DETECTION OF A NEW PROTEIN INVOLVED IN THE ELECTROGENIC LOOP OF GREEN ALGAE

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1. Introduction

In addition to the two photochemical reactions, there exists in green algae [1,2] and higher plants [3,5], a third electrogenic reaction. This electrogenic reaction which occurs in the ms range after photochemical reactions, increases the transmembrane electric field and is revealed by an increase of the electrochromic effect at 518 nm [1–5].

This extra phase occurs when the electrons are transferred through the cyclic pathway, as well as when they are transferred in a linear pathway [1,6] thus indicating that only photosystem I is required.

The amplitude of the slow electrochromic rise, phase b (according to the terminology in [1]), can be varied in four ways: (i) addition of an oxidant; (ii) addition of a reductant; (iii) addition of DBMIB; (iv) preillumination with a few flashes.

The inhibition of phase b by oxidants, which can be suppressed by further addition of reductants, indicates that phase b depends on a compound in the reduced form [2,7].

The sensitivity to DBMIB [2], confirmed in chloroplasts [8], indicates that some quinones are involved in the pathway generating phase b.

The inhibition of phase b by a preillumination with a few flashes in *Chlorella* cells [1,2] was at first interpreted as locating phase b on a sidepath [2]. However, by measuring the stochiometry of the protons in chloroplasts, the electrogenic loop was shown to occur under stationary conditions of illumination [4]. It was concluded [4] that the electrogenic loop was involved in the main pathway of the electron transfer chain. This paper argues in favor of the conclusion in [4].

The inhibition of phase b by a preillumination with a few flashes would occur when the energy liberated by the reactions coupled to the electrogenic step is no more sufficient to draw an electron across the membrane against the transmembrane electric field [7]. The larger the transmembrane electric field, the larger the inhibition [7].

The four independent treatments which vary the amplitude of phase b have been used to correlate the reduction of the donors of photosystem I in the ms range after a photochemical reaction with the occurence of the electrochromic rise in the same time range [2]. This correlation has now been confirmed in chloroplasts [4,8].

These treatments will be used here to correlate the photoinduced redox changes of a new protein with the existence of phase b.

Abbreviations: DBMIB, 2,5-dibromo-3-methyl-6-isoproyl-1,4-benzoquinone; DCMU, 3(3,4-dichlorphenyl)-1,1-dimethylurea; DAD, 2,3,5,6 tetramethyl-*p*-phenylenediamine

2. Materials and methods

Chlorella pyrenoidosa was grown on Knopp medium (9) to which were added Arnon's trace elements A_5 and B_6 [10]; the culture was illuminated by white fluorescent light of 3000 lux. Before use, cells were resuspended in 0.1 M phosphate buffer (pH 7) containing 7% Ficoll. To avoid any redox changes due to photosystem II, hydroxylamine (10^{-4} M) and DCMU (10^{-5} M) were added (except for table 1 and fig.1) to the cells which were preilluminated before dark adaptation [11].

Absorption changes (ΔA) were measured using the flash detector differential spectrophotometer constructed by P. J., D. B. and B. F. [12]. With this appa-

ratus, the standard error on $\Delta I/I$ for n averages is $\sigma = 5 \times 10^{-5}/\sqrt{n}$. The error margin used was $\pm 2\,\sigma$. The sample was renewed before each cycle to get dark-adapted material.

The optical path of the cuvette was 16 mm. The chlorophyll concentration was chosen so that the $\Delta A_{400-600}$ was a linear function of the concentration of algae. The actinic xenon flashes (Verrerie Scientific type CA 972) were screened by red filters (Schott RG 2+ Wratten 35), and had a half-time of 1 μs giving a mean value of 1.2 photochemical turnovers/system I center. Complementary filters (Schott BG 38) protected the photocells.

At any wavelength and at any time, the ΔA due to the redox reactions of photosystem I, are mixed with the ΔA due to the electrochromic effect which thus have to be subtracted. For dark-adapted material it was shown [13,14] that the $\Delta A_{400-600}$, at long times after a flash, are only due to the electrochromic effect. This observation was again tested using agents which slow down (tri-*N*-butyltinchloride) or accelerate (dicyclohexyl-18-crown-6) the decay of the transmembrane electric field (B. Diner, unpublished). At >100 ms after a flash nothing but the electrochromic effect could be observed and 200 ms was chosen as the reference time to draw the spectrum of the electrochromic effect.

The exact kinetics of the electrochromic effect are given by the ΔA difference:

$$\frac{\Delta I}{I}(515\text{ nm}) - \frac{\Delta I}{I}(530\text{ nm}) \qquad [15]$$

Once the spectrum and the kinetics of the electrochromic effect are known, and assuming that its kinetics are independent of the wavelength, it is possible to compute the electrochromic effect at every time and at every wavelength, and subtract it from any measurement.

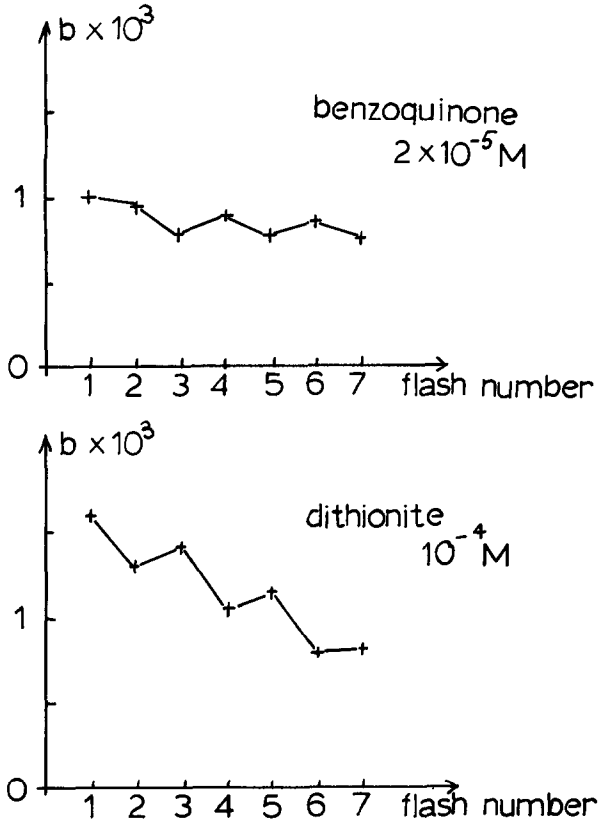

Fig.1. Amplitude of phase b (corrected for the following electric field decay) during a series of flashes spaced by 125 ms on *Chlorella* cells. Average of 256 series fired on the same sample once every 6.4 s. Top trace, benzoquinone 2×10^{-5} M; bottom trace, dithionite 10^{-4} M.

3. Results and discussion

In the absence of DCMU, the amplitude of phase b during a series of closely spaced flashes oscillated with period 2 (fig.1). The maxima occur on even flash numbers in the presence of benzoquinone and on odd flash numbers in the presence of dithionite. The oscillating pattern is suppressed by DCMU. This indicates that, in the absence of DCMU, the electrons arriving on the reductant required for the electrogenic phase, originate from the secondary acceptor of system II, B [16,17]. However, the still efficient generation of phase b in the first few flashes in the presence of DCMU [2] indicates that the reductant may also receive electrons from the acceptors of photosystem I when system I is working under cyclic conditions.

The electrogenic loop is thus located in the main pathway under both linear and cyclic conditions. To this assertion, should be added, according to [7], that the main pathway of the electron transfer under both linear and cyclic conditions may by-pass the electrogenic loop when the energy liberated by the reactions coupled to the electrogenic step is no more sufficient to draw an electron across the membrane against the electric field and proton gradient.

Fig.2 (open circles) presents the ΔA, corrected for

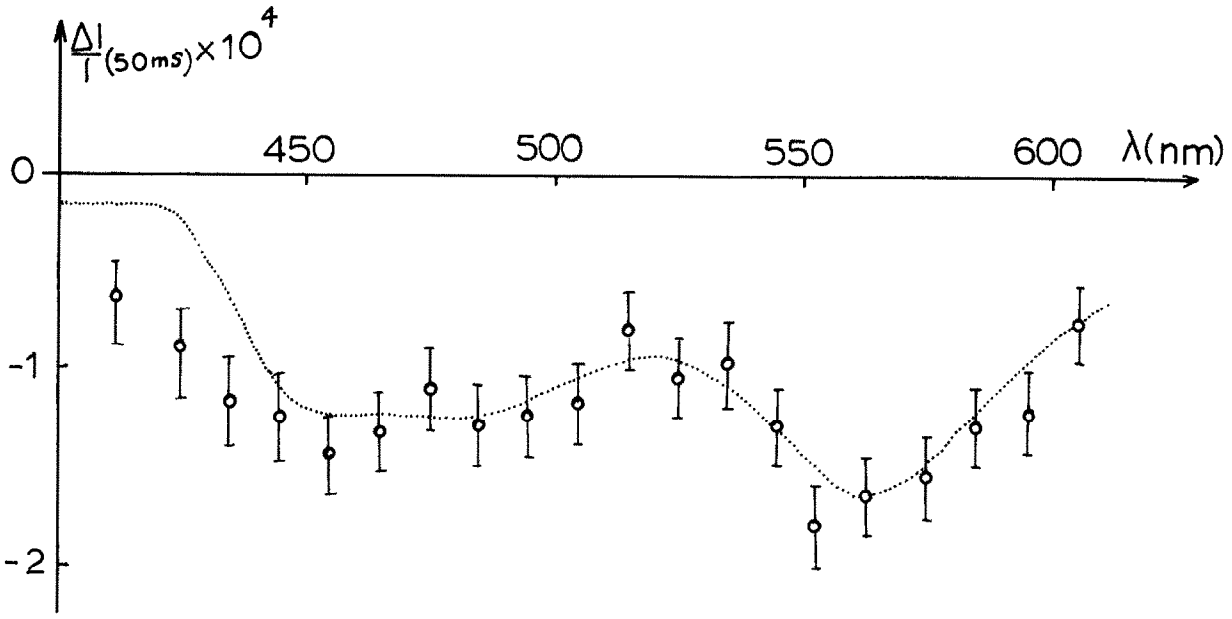

Fig.2. Open circles: ΔA 50 ms after a flash (after correction for the electrochromic effect) as a function of the wavelength. Average of 100 flashes. Hydroxylamine 10^{-4} M and DCMU 10^{-5} M. Dotted line: reduced minus oxidized spectrum of the Rieske protein, drawn from [Rieske, J. S., Maclennan, D. H. and Colemann, R. (1964) Biochem. Biophys. Res. Commun. 15, 338–344].

the electrochromic effect, observed 50 ms after single flash excitation of dark-adapted material.

This signal probably reflects the redox change of only one species as it behaves homogeneously from a kinetic point of view. Let us call C the compound, the redox change of which is revealed by these ΔA.

The photoinduced redox changes of C are inhibited when oxidants are added and restored by further addition of reductants (table 1) as is the electrochromic phase in the ms range (phase b). Like phase b, they are inhibited by DBMIB (table 1) and 50% inhibition is obtained for the two phenomena at $\sim 5 \times 10^{-6}$ M. Further addition of reductant restores none of the two phenomena (table 1). Both phenomena are inhibited by preillumination with a few flashes. In this experiment, tri-*N*-butyltinchloride, which slows the decay of the electrochromic effect (B. Diner, personal communication), had been added to allow better detection of phase b and better inhibition after a few flashes [7].

Table 1
Phase b and signal due to the compound C in dark-adapted *Chlorella* cells with tri-*N*-butyltin chloride 10^{-5} M

Flash no.	Additions	Phase b	C
	None	+	+
	DAD 5×10^{-5} M	–	–
	$+K_3Fe(CN)_6$ 2×10^{-4} M	–	–
1	DAD 5×10^{-5} M $+K_3Fe(CN)_6$ 2×10^{-4} M $+Na_2S_2O_4$ 2×10^{-2} M	+	+
	DBMIB 10^{-5} M	–	–
	DBMIB 10^{-5} M $+Na_2S_2O_4$ 2×10^{-2} M	–	–
9	None	–	–

Table 1 indicates that C redox changes are correlated to the existence of phase b in conditions where the photoreactions themselves are not inhibited, and suggests that C is involved in the section of the transfer chain which includes the electrogenic process.

It was thus interesting to compare the kinetics of phase b of the electrochromic effect, which has already been kinetically linked to the reduction of cytochrome *f* [2,4,8], with the kinetics of the new compound (fig.3). Redox changes of cytochrome *f* are characterized by the change:

$$\frac{\Delta I}{I}(545\text{ nm}) - \frac{\Delta I}{I}(553\text{ nm})$$

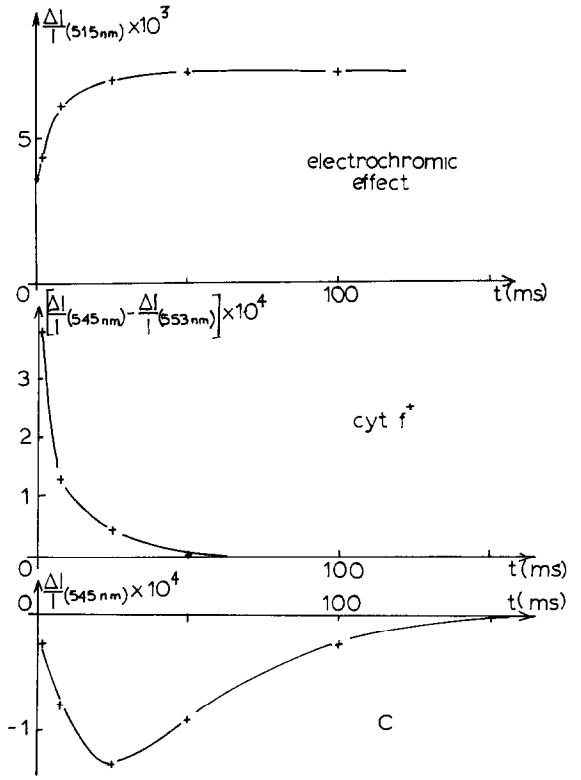

Fig.3. Absorption changes or difference of absorption changes as a function of the time after a flash (after correction for the electrochromic effect). Average of 100 flashes. Hydroxylamine 10^{-4} M and DCMU 10^{-5} M.

after correction for the electrochromic effect [13]. The changes due to the new compound C were detected at 545 nm, an isobestic point of cytochrome *f*. At this wavelength, at >1 ms, there are no ΔA associated with system I acceptors [14]. The reduction of $P700^+$ is also over at 1 ms [15] and the only additional contribution at 545 nm after 1 ms is a small change due to plastocyanin which leads to a slight under estimation of the signal due to C before 20 ms. From fig.3, it then appears that the new signal is formed in the ms range as is the reduction of cytochrome f^+ and phase b of the electrochromic effect. The difficulty of these measurements however does not allow more precise kinetics. The ΔA due to C disappear with a half-time of ~40 ms.

At >100 ms after the flash, no other compounds could be detected under these conditions.

More precise conclusions would require knowledge of whether the bleaching is due to an oxidation or to a reduction of C. A comparison of the difference spectrum of C (fig.2, open circles) with that of the Rieske protein [18] corrected for the 'particle flattening effect' due to the cells [19–21] (fig.2, dotted line) reveals a close resemblance between the two spectra, indicating that C could be a new iron–sulfur protein. The potential of C (C, if bleaching corresponds to a reduction, is oxidized in the dark even when the plastoquinone pool of midpoint potential around 100 mV is mainly reduced) excludes the possibility that C is the Rieske protein (midpoint potential +290 mV [22]).

If bleaching corresponds to a reduction of C which would in particular be the case if C is an iron–sulfur protein, the re-reduction of the donors of system I [2] and the reduction of C are both correlated to phase b. Let us call U the reductant required for phase b [2,7]. These results are difficult to interpret unless U is a two electron carrier. The reactions would then be written, without taking into account the protons :

$$U^{2-} + X_1^+ \rightleftharpoons U^- + X_1 \quad (1)$$

$$U^- + C \rightleftharpoons U + C^- \quad (2)$$

Reduced U would give a first electron to an oxidized donor of photosystem I represented by X_1^+ in reaction (1). X_1 could be the Rieske protein [23–26]. This reaction would lead to a semiquinone form of U which would have a potential sufficiently low to reduce C in reaction [2]. This would be the electrogenic reaction.

A recent titration of the reductant responsible for phase b also indicates that it is a two electron carrier and is consistent with this scheme (D. Crowther, personal communication).

In this reaction scheme, the inhibitory effect of DBMIB, a quinone antagonist [27], suggest that U is a quinone. The potential found for U (40 mV more positive that the plastoquinone pool in *Chlorella* cells [7]), would make U very similar to the bacterial Z [28,29].

If bleaching corresponded to an oxidation of C, C would be the reductant required for phase b [2,7]. This would lead to more complicated reaction schemes and eliminate the analogy with bacteria. Though it is not possible to exclude this hypothesis until the biochemical nature of C has been identified, it seems less likely.

In chloroplasts, cytochrome b_6 was reduced by electrons originating from photosystem II acceptors [30]; cytochrome b_6 could be involved in the electrogenic reaction itself [30]. But no redox changes of cytochrome b_6, correlated with phase b, could be measured in *Chlorella* cells under physiological conditions or in the presence of hydroxylamine and DCMU. This result and the absence of inhibition of phase b by antimycin on the first flash after dark adaptation in chloroplasts (unpublished) indicates that cytochrome b_6 is not involved in the electrogenic reaction itself. The experiments in [30] may be interpreted with the reaction scheme proposed here if, in chloroplasts, cytochrome b_6 can be reduced by C^-. The absence of redox changes of cytochrome b_6 in *Chlorella* cells could indicate either that the physiological acceptor to C^- is not cytochrome b_6 and that cytochrome b_6 reacts with C^- only when the physiological acceptor is inaccessible, or that, in *Chlorella* cells, the reoxidation of cytochrome b_6 is faster than its reduction.

4. Conclusions

A new protein C has been detected by optical spectroscopy in the visible range. It could be a new iron–sulfur protein.

The redox changes of C are correlated to the existence of an electrogenic reaction in the ms range.

A reaction scheme is suggested in which a special quinone U would transfer one electron to the oxidized donors of photosystem I and the second one to C in an electrogenic reaction.

The biochemical identification of C is required to go further.

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