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Electrochromic responses of carotenoid absorbance bands in purified light-harvesting complexes from *Rhodobacter capsulatus* reconstituted into liposomes

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Light-Harvesting Complexes I and II (LHI and LHII) were extracted from chromatophores of *Rhodobacter capsulatus*, purified in Triton X-100 and reconstituted into phospholipid vesicles. Application of membrane potentials (K^+ diffusion potentials) to LHII proteoliposomes led to absorbance changes in the carotenoid bands which were spectrally similar to those in chromatophores. These (electrochromic) absorbance changes were linear with the applied membrane potential between −107 mV and +105 mV. The data were consistent with the existence of two forms of carotenoid in LHII. One form, comprising 2/3 of the total and with a long wavelength absorbance maximum at 510 nm, was not significantly affected by membrane potential. The other component, comprising 1/3 of the total and with a long wavelength absorbance maximum at 516.5 nm, was shifted by approx. 1.6 nm to the red by a membrane potential of 105 mV. Reduction of the B800 bacteriochlorophyll in LHII with $NaBH_4$ before reconstitution did not affect the absorbance spectrum of the carotenoids and it did not affect their response to applied membrane potentials in proteoliposomes. Although the electrochromically-sensitive carotenoids might be associated with B800, interactions with the bacteriochlophyll are perhaps not the cause of the polarisation of the carotenoid that is responsible for the linearity of the response. The carotenoids in reconstituted LHI complexes were not detectably electrochromic. The electrochromic absorbance changes of carotenoids in LHII could be useful for membrane potential measurement in liposomes containing ion-translocating proteins.

Introduction

The change of absorbance of a molecule resulting from the application of an electric field is known as electrochromism [1,2]. Many of the absorbance changes of carotenoids and chlorophylls arising during illumination of photosynthetic membranes are thought to be electrochromic in origin. In some circumstances these absorbance changes are caused by the *transmembrane* electric potential ($\Delta\Psi$) that is generated and consumed during chemiosmotic energy transduction and they can be used as an indicator of this potential [3]. In principle, because electrochromic absorbance changes are very sensitive to the local environment of the chromophore, they might also be useful probes of structure within pigment-protein complexes. In this context, studies on the effects of electric fields in isotropic, non-oriented samples [4,5] and in anisotropic, membrane-associated proteins will give complementary information.

The light-induced electrochromic spectra of chromatophore membranes from *Rhodobacter sphaeroides* can be modelled on the assumption that only a small fraction (between 20% [6] and 25–35% [7]) of the carotenoid population responds to $\Delta\Psi$. The sensitive fraction is probably located in light-harvesting complex II (LHII). Thus, growth of cells at high light intensities to depress the formation of LHII [8,9], incubation of chromatophores with proteases to degrade LHII [10] and mutational inactivation of LHII [11] all result in a decrease in the electrochromic response to $\Delta\Psi$. Measurements of energy transfer efficiency on the isolated protein indicate that there are two pools of carotenoid in LHII, one associated with B800 bacteriochlorophyll

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Abbreviations: LHI and LHII, light-harvesting complexes I (B870) and II (B800–850), respectively.

and the other with B850. It has been suggested that only the carotenoids associated with B800 are sensitively electrochromic [13]. The ratio of bacteriochlorophyll to carotenoid in LHII complexes is thought to be 2.0 [14] and the ratio of B850 to B800, also 2.0 [15]. These ratios are consistent with an earlier model of LHII based on an α_2-β_2 polypeptide structure with two carotenoids associated with a cluster of four B850's and one carotenoid associated with two B800's but they are inconsistent with a later model based on an α_3-β_3 structure with three carotenoids associated with six B850's and either two or three carotenoids associated with three B800's [16].

The distinctly linear response of carotenoid absorbance to $\Delta\Psi$ in chromatophores has been the subject of much discussion. The most widely accepted explanation is that the sensitive carotenoids are polarised by local charges which gives rise to a permanent local field thus bringing the response into a pseudo-linear range [17]. The polarisation effect might originate from adjacent chlorophyll molecules or from charged amino acids in the protein [17,18]. The former possibility was supported by the finding that the relation between absorbance and electric field strength in lutein films was changed from parabolic to linear by complexation with chlorophyll [19]. In view of the arguments above, B800 might exert a major polarising effect on the electrochromically-sensitive carotenoid.

When protein was extracted from chromatophores with detergent, fractionated by gel filtration and then reconstituted into liposomes, electrochromic absorbance changes of carotenoids were observed in response to the generation of K^+-diffusion potentials [20]. More recently these experiments were refined by partially purifying the proteins on sucrose density gradients [21]. A fraction containing photosynthetic reaction centres and light-harvesting complexes I and II was incorporated into liposomes and their carotenoids were found to be electrochromically active. There was also some success with a reconstituted fraction enriched in LHII [21]. However, in that case the reconstitution procedure did not routinely lead to electrochromically-active vesicles (W. Crielaard, personal communication). Moreover, the electrochromic absorbance changes of carotenoids in the RC-LHI-LHII proteoliposomes were distorted relative to those observed in the native membranes. In this report we describe a procedure that reliably yields electrochromically-active proteoliposomes using highly purified and stable LHII. In contrast to the earlier work, our procedures gave electrochromic absorbance changes of carotenoids that were identical to those in the natural membranes. In this reconstituted system the electrochromic properties of the pigments could be studied in the absence of contributions from other proteins. The procedure also opens up the possibility of using LHII as a probe for the characterisation of electrogenically active membrane proteins.

Methods

Rhodobacter capsulatus strain SB1003 (from Dr. D.J. Kelly, University of Sheffield) was grown and chromatophores were prepared in 10% (mass/vol) sucrose, 30 mM NaCl, 2 mM $MgCl_2$, 50 mM Tricine (pH 7.6) ('buffer A') as described [22]. Chromatophores were washed in buffer A supplemented with 2 M NaCl to remove peripheral membrane proteins. The LH complexes were solubilised by the dropwise addition of washed chromatophores (0.2–0.3 g protein) to buffer A containing 0.5% Triton X-100 to give a final concentration of 1 mg ml^{-1} protein, followed by gentle stirring for 0.5 h and centrifugation at $140\,000 \times g$ for 60 min to remove insoluble material. The supernatant was applied to a 35×1.5 cm column of DEAE-Trisacryl-M (IBF Biotechnics) equilibrated with 0.2% Triton X-100, 20 mM Tricine (pH 7.6) ('buffer B'). Proteins were eluted with a 0–0.5 M linear gradient of NaCl in buffer B. The fractions containing LHI and LHII were identified by their absorbance spectra and by SDS-PAGE. For further purification the proteins were applied to a 20×1.3 cm column of hydroxyapatite (HA-Ultrogel, IBF Biotechnics) equilibrated with buffer B and eluted with a 0–0.2 M linear gradient of potassium phosphate in buffer B. All operations in the preparation of chromatophores and purification of the proteins were performed in dim light at 0–4°C. LHI and LHII from either the DEAE column or the HA column could be stored at 4°C for several days or at −20°C for several months without any significant changes in the absorbance spectrum or any decrease in electrochromic activity upon reconstitution.

In the routine reconstitution procedure (performed at 0–4°C) Triton X-100 was exchanged for cholate, as follows. The LHI- or LHII-containing fractions (approx. 5 mg) from either the DEAE column or the HA column were applied to an HA column (6×1.5 cm) pre-equilibrated with 0.05% (mass/vol) cholic acid, 1.0 mM KH_2PO_4 (pH 7.0 with NaOH). The column was washed with either 7 vols. (for LHII) or 14 vols. (LHI) of 0.5% cholic acid, 2 mM KH_2PO_4 (pH 7.0 with NaOH) and then the LH complex was displaced with a step of 0.5% cholic acid, 300 mM (for LHII or 500 mM for LHI) KH_2PO_4 (pH 7.0 with NaOH). A 1:1 mixture of phosphatidylethanolamine and phosphatidylcholine was washed in diethylether and then dispersed at 20 mg ml^{-1} by sonication to clarity (Heat Systems-Ultrasonics W225) in 50 mM KH_2PO_4, 50 mM KCl, 1 mM EDTA, 4 mM Hepes (pH 7.6 with NaOH) ('buffer C') supplemented with 0.5% cholic acid. The LH complex was added to give a lipid to protein ratio of 10:1 (mass/mass) and the suspension was dialysed for 20 h

against 2×1000 vols. of buffer C in a Boehringer Micro-dialyser. The vesicles were then frozen in liquid N_2 and allowed to thaw at room temperature. They were briefly sonicated and then pelleted by centrifugation at $200000 \times g$ for 2 h. The vesicles were finally resuspended by sonication in buffer C to give a bacteriochlorophyll concentration of approx 0.6 mM for LHII or 0.3 mM for LHI. The choice of phospholipid mixture, described above, was based on the earlier successful reconstitution of transhydrogenase from *Rb. capsulatus* [23] which, in turn, was derived from the phospholipid composition of chromatophores from this organism [24]. Phosphatidylglycerol, the other main component of the natural membrane proved not to be necessary for reconstitution of transhydrogenase and was not included in the present work.

In some experiments the reconstitution was carried out with preparations of LHI and LHII in which the Triton X-100 was not replaced by cholate but otherwise using the same procedure.

Treatment with $NaBH_4$ was carried out as follows. After replacement of Triton X-100 for cholate, LHII (2 mg protein) was resuspended in 15 ml of 50 mM KCl, 50 mM K^+-phosphate, 166 mM Tricine (pH 7.4 with NaOH). Solid $NaBH_4$ (approx. 200 mg) was added with stirring for 1 h, whilst maintaining the pH below 9.0 with 0.1 M HCl. In controls the $NaBH_4$ was omitted but the pH increase was duplicated by adding 0.1 M NaOH. Both samples were finally taken to pH 7.4 with 0.1 M HCl, centrifuged at $200000 \times 180\ g \cdot \min$ and incorporated into liposomes, as described above.

Absorbance spectra were recorded using either a Kontron-Uvicon or a Perkin Elmer Lambda-16 double beam spectrophotometer and absorbance changes were measured in a Shimadzu UV3000 dual-wavelength spectrophotometer. All spectra and absorbance changes were measured at 30°C. SDS-PAGE was carried out as described [25].

Electron microscopy of the vesicles was carried out after staining with silicotungstate.

Results

Purification of the light-harvesting complexes and their incorporation into liposomes

A new procedure for the preparation of the LH complexes from *Rb. capsulatus* is outlined in Methods. A rather pure LHII is routinely obtained after just one chromatographic step (DEAE-Trisacryl-M) but residual contaminating bands can be removed by further chromatography on a hydroxyapatite column. Examination of the protein by SDS-PAGE (Fig. 1B) revealed three polypeptides of M_r 14000, 7000 and 5000. The two polypeptides of low M_r are known to bind bacteriochlorophyll and carotenoid in their native state [26]. The polypeptide of M_r 14000 (gamma) has no

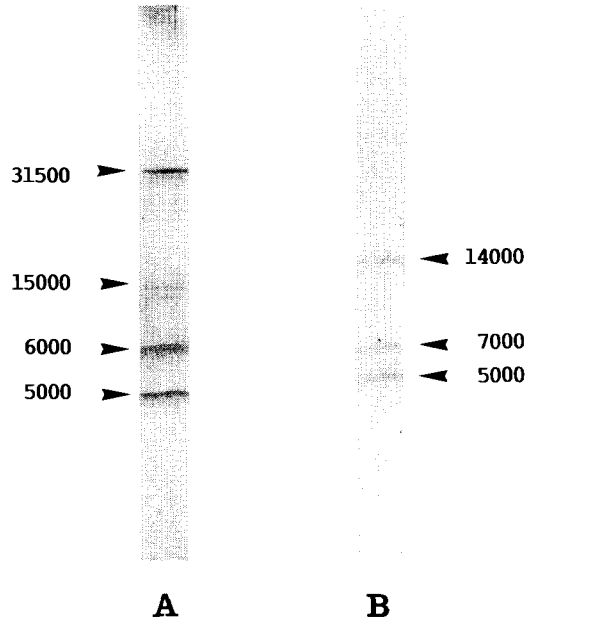

Fig. 1. SDS-PAGE of purified (A) LHI and (B) LHII from *Rb. capsulatus*. Protein samples were purified by DEAE ion exchange chromatography and a step elution from hydroxyapatite, as described in Methods. The M_r values shown in the figure were derived from the best straight lines relating the $\log(M_r)$ of the protein standards with the distance of migration.

pigment and is thought to be involved in the assembly of LHII into the membrane [26]. Fig. 2C shows the absorbance spectrum of the complex. In the near-i.r. the bacteriochlorophyll Q_y transitions had maxima at 802 and 858 nm, which is characteristic of this complex. The addition of K^+-ferricyanide had no effect on the absorbance at 870 nm indicating that the reaction centre content was negligible. The carotenoid absorbance bands were slightly red-shifted in the isolated complex (absorbance maxima at 455, 481.5 and 514.5 nm) compared with those in the native chromatophore membranes (451, 477.5 and 509.5 nm, Fig. 2A), probably reflecting the removal of carotenoids associated with LHI during preparation.

The preparation of LHI is less pure. Analysis by SDS-PAGE indicates that, as well as the polypeptides normally associated with this protein (M_r 6000 and 5000), there were contaminants with M_r 14500, 15000 and 31500 (Fig. 1A). The absorbance spectrum of the complex (Fig. 2B) and the fact that there was no significant bleaching at 870 nm upon addition of potassium ferricyanide suggest that the contribution from functional reaction centre pigments was minimal. The absorbance spectrum in the near i.r. is dominated by the characteristic B870 bacteriochlorophyll band at 880 nm. The origin of the small 800 nm band is not known. It might arise from contamination by LHII or reaction centre. In the visible the carotenoid absorbance bands of the LHI preparation are slightly blue-shifted relative to the chromatophore spectrum, probably the conse-

quence of LHII removal during preparation. The addition of LHI and LHII spectra in the ratio of 1:2 yielded a spectrum (Fig. 2A) that was very similar to that of chromatophore membranes, indicating that the absorbance properties of the pigments are not significantly affected by the solubilisation and purification procedures.

After incorporation of either LHI or LHII complexes into liposomes by a cholate dialysis procedure (see Methods) there was no change in their absorbance spectrum. The mean diameter of the vesicles from electron micrographs was 38 nm ± 9 nm.

Electrochromic absorbance changes of carotenoids in LHII proteoliposomes

The proteoliposomes were prepared in a K^+-containing medium. In the spectrophotometer cuvette they were diluted into a K^+-free buffer to establish a K^+ concentration gradient across the liposome membrane. The subsequent addition of valinomycin (to give a negative-inside diffusion potential) led to a transient absorbance change as shown in Fig. 3A. At 528–511 nm the absorbance promptly decreased upon valinomycin addition and then slowly relaxed over a period of several minutes. The further addition of a high concentration of KCl to generate a positive-inside diffusion potential produced a rapid absorbance change in the opposite direction, again followed by a slow decay (Fig. 3B). The spectra of these absorbance changes, recorded immediately after the addition of the valinomycin and the KCl, are shown in Fig. 4B (the absorbance spectrum of the LHII proteoliposomes is shown in Fig. 4A for comparison). It is clear that the spectra resulting from the negative and positive diffusion potentials were essentially mirror images. It is also evident that the absorbance changes generated by a positive-inside diffusion potential in proteoliposomes (Fig. 4B) were spectrally similar to those generated by a positive-inside potential in chromatophores (Fig. 4C).

The electrochromic absorbance changes illustrated in Figs. 3 and 4 were inhibited by > 90% when experiments were performed in the presence of 10 μM carbonylcyanide-*p*-trifluoromethoxyphenylhydrazone

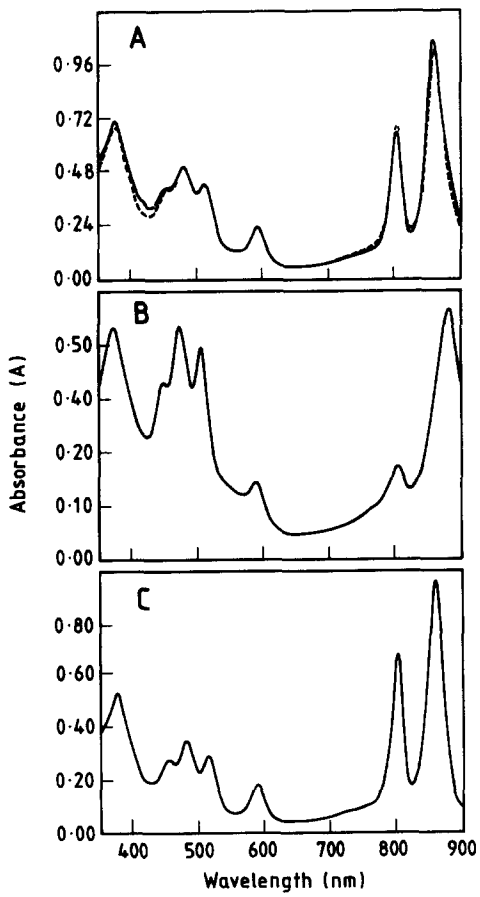

Fig. 2. Absorbance spectra of (A) chromatophores of *Rb. capsulatus*, (B) purified LHI and (C) purified LHII. Chromatophores (10.8 μM bacteriochlorophyll) were suspended in buffer A (see Methods) and LHI (5.8 μM bacteriochlorophyll) and LHII (10.8 μM bacteriochlorophyll) were suspended in 50 mM NaCl, 50 mM NaH_2PO_4, 1 mM EDTA, 4 mM Hepes (pH 7.6 with NaOH). The dashed line in (A) is the sum of the spectra from (B) and (C) in the ratio 1LHI: 2LHII

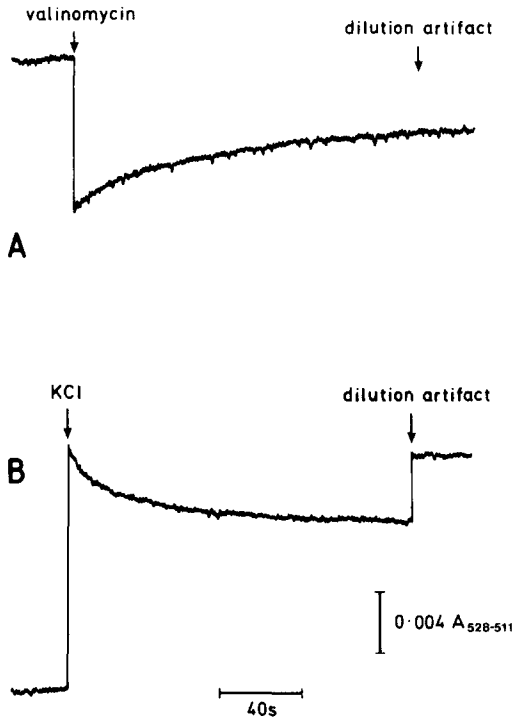

Fig. 3. Electrochromic absorbance changes of carotenoids in response to diffusion potentials generated across the membranes of LHII proteoliposomes. (A) LHII proteoliposomes (10.8 μM bacteriochlorophyll), prepared as described in Methods, were resuspended in a medium containing 50 mM NaCl, 50 mM NaH_2PO_4, 1 mM EDTA, 4 mM Hepes (pH 7.6 with NaOH). Valinomycin (0.15 μg) was added, leading to the absorbance changes at 528–511 nm as shown. (B) 10 min later KCl was added to a final concentration of 94 mM. In each trace the absorbance changes resulting from the addition of an equivalent volume of buffer (the dilution artifact) are also shown.

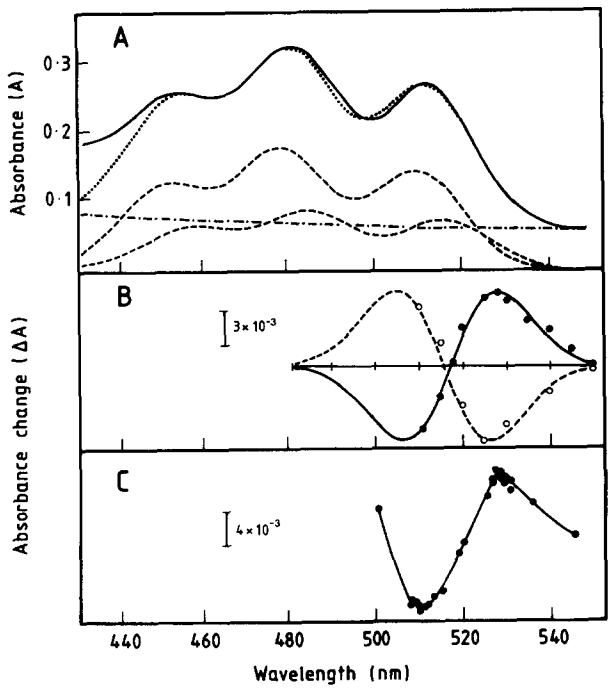

Fig. 4. Absorbance, electrochromic and model spectra of LHII proteoliposomes and chromatophores in the carotenoid region. (A) *Solid line* – the absorbance spectrum of LHII proteoliposomes (10.8 μM bacteriochlorophyll) in the carotenoid region. *Dashed and dotted line* – the scattering spectrum of an equivalent quantity of protein-free liposomes. *Dashed lines* – best fits of the two postulated pools of carotenoid (see text and below). *Dotted line* – the sum of spectra of the two postulated component pools plus the scattering spectrum. (B) *Solid points* – the electrochromic spectrum of LHII proteoliposomes generated in response to positive-inside diffusion potentials from experiments similar to those shown in Fig. 3B. *Open points* – the electrochromic spectrum of LHII proteoliposomes generated in response to negative-inside diffusion potentials from experiments similar to those shown in Fig. 3A. *Solid line* – First derivative spectrum of the longest wavelength band of the postulated 'sensitive' carotenoid pool from Fig. 4A, equivalent to an absorbance band shift of this component of 1.55 nm to the red. *Dashed line* – mirror image of the solid line, equivalent to an absorbance band shift of the 'sensitive' carotenoid pool of 1.55 nm to the blue. (C) The electrochromic spectrum of chromatophores in response to a positive-inside diffusion potential from experiments similar to those shown in Fig. 3B. Modelling parameters. It is assumed that there are two spectrally-distinguishable carotenoid pools in LHII, the long-wavelength component is sensitive to membrane potential, the short-wavelength component, insensitive (see text). The amplitude and width of the red-most (Gaussian) band of the sensitive pool were varied until (a) a shift to longer wavelengths of this band best fitted the electrochromic spectrum and (b) arithmetic addition to this band of the long wavelength band of the insensitive component (with similar width but variable position and amplitude) best fitted the absorbance spectrum. To complete the model shown in A, the two short-wave bands of both components were constructed by varying the amplitudes and peak positions (retaining the same widths and the same peak-peak wavelength intervals for all bands) until the best fit to the absorbance spectrum was obtained.

(not shown). There were no absorbance changes resulting from the addition of valinomycin to proteoliposomes that had been prepared in K^+-containing medium and resuspended in the same medium. When NaCl was added in the conditions shown in Fig. 3B the absorbance changes were about 10% of those observed after KCl addition.

The dependence of the absorbance change upon the value of the applied diffusion potential was investigated (Fig. 5). Using the Nernst equation to calculate $\Delta\Psi$, the relationship was linear within the measurable range, from -107 to $+105$ mV. Similar linear dependences have been observed with chromatophores [27], and with chromatophore protein extracts [20] and LHI/LHII/reaction centre complexes [21] in liposomes.

The experiments described in Figs. 2 to 5 were all performed with proteoliposomes made with LHII prepared following a single chromatographic separation on DEAE-Sephacryl-M after solubilisation. Virtually identical results were achieved using preparations that had been further purified by subsequent hydroxyapatite chromatography (not shown). Interestingly, it was also discovered that good reconstitution of electrochromic activity could be observed using protein preparations in which the Triton X-100 was not replaced by cholate before addition to the dispersion of phospholipids in cholate. Because of its low critical micellar concentration, reconstitution with Triton X-100 is usually successful only if it is subsequently removed for example by exposure to material with a very high affinity for the detergent [28]. In our experiments the extensive treatment in the microdialysis cell and the dilution of the proteoliposomes into the assay

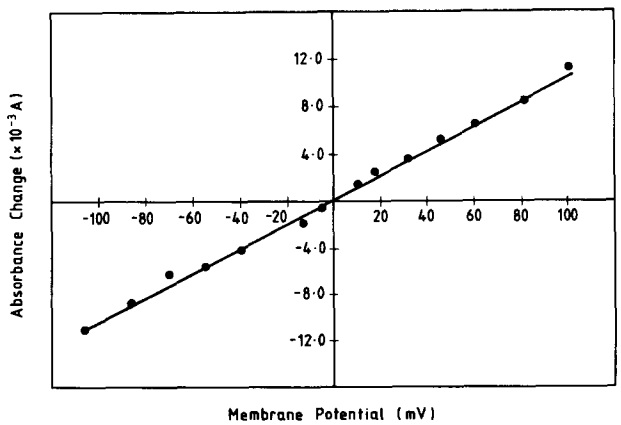

Fig. 5. The linear dependence of electrochromic absorbance changes of carotenoids on diffusion potential in LHII proteoliposomes. Experiments to generate positive-inside diffusion potentials were performed as described as in Fig. 3 but with various concentrations of added KCl. The magnitude of the diffusion potential was calculated as described for chromatophores [27]. Negative-inside diffusion potentials were generated in a similar way except that the initial concentration of external K^+ was adjusted before adding the liposomes. The internal concentration of K^+ was assumed to be similar to that in the liposome isolation medium. Having established that the K^+ concentration gradient was stable, the diffusion potential immediately after the addition of valinomycin was calculated from the Nernst equation.

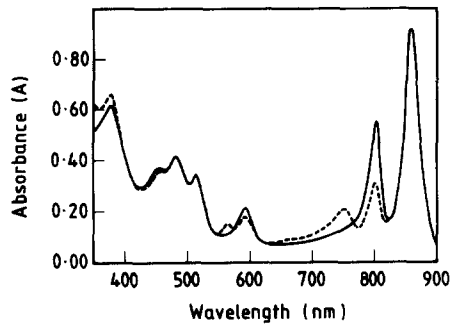

Fig. 6. The effect of $NaBH_4$-treatment of LHII on the absorbance spectrum of subsequently prepared proteoliposomes. See Methods. *Solid line* – control. *Dashed line* – treated with $NaBH_4$.

buffer might both contribute to their good quality but this was not investigated in detail.

Electrochromic absorbance changes in LHII proteoliposomes with selectively inactivated B800

Treatment of LHII with $NaBH_4$ specifically reduces the B800 bacteriochlorophyll without significantly affecting the B850 [29]. This is shown in Fig. 6. Importantly, this figure shows that an extensive decrease in the B800 band was not accompanied by any change in the carotenoid absorbance spectrum, which is in contrast with Ref. 29. The treatment with $NaBH_4$ in Fig. 6 was carried out in the presence of a high concentration of buffer to limit the potentially damaging rise in solution pH. When LHII, treated with $NaBH_4$, was reconstituted into liposomes and subjected to K^+ diffusion potentials, there was no significant difference in the extent of the carotenoid electrochromic absorbance changes relative to controls (Table I).

Carotenoids in LHI proteoliposomes

The application of K^+-diffusion potentials to LHI proteoliposomes did not lead to any detectable electrochromic changes in the carotenoid absorbance bands. That the protein was properly inserted into the membranes and that the liposomes were not highly leaky to ions (and therefore unable to hold a diffusion potential) was shown by parallel measurements of bacteriochlorophyll electrochromism [30].

TABLE I

The effect of reduction of B800 by $NaBH_4$ on electrochromism in LHII proteoliposomes

LHII preparation		Absorbance of B800	Electrochromic absorbance change ($A_{528-511} \times 10^{-3}$)	
			valinomycin	KCl
A	untreated	0.406	−3.1	3.4
	$NaBH_4$-treated	0.198	−2.3	2.6
B	untreated	0.455	−1.3	2.6
	$NaBH_4$-treated	0.273	−1.4	2.6
C	untreated	0.567	−3.0	5.2
	$NaBH_4$-treated	0.319	−4.0	5.3
D	untreated	0.527	−0.6	3.6
	$NaBH_4$-treated	0.272	−3.0	5.0

See Methods section for the procedure for treatment with $NaBH_4$ and the controls and see Fig. 3 for description of the diffusion potential experiments.

Discussion

The experiments described above confirm that the highly sensitive electrochromic absorbance changes of carotenoids in chromatophores from *Rb. capsulatus* arise mainly from LHII. Association with other protein complexes, such as LHI or reaction centres, seems to have no effect on the electrochromism of LHII. The linearity of the carotenoid response to $\Delta\Psi$ in chromatophores was also found in the LHII proteoliposomes (Fig. 5). Thus, LHII could be a valuable probe of electrical events arising from ion pumps co-reconstituted into the same membrane.

The direction of the diffusion potential-induced absorbance changes (Fig. 4) suggests that the predominant orientation of LHII in the proteoliposomes is similar to that in chromatophores. There is an indication that all the protein adopts the same orientation. Thus, for the same value of the applied K^+ diffusion potential, the ratio of the peak-to-trough absorbance change (see Fig. 4) to the absolute absorbance of the long wavelength carotenoid band (see Fig. 2) was about the same in chromatophores (typically 0.044) and in LHII proteoliposomes (0.041). Since, in the natural membrane from cells grown at moderate light intensities, total carotenoid in LHII exceeds that in LHI plus RC complexes [31], this ratio, which reflects the sensitivity of the carotenoids to $\Delta\Psi$, is expected be slightly larger in the proteoliposomes but only if reconstitution were complete and the orientation were homogeneous. Unfortunately, attempts to confirm this conclusion by selectively degrading either the N-terminal or the C-terminal parts of LHII were unsuccessful because of complications arising from changes in the permeability of the liposomes after treatment with proteases (data not shown).

The carotenoid absorbance maxima in the detergent-solubilised LHII preparations and in the proteoliposomes lay at slightly longer wavelengths than in chromatophores (Fig. 2). However, the diffusion potential-induced absorbance spectrum was similar in chromatophores and proteoliposomes (Fig. 4). These findings are consistent with the thesis that the carotenoid spectrum in chromatophores reflects the absorbance of pigments in several different environments and that only one population, whose absorbance bands lie slightly towards the red is sensitively electrochromic. Thus, the purification of LHII selects this long wavelength population and it is this same population that

responds electrochromically in both chromatophores and proteoliposomes. In this context our results differ from those of Crielaard et al.; in [20] it was reported that the potential-induced absorbance changes appeared at shorter wavelengths in LHI-LHII-RC proteoliposomes than in chromatophores. Thus, it was argued that solubilisation and/or reconstitution by their procedures caused a blue shift in the field-sensitive carotenoid even though this was not reflected in the carotenoid absorbance spectra of their preparations.

The electrochromic absorbance changes in the LHII proteoliposomes (Fig. 4) do not arise from a homogeneous band shift of the entire carotenoid complement of the protein – the isosbestic points in Fig. 4B do not correspond to the absorbance maxima in Fig. 4A. However, it has been suggested that there are two pools of carotenoid in LHII complexes, one, accounting for about 1/3 of the total and associated with B800, is located at 'somewhat longer wavelengths' than the other, which is associated with B800 [12]. This arrangement provides a good solution for the data described above if it is assumed that only the carotenoid associated with B800 is electrochromically sensitive. Thus, the absorbance spectrum of LHII proteoliposomes between 430 and 550 nm (Fig. 4A) can be satisfactorily explained by the existence of two pools of carotenoid, each with its characteristic set of three absorbance bands (which are oscillation bands of the same electronically excited state), one, comprising 2/3 of the total, has wavelength maxima at 450, 478 and 510 nm, the other, comprising 1/3 of the total, has maxima at 456.5, 484.5 and 516.5 nm. Fig. 4B shows that the diffusion potential-induced absorbance changes in the long wavelength band can be simulated if the larger pool (associated with B850) is unaffected by $\Delta\Psi$ and the spectrum of the smaller pool (associated with B800) is shifted by about 1.5 nm upon application of a $\Delta\Psi$ of approx. 105 mV. Consistent with the view that 'electrochromically-sensitive' carotenoid is associated with B800, Crielaard et al. [13] found that the blue shift of the electrochromic spectrum, which accompanied the isolation of their LHI-LHII-RC complexes, was matched by a blue shift of the carotenoid fluorescence excitation spectrum of B800. The constraints imposed on the modelling parameters in Fig. 4 were quite restrictive. Thus, simulations on the basis that the pools of 'electrochromically-sensitive' and 'electrochromically-insensitive' carotenoid are of equivalent size did not give a good fit to the data. The data are therefore inconsistent with structural models in which equal numbers of carotenoid molecules are associated with B800 and B850 [16].

Because the permanent dipole moments of carotenoids are usually small their electrochromic absorbance changes in thin capacitors are controlled mainly by polarisability differences between the ground state and the excited state and have a quadratic dependence on field strength [32]. The linear relationship in photosynthetic membranes has been explained by the existence of a permanent field that shifts the dependence into a pseudo-linear region [17]. This is also consistent with the observation that the absorbance spectrum of the electrochromically sensitive carotenoids is red-shifted relative to the insensitive pigment. It was suggested that the permanent field might arise from the polarising influence of the Mg^{2+} atom of an adjacent molecule of chlorophyll [19]. However, the experiments described above may provide evidence against this suggestion, at least in the bacterial system. Thus, reduction of B800, the bacteriochlorophyll probably associated with the electrochromically sensitive carotenoid (see above), with $NaBH_4$ had no effect on either the carotenoid absorbance spectrum of LHII or on its response to $\Delta\Psi$ (Fig. 6 and Table I). If it is assumed that $NaBH_4$ causes reduction of the bacteriochlorophyll to a bacteriophaeophytin derivative and/or its removal from the complex ([33] but see [34]), it must be concluded that the polarising effect originates from the protein itself, perhaps from charged amino acids in the vicinity of the B800-carotenoid binding site [18].

In conclusion, it is worth making the point that these studies support the view that the electrochromic response of carotenoids in vivo (for example in chromatophore membranes or in intact bacterial cells) arises very largely from the electric field effect of the delocalised membrane potential. In the membrane system described here, there can be no local charge separation reactions, such as those that take place in vivo in the electron transfer complexes, and yet the spectra and amplitudes of the absorbance changes in the proteoliposomes closely match those in the natural membranes.

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