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SIDEDNESS OF MEMBRANE STRUCTURES IN *RHODOPSEUDOMONAS SPHAEROIDES*

ELECTROCHEMICAL TITRATION OF THE SPECTRUM CHANGES OF CAROTENOID IN SPHEROPLASTS, SPHEROPLAST MEMBRANE VESICLES AND CHROMATOPHORES

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SUMMARY

The shift of the carotenoid absorption spectrum induced by illumination and valinomycin-K^+ addition was investigated in membrane structures with different characteristics and opposite sidednesses isolated from *Rhodopseudomonas sphaeroides*. Right-side-out membrane structures were prepared by isotonic lysozyme-EDTA treatment of the cells (spheroplasts) and by hypotonic treatment of spheroplasts (spheroplast membrane vesicles). Inside-out membrane structures ("chromatophores") were obtained by treating spheroplast membrane vesicles by French press or sonication.

The membrane structures with either sidedness showed the same light-induced change of the "red shift" type. However, the absorbance change by K^+ addition in the presence of valinomycin in the right-side-out membrane structures were opposite to that in the inverted vesicles, "blue shift" in the former and "red shift" in the latter. The carotenoid absorbance change was linear to membrane potential, calculated from the concentration of KCl added, with a reference on the cytoplasmic side, through positive and negative ranges.

INTRODUCTION

The carotenoid spectrum change in certain photosynthetic bacteria has been shown to be associated with a high-energy state of the photosynthetic membrane [1–4]. Jackson and Crofts [5] found the shift in the carotenoid spectrum of chromatophores, similar to that observed under illumination, was induced when an inside-positive potential was generated. Using the carotenoid shift as an in situ indicator of the membrane potential the nature of energy transduction has been investigated in photosynthetic bacteria [6–8] and in chloroplasts (515 nm absorbance change) [9].

The location and orientation of molecules on the biological membranes,

sidedness of molecular organization and function of membranes have been extensively discussed. In mitochondria, intact right-side-out preparations and sonicated inside-out submitochondrial particles have been used for the investigations of the organization of membranes [10] and the coupling of vectorial translocation of protons to electron transfer [11]. In bacterial systems, asymmetry of membranes has been investigated with electron microscopy, immunochemical method, assay of membrane-bound enzymes or transport of substrate molecules [12–14].

Studies on bacterial photosynthesis have been generally carried out with chromatophores which are considered to be inverted membrane vesicles [15]. Recently, cell-free right-side-out membrane vesicles obtained by mild procedures have been used for the study of localization of a certain membrane component [16, 17] or transportation of amino acids [18].

In this work, we prepared both right-side-out and inside-out membrane vesicles, and studied the spectrum change of carotenoid as an indicator of the membrane orientation. A linear relationship between the carotenoid change and the externally applied membrane potential in a wide range was demonstrated in both types of membrane preparations.

MATERIALS AND METHODS

Rhodopseudomonas sphaeroides was grown at 30 °C in a medium described by De Klerk et al. [19] anaerobically in the light. About 10 g (wet weight) of cells were harvested from 1 l of culture at the exponential growth phase (generation time was about 5 h). The cells were washed once with cold distilled water, and used immediately. Spheroplasts were prepared according to the procedure described by Miura and Mizushima [20]. Cells were incubated in the 80 ml medium containing 20 mg of lysozyme, 25 mM Tris · HCl (pH 8.0), 0.45 M sucrose, and 1.3 mM EDTA at 30 °C for 60 min. The spheroplasts formed were collected by centrifugation ($6000 \times g$, 15 min). The buffer containing 0.5 M sucrose, 5 mM $MgCl_2$, 5 mM sodium phosphate (pH 7.4) and 100 μg deoxyribonuclease was added to the precipitate to make a 14 ml suspension. Hypotonic treatment of the spheroplasts was performed by a 15-fold dilution of the suspension with 5 mM sodium phosphate buffer (pH 7.4) containing 5 mM $MgCl_2$. The hypotonically treated spheroplasts (spheroplast membrane vesicles) were collected by centrifugation ($6000 \times g$, 20 min), washed once with 5 mM $MgCl_2$/5 mM sodium phosphate buffer (pH 7.4), and resuspended in a small volume of the same solution.

French-pressed vesicles were obtained by passing the spheroplast membrane vesicles through a French pressure cell (1000 kg/cm^2). The supernatant obtained by a centrifugation at $10\,000 \times g$ for 15 min was used for experiments. Sonicated vesicles were obtained by treatment of spheroplast membrane vesicles in the 3.5 ml $MgCl_2$ phosphate buffer with a 20 kHz probe-type sonicator at the power level of 25 W. Both French-pressed and sonicated vesicles had the same characteristics as normal chromatophore preparations in the light-induced absorption spectrum change and ion translocation. Therefore these preparations are referred to as "chromatophores".

Bacteriochlorophyll concentrations of the preparations were determined in acetone/methanol (7 : 2, v/v) extracts using the absorption coefficient of 75 $mM^{-1} \cdot cm^{-1}$ at 772 nm [21].

Absorption changes of carotenoid were measured by a dual-wavelength spectrophotometer (Hitachi 356). For the light-induced changes, near-infrared actinic illumination was provided by light from an incandescent lamp filtered through two layers of Wratten 88A filter and a 5-cm water layer. The photomultiplier was protected from the actinic illumination with a Corning 9782 filter.

RESULTS AND DISCUSSION

The time courses of the carotenoid absorbance changes of cells, spheroplasts, spheroplast membrane vesicles, and "chromatophores" induced by illumination or by addition of valinomycin and potassium ions are shown in Fig. 1. Illumination induced an increase in the difference absorbance (523–507 nm) in all of the four types of preparations. The extents of the absorbance increase were about the same. The absorbance change recovered to the initial level when light was turned off. The presence of valinomycin had no effect on the light-induced change with cells, and only a slight decrease with other preparations because of a negligible potassium concentration (broken lines).

Effects of valinomycin addition and subsequent additions of potassium on the difference absorbance, however, were different between preparations. Addition of valinomycin (90 nM) induced no change in cells and only a small decrease took place

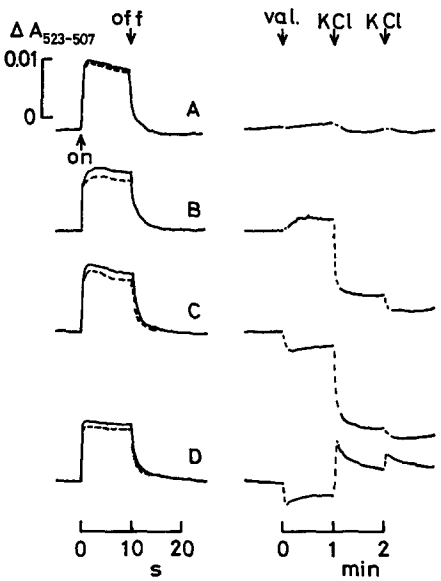

Fig. 1. Absorbance changes of carotenoid induced by light and K^+ gradients. Cells (A), spheroplasts (B), spheroplast membrane vesicles (C), and "chromatophores" (D) were prepared as described in Materials and Methods. The reaction mixture contained bacterial preparation (equivalent to 10 μM bacteriochlorophyll in A, B and C; 8.7 μM bacteriochlorophyll in D), 5 mM $MgCl_2$, and 5 mM sodium phosphate, pH 7.4. In addition, 0.5 M sucrose was present in the spheroplast sample (B). Time courses on the left side are the light-induced absorbance changes (solid lines, no valinomycin; dashed lines, in the presence of 90 nM valinomycin). Changes in absorbance caused by additions of valinomycin and KCl are shown in the traces on the right. Valinomycin added was 90 nM; the first and the second KCl additions were 8.6 mM each.

when potassium was added. It is probably because of the cell wall barrier shielding inner cytoplasmic membranes from external reagents. On the other hand, preparations of other types showed more distinct changes. Spheroplasts showed a small increase of the difference absorbance by valinomycin and a large decrease by KCl addition. Spheroplast membrane vesicles showed a valinomycin-induced small decrease in absorbance and a large KCl-induced absorbance decrease, the latter being the same in the direction and the extent as in spheroplasts. "Chromatophores" obtained by the French press treatment of spheroplast membrane vesicles showed an increase of the difference absorbance by KCl addition which was opposite in direction to that observed in other types of preparations.

Light-induced H^+ translocation was also measured in these four preparations (data not shown). Cells, spheroplasts, and spheroplast membrane vesicles showed the efflux of H^+ by illumination, and "chromatophores" showed the influx. It has been generally accepted that the intracytoplasmic photosynthetic membranes of intact cells are continuous to the cytoplasmic membrane and chromatophores are inverted membrane vesicles [15]. Normal chromatophores of *R. sphaeroides* showed the same direction of the KCl-induced carotenoid absorbance change [5] and light-induced H^+ translocation [6] as our "chromatophore" preparation. From these results we concluded that the lysozyme-EDTA treatment of cells and the hypotonic treatment of spheroplasts did not alter the membrane vesicles orientation, and the French press treatment or sonication of cells or spheroplast membrane vesicles produced smaller vesicles with inverted membranes.

Fig. 2 presents the experiments similar to those in Fig. 1 except for the presence of 5 mM KCl. In the medium containing potassium ions, valinomycin reduced the extents of light-induced absorbance change by half in spheroplast membrane vesicles

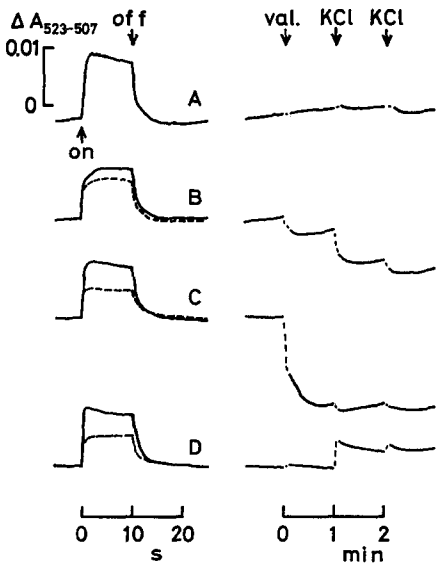

Fig. 2. Absorbance changes of carotenoid in the presence of 5 mM KCl. The conditions were the same as in Fig. 1 except that 5 mM KCl was present throughout preparation procedures for spheroplasts, spheroplast membrane vesicles and "chromatophores", as well as in the reaction medium.

and "chromatophores". Spheroplasts were less affected, and cells were unaffected probably because of the reason described before. The difference between spheroplasts and spheroplast membrane vesicles was also observed during valinomycin-induced absorbance change (Fig. 2, right). The valinomycin-induced decrease in absorbance in spheroplast membrane vesicles was much larger than that in spheroplasts. This difference may be due to a lower potassium concentration inside of spheroplast membrane vesicles than that in spheroplasts. As the hypotonic treatment of spheroplasts was carried out in the presence of 5 mM KCl, the above data indicate that the membrane burst did not take place during the hypotonic treatment. Hellingwerf et al. [18] described previously that the spheroplasts of *R. sphaeroides* were not osmotically lysed, when intracytoplasmic membranes were well developed, due to unfolding of the invagination.

Spectra of the light-induced changes and the KCl-induced changes in the presence of valinomycin in spheroplast membrane vesicles and "chromatophores" are shown in Fig. 3. The light-induced changes of both preparations and the KCl-induced change of "chromatophores" were of the "red shift" type. On the other hand, KCl induced a spectral change of the "blue shift" type in spheroplast membrane vesicles.

In the normal chromatophores, Jackson and Crofts [5] demonstrated that the spectral change induced by addition of valinomycin or by KOH in the presence of carbonylcyanide-*p*-trifluoromethoxyphenylhydrazone was the mirror image of the change induced by light or KCl addition in the presence of valinomycin or HCl in the presence of carbonylcyanide-*p*-trifluoromethoxyphenylhydrazone. They discussed the absorbance changes in terms of the membrane potential produced, in the former cases, negative inside and in the latter, positive inside. This interpretation may be extended to our systems to generalize that the cytoplasmic-side-negative potential

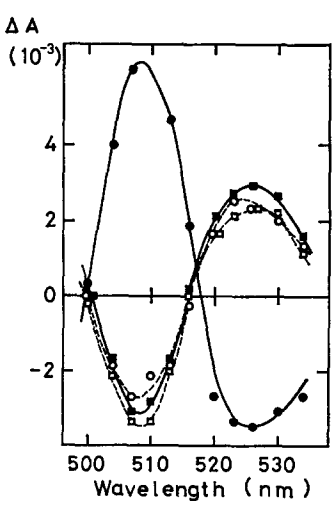

Fig. 3. Absorption spectrum changes caused by light and by K^+ gradient in spheroplast membrane vesicles and "chromatophores". Light-induced changes in spheroplast membrane vesicles (○- - -○) and "chromatophores" (□- - -□), and K^+-induced changes in spheroplast membrane vesicles (● — ●) and in "chromatophores" (■ — ■) were measured in a series of experiments similar to those in Fig. 1. In the K^+-pulse experiments, 8.6 mM KCl was added to reaction mixtures containing 90 nM valinomycin. Bacteriochlorophyll concentration was 10 μM. Reference wavelength, 500 nm.

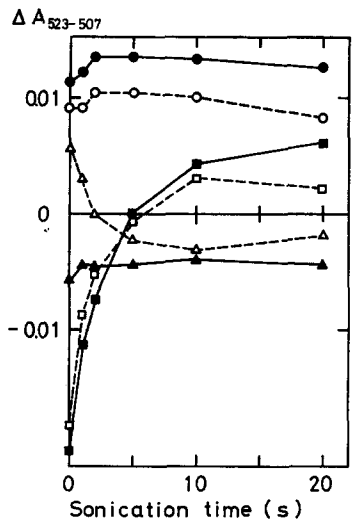

Fig. 4. Effect of sonication of spheroplasts (open symbols) and spheroplast membrane vesicles (closed symbols) on carotenoid changes. Light-induced changes (circles), valinomycin-induced (triangles), and subsequent KCl-induced changes (squares) were measured as in Fig. 1.

change induced by either illumination or ionic gradient causes the "red shift" type spectral change of carotenoid, and the cytoplasmic-side-positive potential change causes the "blue shift" type change.

The inversion of the spheroplast membrane vesicles was caused not only by French press treatment but also by sonication. Fig. 4 shows the sizes of absorbance changes of carotenoid as functions of sonication time. The light-induced changes

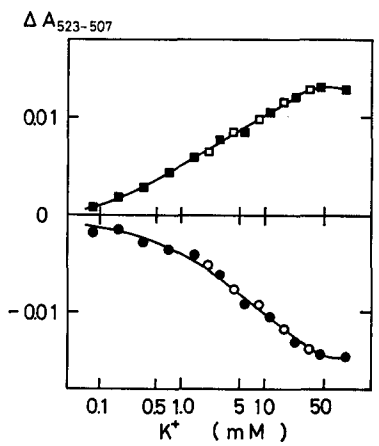

Fig. 5. Dependence of K^+-pulse-induced carotenoid change on concentration of K^+ added. KCl (open symbols) or potassium phosphate pH 7.4 (closed symbols) of various concentrations were added to suspensions of spheroplast membrane vesicles (circles) and "chromatophores" (squares) in the presence of 90 nM valinomycin. Other details are the same as in Fig. 1, except that the bacteriochlorophyll concentration of the samples used was 10 μM.

were little affected by sonication, but the direction of ionic-gradient-induced changes were inverted with sonication time. No distinct difference was observed between isotonic and hypotonic preparations except the initial valinomycin-induced changes.

Fig. 5 shows the change in the difference absorbance (523–507 nm) as a function of concentration of potassium added in the presence of valinomycin in "chromatophores" and spheroplast membrane vesicles. K^+ was added as potassium chloride (open symbols) or potassium phosphate (closed symbols). No difference was observed between anion species. Increasing the concentration of potassium added, the difference absorbance of "chromatophores" increased gradually and that of spheroplast membrane vesicles increased in the opposite direction. Linear relationships between the absorbance change and the logarithm of concentration of K^+ added were observed in a rather wide range of K^+ concentration.

If we neglect permeability of ions except potassium, the membrane potential, under equilibrium conditions, would become:

$$\Delta\psi_{in-out} = \frac{RT}{F} \ln \frac{(K^+)_{out}}{(K^+)_{in}},$$

which will be approximately 60 log $[K^+]_{out}/[K^+]_{in}$ mV where (K^+) and $[K^+]$ are activity and concentration of K^+, respectively. When there is not much difference in $[K^+]_{in}$ before and after the addition of salt, the increase in the membrane potential by the potassium pulse will be:

$$\Delta\psi_{post-pre} = \frac{RT}{F} \ln \frac{(K^+{}_{out})_{post}}{(K^+{}_{out})_{pre}},$$

which will be approximately 60 log $[K^+{}_{out}]_{post}/[K^+{}_{out}]_{pre}$ mV. From Fig. 5, the increase in the membrane potential were calculated assuming that the $[K^+]_{out}$ before the K^+ pulse were 0.1 mM in "chromatophores" and 0.3 mM in spheroplast membrane vesicles. These values were estimated from the intercepts of the extrapolation of linear part of the absorbance change responses on the logarithmic scale of K^+.

The absorbance changes of carotenoid presented in Fig. 5 were replotted against the calculated membrane potential change in Fig. 6. The membrane potential is expressed with a reference on the cytoplasmic side. The absorbance change was linear to the membrane potential change in a range of 0 to +120 mV with "chromatophores" and 0 to −120 mV with spheroplast membrane vesicles with the same inclination. At both ends of the range of membrane potential change, the absorbance changes were somewhat smaller than the linear response, probably limited by factors other than the membrane potential. We sometimes observed a larger inclination with spheroplast membrane vesicles than with "chromatophores" probably due to the difference of inner potassium concentration or incomplete inversion of the membrane vesicles.

Absolute values of the initial membrane potential of the two preparations are probably not much different judged from the similarity of the dark difference absorbance levels before valinomycin addition (dashed lines) which should depend mainly upon the characteristics of the membrane itself and should not be affected by the vesicle orientation.

By comparing the data in Fig. 1 and 2 with that in Fig. 6, it is estimated that the light-induced membrane potential changes in all of the four types of preparations are

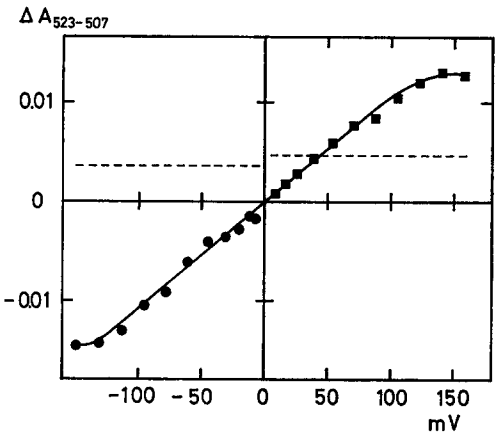

Fig. 6. Absorbance change of carotenoid as a function of calculated membrane potential change. The data presented in Fig. 5 are replotted. Spheroplast membrane vesicles (circles) and "chromatophores" (squares) were subjected to potassium phosphate (pH 7.4) pulses in the presence of 90 nM valinomycin. Membrane potential was calculated as described in text and expressed with a reference on the cytoplasmic side. Dashed lines represent the levels of absorbance before the addition of valinomycin.

in the order of +100 mV with a reference on the cytoplasmic side. In these experiments, the actinic light was not saturated. Somewhat higher values were estimated by Jackson and Crofts for the maximal potential generated by light [5]. In the chromatophores of *Rhodospirillum rubrum*, values of 12 mV and 89 mV were reported as the membrane potential in the dark and under illumination, respectively, determined by the distribution of CNS^- [22].

In view of the appearance of spectral changes of carotenoid in opposite directions, depending on the sign (positive or negative) of the membrane potential, it is suggested that the membrane-potential-sensing carotenoid molecules are oriented asymmetrically in reference to the direction of fluxes of ions. These carotenoid molecules may have definite orientation on the bacterial photosynthetic membranes, in a fashion similar to the cases of other molecules which are oriented and located on the photosynthetic membranes of bacteria with definite sidedness and polarity of structure and function [8, 16, 17, 23].

As an alternative of assuming asymmetrically oriented carotenoid molecules on the membranes, we can postulate the asymmetry of local field in which carotenoid molecules are located. If the carotenoid molecules are subjected to the electrochromic spectral change induced by fixed charges within the membrane, additional change in the electric field, caused by either diffusion of ions or redistribution of charges accompanying the electron transfer, would counteract or reinforce the fixed-charge-induced spectral shift. This mechanism, as well as the first interpretation, is compatible with the linear and quadratic responses of spectra of carotenoids, if we envisage a non-zero field surrounding carotenoid molecules. A small change in the microfield may induce a practically linear response even in the mechanism involving a quadratic term [24].

Regardless of the interpretation of mechanism for spectral responses, sidedness of the membranes or asymmetry of the molecular organization can be clearly demon-

strated in different types of membrane vesicles by electrochemical titration of the spectral change of carotenoids, and independently, by inversion of the vectorial movement of ions by light-activated proton pump.

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