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Photophosphorylation at variable ADP concentration but constant ΔpH in lettuce thylakoids. Effect of ΔpH and phosphate on the apparent affinity for ADP

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There is a wide variation in the literature of the apparent affinity of membrane-bound F_0F_1-ATPases for the substrates of phosphorylation, especially for ADP. Since these measurements are inescapably biased by an increased drop of $\Delta\tilde{\mu}_{H^+}$ when the phosphorylating flow rises with substrate concentration, we have developed a method to keep the proton gradient constant when substrates are varied. Light-induced trapping of hexylamine inside lettuce thylakoids delays the ΔpH decrease when the membrane H^+-conductivity increases. This was used to measure the initial rate of ATP synthesis just after the addition of variable amounts of ADP, without a significant change of the proton gradient due to the phosphorylating H^+-flow. ΔpH was estimated by the quenching of 9-aminoacridine fluorescence, calibrated with the 'phosphate potential' ΔG_p (in State 4), and the rate of phosphorylation was computed from the medium alkalinization using a glass electrode. Saturation curves (rate vs. ADP concentration) at iso-ΔpH were then obtained, and the magnitude of the proton gradient was also adjusted at different levels by varying the light intensity. An inhibitory effect of high concentrations of ADP on ATP synthesis was noticed in the case of a low ΔpH. Within all the present ΔpH range, phosphate, which raises V_{max}, augments the apparent affinity of the coupling factor for the substrate ADP, i.e. K_m is lowered. Finally, the K_m for ADP obeys a complex law with the ΔpH increase: first, K_m slightly decreases down to some kind of plateau ($K_m = 6–7\ \mu M$ around ΔpH 3.4), then sharply increases ($K_m > 20\ \mu M$ at $\Delta pH \gtrsim 3.5$). In the same condition, V_{max} regularly rises, that is V_{max}/K_m declines for high ΔpH values. This cannot be explained by the classical energetic role of the protonmotive force. Rather it could reflect a regulation of substrate affinity by ΔpH, a process also distinct from the well-known ATPase activation by the proton gradient.

Introduction

The mechanism by which the proton ATPases of F_0F_1 type couple the proton flow to ATP synthesis or hydrolysis is not yet elucidated [1–8]. At least in the case of the chloroplast ATPase, it seems well established that the electrochemical proton gradient $\Delta\tilde{\mu}_{H^+}$ activates the enzyme [9,10] in addition to its classical [11] role of energy supply. ADP, ATP and phosphate also regulate the activity [12–16] and then cannot be simply considered as substrates and products.

Determination of the kinetic parameters of the chloroplast ATPase, which are prerequisite for understanding the mechanism of phosphorylation, is made very difficult by the complexity of these phenomena. Considering only the dependence of the rate of ATP synthesis upon the ADP concentration, an extreme variation in the CF_1 behaviour is reported in the literature. The process was found biphasic [17], which was also observed with mitochondrial F_1 [18]. In many cases, however, the rat vs. substrate relationship was found to obey a simple Michaelis-Menten law, but with a considerable scattering in the apparent K_m for ADP [19–28]. The extreme values encountered were about 1 μM [22]

Abbreviations and symbols: Chl, chlorophyll; $\Delta\tilde{\mu}_{H^+}$, ΔpH, $\Delta\psi$, transmembrane difference of proton electrochemical potential, of pH, of electrical potential; ΔG_p, Gibbs' free enthalpy of phosphorylation or 'phosphate potential', here at static head ('State 4'); $\Delta G_p^{o'}$, standard phosphate potential; V, S, rate, substrate of a reaction at a given ΔpH; V_p, V_{max}, actual or maximal rate of ATP synthesis for given or saturating ADP at set P_i and ΔpH; K_m, apparent Michaelis constant for ADP at set P_i and ΔpH; V_e, volume of the external medium; V_i, operational (probe-sensitive) internal volume of the thylakoids; CF, coupling factor or ATPase.

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and almost 200 μM [24]. When the energization of the membrane was increased by varying the redox-linked proton input, the K_m for ADP was generally increased [19,20,23,24]. When the proton gradient was varied by nigericin [19,23,26], uncoupling amines [24,26] or FCCP [27], K_m increased [23,26] or decreased [19,24,27] with the energized state. With a variable concentration of gramicidin D, K_m increased with the proton gradient [24].

Some factors were suspected to influence the affinity of the ATP synthase for its substrates. It was proposed that the electron transfer [19,24,25], especially the Photosystem I activity [24,25], could decrease the affinity for ADP, i.e. increase its apparent K_m, which supposes some interaction between the electron carriers and the ATPase. In another hypothesis, thylakoid swelling or nigericin addition would break a diffusion barrier limiting the access of ADP to the coupling factors [23].

The lack of control of the $\Delta\tilde{\mu}_{H^+}$ (or ΔpH) magnitude in this type of experiment was regularly raised [10,26,29–31]. More precisely, the magnitude of ΔpH may change the affinity of the enzyme for ADP [10], especially in the context of the 'binding change hypothesis' [4,6,32]. But the main criticism is that any increase of the substrate concentration always decreases the ΔpH, in a manner which depends on the energy input, electron transfer control, membrane leaks, lumen volume and internal buffering power [10,26,29–31]. This inescapably alters the dependency of the rate of phosphorylation on the substrate concentrations. Therefore, the most surprising is not to obtain variable K_m, but rather to find simple Michaelis-Menten relationships. By modelling all of the relevant parameters listed above, and assuming constant K_m for ADP and phosphate, it was recently shown [29,31] that it is possible to obtain data apparently consistent with simple hyperbolic laws of saturation, though with erroneous K_m values which vary with the experimental conditions. This approach could account for the discrepancies of previous data [19–28] in a satisfactory way, but it was not possible at the same time to determine the true value(s) of K_m and finally to establish whether it is constant or not. To do that indeed, it is not sufficient to simulate theoretically the effect of ΔpH variations, it is important to suppress them.

In this report, we present a method which allows to study the dependency of the rate of photophosphorylation upon the ADP concentration, without concomitant variation of the ΔpH. It is based on the use of hexylamine, an uncoupling amine of high pK. At low concentrations, amines accumulate in the lumen in response to the ΔpH [33,34]. Recently, it was also shown that they delocalize steady state ΔpH by increasing the lumen H^+-conductivity [35,36]. Here, we have used their well-known capacitive properties [37,38], which damps all the ΔpH variations which follow the various perturbations the system may undergo, such as a change in the energy input or in the membrane leaks. This allowed us to measure the initial rate of ATP synthesis after ADP addition in a time range where no significant ΔpH shift occurred. It was thereby found that the apparent K_m for ADP actually depends on the magnitude of the ΔpH and also varies with the phosphate concentration.

Materials and Methods

Chloroplasts

Envelope-free chloroplasts were extracted from lettuce leaves as in Ref. 39 and stored at 2 mM chlorophyll concentration, on ice and in darkness, in the following medium: 2 mM Tricine, 50 mM KCl, 5 mM $MgCl_2$, 2 or 8 mM K_2HPO_4 (pH 8.0).

Assays

Experiments were carried out in this same medium adjusted at pH 8.2, after addition of pyocyanine 50 μM to ensure a cyclic electron flow around Photosystem I, valinomycin $\geq$ 50 nM, to cancel $\Delta\psi$ (see Discussion), and 9-aminoacridine 4 μM. Hexylamine (300 or 500 μM) was present when indicated. The sample (20 μM chlorophyll, 1.5 ml) was put into a 1×1 cm spectroscopic cuvette, stirred and thermostated at 20 °C. Phosphorylation was estimated from the consumption of 'scalar' protons in the medium (i.e., not crossing the membrane), measured with a glass electrode: $ADP + P_i + mH^+ \rightleftharpoons ATP + H_2O$ ($m = 0.97$ at pH 8.2 [40]). ΔpH was monitored by the fluorescence of 9-aminoacridine [41] (see below). The two signals were simultaneously recorded, using a set-up described elsewhere [39,42]. The ΔpH amplitude was adjusted by the intensity of the red actinic light with neutral filters (maximum intensity available: 1.3 $kW \cdot m^{-2}$). Each sample was illuminated for 30 to 180 s, depending on the light intensity, until a steady-state ΔpH was reached. Then, in a fraction of a second, ADP (4 to 100 μM) was injected in the cuvette. The initial rate of ATP synthesis was measured less than 2 s thereafter (see Results and Fig. 1). For lowest substrate concentration, ADP consumption caused an underestimation of the initial rate of at most 10%. The electrode response time was not limiting and ADP as such had no effect on the pH of the medium, being preadjusted at the same value. To calculate the phosphorylation-dependent H^+ consumption, the suspension was titrated with 10 mM HCl. Owing to its low concentration (< 0.5 mM) and high pK (10.6), hexylamine had insignificant effect on the buffering power of the suspension.

One experiment generally consisted in scanning the ADP range for a single preset value of ΔpH; in some cases, however, two ΔpH could be investigated in the same day. The time required for one experiment was

3–5 hours, during which only a negligible loss of activity was generally noticed. When it was not the case, duplicate samples made in reverse order were averaged to compensate for the deactivation of the material. In the presence of hexylamine, we obtained with different chloroplast preparations so reproducible activities that no normalization was judged necessary before mixing together the results.

'State 4' measurements

For the ΔpH measurements, we estimated the 'operational' internal volume, i.e., that which is sensitive to the probe (see Discussion), by correlating the light-induced 9-aminoacridine fluorescence quenching to the 'phosphate potential' ΔG_p in the 'static head' condition, where ATP synthesis is strictly balanced by its hydrolysis [43]; this is also known as 'State 4' [44]. We considered the standard $\Delta G_p^{o'}$ as established [45]. Assays were carried out as mentioned above, except that phosphate concentration was only 0.2 or 0.5 mM and ADP was always 20 μM in order to reach this state in a reasonable time and to measure ΔG_p with sufficient precision. The latter was computed from the amounts of formed ATP (measured on small aliquotes by the luciferase reaction [39]) and of remaining ADP and P_i. Depending on the light intensity, from 3 to 15 min were necessary to reach State 4, recognizable by constant ATP-induced luminescence levels.

Our preparations sometimes exhibited an adenylate-kinase property, which incidently could not affect the phosphorylation rate as it was measured (no proton imbalance and small activity, if any). To be safe, however, in State 4 calibrations, this activity was fully inhibited by 10 μM diadenosine pentaphosphate, as stated earlier [39].

Results

Conditions for keeping constant ΔpH upon ADP addition

Fig. 1 shows typical recordings of the 9-aminoacridine fluorescence and pH changes, respectively, due to vectorial proton translocation (hence ΔpH) and scalar proton uptake (hence phosphorylation rate V_p). In the absence of hexylamine, addition of ADP in the steady-state light conditions induces a rapid ΔpH decrease to the phosphorylating 'State 3'. This stage is then followed by a slow ΔpH increase accompanying the transition towards 'State 4'. As expected, the initial ΔpH decrease is more pronounced when ADP is more concentrated: compare the traces for 10 and 100 μM. In addition, the initial rate of photophosphorylation, as measured by the external medium alkalinization, is superimposed to a transient vectorial H^+ release due to the opening of H^+ channels in CF_1, especially marked with high amounts of ADP. The disturbing effect of this H^+ output on the initial kinetics of ATP synthesis depends on the light intensity and the ADP concentration: it may consist in a negative signal, in just a lag or even many have a negligible weight. Fig. 1b shows that

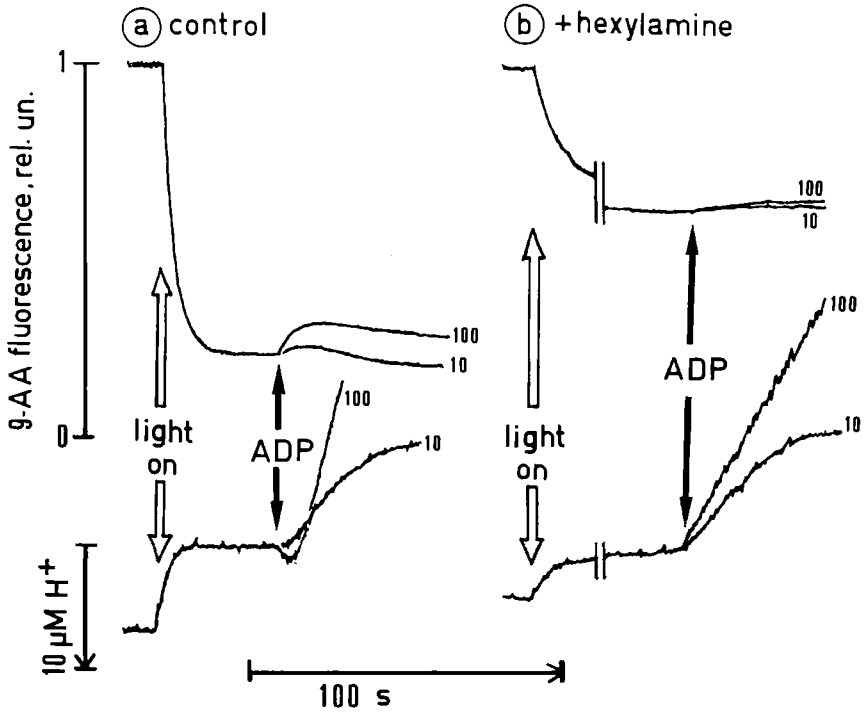

Fig. 1. Typical recordings of 9-aminoacridine (9-AA) fluorescence quenching and external proton consumption or uptake. Conditions as described in Materials and Methods; maximal light intensity. (a) no hexylamine; (b) hexylamine 500 μM. Top traces: 9-AA fluorescence; bottom traces: glass electrode signal, converted into μM H^+. Illumination and ADP injection (in the light) are indicated with vertical heavy arrows; ADP concentrations (10 and 100 μM) are mentioned on the traces.

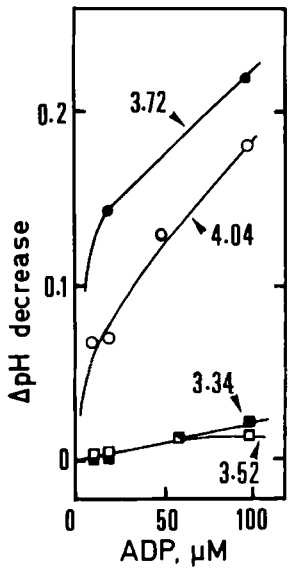

Fig. 2. ΔpH decrease upon addition of ADP at different concentrations. Conditions as in Fig. 1b. Circles (●, ○): no hexylamine; squares (■, □): with hexylamine 500 μM. Open symbols (○, □): strong light; closed symbols (●, ■): low light. The ΔpH decrease is estimated at the beginning of the phosphorylation reaction. The ΔpH amplitude before ADP addition is indicated near the arrow head for each curve.

hexylamine 500 μM suppresses these two disturbing phenomena: no ΔpH decrease and no pH shift due to vectorial protons are now observed. We have even verified that the latter does not occur when ADP addition is replaced by nigericin injections giving rise to a significant ΔpH decrease (not shown). This suggests that, when the membrane conductivity is suddenly increased, each outcoming proton is neutralized in the external medium by a co-migrating molecule of hexylamine. An indirect way to estimate the $\Delta\psi$ role was to use 500 nM valinomycin instead of 50. In this case, to avoid an uncoupling by valinomycin, sensitive in this concentration range, we have have injected the ionophore only ten seconds before ADP, taking advantage of the delaying effect of hexylamine. Indeed, no uncoupling then occurred and control experiments without hexylamine showed that valinomycin had a full effect in less than 10 s.

Fig. 2 shows how varies the extent of this initial decrease of the proton gradient upon ADP addition. The smaller is the initial ΔpH, the greater is this ΔpH drop. It is also clear, as it was qualitatively shown in Fig. 1b, that the presence of hexylamine renders negligible the ΔpH change at the onset of photophosphorylation. With some chloroplast preparations, 300 μM hexylamine instead of 500 was even sufficient to obtain this effect (200 μM was found too low). Since the concentration of hexylamine is not important per se, except to minimize uncoupling (shown Fig. 1 by a ΔpH lowering), experiments carried out with either 300 or 500 μM hexylamine are reported in the following. Theoretically, the internal concentration of an amine of high pK is $10^{\Delta pH}$-times the external concentration, but actually it may be below [36].

Variation of the phosphorylation rate with ADP at different ΔpH and two phosphate concentrations

The internal accumulation of an amine of high pK like hexylamine, which delays ΔpH changes, easily allows to establish rate vs. substrate relationships in iso-ΔpH conditions. This gives curves of the type displayed in Figs. 3 and 4. These figures also indicate the ΔpH value attained at the moment of estimation of the initial rates of ATP synthesis. Experiments were carried out at two phosphate concentrations: 2 mM (Fig. 3), and 8 mM (Fig. 4). Fig. 3a shows that when ΔpH is high enough, the data satisfactory fit a Michaelis-Menten hyperbola. When ΔpH is decreased, here by lowering

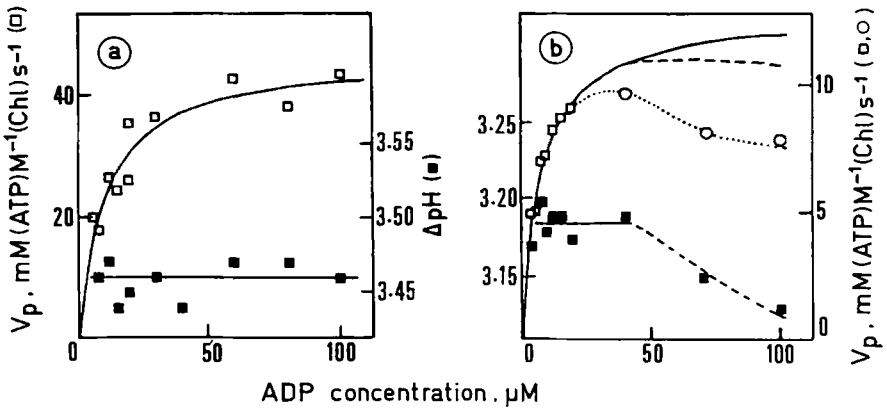

Fig. 3. ΔpH and rate of ATP synthesis as a function of ADP concentration with 2 mM phosphate. Conditions as in Fig. 1b, 500 μM hexylamine; ΔpH estimated as in Fig. 2. (a) Strong light; (b) low light. Open symbols (○, □): rate of ATP synthesis; closed symbols (■): ΔpH. ATP synthesis curves are fitted as indicated Fig. 5. (a): $K_m = 10.6$ μM, $V_{max} = 47.0$ mM ATP·M^{-1} Chl·s^{-1}; (b): $K_m = 7.7$ μM, $V_{max} = 13.0$ mM ATP·M^{-1} Chl·s^{-1}. In (b), only rates at low substrate concentrations (□) were selected to obtain the theoretical curve (full line), the actual curve at high ADP concentrations (○) being indicated by a dotted line (· · · · · ·). The dashed lines (— — —) indicate the ΔpH lowering by the increased H^+ backflow due to ADP phosphorylation, and the expected drop of ATP synthesis. The difference between dotted and dashed Vp lines represents the ADP inhibitory side-effect on ATP synthesis.

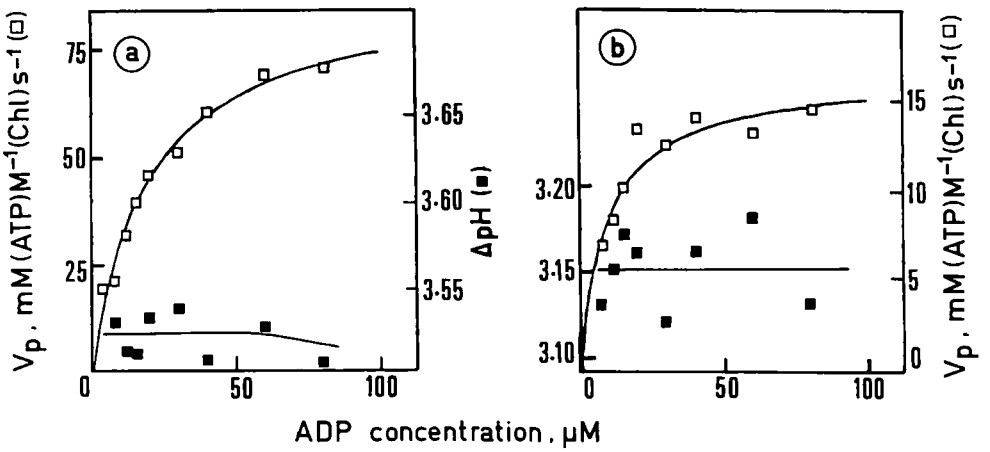

Fig. 4. ΔpH and rate of ATP synthesis as a function of ADP concentration with 8 mM phosphate. Conditions as in Fig. 3, except for phosphate and hexylamine (300 μM) concentrations. (a) Strong light, high ΔpH: $K_m = 20.4$ μM, $V_{max} = 90.1$ mM ATP·M^{-1} Chl·s^{-1}; (b) low light, low ΔpH: $K_m = 9.5$ μM, $V_{max} = 16.5$ mM ATP·M^{-1} Chl·s^{-1}. (■): ΔpH. (□): rates of ATP synthesis, fitted as shown Fig. 5.

the light intensity (Fig. 3b), this does not hold anymore for high ADP concentrations. It may be noticed that in this case ΔpH could not be maintained constant over the full ADP concentration range. However, this ΔpH drop, around 0.05, cannot be taken as the sole responsible of the decrease of ATP synthesis. Indeed, only 10% lessening of V_{max} could then be expected instead of almost 40% which is observed. Thus, it appears that ADP is not only a substrate of ATP synthesis, but also an inhibitor, even with low concentrations at low ΔpH. An inhibitory effect of ADP on ATP hydrolysis, by a high-affinity binding to a regulatory site of the enzyme, is already known [12–15,46,47] and phosphate protects against this inhibition [14,48]; this is also true here for ATP synthesis: see the different curves at 2 and 8 mM phosphate in Fig. 4. Similar inhibition of phosphorylation was also observed, but at much higher ADP concentrations (0.1–10 mM) and likely for much stronger ΔpH, depending on phosphate concentration [28]. All this makes that for low ΔpH, only ADP concentrations below about 20 μM can be used to calculate the kinetic parameters K_m and V_{max}.

Computation of the K_m and V_{max} of phosphorylation for ADP and their variation with ΔpH

Fig. 5 gives an example of fitting of data obtained at iso-ΔpH. Three classical linear representations of a Michaelian process were used: (a) $1/V$ versus $1/[S]$; (b) V versus $V/[S]$; (c) $[S]/V$ versus $[S]$. The parameters K_m and V_{max} were then calculated in each case by linear regression. Direct least squares hyperbolic regression, using an iterative algorithm, was also tested, but it gave unsatisfactory results and thus was rejected. The main difficulty encountered in the fitting procedure was the above-mentioned inhibitory effect of ADP. This made necessary to suppress the corresponding experimental points in the high substrate concentration range. The remaining domain of ADP concentrations, relevant for the kinetic analysis, was from 0–20 to 0–100 μM, that is increasing with the size of the proton gradient. Actually, the estimation of this operational domain obeyed to a double criterion: (1) to keep the greatest number of experimental points in each curve of rate vs. substrate, (2) to obtain values of K_m and V_{max} as close as possible with the three linearizations a-b-c. Once the best compromise was found between these two constraints, four different fits were tested on the direct V versus [S] data: three using the sets of parameters obtained in a-b-c, and

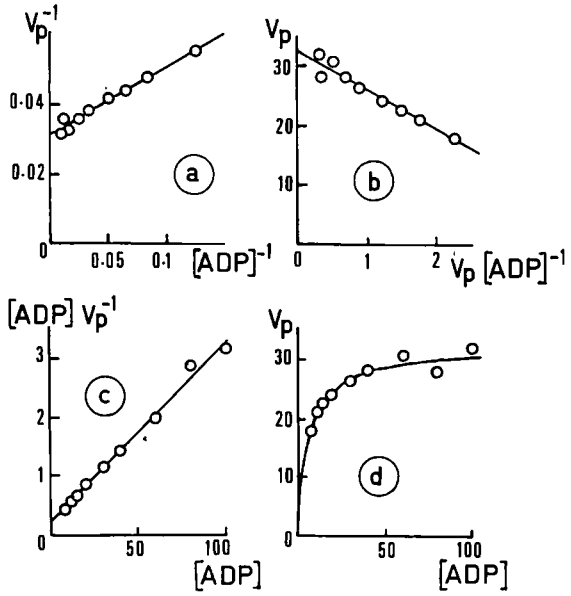

Fig. 5. Example of K_m and V_{max} determination. Conditions as in Fig. 4, 8 mM phosphate, 300 μM hexylamine, ΔpH = 3.32. Same data in a-b-c-d. (a) Lineweaver-Burk plot, i.e., $1/V$ vs. $1/[S]$: V_{max} = 1/intercept = 32.0 mM ATP·M^{-1} Chl·s^{-1}, K_m = slope/intercept = 6.3 μM. (b) Woolf-Augustinsson-Hofstee plot, i.e., V vs. $V/[S]$: V_{max} = intercept = 32.2 mM ATP·M^{-1} Chl·s^{-1}, K_m = −slope = 6.5 μM. (c) Hanes-Woolf plot, i.e., $[S]/V$ vs. $[S]$: V_{max} = 1/slope = 32.7 mM ATP·M^{-1} Chl·s^{-1}, K_m = intercept/slope = 7.1 μM. (d) Direct V vs. [S] plot, fitted with the average of the parameters computed in a-b-c, i.e., V_{max} = 32.3 mM ATP·M^{-1} Chl·s^{-1} and K_m = 6.6 μM.

the fourth using their average. Among these four fits, we have then selected that giving the smallest residue. A typical result is displayed in Fig. 5d. In this example the averaged parameters gave the best fit.

Measuring ΔpH with 9-aminoacridine or other partitioning amines is quite delicate, especially since the actual vesicular volume trapping the probe is difficult to estimate [30,49–51]. Problems such as probe–membrane interactions [50–52], especially electrostatic, should have a limited weight due to the ionic strength of our medium (50 mM KCl, 5 mM $MgCl_2$). A recent study indeed, using 9-aminoacridine in similar conditions as ours, gave a quite satisfactory relationship between the phosphate potential ΔG_p and the proton gradient maintained in 'State 4' by chloroplasts [43]. In this condition, where ATP synthesis and hydrolysis rates are strictly equal, one has (all values being kept positive here):

$$\Delta\tilde{\mu}_{H^+} = \Delta G_p/n, \text{ where } \Delta G_p = \Delta G_p^{o'} + RT \ln([ATP]/[ADP][P_i])$$

where n is the number of H^+ translocated across the ATPase per molecule of ATP synthesized or hydrolyzed, and $\Delta\tilde{\mu}_{H^+}$ ($= F\Delta\psi - 2.3\ RT\ \Delta$pH) is restricted here to the ΔpH term, $\Delta\psi$ being cancelled by valinomycin (see Discussion) or else expressed in equivalent ΔpH terms by this procedure, since ΔG_p is correlated to the whole proton gradient. With $\Delta G_p^{o'} = 33$ kJ · mol^{-1} at pH 8.2 [45], we have calibrated the 9-aminoacridine signal with ΔG_p measurements in 'State 4' at different light intensities. Fig. 6 shows the relationship found between the theoretical ΔpH, computed from ΔG_p, and the quenching of 9-aminoacridine fluorescence ($F^\bullet$ in dark-relaxed thylakoid state, F° in the light-energized):

$$\Delta G_p/2.3\ RT\ (= \Delta G_p^{o'}/2.3\ RT + \log([ATP]/[ADP][P_i]))$$

$$= n \log V_e/V_i + n\ [\log(1 + 10^{pH-pK})(F^\bullet/F^\circ - 1)]$$

(pH refers to the suspending medium). In this equation, the variable is the log function, within brackets, on its right-hand side; the slope of the resulting line gives n and its intercept the external/internal volumetric ratio V_e/V_i. In fact, the points reasonably fit a straightline with $n = 2.91$, i.e. close to the classical H^+/ATP ratio of 3 [43,53–56], and one finds $V_e/V_i = 3350$, also compatible with available data. The result does not signifi-

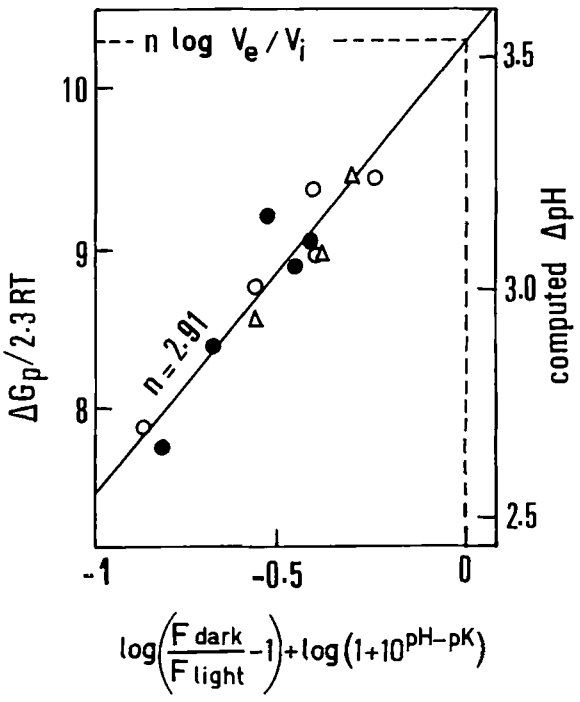

Fig. 6. Calibration of the 9-aminoacridine response by 'static head' (State 4) ΔG_p-ΔpH relationship. Conditions described in Materials and Methods (10 μM diadenosine pentaphosphate present); 20 μM ADP but no ATP before illumination. Hexylamine 300 (△) or 500 (●, ○) μM, valinomycin 50 (△, ○) or 200 (●) nM, phosphate 0.2 (△, ○) or 0.5 (●) mM. ΔpH amplitudes adjusted by light intensity. The 9-aminoacridine fluorescence level is measured in the relaxed (F_{dark}) and energized (F_{light}) states.

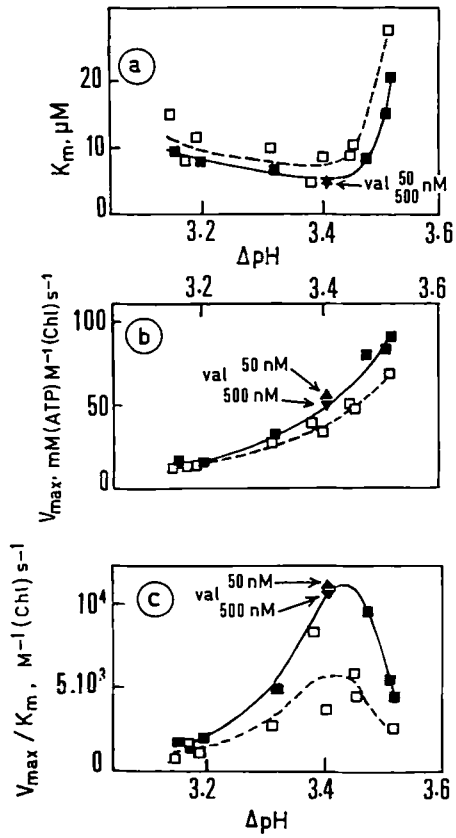

Fig. 7. Michaelis constant (K_m), maximal rate (V_{max}), and V_{max}/K_m ratio for the substrate ADP as a function of ΔpH. Conditions as in Figs. 1–5. Hexylamine 300 or 500 μM, phosphate 2 (□) or 8 (■, ▲, ▼) mM, valinomycin 50 (■, ▲) or 500 (▼) nM. Different chloroplast preparations, except for symbols ▼ and ▲. Each point corresponds to the data of one experiment, treated as shown Fig. 5 for determining its kinetic parameters. ΔpH, adjusted by light intensity, was calibrated from Fig. 6 data. (a) K_m; (b) V_{max}; (c) V_{max}/K_m; notice the drop above ΔpH 3.45 (see Discussion). The fitted curves (c) are the ratio of the fitted curves (b) and (a).

cantly depend on the hexylamine concentration (300 or 500 μM). The volumetric ratio so estimated was used to calculate all the ΔpH values presented in this report.

Finally, Fig. 7 summarizes the results of different experiments at different ΔpH adjusted by light intensity. The apparent K_m for ADP and the phosphorylation rate, extrapolated at infinite ADP concentration (V_{max}), are both plotted vs. ΔpH; they are independent on the increase of valinomycin concentration from 50 to 500 nM (see Discussion). As expected, V_{max} regularly rises with ΔpH, but it is noteworthy that K_m is not constant. It slightly decreases in the low ΔpH range, then dramatically increases. The increase of phosphate concentration lowers the K_m (that is increases the affinity for ADP) in all the investigated ΔpH range. Possible significance of these K_m variations will be discussed below.

Discussion

Iso-ΔpH measurements with hexylamine

The method presented here allows to measure rates of ATP synthesis at variable substrate concentrations without changing the protonmotive force magnitude. A second advantage of this method is the suppression, by the ΔpH-delocalizing virtue of amines in steady state [35,36], of the possible differences which may exist between local (viz. across CF_1) and average values of the ΔpH [35,36,57]. Third, by eliminating the disturbing effects of the vectorial proton flow on the external pH, it makes possible to measure the initial rate of ATP synthesis by a simple pH-metric technique, which allows a simultaneous monitoring of the proton gradient with a fluorescent probe. The third condition is only fulfilled by weak acids as amines of high pK (10.6 for hexylamine), which deprotonated form, since neutral, is freely permeant. This is because each internal H^+ which leaves the internal compartment, especially when it crosses the coupling factor due to ATP synthesis, is accompanied without delay by the neutral amine form, RN, which, once in the external medium, immediately binds the outcoming H^+ to reform RNH^+; this is a consequence of simple acid-base equilibria.

Such a quasi instantaneous equilibration of RN, suggested by the absence of vectorial transient H^+ flow in the external medium, is also the only way to explain the counterparting lack of ΔpH change. Indeed, owing to the considerable difference between amine high pK and lumenal low pH, it is excluded that it may damp internal pH variation by a classical non-permeant buffer property, even at its highest internal concentration. Other type of buffers are also able in principle to damp internal pH changes, but not to conceal the vectorial proton flow. A limitation of our method, however, is some uncoupling by amines which narrows the range of ΔpH we may investigate (highest for the moment: $\approx$ 3.55).

Some experiments in the literature [24,26] were also carried out in the presence of diffusible amines, but then used as uncouplers, and not in iso-ΔpH conditions, because the substrates were added prior to membrane energization, which necessarily induced a partial relaxation of the proton gradient. Indeed, if amines trapped in the thylakoid lumen can delay this inescapable ΔpH drop, at the onset of ATP synthesis, they cannot prevent the steady-state ΔpH to depend upon the ADP concentration.

An iso-ΔpH condition was realized using chloroplast ATPase coreconstituted with bacteriorhodopsin into liposomes [21,22]. This stabilization was possible because the high leakiness of these vesicles minimized the weight of the additional ADP-induced proton output, but at the expense of the yield. The apparent K_m for ADP was then found around 1.5 μM at pH 8.0, a much lower value than obtained here. It is possible that the extraction of the enzyme and its new lipid environment in the liposomes have changed its catalytic properties, not mentioning other problems like electrostatic interactions (ADP is negatively charged). Although not dealing with the K_m problem, one must also mention here the phosphorylation measurements made at fixed initial ΔpH by using artificial pH jumps in a rapid mixing device [58,59].

Lack of $\Delta\psi$ effect

It is important that some hidden and variable $\Delta\psi$ does not participate in our experiments. In thylakoids, steady-state $\Delta\psi$ is dual [50,50–62]. The first, fast-relaxing, phase is fully abolished by valinomycin in presence of K^+ (our conditions). The second, slower, phase is suppressed only by protonophores and is related to ΔpH: it would be due to the protonation of the inner thylakoid compartment, giving rise to a Donnan [64,65] or a Gouy-Chapman [50,63] potential. The slow phase could be increased by the internal accumulation of protonated amine, but not indefinitely [36]. However, it may be, since this valinomycin-resistant component is strictly related to ΔpH, it would exactly vary in the same way, that is it would stay constant upon ADP addition when hexylamine, at proper concentration, stabilizes ΔpH as in the present case. Therefore our iso-ΔpH conditions were in fact iso-$\Delta\tilde{\mu}_{H^+}$. From a quantitative point of view, one must recall that this $\Delta\psi$ is implicitely contained in 'State 4' calibration (Fig. 6) since then ΔG_p is in stoichiometric equilibrium with the whole $\Delta\tilde{\mu}_{H^+}$ and not only with ΔpH.

Finally, the charge imbalance brought about by the proton leakage through phosphorylating ATPases is compensated by parallel outflux of anions or influx of cations. Particularly for the latter, K^+ can easily cross the membrane thanks to valinomycin, already sufficient at 50 nM since a 10-times higher concentration changes neither K_m nor V_{max} (Fig. 7).

Determination of the apparent K_m and V_{max}

The way by which K_m and V_{max} are computed from a given set of experimental data may be important, especially considering the inhibitory effect of ADP. When both ΔpH and phosphate concentration were low, as in Fig. 3b, it was not difficult to select the ADP range adequate for our kinetic analysis, because the inhibitory effect was clear. In the intermediate cases, however, the inhibitory effect was not strong enough to visually decrease the rate of ATP synthesis, but could only lower the apparent saturation plateau. This was revealed by the very different values of K_m and V_{max} sometimes obtained with the three types of linearization used. This is why, in each of the rate vs. concentration curves, we had to suppress the data in the highest ADP range, until we obtained comparable kinetic parameters with the three methods of calculation a-b-c shown Fig. 5. Such a hidden inhibitory effect of ADP may have misled the investigators and resulted in erroneous values of K_m and V_{max} when only one fitting method was used, which generally was the case: direct $V = f([S])$ fitting [16], $1/V = f/[S])$ fitting [19,20,23,25,27–29,31], $V = f(V/[S])$ or $V/[S] = f(V)$ fitting [17,18], or $[S]/V = f([S])$ fitting [21,22,26]. This may be an additional reason for the scattering and apparent discrepancies of the results found in the literature.

Calibration of the 9-aminoacridine signal by ΔG_p in State 4

From the V_e/V_i ratio given, at actual chlorophyll concentration, by the the straight-line of Fig. 6, one gets an 'internal volume' of the thylakoid of about 15 $l \cdot mol^{-1}$chlorophyll. It well agrees with the average values found by centrifugation techniques in analog media at comparable pH, as in Ref. 30 (where pH was 8.0 instead of 8.2 used here): about 11 $l \cdot mol^{-1}$ chlorophyll. Even though, as suggested in many reports [50–52,67], 9-aminoacridine does not ideally distributes according to the early model of Schuldiner et al. [41], all side-effects are taken into account by the 'State 4' calibration procedure which gives an 'operational internal volume'. This parameter is perhaps different from the true lumenal volume but is that which is needed for expressing the ΔpH-dependent 9-aminoacridine accumulation, i.e., the measured fluorescence quenching. It allows a qualitative, and even quantitative, estimate of ΔpH, whatever the exact mechanisms are, and this is the only thing which counts here. One may add that because a simple and reproducible correlation is found between ΔG_p and $\Delta\tilde{\mu}_{H^+}$ (ΔpH), 9-aminoacridine is probably no more artifactual in its basic properties than similar substances, even though the latter are generally ignored in the criticisms which accompanies the use of molecular probes.

Significance of the K_m variations

ATP synthesis by proton ATPases requires three substrates: ADP, inorganic phosphate, and protons, which is expressed for the latter by $\Delta\tilde{\mu}_{H^+}$, restricted here to ΔpH. Even though the 'proton gradient' is not a true substrate, it has all of its characteristics, due to the fact that the translocation of protons is a necessary step of the overall phosphorylating process. From a general point of view, in a ternary system as this, each variable: ADP, phosphate, ΔpH, may be expected to change the apparent K_m of the others, not mentioning possible regulatory events. In principle, any such variation brings an information on the catalytic mechanism.

Fig. 7a shows that the apparent K_m of ADP for the ATP synthesis depends upon the magnitude of the proton gradient. It slightly decreases between ΔpH 3.1 and 3.4, then sharply increases above $\Delta pH \approx 3.45$. The significance of K_m variations is not clear, especially since it increases more than V_{max}, which is illustrated by the strong drop of the V_{max}/K_m ratio for $\Delta pH > 3.45$ (Fig. 7c). This would be in violation of the laws of enzymology unless the substrate had additional effects.

One of these effects could be a regulatory role of the proton gradient besides its energetic function. Indeed, an effector may increase the K_m of a reaction more than its V_{max} [68]. Relevant to this point is the report [69] that ΔpH decreases the affinity of ADP as an inhibitor of the enzyme: it could well be that ΔpH cannot increase K_i without doing the same with K_m. This new type of regulation, however, must not be confused with the well known activation process, which is the switch of the ATP synthase or hydrolase from its inactive form, in non-energized membrane, into an active form, for either of these two reactions, when it senses a sufficient $\Delta\tilde{\mu}_{H^+}$, i.e., here ΔpH (see, for example, Ref. 10).

An alternative hypothesis would be that at high rates of phosphorylation, the diffusion of substrates, namely ADP, becomes limiting [23]. In this case, K_m should increase indefinitely with ΔpH. It is not possible for the moment to know if K_m would level off for ΔpH above the highest attainable here (approx. 3.55); some indirect data suggest that this may be the case, at least when GDP is used instead of ADP [30].

The increase of phosphate concentration lowers the apparent K_m for ADP (Fig. 7a), in agreement with some [21] but not all [28] previous data. Taken alone, this fact nevertheless does not allow to determine the rank of phosphate and ADP intervention in the catalytic mechanism if the elementary steps of the phosphorylation are linearly arranged. Actually, ADP binding could be a primary event, considering the reciprocal case of ATP hydrolysis where ADP release is thought to be the terminal stage [28,70,71] (this last proposal was mainly based on the fact that high amounts of radioactive phosphate may be incorporated into ATP during net ATP hydrolysis and in the presence of an ADP trap [70]). However, a random mechanism as well would be consistent with ATP–P_i exchange experiments, but in all circumstances, phosphate binding would decrease

the ADP dissociation from the catalytic site of the enzyme during phosphorylation.

Whatever it may be, the variations of apparent K_m for ADP, especially with ΔpH, makes more complicated to check its possible dependence on factors like the redox chain activity [19,24,25], thylakoid swelling [23], external pH etc. Not only each experiment has to be carried out at iso-ΔpH conditions, as it was done here, but any comparison between two experiments should be made at identical ΔpH, with a precise control of how the above-mentioned factors may affect ΔpH and how the latter is measured. Firm conclusions will become possible only when all these conditions will be met.

The data of this work are consistent with a single-affinity process. The michaelian nature of our rate vs. substrate relationships makes that it is not necessary to imagine a cooperative or allosteric phenomenon, but we cannot exclude it, since we could not explore ADP concentrations below 4 μM. Various experimental facts support the idea of a cooperativity between different catalytic centers [72,73]. However, we must keep in mind that the non-hyperbolic relationships reported between ADP concentration and the rate of ATP synthesis [17,18] must be cautiously regarded, because the magnitude of ΔpH was not controlled during these experiments.

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