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Dissipation of the Proton Electrochemical Potential in Intact Chloroplasts[1]

II. The pH Gradient Monitored by Cytochrome *f* Reduction Kinetics

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The potency of various uncouplers for collapsing the light-induced pH gradient across thylakoid membranes in intact chloroplasts was investigated by time-resolved optical spectroscopy. The thylakoid transmembrane pH gradient (ΔpH) was monitored indirectly by measuring the rate of cytochrome (Cyt) *f* reduction following a light flash of sufficient duration to create a sizable ΔpH. The results show that the rate of Cyt *f* reduction is controlled in part by the internal pH of the thylakoid inner aqueous space. At pH values from 6.5 to 8.0, the Cyt *f* reduction rate was maximal, whereas at lower pH values from 6.5 to 5.5 the reduction rate decreased to 25% of the maximal rate. The ability of three uncouplers, nigericin, carbonylcyanide *m*-chlorophenylhydrazone, and gramicidin, to accelerate the rate of Cyt *f* reduction was determined for intact chloroplasts isolated from spinach (*Spinacia oleracea*). The efficacy of the uncouplers for collapsing the ΔpH was determined using the empirical relationship between the ΔpH and the Cyt *f* reduction rate. For intact chloroplasts, nigericin was the most effective uncoupler, followed by carbonylcyanide *m*-chlorophenylhydrazone, which interacted strongly with bovine serum albumin. Gramicidin D, even at high gramicidin:chlorophyll ratios, did not completely collapse the pH gradient, probably because it partitions in the envelope membranes and does not enter the intact chloroplast.

The electrochemical potential is composed of an electrical component ($\Delta\Psi$) and a proton chemical component that is proportional to the ΔpH. Evidence that a ΔpH may drive photosynthetic phosphorylation was presented more than 25 years ago (Neumann and Jagendorf, 1964); yet, the mechanism by which the proton electrochemical potential is coupled to bioenergetic events such as ATP synthesis is poorly understood (Boyer, 1988). ATP formation during photosynthesis is driven mainly by ΔpH. The $\Delta\Psi$ contributes significantly to ATP formation when chloroplasts are first excited by light, and the $\Delta\Psi$ is large compared to ΔpH (Ort and Dilley, 1976; Graan and Ort, 1981). The electrochemical potential also provides energy for other cellular processes, including transport of proteins into mitochondria (Pfanner and Neuport, 1986; Verner and Schatz, 1987). It is generally recognized that an electrochemical potential is not required for protein transport across the envelope membranes into chloroplasts (Archer and Keegstra, 1990). Protein transport into or across the thylakoid, however, appears to require a proton chemical gradient (Cline et al., 1989; Mould and Robinson, 1991) but not ATP (Cline et al., 1992).

Ionophores are capable of uncoupling ATP synthesis from electron transport. In most cases the uncoupling is a direct result of a collapse of the proton electrochemical potential across the thylakoid membrane (Good, 1977). Recently, we determined the efficacy of various ionophores at collapsing $\Delta\Psi$ in intact and lysed chloroplasts by monitoring the effect of ionophores on the electrochromic shift measured by the ΔA_{518} (Nishio and Whitmarsh, 1991). The main objective of the present research is to establish procedures that result in the dissipation of ΔpH in chloroplasts under commonly used conditions. However, techniques to monitor ΔpH have inherent limitations. Usually, methods using fluorescent dyes and the partitioning of amines are used to estimate ΔpH across thylakoid membranes (Rottenberg et al., 1972; Rottenberg and Grunwald, 1972; Schuldiner et al., 1972; Pick and McCarty, 1980). The reduction kinetics of P700 have also been used to estimate ΔpH across thylakoid membranes (Rumberg and Siggel, 1969; Tikhonov et al., 1981).

To determine the impact of ionophores on ΔpH in intact chloroplasts, we used the relationship between Cyt *f* reduction following a flash and the internal pH of the thylakoid lumen. The oxidation of plastoquinol by the Cyt *b*/*f* complex limits electron transport (Stiehl and Witt, 1969; Junge, 1977; Witt, 1979). As a consequence, Cyt *f* is oxidized in the light and subsequently reduced in the dark. The rate at which Cyt *f* is reduced in the dark reflects the rate of light-driven electron transport flow and is partially dependent on the pH of the thylakoid lumen, estimated to decrease to about pH 5.5 during the light-driven increase of protons in the lumen (Karlish and Avron, 1971; Rottenberg et al., 1972). Thus,

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Abbreviations: CCCP, carbonylcyanide *m*-chlorophenylhydrazone; ΔA, change in absorbance; ΔpH, transmembrane pH gradient; $\Delta\Psi$, electrical component of electrochemical potential.

monitoring the rate of Cyt *f* reduction provides a simple method for examining ΔpH in illuminated chloroplasts.

To determine the ionophore:Chl ratio adequate for collapsing the pH gradient across thylakoid membranes in intact chloroplasts, we titrated gramicidin, CCCP, and nigericin. Gramicidin, a naturally occurring antibiotic, forms a channel in the lipid bilayer that allows alkali cations and protons to diffuse across the membrane (Reed, 1979). CCCP, a lipophilic, weak acid, is an electrogenic proton carrier (a proton uniporter) that, like gramicidin, collapses both $\Delta\Psi$ and ΔpH (Nichols, 1982). Nigericin, a naturally occurring polyether antibiotic, is a monovalent cation-transporting ionophore (Reed, 1979). When nigericin complexes with a monovalent cation (in our experiments K^+), an H^+ is released. Thus, nigericin is an electroneutral antiporter that responds to a pH or cation gradient. In the presence of adequate K^+, the ionophore equilibrates cations and protons across the membrane, and ΔpH is dissipated (Reed, 1979). As in the previous report dealing with $\Delta\Psi$ (Nishio and Whitmarsh, 1991), guidelines are provided for the use of ionophores to dissipate ΔpH in intact chloroplasts under conditions frequently used to analyze chloroplast energetics and transport phenomena.

MATERIALS AND METHODS

Plant Growth Conditions

Hydroponic

Spinacia oleracea (U.S. 424, Ferry-Morse Seed Co., Modesto, CA) was hydroponically grown using a modified Hoagland nutrient solution (Hoagland and Arnon, 1938). Plants were grown in controlled environment growth chambers with a 12-h light period and a constant irradiation of about 450 μmol of photons of PAR (400–700 nm) m^{-2} s^{-1}, at 22°C during the light period and 16°C during the dark period, and at an RH of about 60 to 70%.

Soil

Spinach plants were also soil grown in controlled environment growth chambers as described previously (Nishio and Whitmarsh, 1991).

Chloroplast Preparation

Intact chloroplasts were isolated from leaves harvested within 2 h of the start of the photoperiod or from leaves stored up to 2 weeks in the dark at 5°C with petioles in deionized H_2O. Intact chloroplasts were isolated from spinach leaves as described previously (Nishio and Whitmarsh, 1991). All procedures were done at 0 to 4°C unless noted otherwise.

Chl

Chl content in 80% (v/v) acetone:H_2O was determined spectrophotometrically at 647 and 664 nm (−730 nm) using the extinction coefficients of Ziegler and Egle (1965).

Chloroplast Intactness

Chloroplast intactness (routinely 85–95%) was determined by phase contrast microscopy (Spencer and Wildman, 1962).

Ionophore Addition

Ionophores were added to the resuspension buffer in cuvettes before addition of intact chloroplasts, as previously described (Nishio and Whitmarsh, 1991). All assays contained 0.1 mM methyl viologen as an electron acceptor. Gramicidin data were collected at least 5 min after addition of the final component. For the other ionophores, measurements were begun at least 2 min after preparation of the complete reaction mixture.

Gramicidin D (mol wt of 1800 was used) and nigericin were purchased from Sigma Chemical Co. (St. Louis, MO); CCCP was from E. I. DuPont De Nemours & Co. (Wilmington, DE). Ethanolic stock solutions of all ionophores were utilized. High concentrations of ethanol are not recommended because of possible artifacts; chloroplast preparations containing 0.33% ethanol did not affect the reduction kinetics of Cyt *f* (data not shown). All additions of ionophores were made with <0.3% ethanol, except for the highest concentration of CCCP (130 μM), which contained 4.3% (v/v) ethanol and the higher concentrations of gramicidin. Gramicidin concentrations of 1.6 to 13 μM contained 0.5 to 3.3% ethanol, respectively. The highest gramicidin concentration, 13 μM, was made by adding 0.1 mL of a 400-μM gramicidin stock to a 3-mL of final volume; all other gramicidin additions were from 300-μM stock solutions. At the highest concentration of CCCP, 130 μM, the maximum ΔA was about 60% of normal. CCCP is known to be inhibitory to electron transport at high concentrations (Good, 1977). An effective nigericin concentration of 1 μM in a 30-μM Chl solution contained 0.33% (v/v) ethanol.

Time-Resolved Optical Spectroscopy of Cyt *f*

Photooxidation of Cyt *f* was determined on a laboratory-built spectrophotometer previously described (Selak and Whitmarsh, 1984). Cyt *f* A changes at 554 nm were monitored following flashes (25–600 ms) provided by a shuttered 250-W tungsten-halogen lamp filtered by a red light-blocking filter (Corning CS 2–58), a hot mirror (Melles Griot 03 MHG 007), and a heat-absorbing filter (Corning CS I-57). Cyt *f* concentration was determined using an extinction coefficient of 20 mM^{-1} cm^{-1}. The optical pathlength was 1 cm, and the half-band width was 2 nm.

The dependence of Cyt *f* reduction on pH was determined by measuring Cyt *f* reduction at six different pH values. The assay medium utilized for these experiments was similar to the resuspension buffer described above in "Chloroplast Preparation," except the pH was varied. For the measurements at pH 7.0, 7.5, and 8.0 (18°C), the assay medium was buffered with Hepes. For the determinations at pH 5.5, 6.0, and 6.5 (18°C), Mes was used as a buffer. Thirty millimolar KCl was added to the low pH solutions. Solutions buffered with Hepes at pH 7.6 (2°C) contained 29 mM KOH, so the pH 8 solution contained a higher K^+ concentration. Addition of >29 mM K^+ to the assay medium did not affect the efficacy

of the alkali ion-transporting ionophore valinomycin (data not shown). To ensure equilibration of the pH across the membranes, 2 μM nigericin was included in the assay medium.

RESULTS

Measurements of Cyt *f* Light-Induced Redox Changes in Intact Chloroplasts

Light-induced oxidation-reduction measurements of Cyt *f* in thylakoids are typically done using a reference wavelength of 540 nm and in the presence of ionophores (Whitmarsh and Cramer, 1979) that eliminate the light-stimulated absorption changes in chloroplasts due to primary charge separation (Witt, 1979) and thylakoid shrinkage (Packer et al., 1970; Murakami et al., 1975). Because we are interested in monitoring Cyt *f* as a probe for ΔpH, it is requisite that the measurements be made in the absence of uncouplers, because they dissipate the pH gradient. Earlier work showed that correction for the electrochromic shift stimulated by single turnover flashes in coupled thylakoids could be accomplished by subtracting a proportional *A* at 531 nm that was determined to be equivalent to the electrochromic contribution (Dolan and Hind, 1974).

We reexamined the problem of deconvoluting the contribution of the electrochromic shift at 554 nm by acquiring light-dependent spectra of chloroplasts in the presence and absence of various ionophores. Light-induced spectra between 500 and 570 nm (560 nm reference) of intact spinach chloroplasts in the presence of various uncouplers are shown in Figure 1. The various treatments represent conditions that dissipate the ΔΨ alone, the ΔpH alone, and the total electrochemical potential. Figure 1 shows that the Δ*A* between 560 and 555 nm is almost identical in all spectra. The Δ*A* between 540 and 555 nm, on the other hand, varied significantly with the various treatments. We tested various reference wavelengths, including 540, 545, 559, 560, and 551 nm (543.5 and 560 nm are isosbestic points for the chemical redox difference of Cyt *f*; Bendall et al., 1971). The data showed that, in coupled chloroplast preparations, the $\Delta A_{554-560}$ was equivalent to the $\Delta A_{554-540}$ in chloroplast preparations that included nigericin and nonactin to dissipate the electrochemical proton gradient (Fig. 2). The Chl:Cyt *f* ratio was 530 in both samples. The major difference between the two treatments was the rate of dark reduction, which was much longer in the coupled chloroplast preparations. The light-dark *A* spectrum is shown in Figure 3. The shape of the spectrum is typical for Cyt *f* (Bendall et al., 1971; Whitmarsh and Cramer, 1979).

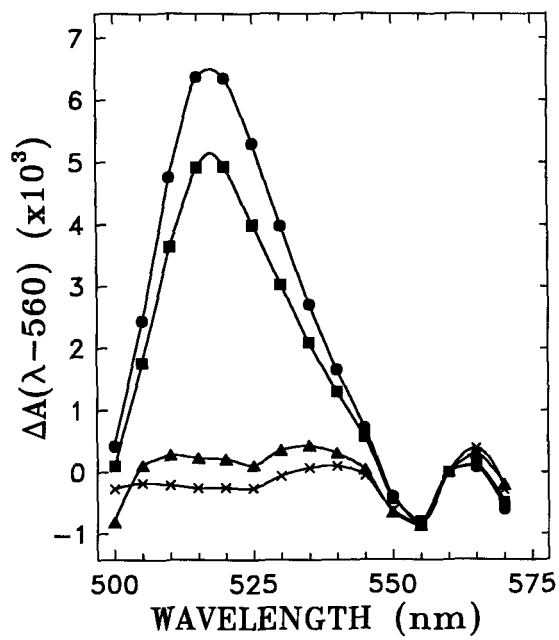

Figure 1. Spectra of light-induced Δ*A* of intact spinach chloroplasts in the absence and presence of nigericin and nonactin. The Δ*A* shown represents the light-induced Δ*A*(λ-560). Each data point represents the average of 32 flashes. Flashes of 350 ms were given at a rate of 0.143 Hz. Temperature was maintained at 18°C. The reaction mixture contained 30 μM Chl, 50 mM Hepes/KOH (pH 7.4), 330 mM sorbitol, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 2 mM EDTA, 2 mg of defatted BSA/mL of buffer, 0.1 mM methyl viologen as electron acceptor, and ionophores as indicated. ●, Control; ■, 2 μM nigericin; ▲, 8 μM nonactin; ×, 2 μM nigericin and 8 μM nonactin.

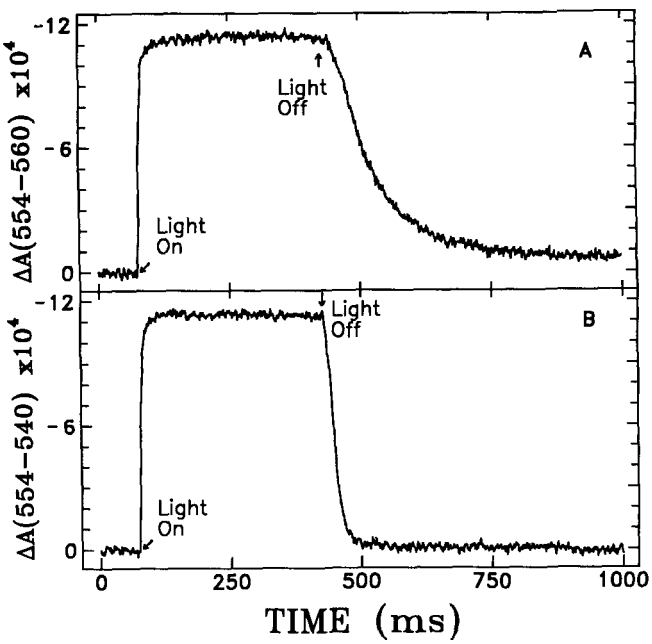

Figure 2. Kinetics of the light-induced Cyt *f* Δ*A* in intact spinach chloroplasts in the absence and presence of nigericin and nonactin. Cyt *f* Δ*A* was induced by a 350-ms flash. Reaction conditions were as described for Figure 1. Each trace represents the average of 64 flashes. A, Control, $\Delta A_{554-560}$; B, 2 μM nigericin and 8 μM nonactin, $\Delta A_{554-540}$.

Dependence of Cyt *f* Reduction on pH

To judge the appropriateness of probing ΔpH across thylakoid membranes by monitoring Cyt *f* reduction kinetics, we tested whether or not pH affected Cyt *f* reduction in intact chloroplasts. The rationale for this experiment is that the

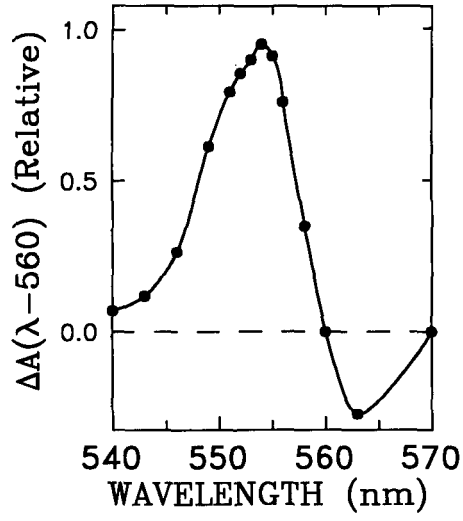

Figure 3. Light-dark *A* spectrum of Cyt *f* in intact spinach chloroplasts. Each data point represents the average of 32 flashes. Flashes of 50 ms were given at 0.33 Hz; a reference wavelength of 560 nm was used. Reaction medium is as described for Figure 1.

oxidation of plastoquinol by the Cyt *b/f* complex is the rate-limiting step in electron transport between PSII and PSI and is a sensitive function of pH (Stiehl and Witt, 1969; Jones and Whitmarsh, 1988). Rumberg and Siggel (1969) took advantage of this phenomenon to determine the ΔpH by measuring P700 reduction. Here, we measure Cyt *f* reduction kinetics to monitor lumenal pH.

The pH of the assay solution affected the kinetics of Cyt *f* reduction in chloroplasts that were treated with 2 μM nigericin (Fig. 4). At pH 5.5, the half-time of decay was slowest. Between pH 6.0 and 6.5, the fastest rates of Cyt *f* reduction were reached. At higher pH the rate of reduction did not accelerate. The inset in Figure 4 shows the reduction kinetics of Cyt *f* at three different pH values: 5.5, 6.0, and 6.5. Note that at low pH (5.5) the $\Delta A_{554-560}$ was decreased by about 25% compared with control samples. The decrease in ΔA may be due to clumping of plastids in the acidic environment. The rise kinetics were similar among treatments.

Dependence of Cyt *f* Reduction on Flash Length

We further examined the possibility of utilizing the reduction kinetics of Cyt *f* to monitor ΔpH by varying the actinic flash length. Under nonphosphorylating conditions, ATP formation will not relieve the ΔpH; therefore, a longer flash should decrease the pH in the lumen more than a short flash. In contrast, the flash length should have little effect on reduction kinetics (possibly decreasing as electron transport is fully activated) under uncoupled conditions. Figure 5 shows the Cyt *f* reduction half-time in coupled chloroplasts as a function of the length of the actinic flash. At 25 ms, the half-time of reduction was about 43 ms, whereas with a flash length of 350 ms or longer, it was about 80 ms. The reduction kinetics after a 25- and 350-ms flash are shown in the inset.

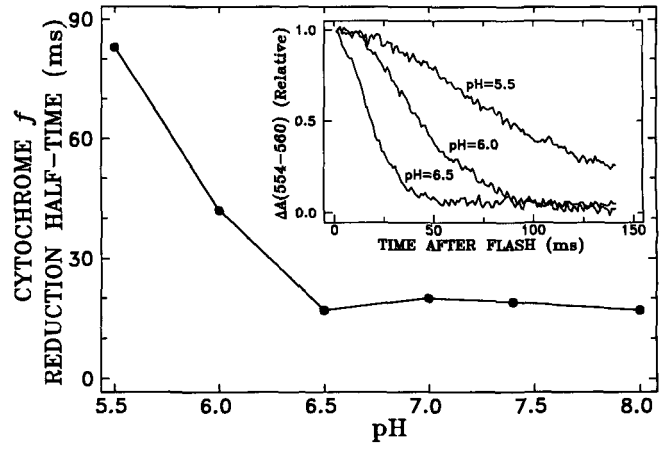

Figure 4. Effect of pH on Cyt *f* reduction kinetics in intact spinach chloroplasts following a 350-ms flash. The pH of the assay medium was varied from 5.5 to 6.5 using Mes and from 7.0 to 8.0 using Hepes, as described in "Materials and Methods." The assay medium contained 2 μM nigericin to ensure equilibration of pH across the thylakoid membrane. Each point represents the reduction half-time in ms of Cyt *f* monitored by the $\Delta A_{554-560}$. Each point is the average of 32 flashes. The inset shows the kinetics of Cyt reduction at pH 5.5, 6.0, and 6.5 after a 350-ms flash. The *A* at the end of the flash was normalized to 1.0. Data were collected as described for Figure 1.

Ionophore Titrations

The results of the ionophore titrations are shown in Figure 6. CCCP was relatively effective at dissipating ΔpH. A CCCP:Chl ratio of 0.07 adequately dissipated ΔpH across thylakoids when BSA was absent. In the presence of BSA, a CCCP:Chl ratio of about 1.1 seemed adequate, but such a ratio is relatively large and is not recommended. Additionally, CCCP was ineffective at collapsing the electrical potential in

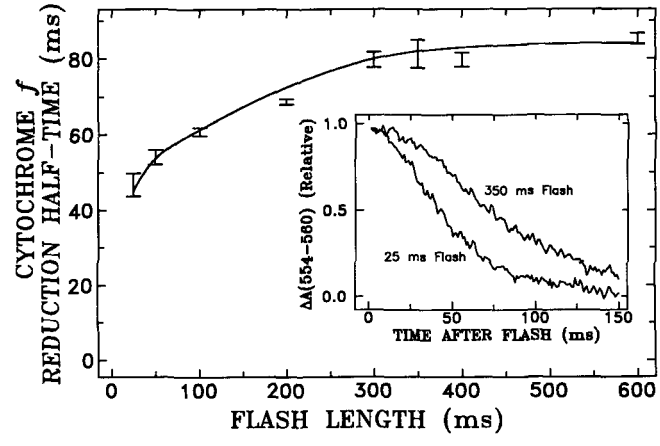

Figure 5. Effect of flash length on Cyt *f* reduction kinetics in intact spinach chloroplasts. The length of the excitation flash was varied from 25 to 600 ms. Kinetics were determined at $\Delta A_{554-560}$. Each bar represents the mean ± SD. The inset shows reduction kinetics after a 25- and 350-ms flash. Data were collected as described for Figure 1.

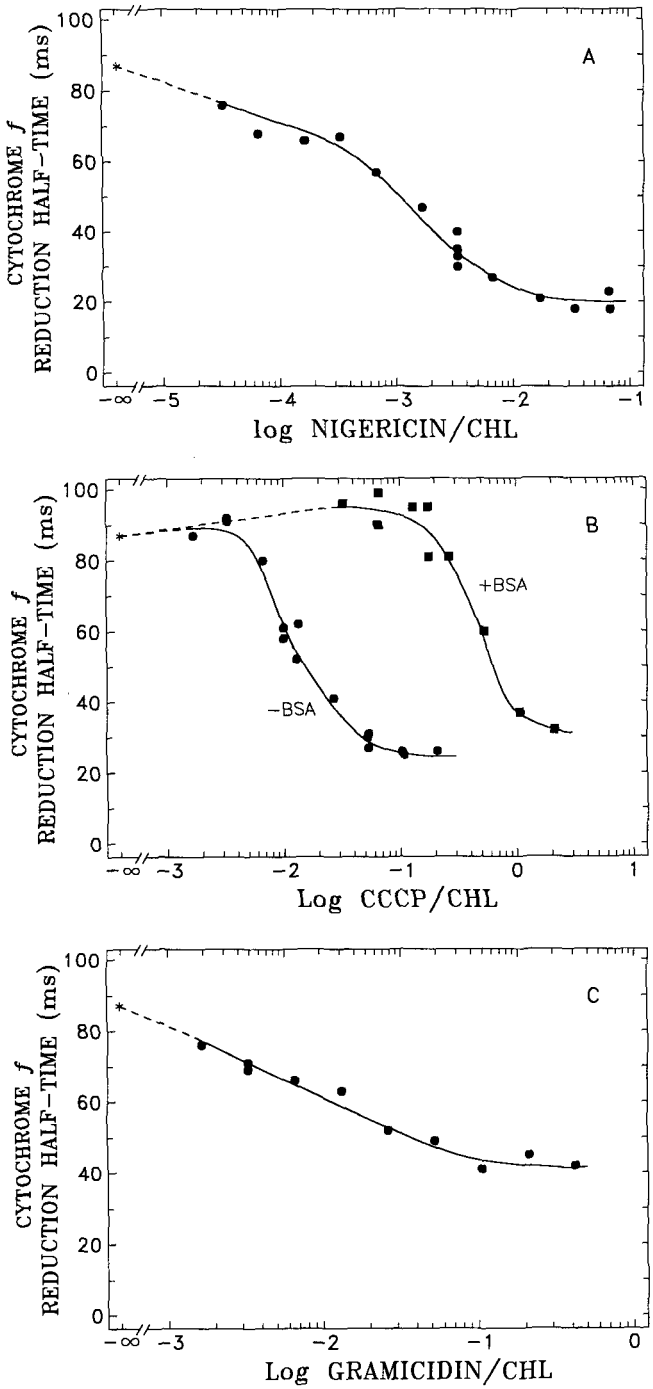

Figure 6. Effect of nigericin, CCCP, and gramicidin D on the reduction half-time of Cyt *f* in intact spinach chloroplasts. Titrations of ionophores illustrate the relationship between ionophore:Chl ratio and Cyt *f* reduction half-time. Each measurement is the average of 32 flashes. Data were collected as described for Figure 1. A, Nigericin; B, CCCP, with 0.1% (1 mg/mL) BSA (●) or without BSA (■); C, gramicidin. The control value (*) was 86 ± 5 (n = 10).

Table I. *Effective ionophore:Chl ratios for collapsing ΔpH in intact spinach chloroplasts*

ΔpH was monitored by the rate of Cyt *f* reduction as described in the text and shown in Figures 6 and 7.

Ionophore	Ionophore:Chl Ratio	Example of [Ionophore] for [Chl] = 30 μm
Nigericin	0.03	1
CCCP	0.07	2
Gramicidin D		>13

chloroplasts (Nishio and Whitmarsh, 1991). Although gramicidin is a potent uncoupler of ATP synthesis in thylakoid membranes, it was ineffective in completely collapsing ΔpH in intact chloroplasts (Fig. 6). At a gramicidin:Chl ratio of 0.3, the half-time of reduction was still 40 ms. In our experiments, a ratio of 0.3 was equivalent to >13 μM gramicidin in a 30-μM Chl solution. Such a high concentration of gramicidin is rarely utilized in bioenergetic experiments. Even if gramicidin entered the chloroplast, it might not completely dissipate the ΔpH, because in thylakoids a gramicidin:Chl ratio of 0.09 ([10 μM gramicidin]/[110 μM Chl]) did not effectively dissipate the pH gradient (Portis and McCarty, 1976).

Nigericin was the most potent ionophore tested for decreasing the reduction kinetics of Cyt *f* in intact chloroplasts, and it did not interact with BSA. Recommended ionophore to Chl ratios are presented in Table I.

The effect of several ionophore:Chl ratios on the dark reduction of Cyt *f* are illustrated in Figures 7 and 8. The control kinetics of the dark reduction of Cyt *f* are shown in Figure 7A. Either a 2-μM nigericin or 2-μM CCCP addition to the 30-μM Chl-containing chloroplast preparation containing no BSA effectively decreased the dark reduction half-time of Cyt *f* (Figs. 7C and 8C). The combination of valinomycin (0.3

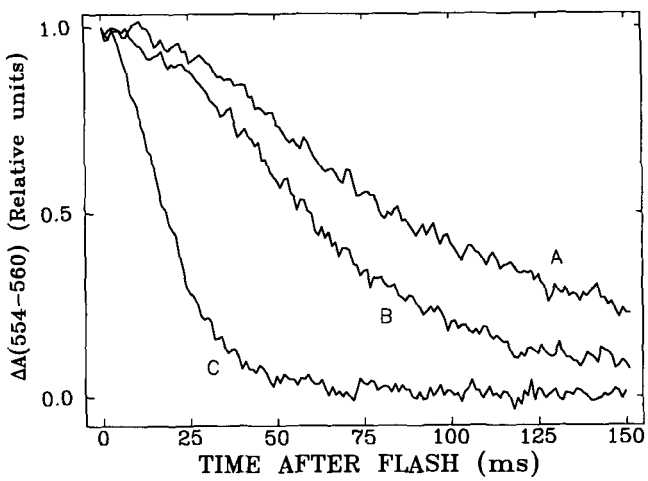

Figure 7. The effect of valinomycin plus CCCP and nigericin on Cyt *f* dark reduction kinetics in intact spinach chloroplasts. Data represent the average of 32 flashes. Data were collected as described for Figure 1. A, Control; B, 0.3 μM valinomycin plus 0.3 μM CCCP; C, 2 μM nigericin.

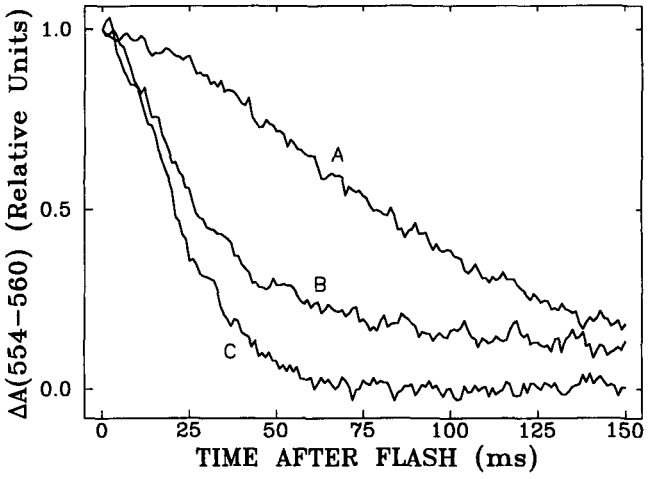

Figure 8. Effect of BSA on the efficacy of CCCP to increase Cyt *f* dark reduction kinetics in intact spinach chloroplasts. Data, collected as described for Figure 1, represent the average of 32 flashes. A, 5.4 μM CCCP plus BSA; B, 64 μM CCCP plus BSA; C, 2 μM CCCP. Defatted BSA, when used, was at a concentration of 0.1% (1 mg/mL).

μM) and CCCP (0.3 μM), which is equivalent to an uncoupler:Chl ratio of 0.1, is shown in Figure 7B.

The ameliorating nature of BSA against the effects of CCCP (Dilley and Schreiber, 1984; Nishio and Whitmarsh, 1991) is illustrated in Figure 8. In the presence of BSA, CCCP at a relatively high CCCP:Chl ratio equivalent to 0.18 had little effect on Cyt *f* reduction (Fig. 8A). Figure 8B shows the kinetics of Cyt *f* reduction in intact chloroplasts in the presence of BSA and CCCP at a much higher CCCP:Chl ratio of 2.1. However, in the absence of BSA, CCCP effectively collapsed the ΔpH at a much lower CCCP:Chl ratio (0.07) (Fig. 8C).

DISCUSSION

We determined a simple method to measure Cyt *f* light-induced redox changes in intact, coupled chloroplasts based on time-resolved optical spectroscopy (Figs. 1 and 2). The subsequent Cyt *f* dark reduction kinetics reflect the pH of the lumen. The data show that Cyt *f* reduction kinetics may be used as an indicator of ΔpH across thylakoid membranes. The method can detect lumenal changes in pH between 5.5 and 6.5 (Fig. 4). A ΔpH of 1 is equivalent to a lumenal pH somewhere between 6.5 and 7.0.

The utility of Cyt *f* reduction kinetics as a probe for ΔpH across thylakoid membranes was investigated by two approaches. In one case, the pH was varied to establish the effect of pH on the reduction of Cyt *f*. The experiment tests pH effects, not ΔpH effects, on Cyt reduction. At pH 6.5, the rate of reduction is about 20 ms, which can account for maximal electron transport flow rates in spinach (Baker et al., 1978; Lee and Whitmarsh, 1989). It is interesting that beef heart Cyt oxidase, which is involved in the transfer of electrons between Cyt *a* and Cyt a_3, was inhibited by the electrochemical proton gradient. The inhibition was caused primarily by the pH on the matrix side of the enzyme rather than ΔpH (Moroney et al., 1984). Our results support the notion that luminal pH rather than ΔpH controls the turnover of Cyt *f* (Rumberg and Siggel, 1969; Rottenberg et al., 1972; Giersch et al., 1980).

In the second case, the length of the flash was varied to determine whether the development of the pH gradient across the thylakoid membrane would affect the reduction kinetics of Cyt *f*. Under nonphosphorylating conditions, the transport of H^+ across the thylakoid during electron transport causes a build up of H^+ in the lumen and a concomitant decrease in pH. As discussed earlier, a low lumenal pH slows the rate of Cyt reduction. Longer flashes create larger pH gradients, slowing the reduction kinetics of Cyt *f*, presumably because more energy is required to deposit protons into the lower pH lumen. As shown in Figure 5, the length of the flash increased the reduction rate of Cyt *f*. The kinetics of the change in reduction rate are similar to the development of the pH gradient in single turnover experiments (Theg and Junge, 1983). Additionally, steady-state ATP synthesis occurs well before 350 ms (Graan and Ort, 1981). Rumberg and Siggel (1969) measured the reduction kinetics of P700 at various pH values (in the presence of gramicidin) to estimate the lumenal pH. They then varied the length of illumination and used the reduction kinetics of P700 to determine the internal pH. Their data showed that the internal pH was minimal after about 500 ms, and they concluded that the ΔpH across the thylakoid membrane was about 3 pH units. However, because gramicidin does not completely dissipate ΔpH, the data should be interpreted cautiously (Portis and McCarty, 1976). The results of Rumberg and Siggel (1969) indicate that measurement of P700 at ΔA_{830} with a pulse amplitude-modulated spectrometer (Harbinson and Woodward, 1987; Schreiber et al., 1988) may provide a simple method for monitoring ΔpH.

The determination of lumenal pH using Cyt *f* reduction compares favorably with other methods. 9-Aminoacridine can detect ΔpH values greater than about 1.5 units (Schuldiner et al., 1972; Pick and McCarty, 1980). The methylamine distribution method (Rottenberg and Grunwald, 1972) is capable of detecting very low ΔpH values and to an accuracy of ±0.05 pH units (Pick and McCarty, 1980). Ammonium uptake measured by electrodes is also possible but is limited by sensitivity (Rottenberg et al., 1972; Pick and McCarty, 1980). Both the methylamine and ammonium distribution methods rely on measurements to determine the volume of thylakoid lumen. The volume determinations are made under conditions different from the pH measurements and require the use of radionuclides.

One interesting difference between the methylamine distribution method for determining ΔpH and the Cyt *f* method is the apparent effect of ionophores on ΔpH. The methylamine method indicates that 1 μM nigericin collapses the ΔpH in samples containing about 446 μM Chl. In our experiments, a similar ratio would only partially collapse the pH gradient. (Based on the half-time reduction rate of Cyt *f*, we estimate that the lumenal pH is about 5.9 at this nigericin:Chl ratio.) Our data suggest that a nigericin:Chl ratio of 0.03 is required to dissipate the ΔpH across the thylakoids (for a Chl concentration of 446 μM, 13 μM nigericin is required). Possibly, the

thylakoids used for the methylamine studies were slightly uncoupled to begin with, because the ΔpH of the controls was about 1.7 (Rottenberg et al., 1972). It may be that Cyt *f* reduction kinetics are more sensitive to ΔpH than the other methods discussed. Alternatively, factors other than lumenal pH may affect the rate of electron transport and consequently the rate of Cyt *f* reduction.

The relation between reduction half-time of Cyt *f* and the nigericin:Chl ratio shown in Figure 6A appears to match the data from a study by Giersch (1983), in which ΔpH, monitored by the fluorescence quenching of 9-aminoacridine, was plotted as a function of the nigericin concentration in thylakoids.

In a previous report, it was shown that the use of ionophores to collapse the electrical component of the electrochemical proton gradient in intact and lysed chloroplasts depended on the ionophore:membrane ratio, the consequence of which was discussed with regard to protein import into chloroplasts (Nishio and Whitmarsh, 1991). The results on the dissipation of the proton chemical component presented here further emphasize that caution in the use of ionophores is required to ensure that the desired effect is obtained (Reed, 1979).

It is generally accepted that a proton electrochemical gradient is not required for protein translocation into chloroplasts, i.e. across the outer and inner envelopes (Grossman et al., 1980; Flugg and Hinz, 1986; Theg et al., 1989). This conclusion is based on protein import studies in the presence or absence of ionophores that are capable of dissipating the proton electrochemical gradient. Although this conclusion is likely correct, we previously pointed out that some of the protein transport studies supporting this conclusion may have been done under conditions that did not completely dissipate the electrical potential across the chloroplast membranes (including the inner envelope) (Nishio and Whitmarsh, 1991).

In the present paper, Figures 7 and 8 illustrate conditions comparable to those used in two such experiments (Flugg and Hinz, 1986; Pain and Blobel, 1987). As discussed previously (Nishio and Whitmarsh, 1991), dissipation of the electrochemical potential across thylakoids by ionophores that rapidly equilibrate across membranes will likely dissipate the potential across the chloroplast envelope as well. We can think of no reason to believe that greater amounts of ionophores would be required. Alternatively, lower concentrations of ionophores may dissipate the electrochemical potential across chloroplast envelopes more than across thylakoid membranes. The following discussion is based on a reasonably conservative assumption that when the proton chemical potential across thylakoids is dissipated by ionophores, it will also be dissipated across the chloroplast envelope. Our results suggest that the two studies cited above were done under conditions that did not completely collapse the proton chemical gradient.

Theg et al. (1989) reinvestigated the import of proteins into chloroplasts using nigericin to collapse the proton chemical gradient. They showed that ATP synthesis is inhibited at a nigericin:Chl ratio of 0.15. Figure 3 shows that such a ratio is adequate to dissipate ΔpH. However, in the presence of nigericin, an electrical potential may persist (Nishio and Whitmarsh, 1991). In another study (Cline et al., 1989), a ratio of 2.7×10^{-4} nigericin:Chl and 5.4×10^{-4} valinomycin:Chl decreased light-driven import of the light-harvesting complex into chloroplasts to 10% of the control. In the presence of ATP, the same amount of nigericin and valinomycin did not affect protein import, and at the highest level of ionophores utilized, 5.4×10^{-3} (nigericin:Chl) and 1.1×10^{-2} (valinomycin:Chl), ATP-driven import remained unaffected. The highest nigericin:Chl ratio (5.4×10^{-3}) utilized seems adequate to collapse the ΔpH (we are recommending a ratio of 3×10^{-2}) and from our previous report (Nishio and Whitmarsh, 1991), a ratio of 3×10^{-2} (valinomycin:Chl) is recommended. Recently, Cline et al. (1992) examined the import of four polypeptides (the light-harvesting Chl protein and the 17-, 23-, and 33-kD polypeptides associated with the oxygen-evolving complex of PSII) destined for the thylakoid membrane. In one experiment they used 1 μM valinomycin and 1 μM nigericin to dissipate the electrochemical potential. The reaction assay (60 μL) contained 20 μg of Chl. The final ratio of ionophore:Chl was 2.7×10^{-3}, and in our studies such a ratio of nigericin:Chl would have resulted in a Cyt *f* reduction half-time of about 35 ms, compared to about 20 ms when completely uncoupled and about 85 ms for controls. Thus, for the light-harvesting complex, it seems that import across the chloroplast envelope does not require an energized membrane. However, we believe that the bioenergetic requirements for the transport of other proteins into the chloroplast should be examined in view of our results to verify that uncouplers were used at adequate concentrations.

The work described here demonstrates the following key points. First, the ionophore:Chl ratio, more than the absolute concentration of the ionophore, determines the ionophore concentration required to collapse the pH gradient in chloroplasts. Recommended ionophore concentrations are given in Table I. The use of gramicidin for intact chloroplasts is not recommended because the envelope creates a barrier to gramicidin (Avron and Shavit, 1965; Schmid and Junge, 1975; Nishio and Whitmarsh, 1991). We suggest the use of nigericin for most studies utilizing chloroplasts and recommend the use of gramicidin with thylakoid preparations only. Finally, monitoring Cyt *f* reduction kinetics provides a simple technique to examine ΔpH in isolated chloroplasts and is likely to be equally successful in intact leaves.

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