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Contribution of electric field (Δψ) to steady-state transthylakoid proton motive force *in vivo*. ±

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Introduction

Proton motive force (*pmf*), which drives the phosphorylation of ADP by the F_1F_O ATP synthase, is sustained in chloroplasts by photoelectron transport. As described by chemiosmotic mechanism, *pmf* is the sum of the chemical and electric potentials, or ΔpH and Δψ, respectively (Mitchell,1966). Experimental observation suggests that a *pmf* of at least 120 to 180 mV (2 to 3 pH units) is required absolutely to sustain steady-state ATP synthesis (Hangarter and Good,1982). The generally accepted view contends that the electric field component does not contribute or at best contributes minimally to *pmf* (van Kooten, et al.,1986). However, if ΔpH is the sole component of *pmf*, then the inferred pH of the thylakoid lumen during photosynthesis (5.8 or lower) is inconsistent with lumenal pH requirements (Kramer, et al.,1999). A significant contribution of Δψ to *pmf* (30 to 60 mV) would satisfy the energetic requirements for ATP synthesis within the observed pH limitations of the biochemical system. This possibility raised a number of questions: 1) Does Δψ contribute significantly to *pmf*, 2) If so, what factors influence the magnitude of Δψ, and 3) To what extent does it contribute to *pmf*, *in vivo*? These issues were addressed through experiments using partially uncoupled thylakoids and a mathematical computer model based on values obtained from the literature. It was concluded that in a relatively low ionic strength environment (<10 mM) and with a moderately large lumenal buffering capacity (25 to 50 mM), Δψ may exist as a significantly large component of *pmf*, during steady state photosynthesis (Cruz, et al.,2001). More recently, we have performed experiments using the freshwater alga, *Chlamydomonas reinhardtii*, to extend these observations.

Materials and Methods

Chlamydomonas strain and growth conditions. It became apparent in our early work that net cell movements were a major contributing factor to long-term drifts. As a remedy, the CC-2853 strain of *C. reinhardtii* deficient in flagellar function (obtained from the *Chlamydomonas* Genetics Center, Duke University, Durham, North Carolina; for strain history see http://www.botany.duke.edu/chlamy/) was used for this study. Growing cells on acetate supplemented medium (TAP medium) greatly increased light-induced light-scattering changes, which likely was due to starch accumulation. Thus, following an initial period of growth on TAP (Tris-acetate phosphate) medium (Harris,1989), cells were transferred to HS (high salt) medium (Harris,1989), supplemented with an additional 10 mM of NH_4Cl, and

grown photosynthetically (~300 μmol photons m^{-2} s^{-1}) for 5-7 days. For spectroscopic assays, cells were concentrated by gentle centrifugation (5 min, ~150 X g, 18°C) and resuspended in HS medium to a final concentration approx. 30-40 μg chlorophyll/ml (Arnon,1949). Following resuspension, cells were allowed to recover for at least 30 min with stirring and under low light (~30 μmol photons m^{-2} s^{-1}), prior to assay.

Kinetic spectroscopy. Light-induced kinetics of the electrochromic shift (ECS) were obtained using a diffused optics flash spectrophotometer (DOFS) (Witt,1975), constructed with modifications that allowed use of glass cuvettes and are detailed in (Cruz, et al.,2001). To obtain reproducible results, it was critical to maintain the CO_2 and O_2 levels of the samples during spectroscopic assays. This was accomplished by computer-controlled, automated mechanical mixing which gently suspended the sample during the intervals between traces. Mixing was achieved by gently, periodic squeezing of a plastic transfer pipette (Fischer Scientific) via a computer-controlled servo motor. Glass cuvettes, which also permitted bubbling of CO_2 enriched air (5% CO_2, 20% O_2, balance N_2) during mixing, were constructed for use with the automated mixer. The samples were allowed to settle for 15 to 30 s before the beginning of each trace.

Results

Spectral contributions from ECS and light scattering in C. reinhardtii strain CC-2853.

Figures 1 (open squares) shows typical absorbance spectral changes that occur after illumination of intact C. reinhardtii cells (strain CC-2853) with 40 ms of 400 μmol photons m^{-2} s^{-1} red light. After such brief illumination, the vast majority of spectral changes in this range can be attributed to ECS, with a peak between 515 and 520 nm and a full bandwidth at half height of about 20 nm and the spectrum was essentially identical to that ascribed earlier to ECS (Kramer and Sacksteder,1998). Figure 1 (closed squares) shows a spectrum taken after 20 s of 400 μmol photons m^{-2} s^{-1} red light.

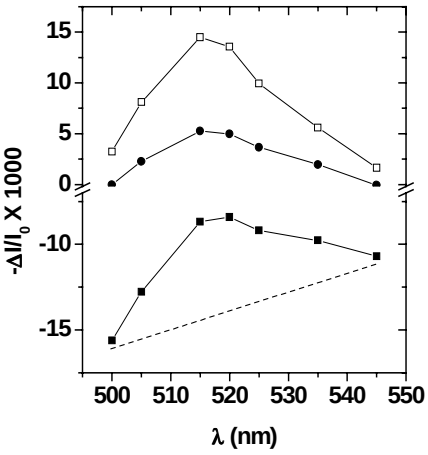

Figure 1. Kinetic analysis of the electrochromic shift during 60 s of continuous illumination. Shown are the ECS spectra after 40 ms (open squares) and 20 s (closed squares) of illumination. Shown also is the 20 s spectrum (closed circles), from which the baseline (dashed line) between 500 nm and 545 nm has been subtracted.

When a baseline taken between the absorbance changes between 500 and 545 nm was subtracted from the data, a spectrum was observed with a line shape essentially identical to that of ECS (Figure 1, closed circles). The ECS signal was completely eliminated by 10 μM nonactin and 25 mM KCl (data not shown). Under these conditions, a component similar to the broad signal, but with altered kinetics and amplitude, remained. Only small, relatively featureless changes were observed in the presence of 100 μM of the protonophore, CCCP (data not shown). These data led us to tentatively ascribe the broad bleaching to light scattering changes. The nearly flat spectral shape of the putative light-scattering signal allowed us to use a relatively simple deconvolution procedure to separate ECS from background signals. For each time point, a baseline was drawn between the changes at 505 and 535 nm. The difference the change at

515 nm and this baseline was taken as an indicator of the extent of ECS. Negligible signals were obtained when this procedure was performed on data taken in the presence of KCl/nonactin or CCCP (as above).

Kinetics of 60 s light pulse-induced ECS in intact cells of C. reinhardtii.

The kinetics of ECS, induced by a 60 s illumination with 400 μmol photons m^{-2} s^{-1} red light were plotted in Figure 2. Light-induced ECS peaked at around 40 ms after onset of illumination and subsequently decreased to a steady-state level. As discussed in (Cruz, et al.,2001), this indicates that light-induced *pmf* can be stored in living *C. reinhardtii* cells for extended periods of time, in this case at least 60 s. Upon switching off the light, the ECS signal decayed with a half time of about 50 ms to a level significantly below the baseline, a point termed the 'field inversion' (Cruz, et al.,2001). The inverted ECS decayed to the baseline level with a halftime of about 20 s.

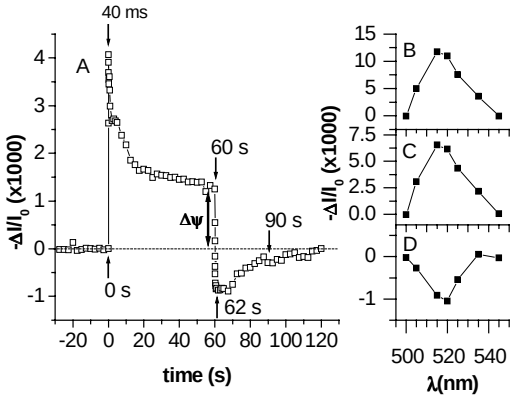

Figure 2. Kinetic analysis of the electrochromic shift during and following a 60 s of continuous illumination. Panel A shows the kinetics of ECS during and after a 60 s actinic interval. Panels B-D show difference spectra taken between selected time points: between time points 40 ms and 0 s (panel B), 60 s and 62 s (panel C), and 62 s and 90 s (panel D); these spectra have been baseline-corrected as in Fig. 1.

This general kinetic pattern was reproduced in five different batches of *C. reinhardtii* cells. Difference spectra taken between selected points on the kinetic curves confirmed that each kinetic phase could primarily be ascribed to ECS (Figure 2 B-D). Overall, the kinetics were very similar to those observed in isolated thylakoids under low ionic strength (Cruz, et al.,2001) or in intact higher plant leaves (Kramer and Sacksteder,1998; Cruz, et al.,2001).

Kinetics of ECS during dark intervals punctuating extended illuminations. Because it is difficult to measure stable signals over times greater than a few min, a different spectroscopic protocol was used to test whether Δψ remained non-zero for longer (i.e. hours) illumination regimes. Cells were illuminated, with mixing and aeration as described above, for an extended period (30 min to a few hours) to establish steady-state photosynthesis. Following this, at 5 min intervals, the light was switched off for one min while the ECS signal was probed (Figure 3). Judging by their spectral signatures, all of the kinetic phases shown in Figure 3 were attributable to ECS (see insets to Figure 3). Baselines were taken during the illumination so that the levels to which ECS decayed in the dark were negative. Upon shuttering of the continuous actinic light, a rapid decrease from steady-state ECS to an inverted ECS was observed, with a half time of approximately 25 ms. As with the experiments in Figure 2, the inverted ECS recovered with a halftime of about 20 s to a value

approximately midway between steady state value of ECS and full extent of the ECS inversion. Assuming that, after a full minute of darkness, ECS had decayed to its dark-stable value, the differences between ECS values at steady state and at the end of the dark interval should reflect the light-induced steady-state $\Delta\psi$. From this we concluded that $\Delta\psi$ during steady-state photosynthesis was significantly positive even after extensive illumination.

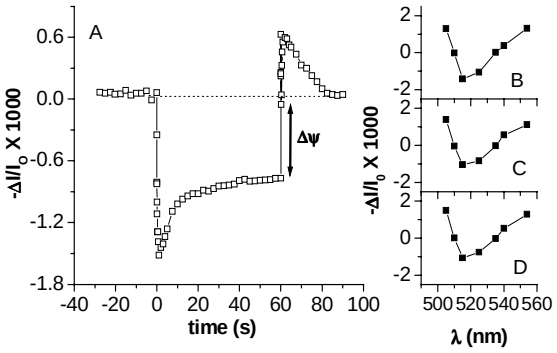

Figure 3. Kinetic analysis of the electrochromic shift during 60 s dark intervals punctuating continuous illumination. Panel A shows the deconvoluted kinetics of ECS during a 60 s dark interval in continuous illumination. Panels B-D show difference spectra taken between selected time points: between time points 40 ms and 0 s, (panel B), 60 s and 62 s, (panel C), and 62 s and 90 s, (panel D); these spectra have been baseline-corrected as in Fig. 1.

Conclusions

Spectral changes characteristic of an ECS are observed in the CC-2853 strain of *C. reinhardtii* upon illumination with an actinic light source (Figure 1). At steady-state ECS is above baseline, indicating that $\Delta\psi$ is significant under steady-state illumination (Figure 2). Experiments in which continuous illumination was punctuated by 60 s dark intervals (Figure 3), also indicated the presence of a substantial $\Delta\psi$ component. In recent work (Cruz, et al., 2001) we concluded that, at least under some conditions, the kinetics of ECS can reveal the relative extent of the $\Delta\psi$ component of steady-state *pmf*. Under ideal conditions, steady-state ECS and the sum of the steady-state ECS and inverted ECS should reflect steady-state values of $\Delta\psi$ and *pmf*, respectively. Based on these criteria, the data is consistent with $\Delta\psi$ comprising about 50% of *pmf*, at steady-state.

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