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Characterization of linear and quadratic electrochromic probes in *Chlorella sorokiniana* and *Chlamydomonas reinhardtii*

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Field-indicating absorption changes have been measured in mutant strains of *Chlorella sorokiniana* and *Chlamydomonas reinhardtii* lacking one or several chlorophyll-protein complexes. Using mutants which lack PS II centers and most of the chlorophyll antenna, we could characterize two types of probe, with linear or quadratic response to the membrane potential. The probes with linear response present an electrochromic spectrum with maxima at 514 and 486 nm and a minimum at 472 nm; those which respond quadratically present a spectrum with maxima at 464 and 504 nm and a minimum at 479 nm. By measuring the relative contribution of these probes upon a weak actinic flash, the offset of the membrane potential may be estimated under various experimental conditions. In anaerobiosis in the dark, a large permanent membrane potential arises from the hydrolysis of ATP, mainly of mitochondrial origin. We have also analyzed the electrochromic absorption changes in other mutant strains lacking either PS II only, or PS I and the major fraction of light-harvesting complexes. The quadratic probes are present to a similar extent in every strain investigated, which suggests that they are not associated with any of the major chlorophyll-protein complexes. These probes are also conserved in higher plants. In contrast, the linear electrochromic changes are roughly proportional to the overall amount of chlorophyll, either associated with the photocenter or with the antenna.

Introduction

The formation of a delocalized membrane potential perturbs the absorption properties of the pigments embedded in the thylakoid membrane and gives rise to the so-called field-indicating or electrochromic absorption change [1]. A one-turnover flash induces the transfer of two electrons across the membrane and the resulting membrane potential was estimated in the range 40–110 mV, which corresponds to an intramembrane field of about 10^5 V/cm [2]. This large field induces an electrochromic shift of the absorption spectrum of several membrane pigments. The major spectra changes correspond to an absorption increase at approx. 515 nm and an absorption decrease at approx. 475 nm [3]. It has been shown that the amplitude of this electrochromic shift is proportional to the membrane potential [4]. This linear response was unexpected, since the theory of electrochromism predicts a quadratic effect. It has been explained on the grounds of a strong pre-existing local polarization of the pigments probes. Sewe and Reich [5] have proposed that a lutein-chlorophyll *b* complex was responsible for the 515 nm peak.

Lavergne et al. [6] have studied the field-indicating absorption changes in several mutant strains of *Chlorella sorokiniana* lacking one or several chlorophyll-protein complexes. These authors have observed that the amplitude of the electrochromic shift in the 450–550 nm range was roughly proportional to the overall amount of chlorophyll-protein complexes, whatever these complexes were. Nevertheless, significant differences in the absorption spectrum of the electrochromic shift characteristic of each of the major chlorophyll-protein complexes have been reported.

In this paper, we report a more detailed analysis of the electrochromic absorption changes in different mutant strains of *C. sorokiniana* and *Chlamydomonas reinhardtii*. We have established that at least two types of probes – linear and quadratic – are involved in the field-indicating absorption changes.

Abbreviations: PS, Photosystem; LHC, light-harvesting chlorophyll *a*/*b* protein; P-700, Photosystem I primary donor; DCHC, dicyclohexyl-18-*crown*-6; DCMU, 3-(3,4-dichlorophenyl)-1,1′-dimethylurea; TBT, tri-*n*-butyltin chloride.

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Material and Methods

Mutant strains of *C. sorokiniana,* isolated by P. Bennoun, were grown in a Tris-acetate phosphate medium under a continuous light intensity of 200 lux. Ultrastructural organization, polypeptide pattern and antenna pigments of their thylakoid membranes were analyzed in Refs. 6 and 7. The polypeptide composition of *C. reinhardtii* mutants BF4 and F34 was described in Refs. 8 and 9, respectively.

Algae were suspended in a 50 mM phosphate buffer (pH 6.5) with 10% ficoll (w/w). Spectrophotometric measurements were performed at room temperature, using an apparatus similar to that described in Ref. 10, with improvements summarized in Ref. 11. Actinic excitation was provided by a xenon flash (3 μs at half-height) filtered through a Schott filter RG8 or by a dye laser flash Candela SLL150. The emission of the laser flash peaks at 692 nm (oxazine) and its total duration (about 700 ns) is short enough not to induce double photoreactions.

Results and Discussion

Characterization of electrochromic probes in mutant S52

The electrochromic absorption change was analyzed 200 μs after a flash in mutant S52, which lacks PS II and a major fraction of LHC I and LHC II. In the spectral range 450–530 nm and at this detection time, the absorption changes linked to the various redox components are of small amplitude. We have observed that the shape of the spectrum of the electrochromic response depends upon the energy of the actinic flash, which suggests that some of the electrochromic probes do not respond linearly to the membrane potential. Let us consider the function $S=f(V)$, where S is the electrochromic signal at a given wavelength and V is the membrane potential. In the case of a linear probe, the first derivative dS/dV is constant, while it depends upon V in the case of a non-linear probe. In Fig. 1A, the spectrum of dS/dV was estimated by measuring the absorption change induced by a non-saturating actinic flash. The experiment was performed under anaerobiosis, i.e., under conditions in which P-700 is rapidly re-reduced. Thus, the non-saturating flash induces the same number of transmembrane charge separations, whatever the value of V when it is fired. Spectrum 1 was obtained in the presence of DCHC, which is known to collapse the membrane potential [12] in the ms range (Diner, personal communication). Spectrum 2, obtained in the absence of DCHC, differs both in shape and in amplitude from spectrum 1. We checked by measuring cytochrome *f* and plastocyanin oxidation that the efficiency of the charge separation was identical in both experiments. It is worth noting that in the presence of TBT, a specific inhibitor of the membrane ATPase, a spectrum identical to spectrum 1 was observed (data not shown). The effect of DCHC and TBT suggests that a permanent membrane potential is induced by the hydrolysis of ATP by the membrane ATPase. Spectra 3 and 4 were also obtained in the absence of addition. Algae were preilluminated by a saturating laser flash and then a non-saturating flash was fired at 80 ms (spectrum 3) or at 185 ms (spectrum 4) after the laser flash. In the conditions of spectrum 3, a maximum value of the laser-induced membrane potential had been reached (after completion of the slow electrogenic phase), while P-700 was already fully re-reduced. In the conditions of spectrum 4, the laser-induced membrane potential was partially collapsed. In Fig. 1B, spectra a, b

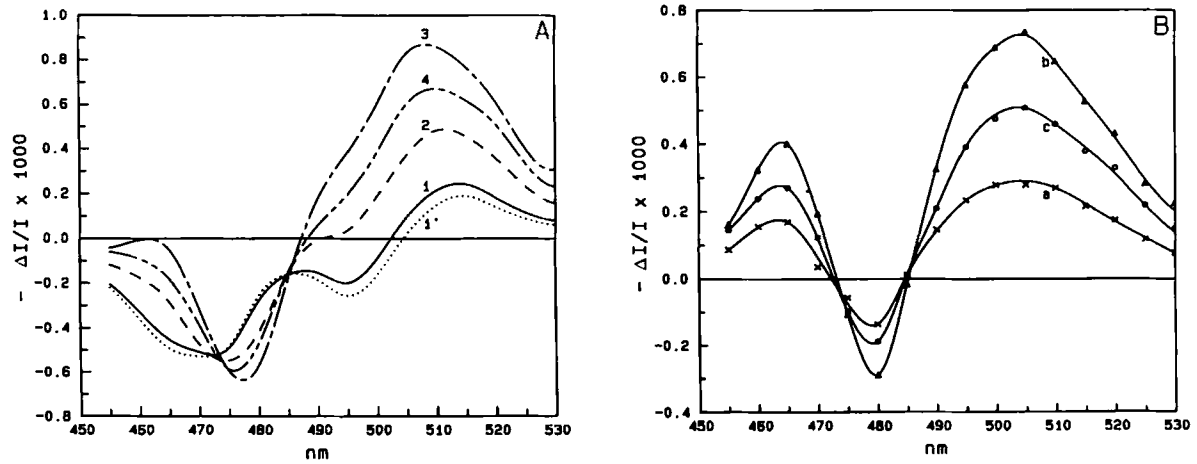

Fig. 1. (A) Electrochromic absorption changes induced by a nonsaturating flash in the S52 mutant strain. The absorption change was measured 200 μs after the sampling actinic flash which hit approx. 50% of the centers. Spectrum 1: dark-adapted algae, 0.2 mM DCHC. A similar spectrum was obtained in the presence of either 20 μM TBT or 0.2 mM DCHC plus 20 μM TBT. Spectrum 1′: same as 1, after subtraction of the minor contribution due to nonlinear probes (see text). Spectrum 2: dark-adapted algae, no addition. Spectra 3 and 4: the sampling actinic flash was fired 80 or 185 ms, respectively, after a saturating laser excitation, no addition. (B) Absorption changes due to nonlinear electrochromic probes. Spectra a, b and c: differences between curves 2 and 1, 3 and 1 and 4 and 1, respectively, from Fig. 1A.

and c were obtained by subtracting spectrum 1 from spectra 2, 3 and 4 (Fig. 1A), respectively. Spectra a, b and c essentially differ by their amplitude, while their shape is about constant. From these experiments, two types of electrochromic probe with different absorption spectra may be distinguished. Spectra a, b, c (Fig. 1B) correspond to non-linear probes, since the amplitude of the signal generated by the sampling flash depends upon the value of the membrane potential at the time the flash is fired. It is worth noting the small shift towards longer wavelengths of the isobestic points of the spectra when the value of the membrane potential increases. Such a shift is predicted by the theory of electrochromism. Spectrum 1 (Fig. 1A) is close to the spectrum of the linear probes. We have measured the amplitude of the linear response by the difference $x = \Delta I/I$(515–494 nm); for this pair of wavelengths, the contribution of the nonlinear probes is negligible (see spectrum b, Fig. 1B). x is proportional to the actual membrane potential value. The amplitude of the nonlinear response was measured by the difference $y = \Delta I/I$(500–484 nm). These wavelengths were chosen so that the function y/x tends towards zero when x tends towards zero.

The theory of electrochromism suggests that the function $y(x)$ takes the form of a parabola $y = ax^2$, which implies that y/x is a linear function. These functions were studied over a large range of membrane potential (Fig. 2). Algae, in the presence of 5 μM TBT, were preilluminated by two oyersaturating xenon flashes 20 ms apart. These flashes induced double PS I reactions and the corresponding slow electrogenic phases. After the second flash, a membrane potential corresponding to the transfer of about 6 electrons through the membrane was reached. We studied the functions $y(x) = ax^2$ and $y/x(x) = ax$ during the dark relaxation of the membrane potential from 40 ms to 20 s after the second flash of the series. As expected, the function $y/x(x)$ is a straight line corresponding to the equation $y/x = 0.21x + 0.15$ (curve 2). The equation of the corresponding parabola (curve 1) is $y = 0.21x^2 + 0.15x$. As a concentration of 5 μM TBT was not sufficient to fully abolish the membrane potential in the dark, the linear coefficient of this parabola differs from zero. For higher concentrations of TBT, the linear coefficient of the parabola tends towards zero but the relaxation of the membrane potential is accelerated, since TBT at high concentrations acts as a charge carrier. It is then no longer possible to build a large membrane potential under repetitive flash illumination.

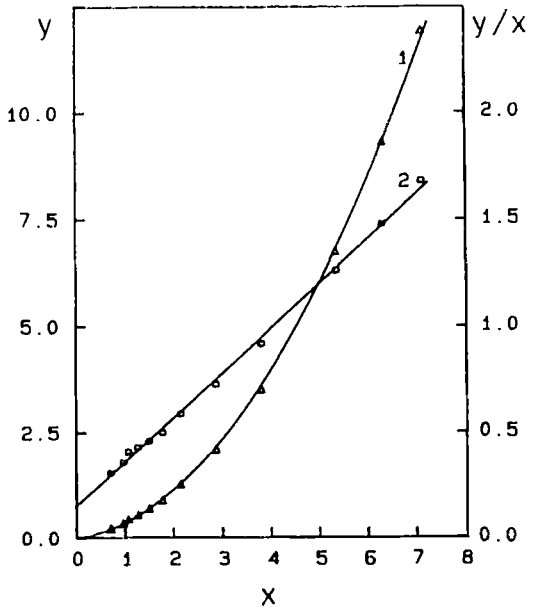

Fig. 2. Functions $y(x)$ (curve 1, left scale) and $y/x(x)$ (curve 2, right scale). $x = \Delta I/I$(515–494 nm)·10^3; $y = \Delta I/I$(500–484 nm)·10^3. S52 mutant strain. 5 μM TBT. The algae were illuminated by two oversaturating flashes 20 ms apart; the absorption changes were measured from 40 ms to 7 s after the second flash.

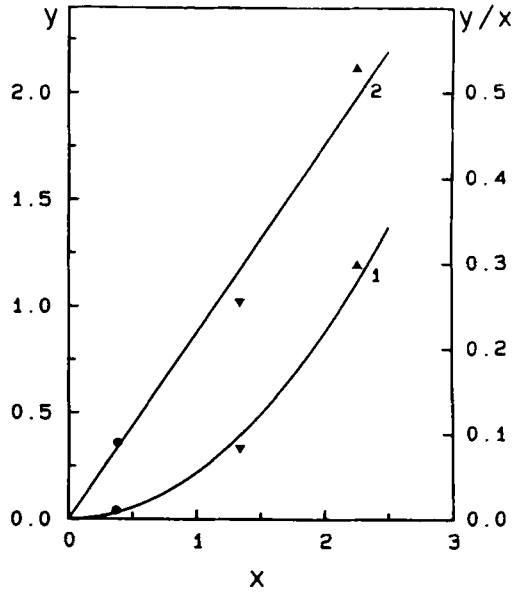

Fig. 3. Functions $y(x)$ (curve 1, left scale) and $y/x(x)$ (curve 2, right scale); x and y as in Fig. 2. S52 mutant strain. 0.2 mM DCHC. The absorption changes were measured 200 μs after flashes of different energies. ●, non-saturating xenon flash; ▼, saturating laser flash; ▲, oversaturating xenon flash which induced double PS I reactions.

In Fig. 3, the functions $y(x)$ and $y/x(x)$ were studied in the absence of permanent membrane potential, i.e., in the presence of 0.2 mM DCHC. Absorption changes were measured 200 μs after flashes of three different intensities given to dark-adapted material. The function $y/x(x)$ is correctly described by a straight line from the equation $y/x = 0.22x$, which corresponds to a parabola from the equation $y = 0.22x^2$. In this particular case (presence of DCHC), the linear coefficient of the parabola is equal to zero.

As the membrane potential induced by the sampling actinic flash is not negligible, spectrum 1 (Fig. 1) includes a minor contribution from the nonlinear probes.

The amplitude of this contribution can be computed from the parabola in Fig. 3; spectrum 1′ thus represents the actual spectrum of the linear probes.

Linear electrochromic signals are generally interpreted by assuming that the involved pigments are submitted to a local electric field generated by charged groups located in proteins or in the polar heads of the membrane lipids. In general, the signal due to a probe submitted to a local field is $S = aV^2 + bV$, where V is the delocalized membrane potential. Thus, the same probe may contribute to both the quadratic and linear responses. The probe will respond linearly to V only if $b \gg aV$, which means that the local field is much larger than the field associated with the delocalized membrane potential. It is worth noting that none of the characteristic peaks of the nonlinear probes appears in spectrum 1′; this suggests that the local field – if any – which could polarize the nonlinear probes is of small amplitude.

Measurement of permanent membrane potential

The value of the membrane potential, whether its origin is light-induced or ATP-induced, can be determined by measuring linear and nonlinear signals generated by a sampling actinic flash. Let us consider the function $y = ax^2$. If a flash is given under conditions where the membrane potential is equal to x_1, the light-induced signals will be

$$\Delta x = x_2 - x_1 \text{ and } \Delta y = y_2 - y_1$$

Then,

$$\Delta y = ax_2^2 - ax_1^2 = a(x_2 - x_1)(x_2 + x_1) = a\Delta x(\Delta x + 2x_1)$$

and

$$x_1 = \frac{1}{2}\left(\frac{\Delta y}{a\Delta x} - \Delta x\right)$$

The coefficient, a, is determined by giving a flash in the presence of DCHC. In this case,

$$x_1 = 0,\ \Delta y = a\Delta x^2 \text{ and } a = \frac{\Delta y}{\Delta x^2}$$

The coefficient a varies slightly ($\pm 10\%$) from one algal culture to the other. This variability could reveal changes in the surface density of the photocenters or/and of the electrochromic probes.

In Table I, the value of the permanent membrane potential is given for various experimental conditions. A large membrane potential is developed in aerobiosis. Addition of antimycin or a 30 min dark incubation under anaerobic conditions largely decreases the value of the membrane potential. These results suggest that the permanent membrane potential is a function of the ATP/ADP ratio in the chloroplast compartment and that the value of this ratio depends upon mitochondrial activity. Interestingly enough, the membrane potential slowly increases during long, dark anaerobic incubation. Addition of glucose also increases the membrane potential under anaerobic conditions, but has no effect under aerobic conditions (data not shown). The only source for ATP synthesis under anaerobic conditions in the dark should be a fermentation process, which would be stimulated by the addition of glucose. We do not know if this glycolytic activity occurs in the chloroplast or in the mitochondria.

TABLE I

Permanent membrane potential under different experimental conditions

S52 mutant strain. The membrane potential was normalized to the light-induced membrane potential measured 200 μs after a saturating laser flash (one charge separation per PS I center) which is estimated in the range 20–50 mV [2].

Experimental conditions	Membrane potential
Aerobiosis, no addition	1.58
Aerobiosis + 2 μM antimycin	0.16
Anaerobiosis + 200 μM DCHC	0.00
Anaerobiosis, 30 min, no addition	0.43
Anaerobiosis, 170 min, no addition	1.12
Anaerobiosis, continuous light (12 $h\nu$/center per s)	2.12
Anaerobiosis, continuous light + 25 s dark	1.64
Anaerobiosis, continuous light + 14 min dark	1.31
Anaerobiosis, continuous light + 34 min dark	1.24
Anaerobiosis, 30 min, 20 mM glucose	1.02
Anaerobiosis, 95 min, 20 mM glucose	1.32

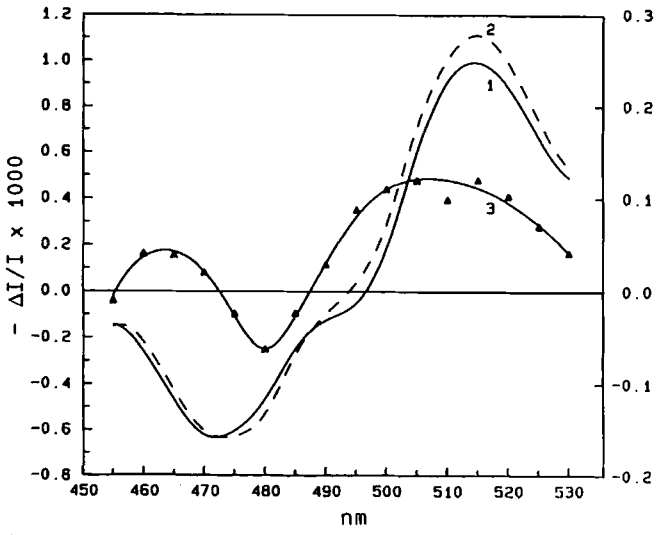

Fig. 4. Electrochromic absorption changes induced by a nonsaturating flash in S8 mutant strain. The absorption change was measured 200 μs after the sampling actinic flash, which hit 50% of the centers. Left scale, spectrum 1: 0.2 mM DCHC; spectrum 2: no addition. Right scale, spectrum 3: difference between spectra 2 and 1.

After continuous illumination for a time longer than approx. 4 min, a large steady-state membrane potential is reached which slowly decreases during a subsequent dark period. The light intensity chosen was sufficiently low (12 photons/center per s) not to induce any significant oxidation of P-700. The long lifetime (several minutes) of the light-induced membrane potential suggests that the cyclic electron flow around PS I significantly increases the ATP/ADP ratio.

It is noteworthy that the membrane potential is only one of the components of the electrochemical proton gradient $\Delta\tilde{\mu}_{H^+}$; nevertheless, the results we have obtained here are qualitatively in agreement with those previously reported [13], when we measured in vivo PS II luminescence or PS II back-reaction, which are stimulated both by the membrane potential and the proton gradient.

Characterization of electrochromic probes in various mutants strains

The flash-induced electrochromic absorption change was measured in the presence or in the absence of a permanent membrane potential induced in the dark by ATP hydrolysis.

Mutant S8 (Fig. 4). This mutant lacks PS II, but in contrast to mutant S52, has a normal chlorophyll antenna content. We observed that the electrochromic signal $\Delta I/I$(515–494 nm) induced by a saturating laser flash is about 6-times larger for mutant S8 than for mutant S52, when normalized to the same amount of reaction centers (P-700). In Fig. 4, spectra 1 and 2 are observed in the presence or in the absence of DCHC, respectively. Spectrum 3, which shows the difference between spectra 2 and 1, is identical to spectrum a in Fig. 1B obtained using mutant S52. The amplitude of the signal due to nonlinear probes after normalization to the same amount of reaction centers is comparable in both mutants. Thus, the weight of nonlinear signal compared to the linear signal is about 5–6-times lower in mutant S8 than in mutant S52. It is important to stress that even in algae with a normal chlorophyll antenna size, the contribution of nonlinear probes cannot be totally neglected. De Grooth et al. [14] have been able to measure both quadratic and linear electrochromic signals in swollen chloroplasts submitted to an externally applied electric field. They conclude that the quadratic response could be generally neglected, a conclusion which can be accepted only for low values of the membrane potential (< 100 mV).

Mutant S56 (Fig. 5). This mutant lacks PS I and the major fraction of the chlorophyll antenna. Diner and Joliot [15] have established that the efficiency of a PS II charge separation decreases in the presence of a membrane potential. We have actually observed that the number of charge separation, as seen by the flash-induced C550 signal, is 0.91-times lower in the absence

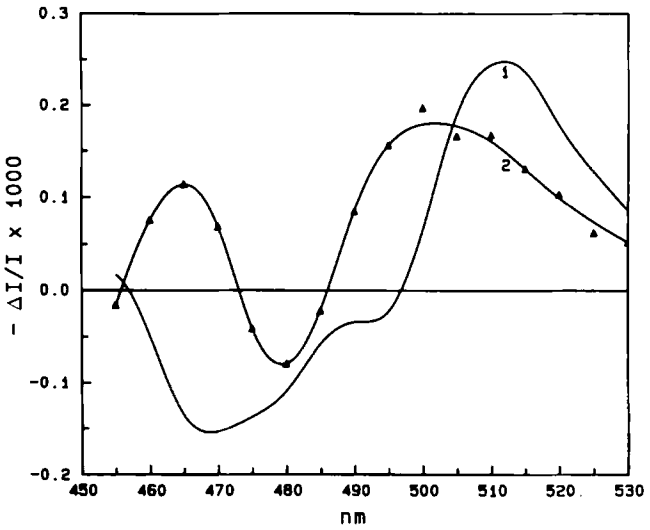

Fig. 5. Electrochromic absorption changes in S56 mutant strain. Actinic excitation and detection were measured as in Fig. 4. Spectrum 1: 0.2 mM DCHC; this spectrum was corrected for the signal due to C550, measured in the presence of 20 μM DCMU; spectrum 2: difference between the spectrum observed without addition (not shown) and spectrum 1. Spectra 1 and 2 are normalized to the same number of charge separations (see text).

than in the presence of DCHC which collapses the ATP-induced membrane potential. Spectrum 2 is the difference between the spectra obtained in the absence and in the presence of DCHC, respectively, after normalization to the same number of charge separations. Spectrum 2 is similar to spectrum a in Figure 1B. As in mutant S52, which also lacks a major fraction of the chlorophyll antenna, a large contribution from nonlinear probes is observed in mutant S56.

The linear and nonlinear probes were also characterized in mutant S30, which has PS I and PS II but lacks the major fraction of the chlorophyll antenna (data not shown). The relative contribution from nonlinear probes was about 3-times lower than in mutants S52 and S56. We can then exclude the nonlinear probes are associated with both photosystems.

We conclude that the nonlinear probes are not associated with any of the major chlorophyll complexes present in the membrane (PS I plus core antenna, PS II plus core antenna, all the presently identified LHC complexes). Therefore, the nonlinear probes must be associated with either a minor pigment-protein complex not affected by the mutation or free pigments embedded in the lipid bilayer. The spectrum of the nonlinear probes, which displays maxima at 464 and 504 nm and a minimum at 479 nm, suggests that the pigment involved is very likely a carotenoid.

The spectra of the linear probes in the various mutant strains (spectrum 1, Figs. 1A, 4 and 5) show certain similarities. The electrochromic probes associated with PS I (mutant S52) present a minimum at 472 nm and a maximum at 514 nm; for the probes associated with PS

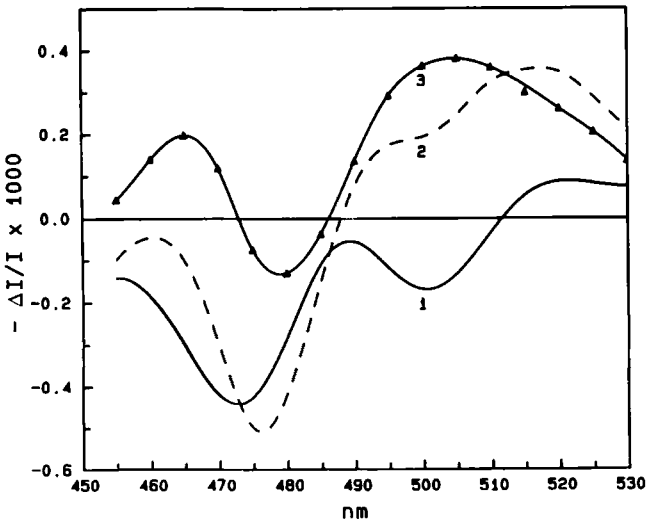

Fig 6. Electrochromic absorption changes in *C. reinhardtii* mutant strain BF4F34. Actinic excitation and detection as in Fig. 4. Spectrum 1: 12 μM TBT; spectrum 2: no addition; spectrum 3: difference between spectra 2 and 1.

II (mutant S56), the minimum and maximum are at 468 and 511 nm, respectively. In both cases, an additional positive peak around 486 nm is observed. In the case of mutant S8, the main contribution is due to the probes associated with LHC-protein complexes, with a minimum at 471 nm and a maximum at 514 nm. As already reported by Lavergne et al. [6], the shoulder around 490 nm is essentially associated with PS I probes.

It is rather surprising that for approximately the same light-induced membrane potential, the amplitude of the linear electrochromic changes are roughly proportional to the number of chlorophyll antennae. This result suggests that similar pigments present at the same concentration with respect to chlorophyll content and submitted to a local electric field of similar value are involved in all types of chlorophyll-protein complexes.

C. reinhardtii mutant BF4F34 (Fig. 6). The flash induced absorption changes were measured on a *Chlamydomonas* double mutant BF4F34, which lacks PS II and LHC complexes. In the case of *Chlamydomonas,* 0.2 mM DCHC does not fully collapse the membrane potential; on the contrary, 12 μM TBT was enough to abolish the permanent membrane potential. The spectrum of the nonlinear probes (spectrum 3) is almost identical to the corresponding spectra measured on *Chlorella.* On the other hand, the spectrum of the linear probes (spectrum 1) significantly differs, especially in the 500–530 nm range.

The characterization of nonlinear probes in the case of chloroplasts isolated from higher plants is a difficult task due to the difficulty to establish permanent membrane potential. When comparing the electrochromic spectra obtained with a weak or saturating flash excitation, we conclude that nonlinear probes contribute slightly to the signal, and preliminary experiments show that their spectrum presents a negative peak at approx. 472 nm. However, the spectrum of the nonlinear probes was determined using chloroplasts excited by external electric-field pulses [16,17]. In these experiments, performed in the absence of the optical device used in Ref. 14, only the quadratic electrochromic signal is detected as half the thylakoid membranes are polarized in either direction. The observed spectrum resembles that of non-linear probes in *Chlorella* and *Chlamydomonas* with a shift lower than 2 nm towards shorter wavelengths. Thus, the pigments involved in the nonlinear probes appear as highly conserved going from unicellular algae to higher plants. Nevertheless, our results obtained in different mutant strains with various antenna contents contradict the assumption proposed in Ref. 17 that all carotenoids in the membrane contribute to the quadratic electrochromic response.

Acknowledgements

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