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A New Electrochemical Gradient Generator in Thylakoid Membranes of Green Algae

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ABSTRACT: Using a new method of delayed luminescence digital imaging, mutants of *Chlorella sorokiniana* lacking the chloroplast CF_0CF_1 ATP synthase were isolated for the first time. Biochemical characterization of these strains indicates a lack of detectable synthesis and accumulation of the ATP synthase subunits α-CF_1 and β-CF_1. Functional characterization indicates the presence of a permanent electrochemical gradient ($\Delta\tilde{\mu}$) across the thylakoid membrane in the dark-adapted state, which is not suppressed under anaerobic conditions. Contrary to what is observed in the presence of the CF_0CF_1 ATP synthase, this gradient is essentially due to an electric field component $\Delta\Psi$ with no detectable ΔpH component, under both aerobic and anaerobic conditions. Neither the CF_0CF_1 ATP synthase nor a respiratory process can thus be responsible for a permanent gradient detected under these conditions. The previous proposal of a new ATP-dependent electrogenic pump in thylakoid membranes is supported by these results that, in addition, indicate a specificity of this new pump for ions other than protons.

Using delayed luminescence measurements, Joliot and Joliot (*1*) showed the existence of a permanent electrochemical gradient across thylakoid membranes in dark-adapted algae under aerobic conditions. This phenomenon was ascribed to the ATP hydrolysis by the chloroplast CF_0CF_1 ATP synthase. However, a permanent gradient was observed as well in the *Chlamydomonas reinhardtii* FUD50 mutant lacking this protein (*2*). The persistence of such a gradient in the absence of the chloroplast CF_0CF_1 ATP synthase was a strong argument favoring the model of chlororespiration, which could also account for the pattern of dark oxidation and reduction of plastoquinone (*2*). In this model, the permanent gradient present in the absence of CF_0CF_1 ATP synthase results from a coupled electron transfer through a chloroplast respiratory pathway, in which plastoquinone is reduced by a chloroplast NAD(P)H dehydrogenase and reoxidized at the expense of oxygen by a chloroplast oxidase. This hypothetical pathway is similar to that prevailing in photosynthetic prokaryotes which associate photosynthesis and respiration in the same membranes (see ref *3* for a review). More recently, however, Bennoun (*4*) analyzed in detail the effects of myxothiazol, a mitochondrial cytochrome bc_1 inhibitor, on the redox state of plastoquinone in darkness and on the permanent gradient. He concluded that the permanent electrochemical gradient present in the absence of CF_0CF_1 ATP synthase could not result from the coupling of the chlororespiratory chain, but rather from a new ATP-driven gradient generator present in the thylakoid membranes.

We have studied the permanent electrochemical gradient ($\Delta\tilde{\mu}$)[1] under strict anaerobic conditions to investigate the role of respiration processes in its formation. In wild-type *Chlorella sorokiniana*, a $\Delta\tilde{\mu}$ has even been observed under anaerobic conditions (*5*). In that case, it was assumed that the CF_0CF_1 ATP synthase was responsible for its formation using ATP provided by fermentation. It was then of interest to evaluate this gradient under anaerobiosis in the absence of CF_0CF_1 ATP synthase. In *C. reinhardtii* strains lacking this complex, a small-amplitude $\Delta\tilde{\mu}$ was still observed under anaerobic conditions. Mutant strains of *C. sorokiniana* lacking this complex were screened recently using the new method of delayed luminescence digital imaging (*6*), and their characterization is reported here. When dark adapted, such mutants display a permanent electrochemical gradient across thylakoid membranes that, under anaerobiosis, is larger than the one observed in *C. reinhardtii* (P. Bennoun, unpublished results) and thus easier to characterize. The permanence of a $\Delta\tilde{\mu}$ under anaerobiosis in the absence of the CF_0CF_1 ATP synthase strongly supports the new ATP-driven gradient generator. Moreover, our experimental evidence demonstrates that this pump generates an electric field across thylakoid membranes rather than a pH gradient.

MATERIALS AND METHODS

Strains, Mutagenesis, and Cultures. The S30 strain was derived from wild-type *C. sorokiniana* as described previ-

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[1] Abbreviations: DCMU, 3-(3,4-dichlorophenyl)-1,1′-dimethylurea; crown, dicyclohexyl-18-(*crown*)-6; HA, hydroxylamine; PBQ, para-benzoquinone; PSI and PSII, photosystem I and II, respectively; $P_{680}{}^+$, primary chlorophyll electron donor of photosystem II; $\Delta\tilde{\mu}$, electrochemical difference potential; $\Delta\Psi$ and ΔpH, electrical component and chemical difference potential for proton component of the transmembrane electrochemical difference potential, respectively.

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ously and is deficient in light-harvesting complex CPII (*7*). The SL6 and SL8 strains were derived from S30 and from the wild type, respectively, and were screened according to their high luminescence yield through digital luminescence imaging as previously described (*6*). The SL20 strain was derived from SL6 and was screened according to its high initial fluorescence yield through digital fluorescence imaging (*6*). SL6, SL8, and SL20 were isolated following 5-fluorodeoxyuridine mutagenesis as described in ref *8*, with the following modifications. The cell density was 10^5 cells/mL, and the concentration of 5-fluorodeoxyuridine was 1.5 mM. After detection, the mutant colonies were subcloned at least twice to eliminate mitotic segregation. Strains were grown at 25 °C in tris-acetate-phosphate or TAP medium (*9*) at a light intensity of 400 lx. All *C. sorokiniana* strains can be kept frozen at −80 °C in TAP medium supplemented with 7% dimethyl sulfoxide. Rapid thawing is achieved by transferring frozen aliquots of 100 μL into 100 mL of liquid TAP medium. After a few cell divisions, the cell suspension is cloned on agar dishes and new clones can be recovered.

Biochemical Characterization of the Mutant Strains. Purification of thylakoid membranes was achieved as described in ref *10*. Gel electrophoretic fractionation using SDS−urea gels was achieved as described in ref *11*. Pulse labeling of proteins with [^{14}C]acetate in the presence of cycloheximide was achieved for 45 min at a light intensity of 1000 lx at 25 °C as described in ref *12*. Immunoblotting was performed as described in ref *12*, using antibodies raised against proteins in bands cut from a gel.

Estimation of the Permanent Electrochemical Gradient through the Rate of PSII Back-Reaction in the Presence of DCMU. Cells were harvested in late exponential phase, washed, resuspended in minimal HS medium (*13*) or in acetate-free buffer as indicated, and placed on a rotatory shaker for at least 1 h before being used. Algae were pre-illuminated in the presence of DCMU (10 μM) to reduce the PSII electron acceptor Q and allowed to stay in darkness for a given time before a second illumination was carried out. The relative concentration of Q reoxidized during this dark time was determined by measuring the area over the fluorescence rise as described previously (*4*). Under these conditions, the reoxidation of Q^- follows satisfactorily the empiric law $[Q_{total}]/[Q^-] = 1 + gt$. The parameter g that can be deduced from these kinetics measurements probes the permanent transmembrane electrochemical gradient (it varies from a basic value of 0.3 s^{-1} in the absence of a permanent gradient to up to 10 s^{-1} in its presence and is the reverse of the half-time of the PSII back-reaction).

Spectroscopic Analysis. Exponentially growing cells were concentrated to 5×10^7 cells/mL in 20 mM HEPES/KOH buffer (pH 7.2) in the presence of 10% Ficoll to avoid cell sedimentation. When used under anaerobic conditions, algae were incubated for at least 1 h under an argon atmosphere in a large reservoir connected to the measuring cuvette. Spectroscopic measurements were performed at room temperature with a home-built spectrophotometer described previously (*14*). Actinic flashes were provided by a xenon flash (3 μs duration at half-height) filtered through red filter (Schott RG 8). Flashes were fired at a frequency of 0.15 Hz.

The transmembrane electric potential was estimated from the amplitude of the electrochromic shift at 515 nm, which yields a linear response with respect to this potential (*15*). Under our experimental conditions, the kinetics of the electrochromic signal display two sequential phases (*16*): a fast phase completed in <1 μs, associated with PSI and PSII charge separations (phase a), and a slow phase in the millisecond time range, associated with the turnover of the b_6f complex (phase b). PSII absorption changes were inhibited by pre-illuminating the samples in the presence of DCMU (10 μM) and HA (1 mM) (*17*). In this case, phase a is a measure of PSI charge separations. Kinetic analysis of phase b requires the deconvolution of the membrane potential decay. This was done assuming that the latter process exhibits first-order kinetics. The kinetics of phase b were then corrected 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 on the order of hundreds of milliseconds and in the time range of seconds (*16*). Therefore, only the fastest one is expected to superimpose with the b phase kinetics.

Measurements of flash-induced changes of chlorophyll fluorescence yield were performed using weak flashes at 480 nm (hitting less than 1% of the photosynthetic reaction centers) and monitoring the induced fluorescence emission in the near-IR region.

pBQ Treatment. Algae were vigorously aerated in darkness and then incubated for 5 min with 500 μM pBQ. They were then centrifuged, washed twice, and finally resuspended in 50 mM HEPES/NaOH for the pH 6.5−8 range and 50 mM MES/NaOH for the pH 5.5−7.0 range, in the presence of 10% Ficoll.

RESULTS

Biochemical Characterization

The pattern of thylakoid membrane polypeptides of wild type and mutant trains of *Ch. sorokiniana* following fractionation by polyacrylamide gel electrophoresis and Coomassie blue staining is depicted in Figure 1A. The highly luminescent strains SL8 and SL6 clearly show a lack of detectable amounts of subunits α-CF_1 and β-CF_1 when compared, respectively, to the wild type and S30 from which they derived (S30 is deficient in light-harvesting chlorophyll proteins or LHCPs). Protein blots were reacted with antisera specific for polypeptides α-CF_1 and β-CF_1. They demonstrate a lack of detectable accumulation of these chloroplast ATP synthase polypeptides in SL8 (Figure 1C) and in SL6 (not shown). Pulse labeling of whole cells with [^{14}C]acetate was achieved in the presence of cycloheximide inhibiting 80S ribosome translation to investigate the labeling pattern of the chloroplast-encoded thylakoid membrane polypeptides. The autoradiogram from SDS−urea PAGE shown in Figure 1B indicates the lack of detectable synthesis of α-CF_1 and β-CF_1 in both SL6 and SL8. The SL20 strain is derived from SL6 following mutagenesis and differs from this strain by an additional deficiencies of two-thirds of the photosystem

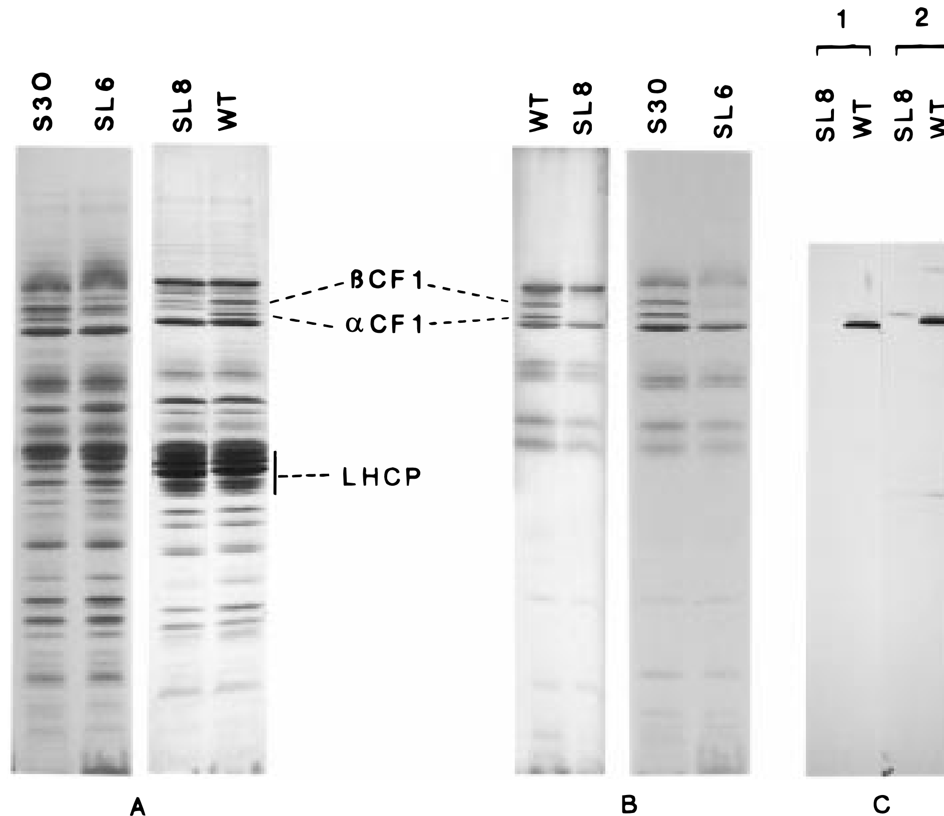

FIGURE 1: Gel electrophoretic fractionation of thylakoid membrane polypeptides from wild-type and mutant cells. Cells were labeled with [^{14}C]acetate for 45 min in the presence of cycloheximide. Coomassie blue staining patterns of thylakoid membrane polypeptides following gel electrophoretic fractionation are shown in panel A. Autoradiographs of the stained gels are shown in panel B. Thylakoid membrane polypeptides were electrophoresed and transferred to Immobilon PVDF membranes (Millipore). Protein blots that reacted with antisera specific for α-CF_1 and β-CF_1 are shown in panel C (groups 1 and 2, respectively). SDS−polyacrylamide (12−18%) gels with 8 M urea.

II (PSII) center polypeptides (not shown). Protein blots that reacted with an antiserum specific for subunit 3 of CF_0 showed no detectable accumulation of this polypeptide in the mutants (D. Drapier, not shown). Altogether, we conclude that the CF_0CF_1 ATP synthase is either completely lacking in the thylakoid membranes of the mutants or present at a very reduced level (the resolution of the immunoblot analysis would allow the detection of 1−2% of the wild-type level).

Functional Characterization

Many light-induced processes related to photosynthetic electron transfer are electrogenic and thus sensitive to the permanent $\Delta\tilde{\mu}$ (preexisting in darkness). If the light-induced $\Delta\tilde{\mu}$ generated by these processes is kept small in comparison with the permanent $\Delta\tilde{\mu}$, they can serve to probe the latter. We made use of such probes in analyzing the permanent $\Delta\tilde{\mu}$ in the wild type and mutants lacking the CF_0CF_1 ATP synthase.

(a) Characterization of the Permanent Electrochemical Gradient under Aerobiosis. The PSII photochemical centers achieve a charge separation between the primary donor chlorophyll P_{680} and the primary quinone acceptor Q_A which are located on either side of the thylakoid membrane (the donor being located on the lumenal side and the acceptor on the stromal side). In the presence of the herbicide 3-(3,4-dichlorophenyl)-1,1′-dimethylurea (DCMU), electron transfer between the primary and secondary quinone electron acceptor is blocked so that the $S_2Q_A^-$ state formed upon illumination recombines to S_1Q_A. The transmembrane location of these charges renders the back-reaction sensitive to the presence of a permanent electrochemical gradient across thylakoid membranes. The kinetics of the back-reaction observed in the presence of DCMU can be satisfactorily approximated by $[Q_{total}]/[Q^-] = 1 + gt$, where the parameter g probes the permanent transmembrane electrochemical gradient (*4*).

Table 1: Estimation of the Permanent Electrochemical Gradient Established under Aerobiosis in the Dark-Adapted State with the Parameter g (s^{-1}) of the PSII Back-Reaction Observed in the Presence of DCMU

	wild type	SL8
no addition	3.8	6
with 2 mM crown	0.3	0.3
with 2 μM nigericin	5	4.1

Table 1 shows the values of this parameter g under various conditions. As previously reported for *C. reinhardtii* (*4*), in both the wild type and mutant strains they were much larger in the absence than in the presence of dicyclohexyl-18-(*crown*)-6 (crown), an ionophore known to disrupt the transmembrane potential (*18*). This indicates that the rate of the back-reaction is enhanced by a transmembrane potential irrespective of the existence of the CF_0CF_1 complex in the thylakoid membrane. The permanent electrochemical gradient ($\Delta\tilde{\mu}$) observed across thylakoid membranes is made up of a pH gradient (ΔpH) and of an electric field (ΔΨ) resulting from the asymmetric charge distribution on both sides of the membranes. It is not known however to what extent g is modulated by each of these components, but it is expected to depend significantly on both the ΔpH and ΔΨ

components. Indeed, Buser et al. (*19*) have shown that, in PSII-enriched membrane fragments (i.e., in the absence of $\Delta\tilde{\mu}$), the back-reaction $S_2Q_A^- \rightarrow S_1Q_A$ is sensitive to pH. Consistent with this, addition of nigericin [a H^+/K^+ exchanger used to selectively disrupt the ΔpH component (*20*)] induced moderate changes of the *g* value in both the wild-type and SL8 strains. However, the parameter *g* is also sensitive to ΔΨ as shown by the drastic effect of crown addition (which collapses both the ΔpH and the ΔΨ components) compared to the small effect of nigericin addition. As the law relating *g* to each of these components is not known, this parameter is not suitable for characterizing the relative amplitudes of both components of the permanent gradient. We thus resorted to other processes that, as explained below, are predominantly sensitive to either component.

(b) Characterization of the Permanent Δ pH Component under Aerobiosis. Illumination of PSII by a short saturating flash allows one charge separation to proceed resulting in the formation of the $P_{680}{}^+Q_A{}^-$ state. The $Q_A{}^-$ state lifetime is increased in the presence of DCMU. The photochemically active chlorophyll P_{680} is reduced by tyrosine 161 of the PSII center polypeptide D_1 that is in turn reduced at the expense of the water-splitting enzyme. This latter reaction may be inhibited by treatments such as addition of hydroxylamine, depleting the catalytic site from its manganese cluster. Under these conditions, hydroxylamine acts as an electron donor to the oxidized YD_1161 in the time range of tens of microseconds, thus precluding the charge recombination $YD_1161^{ox}Q_A{}^- \rightarrow YD_1161Q_A$ reaction from proceeding. The $Q_A{}^-$ state is then long-lived (several tens of seconds). It is well-known however that, under these conditions, the amount of the stable $Q_A{}^-$ state observed after one saturating flash is dramatically decreased upon hydroxylamine addition (*21*, *22*). It has been recently shown that this may be rationalized by the increased lifetime of $P_{680}{}^+$ in Mn-depleted PSII, allowing a significant fraction of centers to undergo charge recombination $P_{680}{}^+Q_A{}^- \rightarrow P_{680}Q_A$ before reduction of $P_{680}{}^+$ by YD_1161 (*23*).

The $Q_A{}^-$ state is characterized by a high chlorophyll fluorescence yield with respect to the Q_A state. Thus, the amount of stable $Q_A{}^-$ formed after a short saturating flash may be probed by measuring the flash-induced change in fluorescence yield. Figure 2 (top panel) shows the effect of nigericin addition on the variation of the fluorescence yield observed in the course of a series of saturating flashes in the wild type and in the SL8 strain lacking the CF_0CF_1 ATP synthase. It is noteworthy that the large effect of nigericin addition observed in the wild type strain is hardly detectable in the SL8 strain. This suggests that at variance with the wild type the SL8 strain displays no ΔpH. A calibration of the pH dependence of the fluorescence yield induced by the first flash of a series was achieved. Figure 2 (bottom panel) shows the pH dependence of the variable fluorescence induced by the first flash of a series observed in the presence of hydroxylamine and of DCMU, normalized to the maximum variable fluorescence (this ratio will be henceforth denoted *R*). The data points were obtained with wild-type cells of *C. sorokiniana* treated with parabenzoquinone (pBQ). This treatment is known to collapse the permanent $\Delta\tilde{\mu}$ (*24*, *25*) and to allow pH equilibration between the pH buffer and each of the cell compartments (*23*). The pH dependence observed is in good agreement with that reported by Lavergne and Rappaport (*23*) using spinach thylakoids. It is consistent with the framework of a competition between the charge recombination reaction $P_{680}{}^+Q_A{}^- \rightarrow P_{680}Q_A$ and the reduction of $P_{680}{}^+$ by YD_1161, since the rate of this latter reaction is decreased when the pH is lowered (*26*−*28*). The pH dependence of the efficiency of $Q_A{}^-$ stabilization thus provides a tool for probing the lumenal pH in vivo.

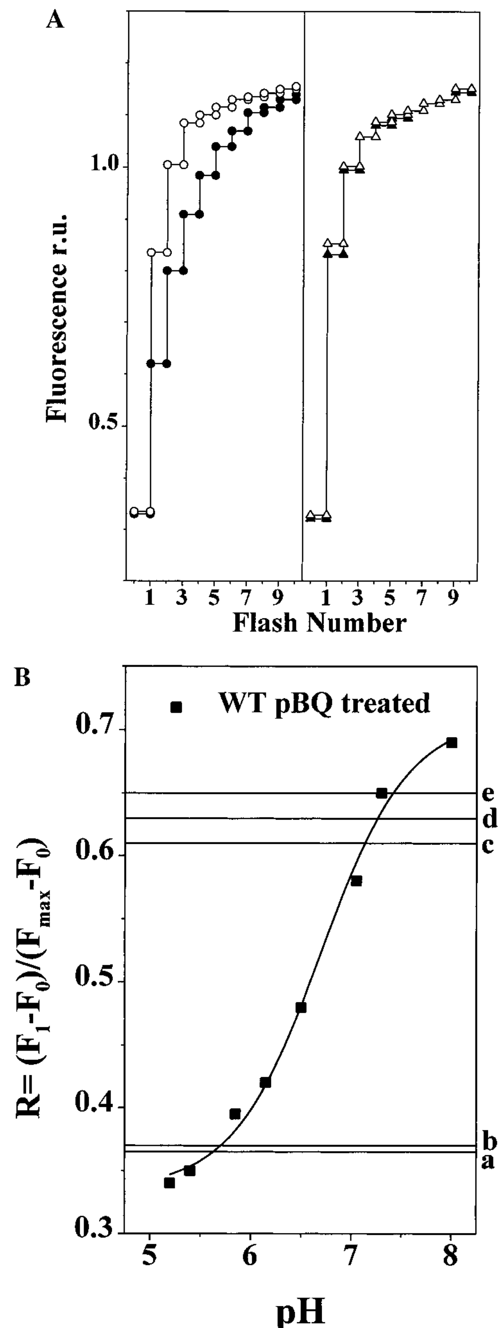

FIGURE 2: (A) Changes of the fluorescence yield induced by a series of saturating flashes in the wild type (circles) or SL8 (triangles) in the presence of DCMU and hydroxylamine in the absence (black symbols) and the presence (white symbols) of nigericin. The time interval between flashes was 200 ms; the fluorescence was sampled 50 and 100 ms after each actinic flash. (B) pH dependence of the fluorescence yield changes induced by the first flash of a series (squares) in wild-type pBQ-treated cells. The letters refer to the value of *R* in the presence of DCMU and hydroxylamine: (a) wild type, (b) S30, (c) SL8, (d) wild type in the presence of 1 μM nigericin, and (e) SL6.

When the cells are not submitted to the pBQ treatment and resuspended in 20 mM HEPES, the pH of the buffer does not modulate the pH of the lumen (*29*). Under such conditions, in the wild-type or S30 strains *R* was close to 0.35, a value that is indicative of a pH of <6. Addition of nigericin resulted in a significant increase of *R* (0.62, indicating a pH ≥7). These data show that the lumenal pH in the dark-adapted state is significantly smaller than the stromal one in the wild type (ΔpH on the order of 1.5 pH units) and that equilibration of the pH of both compartments is achieved most likely at the stromal pH upon addition of nigericin. At variance with this, the parameter *R* is indicative of a pH on the order of 7 in mutant strains lacking the CF_0-CF_1 complex, even in the absence of nigericin. Addition of this compound hardly resulted in a change of the value of *R* (not shown), which is consistent with the absence of a significant ΔpH. The electrochemical transmembrane potential that is nevertheless detected by the rate of the PSII back-reaction in the absence of the CF_0CF_1 therefore consists essentially of an electric field component ΔΨ.

(c) Characterization of the Permanent ΔpH Component under Anaerobiosis. Unfortunately, the above-described experiments do not allow the study of $\Delta\tilde{\mu}$ under anaerobic conditions for oxygen deprivation results in the complete reduction of the plastoquinone pool and thus in the inactivation of the PSII activity. Conversely, these conditions are well suited to studying the effect of the permanent transmembrane potential on the cytochrome b_6f turnover kinetics since they result in the complete occupancy of the plastoquinol oxidation site of this complex. The cytochrome b_6f complex catalyzes a transmembrane charge transfer, thereby contributing as a slow phase to the flash-induced ΔΨ induced by photosystem I and II charge separation. According to the Q cycle model, this transmembrane charge transfer results from electron injection into the cytochrome b_6 hemes after PQH_2 oxidation (*30*, *31*). It has been recently shown that the rate of this charge transfer is controlled mainly by the lumenal pH (*29*). When submitted to a ΔΨ, some of the pigments embedded in the thylakoid membrane undergo an electrochromic shift. This provides a tool for probing the time course of this transmembrane charge transfer (*15*, *16*).

This is illustrated in Figure 3 (top panel), showing that addition of nigericin to a wild-type strain under anaerobiosis resulted in a significant enhancement of the rate of the flash-induced electrochromic shift measured at 515 nm, as previously reported in ref *29*. In the SL8 mutant lacking CF_0-CF_1 ATP synthase (Figure 3, bottom panel), the rate of the 515 nm absorption changes was no more sensitive to nigericin addition but was similar to that observed in the wild type after addition of nigericin. This shows that the value of the lumenal pH in the SL8 mutant strain is similar to that observed in the wild type after pH equilibration between the lumenal and the stromal compartments. Thus, as was observed under aerobiosis, no ΔpH is detectable in the mutant under anaerobiosis. The question that remains is whether a ΔΨ persists under anaerobiosis.

(d) Characterization of the Permanent Electric Field Component ΔΨ under Anaerobiosis. In strains having the wild-type complement of light-harvesting complexes, the flash-induced electrochromic shift is linearly related to the flash-induced ΔΨ (*15*). Joliot and Joliot (*18*) however observed that, in cells lacking the CPII light-harvesting complexes and the PSII center complex, the amplitude of the flash-induced electrochromic shift is quadratically responding to the flash-induced ΔΨ. This provides a useful tool for probing the existence of a permanent ΔΨ. Indeed, the flash-induced charge separation at the level of PSI or PSII results in a ΔΨ component that superimposes with the putative permanent ΔΨ. Thus, to probe the latter, the observed flash-induced signal should be dependent on the value of the transmembrane electric field that exists before the flash. This is precisely the case for the quadratic electrochromic effect, at variance with the linear one. If no or little transmembrane electric field exists previously, the flash-induced electrochromic shift that the quadratic probes undergo is negligible, whereas it is noticeable in the presence of a significant ΔΨ prior to the flash.

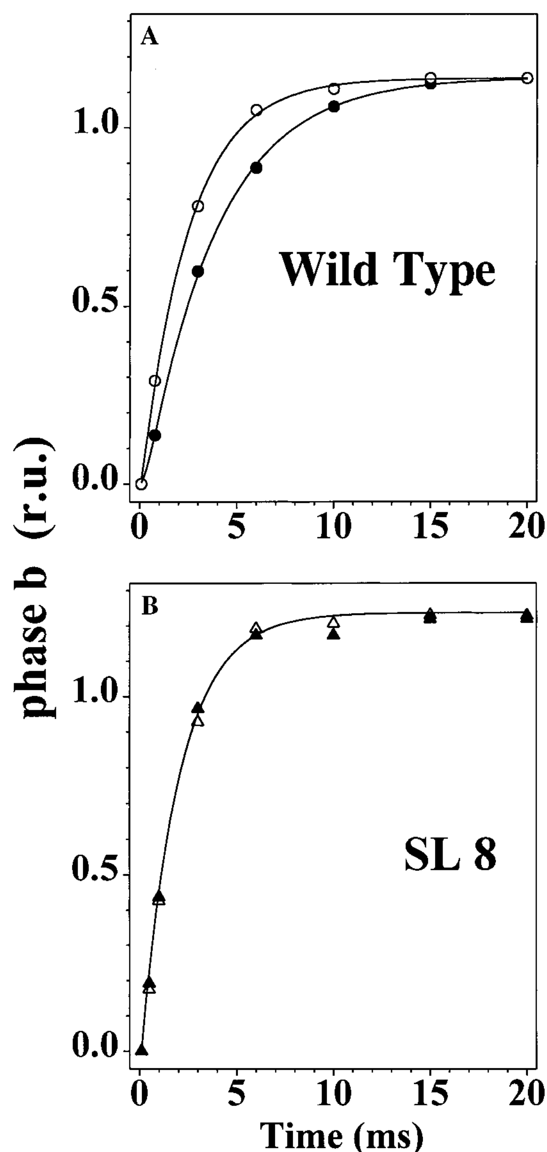

FIGURE 3: Slow electrochromic reaction (b phase) in *C. sorokiniana* wild-type (A) and SL8 cells (B) in the absence (black symbols) and the presence (white symbols) of 1 μM nigericin. Algae were illuminated with nonsaturating red flashes hitting 30% of the centers at a frequency of 0.15 Hz in 20 mM HEPES (pH 7.2). Kinetics were corrected for membrane potential decay as described in Materials and Methods.

We thus selected a mutant strain SL20 deficient in CPII and PSII complexes, and showing no detectable traces of

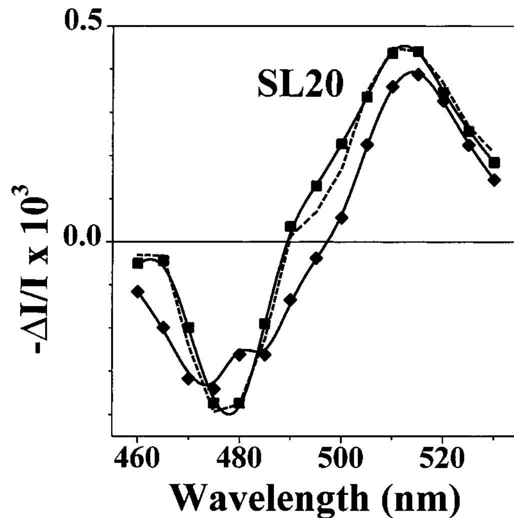

FIGURE 4: Electrochromic absorption changes induced by a nonsaturating flash in *C. sorokiniana* SL20 cells. The absorption change was measured 100 μs after the actinic flash hitting 10−20% of the centers (■ and ◆), in the absence (■) or presence (◆) of 100 μM crown. The dashed line represents the spectrum observed after an oversaturating actinic flash in the presence of 100 μM crown, after normalization at 515 nm.

CF_0CF_1 ATP synthase. Figure 4 shows the spectra observed in anaerobiosis in the SL20 mutant strain 100 μs after a subsaturating flash, in the absence (squares) and presence (diamonds) of the ionophore crown. The two spectra differ significantly indicating that the polarization state of the pigments embedded in the membrane is modified upon crown addition. This shows the permanence in the dark, under anaerobiosis, of a transmembrane electric field independent of the presence of the CF_0CF_1 ATP synthase, a conclusion that is further supported by the following result. Figure 4 shows the spectra measured 100 μs after an oversaturating actinic flash (dashed line) in the presence of crown. Under these conditions, the flash-induced $\Delta\Psi$ resulting from the charge separation at the level of the photosystems is significantly increased. The observed spectrum closely resembles the spectrum measured after a subsaturating flash in the absence of crown. This shows first that the spectral changes induced when collapsing $\Delta\Psi$ by crown addition are reversible, and most importantly that they are mimicked by building a transmembrane $\Delta\Psi$ through charge separation at the level of the photochemical reaction centers.

DISCUSSION

Evaluation of the Permanent Electrochemical Gradient across Thylakoid Membranes under Aerobiosis. The permanent electrochemical gradient ($\Delta\tilde{\mu}$) observed across thylakoid membranes consists of two components, a pH gradient (ΔpH) and an electric field ($\Delta\Psi$) resulting from an asymmetric charge distribution on both sides of the membranes. We first evaluated the consequence of the absence of the chloroplast ATP synthase on the lumenal pH. This pH was probed through measurements of the efficiency of stabilization of the $P_{680}Q_A^-$ state in the presence of hydroxylamine and DCMU following a single-turnover saturating flash. A calibration curve of the lumenal pH was established by this method and appears in Figure 2. To test for the presence of a pH gradient built up across thylakoid membranes, we made use of nigericin, an H^+/K^+ exchanger equilibrating the pH on both sides of these membranes. Figure 2 shows that in the absence of nigericin the efficiency of stabilization of the PSII primary charge separation is much smaller in the wild type than in the mutant. The value observed in the mutant in the absence of nigericin corresponds to that observed in the wild type when nigericin is present, thus showing the absence of a significant ΔpH across thylakoid membranes in the mutant at variance with the wild type. The pH gradient built up in the dark in the wild type is thus the result of the functioning of the CF_0CF_1 ATP synthase. Despite the absence of detectable chloroplast ATP synthase and of significant ΔpH across thylakoid membranes, the mutant analyzed still displayed a permanent electrochemical gradient across thylakoid membranes in darkness under aerobiosis, as detected with the rate of the PSII back-reaction measured in the presence of DCMU (Table 1). This persisting gradient therefore consists of the sole electric field component $\Delta\Psi$. We thus come to the conclusion that removing the CF_0CF_1 ATP synthase leads to a drastic qualitative modification of the permanent electrochemical gradient; the ΔpH component disappeared (or becomes barely detectable) for the sole benefit of the electric field component $\Delta\Psi$.

Evaluation of the Permanent Electrochemical Gradient across Thylakoid Membranes under Anaerobiosis. The permanent electrochemical gradient that was observed in the absence of ATP synthase was thought to originate from chlororespiration (*2*). However, this origin was refuted on the basis of two sets of experiments. Indirect arguments arising from a careful examination of the modifications of the redox state of plastoquinone upon inhibition of mitorespiration led to the proposal that this gradient results from a new ATP-driven gradient generator (*4*). Moreover, under anaerobiosis, a high permanent electrochemical $\Delta\tilde{\mu}$ was observed across thylakoid membranes of wild-type *Ch. sorokiniana*, likely due to immediately active fermentation processes providing ATP to the CF_0CF_1 ATP synthase (*5*). In mutants lacking this ATP synthase, the permanent electrochemical $\Delta\tilde{\mu}$ was also detectable upon oxygen deprivation as depicted in Figure 4. In that case, the formation of the gradient cannot be ascribed either to a respiratory process owing to the absence of oxygen or even to the CF_0-CF_1 ATP synthase. The latter point deserves careful examination since immunoblot analysis would not allow detection of <1−2% of the wild type level of ATP synthase. Further evidence of the absence of this complex in the mutants is provided by the functional characterization of the $\Delta\tilde{\mu}$ in wild type and CF_0CF_1 ATP synthase-lacking strains. Although a large fraction of the CF_0CF_1 ATP synthase is inactivated in the wild type under anaerobic conditions, a large ΔpH is nevertheless observed in the dark-adapted state. Consequently, a small amount of active CF_0CF_1 ATP synthase is sufficient to build up a detectable ΔpH accounting for at least two-thirds of the overall $\Delta\tilde{\mu}$ [1.5 pH units (*29*)]. Would a few CF_0CF_1 ATP synthase complexes remain in the mutants, a significant ΔpH component of their electrochemical gradient would have been expected. At variance with this possibility, under aerobic conditions, the $\Delta\tilde{\mu}$ gradients observed in the wild type and in mutants lacking the CF_0-CF_1 are very different in nature. The mutants do lack any detectable ΔpH so that their $\Delta\tilde{\mu}$ solely consists of an electric field $\Delta\Psi$. We made use of the electrochromic effect in demonstrating independently the existence of a permanent

$\Delta\Psi$ under anaerobiosis in the absence of CF_0CF_1 ATP synthase. A shift of the absorption peak of some of the pigments present in the thylakoid membranes is observed in response to the establishment of an electric field $\Delta\Psi$ across these membranes. The spectra of the electrochromic effect were analyzed in the SL20 strain (deficient in the light-harvesting complex CPII, CF_0CF_1 ATP synthase, and PSII centers) in the absence or in the presence of the ionophore crown (Figure 4). Their comparison clearly demonstrates that crown modifies the polarization state of the pigments embedded in the membrane, and consequently strongly supports the existence of an electric field $\Delta\Psi$ under anaerobiosis in the absence of CF_0CF_1 ATP synthase. This reinforces the conclusion derived from the examination of the rate of the PSII back-reaction. We then analyzed the b phase of the 515 nm electrochromic signal that was shown to be highly sensitive to the ΔpH component of the gradient and slightly sensitive to the $\Delta\Psi$ one (*29*). A significant acceleration of the kinetics of phase b was observed upon uncoupling with nigericin in the wild type under anaerobiosis. However, in the SL8 mutant lacking the chloroplast ATP synthase, the kinetics of the b phase were faster than in the wild type and were not accelerated by nigericin (Figure 3). This strengthens the conclusion drawn from the analysis of the PSII back-reaction and of the spectra of the electrochromic effect that, in the absence of CF_0CF_1 ATP synthase, a permanent electrochemical gradient $\Delta\tilde{\mu}$ is built up across thylakoid membranes under anaerobiosis, mainly as an electric field $\Delta\Psi$.

Bennoun (*4*) observed a collapse of the permanent gradient followed by a reduction of plastoquinone upon specific inhibition of the mitochondrial chain. To account for this observation, he proposed the hypothesis of an ATP-driven electrogenic pump in the thylakoid membranes distinct from the CF_0CF_1 ATP synthase. The permanent electric field $\Delta\Psi$ detected across thylakoid membranes under anaerobiosis in the absence of CF_0CF_1 ATP synthase supports this conclusion and clearly supports a pump X specific for ions other than protons. It is noteworthy however that, when both this pump and the CF_0CF_1 ATP synthase are present (in wild-type strains), the ΔpH component accounts for at least two-thirds of the permanent $\Delta\tilde{\mu}$ (*29*). This suggests that the CF_0CF_1 complex rather than the X pump is predominantly responsible for the building up of the $\Delta\tilde{\mu}$ in the wild type. The competition between the CF_0CF_1 complex and the X pump for the establishment of the permanent $\Delta\tilde{\mu}$ would favor the former enzyme, which thus would display a higher energetic yield than the latter (the number of charges translocated per ATP molecule consumed being higher for the CF_0CF_1 than for the X pump). The ionic specificity of the X pump may not be simply deduced by testing the effect of specific ionophores. If it is assumed that the X pump generates a Na^+ gradient, addition of valynomicin would collapse the $\Delta\Psi$ present in the mutants despite its specificity for K^+ ions. The Na^+ gradient would indeed be canceled out by the building of an opposite K^+ gradient in response to the transmembrane $\Delta\Psi$. The ionic specificity of the X pump thus deserves further studies.

Permanent $\Delta\tilde{\mu}$ and Protein Translocation in the Lumenal Compartment. In higher-plant thylakoids, it has been proposed that the three extrinsic proteins of the PSII reaction center were translocated into the lumen via a ΔpH sensitive pathway (see ref *32* for a review). However, the mutant strains lacking CF_0CF_1 display an active photosynthetic electron transport chain, although they do not display detectable permanent ΔpH. This raises the question of the mechanism of translocation of some of the nuclear encoded subunits of the functional complexes of the photosynthetic membranes. We propose either that a $\Delta\Psi$ would be sufficient to allow lumenal import or that in the absence of a ΔpH a basal level of lumen translocation may occur in the case of these polypeptides. This would be sufficient to allow assembly of PSII in darkness in the mutants characterized here.

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