Flow–Force Relationships in Lettuce Thylakoids. 2. Effect of the Uncoupler FCCP on Local Proton Resistances at the ATPase Level

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ABSTRACT: The relationship between the steady-state proton gradient (ΔpH) and the rate of phosphorylation was investigated in thylakoids under various conditions. Under partial uncoupling by carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone (FCCP), the rate of ATP synthesis was reduced by less than expected from the decrease of ΔpH. This was observed in the case of the pyocyanine-mediated cyclic electron flow around photosystem 1, but not with the $H_2O \rightarrow$ photosystem 2 → cytochrome $b_6f \rightarrow$ photosystem 1 → methyl viologen system. In state 4, a unique relation was found between ΔpH and the "phosphate potential", ΔG_p, regardless of whether the energy level was controlled by light input or FCCP. The anomalous effect of FCCP on the rate of ATP synthesis disappeared when the ATPase was partially blocked by the reversible inhibitor venturicidin, but not in the presence of tentoxin, an irreversible inhibitor. These results are consistent with the existence of a small kinetic barrier for protons, limiting their access to the ATPase. This resistance would be collapsed by FCCP.

Whether the proton gradient is fully delocalized or not in biomembranes remains on open question [for reviews, see Westerhoff et al. (1984a), Ferguson (1985), and Haraux (1985)]. One way to investigate this problem is to examine if the rate of ATP synthesis is univocally related to the macroscopic proton gradient, $\Delta\tilde{\mu}_{H^+}$.[1] Early experiments carried out on thylakoids (Pick et al., 1974) or bacterial chromatophores (Casadio et al., 1978) have shown that when $\Delta\tilde{\mu}_{H^+}$ was diminished to a certain value by adding an uncoupler (FCCP) or by lowering the electron flow, a higher steady-state rate of ATP synthesis was obtained in the partially uncoupled system. The same kind of data was obtained with nigericin in thylakoids (Sigalat et al., 1985). Similar results were published for mitochondria (Mandolino et al., 1983; Zoratti & Petronilli, 1985; Petronilli et al., 1991). In chloroplasts, the relationship between the proton gradient and the phosphorylating flow was also found to depend on the nature of the uncoupler (Pick et al., 1987). However, other investigators found a unique relationship between $\Delta\tilde{\mu}_{H^+}$ and the rate of ATP synthesis, in thylakoids (Davenport & McCarty, 1986) or in mitochondrial membranes (Sorgato et al., 1985; Woelders et al., 1986, 1988). In the latter case, it was also shown that the apparent mode of coupling, localized or delocalized, depended on the probe used to monitor the membrane potential. This led to suspicion of artifactual measurements in the case where a localized pattern was found. This question of artifacts was raised in the case of thylakoids (Davenport & McCarty, 1986), but we have suggested that the conflicting results would be better explained by a delocalizing effect of the amines of high p*K* used to measure ΔpH but not always added at negligible concentrations (Sigalat et al., 1988).

The most widely invoked model of localized coupling is a moderate version of the "direct coupling" hypothesis (Williams, 1961, 1978), which assumes that the proton flow between the $\Delta\tilde{\mu}_{H^+}$ producers and consumers more or less short-circuits the aqueous compartments facing the membrane (Van Dam et al., 1978; Westerhoff et al., 1984a). This idea of a close interaction between redox pumps and ATPases was kept in a model especially stated for thylakoid membranes. Mainly based on kinetic experiments, it postulates that the high-potential sides of the redox and phosphorylating pumps are confined to a common microspace, not exchanging protons with the lumen under normal conditions (Baker et al., 1981; Dilley & Schreiber, 1984; Beard & Dilley, 1986; Dilley et al., 1987). Bound calcium and, in steady state, high ATP synthase activity would maintain the integrity of this microcompartment (Chiang & Dilley, 1987; Renganathan et al., 1991). At variance with this view of a semidirect coupling, we have proposed a model where the proton circulation from primary to secondary proton pumps is limited by lateral resistances, which leads to a small loss of energy [Haraux & de Kouchkovsky, 1983; Haraux et al., 1983; Sigalat et al., 1985; see also Hong and Junge (1983)]. This kinetic restriction would be especially pronounced in the case of PS2-driven $\Delta\tilde{\mu}_{H^+}$, because water-splitting enzymes and ATPases are located in distinct membrane areas [for a review, see Melis (1991)].

The semidirect coupling mechanism implies that the local value of the proton gradient is the same at the level of the redox and phosphorylating pumps and always higher than the macroscopic $\Delta\tilde{\mu}_{H^+}$. This should be true not only during net ATP synthesis but also at the equilibrium between ATP synthesis and hydrolysis, the so-called "state 4" (Chance & Williams, 1955). If this microchemiosmosis scheme is valid, then one expects, in state 4, a variable relationship between Gibbs free energy of phosphorylation (ΔG_p) and $\Delta\tilde{\mu}_{H^+}$, depending on how the energetic state is adjusted, by redox energy input or by artificial proton leaks. In mitochondria, such a variable relationship was observed by some groups (Azzone et al., 1978), whereas others found $\Delta\tilde{\mu}_{H^+}$ and ΔG_p

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[1] Abbreviations: $\Delta\tilde{\mu}_{H^+}$, transmembrane difference in electrochemical potential of protons; $\Delta\psi$, transmembrane difference in electrical potential; ΔpH, transmembrane pH difference; ΔG_p, Gibbs free energy of phosphorylation, or "phosphate potential"; PS1 and PS2, photosystems 1 and 2; (C)F_o, (C)F_1, membranous and soluble catalytic sectors of (chloroplast) F_oF_1-ATPase; DCCD, *N,N'*-dicyclohexylcarbodiimide; FCCP, carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; b_6f, cytochrome b_6f.

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univocally linked (Westerhoff et al., 1981; Woelders et al., 1985). In the case of mitochondria, the problem is complicated by the fact that, due to the energy-dependent translocation of substrates, ΔG_p is not the same inside and outside the matrix compartment. Until now, no such data were available for thylakoids.

In this report, we have investigated flow–force relationships (rate of ATP synthesis vs ΔpH) and force–force relationships (ΔG_p vs ΔpH) in limiting light or under partial uncoupling conditions. Flow–force relationships were also studied in the presence of nonsaturating amounts of energy-transfer inhibitors, reversible or not. The results are consistent neither with a fully delocalized coupling nor with the semidirect scheme. They rather suggest the existence of a kinetic barrier for protons surrounding the ATPase, which would be broken by uncouplers like FCCP.

MATERIALS AND METHODS

Extraction of Thylakoids. Envelope-free chloroplasts were extracted from mature green leaves of market lettuce (*Lactuca sativa* L.) as described in the preceding paper (Sigalat et al., 1993) and stored in the assay medium. The dense preparation, at a chlorophyll concentration of 1 mM, was kept on ice and in darkness before use. The preparation was absolutely stable in activity for at least 8 h.

Ca^{2+}-Treated Thylakoids. When indicated, 1 mM $CaCl_2$ was added to the storage medium. In this case, thylakoids were incubated for at least 2 h, on ice and in darkness, before use.

ΔpH and ATP Measurements under Steady-State Conditions. Phosphorylation rate and ΔpH were simultaneously measured in the same setup (Sigalat et al., 1993) at 20 °C. Unless otherwise indicated, the reaction mixture, slightly buffered to measure pH changes of the medium, contained thylakoids at 20 μM chlorophyll concentration, 0.2 M sorbitol, 2 mM Tricine (pH 7.8), 10 mM KCl, 6 mM $MgCl_2$, 2 mM K_2HPO_4, 10 μM diadenosine pentaphosphate, 0.5 mM ADP, 100 nM valinomycin, and 4 μM 9-aminoacridine. Pyocyanine (50 μM) or methyl viologen (100 μM) plus catalase (1.2 units mL^{-1}) was added to feed the electron-transfer chain. The maximal intensity of the saturating red actinic light was 1.5 kW m^{-2} in the first case, but could not exceed 150 W m^{-2} in the second due to some photoinhibition. When needed, the light intensity was adjusted with neutral-density filters. ΔpH was monitored by the quenching of the 9-aminoacridine fluorescence (Schuldiner et al., 1972). External pH changes were simultaneously recorded with a fast and sensitive glass electrode, and the steady-state rate of ATP synthesis, reached in 90–180 s, was calculated according to Nishimura et al. (1962). ADP was present before the light was switched on, except in the presence of tentoxin. In this latter case, ADP was added only after 3 min of illumination, because the presence of tentoxin and ADP before light led to an unstable residual activity. In state 4 experiments, ΔG_p was estimated from ATP titration by the luciferin-luciferase method (Lemasters & Hackenbrock, 1978), as described in the preceding paper (Sigalat et al., 1993). Thylakoids were assayed in the same medium as above, except that 10 mM Tricine (pH 7.8), 30 μM ADP, and 0.3 mM K_2HPO_4 were added.

Measurements of Light-Induced Proton Uptake. Thylakoids were diluted to 20 μM chlorophyll concentration in the same assay medium as above, except that Tricine and phosphate were omitted. Pyocyanine (50 μM) or methyl viologen (100 μM, in the presence of catalase, 1.2 units mL^{-1}) was used as artificial electron carrier. After a few minutes of incubation in darkness, each sample was illuminated for 2 min (red actinic light, 1.5 kW m^{-2} with pyocyanine, 150 W m^{-2} with methyl viologen) and then allowed to relax in the dark for 3 min. pH was continuously monitored with the glass electrode. To convert pH changes into equivalent protons, the suspension was titrated with HCl injections in each condition. Imidazole (0.5 mM), pyridine (2.5 or 5 mM), and arsenate (2.5 mM) indeed changed the buffering power of the suspension. Coupled conditions were achieved by adding arsenate. The proton flow across the ATPase is the same as with phosphate, which is indicated by the value of the steady-state ΔpH (not shown). The only difference is that the ADP–arsenate complex is cleaved in the medium immediately after being synthesized (Gresser, 1981), avoiding the net consumption of protons (Nishimura et al., 1962) normally linked to the condensation reaction, which would have interfered with the proton-uptake measurement.

Δψ Measurements under Flashing Light. Δψ was estimated from the transient electrochromic shift of carotenoid absorbance following a short saturating actinic flash (Witt, 1979), in an appropriate setup (Agalidis et al., 1987). Chloroplasts at 20 μM chlorophyll concentration were assayed at 20 °C in a medium containing 0.2 M sorbitol, 10 mM Tricine (pH 7.8), 10 mM KCl, 6 mM $MgCl_2$, 2 mM K_2HPO_4, 10 μM diadenosine pentaphosphate, and 50 μM pyocyanine. Other additions are mentioned in the captions of the figures. The sample was not stirred during the measurement. The weak analytic light, selected at 518 nm (bandwidth = 3 nm), was detected by a photomultiplier protected by a Corning 9782 filter (half-width 380–560 nm). The sample was illuminated by a train of 16 short saturating flashes (duration 20 μs) spaced by dark intervals of 3 s. The wavelength of the actinic light was centered around 650 nm with a Corning 2404 filter. Kinetics displayed in the figures are the average of the 16 successive signals.

Uncouplers and Energy-Transfer Inhibitors. Nigericin, valinomycin, venturicidin, and tentoxin were purchased from Sigma; FCCP was from Fluka. All these reagents were added to the thylakoid suspensions in small volumes of concentrated ethanolic solutions (no effect of ethanol alone was noticed).

RESULTS

Variable Relationship between ΔpH and the Rate of ATP Synthesis. Figure 1 shows the different relationships obtained between the steady-state ΔpH, generated by the pyocyanine-mediated cyclic electron transfer around PS1, and the rate of phosphorylation. To get these correlations, ΔpH was lowered from its maximal value, either by attenuating the light intensity or by adding FCCP or nigericin. It is clear that the rate of ATP synthesis is not a unique function of ΔpH but also depends on the way the proton gradient has been adjusted. We had already reported that a given ΔpH limited by nigericin addition drives a higher rate of photophosphorylation than the same ΔpH controlled by light intensity (Sigalat et al., 1985, 1988). This is fully confirmed here, and moreover, nigericin at low concentrations was found to stimulate ATP synthesis, as already observed by others (Giersch, 1983). One should remark that nigericin exchanges H^+ against K^+, which could increase the membrane potential, Δψ, at the expense of ΔpH, even in the presence of valinomycin, used at a low concentration. However, Figure 1 shows that with FCCP, which is a purely protonic uncoupler, ATP synthesis also proceeds at a higher rate than when the proton gradient is limited by the electron flow. These results are similar to those found on bacterial chromatophores (Casadio et al., 1978).

Before going further, one should verify that no side effects occur with FCCP. Interactions between FCCP and valino-

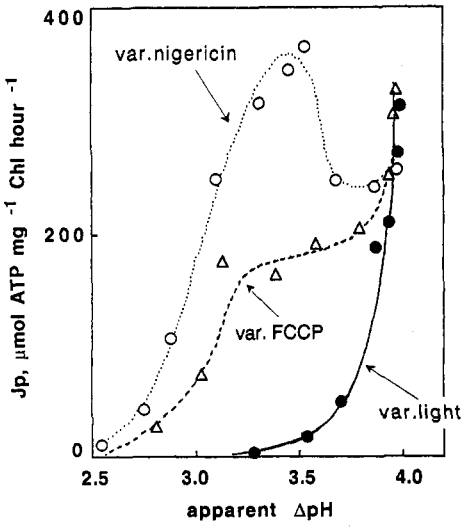

FIGURE 1: Rate of ATP synthesis versus ΔpH with pyocyanine as cyclic electron transfer mediator. Conditions were as described under Materials and Methods: (●) variable light intensity (10–1500 W m^{-2}), no uncoupler; (▲) constant light intensity (1500 W m^{-2}), variable FCCP concentration (10–10 000 nM); (○) constant light intensity (1500 W m^{-2}), variable nigericin concentration (20–2000 nM).

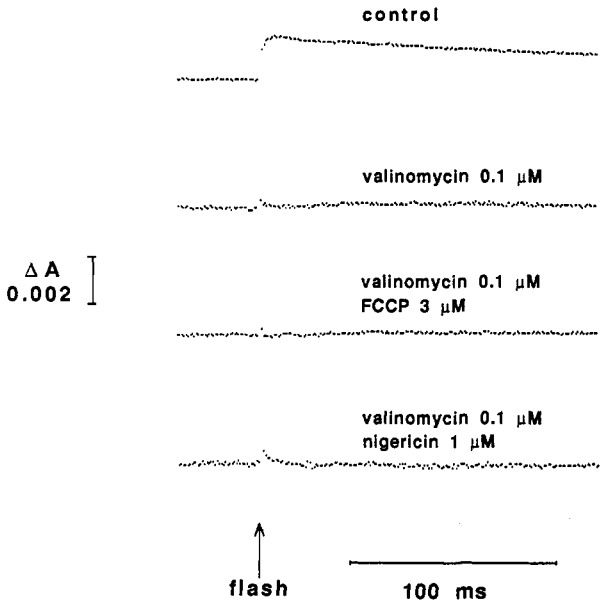

FIGURE 2: Effect of ionophores on kinetics of flash-induced absorbance changes at 518 nm. Conditions were as described under Materials and Methods. Ordinate, absorbance change at 518 nm (ΔA), positive upward; abscissa, time. Data were taken every 1 ms. Each trace is an average of 16 scans, each with a single flash.

mycin have actually been observed under conditions of high K^+ concentration (0.8 M or more), forming a neutral ternary complex, valinomycin·K^+·$FCCP^-$ (O'Brien et al., 1978). In such a case, FCCP could be expected to restore a $\Delta\psi$ abolished by valinomycin (Lerner et al., 1982). But under our conditions, that is, in 10 mM KCl, we would have instead a synergistic effect of the two uncouplers in mediating cation and proton transport through the membrane (Kessler et al., 1977; Yamaguchi & Anraku, 1978; Ahmed & Krishnamoorty, 1990). Nevertheless, we have checked the possibility of $\Delta\psi$ restoration by looking at the effect of different combinations of uncouplers on the light-induced $\Delta\psi$, generated by a short flash and monitored by the absorbance change at 518 nm. The results are depicted in Figure 2. As expected with this time resolution, valinomycin almost completely erased the $\Delta\psi$-related signal. An additional injection of FCCP not only failed to restore the membrane potential but more completely

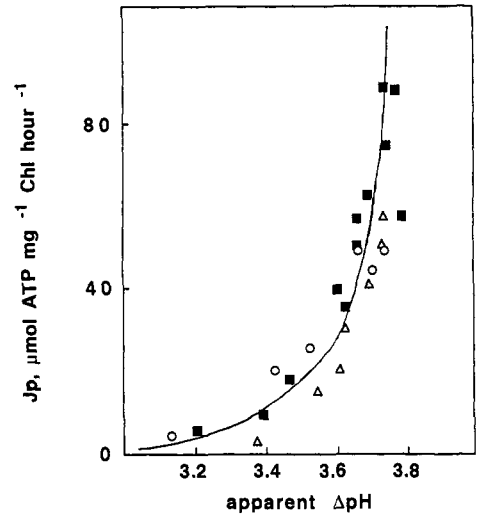

FIGURE 3: Rate of ATP synthesis versus ΔpH with methyl viologen as final acceptor of electrons coming from H_2O through the whole redox chain. Conditions were as described under Materials and Methods: (■) variable light intensity (5–150 W m^{-2}), no uncoupler; (▲) constant light intensity (150 W m^{-2}), variable FCCP concentration (10–600 nM); (○) constant light intensity (150 W m^{-2}), variable nigericin concentration (2–100 nM).

dissipated it, as expected. Figure 2 also shows that nigericin added together with valinomycin slightly restored $\Delta\psi$, which might explain the difference between the nigericin and FCCP curves in Figure 1. In conclusion, the results with FCCP in Figure 1 cannot be due to an inactivation of valinomycin by this uncoupler and more surely reflect the existence of localized proton gradients, due to resistances for protons in the thylakoid lumen. The $\Delta\tilde{\mu}_{H^+}$ across the F_oF_1-ATPases, responsible for ATP synthesis, would then differ from the mean $\Delta\tilde{\mu}_{H^+}$, represented by the measured ΔpH ($\Delta\psi$ being negligible).

Differences between ΔpH Generated by Cyclic and Linear Electron Flows. We have previously shown (Sigalat et al., 1985, 1988) that the apparent efficiency of the proton gradient depends on the proton source, namely, PS1 or PS2 due to their different localizations with respect to the ATPases. It is then important to examine if the localized behavior observed in Figure 1 with a cyclic electron transfer around PS1 is still found with another proton gradient generator. Especially interesting is the whole linear redox chain including PS2, b_6f complex, and PS1. Indeed, we have shown in the preceding paper (Sigalat et al., 1993) that this chain is regulated by ΔpH (or rather by internal pH) in an apparently delocalized mode. So we have checked the relationship between ΔpH and ATP synthesis, as in Figure 1, but with methyl viologen as a terminal PS1 electron acceptor. Methyl viologen was used here preferentially to ferricyanide, because the electron transfer from water to methyl viologen does not give rise to a scalar production or consumption of H^+, which would have interfered with the ATP synthesis measurement. The results of a typical experiment are shown in Figure 3. Contrary to the ΔpH generated by the cyclic electron transfer around PS1, the proton gradient produced by the linear H_2O → methyl viologen chain seems to be delocalized: a unique relationship now exists between the measured value of ΔpH and the rate of phosphorylation. On the other hand, the results obtained in Figure 1 with pyocyanine as an electron carrier were confirmed in this chloroplast preparation (not shown).

A weakness of this comparison is that the maximal phosphorylating proton flow is quite different with the two systems: there is almost a factor of 4 between the highest rate of ATP synthesis generated by the cyclic and linear electron-transport chains. This is because the linear electron flow is

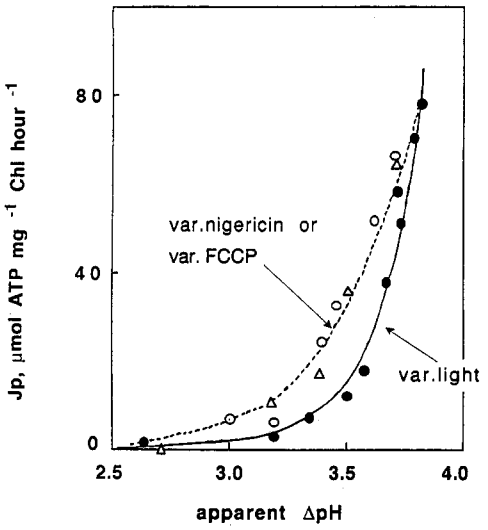

FIGURE 4: Rate of ATP synthesis versus ΔpH with pyocyanine as electron carrier, in low light. Conditions were as described under Materials and Methods: (●) variable light intensity (1–50 W m^{-2}), no uncoupler; (Δ) constant light intensity (50 W m^{-2}), variable FCCP concentration (100–1000 nM); (○) constant light intensity (50 W m^{-2}), variable nigericin concentration (5–70 nM).

strongly regulated by the internal pH at the b_6f level. From elementary ohmic considerations, it is not surprising that $\Delta\tilde{\mu}_{H^+}$ singularities, due to local proton resistances, are minimized when the maximal proton flow generated by the redox carriers is low. So, to make a better comparison between PS1 and PS2+PS1 conditions, we have repeated the experiment with pyocyanine as cyclic electron carrier, but under conditions where the maximal proton flow was the same as that catalyzed by transfer from water to methyl viologen. This was achieved simply by lowering the maximum light intensity by a factor around 30. Then, under this new control condition, ΔpH was diminished either by further decreasing the light input or by adding an uncoupler. The resulting flow–force relationships are shown in Figure 4. The efficiencies of the ΔpH modulated by partial uncoupling and by the electron flux were less different than in Figure 1, as expected from the decrease of the proton currents through the local resistances. However, the localized behavior was only attenuated, and the situation remained different from that observed with the $H_2O \rightarrow$ methyl viologen chain, although the maximum phosphorylating proton flow now was the same. Uncouplers like FCCP seem thus to exert a specific effect on the PS1-driven protonic coupling.

Accessibility of the Acidic Compartment to Amines. We have tried to detect in our thylakoid preparations the presence of sequestrated acidic domains such as those studied by Dilley's group (Baker et al., 1981; Dilley & Schreiber, 1984; Beard & Dilley, 1986; Dilley et al., 1987). An indication for the existence of such microdomains is the lack of stimulation of the light-induced proton uptake by permeant amines such as pyridine, especially under phosphorylating conditions (Renganathan et al., 1991). Such a stimulation should normally occur when the amine is trapped in the acidic compartment in response to ΔpH (Pick & Avron, 1976), and its absence has been interpreted as the existence of distinct compartments for amines and protons; nigericin at very low concentrations (nanomolar) would restore the amine-dependent proton uptake by equilibrating the lumen with the postulated H^+ microdomains (Allnutt et al., 1991). We have therefore measured the light-induced proton uptake by thylakoids in the absence and in the presence of two amines, pyridine (pK = 5.2) and imidazole (pK = 6.9). Under coupled conditions the scalar proton consumption due to phosphorylation was avoided by replacing phosphate by arsenate. Table I shows the results of two typical experiments carried out with the cyclic PS1-driven electron flow in the presence of pyocyanine (expt A) and with the linear PS2+PS1-driven electron flow from H_2O to methyl viologen (expt B). Under all conditions, we obtained a small but sizeable additional proton uptake with pyridine (in experiment B, due to the lower ΔpH, the pyridine concentration was doubled to increase the sensitivity). A more important stimulation of the proton uptake was obtained with imidazole. This was expected because, at given ΔpH, the maximal proton uptake is theoretically obtained when the external pH equals the pK of the amine (Pick & Avron, 1976). Given their pK's, the additional proton uptake induced by imidazole should be 8 times (expt A) or 4 times (expt B) that obtained with pyridine. This was verified with satisfactory accuracy. In all cases nigericin had no effect at this low concentration (1 nM).

Thus, under our conditions, pyridine and imidazole stimulated the light-induced proton uptake in a strictly conventional way. We have repeated the experiments with thylakoids stored for 2 h in the presence of 1 mM Ca^{2+}, which should favor the sequestration of H^+ in microdomains (Chiang & Dilley, 1987). Yet, with cyclic (PS1) or linear (PS2+PS1) electron flow, we found exactly the same result as with our standard protocol (not shown).

Unique Relationship between ΔpH and the Phosphate Potential, ΔG_p, in State 4. At this stage, the anomalous effect of uncouplers may be explained in two different ways: (1) A very localized proton circuit links PS1 pumps and ATPases, which is little sensitive to membrane leaks created by uncouplers; this is the most popular microchemiosmotic model (Van Dam et al., 1978; Westerhoff et al., 1984a,b), very close to the direct coupling idea (Williams, 1978). (2) A diffusion barrier for protons exists between the proton sources and the ATPases (Haraux & de Kouchkovsky, 1983), and like other proton carriers such as amines (Sigalat et al., 1988), FCCP lowers this local resistance, allowing the ATPase to run with a higher efficiency.

In the first case, FCCP fails to dissipate the $\Delta\tilde{\mu}_{H^+}$ across F_oF_1, which is supposed to be higher than the mean $\Delta\tilde{\mu}_{H^+}$. In the second case, FCCP suppresses a $\Delta\tilde{\mu}_{H^+}$ deficit which locally existed at the ATPase level. These two interpretations oppose each other from an energetic point of view and lead to different predictions. If a semidirect coupling exists, one expects that $\Delta\tilde{\mu}_{H^+}$ across F_oF_1 resists uncouplers even in state 4 (Chance & Williams, 1955), i.e., at the equilibrium between ATP synthesis and hydrolysis, when no net proton flow crosses the ATPases. So the relationship between $\Delta\tilde{\mu}_{H^+}$ and the phosphate potential, ΔG_p, in state 4 should be different when the proton gradient is varied by proton output, i.e, uncoupling, or input, i.e., electron flow lowering (Westerhoff et al., 1981). In the case of a proton resistance between the generator and the ATPase, and especially of a proton inlet resistance at the ATPase level, a quite different behavior is expected. Contrary to what is believed to occur during net ATP synthesis, a negligible $\Delta\tilde{\mu}_{H^+}$ deficit should exist at the site of ATPase in state 4. Indeed, for ohmic reasons, such a deficit is linked to the net proton flow through the inlet resistance. FCCP, believed to break this kinetic barrier, should not affect the relationship between $\Delta\tilde{\mu}_{H^+}$ and ΔG_p in state 4.

In Figure 5, experimental values of ΔG_p in state 4 are plotted against $\Delta\tilde{\mu}_{H^+}$, the latter being generated by PS1 (pyocyanine loop). The force–force relationships are very close when the proton gradient is varied by FCCP or light. At first sight, this corroborates our previous view of a H^+ resistance separating redox carriers and ATPases (Haraux & de Kouchkovsky, 1983), but not the semidirect coupling scheme. One may

Table I: Effect of Permeant Amines on Light-Induced Proton Uptake and Apparent ΔpH in Different Steady States[a]

		H^+ uptake, mmol (mol of Chl)$^{-1}$ (glass electrode)			apparent ΔpH (9-aminoacridine)		
expt		control	imidazole, 0.5 mM	pyridine, 2.5 (expt A) or 5 mM (expt B)	control	imidazole, 0.5 mM	pyridine, 2.5 (expt A) or 5 mM (expt B)
A	basal	289	669 [+380]	327 [+38]	4.27	4.08	4.07
	coupled	234	526 [+292]	272 [+38]	4.00	3.96	4.05
	coupled + 1 nM nigericin	252	501 [+250]	290 [+38]	4.01	4.02	4.00
B	basal	161	491 [+330]	235 [+74]	4.17	4.06	4.09
	coupled	166	376 [+210]	224 [+58]	3.90	3.86	3.87
	coupled + 1 nM nigericin	164	360 [+196]	209 [+45]	3.89	3.86	3.85

[a] Conditions were described under Materials and Methods. Experiment A: pyocyanine, 50 μM; light intensity = 1.5 kW m^{-2}. Experiment B: methylviologen, 100 μM; light intensity = 150 W m^{-2}. (Experiments A and B were performed on two different chloroplast preparations.) Pyridine concentration was 2.5 mM (expt A) or 5 mM (expt B). Basal state, without arsenate; coupled state, with 2.5 mM arsenate (ADP present in both cases). Numbers in brackets refer to the additional proton uptake due to the amine; they were obtained by subtracting the value of the control from the total uptake. The precision of each measurement is about 10 mmol of proton per mole of chlorophyll. The apparent ΔpH values in the right columns were computed from the fluorescence of 9-aminoacridine, monitored on the same sample.

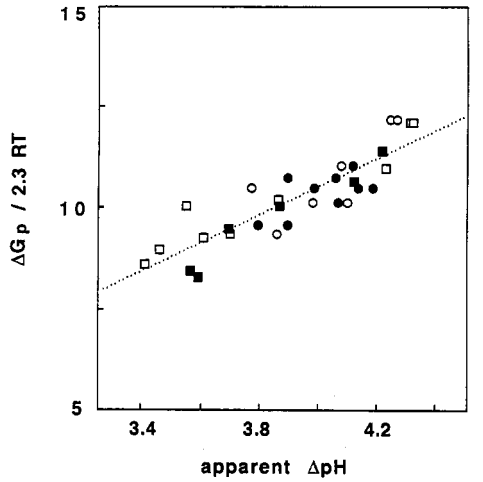

FIGURE 5: "Phosphate potential", ΔG_p, versus ΔpH with pyocyanine as electron acceptor. Conditions were as described under Materials and Methods. (■, ●) variable light intensity (5–1500 W m^{-2}), no uncoupler; (□, ○) constant light intensity (1500 W m^{-2}), variable FCCP concentration (50–1000 nM). Squares and circles refer to two different experiments.

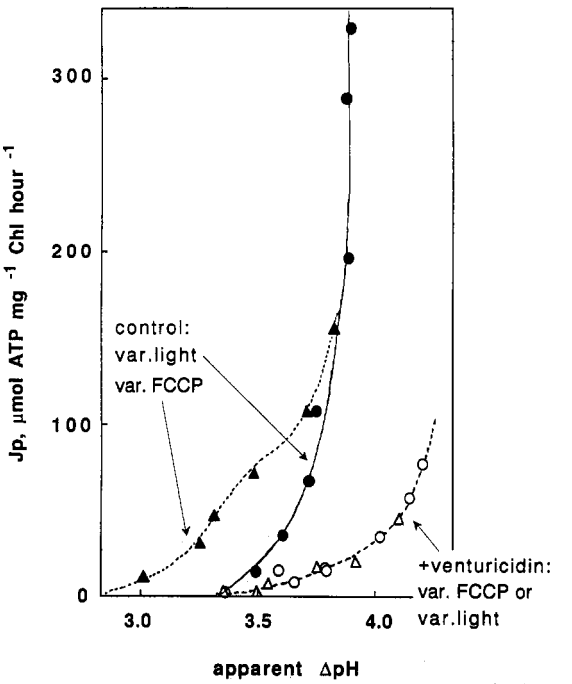

FIGURE 6: Rate of ATP synthesis versus ΔpH with or without venturicidin. Conditions were as described under Materials and Methods, with pyocyanine as electron acceptor: (●, ○) variable light intensity (10–1500 W m^{-2}), no uncoupler; (▲, △) constant light intensity (1500 W m^{-2}), variable FCCP concentration (100–5000 nM); (●, ▲) no inhibitor; (○, △) 0.3 μM venturicidin.

object, however, that the state 4 experiment was not performed under conditions strictly identical to those of Figure 1, that is, the ΔpH range is not the same (3.5–4.5 instead of 2.5–4.0). On the one hand, higher ΔpH's were obtained in state 4, due to the lack of net proton flow through F_oF_1. On the other hand, it was not possible to explore ΔpH below 3.5, because the time required to reach state 4 became too long, and less than 10% of the ADP was phosphorylated, which resulted in important uncertainties in ΔG_p determination. As a consequence, the FCCP concentration could not exceed 1 μM, which is only in the range where the flow–force curves start to diverge in Figure 1. So the data shown in Figure 5 might not have been completely relevant for our purpose. For this reason, we have measured the initial rate of ATP synthesis, easier to estimate than ΔG_p, but now under conditions close to state 4, i.e., with a very low proton flow through ATPase, limited at the F_oF_1 level.

Unique Relationship between ΔpH and the Rate of ATP Synthesis Slowed Down by a Reversible Energy-Transfer Inhibitor. A good way to specifically slow down the proton flow through the ATPase complex is to use a reversible energy-transfer inhibitor at a limiting concentration. It is important, for this purpose, that all the enzymes interact with the inhibitor but at different times. In this case, an 80% inhibition reduces the open probability of each enzyme in this proportion, which does correspond to a true diminution of the proton flow through the complexes. Venturicidin, which interacts with subunit c of the F_o proton channel (Galanis et al., 1989) in a reversible way (Bizouarn et al., 1990), seems to fulfill this condition (Schönknecht et al., 1989; Bizouarn et al., 1990; Valerio et al., 1992). So we have carried out the same experiment as in Figure 1, except that ATP synthesis was dramatically decreased with venturicidin. The results are plotted in Figure 6. Whereas the control exhibits the same behavior as that shown in Figure 1, in the venturicidin-treated samples the rate of phosphorylation now strictly depends on the ΔpH, independently of the presence of FCCP up to 5–10 μM. Thus, FCCP used at the same concentrations as in Figure 1 does not seem to affect the distribution of protons between the ATPases and other domains of the membranes. This is consistent with data obtained at state 4. Once again, this result supports the view of an inlet resistance at the ATPase level which is disrupted by FCCP.

Variable Relationship between ΔpH and the Rate of ATP Synthesis Slowed Down by an Irreversible Energy-Transfer Inhibitor. If an inlet resistance exists at the ATPase level, it would be interesting to know whether it embeds an important pool of enzymes sharing the same proton microspace or if it

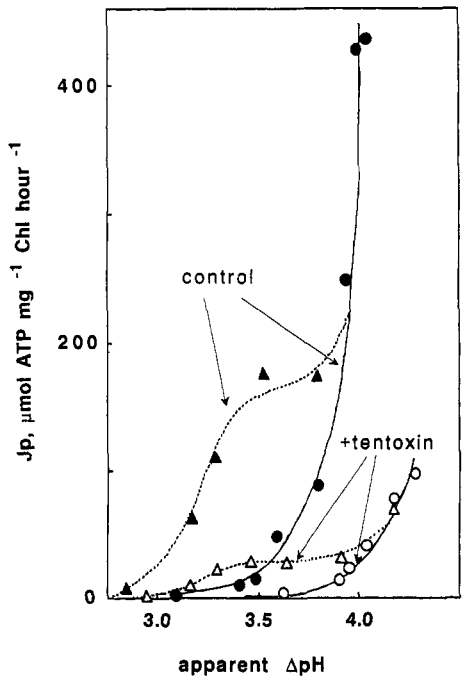

FIGURE 7: Rate of ATP synthesis versus ΔpH with or without tentoxin. Conditions were as described under Materials and Methods, with pyocyanine as electron acceptor: (●, ○) variable light intensity (10–1500 W m^{-2}), no uncoupler; (▲, △) constant light intensity (1500 W m^{-2}), variable FCCP concentration (400–10 000 nM); (●, ▲) no inhibitor; (○, △) 0.4 µM tentoxin.

tends to separate each F_oF_1 complex from others. If the enzymes are pooled, the permanent inhibition of a given ATPase should diminish the local ΔpH deficit across all the enzymes of the same microdomain. So one expects that the specific effect of FCCP on the flow–force relationship disappears when an important fraction of the ATPases has been irreversibly inhibited. To check this possibility, we have used the CF_1 inhibitor tentoxin. At low concentrations (submicromolar range), tentoxin inhibits the chloroplast ATPase. It becomes an activator at much higher concentrations. The activation is reversible (Steele et al., 1978), but not the inhibition (Bizouarn et al., 1990). Thus the difference with an inhibitor like venturicidin is that here 80% inhibition means that 20% of the enzymes remain fully active, the others being closed in a permanent way. Figure 7 shows the effect of FCCP on the flow–force relationship when the main part of the activity has been inhibited by tentoxin. Contrary to the case of venturicidin, the specific effect of FCCP was here preserved: as in the control, when the ΔpH was adjusted by FCCP instead of light, a higher rate of phosphorylation was obtained. A similar pattern was observed in bacterial chromatophores, using the irreversible inhibitor DCCD instead of tentoxin (Casadio et al., 1978). So the full inhibition of an important fraction of the enzymes does not affect the functioning of the others. More precisely, the putative FCCP-sensitive ΔpH deficit subsists across the noninhibited enzymes. This means that inlet resistances tend to separate ATPases from each other.

DISCUSSION

Reliability of ΔpH Measurements. A prerequisite to drawing conclusions from flow–force relationships is a correct estimation of the force, here ΔpH. Numerous criticisms have been directed against 9-aminoacridine as a ΔpH probe [Fiolet et al. (1974), Elema et al. (1978), Grzesiek and Dencher (1988), and many others], especially in the context of the localized versus delocalized question (Davenport & McCarty, 1986). We have already discussed this problem in depth in some papers (de Kouchkovsky et al., 1984; Sigalat et al., 1985, 1988). Only two points must be recalled here: (1) to compare two rates of phosphorylation at identical ΔpH, only a qualitative estimation of ΔpH is needed; (2) for different experimental conditions (here, with or without uncouplers), the only requirement is the constancy of the probe response. Concerning the first point, it has been obvious for many years that even though it does not obey the early model of Schuldiner et al. (1972), and whatever the exact mechanism is, the quenching of 9-aminoacridine fluorescence is at least qualitatively related to ΔpH (Hope & Matthews, 1985). It seems even possible to calibrate the signal in some conditions (Casadio & Melandri, 1985; Strotmann & Lohse, 1988; Bizouarn et al., 1991). The second point is less easy to state. For example, in mitochondria, the uptake of triphenylmethylphosphonium cation, a Δψ-probe, seems to depend on the way the membrane potential is adjusted (Woelders et al., 1988). Although this may be a specific problem, one should wonder, in our case, if FCCP could affect the response of 9-aminoacridine, for instance, by interacting with this probe. We have good reasons to claim that this is not the case, because three relationships between ΔpH and associated energetic events are not affected by FCCP: (1) that between ΔpH and the electron flow at variable membrane H^+ conductance, with native or EDTA-treated thylakoids (Sigalat et al., 1993); (2) that between ΔpH and ΔG_p in state 4 (this report, Figure 5); and (3) that between ΔpH and the rate of ATP synthesis when the latter is slowed down by a reversible inhibitor (Figure 6). In addition, the 9-aminoacridine fluorescence in deenergized conditions is insensitive to FCCP (not shown). To conclude, the nonunicity of the flow–force relationships observed in the present report cannot be due to an artifactual response of the probe.

Microchemiosmotic Model. In the preceding paper (Sigalat et al., 1993), a unique relationship was obtained between the rate of electron flow and ΔpH when the membrane H^+ conductance was varied. This could have been interpreted in a delocalized way. But we also have to explain why the proton gradient appears locally modulated when another criterion is used, i.e., the relationship between the proton gradient and the rate of ATP synthesis, with PS1 as ΔpH generator. This probably reflects the great complexity of the membrane topography of thylakoids, and more especially the heterogenous distribution of the proton pumps. For this reason, only qualitative arguments may be developed to explain all the data.

The proton gradient seems to be localized when generated by the cyclic PS1-mediated electron flow. But this localized behavior disappears in state 4 or when the catalytic turnover is slowed down by venturicidin. So it does not involve a close communication between redox pump and ATPase. A better way to explain these results is the existence of a small inlet resistance at the F_oF_1 level, disrupted by FCCP and limiting the local ΔpH across CF_1 only at high rates of proton transfer. Interestingly, low concentrations of uncouplers, including CCCP, were found to stimulate the ATP synthesis induced by pH jumps, that is, without involvement of the redox pumps (Lobysheva et al., 1987). This effect cannot be attributed to some kind of direct coupling and strengthens our hypothesis.

In the case of the $H_2O \rightarrow$ methyl viologen electron pathway, the phosphorylating flow is always low, due to the low turnover rate of the redox chain. In addition, the main part of the resistance between the electron carriers and the ATPases is spread along the route between appressed and nonappressed domains of the membrane. We may explain the apparently delocalized behavior of the system if we suppose that, at

variance with the inlet resistance at the F_oF_1 level, these lateral resistances are not disrupted by FCCP in the concentration range used here.

The $H_2O \rightarrow$ ferricyanide electron pathway (Sigalat et al., 1993) works like the $H_2O \rightarrow$ methyl viologen system, but here the ΔpH is examined by its effect on the b_6f complex. No localized mode of coupling has been observed: this is not surprising if FCCP mainly exerts its delocalizing effect on the ATPase domain. On average, the b_6f complexes, which are randomly distributed in all the membrane area (Albertsson et al., 1991), have no close interaction with the ATPases. Even though a small ΔpH excess may exist at the b_6f level, it does not depend on the presence of FCCP.

Local Resistance at the F_oF_1 Level and Conductance of the F_o Channel. The existence of a resistance which slows down, even in a limited way, the proton flow through F_oF_1 could seem *a priori* inconsistent with the fact that the conductance of CF_o alone was estimated to be 10^2–10^3 times higher than that of CF_oCF_1 (Lill et al., 1986; 1987). But these estimates were based on the rapid decay of ΔpH or $\Delta\psi$ after a short flash. In such transient experiments, a very small number of protons cross the F_o channel, and they could be picked from a very limited domain of the lumen. So it is not necessary that these protons have to cross our putative H^+-diffusion barrier, which may have a significant proton capacity. In such a case, this additional inlet resistance, in series with F_o at steady state, could not be revealed by this kind of transient experiment.

Anomalous Effects of Uncouplers Related to Localized Proton Gradients. In a preceding paper, we have suggested that amines of high p*K* such as hexylamine, once accumulated in the lumen in response to ΔpH, delocalize the proton gradient (Sigalat et al., 1988), which could explain contradictory data (Haraux et al., 1983; Davenport & McCarty, 1986). In a general way, the delocalizing effects of uncouplers may simply be due to their properties as proton carriers. Amines were thought to delocalize the ΔpH in all the thylakoid vesicle, and especially between appressed and nonappressed regions (Sigalat et al., 1988). Here FCCP seems to affect more precisely the ATPase domain. Moreover, when a fraction of the enzymes is inhibited in a permanent way by tentoxin, the local ΔpH deficit is maintained for the other fraction. So the FCCP-sensitive inlet resistance would not be shared by different ATPase molecules.

Some anomalous effects of uncouplers, more or less related to the ATPase functioning or to localized proton gradients, have been reported. It was earlier shown (Giersch, 1983) that low concentrations of nigericin could enhance ATP synthesis, an effect we have also observed here. Stimulatory effects of uncouplers were even reported in pH jump experiments (Lobysheva et al., 1987; see above). These experiments are fully consistent with our hypothesis.

Using other criteria than ours, another group found a delocalizing effect of nigericin in the nanomolar range, but not of hexylamine (Allnutt et al., 1991). This group has constantly reported effects of uncouplers that they have interpreted as a release of protons from a membranous compartment, normally insulating redox carriers and ATPases from the lumen (Baker et al., 1981; Dilley & Schreiber, 1984; Dilley et al., 1987). In their case, uncouplers at low concentrations are nevertheless expected to decrease the energetic yield, whereas in our hands they increase it. Moreover, this effect seems to exist even at equilibrium [the so-called metastable proton pools; see also Theg and Homann (1982), Johnson et al. (1983), and Horner and Moudrianakis (1986)], in contrast to the FCCP effect reported here, which is only dynamic. Finally, the only common point between the two kinds of localized coupling reported by our two groups is the effect of osmolarity and ionicity (Johnson et al., 1983; Sigalat et al., 1985; Beard & Dilley, 1986; Renganathan et al., 1991), calcium ion playing a key role in the case of metastable proton pools (Chiang & Dilley, 1987; Allnutt et al., 1991). Thus, it is possible that uncouplers act differentially on multiple local resistances, each effect being revealed by particular experiments. It should also be noted that, under our experimental conditions, amines stimulated the external proton uptake in a way perfectly consistent with their free access to the high-potential compartment. According to the observations of Dilley's group (Renganathan et al., 1991; Allnutt et al., 1991; Chiang et al., 1992), under the so-called "localized" conditions, amines enters the lumen, but not the true high-potential compartment relevant for ATP synthesis. So these conditions do not seem to operate in our case.

Another group has recently reported anomalous relationships between ΔpH and the activity of ATPase in the presence of procaine, a local anesthetic, which persisted near state 4 (Laasch et al., 1993), at variance with our present data (Figure 5). Some of their results also favored a close interaction between the b_6f complex and the ATPase, which we observed neither here nor in the preceding paper (Sigalat et al., 1993). We think, however, that comparison of their results to those obtained with classical uncouplers is not straightforward. Indeed, as recognized by the authors themselves, local anesthetics at the concentrations used profoundly alter the chemical composition of the membranes. They may thus reveal, enhance, or even create topographic heterogeneities which cannot be observed with classical uncouplers.

Conclusion. In agreement with our previous reports, we think that small resistances for protons separate redox carriers and ATPases in thylakoids. Some of the resistances would span the lumen and would be sensitive to uncoupling buffers like amines, while some others, more localized in the F_oF_1 region, would be disrupted by FCCP-type uncouplers. A local $\Delta\mu_{H^+}$ deficit through the ATPase, depending on the net proton flow, is the best way to explain the localized functioning during net ATP synthesis and the delocalized behavior in state 4. This contradiction between kinetics and equilibrium studies was discussed, more than ten years ago, in the case of mitochondria (Westerhoff et al., 1981). However, the simple hypothesis that we present here was not yet examined, probably because it was assumed that a localized system should necessarily be energetically more efficient than a delocalized one. It seems that systems like F_oF_1-ATPase can present local diffusion barriers for protons at the very entrance of the proteic complex.

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