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Patch-clamp study on flash-induced secondary electrogenic transport in the thylakoid membrane. Interpretation in terms of a Q-cycle

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Abstract

Photo-electrical signals are studied using a patch-clamp method on single chloroplasts of *Peperomia metallica* in the so-called 'whole-thylakoid' configuration. The thylakoid transmembrane potential after a single-turnover flash shows multiphasic kinetics which can be described as the sum of two components: (1) Reaction R1/RC associated with fast primary charge separation in the reaction centers of photosystems I and II and (2) Reaction R1/Q, a slow rising component most likely associated with secondary electrogenic steps within the cyt. b_6f complex. The latter assignment is supported by the inhibition of the slow rising component by 2.5 μM DBMIB. The dark relaxation time of both R1/RC and R1/Q associated with the field dissipative back flow of ions was independently and unambiguously obtained from the discharge RC-time after current-injection. Reaction R1/Q shows sigmoid rise kinetics which is modelled by two populations of cyt. b_6f complexes incorporating a 2-step consecutive reaction scheme. The average time constants associated with the two steps are 13 ms and 28 ms and might be related to plastoquinol oxidation at the lumenal interface and plastoquinol formation at the stromal interface, respectively. The ratio of charges displaced by R1/Q and R1/RC (Q/RC-ratio) varied between 0.15 and 0.95 depending, amongst others, on pre-treatment with flashes.

Keywords: Kinetics; Membrane potential; *Peperomia metallica*; Photocurrent; Patch-clamp; Q-cycle

1. Introduction

Excitation of the light harvesting complexes of photosystem (PS) I and II results in the formation of the primary charge-separated state in the respective reaction centers within about 300 ps [1,2]. The ensuing redox reactions, including those associated with proton binding and release at the thylakoid membrane interfaces, cause the formation of a transmembrane electrical potential and a proton gradient which serve as the electrochemical driving-force for the formation of ATP by the ATP synthase. In addition to the fast primary electrogenic events in the reaction centers, a slow secondary electrogenic process involving the cyt. b_6f complex has often been discerned, having the same orientation as primary charge separation [3–6]. In this secondary electrogenic step, generally modelled as a Q-cycle [3,7] and modifications thereof

Abbreviations: b_l (b_h), low and high potential *b*-cytochromes in cyt b_6f complex; DBMIB, 2,5-dibromo-3-methyl-6-isopropyl-p-benzoquinone; FeS, Rieske iron-sulfur center; PQ, plastoquinone; PQH_2, plastoquinol; PS, photosystem; Q_p, quinol oxidising site of cyt. b_6f complex; Q_n, quinone reducing site of cyt. b_6f complex.

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[7–10], a difference in redox energy between two *b*-cytochromes is coupled to transverse electron transport from lumen to stroma [11]. Besides these two major electrogenic events other thylakoid membrane related processes might be operative and add to the light-induced transmembrane electrical field, e.g., ATP synthase activity [12,13].

The dispute of a functional Q-cycle in chloroplasts continues unabated. In general, the electrogenic nature of a Q-cycle concept is inferred from the kinetic correlation of the slow electrochromic phase of P515 with the turnover of the b_6f complex, strengthened by the suppression of this slow phase by specific Q_p-site inhibitors like DBMIB or stigmatellin [5,14,15]. The interpretation of the decay kinetics of the P515 response is, however, not unambiguous and is hindered by non-specific flash-induced absorbance changes taking place concurrently. This may lead to an incorrect estimation of the electrogenic reactions associated with the cyt. b_6f complex [16]. For example, the slow decaying phase defined as Reaction 2 [17], a non-electrogenic reaction, needs to be taken into account when extracting a Q-cycle-type component from the slow P515 phase [6].

The Q-cycle concept was challenged by Furbacher et al. [18], who found a too small redox potential difference between the two *b*-hemes to allow for efficient electron transfer in an operative Q-cycle. This was contradicted by Kramer and Crofts [15], who demonstrated the existence of rapid electron transfer between the quinol oxidising site (Q_p) and cyt. b_h as is demanded for by a Q-cycle mechanism. A more straightforward and independent technique will therefore be desirable to settle some of these controversies on the electrogenic steps within the cyt. b_6f complex.

Progress in the detection and analysis of light-induced thylakoid membrane potentials has been achieved recently by the introduction of a patch-clamp method [19,20]. With this method, the extent of the flash-induced membrane potential can be measured on a single chloroplast, along with the decay relaxation kinetics of the created electrical field. The patch-clamp method is particularly suitable for the study of light-induced electrical phenomena using single-turnover flashes because (1) signal-averaging is not required considering the sufficient signal-to-noise ratio and (2) the electrical response is directly proportional to the thylakoid membrane potential, without interfering contaminations from non-electrogenic processes (e.g., P515, see Ref. [17]).

This paper reports on a secondary electrogenic event which results in a multi-phasic photopotential response detected in single *Peperomia metallica* chloroplasts. Emphasis is laid on a detailed kinetic analysis of the slow electrogenic phase. The results are discussed in the light of existing Q-cycle models for b_6f turnover.

2. Materials and methods

All experiments were performed on single chloroplasts isolated from *Peperomia metallica*. The isolation was done by cutting a leaf with a razor blade in isolation medium composed of 330 mM sorbitol, 50 mM KCl, 0.5 mM $MgCl_2$, BSA 0.5% (w/v) and 10 mM Hepes/KOH (pH 7.5). The chloroplasts were dark-adapted for 5 min prior to starting a measurement. A Superfusion Drug Application Device (DAD-12, Adams and List Associates, Westbury, NY, USA) allowed for a controlled flow of medium, where indicated poised with DBMIB, along a single chloroplast.

Electrical potentials and currents were detected with an EPC-7 patch-clamp amplifier (List Electronics, Darmstadt-Eberstadt, Germany) in current-clamp or voltage-clamp mode, respectively. In both measuring modes the responses were filtered electronically by a 10 kHz low-pass Bessel filter and digitised with a sampling rate of 2 kHz. Process control and data acquisition were accomplished by the program MEAM (Lovoan, Wageningen, The Netherlands) running under MS-DOS on a personal computer. Square pulses used for current-injection were obtained from a pulse generator (Tektronix, Oregon, USA). The kinetic pattern of flash-induced responses in current-clamp during the first 1–5 ms is poorly defined because of an as yet unresolved multiphasic rise. In voltage-clamp, however, the response time is much improved to about 0.2–1 ms. The field dissipation rate in voltage-clamp is somewhat accelerated compared to current-clamp because of the additional dissipative branch of the pipette and patch-clamp amplifier (Fig. 1) [20]. The measured photopotentials or photocurrents, albeit for an attenuation by a volt-

age-dividing network (Fig. 1), are a direct measure of the thylakoid membrane potential [20]. Saturating single-turnover flashes (discharge time 6 μs) of white light were provided by a Xenon flash lamp.

All measurements were done at room temperature.

3. Results and interpretation

3.1. A fast and a slow electrogenic pump

The flash-induced changes in potential (photopotential) and current (photocurrent) were measured on single *P. metallica* chloroplasts in current-clamp and voltage-clamp mode, respectively. A representation of the measuring configuration, the so-called whole-thylakoid configuration, is given in Fig. 1 in which the equivalent electrical scheme first introduced by Vredenberg et al. [20] is incorporated. The electrical impedance of the whole-thylakoid configuration is probed by injection with a square current pulse (Fig. 2A). The potential response of the patch-pipette before seal-formation is marked by the horizontal dashed line. Within the limited time resolution of $t < 5$ ms (current-clamp mode) a higher initial potential response upon current-injection is observed after seal-formation. This suggests that the pipette tip and the reference have a low conductance access to the thylakoid membrane. The following mono-phasic slow rise is caused by the charging of the thylakoid membrane capacitance through the load resistances which is composed of the thylakoid membrane resistance (R_M) shunted by the summation of the resistances R_X and R_L (Fig. 1 and see Ref. [20]). Fig. 2A shows that the current-off response is symmetrical to the current-on response. In order to minimise polarisation effects short current pulses had to be employed. As photopotential relaxation times as high as 120 ms were observed, we used the current-off decay for the analysis of the decay kinetics as the relaxation of the potential could be measured for extended periods without polarising the thylakoid membrane. For $t > 15$ ms the slow decay can be described satisfactorily with a single exponential function (Fig. 2C, curve I) to which a field dissipation relaxation time (τ_D) is assigned. For the particular chloroplast of Fig. 2 the calculation yields $\tau_D = 74$ ms. Single-exponential decay kinetics would thus be anticipated for a flash-induced response evoked by fast primary charge separation in PS I and PS II reaction centers.

Fig. 2B shows the flash-induced photopotential response of the same chloroplast. After a fast initial

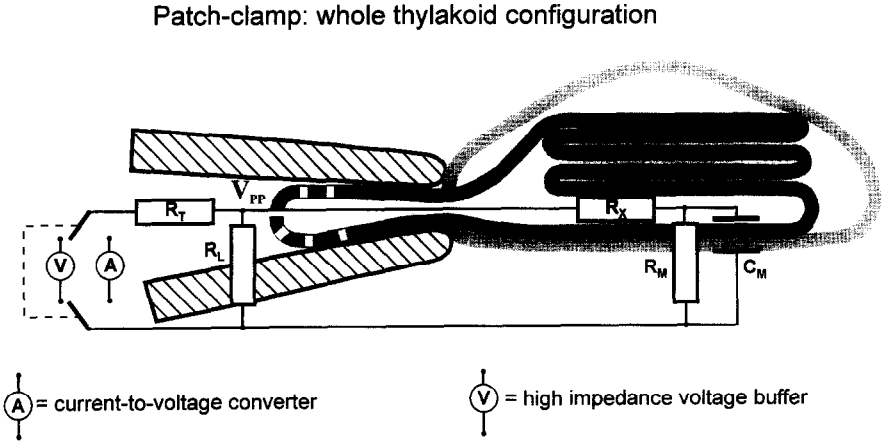

Fig. 1. Representation of the patch-clamp configuration in which a single chloroplast (right) is sucked onto the tip of a fire-polished patch-pipette (left). The envelope and thylakoid membrane fraction inside the tip are assumed to be disrupted as a result of the suction. R_T, tip resistance; R_L, leak resistance along the pipette's tip; R_X, unidentified resistance related to the low conductance phase between pipette and the thylakoid membrane and between the reference and the thyalkoid membrane; R_M, thylakoid membrane resistance; C_M, thylakoid membrane capacitance. The two symbols in the dashed box represent the measuring configurations of the EPC-7 patch-clamp amplifier: V, high impedance voltage buffer (current-clamp mode) and A, current-to-voltage converter (voltage-clamp mode). The measured photopotential response V_{PP} (current-clamp) is a direct measure of the membrane potential V_M after correcting for the attenuation caused by the voltage divider formed by R_L and R_X.

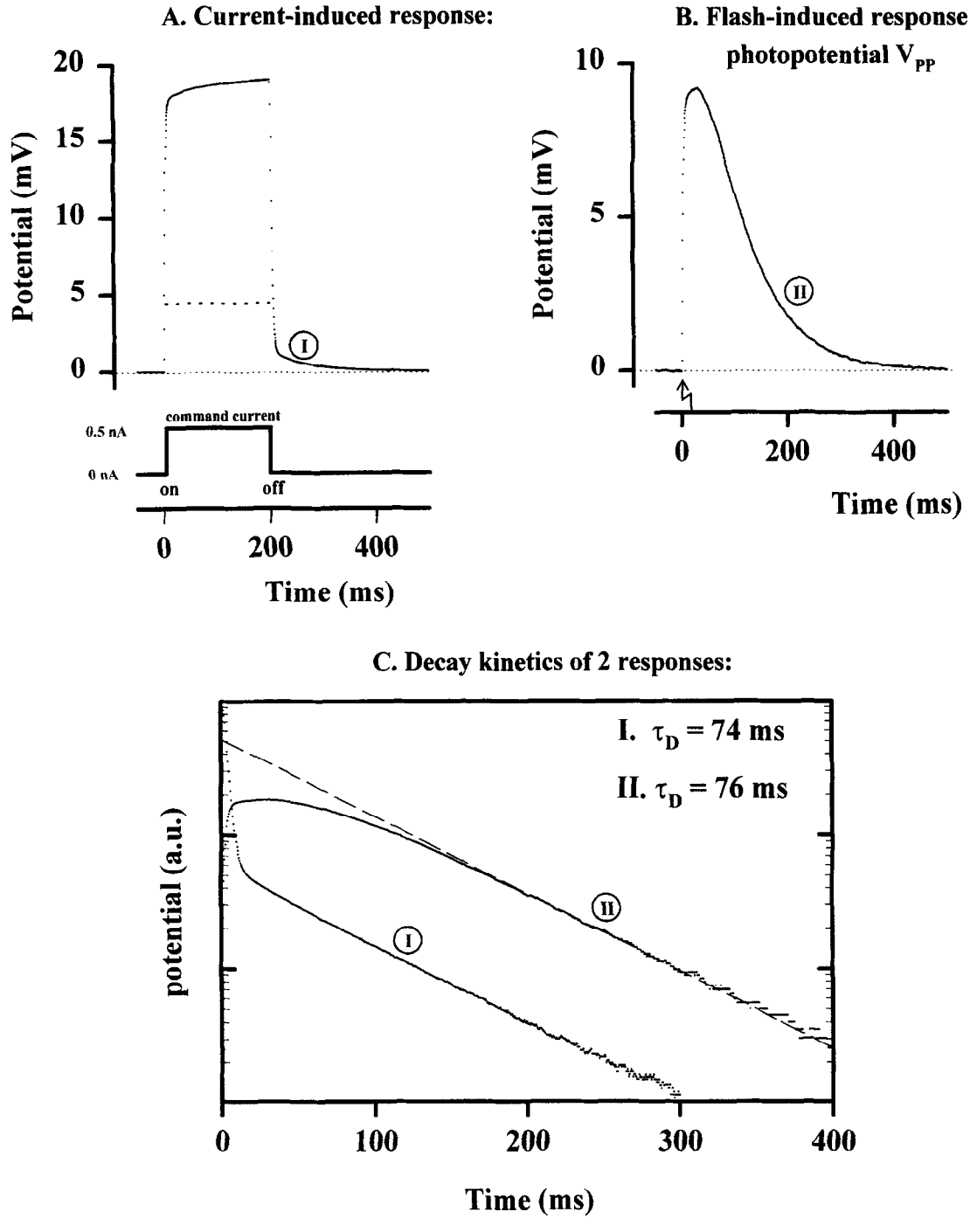

Fig. 2. Potential responses (current-clamp mode) of a patched *P. metallica* chloroplast in the whole-thylakoid configuration. (A) response induced by a square current pulse of 200 ms duration and 0.5 nA amplitude. The horizontal dashed line indicates the potential response of the patch-pipette before seal-formation. The bi-phasic rise and decay kinetics, complemented with a native membrane relaxation time of 130 ms [20], allows the determination of the resistances, membrane capacitance and membrane potential of the equivalent electrical scheme incorporated in Fig. 1 [20]. For this particular experiment the following values were calculated: R_T = 10.0 MΩ, R_L = 35.4 MΩ, R_X = 173 MΩ, R_M = 174 MΩ, C_M = 0.775 nF, V_M = 46 mV. (B) Potential response induced by a saturating single-turnover flash. (C) Semi-logarithmic plot of the decay after current-off (curve I) and after the flash (curve II). The respective relaxation times of both decays for times $t > 200$ ms are τ_D = 74 ms and τ_D = 76 ms.

rise a further slow increase in photopotential is evident and this is followed by a multi-phasic decay. Hence, contrary to what is predicted from the results of the current-injection technique, the photopotential exhibits multi-phasic kinetics (Fig. 2C, curve II). This observation leads to the conclusion that besides primary charge separation a secondary electrogenic event is involved. The photopotential relaxes from 200 ms onwards (Fig. 2C, dashed line) with a relaxation time of 76 ms which corresponds nicely with the relaxation time of the potential decay after current-injection. This close match strongly suggests that the late decay phase of the photopotential is associated with the dissipation of the electrical field by passive ion fluxes through the load resistances. It must be stressed that the secondary rise of the flash-induced photopotential is more obvious in experiments with $\tau_D > 50$ ms. This is the case when R_L or R_X are relatively high (high seal) compared with R_M and τ_D will approach the native membrane relaxation time (τ_M) defined as $\tau_M = R_M \times C_M$. In fast responses with $\tau_D < 50$ ms, i.e., with relatively low seal resistances as in [20] secondary electrogenic events are masked by the fast field dissipation.

Both current and flash-induced responses (Fig. 2A,B) were measured in the same current-clamp mode configuration which allowed for a direct comparison of their field dissipation relaxation times. The current-clamp mode benefits from a good signal-to-noise ratio and from less interference of stray capacitance than voltage-clamp mode but has a relatively poor time resolution in the first milliseconds after the flash. To improve the kinetic resolution in these first milliseconds, the flash-induced current response was measured in voltage-clamp mode on the same chloroplast (Fig. 3). Notice the similarity in the response

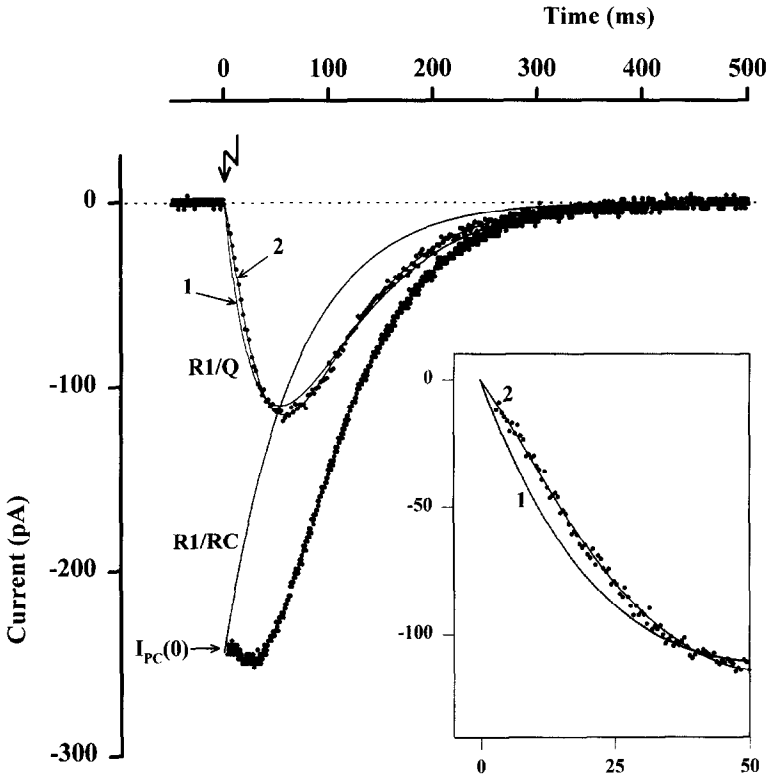

Fig. 3. Flash-induced photocurrent (voltage-clamp mode) of the same chloroplast as Fig. 2. The overall response was deconvoluted into a Reaction R1/RC and R1/Q. R1/RC is a single exponential constructed from the initial amplitude $I_{PC}(0)$ and the relaxation time taken from the current decay for $t > 200$ ms (see Fig. 2). R1/Q (dots) was subsequently obtained by subtraction of the overall response with the constructed R1/RC. The solid lines 1 and 2 refer to the two analytical fits of R1/Q: 1, first approximation fit calculated from a bi-exponential fit of the overall photocurrent transient (see text, Eq. (1)) and 2, analysis based on a partly consecutive reaction scheme shown in Fig. 4. Inset shows R1/Q on a shorter time-scale elucidating the sigmoid rise kinetics.

patterns obtained for both measuring modes (Fig. 2B and Fig. 3). The initial amplitude of the photocurrent is -240 ± 10 pA. The relaxation time calculated from the decay of the photocurrent profile for $t > 200$ ms, about 68 ms, was again found to be in close agreement with τ_D obtained with current-injection (Fig. 2) provided that the additional shunt resistance R_T (Fig. 1), which causes a somewhat increased rate of field dissipation, was taken into account. In a first approximation the photocurrent (I_{PC}) of Fig. 3 can be well fitted as the sum of two exponentials,

$$I_{PC}(t) = a_I \cdot e^{-t/\tau_I} + a_{II} \cdot e^{-t/\tau_{II}} \quad (1)$$

in which a_I and a_{II} are amplitudes of opposite sign and τ_I and τ_{II} are relaxation times with, by definition, $\tau_I > \tau_{II}$. Relaxation time τ_I is thus equal to the field dissipation relaxation time τ_D. The values found for the photocurrent of Fig. 3 are: $\tau_I = 67.7$ ms, $\tau_{II} = 42.8$ ms, $a_I = -903$ pA, $a_{II} = 659$ pA. The initial amplitude of the photocurrent calculated from the bi-exponential fit is $a_I + a_{II} = -244$ pA which is in close agreement with the amplitude directly determined from the current profile. In order to give a physiological interpretation of this bi-exponential analysis, we must realise that the fast primary charge separation in the reaction centers must give a fast initial rise with a subsequent field dissipation governed by τ_D [20,21]. Therefore, the only meaningful deconvolution of the bi-exponential fit of the photocurrent will be the sum of a reaction called R1/RC, associated with fast primary charge separation in the reaction centers, and of a slow secondary reaction which we call R1/Q,

$$R1/RC(t) = (a_I + a_{II}) \cdot e^{-t/\tau_I} = I_{PC}(0) \cdot e^{-t/\tau_D} \quad (2)$$

$$R1/Q(t) = -a_{II} \cdot (1 - e^{-t/\tau_r}) \cdot e^{-t/\tau_D} \quad (3)$$

$I_{PC}(0)$ is the initial amplitude of the photocurrent (Fig. 3) and $1/\tau_r = 1/\tau_{II} - 1/\tau_I$. Fig. 3 shows R1/RC (solid line) and R1/Q (solid line 1) as calculated by this first approximation. The acronym R1/Q specifies the relation to a Q-cycle mechanism [6], a notion which is supported by the inhibition of R1/Q by DBMIB (Fig. 5B). In a second, more general way R1/Q is obtained as follows. Reaction R1/RC can be constructed as a single exponential function with an initial amplitude read from the photocurrent response at $t = 0$ ms and a field dissipation relaxation time τ_D given by the current-injection technique or, equivalently, by the relaxation time deduced from the photocurrent for $t > 200$ ms. Next, R1/Q is deduced as the difference between the measured curve and the constructed component R1/RC (Fig. 3, R1/Q dots).

3.2. A detailed kinetic analysis incorporating a consecutive reaction scheme

The bi-exponential fit of R1/Q is a reasonable first approximation but deviates significantly from the data points, especially in the onset phase (Fig. 3, curve 1). Actually the rise of R1/Q seems to be sigmoid (Fig. 3, inset) and we suggest that at least part of the charge separation proceeds according to a consecutive reaction sequence. Therefore, the data of R1/Q were analysed with the help of the reaction schemes shown in Fig. 4; for a derivation of the analytical solutions see appendix A.

Two populations of cyt. b_6f complexes A1 and A2 are assumed to exist at the start of the flash firing. These complexes produce the electrogenic products Q1 and Q2, respectively. An electrogenic product Q stands for the transmembrane potential (field) or equally for the number of charges separated by b_6f turnover. The formation of Q1 will exhibit a lag

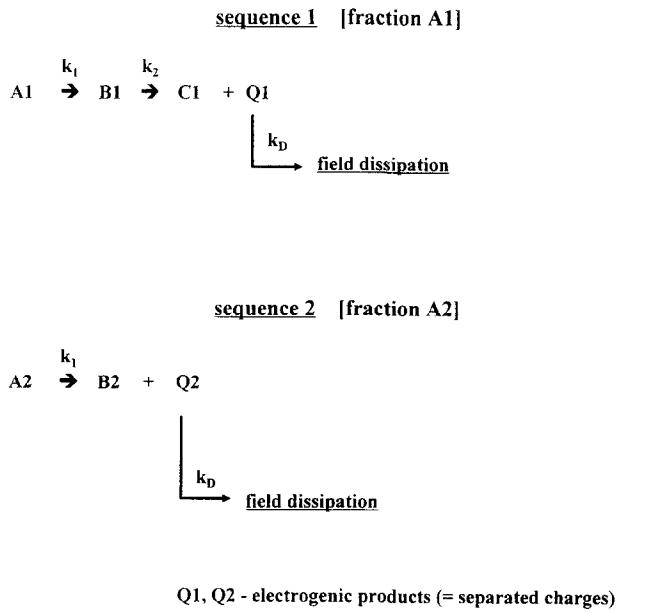

Fig. 4. Two reaction sequences used for describing the sigmoid rise kinetics of reaction R1/Q. Sequence 1 is a consecutive reaction resulting in Q1 formation with an initial lag. Sequence 2 will give direct production of Q2.

Table 1
Quantification of the slow electrogenic component R1/Q of 5 different *P. metallica* chloroplasts

	τ_1 (ms)	τ_2 (ms)	$A1_0$	$A2_0$	Ratio Q/RC
1	13.7	28.3	0.74	0.26	0.15
2	11.0	37.2	0.84	0.16	0.43
3 (Fig. 5A)	16.4	28.6	0.76	0.24	0.50
4	6.3	15.2	0.55	0.45	0.74
5 (Fig. 3)	17.6	27.8	0.66	0.34	0.95

τ_1 and τ_2 are the relaxation times derived from a 3-exponential fit to the experimental data of R1/Q as discussed in Appendix A. These two τ-values correspond to the rate constants k_1 and k_2 of the reaction schemes shown in Fig. 4.

phase, whereas Q2 formation will proceed without a noticeable lag. Consequently, the rise kinetics of the secondary charge separation evoked by the sum of the two b_6f populations will be an average of these two extremes. Fig. 3 (R1/Q, curve 2) shows the fit of R1/Q based on the reaction sequences of Fig. 4 and obviously the data are much better described by the inclusion of a consecutive reaction. The relaxation times corresponding to k_1 and k_2 are found to be 17.6 and 27.8 ms, respectively. The two kinetic rate constants k_1 and k_2 give a description of R1/Q which is independent of the rate of field dissipation and the variations in τ_D, observed when patching different chloroplasts, are thus eliminated. Table 1 presents a survey of the parameters characterising the kinetics and extent of R1/Q as obtained from 5 experiments on different *P. metallica* chloroplasts.

The area under the photocurrent traces allows a direct estimation of the number of charges separated by the two electrogenic reactions. A ratio Q/RC is introduced to quantify the contribution of R1/Q. The Q/RC-ratio is defined as the number of charges displaced by R1/Q relative to the number of charges displaced by R1/RC (originating from both PS I and PS II). This Q/RC-ratio shows large variations among different chloroplasts. The two responses of Figs. 3 and 5A illustrate this diversity and the Q/RC-ratio for these two different chloroplasts were found to be 0.95 and 0.20 (flash 1 in Fig. 5A), respectively.

3.3. Characterisation of R1/Q

It was observed that the extent of R1/Q increased in a sequence of 5 single-turnover flashes given with a flash frequency of 0.5 Hz to a dark-adapted chloroplast (Fig. 5A). A deconvolution of the responses alike the general method discussed for Fig. 3 results

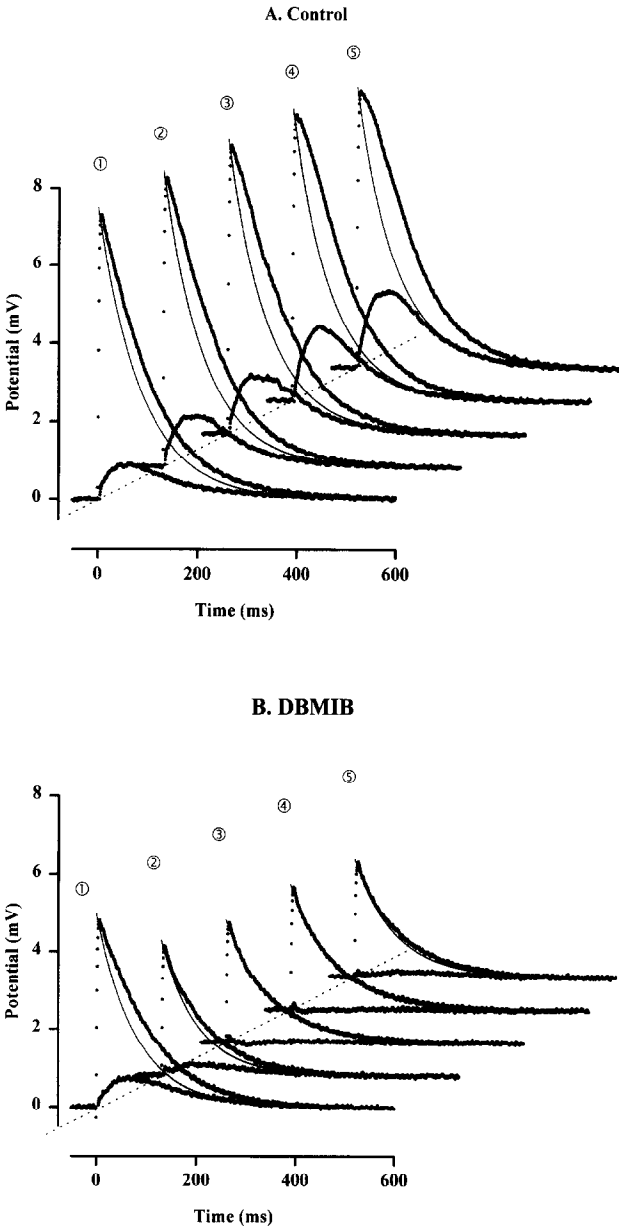

Fig. 5. Photopotentials measured on a single *P. metallica* chloroplast upon five saturating single-turnover flashes given with a flash frequency of 0.5 Hz. Deconvolution into R1/RC and R1/Q as in Fig. 3: solid lines are R1/RC, lower dotted curves are R1/Q. After the recording of the control (A), the same chloroplast was immersed with medium supplemented with 2.5 μM DBMIB by the aid of a superfusion DAD-system (B). BSA was omitted from the isolation medium in this experiment to prevent non-specific adsorption of DBMIB onto BSA, thus preventing a lowering of the effective concentration of inhibitor.

in an *increase* of the Q/RC-ratio from 0.20 to 0.50 for flash numbers 1 to 5, respectively. This is most readily explained by an increase in the concentration of PQH_2 produced by PS II turnover of previous flashes. The increased PQH_2 concentration will subsequently enhance the number of b_6f complexes with bound PQH_2 at the Q_p-site prior to the flash. Contrary, in some chloroplasts which had a relatively high Q/RC-ratio upon a first flash, a *decrease* of the Q/RC-ratio was seen after a few single turnover flashes (data not shown). For example, the Q/RC-ratio of experiment 2 in Table 1 was calculated from the third flash of a chloroplast with an initial Q/RC-ratio of 0.71 upon the first flash (data not shown).

Subsequent addition of 2.5 μM DBMIB (Fig. 5B) and repetition of the 5-flash sequence revealed the disappearance of the secondary phase from flash 2 onwards. The inhibition of R1/Q by DBMIB supports our conclusion that R1/Q arises from cyt. b_6f turnover. Three other characteristics of the slow rising phase were observed (data not shown): (1) the presence of far-red background light eliminated R1/Q, (2) in the presence of the artificial donor duroquinol (DQH_2) a slow secondary rising phase with a relatively high Q/RC-ratio reappeared after initial inhibition by DCMU [6], (3) addition of 1 μM tentoxine, a specific blocker of ATP synthesis [13], was without noticeable effect on R1/Q.

4. Discussion

In this paper a slow light-driven electrogenic reaction in the thylakoid membrane is discussed whose kinetics could be accurately determined. This was realised by the use of a current-injection technique which grants an objective determination of the field dissipation relaxation time τ_D associated with the ion fluxes dissipating the transmembrane electrical field. The unequivocal determination of τ_D can be considered an advantageous property of the patch-clamp method. The simple relaxation kinetics obtained with the current-injection technique (Fig. 2) in conformity with the slow relaxation time of the photopotential/photocurrent for $t > 200$ ms justifies the estimation of R1/RC from the initial amplitude of the flash-induced response and τ_D. Hence a proper kinetic definition of R1/Q is obtained by subtraction of R1/RC from the measured data and this will most likely provide us with the true extent and kinetics of R1/Q. The implicit assumption made in these analyses is that the initial amplitude at $t = 0$ arises solely from R1/RC, which automatically sets the initial amplitude of R1/Q to zero. In voltage-clamp the initial amplitude can be fairly accurately determined within a time resolution of less than 1 ms. The close agreement between the initial amplitude values as calculated with an exponential fit or by direct determination from the current data near $t = 0$ strongly suggests that the initial extent of primary charge separation is safely determined with the patch-clamp method within an acceptable error limit of about 5%.

The initial retarded decay of the photopotential (photocurrent), sometimes even preceded by a slow rising phase, can originate in principle from the following processes:

(1) Non-linearity in the network impedance(s): resistance or capacitance.

(2) Electrogenic proton transfer driven by ATP hydrolysis catalysed by a flash-activated ATP synthase.

(3) Electrogenic steps involving the cyt. b_6f complex.

A 1–5% increase in the chloroplast network resistances have been reported under flashing light conditions [22]. These resistance changes are only detectable if an off-set current is applied which transforms a resistance change into a slow potential change superimposed on the photopotential response. In our measuring conditions of zero current, potential changes related to changing network resistances can be neglected. Since the measured potential is a reflection of the thylakoid membrane potential attenuated by a voltage-divider $R_L/(R_X + R_L)$ (Fig. 1), an alteration of the photopotential profile by changing network resistances might, however, be anticipated. The attenuation factor will shift to a new value in the event that one or both resistances R_X and R_L change after the flash. It can be calculated (not shown) that such an effect will have little impact on the photopotential kinetics unless unreasonably large changes in R_X or R_L are assumed. Such an assumption can be considered unlikely [22]. A similar rationale holds for flash-induced changes in the thylakoid membrane capacitance. Furthermore, the relatively slow kinetics of the flash-induced resistance changes are quite dis-

tinct from those of R1/Q [22]. We therefore conclude that resistance and capacitance changes are not involved in the multiphasic relaxation at zero holding potential/current and the phenomenon is best explained by a slow secondary electrogenic step.

Hydrolysis of stromal ATP can create an electrical field with the same orientation as that evoked by primary charge separation: positive inside [23]. For this to happen, the ATP synthase needs to be activated which might have been realised by the momentary transmembrane potential set up upon the flash. Involvement of ATP hydrolysis is, however, unlikely because tentoxine addition was without effect on the slow rising phase. In addition, the free enthalpy change for ATP hydrolysis shortly after the flash would be about 46 mV (see legend to Fig. 2) less favourable than in the dark and proton pumping by flash-activated ATP synthase is just expected to be lowest shortly after the flash while increasing concomitantly with the decay of the transmembrane potential. In this context, Schreiber and Del Valle-Tascon [24] detected net flash-induced ATP synthesis under ATP hydrolysing conditions.

The sensitivity of the slow secondary rise to DBMIB, a potent Q_p-site inhibitor [5,14,25] suggests that our results are best explained by electrogenic events involving the cyt. b_6f complex. The partial inhibition seen upon the first flash (Fig. 5B) suggests that reduced DBMIB has to be formed first by PS II activity before full inhibition of b_6f turnover results [26]. The concomitant decrease of the amplitude of the photopotential in the flash sequence (Fig. 5B) witnesses partial inactivation of PS II by competitive binding of DBMIB to the Q_B pocket [25,27] and of PS I inactivation probably caused by a shortage of reducing equivalents at the donor side.

One of the characteristics of the slow electrogenic phase as measured with the patch-clamp technique is its sigmoid rise. This S-shaped rise might suggest that in one fraction of b_6f complexes the electrogenic step occurs with an apparent lag phase and in a second population without. Such a typical behaviour was also noticed by Fernández et al. [28] who discriminated a lag in the slow P515 phase by its HQNO sensitivity, suggesting that the electrogenicity associated with the re-oxidation of b_h at the Q_n-site proceeds indeed with sigmoidal kinetics. A proper physiological assignment of the rate constants k_1 and k_2 found from the mathematical analysis (Table 1) cannot be given as yet. One reasonable interpretation would be that k_1 and k_2 are related to the oxidation of PQH_2 at the Q_p-site and the re-reduction of PQ at the Q_n-site, respectively. The two reaction sequences (Fig. 4) are based on this interpretation. Cytochrome b_6f complexes with reduced b_h and oxidised b_l are expected to give an electrogenic phase corresponding to the oxidation of the *b*-hemes and reduction of PQ at the Q_n-site (k_2) but only after non-electrogenic reduction of the low potential *b* heme triggered by PQH_2 oxidation (k_1) has occurred. When both *b*-hemes are in their oxidised state oxidation of PQH_2 at the Q_p-site will immediately induce a slow electrogenic phase corresponding to reduction of the high potential *b*-heme (k_1). The relaxation times of about 13 and 28 ms (Table 1) for k_1 and k_2, respectively, correspond reasonably well with results from others [7,29,30] and differences might be related to the local environment in the native *P. metallica* chloroplasts (e.g., pH, quinol concentration, ambient redox potential).

Alternatively, the sigmoid rise might be related to a heterogeneous oxidation of b_6f complexes by two different pools of plastocyanin [31,32]. In this scenario one pool, in close proximity of PS I and the cyt. b_6f complex, is expected to exhibit an instantaneous onset of secondary potential rise whose kinetics is determined by PQH_2 oxidation. The other, less tightly associated pool, will give a retarded potential rise with a lag time controlled by the average diffusion time of the mobile plastocyanin from PS I to a b_6f complex.

Our results show that flash-induced turnover of the cyt. b_6f complex in *P. metallica* chloroplasts may translocate a variable amount of charges per single turnover of the reaction centers. It was observed that in several cases the Q/RC-ratio converged to a value of about 0.5 at sufficiently high flash frequencies of about 0.5 Hz. This was true when either the Q/RC-ratio upon the first flash was low (e.g., Fig. 5A) or as high as about 1.0 (data not shown). Most of the responses fit in nicely with the traditional Q-cycle concept although some chloroplasts showed a quit large secondary charge separation on a first flash. A semiquinone (SQ)-cycle mechanism might be a possible explanation for the high Q/RC-ratios [8,10]. A prerequisite for full SQ-cycling is the pre-reduction

of both low and high potential *b*-hemes in the dark which requires a considerable low ambient redox potential of at least −200 mV [10,15]. If both *b*-cytochromes would have undergone dark reduction the prevailing ambient redox potential inside the *P. metallica* chloroplasts, isolated from a few cells by mild cutting with a surgery knife, apparently warrants this pre-reduction. Unfortunately, the preparation of suspensions of intact *P. metallica* chloroplasts for biochemical characterisation remains a daunting task at this time and consequently the redox state of the *b*-hemes in *P. metallica* in relation to light history or local conditions is unknown. Double turnovers of PS I during the flash can cause an apparent increase of the Q/RC-ratio which would lead to an overestimation of the Q-cycle activity. However, since PS I and cyt. b_6f turnover are stoichiometrically coupled by a one-to-one relation, the apparent Q/RC-ratio can never exceed 2/3 assuming all that all PS I centers make a double turnover and that there are equal concentrations of PS I and PS II. The about 5% uncertainty in the initial amplitude will cause no more than about 10% uncertainty in the calculated Q/RC-ratio. We therefore conclude that Q/RC observations of < 0.75 could still be explained in the traditional Q-cycle concept but chloroplasts having a larger extent of secondary charge separation must have a fundamentally different cyt. b_6f turnover.

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Appendix A

In this appendix the analytical solution for the number of charges Q(t) translocated by cyt. b_6f turnover is derived using a kinetic model. Fig. 4 shows two reaction sequences describing transient cyt. b_6f states A1, B1, etc. of the cyt. b_6f complex. A fraction A1 of b_6f complexes produces a charge separated state Q1 according to the consecutive reaction sequence 1 which involves two steps characterised by two rate constants k_1 and k_2. In a remaining fraction A2 a charge separated state Q2 is produced with a rate constant k_1. Subsequently, the electrogenic products Q1 and Q2 will be dissipated with a field dissipation rate constant (k_D) given by $1/\tau_D$, the same rate constant as was found after current-injection.

The concentrations of the reactants A1, B1 and of the electrogenic product Q1 of the two-step reaction sequence 1 change in time according to,

$$\frac{dA1}{dt} = -k_1 \cdot A1 \quad \text{(A1)}$$

$$\frac{dB1}{dt} = k_1 \cdot A1 - k_2 \cdot B1 \quad \text{(A2)}$$

$$\frac{dQ1}{dt} = k_2 \cdot B1 - k_D \cdot Q1 \quad \text{(A3)}$$

with t the time. These three differential equations were solved sequentially with initial conditions given by $A1(t=0) = A1_0$, $B1(t=0) = 0$ and $Q1(t=0) = 0$ and the solution for $Q1(t)$ results in a 3-exponential function,

$$Q1(t) = \alpha 1 \cdot e^{-k_1 \cdot t} + \beta 1 \cdot e^{-k_2 \cdot t} + \gamma 1 \cdot e^{-k_D \cdot t} \quad \text{(A4)}$$

The coefficients $\alpha 1$, $\beta 1$ and $\gamma 1$ are functions of the rate constants k_1, k_2 and k_D and of $A1_0$. In a similar way the solution for $Q2(t)$ can be obtained,

$$Q2(t) = \alpha 2 \cdot e^{-k_1 \cdot t} + \gamma 2 \cdot e^{-k_D \cdot t} \quad \text{(A5)}$$

The final fit-function was constructed out of Q1 and Q2 according to,

$$Q(t) = Q1(t) + Q2(t)$$

which results in the general expression,

$$Q(t) = \alpha \cdot e^{-k_1 \cdot t} + \beta \cdot e^{-k_2 \cdot t} + \gamma \cdot e^{-k_D \cdot t} \quad \text{(A6)}$$

Note that α, β and γ are again functions of the variables $A1_0$, $A2_0$, k_1 and k_2. Accordingly, Eq. (A6) was fitted to the data of R1/Q using the Marquardt-Levenberg algorithm (as implemented in SigmaPlot 2.01 for Windows) for finding the optimal solutions of the four parameters $A1_0$, $A2_0$, k_1 and k_2.

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