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FLUORESCENCE INDUCTION IN CHLOROPLASTS ISOLATED FROM THE GREEN ALGA, *BRYOPSIS MAXIMA*

V. pH DEPENDENCE OF THE P-S_1 TRANSIENT

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The effects of uncouplers of photophosphorylation on the P-S_1 transient of the fluorescence induction in dark-adapted intact chloroplasts of *Bryopsis maxima* were studied to examine the mechanism of light-dependent regulatory changes in electron transport. (1) Carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) and nigericin slowed down the fluorescence quenching from P to S_1, whereas the transient was significantly accelerated by the addition of NH_4Cl and methylamine. (2) The P-S_1 decline was slowed down at low pH of the suspending medium, suggesting sensitivity of the transient to the stroma pH. The inhibitory effect of nigericin was markedly enhanced at low pH and at low KCl concentrations, whereas the ionophore stimulated the transient at high pH and at high KCl concentrations. Similar results were obtained on the combined addition of CCCP and valinomycin. (3) Nigericin and the CCCP-valinomycin couple altered the internal pH of intact chloroplast through the H^+-K^+ exchange across the outer limiting membrane. The fluorescence decline was rapid at alkaline internal pH but was suppressed with lowering internal pH below 8.0. (4) A similar internal pH dependence of the transient was obtained when the internal pH was changed by the addition of NH_4Cl and acetate. (5) It is proposed that the photoactivation of electron transport is regulated by the stroma pH. The progress of the photoactivation is slow at acidic or neutral pH but is significantly accelerated by light-induced alkalinization near the light-regulation site of electron transport located on the outer surface of the thylakoid membrane.

Introduction

The induction of chlorophyll *a* fluorescence in higher plants and algae monitors a series of changes in the photosynthetic machinery during the transition from the dark to the light steady state [1–12]. On illumination of dark-adapted cells or intact chloroplasts of the green alga, *Bryopsis maxima*, the fluorescence yield rises from the initial level O to a peak P via intermediary transient features I and D, then decreases to a lower level S_1. The fluorescence induction up to the stage S_1 has been explained in terms of redox changes of Q, the primary electron acceptor of photosystem II which quenches fluorescence in the oxidized form [2]. A series of works published from our laboratory [9–12] have demonstrated that the O-I-D-P-S_1 transient reflects the situation that light exerts a regulatory effect in photosynthetic electron transport. The O-P rise, or the initial rapid reduction of Q, occurs, as electron transport is inactivated at its reducing terminal in dark-adapted chloroplasts. Subsequent decline in the fluorescence yield from P to S_1 reflects the photoactivation of electron transport from its dark-inactivated state.

Satoh et al. [9] have previously shown that CCCP,

Abbreviations: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; Mes,2-(*N*-morpholino)ethanesulfonic acid; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; Chl, chlorophyll.

a potent uncoupler of photophosphorylation, effectively and selectively inhibits the photoactivation of electron transport. They suggested two mechanisms to explain the effect of CCCP. The first mechanism is that the photoactivation of electron transport is related to light-induced changes in the energy state or structure of the thylakoid membranes and is thus sensitive to the uncoupler. The second, which is analogous to the mechanism of photoactivation of enzymes in the carbon-reducing cycle such as glyceraldehyde-3-phosphate dehydrogenase, assumes an involvement of light-generated and CCCP-sensitive dithiols [13] in the photoactivation of electron transport.

The present work was initiated to examine the two mechanisms of photoactivation described above in an attempt to elucidate the mechanism of light-dependent regulation of electron transport. These mechanisms can be distinguished by studying the effects of various uncouplers of photophosphorylation on the P-S_1 transient of the fluorescence induction, a manifestation of the photoactivation of electron transport. All uncouplers are expected to be equally inhibitory to the fluorescence decline in the first uncoupling mechanism, whereas the suppression of the P-S_1 transient should be specific to CCCP in the second dithiol mechanism. However, the results obtained in the present work were compatible with neither mechanism. Instead, they indicated that the uncouplers affected the fluorescence transient by altering the internal pH through H^+ transport across the outer limiting membrane of intact chloroplasts. It is proposed that the photoactivation of electron transport is regulated by the internal pH of intact chloroplasts.

Materials and Methods

Intact chloroplasts were prepared from giant cells of a sea alga, *B. maxima*, as described in the preceding paper [14]. They were suspended in a medium containing 1.0 M sorbitol, 2 mM EDTA, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 2 mM $NaNO_3$ and 50 mM Hepes/NaOH (pH 6.7), and kept in the dark at 0°C for at least 2 h before use.

Fluorescence of chlorophyll *a* was determined at room temperature as described previously [11]. Chloroplasts were illuminated with a broad band of blue light (350–600 nm) with a light intensity of 4.7 mW/cm^2. Fluorescence was monitored at a right-angle to the exciting light beam by a photomultiplier through appropriate combination of a cut-off filter and an interference filter to isolate fluorescence at 685 nm. Signals were stored in a Rikendekii transient recorder TCB-2 000 and displayed on a servo recorder.

Methyl viologen photoreduction was determined by monitoring O_2 uptake with a Clark-type oxygen electrode at 25°C in the presence of 2.5 mM methyl viologen and 1 mM KCN. pH changes were monitored by a combination glass electrode (Horiba 6 028) connected to a Hitach-Horiba F-7ss pH-meter under continuous stirring at 10°C in the dark as described previously [14]. The internal pH of intact *Bryopsis* chloroplasts was determined by the second method in the preceding paper [14]. Light scattering of chloroplasts was determined at a right-angle to a weak modulated measuring beam at 495 nm (where no electrochromic absorbance change occurs). Only modulated signals were amplified and recorded. Non-modulated blue actinic light (350–450 nm, 2.8 mW/cm^2) was provided by a 100 W halogen lamp through a combination of filters (Corning 4-96, Toshiba VV-1A and Hoya B-370). The photomultiplier (Hamamatsu T.V. R-636) was protected from actinic light and chlorophyll fluorescence with a Corning 4-96 and a Hoya VY-48 filter. Measurements were made at room temperature (20–25°C).

Results

Fig. 1 shows early transient of the fluorescence induction in dark-adapted intact chloroplasts of *B. maxima*. During the illumination period of about 7 s, the fluorescence yield rose to the maximum P and, after passing the minimum S_1, nearly attained the second peak M_1 (trace a). In accord with a previous observation [9], the addition of CCCP markedly slowed down the fluorescence decline from P to S_1 (trace b). The subsequent rise from S_1 to M_1, which reflects the formation of an H^+ gradient across the thylakoid membranes [11], was also eliminated by the uncoupler.

The inhibitory effect of CCCP on the P-S_1 decline was explained in terms of either its uncoupling action on the thylakoid membranes, or its interaction with light-generated dithiols [9]. In order to distinguish

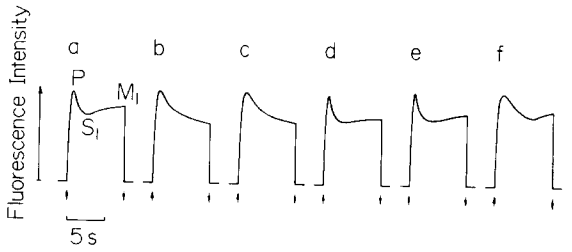

Fig. 1. Effects of uncouplers of photophosphorylation and sodium acetate on the P-S_1 transients of the fluorescence induction in dark-adapted *Bryopsis* chloroplasts. The reaction mixture contained 1.0 M sorbitol, 50 mM KCl, 50 mM Hepes/NaOH (pH 7.5), 2 mM EDTA, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 2 mM $NaNO_3$ and chloroplasts equivalent to 35 μg Chl/ml. Chloroplasts were illuminated with blue light of 4.7 mW/cm^2 and fluorescence was determined at 685 nm. Upward and downward arrows indicate when light was on and off, respectively. (a) No addition, (b) 2.5 μM CCCP, (c) 0.1 μM nigericin, (d) 5 mM NH_4Cl, (e) 30 mM methylamine hydrochloride, (f) 100 mM sodium acetate.

between the two mechanisms, experiments were extended to other uncouplers of photophosphorylation. Trace c shows that nigericin was also effective

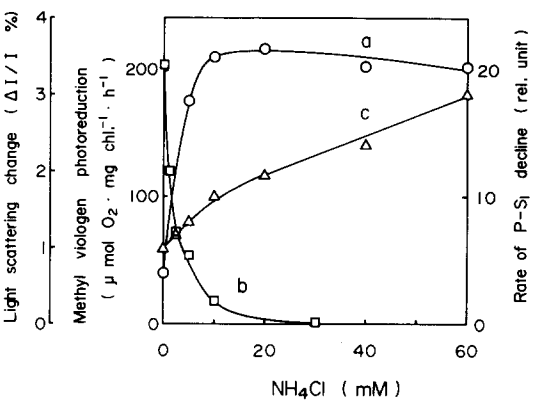

Fig. 2. Effects of NH_4Cl on methyl viologen photoreduction (a), the light-induced increase in light scattering of chloroplasts (b) and the rate of decline of the PS_1 fluorescence transient (c). The suspending medium was the same as in Fig. 1, except KCl was omitted. Chloroplast concentrations were equivalent to 15.1, 19.0 and 24.0 μg Chl/ml for a, b and c, respectively. Oxygen uptake was measured in the presence of 2.5 mM methyl viologen and 1 mM KCN. Extents of light-scattering changes at 495 nm were determined after 10 s of illumination with blue light of 2.8 mW/cm^2. The rate of the P-S_1 decline was expressed as the extent of the decrease in fluorescence yield during 0.5 s after the fluorescence yield passed the maximum P, during which the subsequent rise to M_1 was insignificant.

in suppressing the P-S_1 transient. A marked retardation of the transient was also observed on the combined addition of 2,4-dinitrophenol and valinomycin which is known to uncouple chloroplasts [15], whereas addition of 2,4-dinitrophenol or valinomycin alone was without effect (data not presented). These results cannot be explained by the second mechanism which assumes that the inhibition is specific to CCCP.

However, the first uncoupling mechanism was also challenged by the finding that the P-S_1 decline was affected quite differently by NH_4Cl and methylamine (traces d and e). The amine-type uncouplers did not slow down the transient. Actually they accelerated it. Fig. 2 confirms that NH_4Cl serves as a potent uncoupler in intact *Bryopsis* chloroplasts, accelerating methyl viologen photoreduction and inversely diminishing light-induced increases in light scattering of chloroplasts, indicative of the protonation of the thylakoid membranes [16,17]. Note that whereas maximum uncoupling was attained at about 20 mM NH_4Cl, the rate of the P-S_1 decline was still increasing at 60 mM NH_4Cl. The results are clearly incompatible with the first mechanism which relates the retardation of the fluorescence transient to the uncoupling of chloroplasts.

Thus, we are now in a position to look for the third mechanism to explain the effects of uncouplers in the P-S_1 transient. In this respect, it is a very suggestive finding that the P-S_1 decline is sensitive to the pH of the suspending medium (Fig. 3). The transient was slightly slowed down by lowering the medium pH from 8.2 to 6.5. Heldt et al. [18] have shown that the stroma pH of intact spinach chloroplasts is affected to some extent by the medium pH. It appears, therefore, that the P-S_1 decline depends upon the internal pH of the chloroplasts in such a way that an acidic internal pH suppresses the quenching of fluorescence.

Fig. 3 also indicates that the pH dependence of the transient was strongly emphasized by the addition of nigericin. The ionophore hardly affected the P-S_1 decline at pH 8.2 but almost completely eliminated the fluorescence decline at pH 6.5. This suggests that nigericin affected the P-S_1 transient by altering the pH inside the intact chloroplasts.

It was shown in the preceding paper [14] that nigericin causes an exchange of H^+ and K^+ across the outer limiting membrane of chloroplasts according to the electrochemical potential differences of H^+

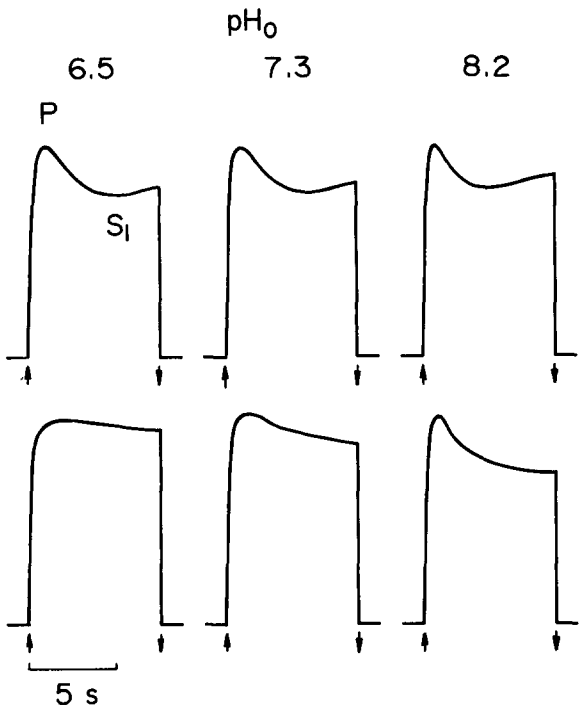

Fig. 3. The pH dependence of the P-S_1 decline. Experimental conditions were as in Fig. 1. Upper and lower traces were determined in the absence and presence of 0.1 μM nigericin, respectively.

and K^+ between the intrachloroplast space and the outer medium. At equilibrium, the relationship shown in Eqn. 1 holds:

$$pH_i = pH_o + \log([K^+]_o/[K^+]_i) \quad (1)$$

where pH_i and pH_o are the internal and external pH values and $[K^+]_i$ and $[K^+]_o$ are the internal and external activities of K^+, respectively.

In accord with this mechanism, the effect of nigericin on the fluorescence transient was found to vary depending not only on pH_o but also on the KCl concentration of the medium (Fig. 4). *Bryopsis* chloroplasts contain an extremely high concentration of K^+ [14]. Note that at pH 8.0 and below, the inhibitory effects of the ionophore became more marked in the presence of low concentrations of KCl, where efflux of K^+ coupled with influx of H^+ is expected to occur. Stimulation was observed at alkaline pH and/or in the presence of a high concentration of KCl, where nigericin will induce alkalinization of the chloroplast interior. In contrast, the P-S_1 decline was independent of KCl concentration in the absence of nigericin.

Similar results were obtained when the outer limiting membranes of chloroplasts were made permeable to both H^+ and K^+ by the combined addition of CCCP and valinomycin (Fig. 4B). The couple caused stimulation or retardation of the transient depending upon the KCl concentration as well as the pH of the suspending medium as did nigericin.

The explanation above cannot be applied to the inhibitory effects of CCCP which does not induce H^+ transport across the outer limiting membrane of *Bryopsis* chloroplasts [14]. Fig. 4C shows that CCCP suppressed the P-S_1 decline approximately equally at all pH values tested. The inhibition was independent of KCl concentrations. We suggest that the P-S_1 decline is accelerated by the alkalinization of the stroma resulting from light-induced H^+ uptake by the thylakoids and that CCCP slowed down the transient by dissipating the pH difference between the stroma and intrathylakoid spaces thus formed.

It has been shown that the internal K^+ activity was equivalent to that of 320 mM KCl [14]. Because pH_o and $[K^+]_o$ are known, pH_i established in the presence of nigericin or CCCP plus valinomycin can be calculated using Eqn. 1. The results of Fig. 4A and B are replotted in Fig. 5 as a function of pH_i thus estimated. It can be seen that all data obtained with widely different cation compositions of the medium fit a single pH curve. The rate of the P-S_1 transient is high at pH_i 8.0 or above, but decreases with lowering pH_i, showing 50% inhibition at about pH_i 7.4.

The effects of the amine-type uncouplers can be explained within the framework of the mechanism above. NH_4Cl or methylamine is known to equilibrate into intact chloroplasts only in the uncharged form and takes up H^+ inside [19,20]. Thus, stimulation of the P-S_1 transient observed in the presence of the amine-type uncouplers can be related to the alkalinization of the intrachloroplast space.

On the other hand, acetate has been used to lower the pH inside intact chloroplasts [21]. The undissociated acid permeates across the outer limiting membrane and once inside release H^+. Fig. 1, trace f, shows that the P-S_1 decline was markedly slowed down on addition of acetate.

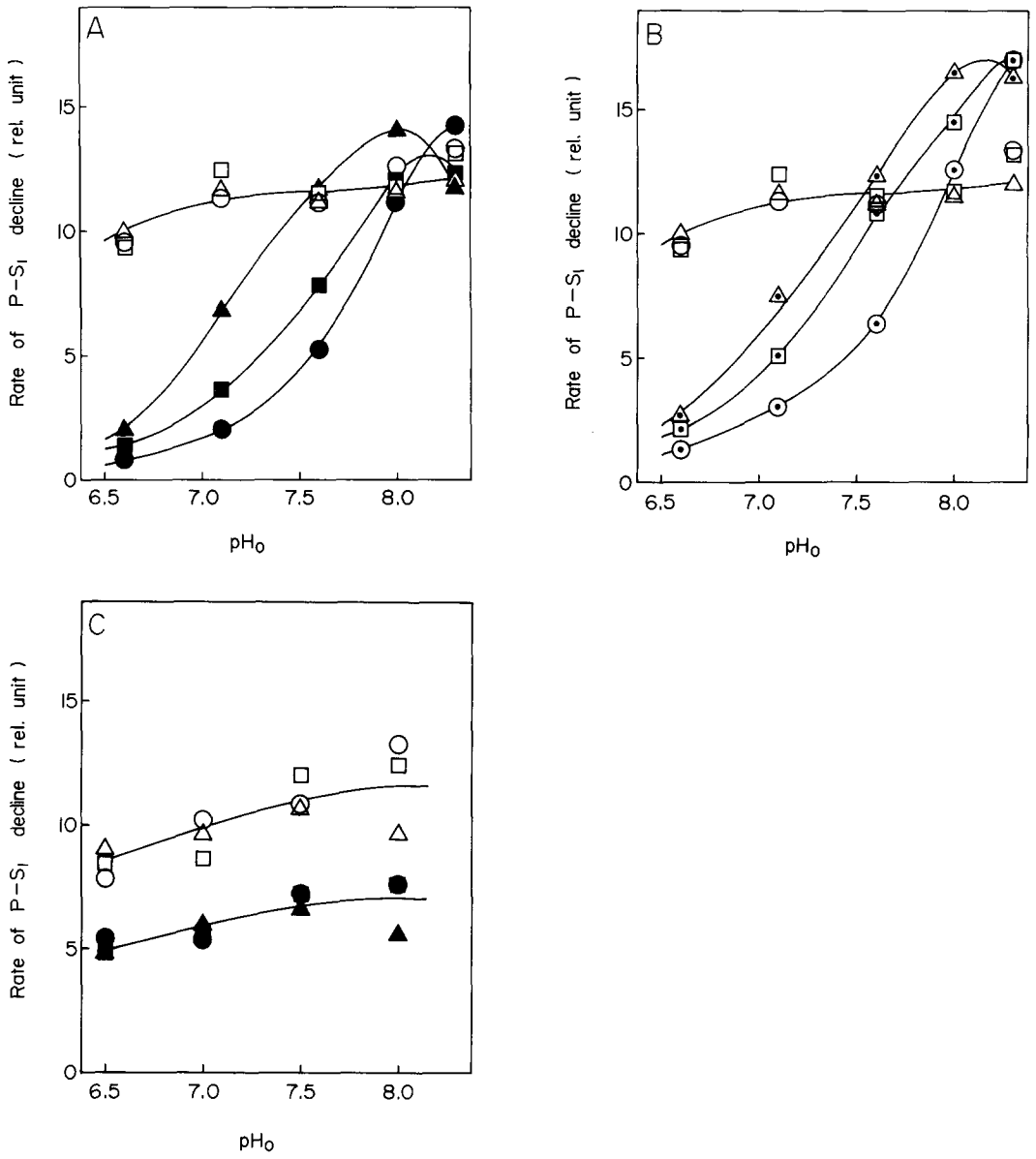

Fig. 4. Effects of nigericin (A), CCCP plus valinomycin (B) and CCCP (C) on the P-S_1 decline at different pH values and KCl concentrations of the media. Experimental conditions were as described in Fig. 3, except that KCl concentrations were 125 mM (○, ⊙, ●), 250 mM (□, ⊡, ■) and 500 mM (△, ⊿, ▲), and the sorbitol concentration was varied to maintain the total osmolality of the media at 1.3 osmol/kg water. Chloroplast concentrations were equivalent to 25.3 μg Chl/ml in A and B, and 32.3 μg Chl/ml in C. Open symbols, no addition. Closed symbols in A, 2.5 μM nigericin. Open symbols with a central dot in B, 5 μM CCCP plus 5 μM valinomycin. Closed symbols in C, 5 μM CCCP.

In order to relate quantitatively rates of the P-S_1 decline to pH_i established in the presence of NH_4Cl and acetate, pH_i was determined by the second method described in the preceding paper [14]. pH changes induced by the addition of a nonionic detergent, Triton X-100, to intact chloroplasts depend only on the pH difference across the outer limiting membranes, provided that the activities of

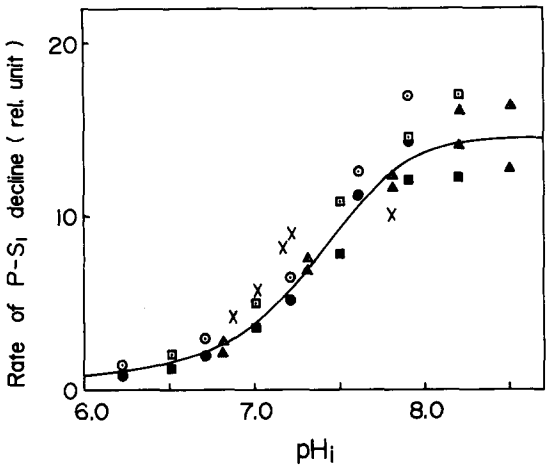

Fig. 5. pH_i dependence of the P-S_1 decline. Symbols: see Fig. 4A and B. (X) Results obtained from Fig. 7.

mono- and divalent cations in the suspending medium are the same as those inside chloroplasts [14]. pH_i is equal to a pH_o where no pH change occurs on addition of Triton X-100 to NH_4Cl- or acetate-treated chloroplasts (Fig. 6). The suspending medium contained 300 mM KCl and 3 mM $MgCl_2$ to minimize the difference in activities between the medium and the intrachloroplast space. Since $pH_o = pH_i$, disruption of the outer limiting membrane with Triton X-100 gave rise to no pH change (trace a). Addition of NH_4Cl caused a significant pH decrease due to the equilibration of neutral NH_3 across the outer limiting membrane of intact chloroplasts (trace b). After the medium pH was brought back to 7.2 with NaOH, addition of the detergent caused a pH increase, indicating pH_i to be higher than pH_o. pH changes of opposite signs were observed on sequential addition of acetate and the detergent (trace c). A rapid transient pH decrease observed on addition of acetate is due to a small pH difference between the medium and the addition (see trace e).

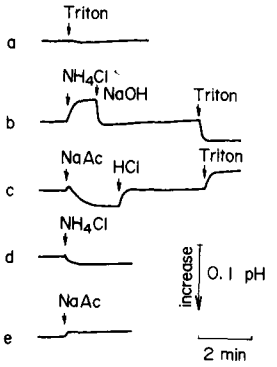

Fig. 6. Time courses of pH changes induced by sequential additions of NH_4Cl or sodium acetate and Triton X-100. The medium contained 660 mM sorbitol, 300 mM KCl, 3 mM $MgCl_2$, 0.5 mM Hepes and 0.5 mM Mes and chloroplasts equivalent to 114 μg Chl/ml. No chloroplasts were present in d and e. The initial pH was adjusted to 7.2 (pH_i). Where indicated, 11 mM NH_4Cl, 22 mM sodium acetate (NaAc) and 0.055% Triton X-100, the pH values of which had been adjusted to approx. pH 7.2, were added. All experiments were carried out at 10°C and in the dark.

Fig. 7 summarizes pH changes induced by the addition of Triton X-100 in the presence of various concentrations of NH_4Cl and acetate at different pH_o values. pH_i was raised from 7.21 to 7.47, 7.67 and 7.95 by the addition of 5.5, 11 and 28 mM NH_4Cl and lowered to 6.85 and 6.52 in the presence of 22 and 55 mM acetate, respectively. The rates of the P-S_1 decline were determined at corresponding NH_3 and acetate concentrations with the same chloroplast preparation and plotted against pH_i in Fig. 5. The pH_i dependence of the P-S_1 decline thus determined fits approximately with that obtained above with the other technique. This proves that the pH_i dependence of the fluorescence transient shown in Fig. 5 is real, and thus renders further support for the above-described mechanism for the uncoupler-induced changes in the P-S_1 transient.

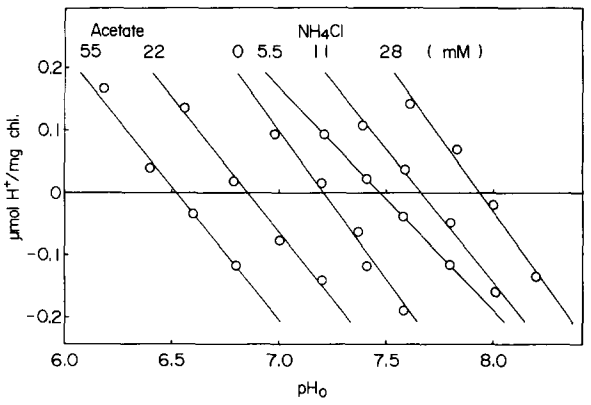

Fig. 7. Determination of pH_i in chloroplasts pretreated with various concentrations of NH_4Cl and sodium acetate. Experiments were carried out as in Fig. 8. Chloroplast concentration was 160 μg Chl/ml.

Discussion

The results obtained in the present work rule out the two possibilities that the quenching of chlorophyll *a* fluorescence from P to S_1 is related to light-induced changes in the energy state or structure of the thylakoid membranes, or to light-generated dithiols [9]. Instead, they demonstrate that the P-S_1 decline is regulated by the internal pH of intact chloroplasts.

The decline in the fluorescence yield from P to S_1 is due to Photosystem I-dependent oxidation of Q [9]. A recent work of Satoh and Katoh [12] on an earlier fluorescence transient, the I-D dip, in intact *Bryopsis* chloroplasts indicated that the actual rate of Photosystem I-dependent oxidation of Q is not only much faster than the rate of the fluorescence P-S_1 transient, but is also unaffected by the addition of CCCP, nigericin or NH_4Cl. Obviously, the P-S_1 transient is rate limited by the slow progress of the light-induced activation of electron transport. Thus, the pH profile of the P-S_1 decline represents the dependence on pH_i of the photoactivation of electron transport.

The photoactivation of electron transport is rapid at alkaline pH but strongly suppressed below pH 8.0. A similar pH_i profile has been reported with the CO_2 fixation in intact chloroplasts [22]. CO_2 fixation is considered to be regulated by the stroma pH which rises in the light to a level optimal for CO_2 fixation through H^+ uptake by the thylakoid. We propose that light-induced redistribution of H^+ across the thylakoid membranes exerts a regulatory effect on the photoactivation of electron transport.

The P-S_1 decline is over within a few seconds of illumination at the light intensity used. The question may arise as to whether or not alkalinization of the stroma proceeds to an extent sufficient to affect the photoactivation of electron transport during such a short period of illumination of dark-adapted chloroplasts. Comparison of Figs. 4 and 5 indicates that the rates of the P-S_1 decline in the absence of ionophores or uncouplers (Fig. 4) correspond to a pH_i of 7.4–7.7, which is significantly higher than the pH_i of about 7.2 in the dark-adapted chloroplasts employed. Thus, alkalinization of the stroma seems to occur to some extent in a few seconds of illumination.

Our interpretation is supported by the inhibitory effects of CCCP on the transient. CCCP dissipates the H^+ gradient between the intrathylakoid and stroma spaces in intact chloroplasts but is incapable of inducing H^+ transport across the outer limiting membranes [14], most probably reflecting the difference in permeability of the thylakoid and the outer limiting membranes to ions. Thus, the stroma pH must have been kept at the dark level when the chloroplasts were illuminated in the presence of CCCP. In fact, CCCP suppressed the P-S_1 decline to low levels which correspond to a pH_i of about 7.2, i.e., the pH_i of dark-adapted and nonilluminated chloroplasts (Fig. 4C).

The stroma pH may not be homogeneous at the early stage of illumination. The electron transport is regulated by light at the region of ferredoxin and ferredoxin-NADP oxidoreductase [10] which are located on the outer surface of the thylakoid membranes. The light-induced H^+ uptake by the thylakoid would cause a rapid rise in the local pH adjacent to the light-regulation site of electron transport.

Rühle and Wild [23] observed induction phenomena of cytochrome *f* photooxidation in leaves of *Sinapis alba* and ascribed them to an energized state of the thylakoid membrane which may be regulated by Mg^{2+} efflux. However, Satoh et al. [9] have shown that the inductive changes of cytochrome *f* in *Bryopsis* chloroplasts are closely related to the fluorescence induction. The transient reduction and subsequent oxidation of cytochrome *f*, respectively, parallel the D-P rise and the P-S_1 decline not only in kinetics, but also in response to electron-transport inhibitors, redox reagents and preconditioning of chloroplasts. Thus, the induction of cytochrome *f* photooxidation is another manifestation of the ligh-dependent regulatory changes in electron transport at the reducing side of Photosystem I and, in the light of the present observations, cannot be related to the energized state of the thylakoid membranes.

Finally, the present work cautions that it should be borne in mind that an ionophore (or a combination of an ionophore and an H^+ carrier) which mediates an exchange of H^+ and a cation across the outer limiting membranes of intact chloroplasts, or any other membrane-bound systems such as cellular organelles, protoplasts or cells, may alter the internal pH and thus may affect the activity inside. Especially, when the outer limiting membrane is permeable to a cation or an anion, addition of an H^+ carrier such as

CCCP, or an ionophore such as valinomycin, would be sufficient to cause pH changes inside. This type of effect of ionophores or H^+ carriers can be minimized by adjusting the pH and the cation composition of the suspending medium to be similar to those inside.

On the other hand, the present work provides a new technique to control the internal pH of organelles or cells. The internal pH can be either raised or lowered by varying the pH and concentration of a cation of the outer medium and adding an appropriate ionophore (and/or an H^+ carrier). When the thermodynamic activity of the cation inside is known, pH_i can be varied to any desirable values according to Eqn. 1.

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