MOBILITY OF PROTEIN MOLECULES IN *MICROCOCCUS LYSODEIKTICUS* MEMBRANES

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1. Introduction

Biological membranes are considered now as somewhat dynamic structures [17]. On the basis of some chromatographic data [15] it seemed to us that the lipids in the bacterial membrane lipoprotein enzyme complexes could be in a state of lateral movement (diffusion). Later on such a diffusion was detected and measured in a number of membrane preparations [9, 12, 16].

The lipid component of a membrane was shown to move not only along the membrane, but also from the outside to inside surface of the membrane in a flip-flop manner [10]. As far as the protein component is concerned, a rapid motion of rhodopsin in photoreceptor membrane [1, 3] and of cytochrome-oxidase in mitochondrial inner membrane [7] was detected with fluorescence polarization. Lateral diffusion of proteins could also be inferred from observations of redistribution of fluorescent antibody to cell surface antigen [4] and from some other experiments [2, 14, 18]. However one could visualize a possibility of protein molecules rotating along an axis parallel to the membrane surface. To assess this possibility we made use of spin labelling of protein molecules. The kinetics of reduction of the label by some agent inside or outside the membrane might shed some light on this type of motion. This paper presents some evidence for such a diffusion of spin-labelled protein molecules in bacterial membranes when a certain site on the protein surface spends some time in the membrane phase and some time in the external medium, the relaxation time being close to 15 min at 24°C.

2. Methods

Membranes of *Micrococcus lysodeikticus* 2665 were isolated from logarithmically growing cells and labelled with 4-(β-*N*-ethylenimino-propionyl)-oxy-2,2,6,6 tetramethylpiperidine-1-oxyl as described elsewhere [8]. Membrane suspension in a pH 6 phosphate buffer was stirred for 12 hr at 4°C with 5×10^{-3} M label, then washed. A suspension of labelled membranes in 0.01 M phosphate, pH 7.4, buffer containing 30–40 mg protein per ml and 0.001 M $MgSO_4$ was placed into a tube 1.5 mm in diameter (total sample volume ~ 10 μl, ~ 10^{15} spins per sample) and its ESR spectrum was recorded at 24±0.25°C. The measurements were carried out on an ESR spectrometer model RE 1301. Rotatory diffusion correlation time (τ_c) was calculated as described by Wasserman [20].

Protein determination [11] and vitamin MQ-9 photoinactivation [5] was performed as described. For protein cross-linking a membrane suspension was incubated in a neutral buffer for 1.5 hr at room temperature with 5% glutaraldehyde.

3. Results

ESR spectrum of spin label attached to membrane proteins reflects the heterogeneity of the label environment (fig. 1a).

When we treat the membrane suspension with 0.1 M $K_3Fe(CN)_6$, which is believed not to penetrate the hydrophobic phase and which subsequently quenches the ESR-signals from the surface localized radicals only, the spectrum becomes simple and there-

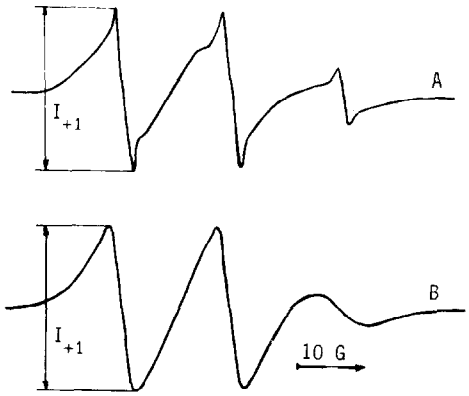

Fig. 1. ESR spectra of spin-labelled *M. lysodeikticus* membranes (A) before, and (B) after the addition of 0.1 M $K_3Fe(CN)_6$.

fore calculable (fig. 1b). Correlation time (τ_c) calculated for the ferricyanide inaccessible portion of the label is 3×10^{-9} sec. To trace the behavior of this supposedly membrane-imbedded label we abolished the paramagnetism of the surface-localized portion by reduction with ascorbate according to Kornberg and McConnall [10] at 0°C when Brownian motion of membrane components is restricted. After 10 min incubation with 2×10^{-3} M ascorbate, membranes were washed with cold buffer and transferred into the ESR measuring unit at 24°C.

The spectrum at first indistinguishable from that in fig. 1b ($\tau_c = 2.7\times10^{-9}$ sec) gradually acquires its original complex form (fig. 1a) corresponding to the dual localization of the label. τ_c Calculated for this changing spectrum and denoted as τ_c^{eff} is not an accurate parameter of the label motion but it can be used as an indicator of the label redistribution in the membrane (fig. 2a). Glutaraldehyde treatment of the labelled membranes prior to ascorbate addition prevents any changes in τ_c^{eff} (fig. 2b) as would be expected from the postulated mechanism of glutaraldehyde action in protein cross-linking [3, 7].

The observed kinetics of τ_c^{eff} may be due to spin-label movement from membrane phase towards the membrane surface. If so, the kinetics of label reduction with ascorbate should be biphasic, the first phase being due to immediate reduction of surface label and the second one due to reduction limited by the

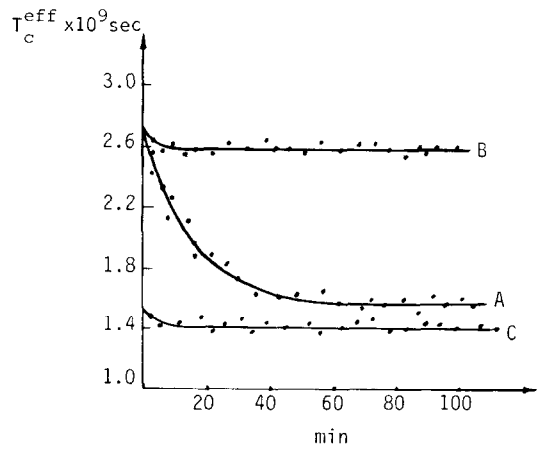

Fig. 2. The kinetics of the spin-label parameter τ_c^{eff} as a function of pretreatment of the labelled membranes: (A) Membranes incubated for 10 min at 0°C with 2×10^{-3} M ascorbate, washed with cold buffer and then transferred into the radiospectrometer kept at 24°C; (B) membranes incubated for 1.5 hr at room temperature with 5% glutaraldehyde, washed and then treated as in (A); (C) control, membranes chilled to 0°C and at 0 min transferred into measuring unit at 24°C.

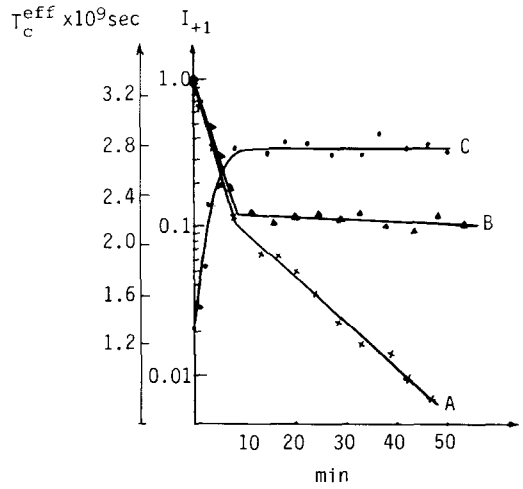

Fig. 3. The time dependence of τ_c^{eff} (C) and of $\tilde{I}_{+1}$ (the low field component of the spectra, relative units) (A, B) for the spin-label attached to *M. lysodeikticus* membranes following 0.05 M ascorbate addition. In (B), labelled membranes were pretreated with 5% glutaraldehyde for 1.5 hr and washed.

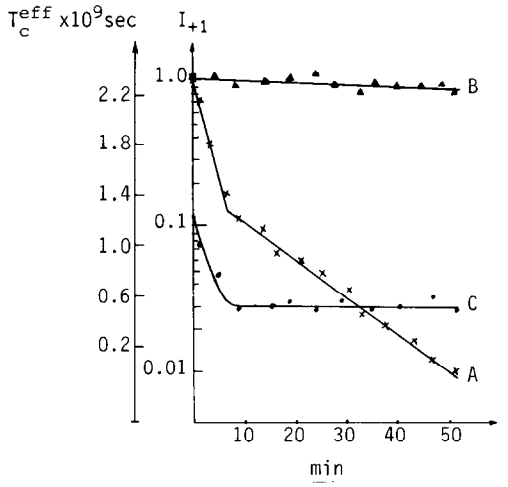

Fig. 4. The time dependence of τ_c^{eff} (C) and of $\tilde{I}_{+1}$ (relative units) (A, B) of the label attached to *M. lysodeikticus* membranes after the addition of malate (8 mM). In (B), the labelled membranes were UV-irradiated before introduction of the substrate.

motion of the protein. On this assumption the biphasic disappearance of the signal after an addition of any respiratory chain substrate is also to be anticipated, provided the motion of protein molecules is slow enough to be detected with the usual ESR-technique. Experiments of this kind are depicted in figs. 3 and 4, where the intensity of the ESR signal $\tilde{I}_{+1}$ on a log-scale and τ_c^{eff} are plotted against time. The decrease of $\tilde{I}_{+1}$ following addition of ascorbate or malate is indeed biphasic as seen from figs. 3a, 4a. Ultraviolet irradiation of membranes under the conditions of selective vitamin MQ-9 inactivation [5] prevents the changes of the signal when malate is a primary substrate. Reduced vitamin MQ-9 is the most probable secondary substrate responsible for nitroxide group reduction since malate itself was shown not to react with the label.

When the labelled membranes are treated with glutaraldehyde before ascorbate addition (fig. 3b) the decrease of $\tilde{I}_{+1}$ follows first phase kinetics and then practically ceases.

Increase of τ_c^{eff} in the case of ascorbate addition (fig. 3c) and its decrease on label reduction by respiratory chain components obviously reflects the selective reduction of the surface-localized label in the first instance and the membrane burried radicals in the second.

4. Discussion

The τ_c^{eff} kinetics and the $\tilde{I}_{+1}$ decrease of protein-attached spin-label seemed to indicate that some sites on the surface of the membrane protein alternatively are in the membrane bulk or face the external medium. Relaxation times of this process calculated from the data of figs. 1, 2, 3 are ~15 min, ~12 and ~15 min respectively. It is not possible yet to describe this kind of protein motion in detail as several variants must be considered as equally probable (fig. 5), one of them being rotation along the axis parallel to the membrane surface (fig. 5, 1).

The values of relaxation time of a protein rotatory diffusion in solution is reportedly 2×10^{-8} sec [19], while rotatory diffusion of rhodopsin in photoreceptor membrane along the axis normal to the membrane surface is a thousand times slower [3]. It is no wonder that the motion across the phase boundary is still more hindered though not so severely as a flip-flop transmembrane diffusion of phospholipids [10].

Let us now consider what kind of biochemical processes would need these types of motion. Certainly membrane dehydrogenases which accept hydrogen from water-soluble substrates and then donate it to some lipophylic intermediate are in need of such a diffusion. There was also a suggestion of a rotatory

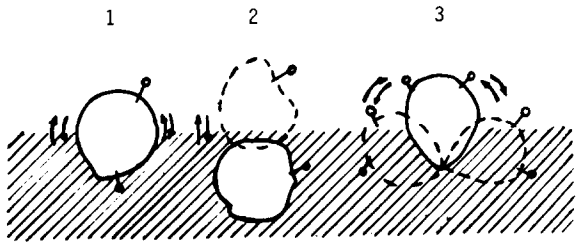

Fig. 5. Various kinds of protein motions in the membrane when a protein molecule (or part of it) exists periodically in the inner membrane phase or faces the membrane environment. (1) Rotation along an axis parallel to the membrane surface. (2) Translational diffusion in the direction normal to the membrane surface. (3) Vibration around a site on a protein molecule.

movement for the ATP-synthesizing machinery [6]. This machinery could be ATPase with its capabilities for structural transitions [13].

The electron transport in the bacterial membrane is however a hundred times faster than the reported diffusion of proteins, but it is not impossible that the relaxation time of respiratory chain components may significantly differ from the averaged data presented. We also think that when in the cell, membrane proteins interacting with natural substrates may undergo conformational changes which facilitate their diffusion. Finally, this type of protein motion may serve as an important instrument of protein rearrangement during the self assembly of membranes in bacterial cells.

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