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REGULATION OF PROTON LEAKAGE FROM BROKEN CHLOROPLASTS BY CF_o

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SUMMARY

Measurements of proton translocation in CF_1-depleted, N, N'-dicyclohexylcarbodiimide-resealed broken chloroplasts were made under different light intensities. Kinetic analysis of the data shows that the outward leakage of accumulated protons through CF_o is still dependent on light intensity with a first-order rate constant equal to mR_o, where R_o is the initial rate of proton uptake which normally increases with light intensity and m is a characteristic constant which is independent of proton gradient and light intensity. Measurements of proton translocation in these modified chloroplasts cross-linked with glutaraldehyde under illumination and in the dark respectively suggest that the light-dependent proton leakage through CF_o is regulated by conformation change in the membrane. It is proposed that the observed regulation of proton leakage through the $CF_1 \cdot CF_o$ complex in native chloroplasts is for optimizing the steady state synthesis of ATP under different light intensities.

INTRODUCTION

Previous kinetic studies of proton translocation in broken chloroplasts demonstrated that protons accumulated inside the thylakoid by light-driven electron transport can leak out via two paths: a light-dependent path through the coupling factor $CF_o \cdot CF_1$ complex and a light-independent path which represents the general proton leakage elsewhere across the thylakoid membrane (1). Since the leakage of accumulated protons through the $CF_o \cdot CF_1$ complex is believed to drive ATP synthesis (2-4), the molecular basis of its regulation is of direct biochemical interest.

In the present work CF_1 was completely removed from chloroplasts by washing with NaBr solution (5). The resulting thylakoids were re-sealed with dicyclohexylcarbodiimide (DCCD) which at low concentrations binds specifically to CF_o (6,7). Kinetic studies of light-driven proton translocation in these

Abbreviations: DCCD, N,N'-dicyclohexylcarbodiimide; $CF_1 \cdot CF_o$, the DCCD-sensitive ATPase complex of spinach chloroplast; CF_1, soluble ATPase moiety of $CF_1 \cdot CF_o$; CF_o, membranous moiety of $CF_1 \cdot CF_o$.

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NaBr-washed, DCCD-resealed thylakoids showed that the accumulated protons can still leak out via two paths, a light-dependent path and a light-independent path. This observation indicates that the light-sensitive regulatory mechanism for proton leakage through the $CF_o.CF_1$ complex directly involves CF_o but not CF_1. The conformational basis and physiological role of this regulation are examined.

MATERIALS AND METHODS

Materials. DCCD and hexanal were from Aldrich Chemical Co. Glutaraldehyde (25% aq., Grade 1) was from Sigma Chemical Co. Pyocyanine was from Schwarz and Mann. All other chemicals used were of the highest grade available. Chloroplasts were prepared from fresh spinach leaves by the method of Avron (8). Broken chloroplasts for proton translocation measurements were prepared as previously described (1).

Removal of CF_1 and resealing with DCCD. Chloroplasts were depleted of CF_1 by washing with 2M NaBr solution according to the procedure of Kamienietzky and Nelson (5). A suspension of chloroplasts in STN buffer (sucrose, 0.25M; Tricine 20mM at pH 7.9; NaCl, 10mM) was diluted with STN buffer containing 5M NaBr to give a final concentration of 2M NaBr and $\sim$1 mg chlorophyll/ml. The mixture was incubated at 0°C under room light for 45 min. The treated chloroplasts were collected by centrifugation (4000 g, 10 min.) and the NaBr was removed by repeated resuspension in STN buffer and recentrifugation. The CF_1-depleted chloroplasts were found to show no net proton uptake under the conditions of light-driven cyclic electron transport with pyocyanine as the mediator and no residual heat-activated Ca^{2+}-ATPase activity (5). The CF_1-depleted chloroplasts were then treated with DCCD (70 μM) for 10-15 min under continuous illumination (250 joules$\cdot m^{-2}sec^{-1}$) at 4°C. Under these conditions the NaBr-washed, DCCD-resealed chloroplasts regained their capacity to take up protons from the medium upon illumination.

Cross-linking with glutaraldehyde. The NaBr-washed, DCCD-resealed chloroplasts were fixed by glutaraldehyde (50 mM) for 6 minutes at 0°C in the dark or under continuous illumination as previously described (1). The excess glutaraldehyde was removed by centrifugation. Treatment of chloroplasts with hexanal was carried out under the same conditions.

Proton translocation. The experimental set-up and method of kinetic analysis were described previously (1). The actinic light was provided by a 500W projector lamp with a red filter, and the light intensity was varied and controlled by the addition of neutral grey filters.

The amount δ of protons taken up by illuminated thylakoids as monitored with a glass electrode was found to vary with time t precisely according to the equation $\ln(1-\delta/\delta_{ss}) = -k_L t$, where δ_{ss} represents nmoles of proton taken up per mg chlorophyll after the steady state has been reached and k_L is a rate constant which is independent of δ and t but varies with light intensity. Differentiation of this equation with respect to t gives $d\delta/dt = R_o - k_L\delta$, where $R_o = k_L \delta_{ss}$ represents the initial rate of proton uptake when the light was turned on. Similarly δ was found to decay after the light was turned off precisely according to $-d\delta/dt = k_D \delta$, where k_D represents the first-order decay constant in the dark. Arbitrary variation of R_o by changing the light

intensity showed that for the same thylakoid sample $k_L = k_D + mR_o$, where m and k_D are independent of the amount of proton taken up, light intensity, concentrations of chlorophyll, pyocyanine, buffer and NaCl provided that [NaCl]≥ 20mM. Because valinomycin + K^+ as well as neutral detergents increase k_D without affecting the value of m whereas at pH 8 DCCD as well as adenylyl imidodiphosphate (AMPPNP) decrease m without affecting k_D (1), it was concluded that the term $-k_L \delta$ in the rate equation represents the outward leakage of protons from illuminated thylakoids via two paths with rate constants mR_o and k_D corresponding respectively to proton leakage through the $CF_o \cdot CF_1$ complex and non-specific proton leakage elsewhere across the thylakoid membrane.

RESULTS AND DISCUSSION

The effects of washing with 2M NaBr solution and subsequent treatment with DCCD on net proton translocation in broken chloroplasts are shown in Fig.1. Fig. 1A shows the experimental trace of medium pH after the light was turned on at t=0 as well as after the light was again turned off due to proton translocation by control chloroplasts under the conditions of cyclic electron transport. Fig. 1B shows that after washing with 2M NaBr solution the CF_1-depleted chloroplasts can no longer accumulate protons under illumination, presumably due to the very rapid outward leakage of protons through the widely opened CF_o gate. Fig. 1C shows that after sealing the CF_o with DCCD the CF_1-depleted chloroplasts regained their capacity to accumulate protons under illumination.

The results of kinetic analysis of proton translocation data at various light intensities for the control and the CF_1-depleted, DCCD-resealed broken chloroplasts are summarized in Fig. 2. The two types of chloroplasts behave very similarly and have within experimental uncertainties the same values for the decay constant under illumination k_L, the decay constant in the dark k_D and the parameter m in the equation $k_L = k_D + mR_o$. It was concluded in our previous study on unmodified thylakoids that the light-dependent term mR_o of the decay constant k_L can be attributed to outward proton leakage through the specific proton channel of $CF_1 \cdot CF_o$ complex . The percentage of $CF_1 \cdot CF_o$ complex which stay in the on (open) or off (closed) conformation depends on the rate of light-driven electron transport. Since these NaBr-washed, DCCD-resealed chloroplasts are practically free of CF_1 but still have essentially

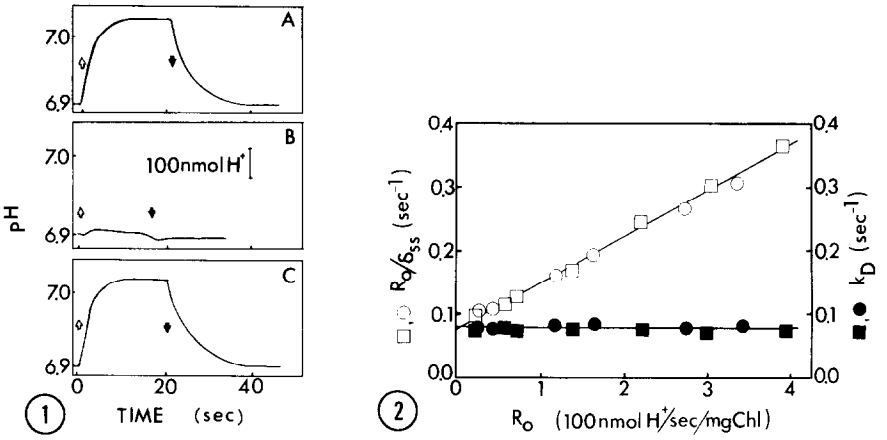

Figure 1. Proton uptake and release by (A) isolated control chloroplasts, (B) NaBr-treated chloroplasts and (C) NaBr-treated and DCCD-resealed chloroplasts. Composition of samples: chlorophyll, 70 μg/ml; [Tricine]= 1mM at initial pH 6.9 and 18°C; [pyocyanine] = 50μM; [NaCl] = 50 mM. Red light (intensity 250 joules.m^{-2} ·sec^{-1}) was turned on at t=0 sec (⇧) and turned off (⇩) after the steady state was reached. The vertical bar indicates the ΔpH scale calibrated by titrating the chloroplast sample at steady state with 5 mM HCl. ATPase activity of the samples at 10°C: control chloroplasts, 425 μ moles P_i/mg chlorophyll·hr; NaBr-treated chloroplast, <0.001 μ mole P_i/mg chlorophyll·hr.

Figure 2. Summary of kinetic parameters for proton translocation in control and in CF_1-depleted, DCCD-resealed chloroplasts. The composition of chloroplast samples is the same as that for Fig. 1. The light intensity was controlled by the addition of neutral grey filters in the path of red actinic light. □ and ■ represent the values of $k_L = R_o/\delta_{ss}$ and k_D respectively of the control chloroplasts. ○ and ● represent the values of k_L and k_D respectively of the CF_1-depleted and DCCD-resealed chloroplasts.

the same vaule of m, we may conclude that CF_1 is not, but CF_o is directly involved in this light-sensitive regulatory mechanism.

The effect of cross-linking with glutaraldehyde on proton translocation in CF_1-depleted, DCCD-resealed broken chloroplasts is shown in Fig. 3. Treatment with glutaraldehyde makes m = 0 and hence k_L or k_D becomes independent of light intensity. However, chloroplasts fixed under constant light intensity (points of Group A in Fig. 3) have a large k_L or k_D which is equal to the k_L of the control chloroplasts under the same light intensity. The same batch of CF_1-depleted, DCCD-resealed chloroplasts fixed with glutaraldehyde in the dark have a small k_L or k_D which is equal to the k_D of the control chloroplasts (points of Group B in Fig. 3). It is also shown in Fig. 3 that the monoaldehyde hexanal has no effect on k_L. These results suggest that the

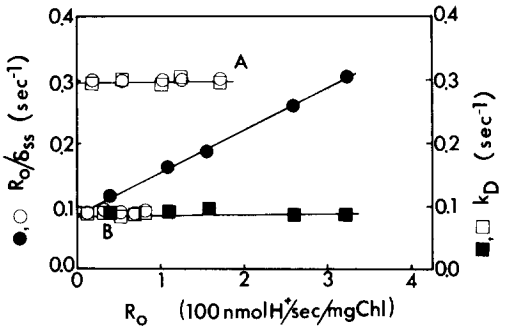

Figure 3. The effect of glutaraldehyde and hexanal on the proton translocation in CF_1-depleted, DCCD-resealed chloroplasts. The preparation of glutaraldehyde-fixed chloroplasts is described under Materials and Methods. Composition of the sample: Chlorophyll, 0.1 mg/ml; [Tricine] = 1mM at pH 6.9 and 18°C; [pyocyanine] = 50 μM; [NaCl] = 50 mM. Experimental points in Group A represent the values of k_L (○) and k_D (□) respectively for CF_1-depleted, DCCD-resealed chloroplasts cross-linked with glutaraldehyde under constant illumination (250 joules·m^{-2}·sec^{-1}), whereas those in Group B represent values of k_L (○) and k_D (□) respectively for CF_1-depleted, DCCD-resealed chloroplasts cross-linked with glutaraldehyde in the dark. ● and ■ represent values of k_L and k_D respectively for the CF_1-depleted, DCCD-resealed chloroplasts treated with equivalent concentration of hexanal.

light-dependent proton leakage through CF_o is regulated by conformation change in the membrane.

The physiological significance of the observed regulation of proton leakage through the $CF_1 \cdot CF_o$ complex may be examined in the following way. The rate of free energy conversion via ATP synthesis driven by proton flux through the $CF_1 \cdot CF_o$ complex at constant ionic strength is given by

$$\frac{dG}{dt} = \left(F\Delta\psi + RT \ln \frac{[H^+]_2}{[H^+]_1}\right)\left(\frac{d\delta}{dt}\right)_{via\ CF_1 \cdot CF_o} \qquad (1)$$

where $\Delta\psi$ is the electric potential difference across the thylakoid membrane, F the faraday, $[H^+]_2$ and $[H^+]_1$ the proton activities on opposite sides of the membrane and $(d\delta/dt)_{via\ CF_1 \cdot CF_o}$ the proton flux through the $CF_1 \cdot CF_o$ complex. For ATP synthesis with definite stoichiometric ratio of H^+/ATP, the electrochemical potential factor in Equation 1 must exceed a threshold value.

Under illumination of constant intensity, the steady state amount of proton uptake is given by $\delta_{ss} = R_o/(k_{CF_1 \cdot CF_o} + k_D)$, where R_o is the initial rate of proton uptake, $k_{CF_1 \cdot CF_o}$ the rate constant for the leakage of accumulated

protons through the $CF_1 \cdot CF_o$ complex and k_D the rate constant for the non-specific leakage of protons elsewhere across the membrane. When the light intensity is diminished, R_o and δ_{ss} would decrease parallelly if $k_{CF_1 \cdot CF_o}$ and k_D were both independent of light intensity. Consequently, the electrochemical potential factor in Equation 1 could decrease to a value below the threshold which would make the synthesis of ATP by $CF_1 \cdot CF_o$ impossible. However, that $k_{CF_1 \cdot CF_o}$ is actually equal to mR_o could partially remedy this unfavorable situation by making δ_{ss} as well as the electrochemical potential factor decrease less rapidly than R_o as the light intensity is lowered. At the molecular level this observed property means that a larger percentage of the $CF_1 \cdot CF_o$ complexes will be turned off under weaker illumination to conserve the electrochemical potential difference across the membrane so that the synthesis of ATP may still proceed albeit at a slower rate. Conversely when the light intensity is raised, possible waste in free energy due to excessive electrochemical potential can be avoided by an increase in $k_{CF_1 \cdot CF_o} = mR_o$ which could lower the electrochemical potential factor to a reasonable value above the threshold and increase the proton flux through $CF_1 \cdot CF_o$ for faster ATP synthesis.

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