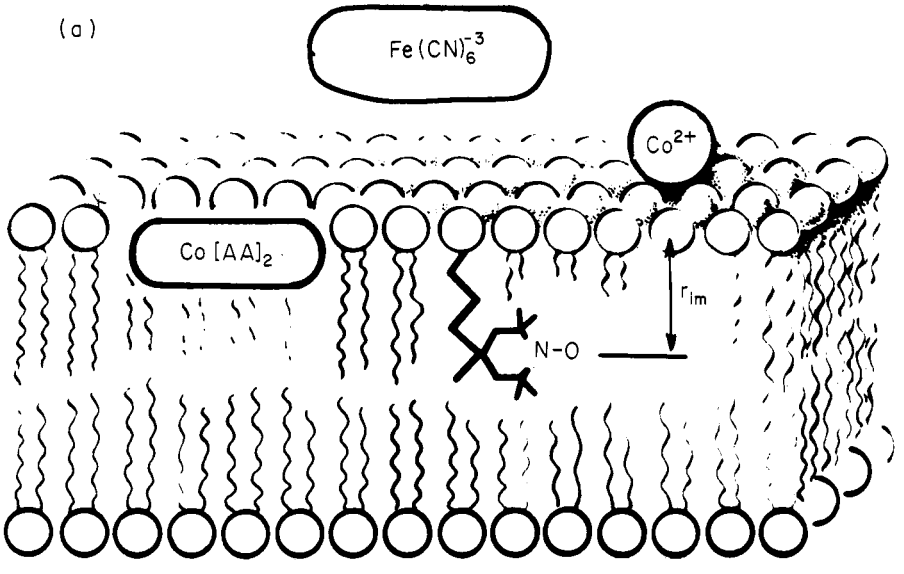
teristic times (10^{-5}-10^{1} s): the spin-lattice relaxation of the NR and quenching of exited triplet molecules by the NR. Another approach is based on parallel use of fluorescent, triplet, mossbauer, radical pairs, redox and electron-scattering labels /5,6/.

The present paper provides a short review of the fundamentals and of main results obtained by new versions of the spin labeling methods in the Laboratory for the Kinetics of Enzyme Action.

THEORETICAL AND EXPERIMENTAL GROUNDS

In the past, the spin-labeling method was used only to observe the EPR lineshape of nitroxyls in the absence of saturation. Under these conditions the spin-spin relaxation time T_2 is the main factor defining the lineshape. The measurement of the spin-lattice relaxation time T_1 of the NR extends the potential of the method. The principal advantages of measuring T_1 is that the quantity T_1 is sensitive to much weaker dipole-dipole and exchange interactions then the quantity T_2 /5-7/. To determine the value of T_1 one may use either pulse radiospectrometers or methods of continous saturation (CS) of ESR spectra /7,8/. Several versions of the CS method application have been developed /5/: 1. Measurement of the distance between NR and other paramagnetic centers (PC) up to 6 nm. 2. Determination of location and the immersion depth of the NR in biological matrix up to 4 nm. 3. Estimation of T_1 values for the PC with fast spin-lattice relaxation ($T_1 \approx 10^{-8}$s). 4. Determination of the spin dynamic exchange of the NR and PC in solution with exchange frequency $\sim 10^{5} s^{-1}$. The procedure of T_1 and T_2 values determination is described in /8/. Data on dependence of T_1 for ferrihemoglobin on temperature has been reported in /9/.

The method of determination of radical location and immersion depth (r_{im}) is based on an analysis of the influence of paramagnetics on the saturation curves of the radicals ESR spectra. Fig. 1 illustrates dependences of spin-lattice relaxation rate of the NR in biomembrane on concentration of PC distributed uniformly in a glassy matrix (ferricyanide ions), on surface (Co^{2+}) and in superficial part (acetylaceton), $Co(AA)_2$ of the membrane /5,11/.



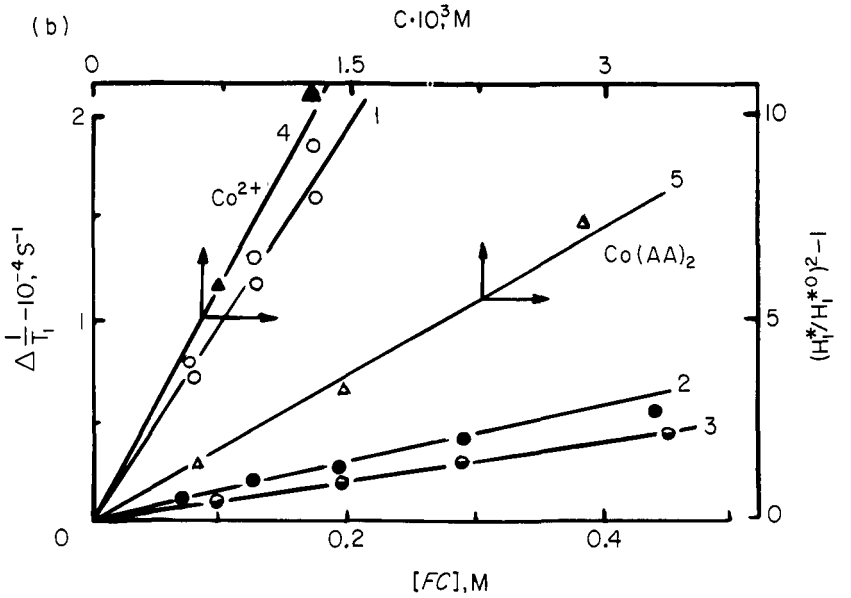


Fig.1. Determination of location and depth of immersion (Γ_{im}) nitroxide probe in biological membrane. a) diagram illustrating the principle of the method. b) dependences of the dipole contribution $(1/T_1)$ for probes with different Γ_{im_2} values on concentration of the PC; PC is the ferricyanide ion, (1-3), Co^{2+} (4) and $Co(AA)_2$ (5); 1-3 relate to membrane of chromatophores from R. rubrum, 4-5 to membrane of M. capsulatus /5,11/. On the right - orbituary units.

From these dependences the distances between the radicals and appropriate places of the PC location calculated according a theory developed in /11/. The distances are in agreement with those predicted on the basis of their chemical formulae.

The lifetimes of phosphorescence τ_{ph} are within a range of 10^{-5} to 10^{1}s. Exited triplet states can be quenched upon interaction with other molecules by the mechanism of triplet-triplet exchange - resonance energy transfer, reversible charge transfer or by catalysis of intercombination transfer by paramagnetics. These essential features of triplet labels extend by 2-8 orders of magnitude the boundaries of investigations of very weak dynamic and static exchange interaction with the phosphorescence quenches including nitroxyl radicals. Dynamic spin exchange effects in very viscous medium or in systems with low concentration of the labels (10^{-7}-10^{-8}M), long distance exchange interaction up to 14-16 Å have been proved to be studied quantitatively by this technique /5/. Figer 2 shows a principal scheme and some results of study of the triplet and spin labels location and translational diffusion in a membrane.

The available data on dependence parameters of spin-exchange on distances between the exchanging centers (NR, ions, triplet molecules) are presented in Fig. 3. All data are grouped on two lines: line 1 for the systems with nonconducting media (saturated bond) and line 2 for the systems in which the exchanging centers are connected by a conducting bridge (conjugate bonds). Both lines are discribed approximately by the formula:

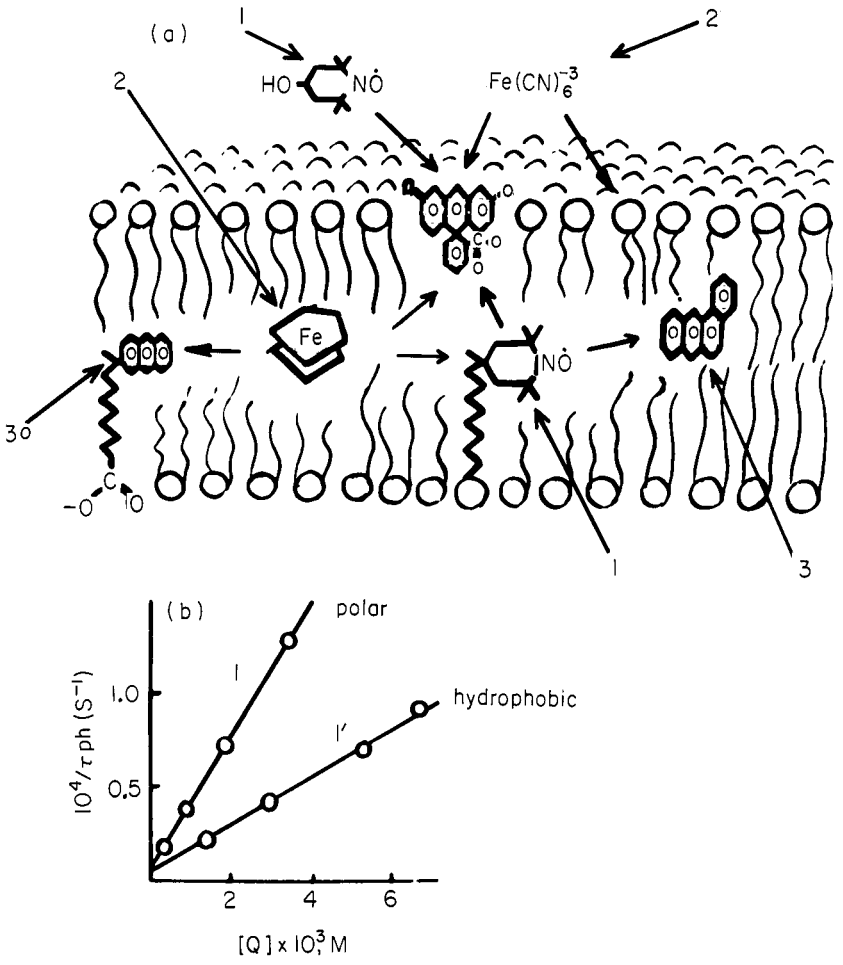


Fig.2. Study of location and translational diffusion of triplet and spin probes in membranes. a) Diagram illustrating the principle of the method. b) Dependences of rate of quenching of eosin phosphorescence on concentration spin probes 1 and 1' in lyposomes /12/.

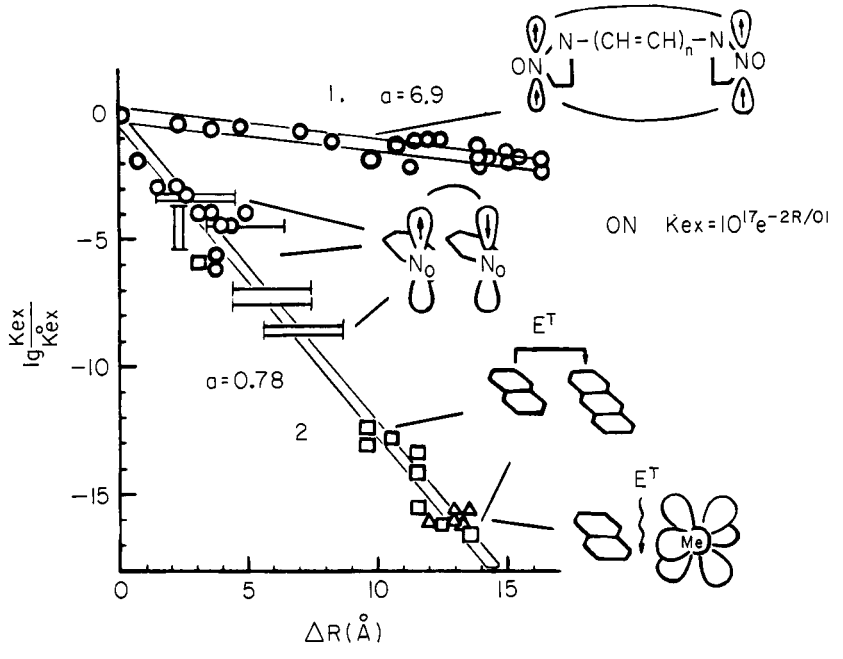


Fig.3. Dependences of spin exchange rate constants of the distance between exchanging centers. 1 - conducting medium; 2 - nonconducting medium /5/.

$$K_e = 10^{17-R/L}\ s^{-1} \qquad (4),$$

where L = 0,9 Å for the nonconducting medium and L = 7,5 Å for conducting one. These data can be used for estimation of the distance between centers and for study of conductivity of systems with the known distances /5,6/.

STRUCTURE? DYNAMICS AND ACTIVITY OF BIOSYSTEMS

The central enzyme in the biological fixation of molecular nitrogen, nitrogenase, catalyses the reduction of N_2 to NH_3, which is coupled to ATP hydrolysis /6,13/. The enzyme consist of two protein components, Fe-protein (4 Fe atoms) and MoFe-protein (2 Mo, 30 Fe). The FeMo-protein includes two molecules of FeMo-cofactor.

To determine the mutual arrangement of metal atoms, the ATP center and the location of these centers on the enzyme macromolecules, the methods of spin labels in combination with other methods was applied (Fig. 4) /4-6,13/.

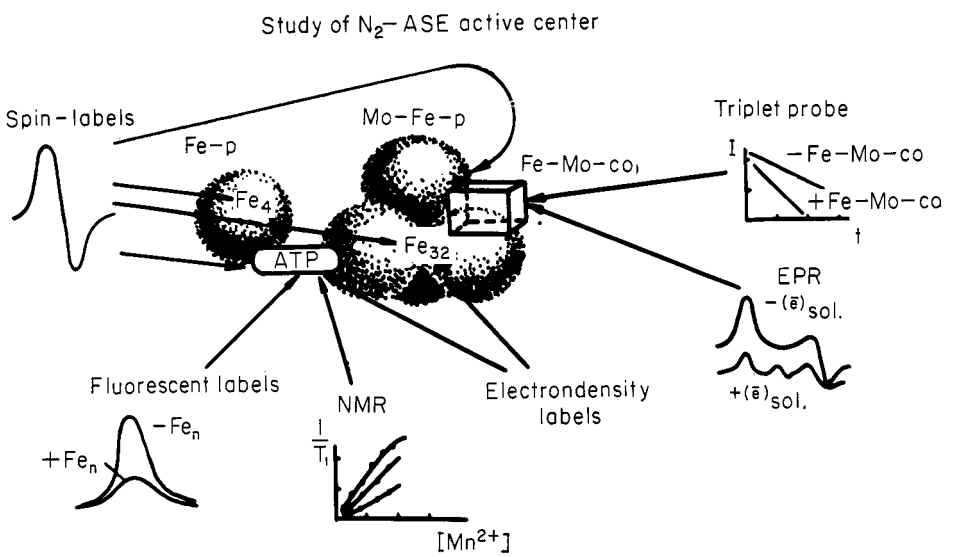


Fig.4. Principal scheme of the nitrogenase structural study by physical labeling methods /5, 6,13/.

It was concluded that the active center of nitrogenase involves an ansemble of Fe_4S_4 clusters and FeMo-cofactor arranged in close proximity to each other and to the ATP center. According to data on FeMo-preparation in which Fe_4S_4 or FeMo clusters were specifically removed two FeMo-cofactor molecules locate near the surface of the protein globule in close vicinity to each other and to Fe_4S_4 cluster, and are bound to an apoenzyme by SH-groups /14/. This model is closely related to the recent X-ray diffraction model of the enzyme. Above mentioned structural information has made a basis for formulation of conceptions of multi-electron mechanism of dinitrogen reaction to a hydrazine derivative and chemical mechanism of coupling ATP hydrolysis and electron transfer in the nitrogenase. The main point of the latter mechanism is the compulsary protonation of the iron clusters effected by the driving forces of ATP hydrolysis energy.

Works /5,6,11,18/ illustrate approach to study of heme location in cytochromes P-450 and b_5 in mikrosomal membranes. The heme group were attacked by whole collection of probes including spin-labeled lysozyme in solution,

ferricyanide in rigid glicerol - aquous phase, Co^{2+} on surface and $Co(AA)_2$ in superficial part of the membrane /11/. Proceeding from the effects of the probes on the saturation curves of the ferrocytochrome P-450 EPR spectrum, the depth of the Fe^{2+} immersion both in the aquous phase (~1,4 nm) and the lipid phase (~1,0 nm) was estimated.

Method of spin and triplet probes it possible to establish localization of the ATP center of Ca^{2+} ATPase and for probing of the photoactive chromophore hidden on hydrophobic packets of human serum albumen (HSA) /15,16/The experimental results on the efficiency of energy transfer from triplet-exited chromophores in protein (tryptophan residues, phosphorescent labels) to acceptor groups (spin labels, heme groups) indicate that the parameters of spin exchange in all cases 5-13 orders magnitude lower than those expected for conducting media (Fig. 3), and the correspond closely to non-conducting media /5,17/ (Fig.3). Thus the protein do not exhibit well-defined anomalous electronic conductivity.

The effects of spin exchange between heme containing proteins (spin-labeled lysozyme) in solution and triplet labeled myoglobin have been studied to estimate the values of geometric factor (fg) /5,18/. The fg value of K_e for cytochrome c was used to estimate other factors that determine the rate of the electron transfer between oxidized and reduced forms of the protein. The ET is characterized by substantial non-adiabaticity ($\varkappa \sim 10^{-6}$) and a relatively slight reorganization of the medium. Such a combination of properties seem to be optimal for the rate of specific processes /5,6,7/.

The introduction of spin, fluorescent, triplet and mossbauer labels and probes, and also of radical pairs, into protein molecule enables to record the dynamics of different domains with correlation times over the entire range of $\tau_c = 10^{2}$-10^{-10}s /5,6/. Several studies have been devoted to the effect of temperature and humidity on the dynamic state, and functional activity of proteins and enzymes (myoglobin, α-chymotrypsin, reaction centers of photosynthetic bacteria, HSA, etc.) /5,6,19-23/.

The suitable model for study of role of molecular dynamics in electron transfer appears to be molecules containing the NR-fragment and a photoactive chromophor group as well /22/. In this system the rates of reversible and unreversible photoinduced electron transfer can be measured by recording of quenching of the chromophor fluorescence and by the NR ESR signal disappearance, respectively. The dynamic state of medium(mobility of the NR and the dipole relaxation of solvent) can be monitored by appropriate ESR and fluorescent technique. Another suitable system is spin labeled proteins, say, α-chymotrypsin in which NR-fragment serves as an acceptor and a label as well, and tryptophane group is a photoactive donor and a fluorescent label /23/.

CONCLUSIONS

The present review considers some advantages and results of approaches based on the phenomena with comparatively long characteristic times (10^{-5}-10 s) (spin-lattice relaxation of nitroxyl radicals, quenching of exited triplet states, radicals pairs recombination). Systematic application of such approaches allowes to solve a number structural and dynamic problems.

These data promoted, in turn, to come to following conclusions: 1. The hard energetics in redox reactions can be overcome by multi-electron mechanisms with the participation of transition metal clusters. 2. The best method for light energy conversion by charge separation is use of the cascade system of donor and acceptor centers at optimum distances. 3. Redox and hydrolysis reaction coupling can be realized through the formation of a H^+-intermediate. 4. Specificity of electron transfer reaction is determined by an optimum penetration of the active center in the protein globule and by the formation of a label complex. 5. The realization of a number of enzymatic processes requires reversible intramolecular equilibrium dynamics of the protein globule in the nanosecond range of correlation time.

It is hoped that the farther application nitroxide radicals in combination with other approaches will continue to make valuable contribution to the solution of elaborate and fascinating problems of chemical biophysics.

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