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LIGHT DEPENDENCE OF THE DECAY OF THE PROTON GRADIENT IN BROKEN CHLOROPLASTS

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Summary

The initial rates and steady-state values of proton uptake by broken chloroplasts have been measured as functions of light intensity at various concentrations of chlorophyll, pyocyanine, supporting electrolyte, buffer, as well as pH and temperature. Kinetic analysis of the data shows that the rate of decay of proton gradient due to backward leakage depends on light intensity. Under steady illumination, the decay constant k_L is equal to $k_D + mR_0$, where R_0 is the initial rate of proton uptake which is a function of light intensity, k_D is the decay constant in the dark and m is a parameter which is independent of light intensity. Treatment of chloroplasts with lysolecithin, neutral detergent, 2,4-dinitrophenol, or valinomycin in the presence of K^+ increases k_D without affecting m. Treatment with *N,N'*-dicyclohexylcarbodiimide or adenylyl imidodiphosphate under appropriate conditions decreases m without affecting k_D. Treatment with glutaraldehyde makes k_L independent of light intensity and hence $m = 0$. These results suggest that the light-dependent part (mR_0) of k_L is due to leakage of protons through the coupling factor (CF_1-CF_0) complex which can open or close depending on light intensity and that the light-independent part (k_D) of the decay constant k_L is due to proton leakage elsewhere.

Introduction

Reversible light-driven proton translocation in isolated chloroplasts has been well documented [1—3]. Its relationship to the mechanism of energy coupling

Abbreviations: Tricine, *N*-tris(hydroxymethyl)methylglycine; DCCD, *N,N'*-dicyclohexylcarbodiimide; Triton X-114, octylphenoxypolyethoxyethanol; AMPPNP, adenylyl imidodiphosphate; CF_1-CF_0, the complex of chloroplast coupling factors.

has been reviewed with different emphasis [4—14]. Definitive measurements on the stoichiometric ratio of proton translocation and electron transport [15—18] as well as ratio of proton to ATP synthesis [15,19—24] have been made recently. The rate of proton uptake and release have been studied using continuous flow methods [25] and by pre-steady-state kinetic analysis [26] to eliminate the error due to slow response of the pH electrode used to monitor the external pH change. In the previous analysis, the rate of proton leakage under illumination was assumed to be the same as that in the dark. Such an assumption is questionable [6] since light-induced conformational change in chloroplasts has been well demonstrated [27—31]. The proton efflux under illumination can be different from that in the dark. In view of this, the kinetic analysis of proton translocation is reexamined.

Treatments with various chemicals alter the extent and kinetics of proton translocation in chloroplasts. *N,N'*-Dicyclohexylcarbodiimide (DCCD), a phosphorylation inhibitor, interacts irreversibly with coupling factor CF_0, increases proton uptake at high pH [32]. Treatment with EDTA at low ionic strength causes the removal of coupling factor CF_1 and uncouples the system [33]. The proton uptake can be subsequently restored either by the addition of DCCD [32] or by reconstitution with isolated CF_1 [34]. In the presence of ATP, an increase in proton uptake has been reported [21,35]. Neutral detergents increase the permeability of proton and other ions through thylakoid membrane and uncouple the system [36]. Both 2,4-dinitrophenol and valinomycin + K^+ were reported to increase the rate of proton translocation without affecting either the extent of proton uptake or the rate of single-stage photophosphorylation [37,38]. Treatment with the cross-linking reagent glutaraldehyde has been shown to inhibit phosphorylation and light-induced conformation change, but has less effect on proton uptake [39]. A quantitative kinetic analysis of proton translocation by chloroplasts under different conditions is of considerable interest because it may reveal some information concerning the translocation mechanism.

In this work, the rate and extent of proton uptake and release by broken chloroplasts treated by different procedure and under different conditions of illumination are examined. A quantitative method is introduced for treating the kinetic data. The results suggest that under normal conditions the rate constant for leakage of protons through the CF_1-CF_0 complex increases with light intensity.

Materials and Methods

Materials. Chloroplasts were prepared from fresh spinach leaves as described by Avron [40]. The chloroplast pellet obtained after the last centrifugation was suspended in the homogenizing medium (sucrose, 0.25 M; *N*-Tris(hydroxymethyl)methylglycine) (Tricine), 20 mM at pH 7.9; NaCl, 10 mM) at 2°C to give a concentration of 3—5 mg chlorophyll/ml. Broken chloroplasts (thylakoids) were obtained by 10—100-fold dilution of the original chloroplast preparation with a sucrose-free buffer of known concentration containing NaCl and pyocyanine at 0°C. The pH of the final suspension was readjusted to the desired value and incubated at 0°C for 40—60 min before use in various experi-

ments. Subchloroplast particles were prepared as described by McCarty [41].

ADP (sodium salt, grade III), ATP (disodium salt), glutaraldehyde (Grade I, 25% aqueous solution) octylphenoxypolyethoxyethanol (Triton X-114), 1-α-lysophosphatidylcholine (egg yoke, type 1, phospholipase A treated), adenylyl imidodiphosphate (AMP-PNP), and valinomycin were obtained from Sigma Chemical Company. Purity of glutaraldehyde was checked spectrophotometrically before use [42]. 2,4-Dinitrophenol and DCCD were from Aldrich Chemical Company. Carrier-free radioactive orthophosphate ($^{32}P_i$) was from New England Nuclear Corporation and was found by paper chromatography to contain no detectable amount of radioactive impurity. Pyocyanine was from Schwarz Mann. All other reagents used were of the highest quality available and were used without further purification.

Illumination. A 500 W projector lamp with a red filter (Edmund Scientific Co., No. 823) was used to illuminate the chloroplast sample in a 2 ml thermostated glass cell. The intensity of actinic light was varied by using additional neutral gray filters and the light intensity was measured with a YSI-Kettering Model 65A radiometer. The duration of illumination was controlled manually by means of a shutter.

Two-stage photophosphorylation. The procedure used was essentially the same as that reported previously [43] except that fresh chloroplasts were used in this work and that the concentration of sucrose in the light-stage mixture was 25 mM. Phosphorylation yield was assayed by ascending chromatography on polyethyleneimine paper [44].

Proton uptake measurements. Proton uptake by broken chloroplasts were measured by means of a combination pH electrode (Beckman 39030) fitted to a thermostated and constantly stirred glass cell which was completely filled with the sample. The output from the electrode was monitored by a Beckman pHaser-I meter fitted with a variable potential offest attachment and recorder. The reproducibility in ΔpH was ±0.0002. For conversion of observed ΔpH to nmol H^+ taken up, a calibration curve was obtained for each set of measurements by titrating the sample at steady state under illumination with 5 mM HCl.

Proton release measurements. The rate of outward leakage of protons after the light was turned off was measured in the usual way. Direct measurement of the rate of outward leakage of protons under diminished light intensity was accomplished by rapidly inserting additional gray filters in the light path after the first steady state has been reached and monitor the change in pH as it decreases continually to a second steady-state value.

Results and Discussion

Kinetics of proton uptake and release

Under the above experimental conditions, the following empirical relationship was always observed for the uptake of protons by illuminated broken chloroplasts:

$$\ln(1 - \delta/\delta_{ss}) = -k_L t \qquad (1)$$

where δ and δ_{ss} denote nmol of H^+ taken up from the medium/mg of chloro-

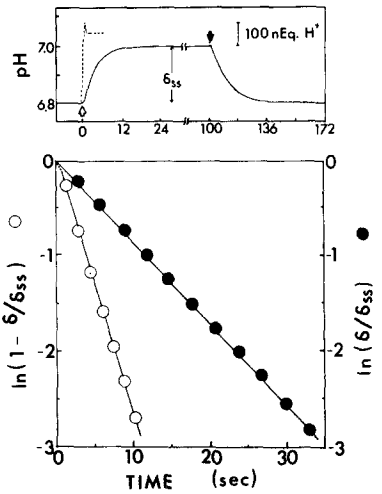


Fig. 1. Proton uptake and release by isolated broken chloroplasts at 18°C. Composition of the sample: chlorophyll, 0.100 mg/ml; [Tricine] = 1 mM, initial pH 6.65; [pyocyanine] = 50 μM; [NaCl] = 50 mM. Top figure: red light (250 J · m^{-2} · s^{-1}) was turned on at t = 0 s (⇧) and turned off at t = 100 s (⇩). · · · · · ·, obtained by injecting an aliquot of 5 mM NaOH solution at t = 0 to estimate the response time of the experimental system. The vertical bar indicates the calibration of ΔpH scale by titrating the sample at steady state with 5 mM HCl. Bottom figure: ○, values of $\ln(1 - \delta/\delta_{ss})$ during the illumination period; ●, values of $\ln(\delta/\delta_{ss})$ taken during the dark decay period which started at t = 100 s in the top figure but adjusted also to t = 0 s in the bottom figure.

phyll at time t and at the steady state, respectively, and k_L is independent of t and δ.

Similarly the subsequent release of protons after the light is turned off was found to obey the first-order decay equation

$$\ln(\delta/\delta_{ss}) = -k_D t \tag{2}$$

The results from a typical experiment are shown in Fig. 1 which also includes an order of magnitude estimate of the instrumental response time.

Differentiation of Eqn. 1 with respect to time gives the rate equation

$$\frac{d\delta}{dt} = k_L(\delta_{ss} - \delta) \tag{3}$$

which at t = 0 reduces to

$$R_0 = k_L \delta_{ss} \tag{4}$$

where R_0 represents the initial rate of proton uptake when the light was turned on. Eqn. 3 may now be written as

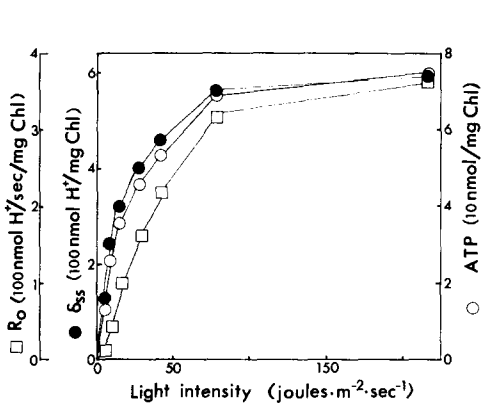
$$\frac{d\delta}{dt} = R_0 - k_L \delta \tag{5}$$

It is often difficult to determine R_0 directly from the limiting slope of the pH or δ vs. t curve shown in the upper diagram of Fig. 1 because of possible instrumental delay. But since δ and δ_{ss} are easily obtainable and k_L can be determined quite precisely from the slope of the linear plot of $\ln(1 - \delta/\delta_{ss})$ vs. t, R_0 can be obtained readily.

It should be mentioned that when the concentration of supporting electrolyte is below 20 mM, the plot of $\ln(1 - \delta/\delta_{ss})$ vs. t is no longer linear, but consists of a more rapid initial phase followed by a slower non-linear phase. This deviation from linearity is probably due to the change of membrane potential during the experiment which tends to retard proton transport. In order to minimize the change in diffusion potential during the course of an experiment, measurements were performed in media containing 50 mM NaCl. The observed linearity of the growth and decay curves in Fig. 1 demonstrates that under our experimental conditions k_L and k_D are indeed first-order constants. Therefore, the membrane potential must have very rapidly reached a steady value after the start of each experiment and remained constant under steady illumination throughout the experimental period.

Effect of light intensity, medium composition and temperature

For a chloroplast sample of given composition and temperature, R_0 and δ_{ss} may be varied by changing the intensity of actinic light. Consequently the corresponding values of $k_L = R_0/\delta_{ss}$ can be readily obtained from the experimental data. The variation of the observed δ_{ss} and R_0 with the intensity of red light is shown in Fig. 2. The corresponding yields of ATP in two-stage phosphorylation experiments are also included in Fig. 2 for comparison. The corresponding values of R_0 and k_L determined through monitoring proton uptake by



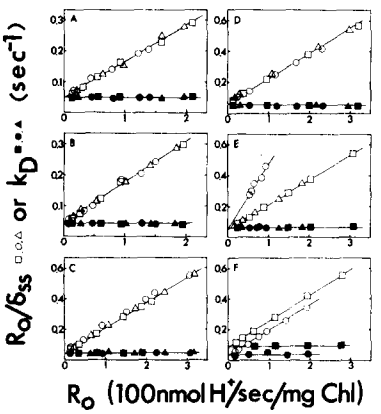


Fig. 2. Dependence of the steady-state value δ_{ss} (●) of proton uptake, the initial rate R_0 (□) proton uptake and two-stage phosphorylation yield of ATP (○) on light intensity. Composition of the sample: chlorophyll, 0.15 mg/ml; [pyocyanine] = 50 μM; [NaCl] = 50 mM; [Tricine] = 1.4 mM at initial pH 6.60 and 18°C. Light intensity was controlled by additional neutral gray filters.

Fig. 3. Proton translocation in chloroplast samples of different composition and temperature. ○, □, △, measured values of $k_L = R_0/\delta_{ss}$; ●, ■, ▲, measured values of k_D. Common feature of all the samples unless stated otherwise for the separate diagrams: chlorophyll concentration = 50—75 μg/ml; [pyocyanine] = 50 μM; [NaCl] = 50 mM; [Tricine] = 1.4 mM at initial pH of 6.3—6.7; t = 20°C. In each of the following sets of experiments, only one variable was changed as indicated and all other variables were held constant for the whole set. (A) Proton translocation at different chlorophyll concentrations: □, ■, 18.9 μg/ml; △, ▲, 37.8 μg/ml; ○, ●, 75.6 μg/ml. (B) Proton translocation at different pyocyanine concentrations: ○, ●, 10 μM; △, ▲, 50 μM; □, ■, 100 μM. (C) Proton translocation at different NaCl concentration: ○, ●, 20 mM; △, ▲, 50 mM; □, ■, 100 mM. (D) Proton translocation at different Tricine concentration: ○, ●, 1.4 mM; △, ▲, 5.4 mM; □, ■, 10.4 mM. (E) Proton translocation at different pH (initial): □, ■, 6.4; △, ▲, 7.2; ○, ●, 8.0. (F) Proton translocation at different temperatures: □, ■, 24°C; ○, ●, 8°C.

broken chloroplasts illuminated with red light of different levels of intensity and at different concentrations of chlorophyll, pyocyanine, NaCl, buffer, temperature and initial pH are summarized in Fig. 3. The corresponding values of the dark decay constant k_D determined through monitoring the proton release in these samples after the light was turned off are included for comparison.

These data obtained over wide ranges of experimental variables lead to the following generalizations:

(A) k_L increases with light intensity and is related to R_0 and k_D by

$$k_L = k_D + mR_0 \tag{6}$$

where m is independent of light intensity, concentrations of chlorophyll, pyocyanine, NaCl and Tricine, but may change with pH.

(B) k_D is independent of R_0 and δ_{ss}, i.e. independent of the intensity of prior illumination. Consequently, k_D is independent of the energy stored.

Similar results were obtained by using subchloroplast particles in place of chloroplasts.

Instead of determining k_L indirectly from the growth curve of proton uptake according to Eqn. 5, k_L can also be measured directly by lowering the light intensity after the steady state has been reached and monitoring the subsequent proton release until a new steady state pH is reached. These experiments are illustrated by inserts B and C of Fig. 4. If δ_{ss} and δ'_{ss} denote the nmol of H^+ taken up at the first and second steady states, respectively, in these measurements, the net leakage rate under diminished illumination is given by $d\delta/dt = -k_L(\delta - \delta'_{ss})$ which upon integration gives $\ln[(\delta - \delta'_{ss})/(\delta_{ss} - \delta'_{ss})] = -k_L t$. Consequently, the leakage constant k_L under the diminished illumination can be determined from the slope of the linear plot of the lefthand side of this integrated equation versus time. Insert A of Fig. 4 shows a series of conventional ΔpH measurements for both determining k_L indirectly from Eqn. 5 and demonstrating that k_D is independent of the amount of energy stored.

The results of a set of such measurements are summarized in Fig. 4. As expected, the direct and indirect methods gave within experimental error the same k_L value.

Increase of k_L with light intensity

The observed increase of k_L with light intensity or R_0 could be explained for the following two cases which are not mutually exclusive.

(I) When light intensity is raised, the rate of proton leakage increases. For this case the term $k_L\delta$ in Eqn. 5 can be interpreted as representing the rate of concurrent backward leakage of protons across the thylakoid membrane under constant illumination. According to Eqn. 6, this leakage of protons takes place via two independent paths, a light-independent path with decay constant k_D which is independent of R_0 and δ_{ss}, and a light-dependent path with decay constant $k_L - k_D$ which is proportional to R_0. The light-dependent path may represent proton leakage through specific channels with conformation-regulated permeability, whereas the light-independent path represents general leakage of protons elsewhere across the thylakoid membrane.

(II) When light intensity is raised, the rate of proton uptake decreases due to the building up of increased membrane potential. For this case Eqns. 5 and 6

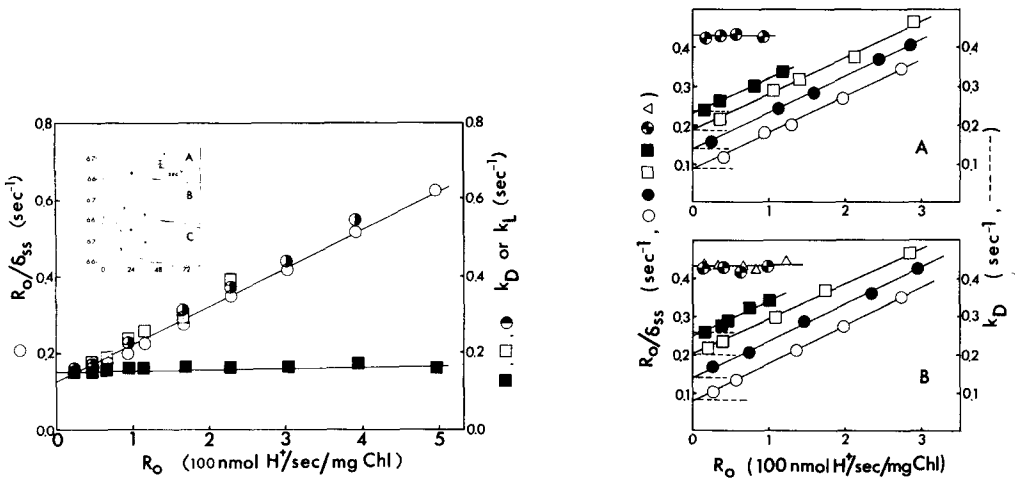


Fig. 4. Correlation between k_L, k_D and R_0. ○, values of $k_L = R_0/\delta_{ss}$ determined by the method of Fig. 1; □, values of k_L determined directly by the method B; ◉, values of k_L determined directly by the method C; ■, values of k_D. Chlorophyll concentration = 58 μg/ml; [pyocyanine] = 50 μM; [NaCl] = 50 mM; [Tricine] = 1.4 mM; t = 18°C. The different methods A—C for measuring k_L and k_D are illustrated in the insert. (a) A chloroplast sample was illuminated by red light of different intensities from t = 0 (⇧) to t = 24 s (⇩). The kinetics of proton uptake and release was analyzed as in Fig. 1. (B) A chloroplast sample was illuminated by red light of different intensities. After each steady state had been reached, two gray filters were inserted to reduce the light intensity (⇩). The pH was then allowed to decay to a new steady-state value before the light was turned off (⇩). (C) A chloroplast sample was illuminated with red light until steady state had been reached. Then three, five or eight gray filters were inserted to reduce the light intensity (⇩), the pH was allowed to decay to the new steady state values and then the light was turned off (⇩).

Fig. 5. Effect of valinomycin and dinitrophenol on the kinetics of proton translocation. Chloroplasts for these experiments were washed with STK buffer (sucrose, 0.25 M; Tricine, 20 mM, pH 7.9; KCl, 0.10 M) and resuspended in the same buffer to a concentration of 4 mg chlorphyll/ml. Broken chloroplasts were then prepared by a 50-fold dilution of the sample with the reaction medium (Tricine, 1 mM, pH 6.6; KCl, 50 mM; pyocyanine, 50 μM). The diluted sample was incubated for 40 min at 0°C before kinetic measurements were made. (A) Effect of valinomycin: ○, 0 μM (control); ●, 2 μM; □, 6 μM; ■, 10 μM; ◉, 25 μM. (B) Effect of 2,4-dinitrophenol: ○, 0 mM (control); ●, 0.2 mM; □, 0.4 mM; ■, 1 mM; ◉, 2 mM; △, 2 μM valinomycin + 0.1 mM dinitrophenol.

may be combined and written as

$$\frac{d\delta}{dt} = (1 - m\delta)\, R_0 - k_D\delta \tag{7}$$

Accordingly, the term $m\delta R_0$ can be interpreted as representing the retardation of proton uptake due to increased membrane potential at high light intensity.

One way to decide between these two possibilities is to conduct experiments in the presence of potassium and valinomycin. According to case II, for a given amount δ of protons taken up by the thylakoids, the presence of valinomycin + K^+ should lower the membrane potential because of facilitated counter-transport of K^+ and hence should decrease the value of mR_0 which represents the retardation of proton uptake. According to case I, valinomycin at low concentration is expected to increase the rate constant k_D for the general leakage of protons across the membrane, but not expected to have much effect on the rate constant $k_L - k_D = mR_0$ for proton leakage through specific channels.

Effect of valinomycin and dinitrophenol

Under constant illumination by red light at 252 joules/m^2 · s, it was observed that the initial rate of proton uptake by thylakoids in the presence of K^+ remained essentially constant at $R_0 = 281 \pm 6$ nmol/s per mg chlorophyll when the concentration of valinomycin was increased from 0 to 6 μM. The data in Fig. 5A show that although k_D was increased as expected, valinomycin up to 6 μM did not change the value of m. These results are consistent with the above discussion for case I, but seem to contradict the interpretation based on case II.

Fig. 5B shows that 2,4-dinitrophenol up to 0.4 mM increased k_D without affecting the value of m. These results can also be explained in terms of the increased rate of general leakage of protons across the membrane. The observation that m is not increased either by valinomycin + K^+ or by 2,4-dinitrophenol at low concentrations suggest that proton leakage by the light-dependent path probably involves the co-transport of negative charge through these specific channels along with the protons, a possibility that may be relevant to the phosphorylation mechanism.

At higher concentrations of valinomycin (25 μM) or 2,4-dinitrophenol (2 mM), the thylakoid membrane became so leaky that proton leakage through the light-dependent channels was no longer detectable.

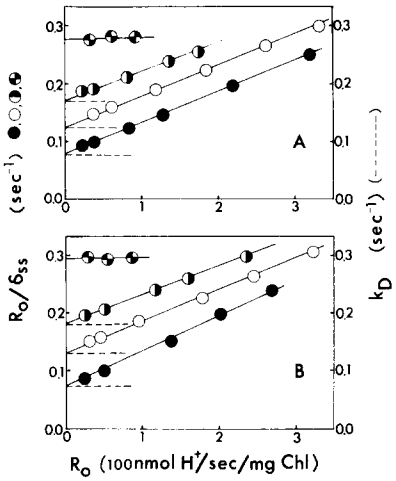
Effect of lysolecithin and Triton X

Neutral detergents such as Triton X [36] and lysolecithin are known to cause non-specific leakage of ions and small molecules across energy-transducing membranes. The effect of Triton X-114 and lysolecithin on the kinetics of proton translocation in chloroplasts are illustrated in Fig. 6A and B. The data indicate that up to a Triton X-114 concentration of 0.0075% (w/w) and lysolecithin concentration of 1 mg/mg chlorophyll, the rate constant $k_L - k_D = mR_0$ for light-dependent proton leakage remains approximately the same, whereas k_D definitively increases with detergent concentration as expected. Since Triton X-114 and lysolecithin are believed to be nonspecifically incorporated in biological membranes, these observations also support the interpretation that the light-independent part involves the general nonspecific leakage of protons across the thylakoid membrane. The absence of any significant change in the value of m suggest that at low concentrations Triton X-114 and lysolecithin are not incorporated into the specific, light-dependent proton channels.

At high concentrations of Triton X-114 and lysolecithin respectively, the broken chloroplasts became very leaky and the light-dependent leakage of protons was no longer detectable.

Effect of DCCD

Previous studies show that removal of the coupling factor CF_1 by washing chloroplasts with low ionic strength buffer containing EDTA deminishes or abolishes the formation of proton gradient under illumination and that gradient formation can be partially restored by DCCD [32,34,48,49]. At low concentrations, DCCD is known to bind specifically and covalently to a single proteolipid of CF_0 and the isolated proteolipid indeed showed expected characteristics of a proton channel [45]. These observations suggest that the CF_1-CF_0 complex may indeed serve as the specific proton channel [46].



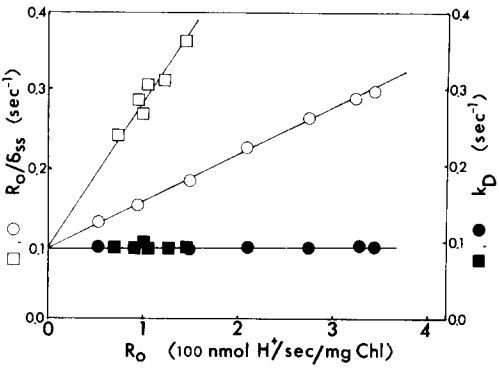


Fig. 6. Effect of lysolecithin and Triton X-114 on the kinetics of proton translocation. Composition of chloroplast sample: chlorophyll concentration = 65 μg/ml; [pyocyanine] = 50 μM; [NaCl] = 50 mM; [Tricine] = 1.0 mM at initial pH of 6.8; t = 18°C. (A) ●, ○, ○ and ○, k_L values for lysolecithin concentration = 0, 0.5, 1.0 and 1.5 mg/mg Chl, respectively. - - - - - -, the corresponding k_D values for each set of experiments. (B) ●, ○, ○ and ○, k_L values for Triton X-114 concentration = 0, 0.005, 0.0075 and 0.010% (w/w), respectively. - - - - - -, the corresponding k_D values for each set of experiments.

Fig. 7. Effect of DCCD on the kinetics of proton translocation by broken chloroplasts at pH 8.0. DCCD (5 μl, 25 mM in ethanol) was added to 1.7 ml of broken chloroplast suspension and illuminated by red light for 5 min before use. The control chloroplast sample was treated in the same way with 5 μl ethanol. Composition of the samples: chloroplast concentration = 70 μg/ml; [NaCl] = 50 mM; [pyocyanine] = 50 μM; [Tricine] at initial pH 8.0; [DCCD] = 70μM. t = 18°C. □, ■, values of k_L and k_D, respectively, for the control chloroplast sample. ○, ●, values of k_L and k_D, respectively, for the DCCD-treated chloroplast sample.

Fig. 7 shows that treatment of chloroplasts with DCCD at pH 8 decreases m without affecting k_D. Under fixed light intensity, treatment with DCCD was found to double the observed value of δ_{ss}. Since at low concentrations DCCD is known to be bound specifically to CF_0, we may conclude from this observation that the light-sensitive path of proton leakage is through the CF_1-CF_0 complex. Because the value of k_D for general nonspecific proton leakage is unaffected by DCCD, we may also conclude that for a given value of R_0, the observed increase of δ_{ss} by DCCD is due only to the slowing down of proton leakage through the CF_1-CF_0 complex.

Effect of Mg^{2+} and adenylyl imidodiphosphate

Previous studies show that ATP can increase the extent of proton uptake by illuminated chloroplasts [21,26]. However, since the synthesis and hydrolysis of ATP also involve proton uptake or liberation and since both of these reactions are light dependent, it is difficult to interpret the proton translocation data in the presence of ATP with confidence. Accordingly, adenylyl imidodiphosphate (AMPPNP), which is not hydrolyzed but has been shown to bind to the ATP sites of CF_1 [47], has been used in the present work to study the effect of nucleotide binding on the kinetics of proton translocation.

The data in Fig. 8 show that while the addition of Mg^{2+} or AMPPNP sepa-

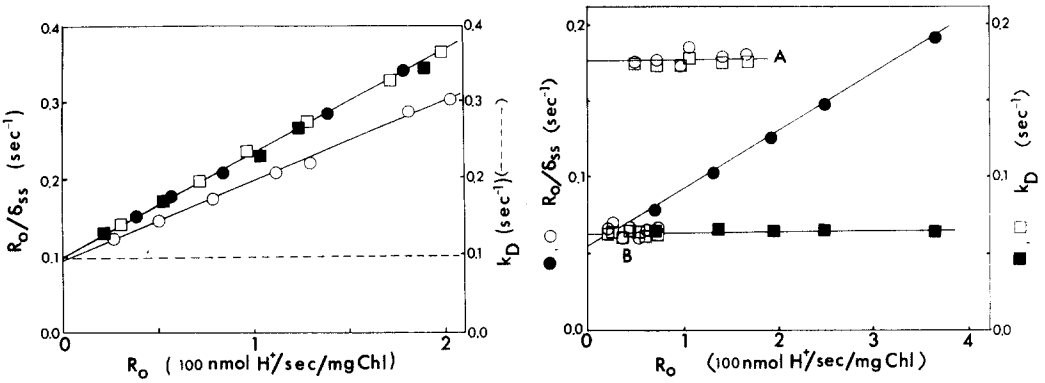


Fig. 8. Effect of adenylyl imidodiphosphate (AMPPNP) on the kinetics of proton translocation by broken chloroplasts at pH 8.0 and 18°C. □, values of k_L for control chloroplasts; ■, values of k_L for control chloroplasts + $MgCl_2$ (5 mM); ●, values of k_L for control chloroplasts + AMPPNP (0.3 mM); ○, values for control chloroplasts + $MgCl_2$ (5 mM) + AMPPNP (0.3 mM). ------, values of k_D for all four sets of experiments.

Fig. 9. Effect of glutaraldehyde on proton translocation by chloroplasts at 18°C. 1 ml of original chloroplast suspension (3—5 mg Chl/ml) was fixed by the addition of 0.1 ml of 0.5 M glutaraldehyde solution for 6 min at 0°C in the dark or under continuous illumination with red light. The reaction was stopped by 30-fold dilution with the homogenizing medium. The fixed chloroplasts were centrifuged down and resuspended in the homogenizing medium. Composition of final suspension: chlorophyll concentration = 0.1 mg/ml; [pyocyanine] = 50 μM; [NaCl] = 50 mM; [Tricine] = 1.4 mM at initial pH 6.80. Experimental points in group A represent the k_L (○) and k_D (□) values for chloroplasts fixed under illumination, whereas those in group B represent the k_L (○) and k_D (□) values for chloroplasts fixed in the dark. ●, ■, k_L and k_D values, respectively, of the control chloroplast sample (not fixed with glutaraldehyde).

rately did not affect the kinetic behavior of broken chloroplasts, the addition of Mg^{2+} and AMPPNP together at pH 8.0 decreased the value of m in Eqn. 6 substantially without changing k_D.

Likewise, δ_{ss} was found to increase 30% under fixed light intensity in the presence of AMPPNP + Mg^{2+} at pH 8.0, although this effect was not observed at pH 6.6. Since no hydrolysis occurred in this experiment, the observed 30% increase in δ_{ss} and decrease in m at pH 8.0 can only be due to the slowing down of light-dependent leakage ($k_L - k_D$). These results also support the above conclusion based on the data in Fig. 7 that the light-dependent proton leakage is through the CF_1-CF_0 complex and that the light-independent decay (k_D) is due to proton leakage elsewhere.

In order to explore whether the light-dependent proton leakage through the CF_1-CF_0 complex is indeed conformation regulated, experiments were conducted using chloroplasts fixed by glutaraldehyde under different conditions.

Effect of glutaraldehyde

Although glutaraldehyde-fixed chloroplasts no longer show photophosphorylation and light-induced conformation change, they still retain the capacity to manifest light-dependent proton translocation [50]. The kinetic data on proton translocation by glutaraldehyde-fixed chloroplasts are compared with those of the control sample in Fig. 9. The first-order rate constant for proton leakage in glutaraldehyde-fixed chloroplasts is no longer sensitivie to light, i.e.

$m = 0$ and hence $k_L = k_D$. However, chloroplasts fixed under continuous illumination have a large k_L or k_D which is equal to k_L of the control chloroplasts under the same illumination intensity used during the fixation with glutaraldehyde. Chloroplasts fixed in the dark have a small k_L or k_D which is equal to the k_D of the control chloroplasts. In other words, the rate constant k_L for proton leakage can be fixed by glutaraldehyde at a value depending on the energy state of the chloroplasts. After fixation, the light-dependent part of k_L disappears.

In view of the information obtained from Figs. 7 and 8, these new results suggest that the light-dependent proton permeability of the CF_1-CF_0 complex may indeed be controlled through protein conformation change and that after fixation by glutaraldehyde this control of permeability disappears. This interpretation is consistent with the observation by Ryrie and Jagendorf [51] on light-induced conformation change in CF_1. Monoaldehydes such as hexanal and valeraldehyde at equivalent concentrations were also used to treat chloroplasts and found to have no effect on the kinetics of proton translocation, although photophosphorylation was inhibited.

Since the glutaraldehyde-fixed chloroplasts are still capable of translocating protons under light, they should be able to build up membrane potential through accumulation or depletion of ionic charges like the control chloroplasts. Therefore, the observed disappearance of the light-dependent part of k_L after glutaraldehyde treatment also contradicts the assumed retardation of proton uptake by increased membrane potential in case II discussed above.

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