In Vivo Characterization of the Electrochemical Proton Gradient Generated in Darkness in Green Algae and Its Kinetic Effects on Cytochrome b_6f Turnover†

Giovanni Finazzi*,‡ and Fabrice Rappaport

CNRS UPR 1261, Institut de Biologie Physico-Chimique, 13 rue Pierre et Marie Curie, 75005 Paris, France

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ABSTRACT: When unicellular algal cells are placed under anaerobic conditions, a large electrochemical gradient is built in darkness across the thylakoid membranes. We have estimated, in vivo, the amplitude of the ΔpH component of this transmembrane potential and shown that the ΔpH is twice as large as the ΔΨ. The amplitude of the $\Delta\tilde{\mu}_{H^+}$ (∼110−140 mV) fits well with estimations based on the ATP/ADP ratio measured in green algae under the same conditions, suggesting that an equilibrium state is established across the thylakoid membrane. Therefore, under anaerobic dark incubation of algae, the electrochemical transmembrane potential is determined only by the cellular ATP content. The existence of this $\Delta\tilde{\mu}_{H^+}$ is expected to result in a constitutive amount of activated CF_o-CF_1 ATPase, thereby facilitating ATP synthesis under low light intensity illumination. We report also on the effects of this dark-existing electrochemical gradient on the cytochrome b_6f complex turnover kinetics. We show that they are largely slowed by the presence of this electrochemical transmembrane potential. The pH component is mainly responsible for the kinetic slowing down of cytochrome b_6f complex turnover, despite the fact that electrogenicity is associated with the reactions taking place within this complex. Therefore, in vivo, owing to the low lumenal pH, the oxidation of plastoquinol at the Q_o site is limiting the turnover of the cytochrome b_6f complex in the presence of the ΔpH, while in its absence the oxidation rate of the b_6 hemes becomes rate-limiting.

In chloroplast membranes, the components of the electron-transfer chain are arranged in such a way that their activity generates a transmembrane electrochemical gradient, $\Delta\tilde{\mu}_{H^+}$, which, according to the chemiosmotic theory (*1*), is the driving force for ATP synthesis. Charge separation at the level of photosystem 1 and photosystem 2, as well as intracomplex electron transfer in the cytochrome b_6f complex, results in the generation of an electric field, ΔΨ (*2, 3*), the magnitude of which has been estimated in the range of 40−100 mV (*4*). In addition to this, water splitting by photosystem 2 and plastoquinol (PQH_2)[1] oxidation by the cytochrome b_6f complex result in the injection of protons into the lumenal space, and thus in the building of a light-induced pH gradient of ∼3 units (*5*−*7*) across the thylakoid membranes. In chloroplasts, the ionic permeability allows a fast relaxation of ΔΨ (*8*), so it is usually considered that the pH component is predominant under steady-state illumination.

In algae, evidence has been provided for the existence of a permanent electrochemical gradient present in the dark (*9*−*11*). According to Bennoun (*11, 12*), this gradient, which cannot be accounted for by the light-induced photosynthetic activity, results from the interaction of the respiratory and photosynthetic apparatus. Under anaerobic conditions as well, an electrochemical gradient is generated in the dark, presumably at the expense of a fermentation process (*13*).

One of the consequences of this permanent electrochemical gradient is the slowing down of the turnover rate of the cytochrome b_6f complex (*14, 15*).

Cytochrome b_6f complex is constituted of seven subunits (*16*): cytochrome b_6, containing two *b* hemes (a low-potential one, b_l, and a high-potential b_h), cytochrome *f*, which binds a *c* heme, subunit IV, the 2Fe2S Rieske protein, and three small subunits of lower molecular mass. Two sites of PQ binding, Q_o and Q_i, respectively close to the b_l and b_h hemes, are recognized. According to the Q cycle hypothesis (*17, 18*), the oxidation of plastoquinol at the Q_o site is associated with the reduction of both cytochromes *f* and b_l and with proton release into the lumen. Oxidation of the *b* hemes then occurs through the reduction of a plastoquinone molecule at the Q_i site. Two steps of this process result in a partial transfer of one charge across the membrane (*19, 20*): electron transfer from b_l to b_h, and b_h oxidation at the Q_i site. Therefore, the cytochrome b_6f turnover rate is expected to depend on both components of $\Delta\tilde{\mu}_{H^+}$, the ΔΨ may result in an electrostatic constraint on the transmembrane electron-transfer reaction between the two *b* hemes, or the plastoquinol oxidation rate at the Q_o site may depend on the lumenal pH.

Until now, very little information was available on the in vivo amplitudes of parameters such as the transmembrane electrochemical gradient and the ΔΨ or ΔpH components. This was most likely due to experimental limitations such as the difficulty in performing quantitative analysis with whole cells or in distinguishing the ΔΨ component from the ΔpH component.

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* Corresponding author.

‡ Permanent address: Centro CNR per la Biologia Cellulare e Molecolare delle Piante, via Celoria 26, 20133 Milano, Italy. Fax: +39-2-26604399. Email: Finazzi@unimi.it.

[1] Abbreviations: FCCP, carbonylcyanide 4-fluoromethoxyphenylhydrazone; PQ, plastoquinone; PQH_2, plastoquinol; PS, photosystem.

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Joliot and Joliot (*12*) performed a detailed study on the electric component of the permanent electrochemical gradient built in algae, which was estimated in the 20−50 mV range. No information, however, was given on the existence of a ΔpH in the same conditions, nor on the relative size on the two components. In this paper, we focus on the study of the ΔpH component of the permanent electrochemical gradient present in darkness, on its relative amplitude. We have studied their respective effects on the kinetics of cytochrome b_6f complex in *C. sorokiniana* cells, taking advantage of the possibility to collapse selectively the ΔpH component by addition of the H^+/K^+ exchanger nigericin and to disrupt both components of $\Delta\tilde{\mu}_{H^+}$ by addition of an efficient uncoupler, FCCP.

MATERIALS AND METHODS

C. sorokiniana S8 cells (devoid of PS2) and S52 cells (devoid of PS2 and of a major fraction of LHC1 and LHC2) were isolated and kindly provided by P. Bennoun. Cells were grown in TAP medium (*21*) under a continuous illumination (intensity 300-lux).

Algae were harvested during the exponential growth phase and suspended in 20 mM HEPES buffer (pH 7.2) with 10% (w/v) Ficoll, to avoid sedimentation. When measuring the pH dependence of the cytochrome b_6f turnover rate, the algae were incubated for 30 min with permeant buffers and in the presence of FCCP. The buffers (sodium acetate, 30 mM, +10% Ficoll in the pH 4.5−6 pH range or sodium-phosphate, 30 mM, +10% Ficoll in the pH 6−7.5 range) are slowly metabolized by the cell, so that both could penetrate the cells, and none has a toxic effect. For these reasons, these buffers were preferred to other permeant buffers such as pyridine, hydroxyethylmorpholine, and imidazole that are often used for in vitro studies (*22*).

Spectroscopic measurements were performed at room temperature, using an apparatus similar to that described in Joliot et al. (*23*), and modified as in Joliot and Joliot (*15*). Actinic flashes were provided by a xenon flash (3 μs at half-height) filtered through a Schott filter (BG8). Actinic flashes were fired at a frequency of 0.15 Hz. Algae were kept in the dark under an argon atmosphere in a large reservoir, connected to the measuring cuvette.

The transmembrane potential was estimated by the amplitude of the electrochromic shift at 515 nm, which yields a linear response (but see below) with respect to the ΔΨ component of the transmembrane potential (*2*). Under our conditions, the kinetics of the electrochromic signal display two phases previously described in (*3*): a fast phase completed in less than 1μs, associated with PS1 and PS2 charge separation (phase a), and a slow phase which develops in the millisecond time range, associated with the turnover of the cytochrome b_6f complex (phase b). In the mutant strains of *C. sorokiniana* used here, which lack PS2, the amplitude of phase a is therefore a measure of PS1 charge separation.

The kinetics of phase b were deconvoluted from membrane potential decay, assuming that the latter process has first-order kinetics. Phase b was then computed assuming that the rate of decay of the membrane potential was linearly related to the actual value of the membrane potential. Although the decay of the transmembrane potential is known to be multiphasic, deconvolution of b phase kinetics assuming multiexponential kinetic decays for membrane potential did not improve significantly the results of the procedure (not shown). This is not surprising since the b phase proceeds in the 5−10 ms time range whereas the transmembrane potential decay displays two phases with half-times in the hundred milliseconds and in the time range of seconds (*3*). Therefore, only the fastest one is expected to superimpose to the b phase kinetics.

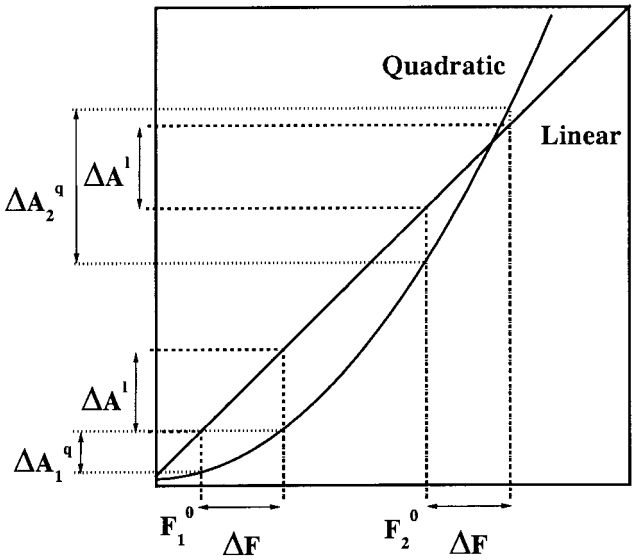

FIGURE 1: Schematic illustration of the method used here to probe the preexisting ΔΨ. The curves represent the amplitude of the electrochromic shift in the case of linearly or quadratically responding probes. Δ*F* stands for the light-induced transmembrane difference potential, F^0_1 and F^0_2 are the values of the electric field preexisting the flash in two different cases, ΔA^l and ΔA^q are the electrochromic responses of the linear and quadratic probes, respectively. It can be seen that ΔA^q depends on F^0 whereas ΔA^l does not.

Cytochrome *f* redox changes were evaluated as the difference between the absorption at 554 nm and a base line drawn between 545 and 573 nm. Cytochrome b_6 redox changes were measured as the difference between the absorption at 564 nm and the same base line.

Continuous red illumination was provided by an LED array placed on both sides of the measuring cuvette.

RESULTS

To better characterize the $\Delta\tilde{\mu}_{H^+}$ built in dark-adapted algae under anaerobic conditions, we took advantage of the possibility to suppress the ΔpH without disrupting the ΔΨ component, by using nigericin, which is an efficient K^+/H^+ exchanger (*24*). To this aim, we addressed the effect of nigericin on the spectrum of the electrochromic shift measured after excitation of the photosynthetic apparatus with a subsaturating flash.

The theory of electrochromism (see, e.g., ref *4*) states that the amplitude of the spectral shift undergone by a pigment when submitted to an electric field (*F*) is proportional to *F* or F^2 depending on the polarization state of the pigment. As schematically illustrated in Figure 1, the amplitude (ΔA^q) of the quadratic response induced by a perturbation of the applied field (Δ*F*; such a perturbation is induced, e.g., by the flash-induced charge separation at the level of PSI and PSII) depends on the value of the field (F^0) which preexists

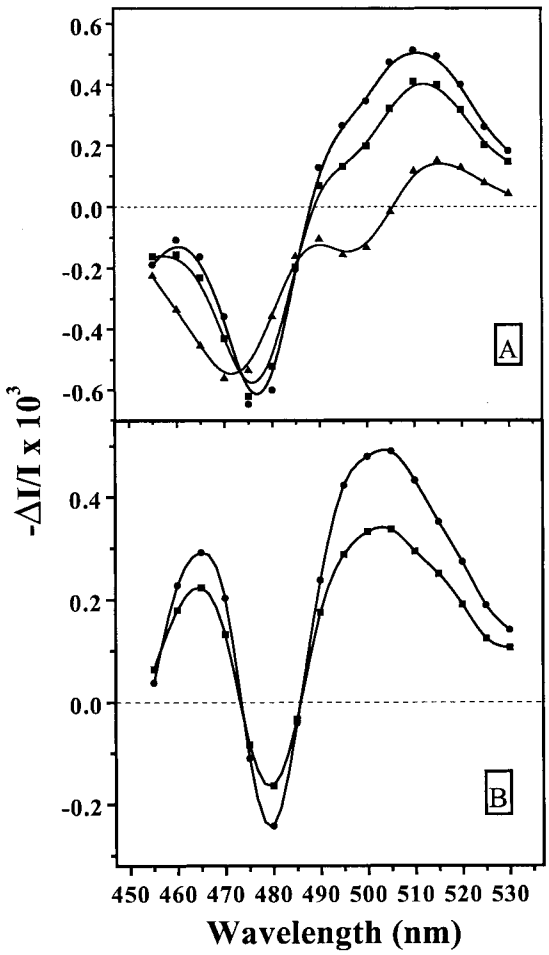

FIGURE 2: (A) Electrochromic absorption changes induced by a nonsaturating flash in C. *sorokiniana* S52 cells. The absorption change was measured 100 μs after the actinic flash hitting 50% of centers. Squares, control; circles, nigericin, 100 nM; triangles, nigericin (100 nM) + FCCP (5 μM). (B) Absorption changes due to quadratic electrochromic probes, obtained by subtracting the FCCP trace from the control (squares) and from the nigericin (circles) spectra.

the perturbation itself (the smaller the preexisting transmembrane potential, the smaller the electrochromic shift). In the case of the linear response, the amplitude of the signal (ΔA^l) is independent of the value of the field preexisting the perturbation.

Thus, the amplitude of the flash-induced electrochromic shift is expected to probe the permanent $\Delta\Psi$ if the quadratic response is not negligible with respect to the linear one. This has been experimentally evidenced (*13*), for example, in the case of the mutant *C. sorokiniana* S52, where most of the pigment binding proteins are absent and both the linear and quadratic probes may be observed, at variance with wild-type and S8 cells, where the linear response is largely predominant.

Figure 2 shows the spectra of the flash-induced electrochromic shift measured after a prolonged anaerobic incubation in the absence of any addition (squares), in the presence of nigericin (circles), or in the presence of nigericin + FCCP (triangles), to relax completely the $\Delta\tilde{\mu}_{H^+}$. As expected from the uncoupling properties of FCCP, the latter spectrum (Figure 2A) is similar to that extrapolated by Joliot and Joliot (*13*) to the 0 value of the preexisting transmembrane potential. We thus conclude that it reflects the electrochromic shift due to the sole linear probes (because ΔA^q is expected to be negligible compared to ΔA^l when F^0 is 0; see Figure 1). Thus, subtracting it from the spectrum measured in the absence of uncouplers should yield the spectra of the quadratic probes (Figure 2B), since the linear and the quadratic ones simultaneously report on the flash-induced change of transmembrane potential in the presence of a permanent $\Delta\Psi$. Addition of nigericin before the actinic flash induced no significant modification of the spectrum when compared to the control but a small increase of its amplitude. This shows that, as expected, nigericin addition does not collapse the component of the transmembrane potential resulting from the combination of the dark and the photoinduced potentials, but increases its amplitude. This increase of $\Delta\Psi$ may be ascribed to a conversion of the ΔpH into a $\Delta\Psi$ upon addition of nigericin (see discussion below). It would then indicate that a ΔpH indeed exists across the thylakoid membranes in dark anaerobic conditions. After addition of nigericin, the amplitude of the electrochromic signal decreased slowly (half-time ~30 min) to a new plateau level, close to that measured in the absence of ionophore (data not shown).

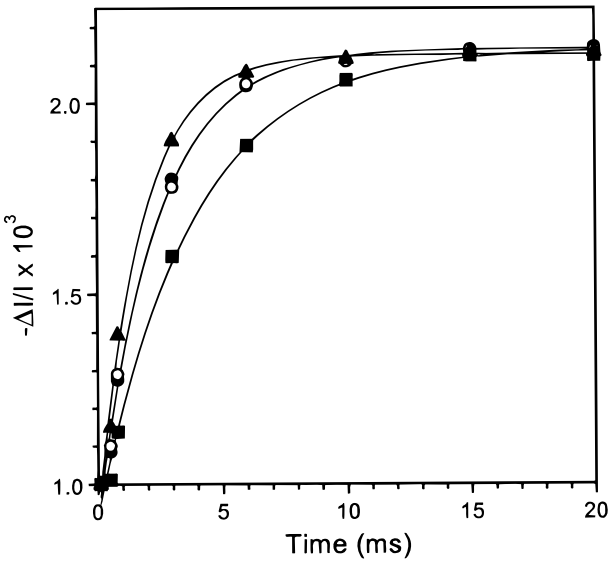

FIGURE 3: Slow electrochromic reaction (b phase) in C. sorokiniana S8 cells. Algae were illuminated with nonsaturating red flashes hitting 30% of the centers at a frequency of 0.15 Hz in 20 mM HEPES pH 7.2. Kinetics were corrected for membrane potential decay as described under the Materials and Methods. Squares, control; circles, nigericin (closed, 100 nM; open, 1μM); triangles, nigericin (100 nM) and FCCP (5 μM). The curves are the best fits of the data using a single exponential.

To better characterize the ΔpH component of the $\Delta\tilde{\mu}_{H^+}$ we have focused on the electron-transfer reactions catalyzed by the cytochrome b_6f complex, which are known to depend on the presence of the electrochemical permanent gradient (*14*, *15*).

The kinetics of the b phase in *C. sorokiniana* S8 cells are shown in Figure 3. All data were normalized to the amplitude of the a phase, i.e., to the charge separation at the level of photosystem 1 (see above). In this strain that, at variance with S52, does not lack the antenna, this amplitude was similar irrespective of the experimental conditions (not shown). In the absence of ionophores, the half-time was ~3 ms. Collapsing the $\Delta\tilde{\mu}_{H^+}$ by addition of FCCP resulted in a smaller half-time (~1.3 ms), in agreement with previous

reports (*15*). The relaxation of the ΔpH by addition of nigericin largely enhanced the kinetics of the b phase ($t_{1/2}$ 1.7 ms), but not as much as upon addition of FCCP. The effect of nigericin addition was maximal at rather low concentrations (100 nM), consistent with its efficient access to the thylakoid membranes. Unfortunately, we were unable to collapse selectively the ΔΨ using ionophores such as nonactin and valinomicin since addition of these compounds significantly increased the proton leakage through the membrane (data not shown).

We then tested the absolute pH dependence of cytochrome b_6f turnover in intact cells. Algae were incubated for half an hour with permeant buffers and in the presence of an H^+ carrier (FCCP) to allow pH equilibration between the external medium and the different compartments of the cells. Figure 4A shows the kinetics of the b phase measured at different pH's. In the presence of FCCP, between pH 7 and 6, we observed only a small variation of the rate of the b phase. At pH 5, however, the kinetics were markedly slowed with respect to those measured at pH 7, and the rate was similar to that observed in the absence of any uncoupler. In Figure 4B, the pseudo-first-order rate constant of the b phase is plotted as a function of pH. Increasing the pH results in a significant enhancement of the rate of the b phase. Surprisingly, when algae were incubated with nonpermeant buffers, no significant pH effect was observed in the pH 4.5−8 range, even when the concentration of uncoupler was increased to 10−15 μM. Above these concentrations, FCCP addition inhibited b phase kinetics in a pH-aspecific manner (not shown). This suggests that permeant buffers are needed to equilibrate the pH of the cellular cytosol with that of the external medium (see discussion below). However, even in the absence of permeant buffers, the observed kinetic effects of nigericin addition on the b phase (Figure 3) suggest that, in the presence of nigericin, equilibration between the stroma and the lumen is achieved. Therefore, we may estimate the value of the stromal pH (∼7) from the rate of phase b measured in the presence of 100 nM nigericin but in the absence of permeant buffer (dotted line in Figure 4B), provided the buffering capacity of the stroma is larger than the lumenal one. The rate observed in the absence of any ionophore is indicated by the dashed line. Since the rate of the b phase is controlled by the absolute value of the pH rather than by the existence of a ΔpH, this rate is indicative of the lumenal pH value in the presence of the $\Delta\tilde{\mu}_{H^+}$ (∼5.5). These results show that a pH difference of at least 1.5 pH units (6.9 − 5.4 = 1.5; 90 mV) exists across the thylakoid membranes upon prolonged anaerobic incubation. This value may be, however, underestimated because of the very small variations measured at pHs above 6.5.

The b phase is triggered by the oxidation of PQH_2 by hemes b_l and f at the Q_o site. None of these processes is electrogenic (*25*), so it is not possible to evaluate their kinetic parameters from those of the b phase. We have thus studied the effect of nigericin or FCCP addition on cytochrome b_l and cytochrome f reduction. These measurements were performed in the S52 strain to allow a more reliable evaluation of the kinetic parameters. In these cells, indeed, the amplitude of the electrochromic signal, which largely overlaps the cytochrome spectral variations, is negligible because of the diminished antennae content. We have checked, in the case of cytochrome f at least, that similar

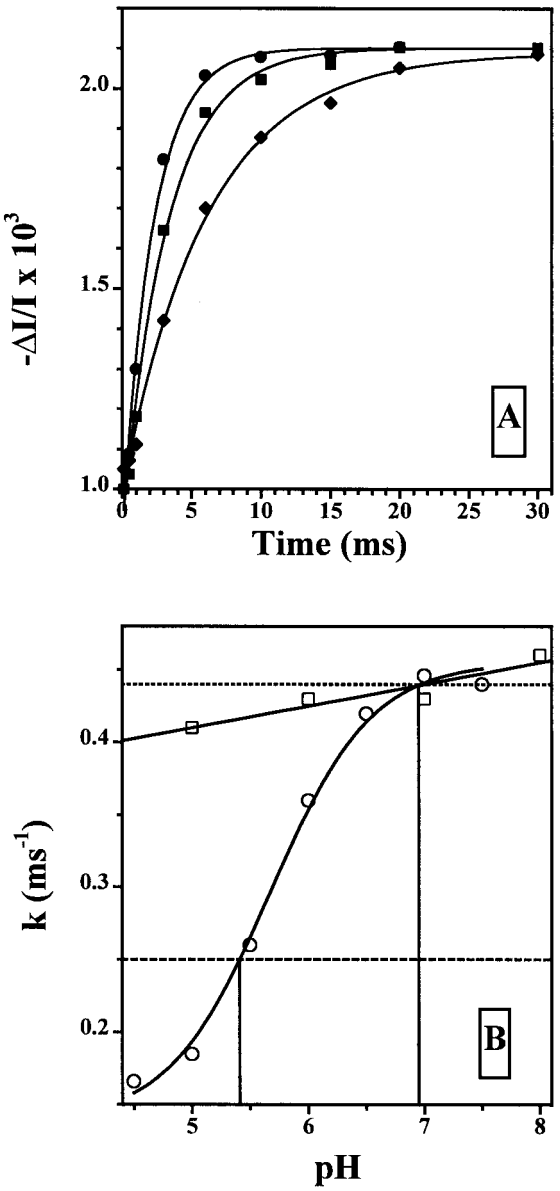

FIGURE 4: (A) Kinetics of the slow electrochromic signal as a function of the absolute pH value in C. sorokiniana S8 cells. Circles, pH 7; squares, pH 6; diamonds, pH 5. FCCP was present at a concentration of 5 μM. Same light conditions as in Figure 3. (B) Absolute pH dependence of the slow electrochromic signal. The pseudo-first-order rate constants were calculated from the data fit of phase b kinetics with a single exponential. Squares, MES or 30 mM HEPES buffer, Ficoll 10% (w/v). Circles, acetate or 30 mM phosphate buffer, Ficoll 10% (w/v). Other symbols: see text. Note that at pH 6 the same rate constant was obtained in the presence of acetate or phosphate.

results could be obtained in S8 cells and wild-type cells (data not shown). This suggests that the amplitude of the dark-existing electrochemical potential and the relative amplitudes of ΔΨ and ΔpH are similar in the wild-type and mutant strains.

In algae, it is difficult to distinguish between hemes b_l and b_h spectral changes, because their spectra are almost identical (*26*). They may, however, be differentiated on a kinetic basis, taking advantage of the separation in time of their respective rate of rereduction in the dark under anaerobic conditions: this rate is in the time range of seconds for the b_h heme, whereas more than 10 min is needed to reduce, at least partially, b_l. Under the conditions of

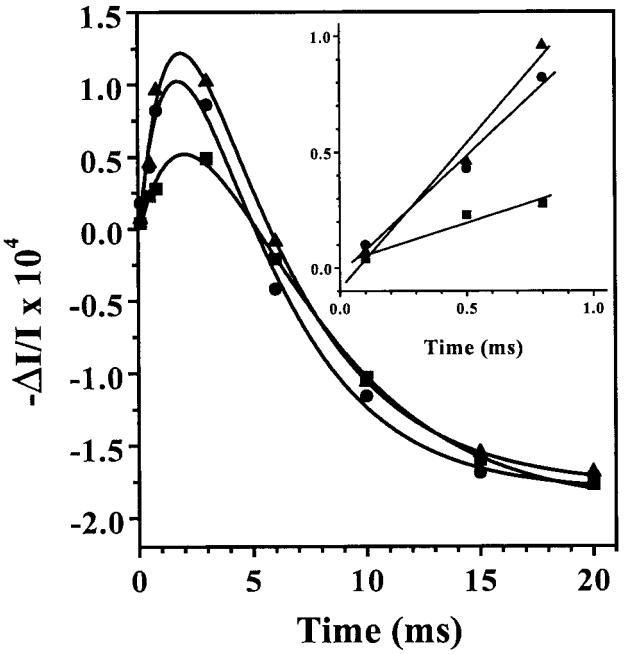

FIGURE 5: Cytochrome b_6 redox changes in *C. sorokiniana* S52 cells. Cytochrome b_6 absorption changes were evaluated as described under Materials and Methods. Squares, control; circles, nigericin 100 nM; triangles, nigericin 100 nM + FCCP 5 μM. Same conditions as in Figure 3. Curves are best fits to data points. The inset shows the linear fit of the reduction kinetics on a smaller time scale.

illumination used in this study (repetitive flashes at a frequency of 0.15 Hz), therefore, heme b_h should be rereduced in the dark time between two consecutive flashes, while heme b_l should not.

The kinetics of cytochrome b_6 redox changes are presented in Figure 5. In the absence of ionophores, a small signal increase (reflecting cytochrome b_l reduction) is observed, followed by a larger oxidation signal. Addition of nigericin or of FCCP induced an increase in the extent of the reduction signal, consistent with an acceleration of the reduction process: a linear fit of the initial rate of the kinetics indicated an enhancement by a factor of ~4 upon addition of nigericin, which was hardly affected by FCCP.

The effect of nigericin or FCCP addition on the kinetics of cytochrome *f* redox changes is shown in Figure 6. Both oxidation of cytochrome *f* by plastocyanin and its rereduction by plastoquinol (through the Rieske protein) were accelerated upon nigericin addition: the oxidation half-time (inset) decreased from 350 to 130 μs upon FCCP addition as previously reported by Delosme (*27*). Again, the nigericin effect on the kinetics was similar to that of FCCP. Similarly, the half-time of rereduction decreased from 3.5 ms in the absence of ionophores to 1.7 ms in the presence of nigericin and to 1.5 ms in the presence of both nigericin and FCCP.

The results presented here show that a good correlation exists between the pH effect on the rate of the b phase and of the hemes b_l or *f* reduction. Furthermore, at low pH, the plastoquinol oxidation is rate-limiting and the rates of cytochrome b_6 and *f* reduction are similar to that of cytochrome b_6 oxidation; at high pH, the plastoquinol oxidation is enhanced and the rate of cytochrome b_6 oxidation becomes rate-limiting with respect to the whole turnover of the complex.

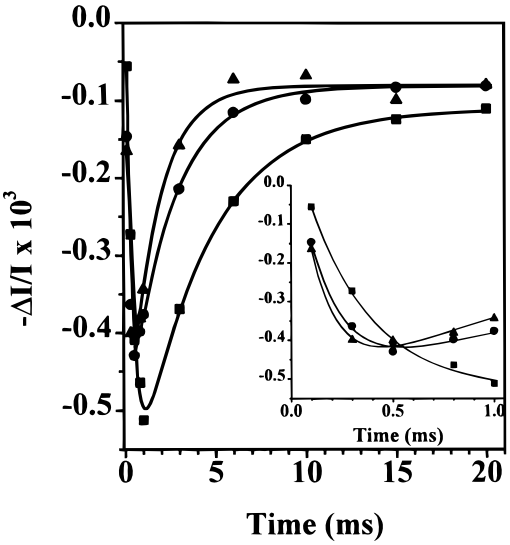

FIGURE 6: Cytochrome *f* redox changes in *C. sorokiniana* S52 cells. Cytochrome *f* redox changes were measured as described under Materials and Methods. Squares, control; circles, nigericin 100 nM; triangles, nigericin 100 nM + FCCP 5 μM. Curves are best fits of the data. The inset shows the oxidation kinetics on a smaller time scale. Other conditions as in Figure 3.

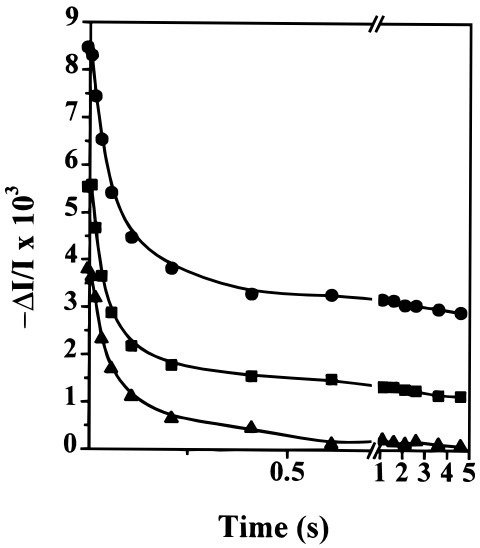

FIGURE 7: Effect of continuous light preillumination on the absorption changes at 515 nm in *C. sorokiniana* S8 cells. Squares, control. Circles, nigericin 1 μM. Triangles, nigericin 1 μM + FCCP 5 μM.

At this stage, one may address the question on the origin of $\Delta\tilde{\mu}_{H^+}$. Most likely, it is built at the expense of ATP (*13*). Therefore, we have investigated the effect of ATP synthesis on $\Delta\Psi$. ATP synthesis was induced by a 3.5 s continuous illumination period. Figure 7 shows the kinetics of absorption changes measured after the light has been switched off at 515 nm in S8 cells. In this strain, the antenna content is identical to that of the wild type; thus the 515 nm absorption change yields a linear response with respect to the $\Delta\Psi$. After the actinic continuous light has been switched off, a transient decay ($t_{1/2}$ ~ 30−40 ms) is observed. In the presence of FCCP, the signal fell to 0 whereas in the absence of uncouplers or in the presence of nigericin the signal decayed to a plateau level. This suggests that at least part of the ATP synthesized during illumination is hydrolyzed, in the dark, to build an additional $\Delta\Psi$. Consistent with the effect of nigericin addition on the $\Delta\Psi$ preexisting in the dark (see

Figure 2), the plateau level observed in the presence of this compound was larger than in its absence.

DISCUSSION

Modulation by $\Delta\tilde{\mu}_{H^+}$ of the Cytochrome b_6f Turnover. The results presented here show that the cytochrome b_6f turnover kinetics are mainly dependent on the lumenal pH. Indeed, even though the $\Delta\Psi$ component is increased upon nigericin addition, the enhancement of the kinetics of the b phase or of the reduction of cytochromes b_6 and f observed upon addition of nigericin is of similar amplitude to that observed upon addition of nigericin + FCCP, that collapse both the $\Delta\Psi$ and ΔpH components of the transmembrane potential. Furthermore, we have been able to mimic this enhancement by varying the pH of the cells (Figure 4). This shows that the absolute value of the lumenal pH rather than the ΔpH proper is mainly responsible for the modulation of these kinetics. This is in agreement with previous findings (*5*) according to which, in vitro, the electron transfer between PS2 and PS1 is controlled by pH. The pH dependence of the rate constant of the b phase is consistent with previous results obtained in vitro with a purified b_6f complex (*28*, *29*) or with a PS1, plastocyanin, and cytochrome b_6f complex reconstituted system (*30)*. Yet, the titration curve found here is shifted toward more acidic pH values. However, our results contradict Bouges Bocquet (*14*), who concluded, from study of the pH dependence of the b phase, that the cytochrome b_6f turnover is controlled by the $\Delta\Psi$ and hardly by the ΔpH. We note that these studies were performed with isolated thylakoids from spinach and that the reduction of the PQ pool was obtained by addition of dithionite. We note that under these conditions the kinetics of the b phase are markedly slower than those reported here or in a previous report (*15*), suggesting that a different mechanism occurs.

A striking feature that also emerges from the present study is the absence of correlation between the kinetics of the b phase and of cytochrome b_6 oxidation. This result is all the more surprising since this electrogenic phase is often considered as the signature of the sole interheme electron-transfer reaction (*18, 31, 32*). That this is not the case and that the b phase is likely at least partially resulting from other events may be illustrated by the absence of an ionophore effect on the rate of cytochrome b_6 oxidation (see Figure 5) which is to be compared to the modulation by nigericin and/or by FCCP of the rate of the b phase (Figure 3). This absence of correlation is striking, since the b phase is completed after 15 ms in uncoupled conditions, whereas the oxidation of cytochrome b_6 is not. These results combined with the good correlation in time between the b phase and the electron-transfer reactions between PQH_2 and cytochromes b_6 or f strongly suggest that phase b results from an electrogenic event which is triggered by the oxidation of the plastoquinol at the Q_o site rather than by the interheme electron-transfer reaction proper.

Estimation of the Amplitude of ΔpH under Anaerobiosis. The data of Figure 4B show that permeant buffers are needed to control the pH in vivo. Indeed, in the presence of nonpermeant buffers, the cellular pH does not equilibrate with the external pH even in the presence of ionophores (squares, Figure 4B). However, equilibration between the stroma and the lumen compartments may nevertheless be achieved, as shown by the effects of nigericin or FCCP on the b phase kinetics (Figure 3). Tentatively, we propose that even in the presence of small amounts of ionophores, the increased passive leak through the membrane may be large enough to allow proton equilibration across the thylakoid membrane. It is known, indeed, that in these membranes a small fraction of the CF_o-CF_1 ATPase pump is active in anaerobic conditions (*33*). On the contrary, other ATPase pumps (e.g. the cytoplasmic membrane ones), which are presumably in their activated state, are expected to maintain a transmembrane potential even in the presence of this slightly increased membrane permeability.

As previously discussed, the data of Figure 4B allow the estimation of the amplitude of the pH component of the permanent electrochemical gradient induced by anaerobic conditions (ΔpH = 1.5 pH units, i.e., 90 mV). Joliot and Joliot (*13*) have estimated the value of $\Delta\Psi$ to be about 20−50 mV. This estimation was based upon the ratio between the electrochromic shifts undergone by the quadratic and linear probes. Since both the spectra and their relative amplitudes reported here are very similar to the one measured in (*13*), we may use this $\Delta\Psi$ figure to calculate the $\Delta\tilde{\mu}_{H^+}$ built in the dark under anaerobic conditions in the 110−140 mV range. This value is smaller than the one built up by the light-induced electron-transfer activity in isolated spinach thylakoids (*6, 7*). Moreover, assuming the free P_i concentration in the chloroplast is in the 5 mM range (*34*) and an ATP/ADP ratio at equilibrium of ∼5 (*35*) yields a $\Delta\tilde{\mu}_{H^+}$ of 120 or 160 mV if an H^+/ATP ratio of 3 or 4 is assumed, respectively. This is in good agreement with the estimation of the $\Delta\tilde{\mu}_{H^+}$ reported here, supporting the idea that the $\Delta\tilde{\mu}_{H^+}$ built by algae in darkness under anaerobic conditions is governed by the ATP/ADP ratio.

A corollary of this hypothesis is that augmentation of the ATP/ADP ratio is expected to increase the electrochemical gradient, provided the membrane leakage is small. This is consistent with the findings (Figure 7) that the ATP synthesis induced by continuous illumination results in an increase of $\Delta\Psi$, measured after the light has been switched off. Conversely, Joliot and Joliot have recently shown that, when the CF_o-CF_1 ATP synthase complex is inhibited by addition of DCCD, the kinetics of the b phase are similar to those observed in the presence of uncouplers (*36*), suggesting that no or little $\Delta\tilde{\mu}_{H^+}$ is built when the CF_o-CF_1 ATP synthase is inhibited.

Effect of the Addition of Nigericin on the Dark Transmembrane Electrochemical Potential. The results presented here (Figure 2) show that nigericin has access to the thylakoid membrane of intact cells of algae and that it does not collapse the $\Delta\Psi$ component of the transmembrane electrochemical gradient built in darkness under anaerobic conditions. This is expected since nigericin activity (K^+/H^+ exchange) is electrostatically neutral. Interestingly, the data presented in Figure 2B show not only that the $\Delta\Psi$ component is not collapsed upon addition of nigericin but also that it is actually increased. The amplitude of the electrochromic spectrum is 1.4 times larger in the presence of nigericin than in its absence; thus, taking into account the quadratic character of the response of the electrochromic probes, the $\Delta\Psi$ component is increased by a factor of 1.2. Since nigericin itself is not an active ion carrier, we propose that, as expected from the chemiosmotic theory, this increase is due to a conversion of the ΔpH into a $\Delta\Psi$ to keep the $\Delta\tilde{\mu}_{H^+}$ constant.

In principle, the increase in $\Delta\Psi$ upon nigericin addition might be a good measure of the preexisting ΔpH. The discrepancy between the pH estimation based on the results previously discussed (1.5 ΔpH units) and those discussed here shows that this is not the case. The increase by a factor of 1.2 of the amplitude of the electrochromic spectrum observed in Figure 2B suggests that the amplitude of the ΔpH is at most on the order of 10 mV ($0.2 \times 50 = 10$ mV; i.e., 0.17 pH unit), i.e., 9 times smaller than the figure of 1.5 pH units estimated from the data of Figure 4B. However, a stoichiometric conversion of the ΔpH into $\Delta\Psi$ may only be expected when the ATP/ADP ratio is equal to the $\Delta\tilde{\mu}_{H^+}$ actually existing. Several nonexclusive possibilities may account for the nonstoichiometric conversion even at equilibrium: (i) the membrane leak in the presence of nigericin is likely to be larger than the proton pumping into the lumen, so that equilibrium is not achieved after nigericin addition, i.e., $\Delta\tilde{\mu}_{H^+} < G_0' + RT \ln([ATP]/[ADP\cdot Pi])$; (ii) the ATP supply could be limiting in the presence of nigericin so that the $\Delta\tilde{\mu}_{H^+}$ value would be reduced in the presence of this ionophore. Indeed, Bulté et al. (*35*) have shown that under anaerobic conditions the intracellular amount of ATP cannot be maintained to its aerobic level for its production occurs at the expense of the cellular energy source which is not inexhaustible. Consistent with this finding, we have observed that the increase in $\Delta\Psi$ measured after addition of nigericin is actually a transient event, which relaxes with a half-time of 30 min. This relaxation presumably reflects the slow exhaustion of the "fuel" supply. However, since after 2 h the $\Delta\Psi$ component has reached a plateau level, some regulatory events presumably allow the cells to keep its energy charge at a minimal nonzero level even in the uncoupled state.

CONCLUSION

We confirm in this work the existence of a strong connection between ATP consumption and generation in green algae. The light-induced $\Delta\tilde{\mu}_{H^+}$, indeed, is combined with the gradient already generated in the dark at the expense of the catabolic process. This is specific of the in vivo conditions. Two thermodynamic consequences of these metabolic interactions can be inferred: first, because of the existence of a nonnegligible proton motive force in darkness, the light-induced $\Delta\tilde{\mu}_{H^+}$ needed to start ATP synthesis would be smaller than usually considered (reviewed in *4*, *37*). This might have relevant physiological consequences, as a very small threshold for ATP synthesis might improve the ATP synthesis efficiency in the case of low light illumination, where, for instance, $\Delta\tilde{\mu}_{H^+}$ of relatively small size are expected. Second, the relative size of the electric component of the proton motive force measured under in vivo conditions is larger than in vitro. This suggests that other processes may generate an electrical gradient across the thylakoid membranes in vivo, but not in vitro. This possibility is confirmed by the data of Bennoun (*12*), who evidenced the existence of a $\Delta\tilde{\mu}_{H^+}$ across the thylakoid membranes in mutants devoid of the CF_o-CF_1 ATPase.

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