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Biochimica et Biophysica Acta 1230 (1995) 77–80

BBA Biochimica et Biophysica Acta

Rapid Report

A patch clamp method for determining single turnover charge separations in the chloroplast membrane

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Received 15 September 1994; revised 23 February 1995; accepted 24 February 1995

Abstract

This report describes the application of the chloroplast patching technique to study the primary electrogenic events in the thylakoid membrane. It is shown that by applying single turnover saturating light flashes the actual number of functional charge transferring reaction center channels in the thylakoid membrane and the voltage generated by this charge transfer can be determined in a single individual chloroplast. Moreover, low conducting barriers between the thylakoid membrane and the adjacent lumenal and stromal phases, respectively, are likely to exist.

Keywords: Thylakoid; Charge separation; Patch clamp; Photocurrent

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The chloroplast inner membrane (thylakoid) of higher plants serves the light-driven generation of electrochemical energy as the primary source for photosynthetic energy production (ATP, carbohydrates). It contains two membrane-spanning photosystems (PSI and PSII), which function as a light-driven transmembrane electron channel. These complexes are the site where an electron is channelled within 3 ps [1] from the reaction center chlorophyll towards an associated primary acceptor. The potential generated by transmembrane charge separation has been studied in various details by electrophysiological (electrode impalement) and optical (P515) methods [2–8]. It has been shown [9] that, by patching of a single chloroplast, the membrane potential and current generated by continuous excitation can be measured with high precision. The present report deals with an analysis of the membrane photopotential evoked by single turnover saturating light flashes in a chloroplast sucked onto a patch electrode. The prospective information content of these responses is illustrated on basis of an electrical model.

The patch clamp experiments were done with giant chloroplasts isolated from *Peperomia metallica* as described by Bulychev et al. [9]. Flash-induced absorbance changes at 518 nm (P515) were measured in an intact leaf of *P. metallica* after infiltration of the leaf with bathing solution. Methods for microscopic observation, light activation, signal detection and dataprocessing were similar as described elsewhere [8–11], except for the use of a Xe flash as actinic light source in the patch clamp set-up.

A chloroplast was gently sucked onto the fire-polished tip of the tapered suction pipette filled with the chloroplast bathing solution (Fig. 1). This procedure in general leads to a 'whole chloroplast' configuration in which the inner thylakoid membrane does not make contact with the patch pipette, as concluded from the absence of light-induced photocurrents and potentials. This is the configuration in which envelope conductance has been studied in relation to the function of ionic channels [12] or to functional aspects of protein import [10]. A brief additional suction leads in most, if not all cases to a configuration in which the thylakoid membrane apparently has made contact with the patch electrode, as inferred from the appearance of light-induced reponses. This final procedure is likely to result in a so-called 'whole thylakoid' configuration in which the membrane patches of envelope and thylakoid inside the pipette have been disrupted. A representation of the 'whole thylakoid' patch clamp configuration, assuming a leaky envelope membrane is also shown in Fig. 1.

The electrode potential reponse upon a square current pulse injection (1 nA, 100 ms) of a chloroplast in the 'whole thlylakoid' configuration is shown in Fig. 2A. The

SSDI 0005-2728(95)00036-4

initial fast potential rise upon current injection is followed by a small but distinct slow rise. The rise occurs with a relaxation of about 17 ms. The time course of the flash-induced photopotential measured under zero current is shown in Fig. 2B. This photopotential response shows an initial amplitude of about 6.4 mV and an exponential decay with a relaxation time of 17.1 ms. The photocurrent response, measured under voltage clamp (not shown) exhibited the same kinetic pattern with a 3–5% lower relaxation time and a current amplitude of about 460 pA. Two characteristics of the response are noteworthy. Firstly, the amplitude of the photopotential is much smaller than the thylakoid membrane potential generated by a single-turnover charge separation (V_M) so far documented by other methods [2–6]. This could be due to the capacitance of the electrode which, in parallel with the membrane capacitance (C_M), will contribute to the overall capacitance to be charged by the turnover of the RC's. However, this cannot be the only reason, because this would not explain the higher instaneous potential response upon current injection after the chloroplast has been sucked onto the pipette (Fig. 2A). This increase suggests a low conductance access of the electrode to the lumen. Moreover, the electrode capacitance has been found to be below 50 pF, which is much less than the values determined for C_M (see below). Secondly, the relaxation of the photopotential is substantially faster than the native (i.e., diffusion-driven) relaxation of V_M as measured with either impaled electrodes [2,3,11] or P515 [6–8,13].

The flash-induced P515 response of a Peperomia leaf shows the multiphasic pattern similar as observed in other leaves and chloroplasts [6]. The decay is biphasic, with a rapid phase, attributed to the decay of the transmembrane potential (Reaction 1), and a much slower phase attributed to localised electrical membrane effects (Reaction 2) [8]. Fig. 3 shows the net Reaction 1 response upon the last

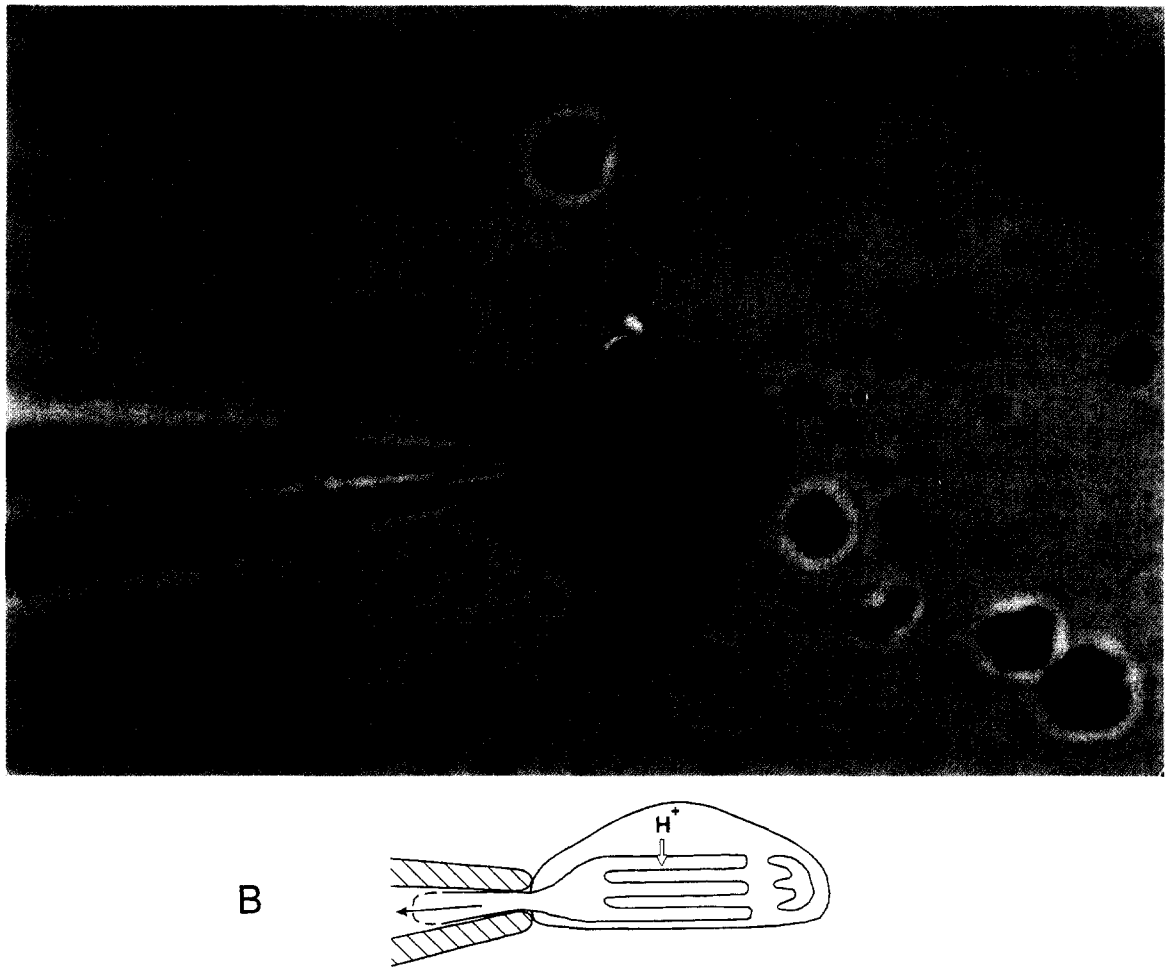

Fig. 1. (A) Light microscopy of a *P. metallica* chloroplast (10 μm in diameter) attached to a patch pipette. Chloroplasts like the one shown were selected from a preparation, obtained by gentle cutting and slicing a leaf in a medium containing (in mM): sorbitol 300; KCl 50; Hepes 10; $MgCl_2$ 0.5 and BSA 0.5% (w/v) (pH 7.5). Suction pipettes (approx. 1 μm open tip) were pulled from Pyrex capillaries, heat polished and mounted in a holder with an internal Ag/AgCl electrode and an outlet for application of suction. The pipette was filled with the isolation medium. The vertical bar corresponds with a length of 5 μm. (B) Schematic representation of the patch clamp configuration, in which the tip of the patch electrode is in contact with the thylakoid lumen ('whole thylakoid' configuration). The arrow marks the proton pump directed from stroma into thylakoid lumen.

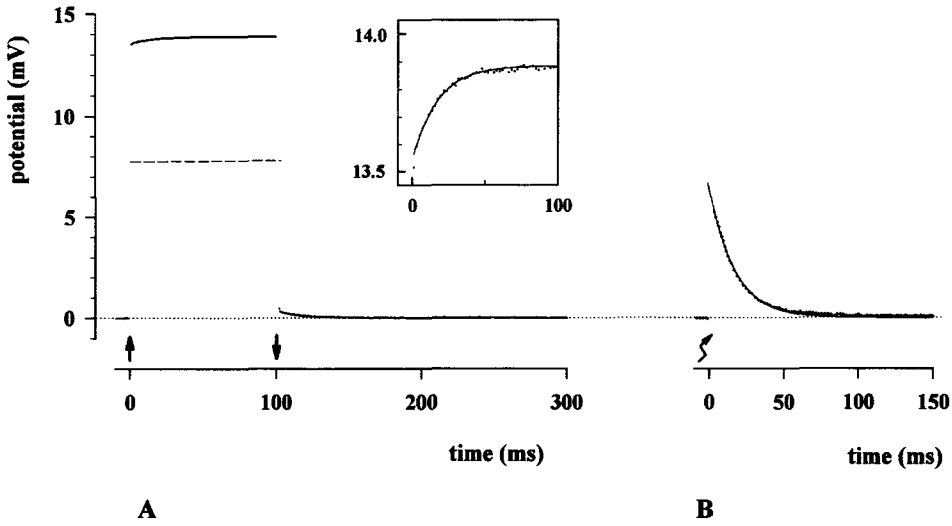

Fig. 2. (A) (left) Profile of the electrode potential response upon a 100 ms (marked by arrows) square current pulse (0.93 nA) through the pipette of a patched *P. metallica* chloroplast. The dashed horizontal line (7.7 mV) is the electrode response before it was sucked onto the chloroplast. The inset shows a magnification of the slow potential rise after the initial fast (< 0.5 ms) response (13.55 mV) towards the final steady state (13.9 mV). (B) (right) Flash-induced photopotential response of the same dark-adapted chloroplast sucked on the electrode. The potential was measured at zero current clamping. The duration of the light flash was 6 μs (half width). Dark adaptation was approx. 5 min. The hatched arrow marks the firing of the flash. The instrumental response time in this mode was set at about 2 ms.

flash in a train of ten, fired at a frequency of 5 Hz, after subtraction of Reaction 2 using a method described elsewhere [6,11].

Assuming a thylakoid membrane with resistance R_M and capacitance C_M, the simplest electrical equivalent scheme which can explain the potential and P515 responses of Figs. 2 and 3 is shown in Fig. 4. A low impedance access of the pipette tip to the lumen is prevented by an unidentified resistance, R_X; current leakage along the electrode tip is represented by a leakage resistance R_L. The additional resistors R_X and R_L act (i) as a voltage divider, explaining the low photopotential and (ii) as an additional load resistor decreasing the relaxation time. As predicted by the model, the relaxations of current- (Fig. 2A) and light-evoked potential (Fig. 2B) are similar, if not identical.

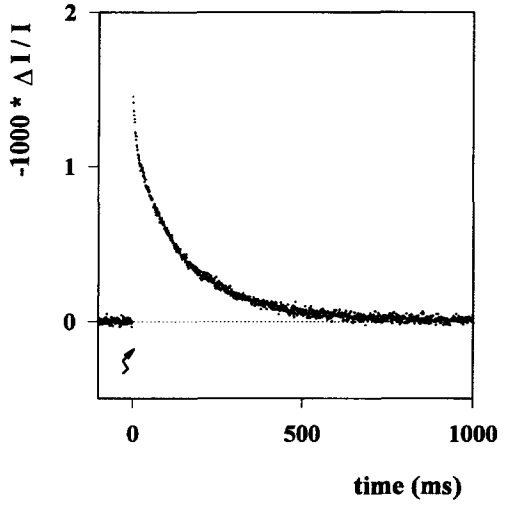

Fig. 3. Flash-induced absorbance changes at 518 nm (P515) in the last flash of a train of ten (darktime interval 200 ms), measured in a dark-adapted leaf of *P. metallica*, after correction for a contribution (approx. 30%) of a slow phase (Reaction 2) with a relaxation of 730 ms. The decay occurs with a relaxation of 130 ms, attributed to reaction 1. Average of 500 flash trains given at a rate of 0.03 Hz. The hatched arrow marks the firing of the flash.

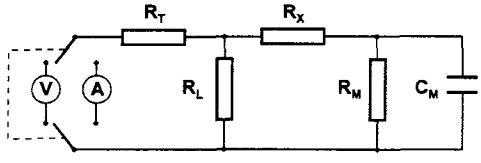

Fig. 4. Electrical equivalent scheme. R_T is the resistance of the patch electrode; R_L is the leak resistance along the electrode tip; R_X is the resistance of a low conductance phase between the electrode and the thylakoid membrane and R_M and C_M are resistance and capacitance, respectively, of the clamped thylakoid. The two symbols in the dashed box indicate the configuration of the EPC 7 patch clamp amplifier in the voltage clamp (A, current–voltage convertor) and the current clamp mode (V, high imput impedance voltage buffer. The capacitance of the electrode has been omitted because of its relatively low value (see text). The amplitude and relaxation values of the response patterns (Fig. 2), combined with a diffusion driven relaxation time of the thylakoid potential (P515) of about 130 ms (Fig. 3) give the following resistance values (in MΩ) of the clamped chloroplast $R_T = 8.2$ $R_L = 6.7$, $R_X = 98$ and $R_M = 690$ ($C_M = 190$ pF). The amplitude of the flash-induced potential (Fig. 2B) then corresponds with an evoked thylakoid potential $V_M = 98$ mV. Assuming a specific capacitance of the thylakoid of 10^{-2} F m^{-2}, the potential is generated by a membrane area of approx. $1.9 \cdot 10^{-8}$ m^2, which is about 60-times larger than the area of the supposedly spherical chloroplast (diameter 10 μm). The number of responding ETC's can be calculated to be $1.0 \cdot 10^8$, assuming a 1:1 ratio of PSII/PSI. This results in an ETC density of $3 \cdot 10^3$ μm^{-2}, which corresponds nicely with estimates from other species [18].

Using the native diffusion-driven relaxation of the thylakoid potential (P515) and the amplitude and relaxations, measured upon current stimulation and flash excitation as input parameters of the model, the number of current-generating reaction center channels involved, the magnitude of V_M, as well as the membrane resistance (R_M) and capacitance (C_M) can be calculated. Assuming a specific membrane capacitance of the thylakoid equal to 10^{-2} F m^{-2} the surface area of the light-activated membrane can be approximated and consequently the density of electron transport chains (ETC).

Values of V_M for individual dark-adapted *P. metallica* chloroplasts were calculated to be in the range between 20 and 100 mV. In addition the area of the thylakoid membrane sensed by the patch pipette in individual chloroplasts is calculated to be manifold larger than the envelope area enclosing the (idealized, 10 μm diameter) spherical chloroplast (Fig. 1). This is consistent with the folded structure of the thylakoid membrane, and illustrates the fact that with this method a large part of the thylakoid membrane in a single chloroplast is measured. The experiment of Fig. 2 yields a calculated patched thylakoid to envelope surface ratio of about 90.

Responses measured in a dark-adapted chloroplast upon a first single flash, as those illustrated in Fig. 2B, show almost single exponential decay kinetics. The kinetics become multiphasic after pre-energization; the pattern is more pronounced at non-zero clamping potentials or currents. This can be interpreted in terms of light-induced changes in the conductance in the boundary phases adjacent to the membranes. Changes of this kind have been noticed before under continuous illumination [9]. It is tempting to suggest that the resistance R_X is connected with these low conducting phases. The small residual slow phase in the relaxation of the photopotential responses sometimes observed in dark-adapted chloroplasts (c.f. Fig. 2B) is presumed to be a manifestation of the light-induced resistance changes. They are under further investigation.

The main features and prospects of the patch clamp method applied to single chloroplasts amongst others are (i) determination of the actual number of reaction centers capable of performing a charge separation, for instance under conditions which are known to lead to antennae quenching, reaction center quenching or photoinhibition [14–16], (ii) study of the behavior of the reaction centers channels at positive and negative offset potentials; (iii) determination of changes in membrane capacitance and resistance as well as in the resistance of the adjacent phases in relation to (pre-)energization of the chloroplast [17]. Finally, the method seems to be promising with respect to its application to spinach and pea chloroplasts which are commonly used in bioenergetic studies.

This research was supported in part by the Netherlands Organisation for the Advancement of Research (NWO).

References

[1] Wasielewski, M.R., Johnson, D.G., Seibert, M. and Govindjee (1989) Proc. Natl. Acad. Sci. USA 86, 524–528.
[2] Bulychev, A.A., Andrianov, V.K., Kurella, G.A. and Litvin, F.F. (1972) Nature 236, 175–177.
[3] Vredenberg, W.J. (1976) in The Intact Chloroplast (Barber, J., ed.), pp. 53–88, Elsevier, Amsterdam.
[4] Schapendonk, A.H.C.M. and Vredenberg, W.J. (1977) Biochim. Biophys. Acta 462, 613–621.
[5] Junge, W. (1977) Annu. Rev. Plant Physiol. 28, 503–536.
[6] Vredenberg, W.J. (1981) Physiol. Plant. 53, 598–602.
[7] Rich, P. (1988) Biochim. Biophys. Acta 932, 33–42.
[8] Ooms J.J.J., Versluis, W., Van Vliet, P.H. and Vredenberg, W.J. (1991) Biochim. Biophys. Acta 1056, 293–300.
[9] Bulychev, A.A., Antonov, V.F. and Schevchenko, E.V. (1992) Biochim. Biophys. Acta 1099, 16–24.
[10] Bulychev, A.A., Pilon, M., Dassen, H., Van 't Hoff, R., Vredenberg, W.J. and De Kruijff, B. (1994) FEBS Lett. 356, 204–206
[11] Van Kooten, O. (1988) Doctoral thesis. Wageningen Agricultural University.
[12] Potossin, I.I. (1992) FEBS Lett. 308, 87–90.
[13] Chylla, R.A. and Whitmarsh, J. (1989) Plant Physiol. 90 765–772.
[14] Krause, G.H., Vernotte, C. and Briantais, J.-M. (1982) Biochim. Biophys. Acta 679, 116–124.
[15] Horton, P. and Ruban, A.V. (1992) Photosynth. Res. 34, 375–385.
[16] Krieger, A. and Weis, E. (1993) Photosynth. Res. 37, 117–130.
[17] Vredenberg, W.J., Snel J.F.H., Dassen, H.J. and Van Voorthuysen, T. (1993) in Research in Photosynthesis (Murata, N. ed.), Vol II, pp. 681–685, Kluwer, Dordrecht.
[18] Haehnel, W. (1984) Annu. Rev. Plant Physiol. 35, 659–693.