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Effect of Hydrophobic Carboxyl Reagents on the Proton Flux through Coupling Factor CF_0 in Thylakoid Membrane†

Yee-kin Ho and Jui H. Wang*

ABSTRACT: Specific carboxyl reagents, 1-(ethoxycarbonyl)-2-ethoxy-1,2-dihydroquinoline (EEDQ) and 1-(isobutoxycarbonyl)-2-isobutoxy-1,2-dihydroquinoline (IIDQ), were found to interact with coupling factor CF_0 of spinach chloroplasts in a way similar to dicyclohexylcarbodiimide (DCCD). At a concentration of 50 μM, both compounds reduced proton leakage out of chloroplasts which were deficient in coupling factor CF_1. The rate of resealing the uncoupled chloroplasts by EEDQ and IIDQ was ~20 times slower than that by DCCD. The regained proton accumulation under constant illumination was 95, 75, and 45% of control chloroplasts for DCCD, IIDQ, and EEDQ, respectively. This is in the same order as the sizes of the resulting groups attached to CF_0. Reaction with EEDQ prior to the addition of [^{14}C]DCCD reduced the incorporation of radioactivity into the DCCD-binding protein in chloroplasts. These compounds at low concentration (50 μM) inhibited photophosphorylation and heat-activated ATPase activity of control chloroplasts but had little effect on either the electron flow from water to 2,6-dichlorophenolindophenol or the ATPase activity of CF_1 extracted into solution. Light-induced proton uptake of control chloroplasts at pH 8.0 was stimulated to different degrees by these reagents also in the order DCCD > IIDQ > EEDQ; however, this effect was not observed at pH 6.6. These results suggest that these hydrophobic carboxyl reagents interact specifically and competitively with the same site in CF_0, presumably a reactive carboxyl group.

The free energy change due to light-driven electron transport in chloroplasts is coupled to ATP formation by the coupling factor complex CF_1·CF_0.[1] The complex has been resolved into the soluble ATPase (CF_1), which provides the catalytic site for an adenine nucleotide, and a membrane-integrated part (CF_0) to which CF_1 is attached. The isolated CF_1 is composed of five subunits, which are denoted by α, β, γ, δ, and ϵ, respectively, in the order of decreasing molecular weight and have a suggested stoichiometry of 2:2:1:1:2 (Baird & Hammes, 1976). CF_0 probably consists of three or four subunits (Pick & Racker, 1979), and it is involved in the transport of protons across the thylakoid membrane.

Investigations of CF_1·CF_0 have been reviewed by Baird & Hammes (1979), McCarty (1977), and Nelson (1976). The CF_1·CF_0 complex acts as a proton channel which utilizes the proton gradient generated by electron transport to synthesize ATP (Mitchell, 1961, 1966). It is well-known that CF_1-depleted chloroplasts obtained by EDTA washing or NaBr treatment lack the ability to accumulate protons under illumination. However, the proton uptake can be restored by reconstitution of purified CF_1 (Lynn & Straub, 1969) or by

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[1] Abbreviations used: Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; DCCD, *N*,*N*′-dicyclohexylcarbodiimide; EEDQ, 1-(ethoxycarbonyl)-2-ethoxy-1,2-dihydroquinoline; IIDQ, 1-(isobutoxycarbonyl)-2-isobutoxy-1,2-dihydroquinoline; EDTA, ethylenediaminetetraacetic acid; DCIP, 2,6-dichlorophenolindophenol; DTT, dithiothreitol; CF_1·CF_0, the complex of chloroplast coupling factors; Dio-9, an antibiotic of unknown structure (McCarty et al., 1965); $NaDodSO_4$, sodium dodecyl sulfate; PEI, poly(ethylenimine).

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the addition of DCCD (Uribe, 1972; Kamienietzky & Nelson, 1975) or other CF_0-associated inhibitors. These observations suggest that CF_0 is a proton channel with CF_1 controlling its gate. The removal of CF_1 increases proton leakage through CF_0, and subsequent addition of DCCD blocks the proton leakage as well as the utilization of the proton gradient to synthesize ATP. A DCCD-binding proteolipid of CF_0 has been isolated and was shown to have proton conductivity in reconstituted vesicles (Nelson et al., 1977). Recent kinetic studies on proton translocation by broken chloroplasts showed a light-dependent proton leakage through the CF_1·CF_0 complex (Ho et al., 1979) which might be controlled by protein conformation change in CF_0 (Ho & Wang, 1979).

Functional groups which participate in proton conduction in CF_0 are generally still unknown. However, studies on yeast, *Escherichia coli*, and *Neurospora* F_0 showed that a carboxyl group of glutamic acid or aspartic acid is involved in the binding of DCCD to F_0 (Sebald & Wachter, 1978). Results obtained in the studies of thermophilic bacterium TF_0 indicate that tyrosyl, arginyl, and carboxyl groups may be involved in the proton conduction (Sone et al., 1979). The amino acid sequences of DCCD-binding proteins from six sources, including that from spinach, have been determined recently (Sepald et al., 1979).

Many inhibitors of energy transduction have been found which interact with either CF_1 or CF_0. The CF_1-associated inhibitors include a variety of compounds: Dio-9 (McCarty et al., 1965); the antiserum of CF_1 (McCarty et al., 1971; Nelson et al., 1973); tentoxin (Steele et al., 1976); phlorizin (Winget et al., 1969); speggazzine (Andreo, 1978); phosphate analogues such as sulfate, selenate, and arsenate (Pick & Avron, 1973); *N*-ethylmaleimide (McCarty & Fagan, 1973); bis(dithionitropyridine) (Andreo & Vallejos, 1976); *o*-iodosobenzoic acid (Vallejos & Andreo, 1976); pyridoxal phosphate (Sugiyama & Mukohata, 1979); phenylglyoxal and butanedione (Schmid et al., 1977). The CF_0-associated inhibitors are all hydrophobic molecules such as DCCD (Uribe, 1972), discarine B (Andreo & Vallejos, 1973), zizyphines A and B (Ravizzini et al., 1977), triphenyltin chloride (Gould, 1976), and tributyltin chloride (Kahn, 1968).

In the present work, the hydrophobic carboxyl reagents 1-(ethoxycarbonyl)-2-ethoxy-1,2-dihydroquinoline (EEDQ) and 1-(isobutoxycarbonyl)-2-isobutoxy-1,2-dihydroquinoline (IIDQ) (Belleau et al., 1968, 1969; Belleau & Malek, 1968; Kiso et al., 1973) are used to study the involvement of carboxyl groups in CF_0. The relative effectiveness of these two compounds as well as DCCD on the observed proton flux through CF_0 in illuminated chloroplasts is reported.

Materials and Methods

Materials. ADP (sodium salt, grade III), ATP (disodium salt), and DTT were obtained from Sigma Chemical Co. DCCD, EEDQ, and IIDQ were from Aldrich Chemical Co. Carrier-free radioactive orthophosphate ($^{32}P_i$) and [γ-^{32}P]ATP were from New England Nuclear Corp. and were found by paper chromatography to contain no detectable amount of radioactive impurity. [^{14}C]DCCD was from Research Products International Incorporated. Pyocyanine was from Schwartz/Mann. Poly(ethylenimine) (PEI) (50% aqueous solution) was obtained from Eastman Kodak Chemical Co. All other reagents used were of the highest grade available and were used without further purification.

Chloroplast Samples. Chloroplasts were prepared from fresh spinach leaves as described in Avron (1960). The chloroplast pellet obtained after the last centrifugation was suspended in a homogenizing medium (sucrose, 0.25 M; Tricine, 20 mM at pH 7.9; NaCl, 10 mM) at 2 °C to give a concentration of 3–5 mg of chlorophyll per mL. Chlorophyll concentration was determined by the method of Arnon (1949). Broken chloroplasts for proton uptake experiments were obtained by 10–100-fold dilution of the original chloroplast preparation with a sucrose-free buffer containing 50 mM NaCl, 50 μM pyocyanine, and 1 mM Tricine at 0 °C as described previously (Ho et al., 1979).

EDTA-Uncoupled Chloroplasts. CF_1 was removed from thylakoid membrane by EDTA washing under hypotonic conditions (McCarty & Racker, 1965). The original chloroplast preparation was diluted 30-fold with 0.75 mM EDTA solution at pH 8.0 and 4 °C. The chloroplasts were uncoupled for 40 min under room light with constant stirring. The CF_1-depleted chloroplasts were collected by centrifugation (4000*g*, 10 min), and the pellet was resuspended in the homogenizing medium. After this treatment, the light-induced proton uptake with pyocyanine as mediator was completely abolished.

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 intensity was measured with a YSI-Kettering Model 65A radiometer. The duration of illumination was controlled manually by means of a shutter.

Proton Translocation Measurements. Proton uptake by broken chloroplasts was measured by means of a combination pH electrode (Beckman 39030) fitted in the thermostated sample cell. For the conversion of observed ΔpH to nanomoles of 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. The kinetic analysis of light-dependent proton leakage through the thylakoid membrane was described previously (Ho et al., 1979).

Incorporation of [^{14}C]DCCD into CF_0. The amount of [^{14}C]DCCD incorporated into CF_0 was determined by a radioactivity assay. The [^{14}C]DCCD-modified chloroplasts were centrifuged down, and the pellet was suspended in a minimum amount of buffer (1 mM Tricine, pH 7.9, and 50 mM NaCl). An aliquot of the sample (100 μg of chlorophyll) was then applied to $NaDodSO_4$–polyacrylamide gel for electrophoresis (Weber & Osborn, 1969). The electrophoresis was performed in 0.1% $NaDodSO_4$ and 50 mM phosphate buffer, pH 7.2, by using 10% polyacrylamide gels (10-mm diameter, 90-mm length). Dansylated bovine serum albumin (M_r 68 000), carbonic anhydrase (M_r 31 000), and ribonuclease (M_r 13 700) were used as standard molecular weight markers. The gels were cut into 1-mm fractions with a Gilson gel fractionator and put into plastic counting vials. To each vial was added 1.5 mL of distilled water and 5 mL of Aquasol (New England Nuclear Corp.). After each mixture was shaken, the sample appeared uniformly suspended. The radioactivity in each vial was assayed with a Beckman LS-233 liquid scintillation counter.

Assay of Electron Transport. The uncoupled electron transport rate from water to DCIP was assayed spectrophotometrically by following the absorbance decrease at 585 nm due to DCIP reduction (ϵ_{ox} of DCIP = 18 228 M^{-1} cm^{-1} in a solution of 20 μg of chlorophyll per mL) using a DW-2 spectrophotometer with a side illumination accessory. The actinic light was passed through a Corning 2403 red filter, and the photomultiplier tube was protected with a Corning 4-94 and an Oriel 5756 filter. A Clark-type oxygen electrode fitted on the top of the reaction cuvette was also used to monitor

oxygen evolution. The two methods give identical electron transport rates. The reaction medium contained 15–20 μg of chlorophyll per mL, 20 mM NaCl, 15 mM Tricine, pH 7.8 at room temperature, 75 μM DCIP, and 2 mM NH_4Cl.

Photophosphorylation. One milliliter of reaction mixture containing 70 μg of chlorophyll, 50 mM Tricine at pH 7.9, 50 mM NaCl, 2 mM $MgCl_2$, 5 mM ADP, 5 mM [^{32}P]P_i, and 50 μM pyocyanine was illuminate under red light for 1 min. After the light was turned of, trichloroacetic acid (4%) was added to stop the reaction. The precipitate was then centrifuged down (Beckman airfuge, 15 psi, 2 min), and 50 μL of the supernatant was put on PEI paper for [^{32}P]ATP assay by ascending chromatography (Wang, 1979).

Extraction of CF_1. Chloroplasts were suspended at concentrations of 70 μg of chlorophyll per mL in 0.75 mM EDTA at pH 8.0 for 20 min at room temperature to extract CF_1 for ATPase assay. The chloroplasts were then removed by centrifugation. The CF_1-containing supernatant (~2 mg of protein per mg of chlorophyll) was used for ATPase study. Protein concentrations were determined by the Lowry method using bovine serum albumin as a standard (Lowry et al., 1951).

Ca^{2+}-ATPase Activity. The Ca^{2+}-dependent ATPase of chloroplasts and soluble CF_1 were activated by heat treatment (60 °C, 10 min) in the presence of 2 mM DTT. The activated sample was then added into the reaction mixture containing 5 mM Ca^{2+}, 5 mM [γ-^{32}P]ATP, 20 mM Tricine at pH 7.9, and 20 mM NaCl at room temperature. After 15 min, the reaction was terminated with trichloroacetic acid. The precipitate was removed by an airfuge, and the supernatant was assayed for [γ-^{32}P]ATP content by chromatography on PEI paper.

Results and Discussion

Effect of EEDQ and IIDQ on EDTA-Uncoupled Chloroplasts. The removal of CF_1 from thylakoid membrane causes fast proton leakage through CF_0 and hence abolishes the light-induced accumulation of protons in the chloroplasts. The proton gradient can be regenerated by the addition of any CF_0-associated energy transfer inhibitor to these uncoupled chloroplasts before illumination. This phenomenon can be used as a convenient assay method for exploring new CF_0-blocking compounds. It has been shown that DCCD labels specifically a carboxyl group in yeast, *E. coli*, and *Neurospora* F_0 (Sebald & Wachter, 1978). Because of the expected similarity of coupling factors from different sources, a carboxyl group may also be labeled by DCCD in CF_0. It is of interest to examine other hydrophobic carboxyl reagents and their effect on the proton flux through CF_0. In the present work, the hydrophobic carboxyl reagents EEDQ and IIDQ have indeed been found to reseal partially the proton leakage through CF_0 in EDTA-uncoupled chloroplasts. The effects of EDTA washing and subsequent treatment with carboxyl reagents on the uptake of protons by the illuminated chloroplasts under the conditions of cyclic electron transport are shown in Figure 1. Figure 1A shows an experimental trace of the change in medium pH due to proton translocation of control chloroplasts, with the light turned on at $t = 0$ and then turned off after the steady-state proton uptake (δ_{ss}) was reached. Figure 1B shows the effect of EDTA washing on proton translocation; the uncoupled chloroplasts no longer accumulate protons. Figure 1C shows the effect of subsequent treatment with DCCD on EDTA-uncoupled chloroplasts, with more than 95% of the proton gradient regenerated within 5 min. Figure 1D shows the effect of EEDQ treatment on the uncoupled chloroplasts. The reaction is slow and reaches a maximum (~45% of control) at 80 min when the sample is incubated in the dark at 15 °C. The reaction is accelerated by light. IIDQ gives similar results except that at maximum, 75% of the gradient can be regenerated. The rate of resealing CF_0 by IIDQ and EEDQ is shown in Figure 2. IIDQ and EEDQ react at similar

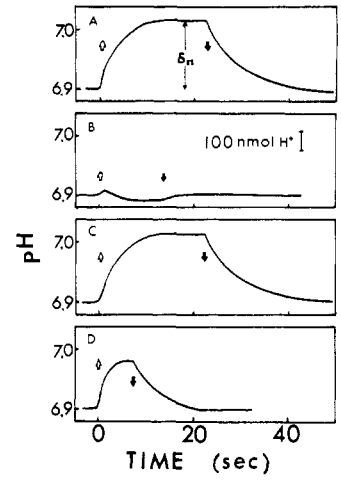


FIGURE 1: Proton uptake and release by (A) control chloroplasts, (B) EDTA-uncoupled chloroplasts, (C) EDTA-uncoupled and DCCD-resealed chloroplasts (reaction with DCCD was completed in 5 min), and (D) EDTA-uncoupled and EEDQ-resealed chloroplasts (reaction with EEDQ was completed in 80 min). Composition of samples: [chlorophyll] = 75 μg/mL; [Tricine] = 1 mM at initial pH 6.9 and 15 °C; [pyocyanine] = 50 μM; [NaCl] = 50 mM. Red light (intensity 250 J m^{-2} s^{-1} was turned on at $t = 0$ (↑) and turned off (↓) after the steady state (δ_{ss}) was reached. The vertical bar indicates the ΔpH scale calibrated by titrating the chloroplast sample at steady state with 5 mM HCl.

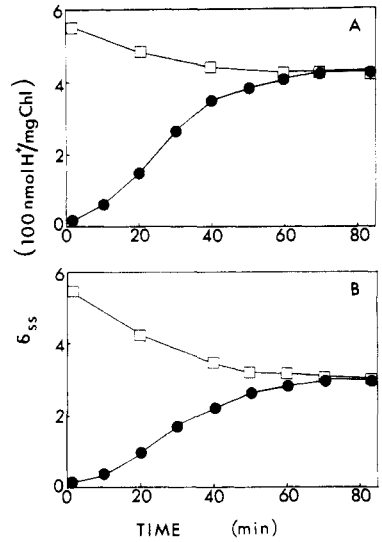
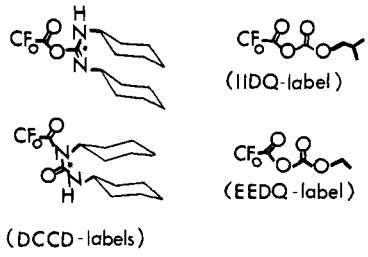


FIGURE 2: Rates of resealing EDTA-uncoupled chloroplasts with IIDQ and EEDQ and the competitive effect of DCCD. Common feature of all samples: sample volume 1.8 mL; [chlorophyll] = 75 μg/mL; [Tricine] = 1 mM; [NaCl] = 50 μM; [pyocyanine] = 50 μM; initial pH 6.9 at 15 °C. Light intensity used was 250 J m^{-2} s^{-1} with a red filter. Each carboxyl reagent (final concentration 55 μM) was added as 4 μL of ethanolic solution. The steady-state proton uptake (δ_{ss}) was obtained by titrating the chloroplast sample with 5 mM HCl under continuous illumination. (A) Effect of IIDQ on EDTA-uncoupled chloroplasts. 50 μM IIDQ was added to each uncoupled chloroplast sample at $t = 0$, and the steady-state proton uptake (δ_{ss}) was measured after an interval of time. (●) δ_{ss} values obtained after reaction with IIDQ for the time indicated; (□) δ_{ss} values obtained after the addition of DCCD to the IIDQ-resealed chloroplasts. These latter values of δ_{ss} were taken 5 min after DCCD addition. (B) Effect of EEDQ on EDTA-uncoupled chloroplasts. EEDQ was added to the uncoupled chloroplast sample at $t = 0$. (●) δ_{ss} values obtained after reaction with EEDQ for the time indicated; (□) δ_{ss} values obtained 5 min after DCCD was added to the EEDQ-resealed chloroplasts.

Chart I



rates with CF_0. The difference in the regenerated gradient is not due to the difference in reactivity, since both compounds have the same $t_{1/2}$ for reaching the maximum δ_{ss}. Further addition of either IIDQ or EEDQ after the steady-state proton uptake (δ_{ss}) has been reached produced no further change in δ_{ss}, which indicates that the smaller regenerated gradient compared with DCCD is not due to the depletion of reagents in the solution. The data are consistent with the assumption that labeling a specific carboxyl group(s) in CF_0 affects proton conduction. The difference in the magnitude of the regenerated gradient by DCCD, IIDQ, and EEDQ respectively (95:75:45%) could be due to the following possibilities.

(1) There is more than one reactive carboxyl group in CF_0, and DCCD, IIDQ, and EEDQ react with different carboxyl groups to produce different effects on the proton channel.

(2) The three compounds interact at the same site, but as shown in Chart I the different size of the resulting label on the modified carboxyl group causes a different percentage of reduction of the proton flux.

(3) The observed difference in δ_{ss} is an artifact due to the nonspecific reaction of IIDQ and EEDQ with thylakoid components other than CF_0. Modification of these other carboxyl groups may have slight uncoupling effects or may reduce the proton pumping rate; both could decrease the apparent δ_{ss}.

Competition of IIDQ and EEDQ with DCCD in Modification of CF_0. Experiments were conducted to examine the competition between these reagents in resealing CF_0 as well as the effect due to double labeling in order to decide between the above three possibilities. Because DCCD seals CF_0 more completely and reacts with CF_0 20 times faster than IIDQ and EEDQ, the addition of DCCD to EEDQ- or IIDQ-treated chloroplasts should have different effects depending upon which of the three possibilities discussed above is true.

(1) If these reagents react with different carboxyl groups, the addition of DCCD to IIDQ- or EEDQ-treated chloroplasts should further reduce the proton leakage and boost the regenerated proton gradient to >95% of the control.

(2) If the DCCD binding site has already been occupied by either the IIDQ or EEDQ label, the addition of DCCD should have no effect on the δ_{ss} of the EEDQ- or IIDQ-treated chloroplasts. Since EEDQ and IIDQ react relatively very slowly, DCCD may increase the proton gradient to a certain extent between 95% (maximum value for DCCD) and the maximum values for the reagent used (45% for EEDQ and 75% for IIDQ) if DCCD is added before all the sites are labeled. Data of such experiments are included in Figure 2. The results are consistent with the second possibility discussed above but seem to contradict the first possibility because DCCD has no effect on δ_{ss} after the reaction with IIDQ or EEDQ is completed but increases δ_{ss} to a certain extent before all the sites are labeled by IIDQ or EEDQ.

(3) When IIDQ or EEDQ is added to DCCD-resealed chloroplasts, it should have no effect on the proton gradient if all these carboxyl reagents compete for the same carboxyl groups but should decrease the proton gradient if IIDQ or

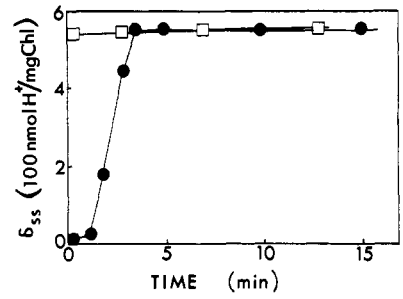


FIGURE 3: Rate of resealing EDTA-uncoupled chloroplasts with DCCD and effect of IIDQ and EEDQ on the DCCD-resealed chloroplasts. The composition of samples is identical with that in Figure 2. DCCD was added to the EDTA-uncoupled chloroplasts at $t = 0$, and values of steady-state proton uptake (δ_{ss}) were determined as a function of time. (●) δ_{ss} values of DCCD-resealed chloroplasts as a function of reaction time with DCCD; (□) δ_{ss} values of chloroplast sample, where either IIDQ or EEDQ was added to the DCCD-resealed chloroplasts. The values of δ_{ss} were determined 60 min after the addition of IIDQ or EEDQ.

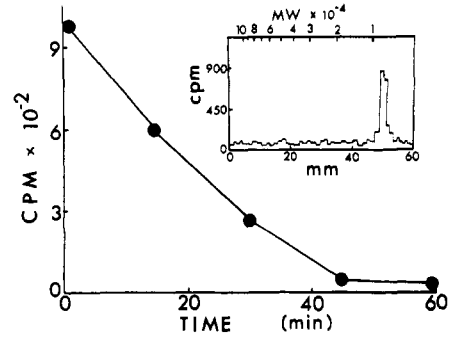


FIGURE 4: Effect of EEDQ on the incorporation of [^{14}C]DCCD into DCCD-binding protein in EDTA-uncoupled chloroplasts. The insert shows a radiogram of the $NaDodSO_4$ gel of [^{14}C]DCCD-labeled EDTA-uncoupled chloroplasts. The molecular weight scale was calibrated by using dansylated bovine serum albumin, carbonic anhydrase, and ribonuclease as markers. EEDQ was added into EDTA-uncoupled chloroplasts at $t = 0$, and [^{14}C]DCCD was then added at different EEDQ reaction times. The chloroplast samples were centrifuged 5 min after the addition of [^{14}C]DCCD, and a sample containing 100 μg of chlorophyll was removed from the pellet for $NaDodSO_4$ gel electrophoresis. ● represents the radioactivity found in the 8300 molecular weight peak, i.e., the DCCD-binding protein.

EEDQ inhibits the proton pump or uncouples the system slightly. The data in Figure 3 again support the second but contradict the third possibility.

It was well demonstrated that at low concentrations [^{14}C]DCCD incorporates only into a single proteolipid in CF_0 of 8300 molecular weight in whole chloroplasts (Nelson et al., 1977). Based on the above results, we expect EEDQ or IIDQ to reduce the incorporation of [^{14}C]DCCD into CF_0. Figure 4 shows that this is indeed the case. Reaction with EEDQ prior to the addition of [^{14}C]DCCD to the chloroplast sample decreases the incorporation of radioactivity into the DCCD-binding protein. Together with the data in Figure 2, these results strongly suggest that IIDQ, EEDQ, and DCCD compete for the same carboxyl group in CF_0.

Effect on Other Properties. In spite of the slow reaction rate of IIDQ and EEDQ, both compounds also hinder the proton flux through CF_0 at a concentration (50 μM) at which DCCD acts as an energy transfer inhibitor. Table I shows the effect of IIDQ, EEDQ, and DCCD on electron transport, photophosphorylation, ATPase activity, and proton uptake of chloroplasts. After a long incubation period (4 to 5 h at 0 °C), both IIDQ and EEDQ behave as a CF_0-associated energy transfer inhibitor. Both compounds inhibit photo-

Table I: Effect of Hydrophobic Carboxyl Reagents on Photophosphorylation, Electron Transport, Proton Uptake, and ATPase Activity of Chloroplasts (CP's)[a]

	control CP's	DCCD-treated CP's	IIDQ-treated CP's	EEDQ-treated CP's
photophosphorylation[b]	675	110	160	175
electron transport:[c] $H_2O \rightarrow$ DCIP + 2 mM NH_4Cl	314	307	320	314
proton uptake (δ_{ss}):[d] +pyocyanine				
pH 6.6	970	955	960	970
pH 8.0	420	809	672	525
Ca^{2+}-ATPase:[e] heat activation + DTT				
CP's	420	98	105	134
soluble CF_1[f]	378 (2.1)	322 (2.2)	312 (2.0)	307 (2.0)

[a] Chloroplast (CP) samples were prepared as described under Materials and Methods. Carboxyl reagents (5 μL, 25 mM in ethanol) were added to 2.2 mL of chloroplast suspension at pH 7.9 (Tricine, 20 mM; NaCl, 20 mM; sucrose, 0.25 M; chlorophyll, ~0.5 mg/mL). The sample was then incubated at 0 °C in the dark for 4 h. For control chloroplast samples, the same volume of ethanol was added. Light intensity used in various measurements was 250 J m^{-2} s^{-1}. [b] Assay procedure was described under Materials and Methods. The reaction was done at 15 °C. Values are given in units of μmol of ATP per mg of chlorophyll. [c] Values are given in units of μmol of DCIP reduced per mg of chlorophyll per h. [d] Proton uptake was assayed at 15 °C. Composition of sample: [Tricine] = 1 mM, [NaCl] = 50 mM, [pyocyanine] = 50 μM, and [chlorophyll] = 70 μg/mL. Values are given in units of nmol of H^+ per mg of chlorophyll. [e] Heat activation was completed by incubating chloroplast or CF_1 samples at 60 °C for 10 min. The activated samples were transferred to the reaction mixture containing 5 mM Ca^{2+}, 5 mM [γ-^{32}P]ATP, 20 mM Tricine at pH 7.9, and 20 mM NaCl. Values are given in units of μmol of ATP per mg of chlorophyll per h. [f] Soluble CF_1 was extracted by EDTA washing as described under Materials and Methods. Values in parentheses indicate the protein concentration of the EDTA extract in units of mg of protein per mg of chlorophyll.

phosphorylation and heat-activated ATPase activity of chloroplasts but have very little effect on either the uncoupled electron transport rate or the ATPase activity of the extracted CF_1. All three reagents stimulate light-induced proton uptake (δ_{ss}) at pH 8.0 but have no detectable effect on δ_{ss} from pH 6.6 to 7.1. These effects are all typical features of a CF_0-associated energy transfer inhibitor.

Recent kinetic analysis of proton translocation in broken chloroplasts (Ho et al., 1979) showed that protons can leak through thylakoid membranes via two paths, a light-independent path which represents the general leakage through the membrane and a light-dependent path which represents the leakage through the CF_1·CF_0 complex. The first-order rate constants are related by

$$k_L = mR_0 + k_D$$

where k_L represents the proton leakage constant under continuous illumination, 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 which is independent of the intensity of previous illumination but can be decreased by treatment with detergents, 2,4-dinitrophenol or valinomycin + K^+, and m is a parameter which is independent of light intensity but decreases with pH from pH 7.4 to 8.0. Compounds such as DCCD or Mg^{2+} + adenylyl imidodiphosphate (AMPPNP), which are bound specifically to the CF_1·CF_0 complex, were found to increase the value of m at pH 8.0 but have no effect at pH 6.6 (Ho et al., 1979). Consequently, the term mR_0 has been interpreted as the first-order constant for proton leakage through the CF_1·CF_0 complex.

Figure 5 shows that the effects of IIDQ and EEDQ on the kinetics of proton translocation in normal chloroplasts are similar to that of DCCD. Together with the previously published information, these results indicate that all three hydrophobic reagents compete for the same carboxyl group in CF_0. Since even at 100% labeling at this carboxyl group the proton channel of CF_0 in thylakoid membrane can still open or close in response to variations in light intensity, we must conclude that proton conduction through this channel does not require the protonation or ionization of this carboxyl group. The different extent of resealing of the EDTA-uncoupled chloroplasts by DCCD, IIDQ, and EEDQ respectively reflects the different size of the corresponding labels.

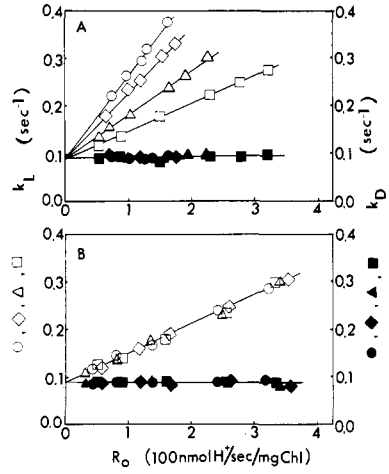


FIGURE 5: Effect of DCCD, IIDQ, and EEDQ on the kinetics of proton translocation by chloroplasts at pH 8.0 and 6.6. General features of all the samples: chloroplast samples were modified with carboxyl reagents (final concentration 55 μM) at 0 °C for 4 h at pH 7.9. The pH was adjusted before kinetic data were taken at 15 °C. Composition of sample: [chlorophyll] = 75 μg/mL; [Tricine] = 1 mM; [NaCl] = 50 mM; [pyocyanine] = 50 μM. Light intensity was controlled by the addition of neutral gray filters on the light path. Kinetic analysis was carried out as described previously (Ho et al., 1979). (A) Measurements at pH 8.0; (B) measurements at pH 6.6. ○, ◇, △, and □ represent the k_L values (decay constant under illumination) of control and EEDQ-, IIDQ-, and DCCD-treated chloroplasts, respectively, obtained at a different light intensity. ●, ◆, ▲, and ■ represent the corresponding values of k_D (the dark decay constant).

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