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Dependence of Kinetic Parameters of Chloroplast ATP Synthase on External pH, Internal pH, and ΔpH

Tania Bizouarn, Yaroslav de Kouchkovsky,* and Francis Haraux

Biosystèmes Membranaires (UPR 39), CNRS, 91198 Gif-sur-Yvette, France

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ABSTRACT: ATP synthesis by the membrane-bound chloroplast ATPase in the oxidized state of its γ disulfide bridge was studied as a function of the ADP concentration, ΔpH, and external pH values, under conditions where ΔpH was clamped and delocalized. At a given pH, the rate of phosphorylation at saturating ADP concentration (V_{max}) and the Michaelis constant K_m (ADP) depend strictly on ΔpH, irrespective of the way the ΔpH is generated: there evidently is no specific interaction between the redox carriers and the ATPase. It was also shown that both K_m (ADP) and V_{max} depend on ΔpH, not on the external or internal pH. This suggests that internal proton binding and external proton release are concerted, so that net proton translocation is an elementary step of the phosphorylation process. These results appear to be consistent with a modified "proton substrate" model, provided the ΔG_0 of the condensation reaction within the catalytic site is low. At least one additional assumption, such as a shift in the pK of bound phosphate or the existence of an additional group transferring protons from or to reactants, is nevertheless required to account for the strict ΔpH dependence of the rate of ATP synthesis. A purely "conformational" model, chemically less explicit, only requires constraints on the pK's of the groups involved in proton translocation.

In chloroplasts, the electrochemical proton gradient, generated by an electron transfer chain, gives the energy required for ATP synthesis (Mitchell, 1961). This energy is stored in phosphate bonds within the F_0F_1 ATP synthase, which consists of a transmembraneous part, the proton channel F_0, and an extrinsic part, F_1, responsible of the catalytic activity.

The detailed mechanism of ATP synthesis, as well as the different roles of the proton gradient, are not yet elucidated. With regard to the energy-coupling events during ATP synthesis, two main models have been described. On the one hand, the proton is considered as a true substrate, which directly interacts with P_i, allowing ATP formation, so its energy is

* To whom correspondence should be addressed.

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partially used for the catalytic process itself (Mitchell, 1974, 1985). On the other hand, the proton translocation through the ATP synthase only brings the energy required for the fixation of substrates and the dissociation of products by conformational changes (Boyer et al., 1973; Boyer, 1977). Experimental arguments were brought in favor of an almost or completely isoenergetic ATP synthesis at the catalytic site (Feldman & Sigman, 1982; Al-Shawi & Senior, 1988, Fromme & Gräber, 1990), which could be explained by an hydrophobic environment in the catalytic site shifting the equilibrium toward ATP formation (H_2O is a product of the reaction). Actually, there is no necessary link between the determination of the energetic steps of the reaction and the question of the direct interaction of protons with the substrates.

It has been observed that decrease of the proton gradient, by lowering the light intensity or adding an uncoupler, decreases the maximal rate of ATP synthesis but has some opposite effects on the apparent Michaelis constant for ADP (K_m)[1] (Vinckler, 1981; Aflalo & Shavit, 1983). Furthermore, K_m has been proposed to vary as a function of the redox activity of photosystem I (Loehr et al., 1985). Important K_m variations were also reported in submitochondrial particles (Heckman et al., 1988; Matsuno-Yagi & Hatefi, 1989). These data have usually been interpreted as a proof of a localized coupling between redox and phosphorylation pumps (Loehr et al., 1985), in contrast to the delocalized electrochemical potential difference of protons across the membrane, which was originally described by the Mitchell hypothesis. A proton "slippage" through the ATPase was also invoked to explain some data (Matsuno-Yagi & Hatefi, 1989). Actually, it was pointed out that, without particular precautions, $\Delta\tilde{\mu}_{H^+}$ decreases when ADP concentration is increased to determine K_m, that is, when the rate of ATP synthesis is increased (Schlodder et al., 1982; Davenport & McCarty, 1986; Biaudet & Haraux, 1987; Quick & Mills, 1987; Bizouarn et al, 1989; Heinen & Strotmann, 1989; Strotmann et al., 1990). It was then suggested that, depending on the conditions, this could lead to more or less underestimation of the K_m for ADP, the decrease of the proton gradient being significant at high ADP concentration (Quick & Mills, 1987). Furthermore, we have recently shown that, when properly estimated, the apparent K_m for ADP does depend on $\Delta\tilde{\mu}_{H^+}$ (restricted here to ΔpH, the usual situation), so comparison between two conditions must be done at a same ΔpH (Bizouarn et al., 1989). In these experiments, the constancy of ΔpH over the ADP concentration range was achieved by delaying the ΔpH variations due to ADP injection by the light-induced trapping of large amounts of hexylamine, an amine of high pK (Bizouarn et al., 1989).

This work is focused on two problems: (1) the possible influence of the redox activity on the parameters K_m (ADP) and V_{max}, which was checked by studying photophosphorylation at a given ΔpH obtained with different modes of functioning of the redox chain; (2) the kinetic role of protons, which was investigated by measuring the parameters K_m and V_{max} as a function of external pH, internal pH, and ΔpH. Until now, there were no data concerning this problem, due to the difficulty in obtaining reliable values of K_m for ADP, for above-mentioned reasons, and to the problem of measuring ΔpH at different external pH (Biaudet & Haraux, 1987).

Materials and Methods

Preparation of the Thylakoids. Envelope-free chloroplasts (thylakoids) were extracted from lettuce leaves as previously described (Sigalat et al., 1985) and stored at high chlorophyll concentration (2 mM), on ice and in darkness, in 2 mM Tricine, 50 mM KCl, and 5 mM $MgCl_2$ (pH 7.8).

The experiments were carried out in the same medium supplemented with 2 or 8 mM K_2HPO_4, and 50 μM pyocyanine to ensure a cyclic electron flow around photosystem I or 500 μM dimethylquinone to catalyze PSII-driven water oxidation, 0.05–0.25 μM valinomycin to suppress $\Delta\Psi$, 4 μM 9-aminoacridine to measure ΔpH, and 250–500 μM hexylamine; the pH was 8.2 unless otherwise indicated.

Photophosphorylation. The conditions were identical with those established in our previous work (Bizouarn et al., 1989). The sample (20 μM chlorophyll, 1.5 mL) was put into a 1 × 1 cm spectroscopic cuvette, stirred and thermostated at 20 °C, in a setup described elsewhere (Sigalat et al., 1985). Each sample was illuminated for 90–180 s until a steady-state ΔpH was reached; when necessary, the light input was limited by using neutral filters. Then, in a fraction of second, ADP (4–70 μM) was injected. The initial rate of phosphorylation was immediately measured by the disappearance of scalar protons using a fast glass electrode [0.90 H^+ per molecule of ATP synthesized at pH 7.6, 0.97 H^+ at pH 8.2, and 0.99 H^+ at pH 8.6 (Nishimura et al., 1962)]. The capacitive effect of hexylamine accumulated inside the lumen prevented a significant ΔpH drop and vectorial H^+ release during the recording (Bizouarn et al., 1989).

ΔpH Estimation. ΔpH was monitored by the light-induced quenching of 9-aminoacridine fluorescence (Schuldiner et al., 1972). The signal was calibrated by ΔG_p in static-head conditions (Strotmann & Lohse, 1988), as previously described (Bizouarn et al., 1989). Calibration experiments were carried out in the same medium as other assays (pH 7.6, 8.2, and 8.6), except that phosphate concentration was only 0.03–1 mM to minimize uncertainties due to an almost complete transformation of ADP into ATP; 10 μM diadenosine pentaphosphate was added to inhibit possible adenylate kinase.

The kinetic parameters V_{max} and K_m were computed by using the nonlinear regression data analysis program Enzfitter (Elsevier Biosoft) based on the Marcquardt algorithm.

Results

Calibration of the 9-Aminoacridine Fluorescence Signal. A prerequisite for a kinetic study of the ATPsynthase in various external pH conditions is to calibrate the 9-aminoacridine signal in each medium. Indeed, due to membrane interactions or to internal volume changes, the response of this probe may depend, in a complex manner, on the medium properties, especially pH, ionicity, and osmolarity (Bizouarn et al., 1990b). The actual ΔpH was then estimated by another method, in the so-called static-head or "state 4" conditions (Chance & Williams, 1955), where thermodynamic equilibrium is reached between ATP synthesis and hydrolysis. The following relationship was used:

$$\Delta G_p = \Delta G_p^{\circ\prime} + RT \log ([ATP]/[ADP][P]) = -n\Delta\tilde{\mu}_{H^+} \; (= 2.3RTn\Delta pH \text{ here})$$

where R and T have their usual meanings; n is the H^+/P stoichiometry, i.e., the number of H^+ translocated across the ATPase per molecule of ATP synthesized or hydrolyzed [the most commonly admitted value for n is 3 (Strotmann & Lohse,

[1] Abbreviations: F' and F°, 9-aminoacridine (9-AA) fluorescence with relaxed and energized thylakoids; V_e and V_i, volumes of suspending medium and of thylakoid lumens; ΔG_p, Gibbs free enthalpy of phosphorylation, normally in thermodynamic static head; $\Delta\mu_{H^+}$, ΔpH, and $\Delta\Psi$, transmembrane differences of proton electrochemical potential, of pH, and of electrical potential; K_m and V_{max}, Michaelis–Menten parameters of phosphorylation for ADP at a given $\Delta\tilde{\mu}_{H^+}$ (ΔpH).

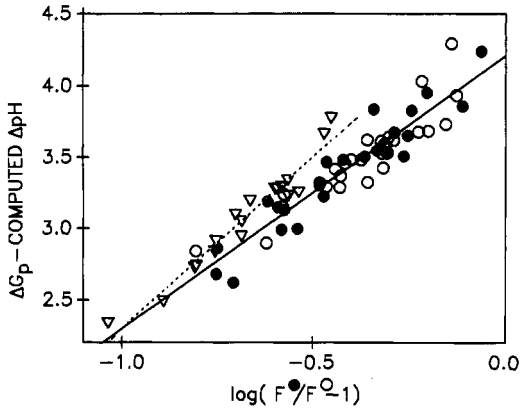

FIGURE 1: ΔpH, computed from static head ΔG_p, as a function of the light-induced 9-aminoacridine fluorescence quenching. ΔpH = $\Delta G_p/2.3nRT$, with $n = 3$ (see text). Conditions were as described under Materials and Methods. Six experiments carried out with different chloroplast preparations. External pH was 7.6 (▽), 8.2 (○), or 8.6 (●). ΔpH magnitude was adjusted by light intensity. The 9-aminoacridine fluorescence level was measured in the relaxed $F^•$ and energized $F^°$ states. Linear regression between ΔpH and log $(F^•/F^° - 1)$ gave the following coefficients: at pH 7.6, slope = 2.41 and intercept = 4.71; at pH 8.2–8.6, slope = 1.77 and intercept = 4.13.

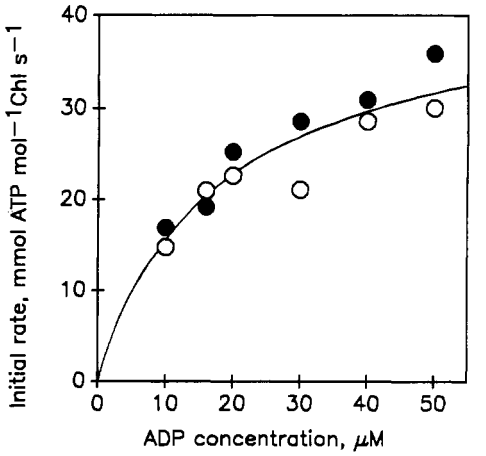

FIGURE 2: Initial rate of phosphorylation as a function of ADP concentration with a cyclic electron transfer around PSI (●) or with a linear electron transfer including only PSII (○). Conditions were as described under Materials and Methods, with 8 mM phosphate, pH 8.2, and, for PSI, 50 μM pyocyanine and 130 W m^{-2} red light intensity or, for PSII, 500 μM dimethylquinone and 1300 W m^{-2} red light intensity; ΔpH = 3.84 in both cases. V_{max} = 50 ± 2 mmol of ATP per mole of chlorophyll per second and K_m = 9 ± 1 μM in both cases.

1988; Rathenow & Rumberg, 1980)]; $\Delta G_p^{°\prime}$ (pH 7.6) = 30.6 kJ·mol^{-1}, $\Delta G_p^{°\prime}$ (pH 8.2) = 33.9 kJ·mol^{-1}, and $\Delta G_p^{°\prime}$ (pH 8.6) = 35.4 kJ·mol^{-1} (Rosing & Slater, 1972). $\Delta\tilde{\mu}_{H^+}$ (= $F\Delta\Psi$ −2.3RTΔpH) is restricted here to the ΔpH term, ΔΨ being cancelled by valinomycin in presence of K^+. Theoretically, ΔpH is related to the light-induced quenching of 9-aminoacridine according to (Schuldiner et al., 1972)

$$\Delta pH = \log (F^•/F^° - 1) + \log (V_e/V_i) + \log (1 + 10^{pH_e - pK})$$

where $F^•$ is the fluorescence level in the relaxed state, $F^°$ the fluorescence level in the energized state, V_e the volume external to thylakoids, and V_i the summed internal volumes of thylakoids. Since 9-aminoacridine pK = 10, the log term containing pH_e, external pH, varies in a negligible manner from pH 7.6 (0.002) to pH 8.6 (0.017) and cannot be responsible of any pH dependency of the signal.

Figure 1 shows the correlation between theoretical ΔpH, computed from ΔG_p assuming $n = 3$, and light-induced 9-aminoacridine fluorescence quenching. Contrary to the previous situation (Bizouarn et al., 1989), but in accordance with further results in various ionic conditions (Bizouarn et al., 1990b), the slope of the correlation, drawn from six experiments, is greater than the expected value of 1. The reason for this variation is not fully understood. During our investigations, however, we found that not only the absolute response of 9-aminoacridine, but also its variation with ΔpH (i.e., the slope of graphs, as in Figure 1), was very sensitive to probe concentration. In particular, the slope tends to 1 for 6 μM 9-AA (not shown). For this reason, in the present work, we paid special attention to the actual probe concentration, which was controlled by spectroscopy, especially since ethanolic solutions tend to become more concentrated with time. We also consider that the true H^+/P stoichiometry is 3 (hence the slope of the graph should be 1) and that higher slopes obtained here are due to side effect, maybe to incomplete saturation of binding sites for 9-AA (Hope & Matthews, 1985). Whatever the mechanism, the principle of this calibration remains valid, provided medium composition, chlorophyll amount, and probe concentration are maintained identically. Figure 1, drawn with 6 different chloroplast preparations, especially shows a good reproducibility of the calibration. The main result of Figure 1 is that, under these conditions, the correlation between ΔG_p (hence ΔpH) and the light-induced quenching of 9-aminoacridine fluorescence is the same at pH 8.2 and 8.6. So a unique relationship will be used to compute ΔpH at these two pH values. In contrast, at pH 7.6, the signal is shifted toward lower values.

Microchemiosmosis. Different experiments made in our laboratory support the existence of a lateral resistance along the thylakoid membrane between the points of H^+ entry (electron transfer chain) and exit (coupling factor and basal membrane leaks). This would maintain a small lateral $\Delta\tilde{\mu}_{H^+}$ superimposed on the transmembrane one (de Kouchkovsky et al., 1984). An ambiguity may thus exist between average $\Delta\tilde{\mu}_{H^+}$, as estimated by probes, and its local value through the ATPases during continuous phosphorylation. However, it was shown that the internal trapping of amines of high pK, like hexylamine, ensures a complete delocalization of the steady-state proton gradient (Sigalat et al., 1988). A rather high KCl concentration and the absence of an osmoticum like sorbitol or sucrose also favor the delocalization of $\Delta\tilde{\mu}_{H^+}$ (Sigalat et al., 1985; Beard & Dilley, 1986). So all is made, in the present report, to avoid any problem linked to the possible existence of local ΔpH.

Lack of Effect of the Redox Chain Activity on K_m ADP and V_{max}. There is not yet a full consensus on the existence or not of a specific interaction between a part of the photosynthetic electron transfer chain and the ATPase (Vinckler, 1981; Loehr et al., 1985). Such interactions have also been proposed for other systems like *Paracoccus denitrificans* (Pérez & Ferguson, 1988, 1990). In chloroplasts, this problem must be clearly distinguished from the well-known thiol reduction of the ATPase by the thioredoxin system, fed by electrons from PSI (Mills & Hind, 1979). It must also be separated from the localized $\Delta\tilde{\mu}_{H^+}$ problem, which does not imply protein–protein interactions. If a direct control of phosphorylation by the redox chain exists, K_m (ADP) and V_{max} parameters should depend not only on ΔpH but also on the rate of the electron transfer and on the redox carriers involved in the electron flow. Only PSI, which is located in the nonappressed regions of membranes, might be expected to interact with ATPases, also restricted to this area. An increase of K_m (ADP) by PSI

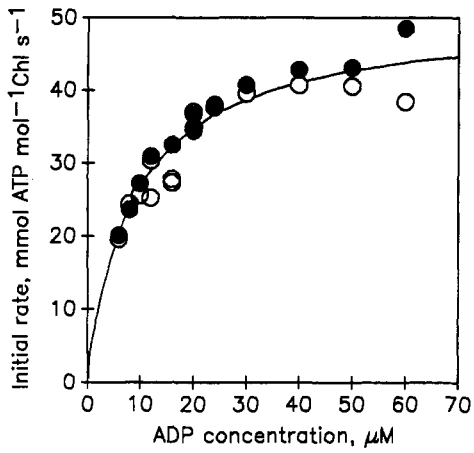

FIGURE 3: Initial rate of phosphorylation as a function of ADP concentration for control (●) and nigericin-supplemented (○) thylakoids. Conditions were as described under Materials and Methods with 8 mM phosphate, pH 8.2. The control had a 60 W m^{-2} red light intensity, and the nigericin case (50 nM) had a 1300 W m^{-2} red light intensity. ΔpH = 3.79 in both cases. V_{max} = 44 ± 5 mmol of ATP per mole of chlorophyll per second. K_m = 18 ± 4 μM in both cases.

activity was indeed proposed (Loehr et al., 1985). However, this hypothesis is ruled out by data of Figure 2, which show that, at a given ΔpH, neither K_m (ADP) nor V_{max} depend on the photosystem used to generate the proton gradient, provided the proton gradient is delocalized.

A second type of experiment, where the rate of electron transfer around PSI was increased by a small addition of nigericin, confirms this lack of direct interaction between redox chains and ATPases. The ΔpH of the control (without nigericin) was adjusted to the same value by decreasing light intensity. Figure 3 shows again no change of K_m and V_{max}. Yet, the initial rate of recovery of 9-aminoacridine fluorescence, upon switching off the light, indicates that the steady-state electron flow was 2.5 times faster with increased H^+ leaks than with limiting light (data not shown). The experiments of Figures 2 and 3 thus prove that K_m and V_{max} depend only on the ΔpH magnitude, not on the manner in which it is obtained, and especially not on the rate of electron transfer in itself.

Effect of External pH, Internal pH, and ΔpH on ATPase Activity. There are two possible approaches to investigating the ΔpH dependence of photophosphorylation at different external pH: to impose the ΔpH magnitude by acid–base jumps (Junesch & Gräber, 1987) or to measure the light-induced ΔpH with a probe, calibrated at each external pH. The first method would appear, at first sight, more straightforward; actually, it requires complex corrections for the inactivation of enzymes during the acidic stage (Gräber et al., 1984), not to mention a possible modification of kinetic parameters such as K_m. So we preferred the second approach.

To distinguish between a dominant role of internal pH or of transmembrane pH difference in the catalytic process, it is necessary to explore an external pH range as large as possible. Unfortunately, in presence of $MgCl_2$, required for the reaction, phosphate was found to precipitate in alkaline media. Sometimes directly seen, this insolubilization could often be detected only by the marked negative pH drift monitored with the glass electrode. This led us to limit the phosphate concentration to 2 mM and the external pH to 8.6. On the other hand, a rather severe uncoupling occurred at pH 7.6. So, even though we have calibrated the 9-aminoacridine response at pH 8.6, 8.2, and 7.6 (Figure 1), with the above discussed results, we have practically restricted our analysis to pH 8.2–8.6. As shown below, this span is large enough to decide whether the kinetic parameters V_{max} and K_m depend on internal pH or on ΔpH.

At a moderate phosphate concentration (2 mM), the data are more scattered than at higher concentration (8 mM) for the following reasons: First, V_{max} values are lower and K_m is higher, resulting in lower phosphorylation rates (from 30% to 50%); second, at this low phosphate concentration, an inhibition by ADP was observed above 20–50 μM, depending on ΔpH (Selman & Selman-Reimer, 1981; Bizouarn et al., 1989), which obliged us to restrict our analysis to the lowest ADP concentrations, thereby diminishing the accuracy of V_{max} and K_m determinations.

Despite these difficulties, it seems quite obvious from Figure 4 that, for a given ΔpH, V_{max} remains the same at pH 8.2 and 8.6 (the single value obtained at pH 7.6, for a low ΔpH, is also plotted on the graphs). In other words, V_{max} depends on ΔpH (Figure 4a), not on internal pH (Figure 4b). To better show the general shape of the curves, less scattered data obtained with 8 mM phosphate at pH 8.2 are also shown Figure 4c. It has already been proposed that the rate of photophosphorylation at saturating ADP concentration depends only on the pH difference across the membrane, but in conditions where the pH dependence of the probe response could not be really checked (Biaudet & Haraux, 1987) or was not even mentioned (Gräber & Witt, 1976; Davenport & McCarty, 1986). Moreover, one should note that opposite results were also reported (Takabe & Hammes, 1981; Pick et al., 1974; Rumberg & Heinze, 1980) for the side reasons discussed

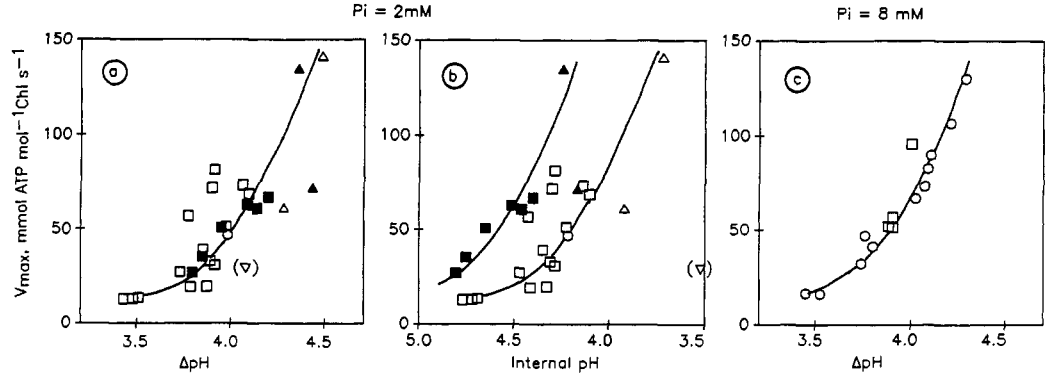

FIGURE 4: Maximal rate of phosphorylation (saturating [ADP]) vs ΔpH or internal pH. ΔpH, adjusted by light intensity, was calibrated with Figure 1. (a) V_{max} versus ΔpH, 2 mM phosphate, pH 7.6 (▽, single point, 500 μM hexylamine, 8.2 (□, ○, △), or 8.6 (■, ▲); data from 16 experiments. Hexylamine, 250 μM (△, ▲), 300 μM (○), or 500 μM (□, ■). (b) V_{max} vs internal pH, same data as in a (c) V_{max} vs ΔpH, 8 mM phosphate, pH 8.2, hexylamine, 300 μM (○) or 500 μM (□); data from 13 experiments.

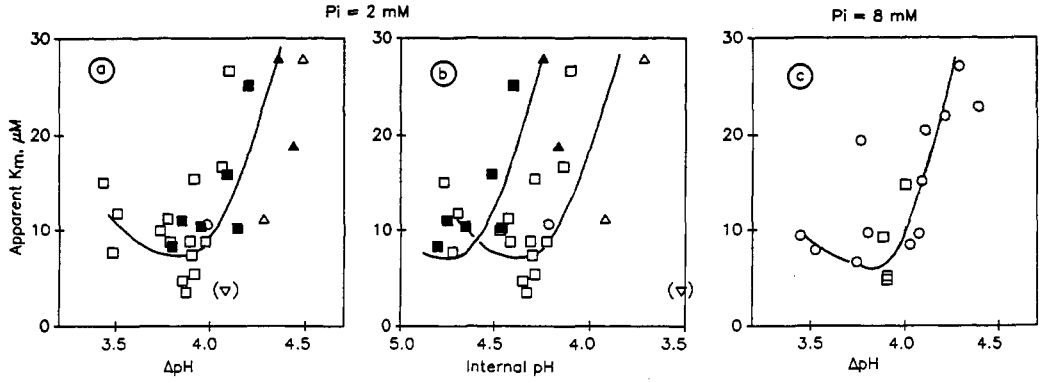

FIGURE 5: Michaelis constant (K_m) for ADP vs ΔpH or internal pH. Computed from the same experiments as in Figure 4. (a) K_m vs ΔpH, 2 mM phosphate, pH 7.6 (▽, single point, 500 μM hexylamine), 8.2 (□, ○, △), or 8.6 (■, ▲). Hexylamine 250 μM (△, ▲), 300 μM (○), or 500 μM (□, ■). (b) K_m vs internal pH; same data as in panel a. (c) K_m vs ΔpH, 8 mM phosphate, pH 8.2, hexylamine 300 μM (○) or 500 μM (□).

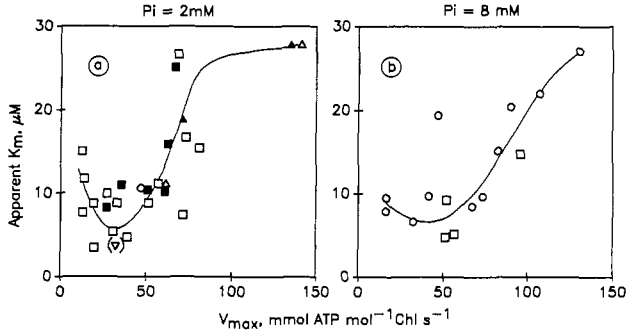

FIGURE 6: Michaelis constant (K_m) as a function of the maximal rate of phosphorylation (V_{max}). Data are from Figures 4 and 5. (a) 2 mM phosphate, pH 7.6 (▽, single point, 500 μM hexylamine), 8.2 (□, ○, △), or 8.6 (■, ▲). Hexylamine 250 μM (△, ▲), 300 μM (○), or 500 μM (□, ■). (b) phosphate 8 mM, pH 8.2, hexylamine 300 μM (○) or 500 μM (□).

elsewhere (Biaudet & Haraux, 1987). Now, it seems firmly established that V_{max} depends on ΔpH and not on internal pH.

Figure 5 shows that this is also the case for K_m (ADP). As in Figure 4, data with 8 mM phosphate at external pH 8.2 were also plotted to clearly establish the curve shape. A first meaning of this result is that the negative form Mg·ADP⁻, predominant in this external-pH range, is really the substrate of the reaction. Our previous data showing that the K_m for ADP seems independent of the screening of the negative surface charges by cations (with background concentrations of 8 mM KCl and 0.5 mM $MgCl_2$) suggested that possibly the neutral form Mg·ADP is the true substrate (Bizouarn et al., 1990b). The present data show that it is not the case, otherwise the apparent K_m at pH 8.6 should have been 2.5 times higher than at pH 8.2, owing to the concentration ratio of the neutral form between these two pH's (pK ~5). This point is important for the molecular mechanism of ATP synthesis.

The ΔpH dependence of K_m (ADP) also gives interesting insight into the role of protons in the catalytical mechanism, which will be discussed further. A consequence of ΔpH dependence of both V_{max} and K_m is that, whatever the external pH, these two parameters remain strictly correlated (see Figure 6). The advantage of this representation is that it is not affected by the problems linked to ΔpH measurement. The complexity of this curve reflects a regulation of the ATP synthase activity, with a maximal value of the K_m reached for V_{max} = 100 mmol of ATP per mol of chlorophyll per second, i.e., well below the largest attainable activity, some 4 times higher (Junesch & Gräber, 1987). Beyond this value, ΔpH acts only on the concentration of active ATPases.

Discussion

Strict Control of Phosphorylation by the Energetic State of the Membrane. The same kinetic parameters K_m (ADP) and V_{max} were found when ΔpH was adjusted either by light or by an uncoupler. A similar result, obtained with a different approach, was reported for thiol-reduced spinach membrane-bound ATPases (Heinen & Strotmann, 1989); in our hands, however, an artificial delocalization of the proton gradient by hexylamine was necessary to obtain this result. In addition, Figure 2 shows the important point that protons released into the thylakoid lumen by the PSII-mediated water oxidation or translocated by the PSI-mediated pyocyanine loop are equivalent in promoting ATP synthesis. These two kinds of data definitely rule out any direct coupling between the redox carriers and the ATPases, too often proposed without strict experimental control.

Variation of K_m (ADP) with ΔpH. A complex law links ΔpH and the Michaelis constant for ADP. This problem has already been tackled (Bizouarn et al., 1989, 1990a), and only a brief survey of the underlying hypothesis will be made.

A first interpretation would be a classical multisubstrate effect. Several protons (probably three) are translocated during one catalytical cycle. If ADP binding is preceded and followed by H^+-translocation steps, it is possible, with some assumptions on the kinetic constants, to get a situation with successive decrease and increase of the K_m for ADP during ΔpH raise. But one can never so obtain a decrease of V_{max}/K_m that seems to occur at high ΔpH (Bizouarn et al., 1989), although additional data are needed to establish it definitely.

A second interpretation is that, at high ΔpH, the reaction becomes limited by ADP diffusion to the enzymes, which would lead to an overestimation of K_m and could explain its sharp increase. But this actually seems improbable, because inhibition of 80% of the enzymes by the irreversible inhibitor tentoxin does not modify the apparent K_m (Bizouarn et al., 1990a). So diffusion-limited kinetics remains conceivable only if each enzyme modulates its local concentration, regardless of whether the neighboring enzymes are working or not. This seems unrealistic, considering the very high density of ATPases that border the nonappressed regions of thylakoids (Garber & Steponkus, 1974). Moreover, a diffusion-limited reaction cannot explain a decrease of V_{max}/K_m at high ΔpH (Goldstein, 1976).

A third interpretation is a regulatory role of ΔpH, which could switch the enzyme from a high- to a low-affinity form for ADP. This might be related to the well-known decrease of the inhibitory effect of ADP that occurs during membrane energization (Strotmann et al., 1987; Biaudet et al., 1988;

Bizouarn et al., 1989; de Kouchkovsky et al., 1990).

At variance with our results, it was reported that the K_m for ADP is around 50 μM and practically independent of ΔpH (Heinen & Strotmann, 1989). But this was obtained with thiol-reduced ATPases, which may have kinetic properties different from oxidized ATPases, and in a ΔpH range (below 3.2) different from that which was scanned here.

ΔpH Dependence of K_m (ADP) and V_{max}. Whatever the reasons of K_m variations, it seems obvious that this parameter depends on ΔpH, irrespective of external (hence internal) pH. Such a dependency has already been shown for the rate of ATP synthesis at a saturating ADP concentration (Davenport & McCarty, 1986; Biaudet & Haraux, 1987). Our interest was then focused on the mechanism of activation more than of catalysis, especially since this strict ΔpH dependency was also observed for activating thiol-reduced ATPases whose hydrolytic function was measured after membrane discharge (Biaudet et al., 1988; Diedrich-Glaubitz et al., 1988). The strict ΔpH dependency, which is now also observed with limiting ADP, has mechanistic implications for catalysis itself, as K_m is by nature independent of the concentration of active enzymes.

At first sight, one may say that the different steps of the translocation process(es) (internal proton uptake, membrane proton crossing coupled to chemical reactions or conformational changes, and external proton release) cannot be separated within the time scale of the catalytic cycle. Proton translocation occurs practically as an elementary event, otherwise an internal pH dependency of V_{max} or K_m should have appeared. This especially rules out any sequential scheme where a rate-limiting ADP binding would be located between internal proton uptake and external proton release. However, it is not necessary to imagine that all protons involved in one catalytic turnover are translocated in a single step.

Mechanism of ATP Synthesis. Two main hypotheses exist. The "binding change" model (Boyer et al., 1973; Boyer, 1977), which is chemically not defined, offers no prediction about internal pH vs ΔpH dependence of ATP synthesis. The "proton substrate" model [Mitchell (1974, 1985) and also Scarborough (1986)] is more explicit and so will be considered here.

A strict ΔpH dependency of the rate of ATP synthesis imposes that the same number of protons is consumed inside and released outside, practically as a single step between binding of substrates (ADP, P) and release of product (ATP). Figure 7 is an adaptation of Mitchell's mechanism, which takes into account the actual dissociation degree of phosphate and nucleotides (bound to Mg^{2+}) in usual slightly alkaline media. Thus, considering the most commonly accepted H^+/P stoichiometry, three protons are taken up from the high-potential compartment (in) to form H_2PO^{3+} and two released in the low-potential compartment (out) to give $MgATP^{2-}$. This is equivalent to the release of three "vectorial" and the consumption of one "scalar" H^+.

This overall process can be approximated by a single step if the concentration of the intermediate states is always very low, which implies that the pK's of acidobasic reactions are markedly lower than the pH on both sides of the enzyme. This is self-evident for the external deprotonation of MgATP (pK $\leq$ 5 in solution) but is disputable for the internal protonation of phosphate (around 7 for the reaction $HPO_4^{2-} + H^+ \leftrightarrow H_2PO_4^-$ in solution, not prejudging how it may shift for bound phosphate). An additional requirement is a low ΔG_0 of the condensation reaction itself, which indeed is possible in the enzyme environment due to the exclusion of water (Mitchell,

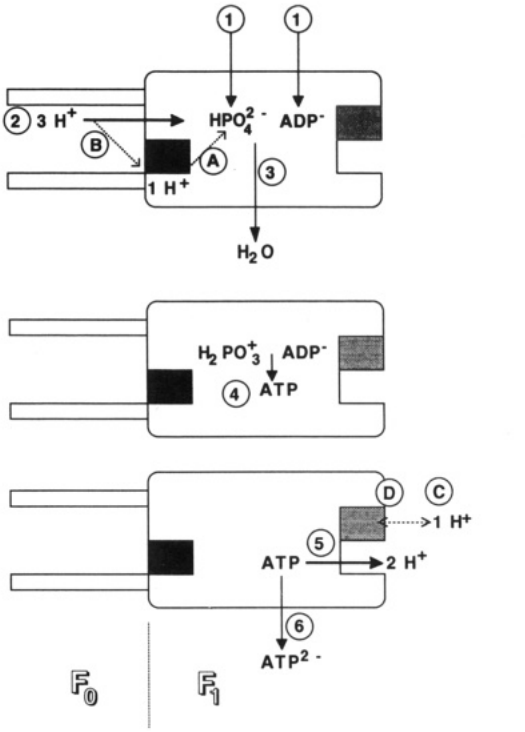

FIGURE 7: "Proton-substrate" hypothesis, adapted from Mitchell (1974, 1985) and presented in the direction of ATP synthesis. Protons are transferred from the high-potential (internal, F_0) compartment to the low-potential (external, F_1) compartment, and Mg^{2+} (not represented) is bound to ADP and ATP. (Step 1) substrates binding (ordered or random), (2) internal proton binding (in fact, three elementary steps), (3–4) condensation reaction (H_2O withdrawn in either compartment), (5) external proton release (two elementary steps), and (6) ATP release. A mechanism with steps 1–6 alone is incompatible with a strict ΔpH dependency of the reaction, due to the imbalance between internal and external H^+ during one turnover, so a "relay" should transiently insure this equality. If the relay is internal (left black box only), it would be deprotonated following step 1 and thus add 1 proton (A) to the 2 removed from F_0 side (step 2), these having their counterpart in the two protons delivered on F_1 side (step 5). If the relay is external (right stippled box only), it would be deprotonated following step 4 and thus add a third proton (C) to the two released by the dissociation of ATP (step 5). In both cases, after step 6, the relay would rebind a proton from F_0 (B) or F_1 (D), in a non-rate-limiting way. One thus has a kinetically limiting translocation of 2 H^+ in the first case and three in the second, but in both the vectorial H^+/P ratio is 3 for a cycle.

1974; Boyer, 1977; Feldman & Sigman, 1982; Al-Shawi & Senior, 1988; Fromme & Gräber, 1990). That is, the strict ΔpH dependency imposes that the condensation step is isoenergetic, if not spontaneous.

Finally, to insure the obligatory balance between internal and external H^+ concentration, a relay (probably a protein group) should transiently be protonated then deprotonated, inside or outside depending on its position (see the legend and shaded boxes of Figure 7). An important pK increase should follow the product release in both cases to rebind H^+.

Concerning the "binding change" hypothesis, one should remark that, although the chemical species involved in proton transfer are not there characterized, the above-mentioned constraints on pK's still apply: pK must be markedly lower than pH on both sides.

In conclusion, even though the internal pH or ΔpH dependency of ATP synthesis rate cannot alone choose definitely between the current hypotheses of the catalytic mechanism of F_0F_1 ATPase, its knowledge is a prerequisite to elucidate the intimate role of protons in this process.

Registry No. ADP, 58-64-0; ATPase, 9000-83-3; H^+, 12408-02-5; ATP, 56-65-5; ATP synthase, 37205-63-3.

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