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Voltage-dependent absorbance change of carotenoids in halophilic archaebacteria

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Abstract

Membrane vesicles of wild-type *Halobacterium* sp. mex strain show a wavy absorbance change which has not been so far reported in halophilic archaebacteria. A white mutant strain lacking carotenoids did not show the wavy absorbance change. The wavy absorbance change in the range of 440–590 nm was induced by a red flash (600–640 nm), which photoexcited electrogenic ion pumps, mex bacteriorhodopsin and mex halorhodopsin but not carotenoids. The wavy change was also caused by K^+ diffusion potentials without light. These results suggest that the wavy absorbance change in the membrane vesicles is the voltage-dependent absorbance change of the carotenoids.

Keywords: Membrane potential; Carotenoid; *Halobacterium*; Ion pump; (Archaebacterium)

1. Introduction

Extremely halophilic bacteria are found in natural salt lakes and areas where salt concentration is sufficiently high to produce salt. They belong to archaebacteria [1]. Most halophilic bacteria show red color due to the presence of carotenoids. The first study on the red color shows that the red pigment is fat-soluble [2]. The structures of the red carotenoid pigments were determined [3–7]. The major carotenoid (85% of the total carotenoids) is a C_{50}-tetraol carotenoid, bacterioruberin in *Halobacterium salinarium*. C_{50}-triol, C_{50}-diol carotenoids and C_{40} carotenoids were also identified in the cells. The C_{50} carotenoids characteristic of extremely halophilic bacteria are synthesized from C_{40} carotenoid, lycopene [8,9]. Another C_{40} carotenoid, β-carotene is cleaved into two retinal molecules [10–12]. Retinal forms the complexes with proteins in halobacteria [13]. One of the most studied halobacteria, *Halobacterium salinarium*, have four kinds of retinal-containing chromoproteins in the cell membrane. Two of them are light-driven ion pumps which convert light energy into chemical energy. Bacteriorhodopsin (bR) transports protons from inside to outside of the cell [13,14] and halorhodopsin (hR) transports chloride anions from outside to inside [15,16]. These ion pumpings generate the transmembrane difference in the electrochemical potential for ions. A cycle of conformational changes of the ion pumps driven by photoexcitation transports ions [17]. The cycle begins with the photoisomerization of retinal from all-*trans* to 13-*cis* form. This cyclic conformational change, called photocycle, is spectroscopically detected as a series of intermediates with distinct absorption maxima [14,18].

A new halobacterial isolate, *Halobacterium* sp. mex from crude solar salt produced in Mexico has strong red color. Wild-type mex strains have electrogenic ion pumps: mex bacteriorhodopsin and mex halorhodopsin which are a proton pump and a chloride pump, respectively [19]. Mex bR and mex hR in the cell membranes have photocycles with half-times of about 10 ms. Mex sensory rhodopsin is also found in the cell membranes whose photocycle is 3 orders of magnitude slower than those of mex bR and mex hR [19]. We report here that the membrane vesicles of wild-type mex cells show a wavy absorbance change

Abbreviations: bR, bacteriorhodopsin; hR, halorhodopsin; sR, sensory rhodopsin; mex bR, mex bacteriorhodopsin; mex hR, mex halorhodopsin; mex sR, mex sensory rhodopsin; FCCP, carbonylcyanide p-trifluoromethoxyphenylhydrazone; Pipes, piperazine-1,4-bis(2-ethanesulfonic acid); DMSO, dimethylsulfoxide; MEGA-9, n-nonanoyl-N-methylglucamide; TPT, triphenyltin chloride.

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which has not been previously reported in halobacteria and that the wavy change is a voltage-dependent absorbance change of carotenoids.

2. Materials and methods

2.1. Strains

Two strains were used: a halobacterial isolate, *Halobacterium* sp. mex that was isolated from the crude solar salt produced in Mexico [19] and a white mutant, mex W1. The mutant, mex W1 was isolated from wild-type mex cells which were irradiated with ultraviolet light (254 nm) from a low-pressure mercury discharge lamp (GL-15, National, Japan, distance 30 cm) for 24 s.

2.2. Medium and culture conditions

Complex medium contains the following per liter: NaCl, 250 g; $MgSO_4 \cdot 7H_2O$, 20 g; KCl, 2 g; Na_3citrate, 3 g; $CaCl_2 \cdot 2H_2O$, 0.2 g; $FeSO_4 \cdot 7H_2O$, 5.01 mg; $MnSO_4 \cdot H_2O$, 190 μg; bacteriological peptone (L34, Oxoid), 10 g. Cells were grown aerobically at 40°C for 5–6 days. The cell density in the culture was monitored at 660 nm with a photometric colorimeter (AE-11W, Erma, Tokyo). When the culture reached the early stationary phase, aeration was reduced. After 1–1.5 days under the limited aeration, the cells were harvested by centrifugation (9800 × *g*, 2°C, 15 min). The cell pellet was resuspended in 4 M NaCl. Since the white mutant, mex W1 cells do not synthesize retinal, all-*trans*-retinal (1.0 mg/ml in 95% ethanol) was added to the culture in the early exponential phase [20]. The final concentration of the retinal was 3 μM.

2.3. Preparation of cell membrane vesicles

Cell membrane vesicles were prepared in two ways: the first is a freeze-thaw method. The other is a sonication method. *The freeze-thaw method:* the resuspended cells were frozen with liquid nitrogen and then thawed in a flow of tap water. The viscosity of the cell lysate was reduced by mechanical forces in a waring blender (Model MJ-C22, National, Japan) for 50 s (five 10-s blendings at time intervals of 1 min). The suspension was centrifuged at 9800 × *g* for 15 min at 2°C to remove undisrupted cells as precipitate. The supernatant was centrifuged at 137 900 × *g* for 60 min at 4°C. The collected red pellet was washed until the supernatant became clear. *The sonication method:* the cell suspension was washed with 4 M NaCl by centrifugation (9800 × g, 2°C, 20 min) and resuspended in 4 M NaCl. The cell suspension was sonicated for 50 s (five 10-s sonication at time intervals of 1 min) with a sonicator (UD-201, Tomy Seiko, Japan) at the power setting of 9 at 0°C. The sonicated suspension was centrifuged (137 900 × *g*, 4°C, 60 min). The pellet was resuspended in 4 M NaCl and centrifuged at 11 300 × *g* for 10 min at 2°C. The supernatant was centrifuged at 27 000 × *g* for 30 min at 2°C. Then the precipitate was suspended in 4 M NaCl and centrifuged (27 000 × *g*, 2°C, 30 min). The washing with 4 M NaCl was repeated until the supernatant became clear. Protein concentrations of the prepared membrane vesicles were determined by the Lowry method with bovine serum albumin as a standard [21].

2.4. Flash spectroscopy

Apparatus and procedure of flash spectroscopic measurement were described previously [22]. An actinic red flash (620 ± 5 nm) was obtained through an interference filter (B-620 Interference filter, Asahi Spectra, Japan) for photoexcitation. Another red flash (618 ± 10 nm) passed through an interference filter (03FV046, MELLES GRIOT, USA) and a short cut filter (R61, Toshiba, Japan) was also used as an actinic flash. Absorbance changes were collected and averaged 5–50 sweeps at 30-s intervals. All measurements were done at 20°C.

2.5. Light-induced pH changes

The membrane vesicles were pelleted (264 000 × *g*, 4°C, 15 min) and suspended in 3 M KCl. The suspension was stirred overnight in the dark at 4°C and centrifuged at 264 000 × *g* for 15 min at 4°C. The collected vesicles were suspended in 3 M KCl. A total of 2.5 ml of the membrane vesicle suspension (1.0 mg protein/ml) was stirred in a glass vial (3.0 ml, GL Sciences, Japan). Nitrogen gas bubbled (15–20 bubbles/min) out of a needle in the vesicle suspension. The pH changes were measured with a pH meter (F-15, Horiba, Japan) with a glass electrode (GK2421C, Radiometer). A 12 V 100 W tungsten light (Techno Light, Kenko, Japan) was used as an actinic light source. The light was focused and passed through an interference filter (620 ± 5 nm, Asahi spectra, Japan). The irradiation were controlled by an electronic shutter. Temperature was kept at 25°C with a water-jacketed vial holder. pH was calibrated by the addition of HCl.

2.6. Extraction of total lipids

Cell suspension was washed by centrifugation (9800 × *g*, 2°C, 15 min) to remove the culture medium and resuspended in 4 M NaCl. The total lipids were extracted from the resuspended cells or the membrane fraction by the method of Bligh and Dyer [23]. UV-VIS spectra were measured with a spectrophotometer (U-3000, Hitachi, Japan).

2.7. K^+ diffusion potential

Potassium diffusion potentials were induced in the membrane vesicles as described [24,25]. The sonicated

vesicles in 4 M NaCl were loaded with 3 M KCl by the method described in Section 2.5. For the K^+ diffusion potential experiments, these KCl-loaded vesicles were diluted into 2 ml of 3 M salt at various ratios of KCl/NaCl concentrations. The protein concentrations of the vesicle suspensions were 1.0 mg/ml and the pH of the suspensions were adjusted to pH 7.2 with 25 mM Pipes/NaOH. 10 μl of 75 μM valinomycin (Wako Junyaku, Japan) in DMSO was added to a final concentration of 0.375 μM. An equal volume of DMSO was added into the reference suspensions. The difference spectra were measured at 30 s after the addition. K^+ diffusion potentials were calculated from the Nernst equation. All measurements were done at 21°C.

2.8. Effects of ionophores

FCCP (Sigma, USA) and gramicidin D (Sigma, USA) were dissolved in DMSO. 10 μl of the ionophore solutions were added to 1 ml of the membrane vesicle suspensions in 4 M NaCl, 25 mM Pipes/NaOH (pH 7.2). An equal volume of DMSO was added to the reference suspensions. Absorbance changes at 565, 545, 525, and 505 nm were observed after the red flash.

3. Results

3.1. Flash-induced absorbance difference spectra of membrane vesicles from wild-type mex cells

Cell membrane vesicles of wild-type mex cells were prepared and absorbance changes induced by a red flash (600–640 nm) were measured. A flash-induced absorbance difference spectrum at 0.1 ms after the red flash (Fig. 1A, open circles) had a negative band around 580 nm and positive bands around 410 nm and 660 nm which are characteristic of the photocycle of mex bR [19]. Fig. 1B shows a transient absorbance change at 580 nm monitoring the original form of mex bR. It is clear that the photocycle of mex bR was completed within 45 ms after the flash. Another flash-induced absorbance difference spectrum at 45 ms (Fig. 1A, closed circles) had a wavy absorbance change in the range between 440 nm and 590 nm. This wavy change was more clearly observed in the slower time range. At 100 ms after the flash, the difference spectrum had several positive and negative peaks every 15–20 nm, a broad negative band longer than 590 nm and a broad positive band shorter than 420 nm (Fig. 2A). Fig. 2B shows transient absorbance changes at selected peak wavelengths of the wavy change. In the later time range than 100 ms after the flash excitation, all of the transient absorbance changes can be resolved into two kinetic components with half times of about 200 ms and about 10 s. In the regions longer than 590 nm and shorter than 420 nm, the only slower component was detected (data not shown).

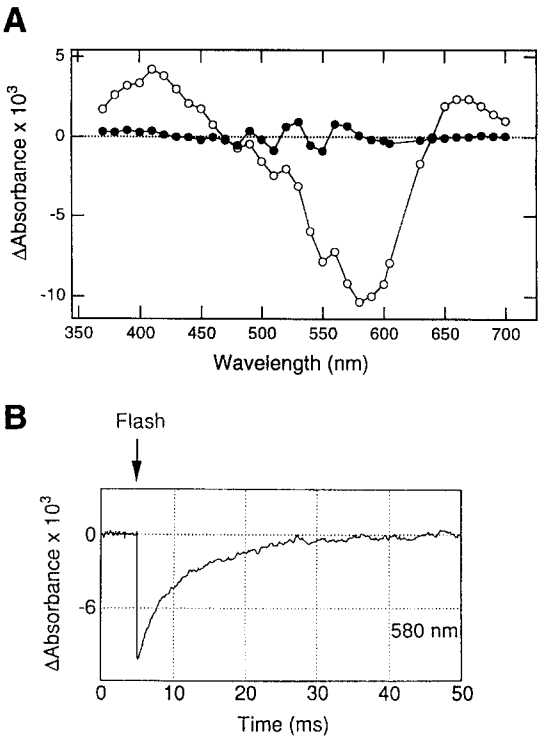

Fig. 1. Flash-induced absorbance changes in membrane vesicles from wild-type mex cells. The membrane vesicles were suspended in 4 M NaCl, 25 mM Pipes/NaOH (pH 7.2). Protein concentration of the suspension was 1.0 mg/ml. Flash light (618 ± 10 nm) was provided for photoexcitation. Temperature was 20°C. (A) Flash-induced absorbance difference spectra at 0.1 ms (○) and 45 ms (●) after the flash. (B) Time course of the absorbance change at 580 nm.

The slower component with the broad spectral bands is attributed to the photocycle of mex sR [19]. These results show that the wavy absorbance change can be attributed to a photochemical activity of a pigment different from the retinal chromoproteins in wild-type mex cells.

3.2. A carotenoid-deficient mutant, mex W1

Fig. 3A shows an absorption spectrum of the membranes from wild-type mex cells with strong red color. Intense absorption bands with three peaks, 538, 503, and 476 nm are characteristic of C_{50} carotenoids in extremely halophilic archaebacteria [3]. This band in the visible region causes the red color of wild-type mex cells. Since the spectral region of the carotenoid bands coincide with that of the wavy absorbance change, we isolated a white mutant from wild-type mex cells. Fig. 3B shows an absorption spectrum of the membrane fraction from the white mutant, mex W1. There were no carotenoid absorption bands. Two small bands around 560 nm and around 410 nm were observed, which are attributable to mex bR and cytochromes, respectively. In order to confirm the deficiency of the carotenoids in the mutant mex W1, we extracted the total lipids using the method by which

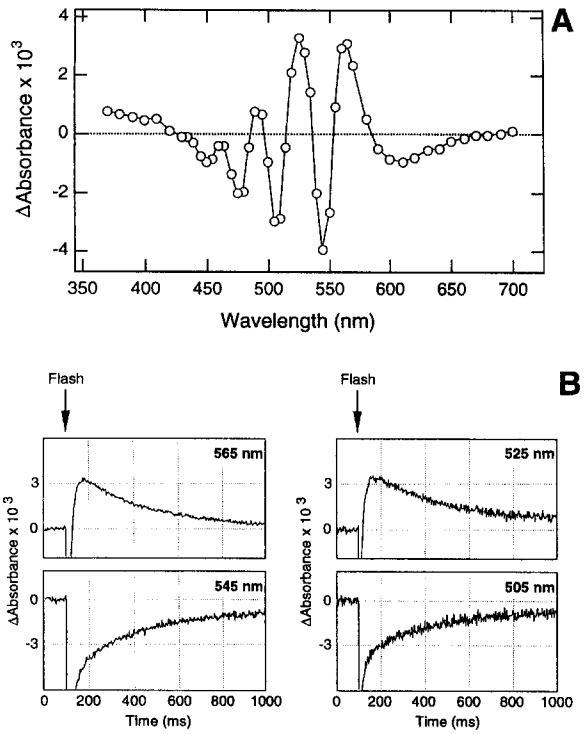

Fig. 2. Flash-induced absorbance changes in membrane vesicles from wild-type mex cells. A red flash (620±5 nm) was used as an actinic light. The other conditions were the same as in Fig. 1. (A) The absorbance difference spectrum at 100 ms after the flash. (B) Time courses of the absorbance changes induced by the red flash at selected positive and negative peak wavelengths: 565 nm, 545 nm, 525 nm and 505 nm.

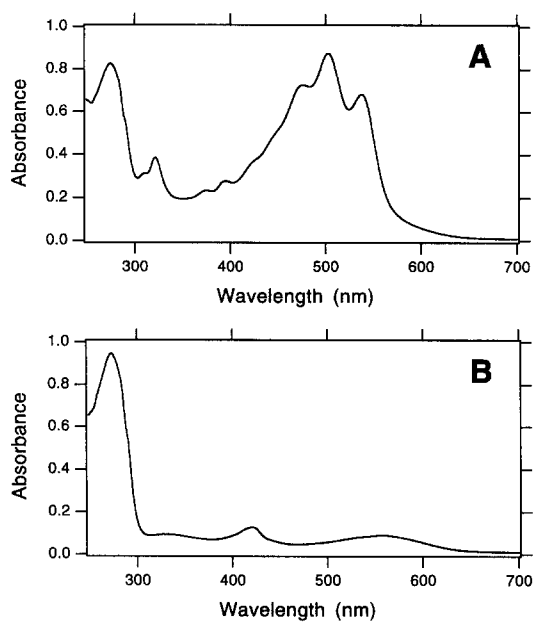

Fig. 3. Absorption spectra of membrane fraction from wild-type mex cells (A) and white mutant mex W1 cells (B). The membranes were suspended in 0.5% MEGA-9, 4 M NaCl, 25 mM Pipes/NaOH (pH 7.2). Protein concentrations were 4.2 mg/ml (A) and 6.1 mg/ml (B), respectively.

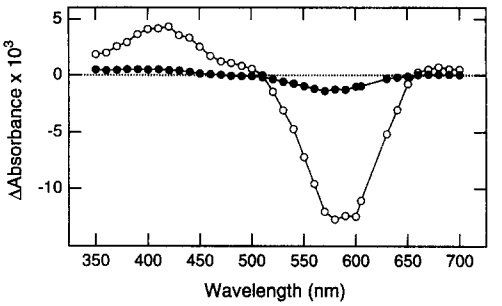

Fig. 4. Flash-induced absorbance difference spectra in the membrane vesicles from white mutant mex W1 cells at 0.1 ms (○) and 45 ms (●) after a red flash (618±10 nm). The absorbance changes were measured as shown in Fig. 1.

carotenoid also can be extracted should they exist, and the absorption spectrum of the total lipids was measured. In the range from 480 nm to 700 nm, there was no absorption (data not shown). These data show that the white mutant, mex W1 has no carotenoids.

We measured red flash-induced absorbance difference spectra of the membrane vesicles from the carotenoid-deficient mutant, mex W1 (Fig. 4). Both difference spectra at 0.1 ms and at 45 ms after the red flash have no detectable wavy absorbance change. The spectrum at 0.1 ms is characteristic of mex bR except a small shoulder around 500 nm which is attributable to mex hR [26]. These results strongly suggest that the wavy absorbance change with the array of peaks and troughs in the range between 440 nm and 590 nm for wild-type mex cells can be attributed to the carotenoids.

3.3. Absorption spectrum of carotenoids in wild-type mex cells

Carotenoids so far reported in extremely halophilic bacteria have no absorption in the wavelength region longer than 600 nm [3,5]. The red flash (600–640 nm), however, causes the wavy absorbance change of the carotenoids in the membrane vesicles from wild-type mex cells. Spectral properties of the carotenoids in wild-type mex cells were measured. Fig. 5 shows an absorption spectrum of the total lipids extracted from wild-type mex

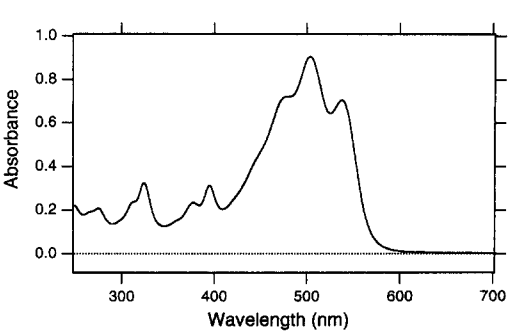

Fig. 5. Absorption spectrum of the total lipids from wild-type mex cells. The total lipids were dissolved in chloroform.

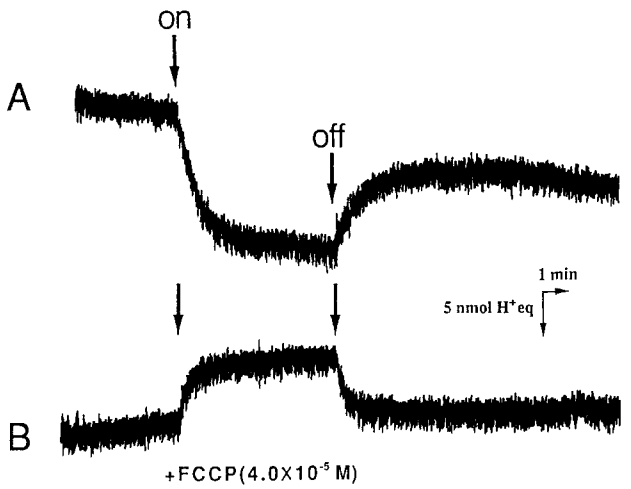

Fig. 6. Light-induced pH changes of the membrane vesicle suspension from wild-type mex cells. 3 M KCl-loaded membrane vesicles were suspended in 2.5 ml of 3 M KCl, 0.25 mM Pipes/NaOH (pH 7.2). Protein concentration was 1.0 mg/ml. All measurements were done at 25°C. An actinic light (620 ± 5 nm) was delivered for the times indicated by arrows. (A) No addition to the membrane vesicle suspension. (B) 10 μl of FCCP dissolved in DMSO was added to the vesicle suspension. The final concentration of FCCP was 4.0×10^{-5} M.

cells. The main band has three maxima at 538, 504 and 479 nm characteristic of C_{50} carotenoids, bacterioruberins which were also identified in *Halobacterium salinarium* [3,5]. It is clear that the carotenoids in wild-type mex have no absorption in the region longer than 600 nm. A major carotenoid was isolated and analysed by NMR, IR and mass spectrometry, all spectrometric data supported that the major (> 80%) carotenoid was bacterioruberin (data not shown). These spectral properties of the carotenoids in wild-type mex cells suggest that the wavy change of the carotenoids is not caused by the direct photoexcitation of the carotenoids by the red flash.

3.4. Membrane potential generated by light-driven ion pumps

Two light-driven ion pumps, mex bR and mex hR exist in the membrane of wild-type mex cells [19]. Mex bR translocates protons from the inside to the outside of the cell. This extrusion by mex bR was observed on the red light illumination (600–640 nm). Trace A in Fig. 6 shows that the red light illumination caused acidification of the outside medium of the membrane vesicles. Addition of a protonophore, FCCP to the vesicle suspension caused alkalinization of the medium (Trace B in Fig. 6). As shown previously, this proton uptake by the vesicles in the presence of FCCP is chloride-dependent and is caused by the passive proton movements driven by the inside-negative membrane potential that is generated by the chloride pump, mex hR [26]. These results show that both ion pumps, mex bR and mex hR can be photoexcited by the red light and generate the inside-negative membrane potential in the membrane vesicles.

3.5. A valinomycin-induced potassium diffusion potential

As mentioned in the previous paragraph, two ion pumps can be excited by the red light (600–640 nm) and generate the inside-negative membrane potential. These results imply that the wavy absorbance change of the carotenoids is caused by the generation of the membrane potential. To demonstrate this, K^+ diffusion potential, not using light, was applied to the membrane vesicles and the absorbance changes were measured. Fig. 7A shows the absorbance difference spectrum induced by the inside-negative K^+ diffusion potential (−88.8 mV). It is clear that the difference spectrum is spectrally similar to the spectrum induced by the red flash (Fig. 2A) except that the peaks are slightly blue-shifted. The absorbance change of the carotenoids was dependent on the applied membrane voltage (Fig. 7B). There is an approximately linear relationship between the carotenoid change and the membrane potential in the range from −20 to −90 mV. In the range from 0 to −20 mV, the change of the carotenoids was nearly zero, indicating that the carotenoid change has a threshold value. These results strongly suggest that the wavy absorbance change of the carotenoids in wild-type mex is caused by the membrane voltage.

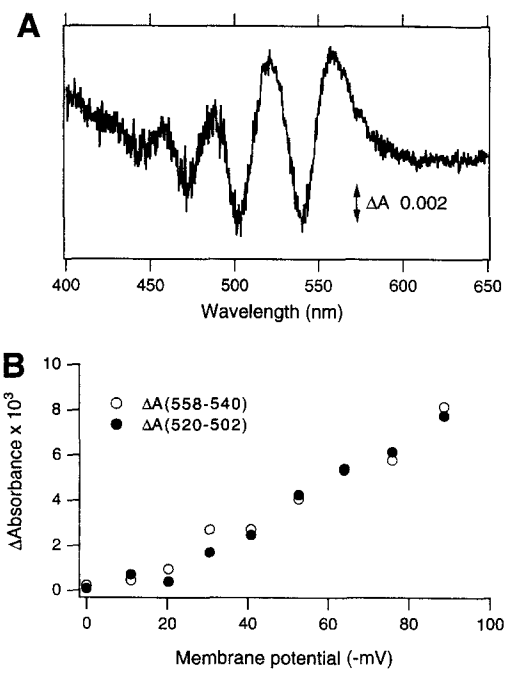

Fig. 7. (A) Absorbance difference spectrum induced by K^+ diffusion potential in the membrane vesicles from wild-type mex cells. Protein concentration of the vesicle suspension was 1.0 mg/ml. Difference spectrum was measured at 30 s after the addition of valinomycin to a final concentration of 0.375 μM. Applied diffusion potential was −88.8 mV. Temperature was 21°C. (B) Dependence of the carotenoid absorbance change on the membrane potential. Absorbance difference between the positive and negative peaks: ΔA(558–540) and ΔA(520–502) were plotted against the applied membrane potential. The other conditions were the same as in Fig. 7A.

3.6. Effects of ionophores on the wavy absorbance change of the carotenoids

We examined the effects of ionophores which dissipate the membrane potential on the wavy absorbance change of the carotenoids. First, the effect of a protonophore, FCCP is shown. In Fig. 8A, the carotenoid absorbance change induced by the red flash is monitored at 565 nm, one of the positive peaks. The carotenoid change was abolished in a dose-dependent manner. At 1×10^{-4} M of FCCP, the carotenoid change disappeared completely. In Fig. 8B, the amplitudes of the carotenoid absorbance change at selected peak wavelengths were plotted against FCCP concentration. The dose–response curve has a half effective dose of about 1×10^{-6} M of FCCP. Fig. 9A and Fig. 9B show the effect of a cation-ionophore, gramicidin D on the carotenoid absorbance changes. Gramicidin D abolished the carotenoid absorbance change in a dose-dependent manner with 50% effective dose at 3×10^{-8} M. We also examined the effect of an Cl^--OH^- exchanger, TPT which collapses anion concentration difference across the membrane but does not affect the membrane potential, the carotenoid absorbance change was not affected with TPT (data not shown). These data confirm further that the carotenoid absorbance change is caused by the membrane potential.

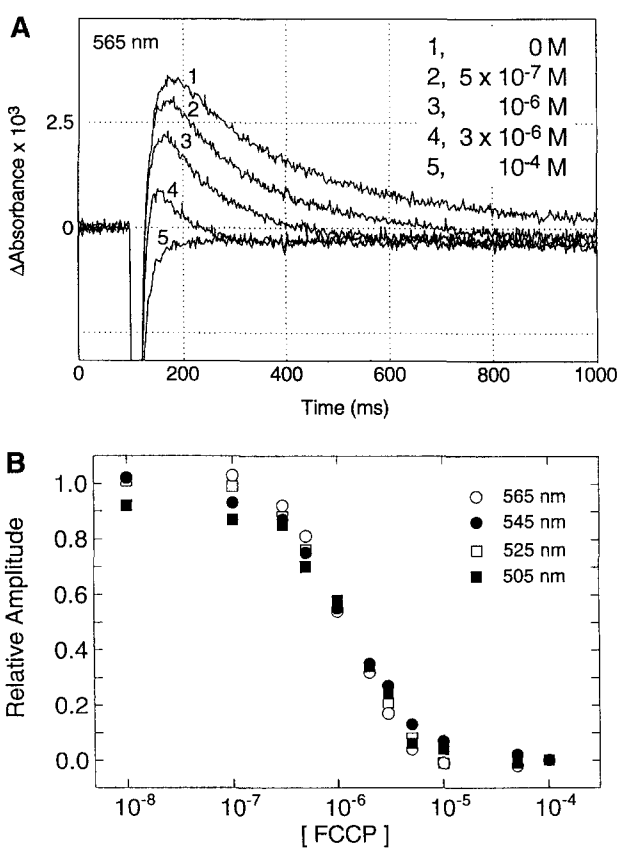

Fig. 8. Effect of FCCP on the carotenoid absorbance change. (A) Effect on the absorbance change at 565 nm which is one of the positive peak wavelength. 10 μl of various concentrations of FCCP in DMSO was added to the vesicle suspension. Final concentration of FCCP was described in figure. 10 μl of DMSO was added as a control (Trace 1 in A). The other conditions were the same as in Fig. 2. (B) The amplitudes of carotenoid absorbance changes at 100 ms after the flash were plotted against FCCP concentration at selected peak wavelengths.

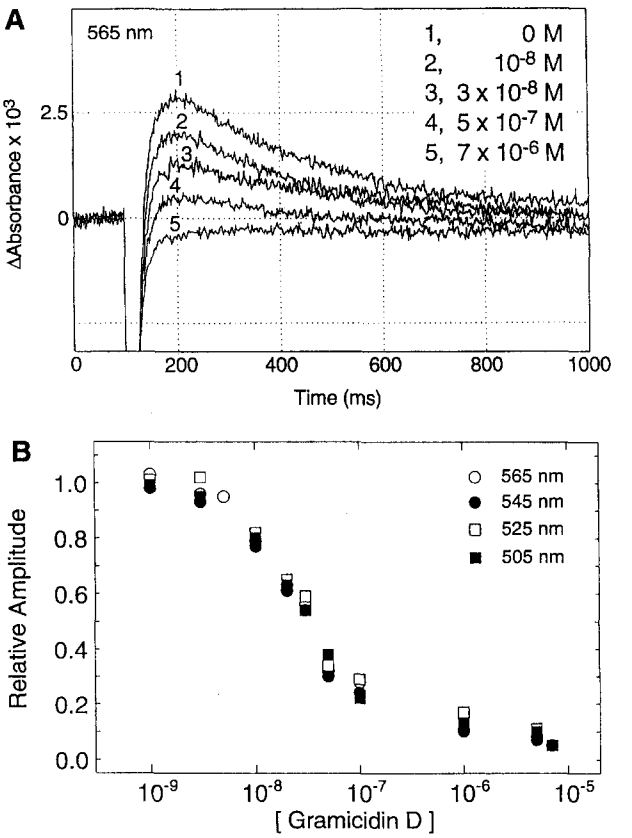

Fig. 9. Effect of gramicidin D on the carotenoid absorbance change. (A) Effect on the absorbance change at 565 nm. 10 μl of various concentrations of gramicidin D in DMSO was added to the vesicle suspension. Final concentration of gramicidin D is described in the figure. 10 μl of DMSO was added as a control (trace 1 in A). The other conditions were the same as in Fig. 2. (B) The amplitudes of the carotenoid absorbance changes at 100 ms after the flash were plotted against gramicidin D concentration at selected peak wavelengths.

4. Discussion

The results presented here indicate that the carotenoids in mex cells show the voltage-dependent absorbance change. This type of absorbance change of carotenoids has been extensively described in photosynthetic systems [27], but it has not been so far reported in halophilic archaebacteria. A well-characterized species, *Halobacterium salinarium* cells, have the same kinds of the carotenoids as mex cells, but do not show the absorbance change of the carotenoids. This difference between them may be caused by that between the environments of the carotenoids in the cell membranes. Further study may clarify the environmental conditions essential for the voltage-sensitivity of the carotenoids. This clarification makes it possible that the

carotenoids are probes of the membrane potential generated by ion pumps.

An absorption spectrum of mex bR is not resolved in the cell membranes of wild-type mex because the intense absorption bands of the carotenoids overlap with the absorption bands of mex bR (Fig. 3A). A carotenoid-deficient mutant, mex W1, is useful in obtaining the absorption spectrum of mex bR. An absorption maximum of mex bR is around 560 nm although the traces of mex hR and cytochromes contribute to the absorption band (Fig. 3B). The absorption maximum of mex bR lay at slightly shorter wavelengths than that of bR from *Halobacterium salinarium*.

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