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ABSORBANCE CHANGES OF CAROTENOIDS AND BACTERIOCHLOROPHYLLS RESPONDING TO THE CHANGE OF LOCAL ELECTRICAL FIELD INDUCED BY HYDROPHOBIC ANION, TETRAPHENYLBORATE IN MEMBRANES OF PHOTOSYNTHETIC BACTERIA

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Large blue-shifts of carotenoid absorption bands were induced by dark addition of a hydrophobic anion, tetraphenylborate, in chromatophores and cell membranes of photosynthetic bacteria, *Rhodopseudomonas sphaeroides* and *Rhodopseudomonas capsulata*. Tetraphenylborate also induced a red-shift of the 850 nm absorption band and a blue-shift and broadening of the 800 nm band of bacteriochlorophyll. From the analysis of the relation between the magnitude and isosbestic wavelength of the absorbance changes the tetraphenylborate-induced carotenoid band shift were assumed to reflect the change of local electrical field close to each carotenoid molecule which exists as a minor pool on the light-harvesting pigment-protein complex II (LHC II). Absorbance changes of carotenoid and chlorophylls were also induced by tetraphenylborate in membranes of spinach chloroplasts.

Introduction

Electrochromic absorbance changes of carotenoids in membranes of photosynthetic bacteria or of chloroplasts have been widely used to monitor the change of electrical field within the membranes [1–3]. Each reaction step of photosynthetic electron transport is assumed to initially generate a local-field change in its neighbourhood inside the membrane. This local field is assumed to be delocalized all over the membrane through the movements of charges inside the membrane and in the outer aqueous phase [1,2], and to induce the electrochromic shifts of absorption bands of special carotenoids which exist in other domains of the membrane. The field-sensing carotenoic molecules in *Rhodospillacea* bacteria are assumed to be attached to the light-harvesting pigment protein complexes as a minor pool [1,4] and to be specially orientated within the membranes under the local permanent electrical field. Because of the local field, the absorption bands of the carotenoids are shifted about 20 nm to the red from those in organic solvents even in the absence of externally applied field. The field around the pigments is assumed to be partially changed upon the application of membrane potential or surface potential change and rather linear responses of the absorbance change occur on applications of both negative and positive membrane potential [1–10].

Modifications of the local field also seem to induce carotenoid band shifts [8]. Oxidation of bacteriochlorophyll by ferricyanide [9], or destruction of the membrane structure by detergents or by protease [11] induces the band shifts of the carotenoid pool which is probably similar to that responding to illumination. Absorption bands of bacteriochlorophylls are also known to respond to the local and delocalized electrical field changes [1,12]. Studies of these local field effects can give

information about the profiles of electrical fields within the membrane, which is necessary for understanding the mechanisms of the couplings of movements of electrons and ions in the membranes, as well as information about the structure of the field-sensing pigment-protein complex itself. Modulations of the local field by membrane-permeable anions were examined in the present study.

Materials and Methods

A green carotenoid mutant of *Rhodopseudomonas sphaeroides* (provided by Dr. S. Morita of the University of Tokyo Agricultural Technology), and wild-type cells of *R. sphaeroides* and *Rhodopseudomonas capsulata* were grown under white incandescent light in synthetic medium as described previously [13]. Cells of *Chromatium vinosum* were grown photoautotrophically in the medium of Bose as previously described [14]. Chromatophores were prepared after disruption of cells in a French pressure cell in a medium containing 5 mM $MgCl_2$/50 mM Tris-HCl/100 mM NaCl, as described previously [13]. Chromatophores obtained after centrifugations were washed and dispersed in the same medium and stored at 0°C. Sonically disrupted spinach chloroplasts were prepared as described previously [15]. Sodium tetraphenylborate and its fluoro derivative, sodium tetrakis(4-fluorophenyl)borate were purchased from Dojindo Laboratories, Kumamoto.

Bacteriochlorophyll was determined in acetone/methanol (7 : 2, v/v) extracts by the method of Clayton [16]. Absorption spectra were measured either with a Hitachi 557 spectrophotometer at a 0.5 nm slit bandwidth (its precision of set wavelength was less than ±0.4 nm, data in Figs. 1, 3 and 4) or with a Shimazu UV 240 double beam spectrophotometer at a 0.5 nm slitwidth (the precision of ±0.3 nm, data in Figs. 2 and 7). In both types of spectrophotometers digital baseline correction modes were used to obtain difference absorption spectra. Flash-induced absorbance changes were measured with a split beam spectrophotometer, constructed in this laboratory, equipped with a signal averager (Kawasaki M-100E and TMC-200) and a flash lamp (half intensity duration of 2 μs, Sugawara PS-271).

Results

Absorption changes of carotenoid induced by tetraphenylborate

Addition of the membrane permeant anion, tetraphenylborate, depressed the light induced absorption change of carotenoids (red shift of the absorption peaks) in chromatophore membranes of *R. sphaeroides* (Fig. 1) as expected [1,2]. In addition to this expected effect, large blue-shifts of absorption bands of carotenoid (Figs. 1 and 3) were induced by the addition of tetraphenylborate in the dark. This effect occurred very rapidly and was not transient. The absorption change became larger as the concentration of tetraphenylborate was increased. This effect of tetraphenylborate was also seen in the presence of a detergent, Triton X-100 (lower trace of Fig. 1a), although the absorbance changes induced by additions of low concentrations of tetraphenylborate were a little decreased in the presence of the detergent. The detergent depressed the light-induced absorption change as tetraphenylborate did, but did not in-

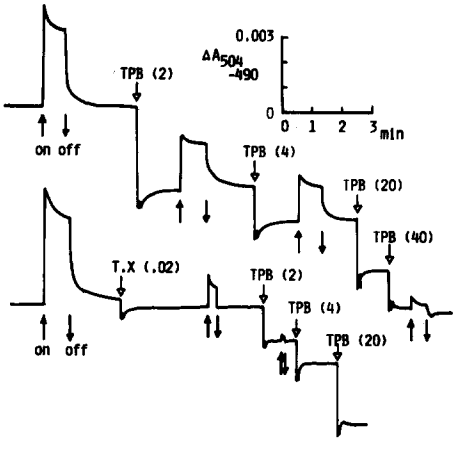

Fig. 1. Tetraphenylborate-induced absorption change of carotenoid in the presence and absence of Triton X-100 in chromatophores of *R. sphaeroides* green mutant. Absorption increase and decrease correspond to the red and blue shifts of carotenoid, respectively. Up and down closed arrows indicate the on and off turning of the continuous illumination light. Tetraphenylborate (TPB, expressed in micromolar final concentration increase) and Triton X-100 (T.X., expressed in percentage of w/w final concentration increase) aqueous solutions were added as indicated in the figure. The reaction mixture contained 100 mM NaCl/5 mM $MgCl_2$/50 mM Tris (pH 7.8) and chromatophores (10 μM bacteriochlorophyll).

duce absorbance change when added in the dark. The depressions of the light-induced carotenoid responses by tetraphenylborate and by Triton X-100 can be explained by the faster dissipation of membrane potential due to the increased ion permeabilities of the membrane [3]. However, the carotenoid shift induced by the dark addition of tetraphenylborate does not seem to reflect the change of membrane potential.

Fig. 2 shows the difference spectrum induced by tetraphenylborate addition in chromatophores and intact cells. Blue-shifts of absorption bands of carotenoids giving absorption changes with positive peaks at 421, 451 and 483 nm and negative peaks at 437, 467 and 498 nm were observed in chromatophores (Fig. 2, trace b). These shifts of carotenoid bands resemble those induced by light, but were in the opposite direction. A blue-shift of the bacteriochlorophyll absorption band around 582 nm showing positive and negative difference peaks at 573 and 589 nm, respectively, and the red-shift of absorption band around 850 nm, showing negative and positive difference peaks at 841 and 868 nm, respectively, were also induced by tetraphenylborate. In addition, the 800 nm band was broadened and shifted towards the blue, producing positive 779 and negative 801 nm difference absorption peaks. The red-shift of bacteriochlorophyll band around 850 nm is very similar to that induced by light [12]. The shift of the 800 nm band is also in the same direction (blue-shift) to that induced by light [12], but is much larger than the latter. The extents of absorbance changes of carotenoids and bacteriochlorophyll induced by tetraphenylborate showed almost the same dependence on the concentration of tetraphenylborate (not shown). These tetraphenylborate-induced absorbance changes were, at least partially, reversible when the effective tetraphenylborate concentration in the medium was reduced by addition of potassium ion or tetraphenylphosphonium cation, each of which forms a complex with tetraphenylborate. Long incubation of chromatophores with tetraphenylborate (several hours) increased the relative extent of absorbance changes around 800 nm and seemed to form bacteriopheophytin. A portion of absorbance change at 800 nm may occur from different mechanism from the shift of bacteriochlorophyll accompanied by the carotenoid shift.

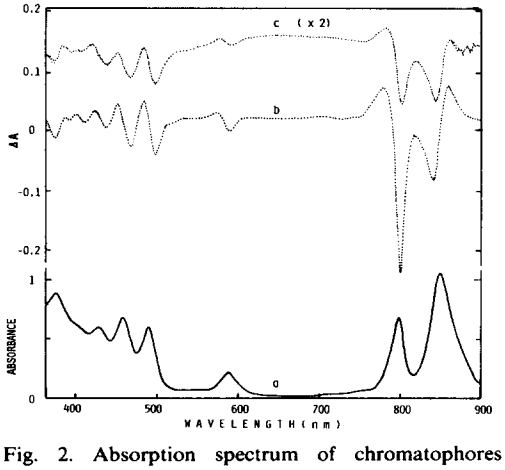

Fig. 2. Absorption spectrum of chromatophores of *R. sphaeroides* green mutant (a) and difference absorption spectrum induced by additions of 40 μM tetraphenylborate in dark-adapted chromatophores (b) and intact cells (c). Concentrations of bacteriochlorophyll were 10.5 μM for (a) and (b), and 3.3 μM for (c). Other conditions were similar to those in Fig. 1.

Addition of tetraphenylborate induced the same type of absorption change (Fig. 2c) in intact cells of *R. sphaeroides,* which have an opposite membrane sidedness to chromatophores. The result indicates that the absorbance changes of carotenoids and bacteriochlorophyll induced by tetraphenylborate addition do not depend on the sidedness of the membrane. Therefore, it can be concluded that the absorption change induced by tetraphenylborate addition was not caused by the change of membrane potential. The bleach of the 800 nm band in intact cells was relatively small compared to that in chromatophores, indicating that not all the absorption changes of bacteriochlorophylls are necessarily related to the carotenoid band shifts.

It seemed that the tetraphenylborate-induced absorbance change is different in its mechanism from that seen upon the application of delocalized intramembrane electrical field by imposing membrane- [3] or surface-potential change [10].

Fig. 3 shows the dependence of the extent of absorbance change upon the tetraphenylborate concentration added. The extent of absorbance change did not saturate within the concentration range tested. Apparently two types of binding sites existed with high and low affinities for tetraphen-

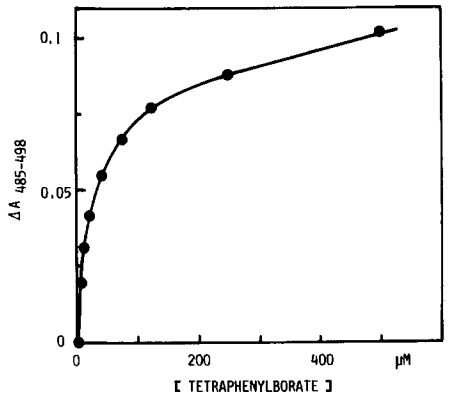

Fig. 3. Dependence of the absorbance change induced by addition of tetraphenylborate in the dark on the concentration of tetraphenylborate in chromatophores of *R. sphaeroides* green mutant. The difference absorbance changes were obtained in the experiments as in Fig. 2.

ylborate (apparent K_m values of 14 and 67 μM, respectively) when the dependence was replotted in a Scatchard plot (not shown). The low affinity site may simply reflect the decrease of tetraphenylborate accessibility to the membrane surface due to the increased negative surface potential created by tetraphenylborate binding itself.

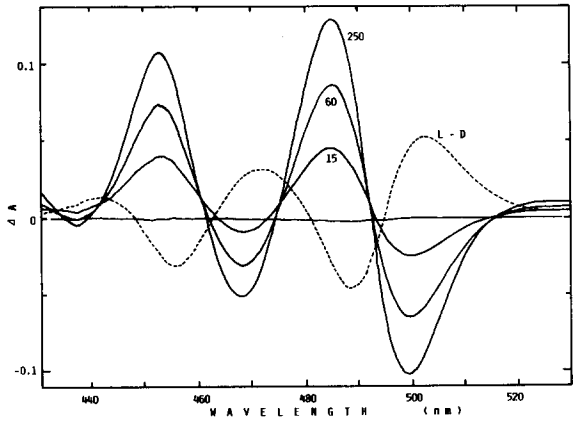

Fig. 4. Difference absorption spectra of carotenoid induced by tetraphenylborate additions in chromatophores of *R. sphaeroides* green mutant. Solid lines, difference absorption spectrum induced by tetraphenylborate addition in the dark. Numbers in the figure indicate final concentrations of tetraphenylborate, added in μM. Broken line, light-minus-dark difference absorption spectrum in the absence of tetraphenylborate. Other conditions were similar to those in Fig. 1.

Relation between the magnitude of absorbance change and the shift of isosbestic point

Fig. 4 shows the difference absorption spectrum of tetraphenylborate-induced band shift of carotenoids. The magnitude of the absorption change increased as the concentration of tetraphenylborate was increased. However, the isosbestic wavelength, which is about 2.5 nm shorter than that of the light-induced absorbance change, remained at an almost constant wavelength.

Tetraphenylborate did, however, change the isosbestic point of the light-induced carotenoid band shift. Since it was difficult to get the correct isosbestic point from the small absorbance change induced by continuous illumination in the presence of tetraphenylborate, absorbance changes were measured over a shorter time range (within 0.1 ms after the flash excitation of chromatophores) by using a flash spectrophotometer (insert in Fig. 5). The initial extent of flash-induced absorbance change was a little decreased in the presence of tetraphenylborate. The isosbestic wavelength of the flashlight-induced absorbance change shifted to shorter wavelengths as the concentration of tetraphenylborate increased (Fig. 5).

The relation between the magnitude of the carotenoid absorbance change and the shift of the isosbestic wavelength induced by tetraphenylborate was carefully analyzed (Fig. 6) and compared with that induced by light. As seen in Fig. 6, the isosbestic point remained at almost the same wavelength (491.4–491.5 nm) on addition of tetraphenylborate up to a concentration of 500 μM. The absorbance change of carotenoid induced by addition of tetraphenylborate appears to have a fixed isosbestic wavelength at the lower concentration range. At about 1000 μM tetraphenylborate, the isosbestic point of the absorbance change begins to shift to the shorter wavelength (Fig. 6). This also occurred with other isosbestic points at shorter wavelengths (not shown).

The relation between the magnitude of the absorption change and the isosbestic point was also analyzed for light-induced changes under varied intensities of illumination. In the absence of tetraphenylborate the isosbestic point shifted as the absorbance change increased. The relationship was linear (open circles in Fig. 6) as reported by Symons et al. [4]. From this relation we can esti-

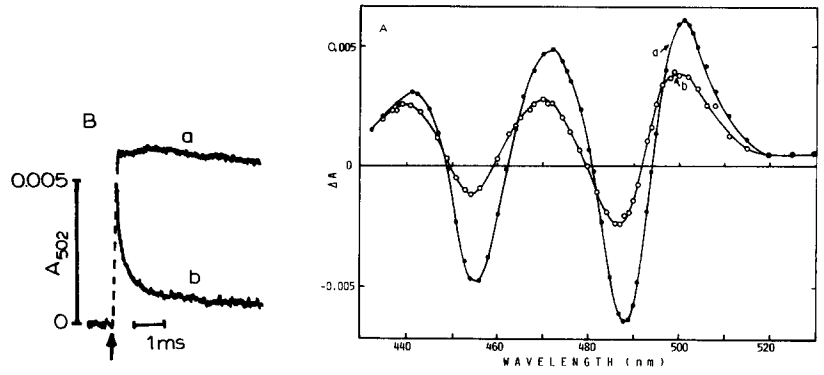

Fig. 5. (A) Flash-induced absorbance change of carotenoid in the absence (a) and presence (b) of tetraphenylborate in chromatophores of *R. sphaeroides* green mutant. (B) Time-courses of absorption change at 502 nm induced by excitations with single-turn-over flashes. Other conditions were similar to those in Fig. 3. Concentrations of chromatophores were 10.5 μM bacteriochlorophyll.

mate that the longest wavelength peak of the carotenoid pool which responds to a light-induced intramembrane electrical field has a peak absorption wavelength of 494.0 nm in the absence of an externally applied electrical field. The isosbestic point shifted to longer wavelengths upon the application of an inside positive membrane potential, in a linear relationship with the magnitude of the absorbance change. The isosbestic point at zero-external field estimated here is assumed to be the absorption peak of the field-sensitive minor carotenoid pool. This wavelength was about 2.6 nm longer than the isosbestic point seen in the case of a tetraphenylborate-induced absorbance change. The isosbestic wavelength–magnitude relation was very different in the latter case. This suggests that a tetraphenylborate-induced absorbance change is different in its mechanism from those seen upon the change of delocalized electrical field induced by the change of membrane potential [4] or surface potential [10]. Tetraphenylborate addition may change the local field close to carotenoids or bacteriochlorophyll.

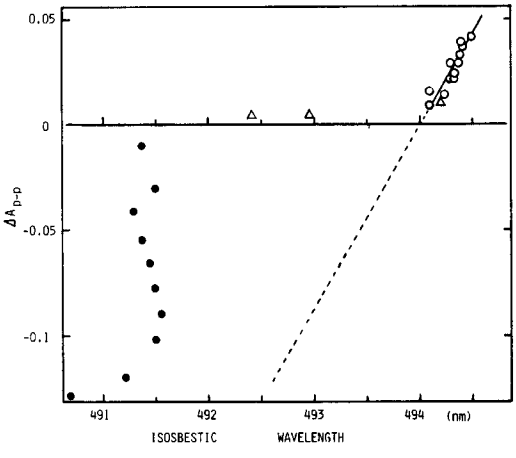

Fig. 6. Relation between the absorption change (difference between the peaks at 498–504 nm and at 484–489 nm) and the shift of isosbestic wavelength, induced by light illumination (open circles) and by tetraphenylborate dark additions (closed circles). Data were obtained in the experiments as in Fig. 4 under various illumination intensities or on additions of various concentrations (5, 8, 20, 40, 75, 125, 250, 500, 1000 and 2000 μM) of tetraphenylborate in the dark. Open triangles indicate the relationship observed in the case of absorbance changes induced by single-turn-over flashes in the presence of 0, 10 and 100 μM tetraphenylborate in a similar experiments to Fig. 5. The isosbestic point of the absorption changes was estimated after corrections of the contributions of dilution effect and light-scattering change induced by additions of tetraphenylborate.

On the other hand, the magnitude-shift relation of the light-induced absorbance change in the presence of tetraphenylborate was outside the relation obtained under continuous illumination in its absence. As the tetraphenylborate concentration was increased, the isosbestic point of the light-induced change shifted to shorter wavelengths (Fig. 6, triangles). A similar tendency was also observed in the case of absorption changes under continuous illumination (not shown). This effect may be explained by postulating two types of light-responding carotenoid pools in the presence of low concentrations of tetraphenylborate, one with an absorbance peak at the original wavelength and

the other with the tetraphenylborate-shifted absorbance peak. Both types of carotenoid pool may respond to the change of light-induced membrane potential and may show red shifts of the original absorption bands. In that case, the mixture of the absorbance changes of these two carotenoid pools will result in apparent light-induced absorbance changes with isosbestic points at shorter wavelengths. In contrast to the progressive shift of the absorbance bands upon the application of delocalized electrical field, the tetraphenylborate-addition seems to induce all-or-none type changes of the electrical field on each carotenoid molecules.

Effects of other hydrophobic ions

Addition of a fluoro-derivative of tetraphenylborate, tetrakis(4-fluorophenyl)borate also induced similar absorption changes of carotenoid and bacteriochlorophyll at lower concentration ranges than tetraphenylborate. Another type of hydrophobic anion, dipicrylamine showed similar effects. On the other hand, salts of membrane-adsorbing alkyl anions, such as sodium laurylsulfate, sodium heptylsulfate and sodium propylsulfate, which have localized anion groups on one end, only induced the band shift of the bulk carotenoid pool and induced the bleaching of bacteriochlorophylls when added in high concentrations. Additions of carbonylcyanide-*m*-chlorophenylhydrazone (CCCP), gramicidin S or hydrophobic cation, tetraphenylphosphonium did not induce the large carotenoid shifts that tetraphenylborate did. These results suggest that tetraphenylborate and hydrophobic anions with delocalized negative charges have special effects on the pigment-protein complexes within the membrane.

Discussion

Three types of carotenoid absorbance changes have been reported in chromatophores [1,3–9]. The first type is an electrochromic one responding to the change of intramembrane delocalized electrical field [3–6] induced by membrane potential or surface potential changes [10]. This type of band shift can be characterized by the change of isosbestic wavelength which is linear with increasing magnitude of the absorbance change. Symons et al. [4] have shown this relationship and concluded that only a minor pool of carotenoid is sensitive to the change of delocalized electrical field. The results in the present study indicate that a minor pool of carotenoid with a longest wavelength absorbance maximum at 494.0 nm at zero applied membrane potential is responsible for this type of absorbance change (Fig. 6). Under continuous illumination which gives an inside positive membrane potential, this peak shifted about 1 nm to the longer wavelength judged from the 0.5 nm shift of the isosbestic point. The peak wavelength estimated above was longer than the absorption maximum (490 nm) of the bulk of carotenoids. It indicates the existence of a minor pool of carotenoid responding to the light-induced delocalized field change essentially in agreement with the results of Symons et al. [4] who used Glc mutant strain of this bacterium with a similar, but slightly simpler, carotenoid composition.

The second type of absorption change is an electrochromic shift caused by the change of local field close to each carotenoid pigment [8] as reported to occur with the bleaching of bacteriochlorophyll on addition of ferricyanide [9] or by the action of pronase [11]. The minor pool of carotenoid, which probably responds to the light-induced field change, also seems to be responsible for this type of absorption change [8]. The third type is the absorption change of bulk of carotenoid which can be seen when the membrane structure is destroyed by the use of detergent or pronase [11].

The characteristics of the tetraphenylborate-induced carotenoid band shift observed in this study seem to fall into the second type, since the isosbestic point (491.4 nm), which was shorter than that of the light-induced one, did not change until the absorbance change became very large. However, the characteristics of the absorption change induced by tetraphenylborate are very different from those reported for ferricyanide-induced or pronase-induced changes. Addition of tetraphenylborate induced shifts and broadening of the bacteriochlorophyll absorption bands, very similar to those induced by light. The pool of carotenoid responding to tetraphenylborate seems to be different from the bulk of carotenoids, since the isosbestic point of the blue shift was longer than the peak wavelength of the bulk of carotenoids (490 nm). The same carotenoid pool seems to

respond to both light and tetraphenylborate, since the isosbestic point of flash light induced absorbance changes shifted to shorter wavelengths in the presence of tetraphenylborate. Thus, it may be concluded that illumination shifts the carotenoid peak about 1 nm to the red from the original 494.0 nm peak, while tetraphenylborate-addition shifts it 5.1 nm to the blue.

In the presence of low concentrations of tetraphenylborate, the carotenoid pool responding to the light-induced delocalized field seems to be divided into two types, one with a wavelength maximum at 494.0 nm (original peak wavelength) and the other with a tetraphenylborate-shifted peak at 488.9 nm (original peak wavelength minus 2 × wavelength difference between the isosbestic point and the original peak). The shift of the isosbestic point of the flash-induced absorbance change in the presence of low concentrations of tetraphenylborate can be explained as the sum of smaller red-shifts of these two types of carotenoid molecules. It may be concluded that the permanent local field induced by charges of the intrinsic molecules close to each carotenoid is changed by the binding of tetraphenylborate, which induces the blue-shift of carotenoid and the absorbance changes of bacteriochlorophylls. Even after the binding of tetraphenylborate, the carotenoid pool still responds to the change of delocalized field in an almost similar way. According to the recent quantum mechanical study by Kakitani et al. [7], a strong field of a charge, placed at the Van der Waals distance from the carotenoid, can induce a large dipole and quadrupole moments in the molecule and induce a strong field (approx. $5 \cdot 10^7$ V/cm) assuming a dielectric constant of 2, in the interior of the membrane. This explains the large 25 nm red-shift of the peak wavelength of neurosporen compared to the one in organic solutions. On the other hand, a charge placed a little further from the carotenoids causes a smaller shift which can also be explained by the use of classical induced-dipole theory, assuming a delocalized field change (approx. $5 \cdot 10^6$ V/cm). The tetraphenylborate effect observed may be explained by this type of mechanism, since the field change induced by tetraphenylborate-binding can be estimated to be $(6.9–14) \cdot 10^5$ V/cm (depending on the angle of carotenoid molecule to the plane of membrane), assuming that a 1 nm shift of the carotenoid peak corresponds to 137 mV light-induced membrane potential change ($2.7 \cdot 10^5$ V/cm intramembrane field change [4]), although the effect may depend on the site of binding. However, it is not clear whether this type of theoretical approach can also explain the large absorbance changes of

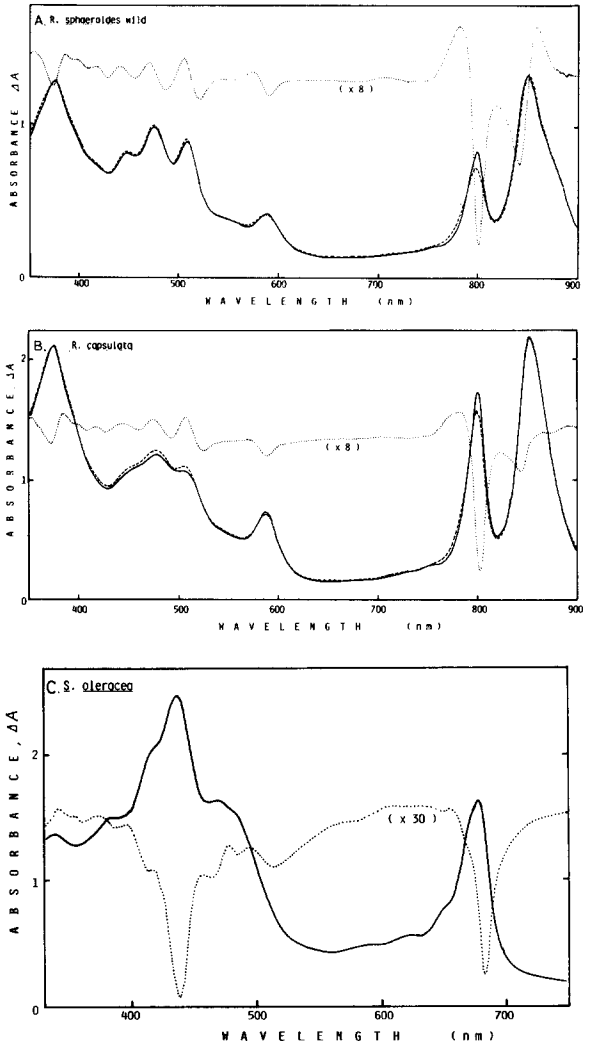

Fig. 7. Absorption changes of carotenoid and chlorophylls induced by addition of tetraphenylborate in darkness in chromatophores of *R. sphaeroides* wild-type cells (A) and of *R. capsulata* wild-type cells (B), and in sonically disrupted spinach chloroplasts (C). Solid and broken lines indicate absorption spectrum in the absence and presence of 20 μM tetraphenylborate. Dotted lines indicate difference absorbance spectra induced by tetraphenylborate additions. Broken line in (C) is almost the same as the solid line and is not shown in the figure.

bacteriochlorophylls as observed in the present study.

Addition of tetraphenylborate also induced similar shifts of carotenoids and bacteriochlorophylls in cells and chromatophores of wild type strains of *R. sphaeroides* and *R. capsulata* (Fig. 7A and B). The difference absorption spectrum varied depending on species, but showed no dependence on the membrane sidedness (not shown). The absorbance change of bacteriochlorophyll in the 850 nm region was relatively small in *R. capsulata* compared with that in *R. sphaeroides.* This suggests differences in the interaction of carotenoids and bacteriochlorophylls in different types of bacteria, or the existence of multiple action sites of tetraphenylborate on the light-harvesting pigment protein complex (LHC II). On the other hand, in chromatophores and cells of *Chromatium vinosum,* which show very small field-responding carotenoid band shift [1,14], almost no absorbance changes of carotenoids and bacteriochlorophylls were induced by tetraphenylborate (Itoh, S., unpublished data). These results also suggest that the local field close to pigments on LHC II [1,6] are specifically affected by tetraphenylborate. Actually addition of tetraphenylborate to the solution of detergent-isolated LHC II causes similar absorption changes of carotenoids and bacteriochlorophylls (Itoh, S., unpublished data).

Tetraphenylborate addition also induced absorbance changes around 518 nm and the bleaching of chlorophylls *a* and *b* in spinach chloroplasts (Fig. 7C), in which large electrochromic shifts of carotenoids and chlorophylls are known to be induced [1,2]. On the other hand, no such absorbance changes were induced by tetraphenylborate addition in thylakoid membranes of cyanobacterium, *Anacystis nidulans,* which lacks field-responding pigments (Itoh, S., unpublished data).

It may be concluded that the hydrophobic anions are absorbed into the membrane and change the permanent local field around the field-responding pigments in photosynthetic membranes. Tetraphenylborate seems to work as a field-modifying point charge by binding to the LHC II complex in photosynthetic bacteria or in the light-harvesting complexes of chloroplast. The binding sites seem to be in a hydrophobic interior of the membrane [17] but may not be very far below the surface [14]. It seems that tetraphenylborate affects carotenoids and bacteriochlorophylls in different ways. Binding of tetraphenylborate into the membrane will change the local field near to binding sites. Reactions bteween electron-transport components or movements of ions inside the membrane will be affected by tetraphenylborate in a slightly different way from other membrane permeant cations or uncouplers; tetraphenylborate will bind not only to the light-harvesting molecules but also to the other membrane constituent molecules and will strongly change the local field. These points were studied elsewhere [17].

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