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The electrical and chemical components of the protonmotive force in chloroplasts as measured with capillary and pH-sensitive microelectrodes

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After impaling an isolated giant chloroplast of *Peperomia metallica* with a glass-insulated antimony pH-microelectrode, the potential difference between this microprobe and the external reference electrode was monitored during light activation and compared with the light-induced change of the membrane potential. The signal recorded with the pH-sensitive microelectrode during illumination was composed of a change of the membrane potential and an additional component, consisting of a slow (20–30 s) voltage increase up to +80–+120 mV with respect to the initial dark level. The superposition of two components was interpreted as being due to the location of the tip of the pH-sensitive microelectrode inside the thylakoid. The slow component in the pH-microelectrode signal reflects presumably an acidification of the internal thylakoid by 1.8 pH units at saturating light intensity. An addition of 5 mM NH_4Cl to the medium resulted in a suppression of the slow component, but it also produced a significant increase in the stationary level of the membrane potential. The results are consistent with the suggestion that a decrease of a pH gradient in the presence of NH_4Cl is compensated by an increase in the membrane potential. It appears that the photo-induced voltage recorded with pH-sensitive antimony electrode inside the chloroplast reflects the time-course of the protonmotive force, provided that the change of pH in the chloroplast stroma is relatively small.

Introduction

It is generally accepted that the light-driven electron transport in chloroplast memmbranes is coupled to ATP synthesis through the H^+ electrochemical potential difference, $\Delta\tilde{\mu}_{H^+}$. In order to evaluate the contribution of the electrical ($\Delta\psi$) and the chemical (ΔpH) components to the overall $\Delta\tilde{\mu}_{H^+}$ it is always required to measure the membrane potential and the pH gradient in chloroplast thylakoids under comparable conditions with similar methods. Membrane potential, $\Delta\psi$, established in continuous light is commonly followed by electrochromic absorbance changes of photosynthetic pigments at 515 nm [4] and by absorbance changes of a potential-sensitive dye, oxonol VI [5], whereas a pH gradient, ΔpH, is assessed form fluorescence quenching of 9-aminoacridine and other fluorescent acridine derivatives [6]. The internal pH of thylakoids was also measured with permeable pH indicators [7] and from a distribution of amines [8,10]. Despite some drawbacks of indirect methods, such as light-scattering artifacts imposed over the ab-

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sorbance change at 515 nm [11], electrostatic interaction of charged forms of acridine derivatives or pH probes with fixed negative charges on thylakoid membranes [9], uncoupling effect of amines [10], these methods proved to be useful for qualitative estimates of $\Delta\psi$ and ΔpH.

Direct measurements of $\Delta\psi$ with microcapillary electrodes can be performed successfully on giant chloroplasts of some species [1,2,16]. It is not clear, however, whether the area of application of the microelectrode technique could be extended such as to allow direct measurements of the pH inside chloroplast thylakoids by a pH-sensitive microelectrode. From a technical ground it is possible to produce glass-insulated fast-responding antimony pH-microelectrodes with a tip-sensitive portion of about 1 μm in diameter [14]. Because of comparable tip dimensions of antimony and microcapillary electrodes, there is a high probability that, after hitting the granum stack, the tip of a pH-sensitive microelectrode would be located in the same compartment from which a light-induced $\Delta\psi$ is recorded by conventional capillary electrodes, that is in the inner space of thylakoids.

In this work, the electrical recordings were made with antimony pH-microelectrodes inserted in isolated individual chloroplasts. It is shown that the electric signal monitored by a pH-microelectrode after a sudden illumination of a chloroplast is composed of a change of $\Delta\psi$ and a slowly rising component which is inhibited by NH_4Cl and is presumably caused by an internal acidification of thylakoids. It is suggested that the photo-induced electrical signal recorded with a pH-microelectrode in chloroplast reflects qualitatively the time-course of $\Delta\tilde{\mu}_{H^+}$ formation.

Materials and Methods

Experiments were performed on intact chloroplasts of a higher plant, *Peperomia metallica*. The experimental solution contained 0.25 M sucrose/25 mM Tris-HCl buffer (pH 7.5)/Ficoll (25 g/l)/bovine serum albumin (1 mg per 10 ml). The method of chloroplast isolation was described elsewhere [1].

The method of fabrication of antimony pH-microelectrodes was similar to that previously described [14]. Glass capillary tubings of 1 mm external diameter, made from Pyrex glass, were filled with a melted antimony by means of a manual pump under 100 kPa of nitrogen. A copper wire was fused into the antimony from one end of the capillary in order to provide an electrical connection of the probe with an external circuit. Capillaries filled with antimony were used to draw pH-microelectrodes by means of a semi-automatic microelectrode puller. The pH-microelectrodes having tip diameters of 0.5–1 μm and an electrical resistance of about 10^9 Ω were selected and used for experiments.

The electrode function of antimony pH-microelectrodes was linear in the range from pH 2.0–10.0 (see also Ref. 14). A portion of the calibration curve in the range of pH 6.0–8.0 is shown in Fig. 1. The calibration of the pH-microelectrode in this pH range was done by means of 0.1 M Tris-HCl buffer. The slope of the dependence of the electrode potential on pH was 58.5 mV per 1 pH unit. At the illumination of the tip of the pH-microelectrode, in the absence of chloroplast, potential changes did not appear.

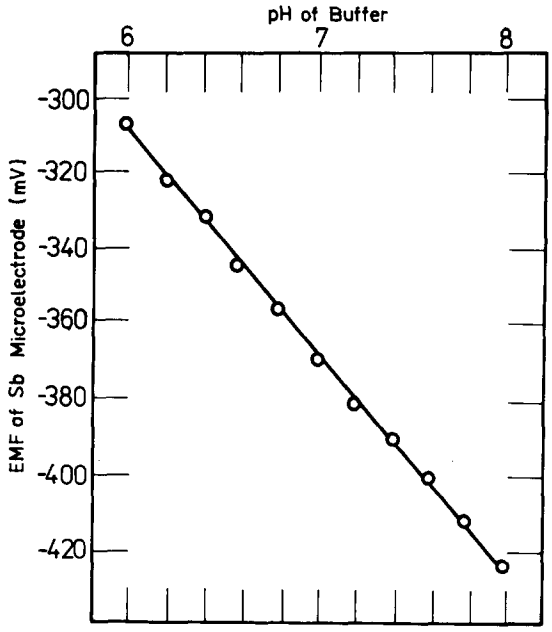

Fig. 1. The dependence of electromotive force (EMF) of the antimony microelectrode on pH in the range pH 6–8. The electromotive force was measured as the potential difference between the antimony microelectrode and the external reference calomel silver/silver-chloride half-cell.

Membrane potential was measured with microcapillary electrodes filled with 1 M choline-chloride. During the measurements, a chloroplast was fixed at the end of a fire-polished suction micropipette. Microelectrodes were inserted into the chloroplast by means of a step-wise driving system. The electrical potential difference between the microelectrode (either a pH-sensitive or a microcapillary electrode) and the external reference electrode was fed into a high-impedance amplifier (input resistance, 10^{12} Ω) and recorded on an oscilloscope. Chloroplasts were illuminated with white light of an incandescent lamp at an intensity of 17 $W \cdot m^{-2}$. Each experiment was repeated at least four times.

Results

In Fig. 2A are shown typical photo-induced changes of membrane potential, $\Delta\psi$, of one isolated chloroplast measured with conventional microcapillary electrodes and in Fig. 2B the changes of electric potential measured by a pH-microelectrode inside the chloroplast with respect to the external reference electrode. Photo-induced changes of $\Delta\psi$ were described before [1,2,16]. Upon illumination, the membrane potential rises quickly to +60–+80 mV and then declines to a steady level not exceeding +15 mV (Fig. 2A). When a pH-microelectrode was inserted into a chloroplast, a similar potential change, i.e., light-induced $\Delta\psi$ generation, was frequently measured, but it was followed by an additional slow-rising phase of the potential (Fig. 2B). We believe that signals detected with a pH-microelectrode as shown in Fig. 2B, should be ascribed to a situation where the tip of the pH-microelectrode is located in the intrathylakoid space. It may be clear that only under these conditions the photo-induced voltage between the pH-microelectrode and the outer reference electrode will include light-induced $\Delta\psi$, superimposed on the potential change of the pH-electrode. It seems likely, therefore, that the secondary rise of the voltage of 60–110 mV during 20–30 s of illumination, which is measured with the antimony pH-electrode, reflects the acidification of the internal thylakoid space.

Apart from typical responses shown in Fig. 2A and B, photo-induced electric signals of opposite

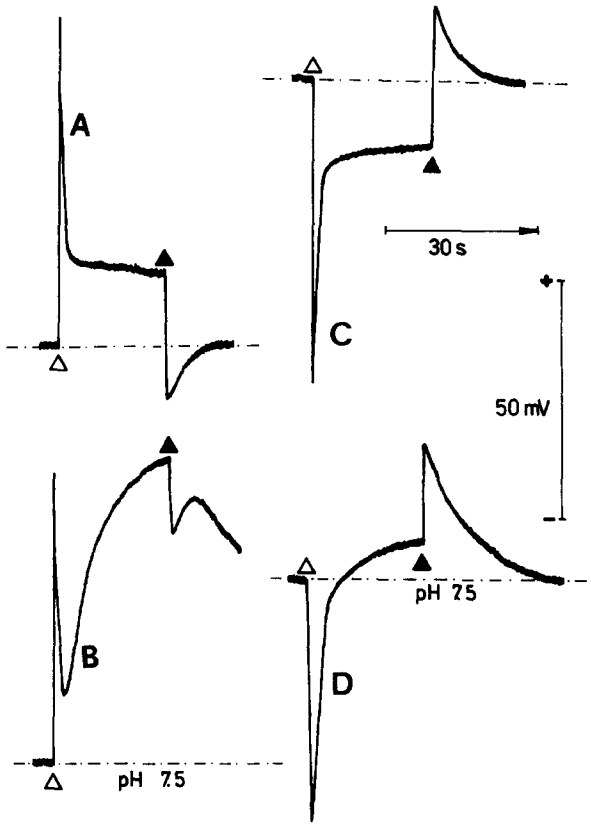

Fig. 2. (A) Light-induced membrane potential of a single isolated *Peperomia metallica* chloroplast, recorded with capillary microelectrodes. (B) Light-induced voltage signal recorded from an isolated chloroplast with an antimony pH-microelectrode. (C) An example of a reversed-polarity record of the membrane potential. (D) An example of a reversed-polarity signal recorded with the pH-sensitive microeelectrode. Open and filled triangles denote the beginning and the end of illumination, respectively.

sign were recorded sometimes both with capillary and pH-sensitive electrodes (Figs. 2C and D, respectively). Occasional variation in the sign of chloroplast photoelectric responses was reported previously [12] and it was interpreted as due to the location of the microelectrode tip in different compartments of the thylakoid structure within the chloroplast [12]. When the chloroplast was impaled with antimony pH-microelectrodes, the photo-responses of negative sign were measured more often (in 16 experiments out of a total of 46) than with conventional microelectrodes (less than 10% of recordings). This may be due to less fre-

quent penetrations of a thicker tip of an antimony pH-microelectrode into the internal thylakoid space. Only records showing positive light-induced potential changes were considered as meaningful.

In Fig. 3 is shown a schematic deconvolution of the overall photo-induced signal monitored with a pH-microelectrode into two separate components, $\Delta\psi$ and the pH change of a thylakoid lumen.

The assumed relation of the slow component to an acidification of a thylakoid interior was tested by studying the effect of NH_4Cl on both the light-induced $\Delta\psi$ and the photovoltage recorded with pH-microelectrodes. Ammonium salts are known to inhibit the formation of a pH gradient, both in isolated thylakoids and intact chloroplasts [10]. As shown in Fig. 4A the photoelectric response of a chloroplast in the presence of 5 mM NH_4Cl comprised two maxima of $\Delta\psi$. A stationary level of the membrane potential in the presence of NH_4Cl was high and it was established slower than under control conditions in the absence of the uncoupler (Fig. 2A). A similar increase of light-induced $\Delta\psi$ was also measured on *Anthoceros* chloroplasts in situ after addition of 2 mM NH_4Cl, although a significant stimulation of $\Delta\psi$ occurred only in the presence of $NaHCO_3$ or methylviologen as electron acceptors [13].

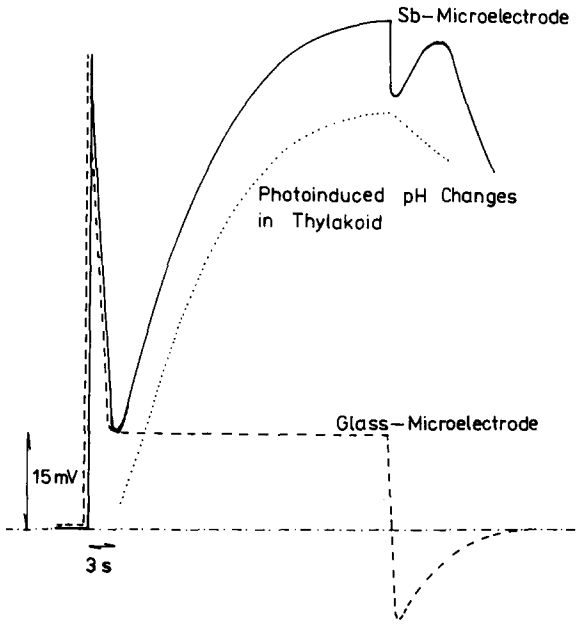

Fig. 3. A schematic representation of the signal recorded with antimony pH-microelectrode as a sum of two components, the membrane potential and the pH change in the thylakoid lumen.

In the presence of NH_4Cl, light-induced signals recorded with pH-microelectrodes (Fig. 4B) and capillary microelectrodes (Fig. 4A) were identical. Under control conditions (Fig. 2B), the dark decay of the signal of the pH-microelectrode occurred in 60–80 s after switching off the light, but the period of dark decay was shortened to 4–6 s after addition of NH_4Cl. The decay of the membrane potential after prolonged illumination occurred within 4–10 s, both in the absence and in the presence of NH_4Cl. These results show clearly that the light-induced $\Delta\psi$ is increased in the presence of NH_4Cl, whereas the slow component in the signal recorded by pH-microelectrodes is severely inhibited.

The photo-induced signals recorded with pH-sensitive and microcapillary electrodes were eliminated after addition of 10 mM 3(3,4-dichlorophenyl)-1,1-dimethylurea, an inhibitor of electron transport (not shown).

Discussion

Two main conclusions were drawn from this study. (1) The properties of a slow component of the photo-response recorded with pH-microelectrodes, particularly its sign and extent as well as its sensitivity to NH_4Cl, are consistent with the suggestion that this component reflects an internal acidification of thylakoids in the light. (2) A reduction of the pH gradient across the thylakoid membrane by the action of NH_4Cl is accompanied by an increase of the membrane potential.

Some comments should be done with respect to the interpretations of signals recorded with pH microelectrodes. If the tip of an ideal pH-microelectrode is located inside the thylakoid, then the light-induced change of the voltage between the pH-microelectrode and the external reference electrode (ΔV) is described by the following equation:

$$\Delta V = \Delta\psi - 2.3RT/F(pH_i - pH_d)$$

where $\Delta\psi$ is the light-induced membrane potential, pH_i is the internal pH of illuminated thylakoids, pH_d is the internal thylakoid pH in

the dark and R, T and F have their usual meanings (gas constant, absolute temperature and Faraday's constant, respectively). In chloroplast stroma the pH increases upon illumination by 0.4 pH unit at saturating light intensity [15]. Since the pH change in the thylakoid is at least 4–5-times higher than the pH change in stroma, it may be assumed that the term $(pH_i - pH_d)$ is almost equal to the pH difference across the thylakoid membrane. Then, the electrical signal ΔV, recorded with pH-microelectrodes, reflects roughly the time course of $\Delta\tilde{\mu}_{H^+}$ formation across the thylakoid membrane.

According to our measurements (Fig. 2), the intrathylakoid pH decreases by 1.8 unit upon illumination. Taking into account a pH change by 0.4 unit in chloroplast stroma, we obtain an estimate of 2.2 pH unit for the pH gradient, which is in harmony with a value of 2.5 pH unit determined from the distribution of NH_4^+ under at saturating light intensity [15]. The values of pH 3.6–4.2 reported on the basis of 9-aminoacridine fluorescence [6] quenching seem, therefore, to be overestimated.

From Fig. 4 it appears that the total level of $\Delta\tilde{\mu}_{H^+}$ is about the same both in the absence and in the presence of NH_4Cl, although the contribution of ψ and ΔpH is essentially different. This could be taken as evidence in support of the suggestion in Ref. 15 that the decrease of pH in the presence of NH_4Cl is compensated, at least partially, by an increase of the membrane potential. Such an increase of a stationary level of $\Delta\psi$ was not evident from measurements of electric-field-indicating absorbance changes at 515 nm [11], although the potential-sensitive absorbance changes of oxonol VI were stimulated in the presence of NH_4Cl by 30–80% [15]. These contradictory results, obtained by various methods, could be reconciled by taking into account possible changes of the surface potential in the thylakoid lumen after addition of NH_4Cl that acts as a H^+-binding agent. It is possible that a simultaneous increase of a positive $\Delta\psi$ and a negative displacement of the surface potential produce no significant alteration in the overall electric-field strength. An increase of the electric component of $\Delta\tilde{\mu}_{H^+}$, occurring concomitantly with a reduction

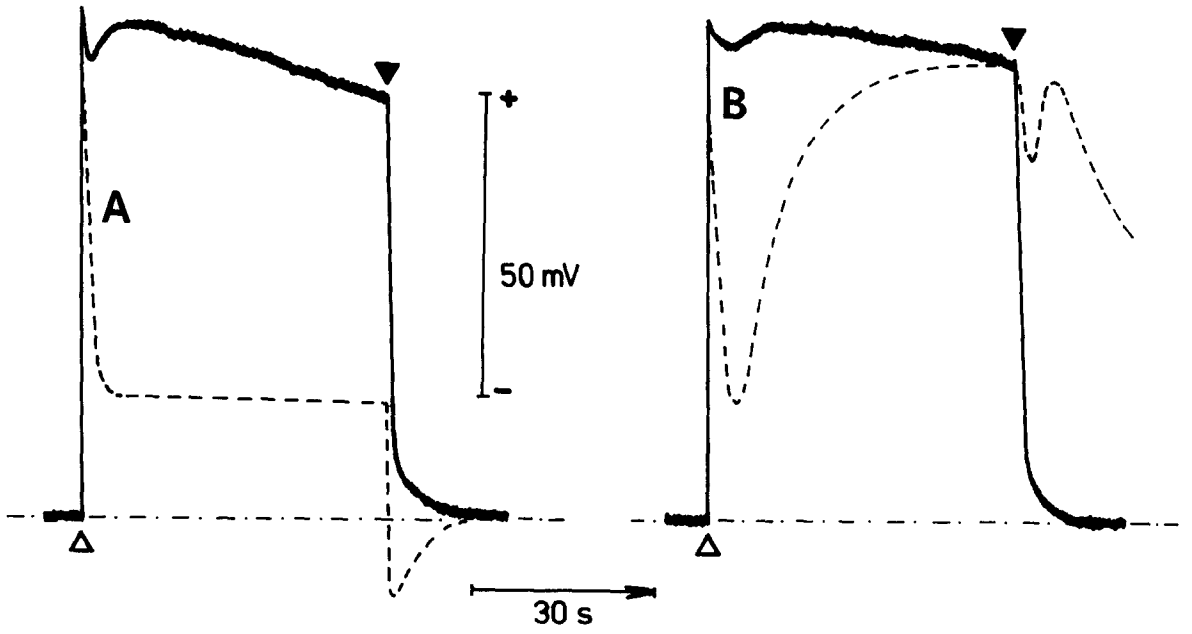

Fig. 4. (A) Photo-induced changes of the membrane potential of an isolated chloroplast measured with capillary electrodes in the presence of 5 mM NH_4Cl; a broken line denotes the change of the membrane potential under control conditions in the absence of NH_4Cl, (B) Photo-induced voltage signal recorded with pH-microelectrodes on chloroplasts treated with 5 mM NH_4Cl. The broken line shows the signal of the pH-microelectrode under control conditions.

of pH after addition of ammonium salts may possibly explain the relative insensitivity of photosynthesis of intact chloroplasts to NH_4Cl.

Quantitative estimates of pH inside the thylakoid are complicated due to interfering factors such as a dependence of the potential of antimony pH-microelectrodes on the oxygen pressure and the protein concentration. It is not yet excluded that other electrochemical processes could occur at the antimony electrode placed in such a heterogeneous system as a chloroplast. Nevertheless, light-induced signals of large amplitude could not be determined by a response of antimony electrodes to changes in O_2 concentration, because 10-fold increase in pO_2 leads to a change of the electrode potential by 10 mV only [17]. Perhaps the most serious complication could arise from the general sensitivity of metal electrodes to oxidation–reduction potential of the medium. However, the inhibition of the slow component in the signal of the pH-microelectrode by NH_4Cl argues against the possible involvement of redox potential change in the signal recorded in our system.

There is a qualitative agreement between the light-induced signals recorded with antimony pH-microelectrodes in chloroplasts and the calculated time-course of $\Delta\tilde{\mu}_{H^+}$ formation [18]. It appears that a combined application of capillary and pH-sensitive microelectrodes may become a promising tool for studying the kinetics of the proton motive force and of its separate components in chloroplasts.

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