EFFECT OF MEMBRANE POTENTIAL ON THE CONFORMATION OF BACTERIORHODOPSIN RECONSTITUTED IN LIPID VESICLES

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ABSTRACT The effect of applied diffusion potential on circular dichroism (CD) of bacteriorhodopsin, reconstituted in lipid vesicles, was measured. The change in CD indicates that the applied electrical field, irrespective of its direction, decreases the α-helical fraction and increases the random fraction of the protein. The results are interpreted by unfolding of edges of the helices, upon their submerging into polar environment when the lipid bilayer is electrostricted or (and) the helices are stretched by the electrical field across the membrane.

INTRODUCTION

Circular dichroism (CD) spectra of reconstituted bacteriorhodopsin-lipid systems were measured before (1) and they do not differ substantially from the original spectra of purple membrane fragments except for the reduction of the scattering and flattening artifacts. On the other hand, structural changes in the α helix of bacteriorhodopsin were observed upon its reconstitution in lipid vesicles, using infrared techniques (2, 3). The effect of the bacteriorhodopsin molecules on the cooperativity and enthalpy of melting of different phospholipids were measured by calorimetric methods (4).

Electric field effects on membrane proteins, such as the effects of external field on purple membrane fragments orientation (5) and on the linear dichroism of bacteriorhodopsin in oriented membrane fragments were also investigated (6). Conformational changes of bacteriorhodopsin in purple membranes induced by the external electric field were investigated by Tsuji et al. (7, 8). As far as we know, there are no reported data on the effect of the diffusion potential on the structure of membrane proteins, even though it was found to affect the fluidity of the lipid model membranes (9).

Here we present some results concerning the modifications of the far ultraviolet (UV) CD spectra of bacteriorhodopsin reconstituted in phospholipid vesicles under a diffusion potential induced by an ionic gradient across the vesicle membrane.

MATERIALS AND METHODS

Purple membrane fragments were obtained from Halobacterium Halobium bacteria, cultured according to Becher and Cassim (10) and then kept in the deep freezer.

The purification of membrane fragments was done according to the method of Becher and Cassim (10). The final absorbtion and CD spectra of the purified fragments were in good agreement with previous results (11–13), indicating a high degree of purity. The equivalent protein concentration of the stock suspension of purple membrane fragments was 1.8 mg/ml determined from absorbtion data with an extinction coefficient corrected for scattering of 63,000 cm^{-1} mol^{-1} at 568 nm (14) and a molecular weight of 26,000 dalton (15).

The purple membrane fragments were used to form reconstituted vesicles with a mixture of phospholipids containing 80% egg phosphatidylcholine and 20% bovine phosphatidylserine (Lipid Products, Nuttfield, England) The lipids in chloroform methanol solutions were mixed to the desired composition, dried in a stream of nitrogen, liophylised for 3 h, and then vortexed in the buffer solution at a total concentration of 5 mg/ml. The lipid suspension was then sonicated in a bath sonicator (Laboratory Supplies Co.) until clear.

The pure phospholipid dispersion was then mixed with purple membrane fragments to a final lipid concentration of 0.4 mg/ml, and a protein concentration of ~0.045 mg/ml (a molar ratio of ~300 lipids per bacteriorhodopsin). The final mixture was frozen in liquid nitrogen, thawed, and large unilamelar vesicles (of ~0.12 μm to 0.3 μm) were obtained after a final short (~1 min) sonication. The vesicle suspension was not affected by centrifugation at speeds of which purple membrane fragments tend to precipitate.

The absorbance of bacteriorhodopsin at the experimental concentrations (40–50 μg/ml) varied between 0.6 and 0.8 in a 1-mm thick cell at 198 nm, which allowed a correct CD measurement down to this wavelength.

Typical buffers were 2 or 20 mM phosphate (K_2HPO_4) buffers with 0.3 M K_2SO_4 (which will be called K buffer) or 0.3 M Na_2SO_4 (which will be called Na buffer). Some vesicles were prepared with K buffer (having inside 0.3 M K_2SO_4) and some with Na buffer (having inside 0.3 M Na_2SO_4). From each kind of vesicles, half was dialysed against K buffer for 24 h at 4°C and half against Na buffer in the same conditions. Finally we obtained vesicle suspensions with: high K^+ inside and high Na^+ concentration outside, high Na^+ inside and high K^+ outside and control suspensions having equal concentrations inside and outside either high Na^+ or high K^+. The diffusion potential was obtained by adding valinomycin (10^{-7}M) to the both samples. Also in the controls without ion gradient the CD was measured in the presence of 10^{-7}M valinomycin. The diffusion potential was determined by addition of 10 μM of a potential sensitive fluorescent dye, 3-3'-diethylthiodicarbocyanine iodide -dis-C_2-[5]- (Molecular Probes, Inc., Eugene, OR) (16). Since this dye is

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sensitive only to negative potentials inside, only diffusion potentials of vesicles with high K^+ concentration inside and high Na^+ outside were measured (at the final lipid concentrations of 0.4 mg/ml). When valinomycin was added to the vesicles diluted in the dye containing external buffer, the fluorescence was quenched. The degree of quenching served as a measure of the membrane potential. The fluorescence quenching in pure lipid vesicles without bacteriorhodopsin provided us with a calibration curve assuming absolute selectivity and Nernst potential with respect to K^+. The fluorescence measurements were carried out on an LS-5 luminescence spectrophotometer (Perkin-Elmer Corp., Instrument Div., Norwalk, CT).

The CD spectra were obtained with a J-500 spectropolarimeter (Jasco Inc., Easton, MD) with a 1-mm pathlength cylindrical cuvette for UV range and a 1-cm one for the visible range.

RESULTS

Our visible CD spectra show a nonconservative positive peak (near 540 nm) indicating that the bacteriorhodospsin is dissociated into monomers (17). This signifies uniform dilution of the added bacteriorhodopsin in the lipid vesicles.

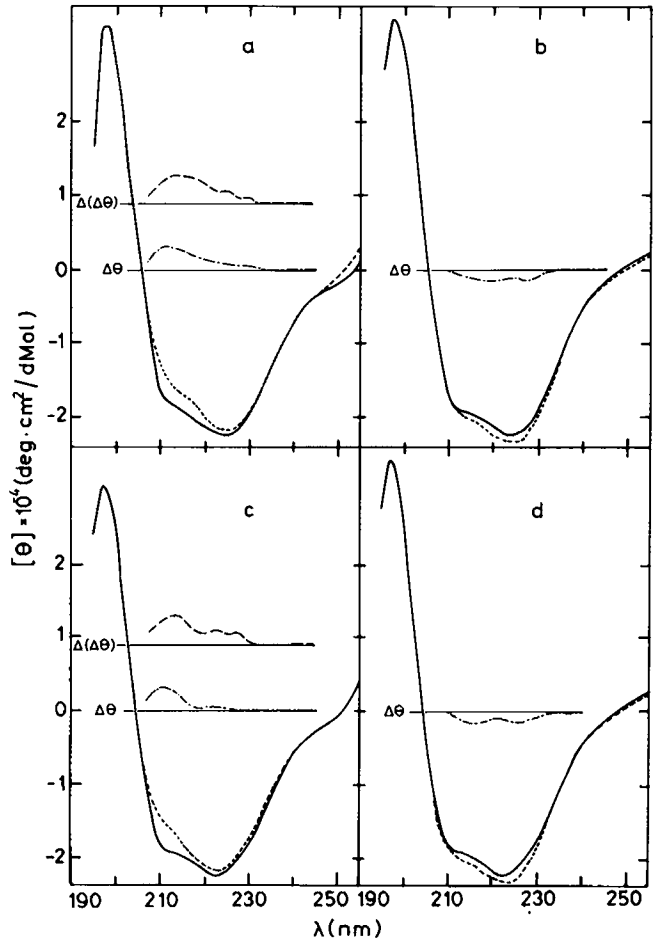

FIGURE 1 CD spectrum of bacteriorhodopsin reconstituted vesicles in the far UV region. Lipid:protein weight ratio is ~10:1. (*a*) Inside 0.6 M K^+ ions and 2 mM Na^+ ions, outside 0.6 M Na^+ ions and 2 mM K^+ ions; (*b*) 0.6 M K^+ ions outside and inside; (*c*) inside 0.6 M Na^+ ions and 2 mM K^+ ions, outside 0.6 m K^+ ions and 2 mM Na^+ ions; (*d*) 0.6 M Na^+ ions outside and inside. *Full line:* without valinomycin; *dotted line* with valinomycin. Curve $\Delta\theta$—differences between valinomycin and valinomycin free spectra. Curve $\Delta(\Delta\theta)$—difference between the difference spectra in *a* and *b*, respectively in *c* and *d*, showing the total field effect. The combined error was <500 deg · cm^2/d mol at 210 nm.

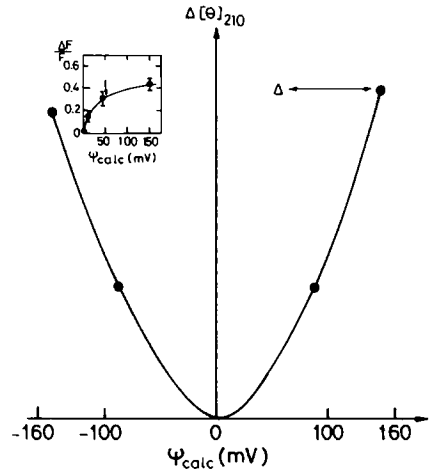

FIGURE 2 The dependence of the elipticity of bacteriorhodopsin reconstituted in vesicles on the electric diffusion potentials. Ordinate: the decrease of the C.D. signal at 210 nm induced by 10^{-7}M valinomycin. Abscissa: electrical potentials calculated by Nernst equation. The sign indicates the polarity inside the vesicle. The triangle is set at the potential measured by fluorescence quenching of DiS-C_2-[5] instead of the potential calculated by the Nernst equation (146 mV) as indicated in the figure. (*Insert*) calibration curve of the fluorescence quenching of Dis-C_2[5] dye as a function of the Nernst potential in pure lipid (80% PC 20% PS) vesicles in the presence of K^+ gradient and 10^{-7} valinomycin. The potential for the bacteriorhodopsin containing vesicles is indicated with an arrow.

Fig. 1 shows the effect of the valinomycin on the far UV CD spectra of the bacteriorhodopsin in lipid vesicles. In the presence of a potential gradient, irrespective of direction (with high K^+ inside and high Na^+ outside or with high Na^+ inside and high K^+ outside), the CD signal at ~210 nm is smaller in the presence of valinomycin than in its absence. The contribution of valinomycin at (10^{-7}M) on the CD spectra is small and is taken into account by deducting the change in the CD spectra of the controls with equal ionic composition on both sides of the membrane, upon addition of 10^{-7}M valinomycin.

When we prepared and dialysed the vesicles in buffers containing 20 mM K_2HPO_4 and NaH_2PO_4 instead of 2 mM, which produced smaller concentration ratios of K^+ across the membrane, the results were similar, but the effects were smaller. Fig. 2 shows the effect of the electrical potential on the CD intensity of bacteriorhodopsin reconstituted vesicles. The potentials on the abscissa are calculated assuming unity for K^+ transport number and the sign refers to the polarity of the interior of the vesicles.

The potential inferred from fluorescence quenching for K^+ vesicles with the maximal K^+_{inside}/K^+_{out} ratio is 50 mV, instead of 146 mV computed assuming Nernst potential.

DISCUSSION

It is known that valinomycin is a K^+ selective ionophore, so, its addition may create an electrical potential across a

lipid membrane, depending on concentrations of K^+ and Na^+ in each side of the membrane and on their relative permeabilities. To calculate the diffusion potential induced by valinomycin, we use the Goldman Eq. (18).

$$V_o = \frac{RT}{F} \ln \frac{\Sigma_i P_i a_i'}{\Sigma_i P_i a_i''} \approx \frac{RT}{F} \ln \frac{a_{K^+}'}{a_{K^+}''}, \quad (1)$$

where the ′ and ″ indicate the inside and the outside of the vesicle, and P_i and a_i are the permeability and the activity of the respective ion i.

For the valinomycin in phospholipid layers, $P_{Na}/P_K \cong 10^{-5}$ (18), and the permeabilities of the other ionic species are also negligible, thus, Eq. 1 reduces to the Nernst equation. We used it to compute the membrane potentials in pure lipid vesicles, taken then for calibration of the membrane potential sensitive fluorescence dye. The inset in Fig. 2 gives the calibration curve for fluorescence quenching/membrane potential relation. The main Figure gives the decrease in ellipticity at 210 nm as a function of membrane potential calculated by the Nernst equation. The triangle gives the potential value inferred from fluorescence quenching. It is only slightly more than one third of the calculated value (146 mV). The difference may be due to the leakiness of the vesicles containing bacteriorhodopsin. Either the K^+ gradient across the membrane was partly abolished during the extended dialysis, or the bacteriorhodopsin induces some permeability to Na^+, H^+, and anions which compel the use of the complete Goldman equation taking into account all the ionic activities and permeabilities. There is also a remote possibility that interaction with bacteriorhodopsin affects the fluorescence and its quenching. The dye is subject to fluorescence quenching only when the interior of the vesicle is negative (16), so we could not get any "experimental potential values" for vesicles with high Na^+ concentration inside and high K^+ outside. However, since the conditions are identical, we may assume that the measured potential would be the same with opposite polarity.

In Fig. 1 the CD spectra in the presence and in the absence of valinomycin as well as the difference spectra are presented. The corrected difference spectra obtained, by subtraction of the difference spectrum in the absence of an ion gradient (Fig. 2, *b* and *d* curves $\Delta\theta$) from the difference spectrum in the presence of ion gradient (Fig. 2, *a* and *c* curves $\Delta\theta$) are also presented (Fig. 2, *a* and *c* curves $\Delta[\Delta\theta]$). Taking into account the accumulated error the values of $\Delta(\Delta\theta)$ are reliable within 10–15% through most of the range. The decrease of the negative ellipticity between 205 and 235 mm indicates that the applied membrane potential irrespective of its direction decreases the helicity of the protein. For this evaluation, we used fitting methods employing the basis spectra for the α helix, random coils, and β forms presented either by Chen et al. (19) or by Chang et al. (20). The CD spectra of the bacteriorhodopsin reconstituted in vesicles without any diffusion potential nearly agreed with the published spectra of the protein reconstituted in DMPC (12). The spectra of bacteriorhodopsin embedded in lipid vesicles differ to some extent from those in purple membrane fragments because of the difference in the scattering and the consequent optical flattening (21, 22). Using a nonlinear least square technique with basis spectra from Chen et al. (19), we got 74% α helix and 17% random coil without any field and only 67% α helix and 26% random coil when the field, regardless of the polarity, is applied. Using the spectra from Chang et al. (20) we obtained without applied electrical field 68% α helix, 23% β sheets and β turns, and 9% random coil. Analyzing the modified spectra upon applied Nernst potential of 146 mV we obtained, irrespective of the field direction, 61% α helix, 24% random coil and 15% β turns and β structure.

The analysis of the conformation of membrane proteins based on spectra of globular proteins may not be completely justified. However, it seems to be satisfactory for inferement of the trend of the conformation changes induced by the electrical field from the change of the CD spectra.

The calculated modifications in the β structures are less reliable, since the molar elipticities of these structures are considerably lower than the helical ones. Using the same nonlinear least square method we obtained nearly same fits assuming no change in the β structure.

The calculations indicate clearly that the field induced a lower α helicity together with a corresponding increase of random coil. It is not yet certain how the membrane potential affects the protein conformation. The membrane potential acts on the lipid bilayer as well as the embedded proteins. The effect on the lipid bilayer is by electrostriction. This by itself may expose the edges of the α helices spanning the membrane to polar environment with a consequent decrease in helicity. The other effect may be on the permanent or on the induced dipoles of the helices themselves. Since the direction of the field does not matter, the effect on permanent dipoles can be neglected. Both possibilities are in keeping with the conclusions of Tsuji and Neuman that imposed electrical field transfer up to 5 tyrosine residues or up to 3 tryptophane residues from the nonpolar to the polar environment (7). The question is whether the α helices are polarizable enough to induce sufficient polarization with an appreciable subsequent stretching by the electrical field. In this case, polarizability may be considered equivalent to field dependent conformational states (7, 8). In either case the decrease in helicity is accompanied by a decrease in $\bar{n}$—the number of amino-acids in the α helix. For semi quantitative evaluation of the decrease in α helicity, we assume that the amino-acids dissociating from the edges of the α helices decrease their length uniformly and increase the random fraction. Electrical potentials of the order of 50 to 150 mV are common in biological membranes. Changes in conformation following variations in potential may regulate physiological

functions of membrane proteins in general and channels in particular. This warrants the incentive to pursue further the investigation of the electric potential—molecular structure—physiological function relation.

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