Light-Induced Proton Gradient Formation in Intact Cells of *Dunaliella salina*

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ABSTRACT

A light-induced proton gradient (ΔpH) increase as exhibited by an increase of 9-aminoacridine fluorescence quenching is demonstrated between the external medium and the interior of the halophytic green alga *Dunaliella salina*. The formation and maintenance of the ΔpH is sensitive to electron transport inhibitors and to uncouplers. It is inhibited by p-chloromercuribenzenesulfonic acid (50% inhibition at 3 micromolar), which does not affect photosynthetic O₂ evolution. It is concluded that the observed ΔpH is located across the plasmalemma or the chloroplast envelope. The formation and maintenance of the light-induced proton gradient requires the presence of Na⁺. Substitution of NaCl by KCl or glycerol results in inhibition of the ΔpH formation. The proton gradient is also sensitive to ATPase and energy transfer inhibitors. It is suggested that a Na⁺/H⁺ pump mechanism may be involved in the formation of the proton gradient in intact *Dunaliella* cells.

The formation of a light-induced ΔpH was monitored by observing changes in fluorescence of 9-AA² (25). The experimental apparatus was described by Avron and Schreiber (1). Four hundred μl cell suspension or cell-free preparation were introduced into the instrumental chamber in the dark. Addition of inhibitors or changes of NaCl concentration were made by injecting into the chamber using the connected syringe. Sources of light and the method used to determine 9-AA fluorescence yield were those described in detail by Avron and Schreiber (1). Data are presented on the basis of relative changes in fluorescence intensity.

RESULTS

Response of 9-Aminoacridine Fluorescence to Light, PMS, DCMU, and Uncoupler. The induction by light of 9-AA fluorescence quenching, which reflects the uptake of protons by the intact *Dunaliella* cells is stimulated by the presence of PMS (Fig. 1). In the absence of PMS, relatively small changes of 9-AA fluorescence were detected upon light to dark transitions. The initial rate of the light-induced proton uptake is very slow in dark-adapted *Dunaliella* cells and becomes faster upon preillumination (Fig. 1A). Three light to dark cycles of 2 min each are usually sufficient to “activate” the system resulting in a repeatable light to dark transition of 9-AA fluorescence signal. The addition of DCMU (5 μm) causes a complete dissipation of the light-induced proton gradient, with kinetics similar to the one observed for a light to dark transition (Fig. 1B). This effect of DCMU could be reversed by the addition of ascorbate (1 μm) which stimulated the initial rate and the steady-state signal up to 3-fold compared to the control (Fig. 1B), both in the presence and absence of DCMU. The observed proton uptake is also sensitive to uncouplers such as SF₆₈₄₇ (27) the addition of which resulted in an immediate restoration of the fluorescence signal to the dark level or even slightly above it (data not shown), indicating that in the dark the ΔpH between the cells and the surrounding medium (pH 7.5) is rather small.

Comparison between Light-Induced 9-AA Fluorescence Quenching in Intact and Osmotically Shocked Cells. The light-induced proton uptake in intact *Dunaliella* cells resembles the light-induced ΔpH formation across the thylakoid membranes of osmotically burst cells. However, the kinetics of the ΔpH formation in osmotically burst *Dunaliella* (Fig. 2) are different from the ones observed in intact cells. It neither requires light “activation” nor PMS, although stimulation of the rate of formation and extent does occur in its presence (not shown).

The light-induced proton gradient in intact *Dunaliella* cells is strongly inhibited by PCMBs (Figs. 3 and 7). This effect of PCMBs can be completely reversed by 0.5 mM DTT (Fig. 3). The light-induced 9-AA fluorescence quenching in a cell-free prepa-

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Abbreviations: 9-AA, 9-aminoacridine; PMS, methylphenazonium methosulfate; PCMBs, p-chloromercuribenzenesulfonic acid.

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Fig. 1. Light-induced quenching of 9-AA fluorescence in intact D. salina. A, dependence on addition of PMS and increase in quenching rate with “light activation” (curves I, II, III). 9-AA concentration, 50 μM PMS concentration, 10 μM. Curves I, II, and III were separated by a 2-min dark period. B, effect of DCMU and ascorbate on the 9-AA fluorescence quenching. DCMU concentration, 5 μM, ascorbate concentration, 1 mM. Samples contained 10 μM PMS and were activated through three light to dark cycles.

Fig. 2. Light-induced 9-AA fluorescence quenching in osmotically ruptured D. salina. 9-AA concentration is 50 μM.

Fig. 3. Inhibition of light-induced 9-AA fluorescence quenching by PCMBs and its reversal by DTT in intact cells of D. salina. PCMBs concentration, 5 μM; DTT concentration, 0.5 mM. Curve A, control; B, in the presence of PCMBs, DTT was added where indicated.

Fig. 4. CO₂-dependent O₂ evolution in D. salina. NaHCO₃ concentration, 25 μM.

Fig. 5. Dependence of light-induced 9-AA fluorescence quenching on the suspension medium of intact D. salina. Cells were suspended in: A, growth medium containing 1.5 M NaCl; B, growth medium, but with 1.5 M KCl instead of NaCl; C, growth medium with 3 M glycerol instead of NaCl; D, curve measured with the sample from C after resuspension in growth medium containing 1.5 M NaCl. Other conditions as in Figure 1B.

Ration is also sensitive to PCMBs. The concentration of PCMBs required for 50% inhibition of the 9-AA fluorescence change however, is higher in osmotically burst cells as compared with intact ones (7 and 3 μM PCMBs, respectively).

In contrast with the immediate response of 9-AA fluorescence quenching to PCMBs, CO₂-dependent photosynthetic O₂ evolution (Fig. 4) is hardly affected by 10 μM PCMBs, a concentration which completely inhibited the proton uptake in intact cells (not shown but see Fig. 7).

Response of the Light-Induced 9-AA Fluorescence Quenching to Na⁺ and Inhibitors of ATPase and Energy Transfer. The light-induced proton uptake in intact Dunaliella cells is strongly dependent on the presence of Na⁺ in the medium and on Na⁺ concentration (Figs. 5 and 6). 9-AA fluorescence change in the light is 60% inhibited when Dunaliella cells were transferred from medium containing 1.5 M NaCl to KCl or glycerol (of the same osmotic potential). Complete recovery of the 9-AA fluorescence signal difference is obtained when cells are resuspended from medium containing glycerol to NaCl (Fig. 5). Raising or lowering the NaCl concentration in the medium resulted in an increase or decrease of 9-AA fluorescence quenching, respectively (Fig. 6).

The proton uptake observed in intact Dunaliella cells is sensitive
The light-induced proton uptake in intact Dunaliella cells is closely related to the activity of the photosynthetic electron transport chain. This is indicated by the inhibition of the light-induced 9-AA fluorescence quenching by DCMU (Fig. 1B) and dicyclohexylcarbodiimide (not shown). The stimulation of proton uptake by ascorbate, in the presence of DCMU (Fig. 1B), may indicate a direct involvement of PSI in the proton uptake process. The proton uptake observed in intact Dunaliella, however, does not appear to reflect the build up of the proton gradient across the thylakoid membrane directly (see below). We therefore suggest that the involvement of the photosynthetic machinery in the proton uptake in intact Dunaliella is related to the supply of energy, possibly in the form of ATP.

The light-induced proton uptake in intact cells could reflect an increase of ΔpH across the thylakoid membrane, a ΔpH across the chloroplast envelope or across the plasma membrane. The experiments with PCMBs may allow one to distinguish between these possibilities. Presumably PCMBs does not permeate biological membranes (8, 23). Its effect on the proton uptake in intact cells may therefore suggest that the proton gradient is formed across the plasma membrane. Ginzburg and co-workers (11), however, suggested that the Dunaliella cell membrane is rather impermeable to molecules as large as inulin. This could mean that in the case of Dunaliella PCMBs might penetrate the cell membrane. The lack of response of CO2-dependent O2 evolution to PCMBs in intact cells (Fig. 4), however, clearly suggests that PCMBs does not penetrate as far as the thylakoid membrane. Otherwise, inhibition of the ΔpH formation across the thylakoid membrane should have inhibited CO2-dependent O2 evolution. This conclusion is further supported by the comparison of kinetics of the increase of ΔpH, requirement for light “activation,” and for PMS and the different sensitivity to PCMBs concentrations in intact versus osmotically ruptured cell preparations. If PCMBs penetrates the cell membrane of Dunaliella then the proton gradient observed here could be located across the chloroplast envelope. A light-induced ΔpH which requires PMS has been demonstrated in intact spinach chloroplasts (13). However, had PCMBs penetrated as far as the chloroplast envelope it would be surprising if its presence in the cytoplasm did not affect the exchange of O2 by inhibiting the activity of cytoplasmic enzymes.

The magnitude of the ΔpH is strongly dependent upon Na+ concentration in the medium (Figs. 5 and 6). This response to Na+ concentration correlates with the specific requirement for Na+ for growth of Dunaliella (4) and the metabolic shift from carbohydrates to glycerol formation which is specifically induced by raising Na+ concentration in the medium (15, 16). These responses of Dunaliella to Na+ concentration may be related to the activity of an Na+/H+ exchange system which has been invoked in Dunaliella, as a possible mechanism by which the Na+ concentration gradient (between the cell interior and the surrounding medium) is maintained (14, 18). The inhibition of the ΔpH by ATPase inhibitors (Fig. 7) may indicate a direct involvement of an ATPase in the transport of protons. This inhibition also could result from the effect of ATPase inhibitors on energy supply in the form of ATP formation, as is the case with the energy transfer inhibitor phlorizin which also inhibited the formation of the ΔpH.

It is suggested that an (Na+/H+)-ATPase may be involved in the formation of the observed proton gradient in the light. Whether Na+ or H+ are actively transported is not yet clear as the electrochemical potential gradient of both Na+ and protons across the membrane and possible involvement of other ions has not been determined. Further, since Na+ transport was not measured, it is not known whether and to what extent stimulation of Na+ efflux will result in an increase of the rate of 9-AA fluorescent quenching. The involvement of a proton pump in energization of ion transport across cell membranes has been invoked (5, 6, 21, 22, 26). Harold and Papineau (11) demonstrated that Na+ efflux

**DISCUSSION**

The light-induced 9-AA fluorescence quenching observed here confirms the report of Ben-Amotz and Ginzburg (2) who demonstrated a light-induced proton uptake in Dunaliella. Their findings have been criticized by Neumann and Levine (20) who attributed the change of pH in the medium to CO2 uptake. We measured O2 evolution simultaneously with pH changes in the medium and concluded that the former (which parallels CO2 uptake, if NaCl concentration is kept constant, e.g. 14, 16) cannot quantitatively account for the pH changes in the medium (Kaplan, unpublished data).

**FIG. 6.** Dependence of light-induced 9-AA fluorescence quenching on the NaCl concentration in the suspension medium of *D. salina*. NaCl concentration in the experimental apparatus was modified by similar additions of 150 μl distilled H2O (A), 1.5 m NaCl (control, B), and 5 m NaCl (C), in order to keep cell concentration constant. The volume of the cell suspension is kept constant at 0.5 ml. Other conditions as in Figure 1B.

**FIG. 7.** Inhibition of light-induced 9-AA fluorescence quenching in intact cells of *D. salina* as a function of the inhibitor concentration. Inhibitors were applied after light activation (by three light to dark treatments) in the presence of 10 μM PMS. Data presented are those obtained at steady state 9-AA fluorescence signal.

to ATPase inhibitors such as quercetin and rutin which are known to inhibit F1-type ATPase (17) (Fig. 7). Ouabain (10 μM) a (Na+/K+)-ATPase inhibitor, on the other hand, had no effect on the 9-AA fluorescence quenching (data not shown). The energy transfer inhibitor phlorizin (see 18) also inhibited the light-induced proton uptake in intact cells of Dunaliella (50% inhibition at 1 mm, data not shown).

**REFERENCES**

in *Streptococcus* is driven by the pH gradient formed by an H⁺ pump. An Na⁺/H⁺ antiport mechanism has also been suggested for *H. halobium*. In contrast to the proton transport in *Dunaliella*, however, the Na⁺/H⁺ exchange in *H. halobium* is insensitive to ATPase inhibitors which may indicate a different mode of energization (7). Whether the postulated (Na⁺/H⁺)-ATPase is involved in the formation and maintenance of an Na⁺ gradient between the *Dunaliella* cell and the surrounding medium (3, 18 versus 9–11) remains to be clarified. The impact of the light-induced proton uptake on the regulation of the internal pH in *Dunaliella* should also be considered. At present, however, it is very difficult to establish a quantitative evaluation of the effect of the postulated Na⁺/H⁺ exchange mechanism on the internal pH. If the internal pH is largely regulated by the activity of a proton extrusion pump (21) then the rate of H⁺ pumping will presumably be affected by the Na⁺ fluxes.

The requirement for PMS of the light-induced ΔpH is not yet understood. Similar response of ΔpH to PMS has been reported in intact chloroplasts (12). PMS is known to stimulate cyclic electron transport which may result in a higher amount of ATP available for the formation of proton gradient. PMS treatment of *Dunaliella* cells, however, resulted in a 30% decrease in ATP pool size in intact cells (data not shown). It seems that if ATP and (Na⁺/H⁺)-ATPase are involved, PMS must somehow accelerate the turnover of ATP, as a consequence of which H⁺ uptake is increased.

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**LITERATURE CITED**

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