Unreliability of carotenoid electrochromism for the measure of electrical potential differences induced by ATP hydrolysis in bacterial chromatophores

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Abstract ATP hydrolysis induces the activation of the proton ATPase in chromatophores of *Rhodobacter capsulatus* supplemented with nigericine and 50 mM K^+ (i.e. when $\Delta pH < 0.2$ units). The value of transmembrane electric potential ($\Delta\varphi$) driving this activation was measured using three different approaches: carotenoid electrochromism, uptake of SCN^- and responses of the dye oxonol VI. The value of $\Delta\varphi$ calculated from the SCN^- uptake, on the basis of an internal volume determined experimentally, was about 140 mV, while that indicated by the electrochromic signal ranged between 35 and 70 mV. Only the value indicated by SCN^- distribution is consistent with the energetic requirement for the activation of H^+-ATPase.

Key words: Membrane potential; Carotenoid electrochromic response; Thiocyanate uptake; H^+-ATPase activation

1. Introduction

Measurement of the electrochromic response of endogenous carotenoids is an extensively used method for evaluating the transmembrane electric potential difference in photosynthetic membranes ([1,2] and references therein). In chromatophores of purple photosynthetic bacteria (specifically of *Rhodobacter sphaeroides* and *Rb. capsulatus*) this method is particularly advantageous due to its speed of response, the osmotic stability of the vesicles and the absence of interferences by other optical signals. For these reasons carotenoid electrochromism has been used very extensively for kinetic and thermodynamic studies on primary and secondary photosynthetic electron transfer reactions, on the energetics of photophosphorylation [3–5] and on ion conductivity of chromatophore membranes in general [6,7].

The large electrochromic signal observed in bacterial chromatophores is due to the carotenoids present in the LH II antenna complex, and corresponds to a red shift of the absorption spectrum. The extent of the shift, and consequently of the amplitude of the absorption changes measured at appropriate wavelengths, is proportional, within a wide range, to $\Delta\varphi$. This linear response is thought to be due to pigment molecules electrically polarized when associated to the LH II apoprotein [8,9].

Calibration of the electrochromic optical signal in terms of electric potential difference is usually obtained by generating K^+-diffusion potentials in chromatophores treated with valinomycin [4,10], a procedure normally yielding a linear dependence of the absorption change on the Nernst's potential. It is assumed that the electrochromic signals generated by diffusion potentials or by the electrogenic reactions in chromatophores essentially reflect a potential difference between an inner and an outer phase isopotential throughout their volumes and, therefore, independent on the nature of the electrogenic reaction causing it. This view neglects any electrostatic phenomenon at the interfaces and any possible dishomogeneity along the membrane plane. It is on this basis that the use of electrochromism for energetic studies has been justified also for membrane enzymes other than the photosynthetic reaction center (a complex structurally related to the LH II voltage sensor). This model however has been challenged by several authors who have observed systematic inconsistencies between the evaluation of $\Delta\varphi$ by electrochromism and by more conventional ion distribution methods [11,12 and references therein] (see also section 4).

In this paper we demonstrate that in chromatophores of *Rb. capsulatus* the $\Delta\varphi$ values induced by illumination or by ATP-hydrolysis, as evaluated by electrochromic signals of carotenoids, seriously disagree with those evaluated using other methods. We show, moreover, that the electric potential difference generated by the ATPase reaction is sufficiently high to activate H^+-ATPase in the absence of any transmembrane ΔpH. A comparison of the $\Delta\varphi$ values measured with the different methods and the energetic requirement for ATPase activation clearly indicates that electrochromism grossly underestimates the value of $\Delta\varphi$ induced by ATP hydrolysis.

2. Materials and methods

Rhodobacter capsulatus strain GA was grown photoheterotrophically at high light intensity, harvested after 16 hours of growth and chromatophores were prepared and stored at −18°C as described in [13]. Due to the interference of Cl^- ions with the SCN^- reversible electrode, in all buffers used chloride salts were substituted with the corresponding acetates.

ATPase activity was measured with a glass electrode (Schott Thalamid Model 9218/7.0/300 MΩ) in an apparatus similar to that described in [14] and, occasionally, colorimetrically [13].

SCN^- uptake was measured potentiometrically with a SCN^--sensitive electrode (Orion, Model 94–58) mounted on the same apparatus used for the ATPase assay. Actinic illumination, provided through the bottom of the measuring cuvette and filtered through 1 cm of water and a Kodak Wratten 88A gelatine filter, had an intensity of 175 $W \cdot m^{-2}$. Stepwise concentration of thiocyanate from 1 to 15 μM were added to every assay in order to calibrate the response of the electrode under each experimental condition.

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Abbreviations: $a_{in,out}$, activities in the internal, external compartments; Bchl, bacteriochlorophyll; H^+-ATPase, ATP-synthase (EC3.6.1.34); LH II, light harvesting complex II; nig, nigericine; oli, oligomycin; val, valinomycin; $\Delta\mu_{H^+}$, transmembrane electrochemical potential difference for protons; $\Delta\varphi$, transmembrane electric potential difference.

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The carotenoid band shift was measured at 503–486 nm utilizing a dual wavelength spectrophotometer (Sigma Biochem ZWS-II) equipped with a cross illuminator identical to the one described above. Care was taken that the illumination of samples in this apparatus and the SCN^- electrode were comparable; this was obtained by compensating the optical paths of the samples in the two apparatus and adjusting the chromatophore concentrations. The intensity of the measuring light was kept as low as possible in order to avoid any actinic effect. Calibration of the electrochromic signal was performed with K^+-diffusion potentials, induced at high and constant ionic strength to minimize surface charge artefacts.

Oxonol VI absorption changes [15] were recorded in the same spectrophotometer at 587–601 nm with a final concentration of 3 μM oxonol VI.

The transmembrane pH difference was measured using 9-amino-6-chloro-2-methoxy acridine calibrated as described in [16].

EPR spectra of the nitroxide probe tempone (4-oxo-2,2,6,6 tetramethylpiperidine *N*-oxyl), employed for the determination of the internal volume of chromatophores, were recorded on a Bruker Model ESP 300 spectrometer.

3. Results

3.1. Activation of the ATPase by $\Delta\varphi$

H^+-ATPase in chromatophores of *Rb. capsulatus* can be activated by $\Delta\mu_{H^+}$ [14,17]. The activation can be observed upon addition of uncouplers as a transient high rate of the ATPase reaction that within tens of seconds decays to the low rate characteristic of the inactive, dark-adapted state. The dependence of this activation on $\Delta\mu_{H^+}$ is clearly demonstrated by the observation that addition of uncouplers prior to the generation of the protonic gradient prevents the onset of the activated state. Studies on the energetics of the activation process performed with artificially induced $\Delta\mu_{H^+}$ (acid-base transitions plus ion-diffusion potentials) have indicated that the stimulation of ATPase activity has a strongly sigmoidal dependence on $\Delta\mu_{H^+}$, with a threshold around 100–120 mV, below which no activation occurs [14].

The activation, besides being induced by the photosynthetic electron transfer or by artificial proton gradients, can be elicited by the hydrolysis of ATP catalyzed by the largely inactivated enzyme present in dark-adapted membranes. In previously published experiments [14], at pH = 8.5 and 20°C a maximum activity, equal to about nine times that of the inactive enzyme, was reached only after about three minutes of ATP hydrolysis. This behavior was observed in the absence of ionophores, when $\Delta\mu_{H^+}$ is partly formed by ΔpH and partly by $\Delta\varphi$. A similar activation kinetics is also observed in the presence of 50 mM K^+ and 2 μM nigericine (Fig. 1), namely under experimental conditions in which ΔpH is lower than 0.2 units (Fregni and Melandri, unpublished data). This functional test clearly indicates that in bacterial chromatophores the hydrolysis of ATP can promote a $\Delta\varphi$ well above 120 mV, the threshold value for activation.

3.2. Evaluation of the transmembrane electric potential

3.2.1. Carotenoid electrochromism. The hydrolysis of ATP induces a significant electrochromic signal (Fig. 2A) [18], whose extent is considerably lower than that induced by the photosynthetic reactions. On the basis of a calibration with K^+-diffusion potentials in the presence of 1–2 μM valinomycin, the value of $\Delta\varphi$ ranges between 35 and 70 mV, depending on the chromatophore preparation, while the membrane potential during actinic illumination transiently reaches values as high as 220 mV and

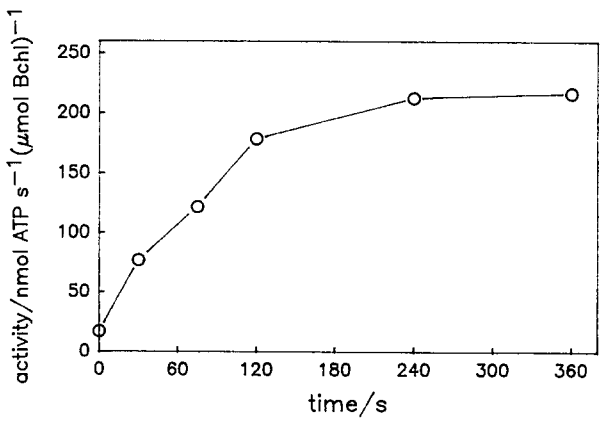

Fig. 1. Activation of H^+-ATPase by ATP hydrolysis in the absence of ΔpH. The assay conditions were: 2 mM Tricine, 50 mM K^+-acetate, 0.2 mM Na^+-succinate, 2 mM Mg^{2+}-acetate and 0.1 mM K_2HPO_4, 25 μM bacteriochlorophyll, 2.5 mM ATP, 1 μM nigericine, pH = 8.5. The temperature was 25°C. Initial rates of ATP hydrolysis were measured after the addition of 2 μM valinomycic. To calibrate the response of the electrode under each experimental condition, three additions of 160 or 320 nmol of HCl were done after every assay. Phosphate, added for stabilizing the active state of ATPase, was kept at suboptimal concentration to minimize interferences with the SCN^- electrode.

declines during prolonged illumination. In spite of its small amplitude the ATP-induced electrochromic signal meets all the requirements to be considered as being related to H^+-ATPase, since it is suppressed by uncouplers and oligomycin and is stimulated in rate and extent by nigericine.

3.2.2. Uptake of thiocyanate. A very large uptake of SCN^- can be observed during illumination or during ATP hydrolysis in bacterial chromatophores [12]. This uptake is consistent with the equilibration of this anion to an electric potential difference, positive inside the vesicles and generated by electrogenic ion pumps, insofar as it is prevented by uncouplers or by 2 μM valinomycin and 50 mM K^+, and inhibited by specific inhibitors of the pumps (Fig. 2B). Contrary to the electrochromic signals, the extents of the uptake induced by light or by ATP are comparable, suggesting that the membrane potential generated by the H^+-ATPase reaction in chromatophores is significantly larger than that evaluated on the basis of the carotenoid electrochromism. The addition of oligomycin completely prevents SCN^- uptake induced by ATP hydrolysis, whereas the uptake induced by light is strongly stimulated.

3.2.3. Optical changes of oxonol VI. The anionic dye oxonol VI also responds to illumination or to ATP hydrolysis with a very large optical signal and exhibits inhibitor characteristics consistent with a $\Delta\varphi$-sensitive response without interferences by the endogenous pigments [15]. Also in this case the amplitudes of the responses to light or to ATP are comparable, indicating that the two pumps operating in chromatophores (i.e. the photosynthetic chain and H^+-ATPase) can generate comparable membrane potentials (Fig. 2C).

The three sets of measurements of the $\Delta\varphi$ generated in bacterial chromatophores are therefore in disagreement, since the electrochromic signals indicate a very large potential induced by illumination and a very low potential generated by the hydrolysis of ATP, while both the uptake of SCN^- and the optical response of oxonol VI suggest that the potential maintained in the steady state by the two pumps is rather similar. It becomes

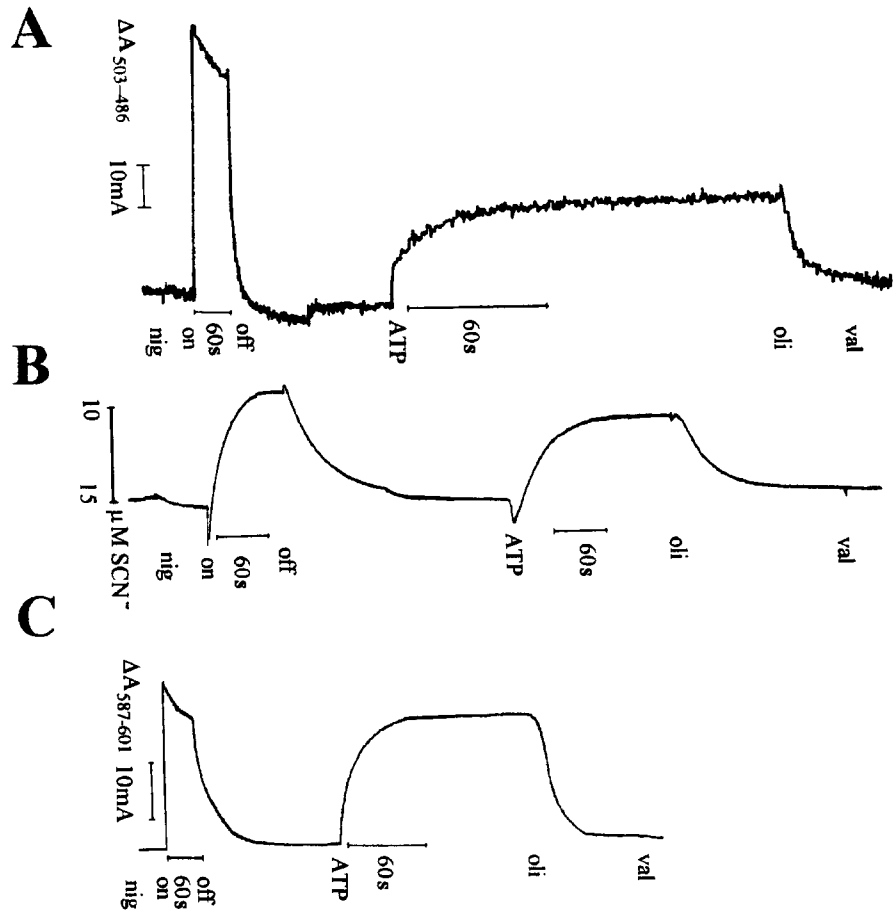

Fig. 2. $\Delta\varphi$ formation induced by light and by ATP hydrolysis in the presence of 1 μM nigericine measured with three different methods. Assay conditions as in Fig. 1 except that the tricine concentration was increased to 10 mM. bacteriochlorophyll was 20 μM, 100 μM or 30 μM in experiments A, B and C respectively. Additions: 2.5 mM ATP, 15 μg/ml oligomycin, 2 μM valinomycin. (A) Carotenoid electrochromism; (B) thiocyanate uptake, KSCN concentration equal to 15 μM. Light *on* and *off* caused electric artifacts on the electrode and ATP addition caused the lowering of the baseline; these artifacts were subtracted in the calculation of the final concentration of thiocyanate inside the chromatophores. (C) oxonol VI absorption changes measured at 587–601 nm.

therefore very important to evaluate quantitatively the $\Delta\varphi$ measured by one of the two methods other than electrochromism. Given the intrinsic difficulties in calibrating the response to $\Delta\varphi$ of a lipophilic anionic dye like oxonol VI [15], the SCN^- response was studied in greater detail.

3.3. Testing of thiocyanate as a probe of $\Delta\varphi$

The cross-membrane distribution of a permeant monovalent anion, such as SCN^-, is based on the very definition of electrochemical potential , the activity ratio between the inner and outer compartments being a direct measure of $\Delta\varphi$ according to the equation:

$$\Delta\varphi_{(in-out)} = RT/F \ln(a_{in}/a_{out})$$

Our method is based on the potentiometric measurement of the activity of SCN^- in the outer buffer. The estimation of the activity ratio rests on a mass balance for total SCN^-, from which the amount of internalized anion is obtained. The activity in the inner lumen of the vesicles can be consequently calculated if the inner aqueous volume of the chromatophores is known, if the amount of anion bound to the membrane lipids can be estimated or neglected and if the activity coefficient for SCN^-, in a concentration range spanning the outside and inside concentrations, can be at least approximately evaluated.

Calibration of the SCN^- electrode in the assay buffer demonstrated that the response of the electrode is nearly Nernstian (approximately 46 mV per concentration decade) from 10 μM to 30 mM suggesting that in this concentration range and in acqueous solution the activity coefficient is rather constant.

The internal volume of the same preparation of chromatophores utilized in the experiments described above was measured with the nitroxide probe tempone using $K_3Fe(CN)_6$ as an external quencher [19]. The EPR spectrum of the internal tempone (less than 1 percent of the total) did not show any line broadening, indicating that the inner lumen of chromatophores contains a true aqueous compartment, in which a hydrophilic solute like tempone does not interact with other molecular structures.

The osmotic behavior of the vesicles, in the presence of $K_3Fe(CN)_6$ ranging from 25 to 150 mM, is demonstrated in Fig. 3: at high osmolarity (>300 milliosmolar, corresponding to 75 mM ferricyanide) the inner volume is proportional to the reciprocal of the osmolarity, indicating osmotic shrinking of the chromatophores. At lower osmolarity the volume is progres-

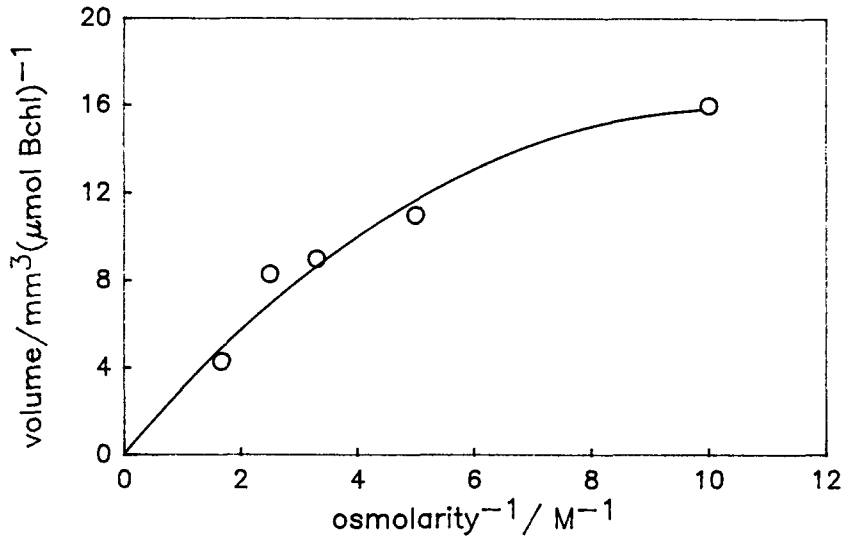

Fig. 3. The internal volume of chromatophores at different medium osmolarity. Chromatophores were washed to remove the glycerol and resuspended in a buffer containing 10 mM Tricine, pH 8.5, 50 mM K^+-acetate, 2 mM Mg^{2+}-acetate with 1 mM Tempone and concentrations of the quencher $K_3Fe(CN)_6$ ranging from 25 to 150 mM.

sively lower than the ideally expected value, possibly due to the counteraction of the surface tension to the osmotic stretching of the membrane bilayer. At low osmolarities (< 100 milliosmolar) a limit value of 16 ± 3 mm^3 (μmol bacteriochlorophyll)$^{-1}$ is approached. This limit value was used to calculate the inner concentrations.

The permeability of the chromatophore membrane to SCN^- was evaluated by measuring the decay of the electrochromic signal generated by one single turnover flash; the acceleration of the decay rate at increasing SCN^- concentrations indicated a rather low permeability ($P_i = 2–3 \times 10^{-8}$ cm·s^{-1}). Measurements of the resistivity of planar lipid membranes confirmed that the permeability of SCN^- in this model system was not significantly different from that of Cl^- (A. Gliozzi, unpublished data). This small permeability coefficient, being proof of a low solubility of SCN^- in the membrane lipids, reinforces our conclusions on the reliability of a distribution ratio based on the mass balance of the anion. It demonstrates however that SCN^- is a poorly permeant anion that will equilibrate only slowly with $\Delta\varphi$ [11,12]. The traces in Fig. 2 show that the half time for the uptake induced by ATP hydrolysis is about 30 seconds, and 10 seconds during illumination. Under steady state conditions, the extent of the uptake is, however, stable for minutes and decays completely only when the action of the proton pump is stopped or the $\Delta\varphi$ dissipated. These characteristics of the SCN^- uptake make this technique totally unsuited for $\Delta\varphi$ transient, for which the only kinetically competent tool remains the carotenoid electrochromism. The sluggishness of the SCN^- method does not affect, however, its validity during steady states. The low solubility of SCN^- in the membrane was also demonstrated in partition experiments performed by centrifugation of the vesicles.

The equilibration of SCN^- with $\Delta\varphi$ was tested by two additional experiments:

(a) $\Delta\varphi$ was measured at different concentrations of SCN^- ranging from 10 to 100 μM. The amount of thiocyanate taken up during steady state was approximately proportional to the initial SCN^- concentrations, yielding comparable $\Delta\varphi$ values. A progressive but small decrease of $\Delta\varphi$ was evident at increasing SCN^- concentration, indicating a limited uncoupling effect of this anion (data not shown);

(b) $\Delta\varphi$ was evaluated in the presence of 0.33 M sucrose, i.e. with an internal volume two-fold reduced due to the osmotic shrinkage of the vesicles (Fig. 3). Under these conditions uptake was also proportionally reduced, yielding values of $\Delta\varphi$ coincident with those measured at low osmolarity. This critical experiment strongly supports the view that the anion taken up is mainly transferred into an osmotically active internal lumen.

The values of $\Delta\varphi$ in the steady state during illumination or ATP hydrolysis in chromatophores evaluated on the basis of the electrochromic responses or of the uptake of SCN^- are compared in Table 1. It is clear that the two sets of data are unreconcilable.

Table 1
Comparison of the $\Delta\varphi$ evaluated from the electrochromic signal and from the uptake of thiocyanate under different experimental conditions

	$\Delta\varphi$/mV	
	Electrochromism[a]	Thiocyanate uptake
Light (t = 2 s)[b]	236 ± 15	–
Light (t = 60 s)[b]	191 ± 15	160 ± 7
ATP	67 ± 7	148 ± 7
Light + oligomycin (t = 60 s)[b]	228 ± 15	173 ± 8
ATP + oligomycin	< 15[c]	< 50[c]

[a]In all measurements of electrochromism 15 μM SCN^- was present; this addition was however uninfluential on the extent or kinetics of the electrochromic responses. [b]The values of $\Delta\varphi$ from the electrochromic signals are indicated at two different times of illumination in order to allow a direct comparison with the slowly responding thiocyanate uptake. [c]Estimated lowest detectable values.

4. Discussion

The results reported here demonstrate the marked disagreement existing in the values of $\Delta\varphi$ measured in chromatophores using different techniques; the disagreement is particularly evident when $\Delta\varphi$ is induced by the hydrolysis of ATP. It is our opinion that the results obtained with the SCN^- electrode are more reliable, especially during steady states or for slowly formed $\Delta\varphi$ like those induced by ATP, since they are based on the following testable conditions:

(a) chromatophores possess an inner aqueous compartment whose volume was directly measured;

(b) SCN^- behaves as a slowly permeant anion, and its partitioning into membrane lipids is small enough to have a negligible effect on the mass balance on which the value of $\Delta\varphi$ is based;

(c) the activity coefficient of SCN^- is rather constant between 10 μM and 30 mM, so that its ratios for the inner and outer compartments remains about one for all values of $\Delta\varphi$ measured.

On this basis it can be inferred that the value of $\Delta\varphi$ generated by the H^+-ATPase is rather large (about 130 mV), but that this potential is scarcely sensed by the electrochromism of LH II carotenoids. This conclusion is further strengthened by the observation, in our opinion decisive, that the activation of ATPase can be promoted when the ATP-induced $\Delta\varphi$ is the only component of $\Delta\mu_{H^+}$. The activation of H^+-ATPase occurs in fact only at $\Delta\mu_{H^+}$ higher than 120–130 mV.

The disagreement between the potentials indicated by the electrochromic signals and by the distribution of permeant ions in bacterial chromatophores has been documented previously [11,12]. Similarly, in intact cells the uptake of butyl-triphenylphosphonium indicated a light- or respiration-induced $\Delta\varphi$ significantly lower than that measured by carotenoids [20]. The novelty of our contribution consists in the direct comparison made here between the values of $\Delta\varphi$ generated by a single H^+ pump, and estimated using different approaches, and a functional test of the same pump sensing $\Delta\varphi$.

A heterogeneity in the distribution of ion pumps and of $\Delta\varphi$-sensing carotenoids in the vesicle population has been often considered as a possible explanation for the low electrochromic signal induced by ATP hydrolysis [12]. In the chromatophores used in this work the rate of light-induced ATP synthesis was approximately 500 nmol ATP$\cdot s^{-1}$(μmol Bchl)$^{-1}$, close to the maximal rates of hydrolysis when the enzyme was fully activated. This very high rate is incompatible with the existence of a large fraction of vesicles possessing H^+-ATPase but deprived of LH II, the only possibility to reconcile large SCN^- uptakes and small electrochromic signals. Moreover, an extensive delocalization of the coupling mechanisms has been previously demonstrated with double inhibition experiments performed with such highly active chromatophores [21], indicating the coexistence of several H^+-ATPases and photosynthetic chains in the same vesicles. The possibility of a heterogeneity in the vesicle composition should be however directly investigated. Such a heterogeneity would possibly aggravate, rather than reduce, the disagreement between electrochromism and ion distribution.

The demonstration that large amounts of SCN^- are accumulated in an osmotically active internal volume, yielding distribution ratios rather independent of the external concentration, represents strong evidence that the $\Delta\varphi$ generated is delocalized in the whole internal lumen. Yet this bulk-to-bulk electric potential difference seems only marginally sensed by the electrochromic response of carotenoids, although the same response has been calibrated with a diffusion potential, supposedly also between bulk phases. Suggesting a solution for this paradoxical conclusion is difficult. What seems possible to us is that the electrical field within the membrane (and at the water-membrane interfaces) might not be constant, but might depend on the actual distance from sources and sinks of ionic currents [22]. This dishomogeneous potential might be sensed to a different extent by localized voltage sensors like LH II carotenoids. It is moreover likely that the voltage profiles within the membrane are significantly perturbed by the K^+-valinomycin complexes during calibration (as they certainly are by other lipophilic ions [23]), so that the Nernstian response of electrochromism is only apparent.

In a previous paper from our laboratory [24] the carotenoid signals induced by a flash in phospholipid-enriched chromatophores were used for the evaluation of the charging capacitance of the vesicles as a function of the lipid-protein ratio. A reasonable value of the capacitance equal to 0.5–0.6 $\mu F\cdot cm^{-2}$ was obtained. The interpretation of these experiments required that carotenoids sense a flash-induced delocalized $\Delta\varphi$. The conclusions of this study are not invalidated, in any case, if the voltage profile along the membrane plane were not homogeneous.

The observations reported here impose, in any case, a critical revision of the use of carotenoid electrochromism as a generalized voltmeter and amperometer across chromatophore membranes.

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