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Cooperative transient trapping of photosystem II protons by the integral membrane portion (CF0) of chloroplast ATP-synthase after mild extraction of the four-subunit catalytic part (CF1)

(photosynthesis/water oxidation/photophosphorylation/proton pumping/proteolipid)

WOLFGANG JUNGE*, YU QUN HONG*†, LU PING QIAN*†, AND ALEXANDRO VIALE‡

*Biophysik, Fachbereich Biologie/Chemie, Universitaet Osnabrueck, Postfach 4469, D-4500 Osnabrueck, Federal Republic of Germany; and ‡Centro de Estudios Fotosinteticos y Bioquimicos, Consejo Nacional de Investigaciones Cientificas y Technicas, Fundacion Miguel Lillo, Universidad de Rosario, Suipacha 536, 2000 Rosario, Argentina

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ABSTRACT **The ATP-synthase in chloroplasts is built from two blocks, CF0, which is integral to the thylakoid membrane and which serves as a proton channel, and CF1, attached to CF0, which is catalytically active. This study is aimed at understanding proton conduction through CF0. By a mild procedure we extracted <10% of total CF1, predominantly the four-subunit CF1 without the δ subunit. Extracted chloroplasts were excited with short flashes of light and the time course of the transmembrane potential and of the pH changes in both phases was measured spectrophotometrically. Mild extraction of CF1 caused two effects. (*i*) Up to 50% of the protons rapidly released from water oxidation transiently escaped detection in the thylakoid interior. (*ii*) The initial extent of the transmembrane potential was decreased by some 10% (20-μs resolution). Protons that were not detected inside appeared in the external phase after having passed the thylakoid membrane. pH titrations of the transient loss of protons produced an extremely sharp transition (near pH 7.5) as if six protons were buffered in a strictly cooperative manner. These effects were reversed upon addition of *N,N'*-dicyclohexylcarbodiimide, which, among other actions, blocks the proton channel through CF0. We interpret these observations as follows. (*i*) CF0 incorporates proton binding groups, which can act in a hexacooperative way. These groups are located near the middle of the membrane. (*ii*) After extraction of CF1, protons produced during water oxidation have very rapid access to these groups, but they pass the full span of the membrane more slowly: buffering precedes conduction through CF0.**

Light-dependent synthesis of ATP of chloroplasts is catalyzed by the enzyme complex CF1–CF0. CF0, an intrinsic membrane protein, serves as a proton channel. CF1, a peripheral protein, catalyzes phosphorylation of ADP. Together they function as a proton-translocating ATP-synthase (or ATPase). Their structure and function have been reviewed recently (1–3). CF1, which can be readily detached from the thylakoid membrane, is composed of five subunits: α to ε with molecular masses of approximately 59, 56, 37, 17.5, and 13 kDa (4). Its subunit stoichiometry is probably 3:3:1:1:1 (5, 6) and its molecular mass is ≈400 kDa (7). Subunits α and β both interact with nucleotides (8) but the catalytic site(s) seems to be located on subunit β (9). Subunit γ may be involved in the binding of the regulatory subunit ε (4) and its modification alters the proton permeability of the complex (10–12). The role of the δ subunit is not clear. In earlier work it was suggested that it may help to bind CF1 to CF0 (13), but in a detailed study on this question Andreo *et al.* (14) found the opposite. However, Roos and Berzborn (15) reported that δ alone can bind to CF0, without aiding the binding of CF1, in agreement with ref. 14.

The properties of CF0 were reviewed by Nelson (3). Further details also for F0 from other membranes may be found in an article by Sebald and Hoppe (16). CF0, which was never purified *per se*, could be recovered together with CF1 (17, 18). It is composed of at least three subunits I, II, and III, with molecular masses of 15, 12.5, and 8 kDa (19). Subunit III, a proteolipid, was isolated and purified. It is one of the *N,N'*-dicyclohexylcarbodiimide (DCCD)-binding proteins of thylakoids. Its reconstitution into vesicles from chloroplast lipids produced enhanced proton conduction (20). In spinach the proton-translocating ATP-synthase could be deactivated if only one of six of the potentially DCCD-binding subunits III was labeled with DCCD (21). This suggested that six copies of subunit III were required to form the proton channel through CF0. Recently, proteolipid from yeast mitochondria was incorporated into bimolecular lipid membranes to study the channel-forming properties in a quantitative way (22). For technical reasons these studies had to be carried out at low pH (2.2). Under these conditions dimers seemed to be the minimal unit for proton conduction. The role of the other subunits, I and II, for the binding of CF1 to CF0 or for regulation of proton flux still awaits elucidation.

In previous studies we found high electric conductivity of, and proton leakage through, the thylakoid membrane after extraction of CF1 (23, 24). These studies were carried out by flash spectrophotometry via the electrochromic absorption changes of certain chloroplast pigments (25) (reviewed in refs. 26, 27) and via pH-indicating dyes. We found that proton leakage could be blocked by reincorporation of isolated CF1 into CF1-depleted membranes or, alternatively, upon addition of DCCD. However, electric leakage remained high. This was attributed to unspecific damage that was perhaps unrelated to CF0.

In this study we attempted to characterize proton binding by membrane-bound CF0 and proton conduction through CF0 under mild extraction of CF1 to minimize electric leaks as side effects of the extraction procedure.

MATERIALS AND METHODS

Chloroplasts. Chloroplasts were prepared from winter spinach, obtained from the local market (exception: pea chloroplasts in Fig. 5). We prepared "broken chloroplasts" according to standard procedures (see ref. 28), but without

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Abbreviations: CF0 and CF1, integral membrane portion and catalytic part of chloroplast ATP-synthase, respectively; DCCD, *N,N'*-dicyclohexylcarbodiimide; $PhMeSO_2F$, phenylmethylsulfonyl fluoride.

†Permanent address: Institute of Plant Physiology, Academia Sinica, 300 Fonglin Road, Shanghai, China.

addition of divalent cations to the blending medium. Broken chloroplasts were pelleted by centrifugation at 12,000 × *g* for 10 min. The pellet was resuspended in 10 mM tricine/NaOH (pH 8) to yield a chlorophyll concentration of 2–4 mg/ml in the stock solution. The stock was stored on ice until needed.

Mild Extraction of CF1. Mild extraction of CF1 was induced as follows. Chloroplasts from the concentrated stock solution were suspended in distilled water and gently stirred for 2 min at room temperature. EDTA was present if indicated. The pH was adjusted to 7.5 by addition of NaOH. The chlorophyll concentration in the dilute suspension was 100 μM for the biochemical assay and 10 μM for the spectrophotometric measurements. After incubation for 2 min, $MgCl_2$ (1–2 mM) was added to stop extraction. Control samples underwent the same incubation except that $MgCl_2$ was added together with EDTA.

Amount of CF1 Extracted. The amount of CF1 extracted was determined for spinach. The sample was centrifuged for 10 min at 15,000 × *g*. To the supernatant, the following chemicals were added from a concentrated stock solution to yield the given final concentrations: Tris sulfate (pH 7.5), 20 mM; EDTA, 2 mM; NH_4SO_4, with NH_4OH to yield pH 7.5, 100 mM; ATP, 1 mM; and phenylmethylsulfonyl fluoride ($PhMeSO_2F$) as protease inhibitor, 10 μM (Sigma; at 100 mM in a 100% dimethyl sulfoxide stock solution). For concentration, the solution was passed over a DEAE-Sephadex column (A-50, 2.5 × 6 cm) that was equilibrated with the same buffer (see ref. 29). Protein was eluted with 50 ml of the stock solution and it was concentrated (20-fold) by Amicon ×100 ultrafiltration. The concentration of CF1 was determined by immunoelectrodiffusion with anti-CF1 (rabbit) according to Laurell (30). The only modification of this technique was the addition of 1% Triton X-100 to the agarose. Purified spinach CF1 served as standard.

For samples used in the spectrophotometric experiments, the degree of extraction was determined by assay of Ca^{2+}-ATPase activity, though, necessarily at lower precision.

Subunit Composition of Extracted CF1. For analysis of the subunit composition, CF1 was separated from the protein extract and was further concentrated by precipitation with anti-CF1 serum. The titer of the antiserum was adjusted properly by comparison with purified CF1. The solution was centrifuged and washed three times with 50 mM Na_3PO_4 (pH 7.0), to which was added 10 μM $PhMeSO_2F$, in a total volume of 5 ml. The pellet from the last wash was dissolved in 1% $NaDodSO_4$/10 M urea and homogenized. Insoluble material was removed by centrifugation. Aliquots of the supernatant (adjusted to contain 20–40 μg of CF1) were applied to the sample compartments of a $NaDodSO_4$/PAGE slab gel [10 × 10 × 0.1 cm; after Laemmli (31)]. Electrophoresis was run for 20 hr at 15 mA and at 22°C. After the run the gel was fixed and stained with Coomassie blue following standard procedures (32, 33).

The subunit composition was determined in two ways: (*i*) by photometrical scan of the gel (Gilson–Holochrome) at 595 nm and (*ii*) after cutting the gel and dye extraction by photometry of the extract after Binder *et al.* (34). The factors for binding of Coomassie blue were taken into account.

Electric Potential Difference, Proton Deposition, and Proton Uptake. The extent and kinetics of the electrical potential difference, proton deposition, and proton uptake were measured flash-spectrophotometrically. Chloroplasts were suspended in buffer medium: 10 μM chlorophyll, 1 mM $MgCl_2$, and 10 μM benzyl viologen, as electron acceptor. Further additions and the pH values are indicated in the figure legends. The cuvette for optical absorption measurements had a 2-cm path length. Excitation was provided by a Xenon flash (half-duration, 15 μs; energy output, 1 mJ/cm^2 at a wavelength > 615 nm) typically at 0.1 Hz repetition frequency. The measuring light was applied only during the actual sampling interval. Its intensity was set reciprocal to the exposure interval to yield <5% excitation of reaction centers by measuring light pulse. Repetitive transient absorption changes were averaged on a TRACOR TN 1500 computer.

The electric potential difference was measured via the electrochromic absorption changes at 515 or 522 nm as described (25, 35). pH transients in the inner phase of thylakoids were measured via the "pH_{in}-indicating absorption changes of neutral red." The outer phase was selectively buffered by addition of bovine serum albumin at 1.3 g/liter. Absorption changes were recorded at a wavelength of 522 or 548 nm as the difference between transient signals obtained in the presence (13 μM) and in the absence of neutral red as described (36, 37). The exclusive response of neutral red to pH transients in thylakoids and the absence of any response to other events (e.g., redox changes) have been demonstrated (37). pH transients in the outer phase were measured via the absorption changes of phenol red at 548 nm (38).

RESULTS

Extraction of CF1. Extraction of CF1 from spinach was measured as a function of the EDTA concentration present during the 2 min of incubation in distilled water. The extraction curve showed a sigmoidal shape with the following properties. At a very low EDTA concentration (e.g., 10 μM) it was essentially flat, with an extraction of between 1% and 5% of total CF1. At 40 μM EDTA a rise became apparent, which saturated around 100 μM with a midpoint at 70 μM. The saturation level was checked at 750 μM EDTA. It varied between 70% and 90% of total CF1 depending on the preparation. The degree of extraction at a very low EDTA concentration and the shape of the sigmoidal curve differed greatly as a function of the starting material, the magnesium concentration, and the pH during preparation. The EDTA concentration had to be adjusted for each batch of spinach or peas.

Subunit Composition of Extracted CF1. The subunit composition of extracted CF1 was analyzed as described. We obtained the following molar proportions of the five subunits (α, β, γ, δ, and ε, with the amount of γ normalized to 1): at 25 μM EDTA, 1.6, 2.0, 1.0, 0.2, and 1.4; and at 750 μM EDTA, 1.8, 2.0, 1.0, 0.9, and 1.3. Under mild extraction—i.e., at a low EDTA concentration—the proportion of δ was lower by a factor of 4.5.

Flash-Induced Transients of the Electric Field, Proton Deposition Inside, and Proton Uptake from Outside. Fig. 1 shows the time course of the electric field (top traces) and of the pH changes in the inner phase of thylakoids (middle traces) in response to a Xenon flash. Inspection of the internal pH transients of the control (middle traces, −EDTA) shows the biphasic rise of the internal acidification known from previous work (36). The slow rise was attributed to the oxidation of plastoquinol by photosystem I via the cytochrome b_6–f–complex. The rapid rise was attributed to water oxidation. Proton release by water oxidation shows a complex kinetic behavior that is the result of contributions of several partial reactions (for details see ref. 39). It is apparent that incubation with EDTA (20 μM) selectively eliminated one part of the *rapid* acidification. The extent of the electric potential difference was diminished to a lesser extent and the acceleration of its decay was negligible in the given time domain (30 ms). The difference between the middle traces, which represent the "lost protons," is shown in the bottom trace of Fig. 1. It revealed high selectivity for rapidly liberated (water oxidation) protons. Repetition of this experiment under conditions in which proton deposition was entirely due to water oxidation (upon addition of 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone and with ferricyanide) confirmed this view (data not shown).

Fig. 2 shows the time course of the electric potential dif-

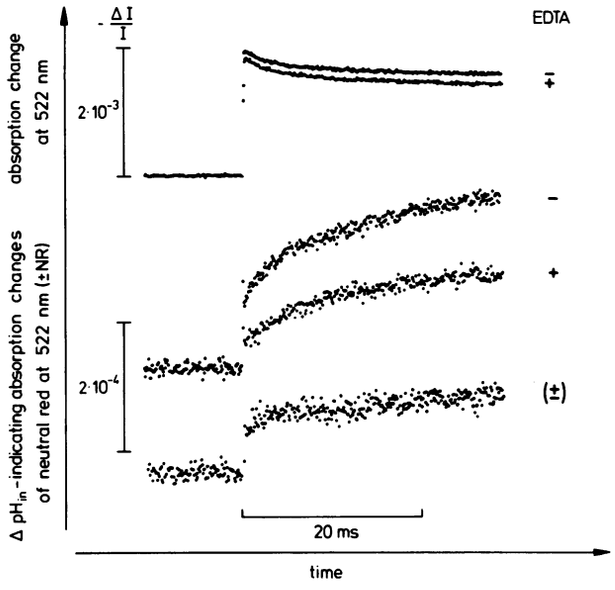

FIG. 1. Electrochromic absorption changes (top traces) and pH_{in}-indicating absorption changes of neutral red (NR) (middle traces) in EDTA-treated (20 μM) spinach chloroplasts and controls (−EDTA). The bottom trace represents the arithmetic difference of the traces in the middle. pH 7.6; bovine serum albumin at 1.3 g/liter.

ference (top traces) and of the internal acidification (bottom traces). Note that the time domain is about 10-fold wider than in Fig. 1 to demonstrate the effect of EDTA on the decay of the electric potential difference. All traces were obtained for material from which CF1 was mildly extracted by addition of EDTA. When DCCD was added, the effects of EDTA treatment were reversed. DCCD restored the detectability of proton release inside, it eliminated the small diminuation of the extent of the electric potential difference, and it reversed the slight acceleration of its decay.

At two sites chloroplasts take up protons from the outer medium. Each site is associated with one of the two photosystems (see ref. 26 for review). The time course of the ex-

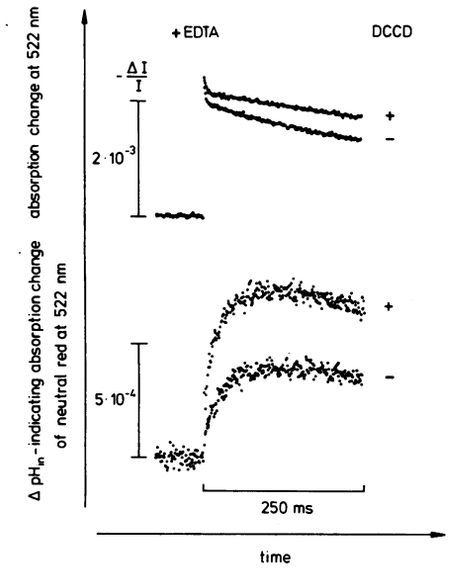

FIG. 2. Electrochromic absorption changes (top traces) and pH_{in}-indicating absorption changes of neutral red (bottom traces) in EDTA-treated (20 μM) spinach chloroplasts in the absence and presence of 10 μM DCCD. Other conditions as in Fig. 1.

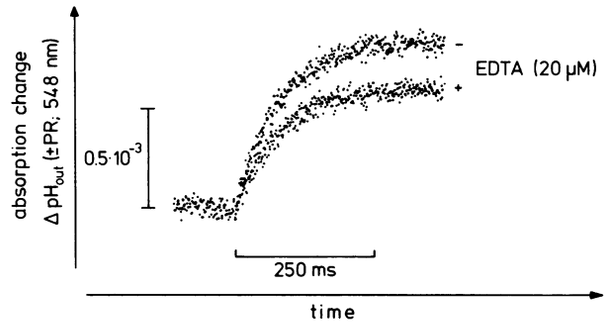

FIG. 3. pH_{out}-indicating absorption changes of phenol red (PR) in EDTA-treated and control chloroplasts. (These traces were recorded in the absence of bovine serum albumin in the suspension medium.)

ternal alkalization reflects this uptake and its compensation by the leakage of inside-released protons into the outer phase across the thylakoid membrane. We measured the external alkalization via phenol red. As shown in Fig. 3 the extent was diminished in EDTA-treated chloroplasts. The apparent rise of the external alkalization was rather slow, as usual (38). Numerical simulation showed that the enhanced membrane permeability for protons did account for the apparent diminution of proton uptake from outside. To find the total number of protons that were produced inside (in EDTA-treated chloroplasts), we repeated the measurement that is shown in Fig. 3, with the addition of 10 mM gramicidin. This speeded up proton leakage across the thylakoid membrane (not shown). We found that the net proton production canceled to zero. These two observations demonstrated that protons, which appeared to be lost when measured inside, were in fact produced. They had transiently escaped detection inside, but they later appeared outside.

With the idea that the protons that transiently had escaped detection were buffered away, we titrated the buffering capacity. The result is shown in Fig. 4. Points were obtained for two different chloroplast preparations (spinach and pea). Lines were calculated according to the following equation:

$$[A^{n-}]/[A_t] = [1 - 10^{n(pK-pH)}]^{-1},$$

which describes the deprotonation of an acid with n equivalent groups that can be protonated in strict cooperativity. $[A^{n-}]$ denotes the concentration of the base, $[A_t]$ is the total concentration, and K is the (geometrical) average dissociation constant of one group. The fit curves were obtained for $n = 6$ and for pK values of 7.35 and 7.70, respectively. For comparison, the slope of a univalent pH titration is indicat-

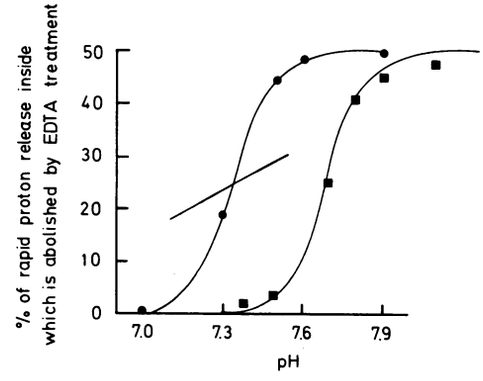

FIG. 4. Relative extent of rapid proton release that became undetectable upon treatment of chloroplasts with EDTA (mild extraction conditions) as a function of medium pH during a spectrophotometric experiment. ●, Spinach; ■, peas.

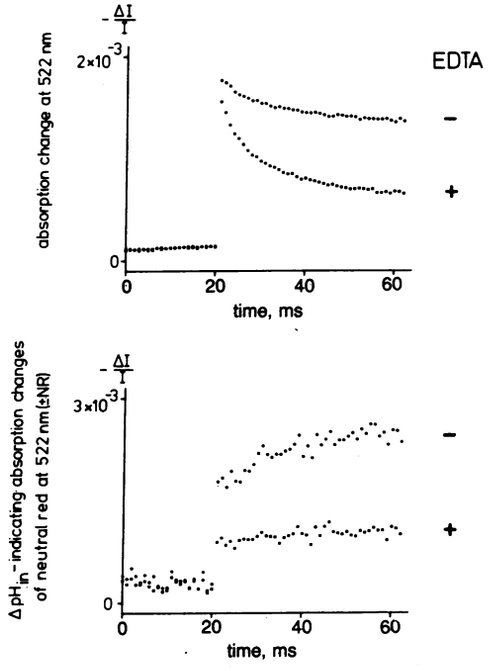

FIG. 5. Electrochromic absorption changes (top traces) and pH_{in}-indicating absorption changes of neutral red (NR) (bottom traces) in pea chloroplasts, which were about 20% extracted by treatment with 390 μM EDTA, and in the control. pH 7.9; bovine serum albumin at 2 g/liter.

ed. It should be mentioned that the steep pH dependence could not be attributed to the pH-indicating dye, neutral red, because its titration curve was univalent (18).

We also followed the described effects at higher degrees of extraction. The experiments were carried out with pea chloroplasts, which required higher concentrations of EDTA than spinach chloroplasts to yield similar effects. Under the conditions shown in Fig. 5, 20% of total CF1 was extracted. The two major features of the situation at milder extraction were reproduced: selective disappearance of protons from water oxidation (bottom traces) and slight reduction of the initial extent of the electric potential difference (top traces). However, the decay of the electric potential difference was accelerated. Under more severe extraction conditions, after treatment of chloroplasts with NaBr, >90% of total CF1 was removed from the membrane. We observed no proton release while the decay time of the electric potential difference became as short as 15 ms (unpublished data). A common feature of these observations was that protons liberated inside escaped detection by neutral red more rapidly than the electric charge crossed the membrane—in short, "buffering by CF0 preceded transport."

One might argue that the reported apparent losses in proton liberation and electric potential generation were caused by inactivation of some photosystems II and that they were not the unique consequence on CF0 of CF1 extraction. To test this possibility we measured the oxygen-evolving capacity of EDTA-treated chloroplasts in the presence of 10 nM gramicidin with 3 mM NH_4Cl as uncouplers. Under saturating light the rates varied between 190 and 240 μequivalents/mg of chlorophyll per second from one preparation to the next. However, there was <10% difference between controls and those samples that were subjected to mild EDTA treatment.

DISCUSSION

We applied *spectrophotometric techniques* to measure electric events and proton deposition into thylakoids. They were based on membrane-bound indicators: the electrochromic absorption changes result from lutein–chlorophyll *b* complexes (40) that are associated with photosystem II. The pH indicator that we used for the internal pH, neutral red, is highly membrane soluble and it intrinsically responds to the surface pH (28). Interpretation of the data relies on whether these two indicators respond to localized or delocalized events. We have previously demonstrated (25) that the electrochromic absorption changes around 520 nm reflect an event that is delocalized over a membrane patch containing at least 10^5 chlorophyll molecules. We do not know the velocity for the lateral spread of a pH difference. However, we showed that neutral red responded to pH transients in the inner aqueous bulk phase within <100 μs (28).

The experiments showed that EDTA treatment of chloroplasts caused proton release from water oxidation transiently to escape detection and that the initial extent of the electric potential difference was diminished. Two properties led us to attribute this to CF0 proper: (*i*) DCCD, which in addition to binding to other chloroplast proteins, blocks the proton channel in CF0, reversed the effects. (*ii*) The effects showed a most peculiar pH dependence. The only other chloroplast reaction for which a cooperativity of 6 was claimed was the block of the proton channel in CF0 by DCCD (21). The kinetics of the measured pH changes in the external phase could be simulated by assuming that they reflected proton uptake and proton efflux from inside with a velocity equal to the slightly accelerated decay velocity of the electric potential difference. Net proton production canceled to zero. In conclusion, the transient disappearance of protons from water oxidation, which was caused by mild EDTA treatment, reflected the opening of a very efficient extra buffering capacity in CF0. It was not caused by inactivation of their source.

What are the properties of the extra buffering capacity in CF0 and of the conducting channel?

(*i*) At first we asked whether the number of protons that had transiently escaped detection were accounted for by the number of CF1 units that were extracted. Under the experimental situation of Fig. 1, we observed disappearance of ≈50% of proton release from water oxidation and this was paralleled by extraction of 10% of CF1. Taking the published numbers for the ratio of chlorophyll to photosystem II and for the ratio of chlorophyll to CF1 (42, 43), we find that the ratio of photosystem II to CF1 for mature spinach is between 1.5 and 1.0. Under excitation with short light flashes, each photosystem II liberates 1 proton per flash (reviewed in ref. 26). In EDTA-treated chloroplasts the equivalent of 0.5 proton per photosystem II escaped detection. This is then equivalent to 5–7.5 lost protons per extracted CF1. This corresponds to the observed cooperativity of 6.

(*ii*) The above considerations suggested that each CF1-free CF0 rapidly captured several protons and that it selected protons originating from water oxidation. Even with the steep pH titration profile (demonstrated in Fig. 4), this can be expected only if the flash-induced pH jump, which is experienced by each active CF0, exceeded 0.5 unit. This implies that the local pH change in the vicinity of photosystem II is transiently much higher than the average change in the lumen [0.06 unit (37)]. How can one visualize an "injection of protons from water oxidation into CF0? For well-stacked thylakoids, CF1 and photosystem II (with the water-oxidizing enzyme) are in different portions of the membrane (reviewed in ref. 44). There are two possibilities for special contact between photosystem II and CF0. (*a*) When bulky CF1 is extracted, CF0 may move into the appressed membrane domains, where it may come into close contact with photosystem II. (*b*) Protons may be laterally conducted from photosystem II into CF0 through special domains in the membrane. Proton-conducting domains were previously pro-

posed by Prochaska and Dilley (45) after they observed that photosystem II protons modulated the degree of derivatization of subunit III of CF0 by acetic anhydride. The first possibility has the advantage that it can be verified or rejected with established separation techniques for apressed and unapressed membranes (46).

(*iii*) In EDTA-treated chloroplasts the initial extent of the electrochromic absorption changes was always lower than in the controls (or in material, where the effects were reversed by DCCD). From previous work it is known that the electrochromic absorption changes are pseudolinear in terms of their extent over charges translocated (reviewed in refs. 26, 27, 35). In controls the voltage induced by one saturating flash corresponds to the translocation of two unit charges per 550 chlorophyll molecules (47, 48). The same set of molecules produces one proton from water oxidation. Note that there was no slow rise of the electric potential and no cyclic electron flow in our chloroplasts and with the electron acceptors used! The observed 10% decrease of the electric potential is equivalent to the passage to 0.2 charge across the membrane, whereas the observed 50% disappearance of rapidly released protons from water oxidation is equivalent to 0.5 charge. More protons disappeared in the inner phase than can be accounted for by their passage across the full span of the membrane capacitor. In other words, the extra buffering capacity in CF0 may be near the middle of the membrane.

(*iv*) It is tempting to identify the hexacooperative buffering groups with the DCCD-binding glutamyl residue of subunit III of spinach CF0 (see ref. 16). Based on a hydropathy analysis, Sebald and Hoppe (16) placed this residue near the middle of one of the two lipophilic stretches of this molecule. This would conform with the above postulated location of the buffering groups in the membrane.

(*v*) We mention an interesting tendency apparent from our experiments. When comparing the effects of low (Fig. 1) and high extraction (e.g., Fig. 5), we found that once a level was reached where approximately half of total proton release by water oxidation became invisible to neutral red, further extraction only accelerated the decay of the electric potential difference. This acceleration was correlated with the appearance of the δ subunit with extracted CF1. It is possible that δ acted as plug for the electric leak through CF0.

(*vi*) Previously we had observed another transient trapping of protons from water oxidation, which we attributed to special domains in thylakoids (41). The extra buffering capacity that was found in those other experiments was distinguished from the one reported in this work in three ways: (*a*) it became accessible only through gramicidin or other uncouplers; (*b*) it lacked DCCD sensitivity; and (*c*) its pH titration showed lower cooperativity (of 2). However, it cannot be ruled out that the two types of selective buffering capacities are related to each other.

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