Biochimica et Biophysica Acta 893 (1987) 544–556
Elsevier

BBA 42624

ΔpH-dependent activation of chloroplast coupling factors and external pH effects on the 9-aminoacridine response in lettuce and spinach thylakoids

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(Received 1 April 1987)
(Revised manuscript received 13 July 1987)

Key words: Chloroplast coupling factor; Proton gradient; ATPase activation; 9-Aminoacridine; ATP synthesis; (Lettuce and spinach thylakoid)

Investigation of steady-state ΔpH and photophosphorylation in envelope-free chloroplasts extracted from lettuce or spinach leaves gave the following results. (1) The ratio of the rates of photophosphorylation at two nucleotide concentrations but at the same ΔpH adjusted by light intensity, and measured by 9-aminoacridine distribution, is independent of the magnitude of this ΔpH. Thus the flow–force relationships only represent the $\Delta\tilde{\mu}H^+$ activation of the coupling factors, each activated enzyme running at its maximum turnover rate. (2) Flow–force curves at pH 8.0 and pH 8.5 have similar shapes when ΔpH is delocalized with 200 μM hexylamine. Thus, activation of the coupling factors (CF) depends on the absolute value of ΔpH, regardless of the respective external and internal pH. This would mean that the basic process of activation is either voltage-dependent, or is due to the strictly coupled deprotonation of an external site of CF and protonation of an internal group. (3) The above result is not modified when GDP is used instead of ADP. This suggests that the shift in the equilibrium between active and inactive CF, due to tight binding of ADP, does not depend on the external pH. (4) With lettuce thylakoids, flow–force curves at pH 8.0 and 8.5 become identical only after a translation of the former by +0.15 along the ΔpH axis. Thus the response of 9-aminoacridine depends on the external pH, probably due to a change in the osmotic volume. The internal volume would be 40% higher at pH 8.5 than at pH 8.0. (5) Shifting the external pH from 8.0 to 8.5 has the same effect on 9-aminoacridine and imidazole responses. This confirms that the main effect is an increase of the lumenal volume at high pH. Direct estimations of this volume are qualitatively consistent with this view. (6) With spinach thylakoids, the effect of external pH on the 9-aminoacridine response is minimized. The 9-aminoacridine space change would be only 17% between pH 8.5 and pH 8.0. At pH 8.0, the light-induced uptake of 9-aminoacridine by lettuce thylakoids is insensitive to the increase in sorbitol concentration above 200 mM, whereas it decreases, as expected, with spinach thylakoids. (7) When the ΔpH is delocalized with 200 μM hexylamine, ΔpH and phosphorylation rates at low light intensities do not depend on the external pH. This means that not only the coupling factors, but also basal leaks, would be regulated by ΔpH and not by internal pH or a complex combination of external and internal pH. The results are discussed with

Abbreviations: CF, chloroplast coupling factor; CF_0, CF_1, membrane sector and catalytic moiety of the chloroplast coupling factor; NDP, NTP, nucleotide disphosphate, triphosphate; Chl, chlorophyll; FITC, fluorescein isothiocyanate; $\Delta\tilde{\mu}_{H^+}$, transmembrane difference of electrochemical potential of H^+; ΔpH, transmembrane pH difference; $\Delta\psi$, transmembrane electrical potential difference; PS I, Photosystem I.

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reference to previous reports on the pH and ΔpH dependency of ATP synthesis by thylakoids. It seems likely that most early discrepancies were due to the different origins of the chloroplasts and to specific behaviour with respect to the ΔpH probe. This should be considered as a warning when one compares data obtained with membranes from different plant species.

Introduction

According to the chemiosmotic theory [1,2], the ATP synthesis in bacteria, mitochondria and chloroplasts is coupled to a flow of H^+ ions through an ATP synthase complex called F_0-F_1, down a transmembrane electrochemical proton gradient ($\Delta\tilde{\mu}_{H^+}$) generated by an electron transfer chain. It is now recognized [3,4] that $\Delta\tilde{\mu}_{H^+}$ is necessary not only to drive a chemically useful proton flow through the ATP synthases (or coupling factors), but also to maintain these enzymes in an active conformation. This is especially obvious with chloroplast coupling factor (CF_1) working as an ATP hydrolase, since, despite its negative thermodynamic back-pressure effect [5], $\Delta\tilde{\mu}_{H^+}$ is required for the activity [6]. Actually, high rates of ATP hydrolysis may be measured only in the case of specific treatments of the coupling factors, such as reduction by thiol reagents [5,7–9], which is believed to make the enzyme susceptible to activation by lower $\Delta\tilde{\mu}_{H^+}$ [4,7,10,11]. Only in this case, $\Delta\tilde{\mu}_{H^+}$ may be both high enough to activate the ATPases, and sufficiently low to allow the proton pumping related to ATP hydrolysis. The activation of the coupling factor is accompanied by the release of tightly bound ADP [12–15]; thus, ADP binding is thought to displace the equilibrium between active and inactive coupling factors.

The primary processes of activation by $\Delta\tilde{\mu}_{H^+}$ have been proposed to involve protonation of some groups on the high-potential pole of the enzyme and/or deprotonation of some others on the low-potential side [4,11,16]. The number of protons involved in such a process and the cooperativity between events on the two poles of the enzyme have been discussed elsewhere [4,11,16]. A prediction from the proposed models is that if internal protonation and external deprotonation of the enzyme are strictly coupled processes, the amount of activated coupling factors should depend on $\Delta\tilde{\mu}_{H^+}$, regardless of the external pH [11]. A strict control of the rate of ATP synthesis by the proton gradient, independent of external pH, was observed using spinach chloroplasts, with ΔpH [17,18] or $\Delta\psi$ [19] as the major component of the driving force. Some other groups, however, found opposite results with lettuce chloroplasts [20] or with CF_0-CF_1 coreconstituted with bacteriorhodopsin into liposomes [21]. To explain some of the discrepancies, it has been proposed that the rate of phosphorylation could be strictly determined by $\Delta\tilde{\mu}_{H^+}$ only for low amplitudes of the proton gradient [17]. This logical hypothesis is now untenable, since ΔpH control independent of the external pH was found even for high values of the protonmotive force [18]. Another difficulty is that it was not proved that the driving force effect of the proton gradient was saturating in these experiments, although this was suggested by the proportionality between the release of tightly bound ADP and the initial rate of phosphorylation induced by short flashes or voltage pulses [22]. Thus, it is not sure that the rate of ATP synthesis reflects the amount of active coupling factors.

Therefore, two problems remain to be elucidated. Firstly, one needs to discriminate fully between the driving force and the activator roles of the electrochemical proton gradient. Secondly, it is essential to understand why some authors have found a dependency of ATP synthesis on both external pH and $\Delta\tilde{\mu}_{H^+}$, while a strict control by $\Delta\tilde{\mu}_{H^+}$ alone now seems the most probable hypothesis [18].

In this report, we present new data suggesting that in non-thiol-treated chloroplasts the rate of ATP synthesis directly represents the extent of coupling factor activation by ΔpH ($\Delta\psi$ being cancelled by valinomycin). Our approach, different from that previously used [22], is based on the comparison of flow–force relationships at two substrate concentrations and on simple enzyme kinetics. We also show that the amount of active ATP synthases probably depends simply on ΔpH if the response of 9-aminoacridine depends on the

external pH. We develop arguments according to which this behaviour would not be specific to this probe. In addition, we have compared spinach and lettuce chloroplasts as regards the 9-aminoacridine response and found that deviations due to the external pH are minimized in the first case. The osmotic behaviour is also different between the two species investigated. Therefore, previous discrepancies could be explained mainly by the origin of the biological material. Other experiments indicate that, at low light intensities and in the presence of hexylamine, the rate of phosphorylation and ΔpH are insensitive to external pH between 7.8 and 8.7. This would mean that not only CF_1, but also other proton leaks, are controlled by the ΔpH and not by the internal pH.

Materials and Methods

Envelope-free chloroplasts were extracted from market lettuce or spinach as in Ref. 23 and stored on ice and in darkness in 50 mM KCl/0.5 mM K_2HPO_4/2 mM Tricine (pH 8.2). Phosphorylation and ΔpH were simultaneously assayed as described elsewhere [24].

ΔpH estimations

ΔpH was estimated by the light-induced quenching of 9-aminoacridine fluorescence [25], using the classical formula:

$$\Delta\mathrm{pH} = \log\left\{(1+10^{\mathrm{pH}-\mathrm{p}K})\left[\frac{F^{\bullet}}{F^{\circ}}\left(\frac{V_0}{V_i}+1\right)-\frac{V_0}{V_i}\right]-10^{\mathrm{pH}-\mathrm{p}K}\right\}$$

$$\simeq \log(1+10^{\mathrm{pH}-\mathrm{p}K})+\log\frac{V_0}{V_i}+\log\left(\frac{F^{\bullet}}{F^{\circ}}-1\right)$$

Where $F^{\bullet}$ is the fluorescence of 9-aminoacridine in the dark (non-energized conditions), F° the fluorescence level under illumination, V_o the volume outside the thylakoids, and V_i their internal volume. pK is that of 9-aminoacridine ($= 10$) and pH refers to the external medium. Since the above relationship is probably only qualitatively valid [26], especially as the actual internal volume trapping the probe is difficult to estimate [27,28], we have used for the figures the expression: $\log R = \log(F^{\bullet}/F^{\circ} - 1) + \log(1 + 10^{\mathrm{pH}-10})$ instead of ΔpH. The two remain linearly related unless V_i is varied.

In some experiments, ΔpH was also estimated by the increase of light-induced proton uptake due to imidazole addition [29]. The formula used was:

$$\Delta\mathrm{pH} = \log\frac{\Delta[\mathrm{H}]}{[\mathrm{A}]}+\log\frac{(1+10^{\mathrm{pH}-\mathrm{p}K})^2}{10^{\mathrm{pH}-\mathrm{p}K}}+\log\frac{V_o}{V_i}$$

Where [A] is the concentration of imidazole in $\mathrm{mol}\cdot l^{-1}$, Δ[H] the additional proton uptake in $\mathrm{mol}\cdot l^{-1}$, and pK is that of imidazole (6.95). V_o, V_i and pH have their usual significance defined above. As for 9-aminoacridine, we have used a reduced expression: $\log R' = \log(\Delta[\mathrm{H}]/[\mathrm{A}]) + \log[(1 + 10^{\mathrm{pH}-6.95})^2/10^{\mathrm{pH}-6.95}]$, in which the internal volume contribution is omitted. Since imidazole had an uncoupling effect at the concentration used (100 μM), the Δ[H] could not be directly measured. Thus, the proton uptake due to the imidazole was obtained by the difference between the proton uptake with and without imidazole at identical log R. The latter was varied by light intensity. These experiments were carried out with chloroplasts, 30 μM chlorophyll in 1 mM Tricine/50 mM KCl/5 mM $MgCl_2$/1 mM K_2HPO_4/50 μM pyocyanine/4 μM 9-aminoacridine. ΔpH measurements at variable osmolarity were made in a medium comprising 2.5 mM Tricine/10 mM KCl/5 mM $MgCl_2$/50 μM pyocyanine/50 nM valinomycin/4 μM 9-aminoacridine with sorbitol at indicated concentrations.

Estimation of the internal volume of thylakoids

The total volume of thylakoids (membrane + lumen) was measured as follows: chloroplasts were suspended at a final concentration of 1 mM chlorophyll in 30 mM Tricine/50 mM KCl/5 mM $MgCl_2$ containing in addition fluorescein isothiocyanate covalently bound to Dextran (FITC-Dextran, M_r 150000) at a concentration of 45 μM. The suspension was adjusted to the required pH, centrifuged at $3000 \times g$ for 30 mn, and the pellet was resuspended in a FITC-Dextran-free medium. FITC-Dextran initially present in the water surrounding the chloroplasts was titrated by fluorescence (excitation, 490 nm; detection, around 550 nm). The estimated external aqueous volume was subtracted from the volume of the pellet to obtain the thylakoid volume.

To measure the volume of the membranes, 0.5

ml of chloroplasts at high chlorophyll concentration (3 to 5 mM) was dried to 24 h in a desiccator at room temperature. The volume of total (external + internal) water was estimated from the weight difference before and after desiccation, assuming a density of 0.998. The membrane volume was calculated by the difference between the volume of the sample and this aqueous volume.

Phosphorylation rate measurements

The phosphorylation rate was deduced from the scalar proton consumption, measured with a glass electrode, and corrected according to the external pH with appropriate coefficients [30]. Experiments were carried out with chloroplasts 30 μM chlorophyll in 2 mM Tricine/50 mM KCl/5 mM $MgCl_2$/2 mM K_2HPO_4/50 μM pyocyanine/ 50 nM valinomycin/4 μM 9-aminoacridine + ADP or GDP at indicated concentrations. Other additions are mentioned in the legends when necessary. The 1×1 cm spectroscopic cuvette, magnetically stirred, was maintained at 20 °C. To establish flow–force relationships, the activity was varied by actinic light intensity modified with neutral filters. The maximum intensity available (red light) was about 750 $W \cdot m^{-2}$. In experiments with limiting substrate, we took special care concerning the precise moment when the rate of phosphorylation and ΔpH were estimated. It was chosen when the ΔpH had just reached a pre-steady state, before any significant decrease of substrate concentration. This decrease was always less than 10% and generally around 5%, therefore no correction was judged necessary.

In each type of experiment, the sample was adjusted to the required pH a few minutes before the measurement.

Results

Rate of photophosphorylation as a function of ΔpH and nucleotide concentration

In order to study the role of external and internal pH in the coupling factor activation, we need to take into account the driving force effect of ΔpH on the rate of phosphorylation. It was suggested [4,11,22,31] that in non-thiol-modulated chloroplasts, this driving force effect is always saturating and that the ATP synthesis rate is directly proportional to the amount of activated ATP synthases. The most convincing argument for this is that the initial rate of ATP synthesis induced by short flashes or external voltage pulses was found to be proportional to the amount of nucleotide released [22], thought to represent the enzyme activation [32–34]. However, some people prefer to consider flow–force relationships as the expression of the order of the phosphorylation rate with respect to internal protons [18,35].

In the case of a saturated turnover for internal protons, one expects that the magnitude of the ΔpH would not change the relative increase of reaction rate with ADP concentration. Thus we have measured the rate of GTP synthesis versus the apparent ΔpH at GDP 50 μM and 500 μM. GDP was used instead of ADP because tight binding of GDP does not seem to inactivate coupling factors [34]. Fig. 1 shows that the ratio of the rates of GTP synthesis at these two substrate concentrations may be reasonably considered as independent of ΔpH. As V_p is plotted on a log scale, a constant rate ratio corresponds to a constant distance along the ordinate axis. This ratio is approx. 2.8, which would give, in the simplified

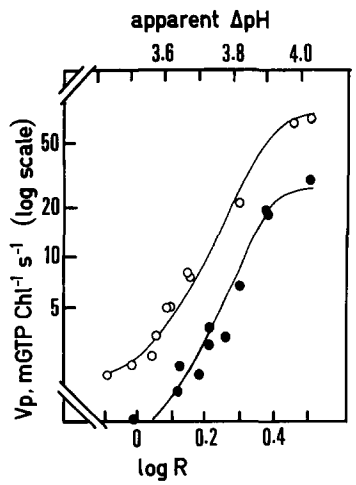

Fig. 1. Rate of GTP synthesis (V_p) as a function of 9-aminoacridine fluorescence quenching (log R) at two GDP concentrations: 50 (●) and 500 (○) μM. Conditions as in Materials and Methods. Lettuce chloroplasts were assayed at pH 8.2. 9-aminoacridine fluorescence quenching is represented by the expression: $\log R = \log(F^{\circ}/F - 1) + \log(1 + 10^{pH-10})$ (see Materials and Methods). Apparent ΔpH are calculated assuming a V_0/V_i ratio of about 2200.

case of a Michaelian process, a K_m of about 130 μM for GDP, not far from previously reported values of 115 μM [36] and 160 μM [37]. However, the two latter values were not obtained at constant $\Delta\tilde{\mu}_{H^+}$, since increasing the substrate concentration decreases the proton gradient even when the energy input is constant. To conclude, although other interpretations cannot be fully excluded (see Discussion), the data displayed in Fig. 1 strongly suggest that phosphorylation is of zero order with respect to ΔpH in the range investigated: in this case, correlation between V_p and ΔpH directly reflects the activation of the enzymes by the proton gradient.

Rate of photophosphorylation as a function of ΔpH at two external pH values

To determine whether the level of activated coupling factors is governed by ΔpH [4] or by a complex combination of external and internal pH [11], we have measured the rate of phosphorylation as a function of 9-aminoacridine fluorescence (related to ΔpH) at pH 8.0 and pH 8.5 under different conditions. The results are plotted in Fig. 2. The permeant buffer hexylamine (200 μM) was present (a,b,c) to ensure a complete delocalization of the steady-state proton gradient [38] which avoids any problems linked to the possible existence of local pH [39]. ADP (a) and GDP (b,c) were used as substrates, and in one case (c), valinomycin, needed to collapse $\Delta\psi$, was omitted. In all cases the curves at pH 8.0 and 8.5 are superimposable if one translates the former by about +0.15 along the ΔpH scale (here represented by the expression log R: see Materials and Methods).

Before going deeper into this result, the following three points should be noted: (1) When one operates at a constant nucleotide concentration, the use of ADP or GDP is indifferent. (2) $\Delta\psi$ has a negligible weight in the results depicted in Fig. 2, or, if any, the relationship between $\Delta\psi$ and ΔpH does not depend on the external pH. This may be understood if the main part of $\Delta\psi$ is due to the internal accumulation of charged hexylamine, strictly correlated to ΔpH regardless of the external pH. (3) In the absence of hexylamine the parallelism of the two curves at pH 8.0 and pH 8.5 following the abscissa axis is not conserved (data not shown): a localized ΔpH probably played a significant role here, despite the high KCl concentration (50 mM) of our medium, favouring the delocalization of the proton gradient [23].

At first sight, the results of Fig. 2 could be interpreted as an ATP synthase activity dependent not only on ΔpH but also on the external pH. In fact, we rather suspect that the systematic shift of 0.15 on the ΔpH scale is due to an effect of the external pH on the 9-aminoacridine response. An indication for this may be found in Fig. 3, which represents the rate of phosphorylation (a) and the 9-aminoacridine fluorescence quenching (b) as functions of light intensity, in a typical experiment like those used to give Fig. 2. It is obvious that, at least in the low light range, the rate of phosphorylation is the same at pH 8.0 and pH 8.5; then, at higher light intensities, the two curves diverge slightly (Fig. 3(a). On the other hand, whatever the

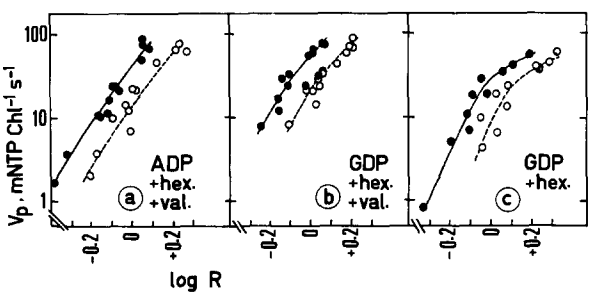

Fig. 2. Rate of NDP phosphorylation (V_p) versus 9-aminoacridine fluorescence quenching (log R) at two external pH for lettuce chloroplasts. ●———●, pH 8.0; O- - - - -O, pH 8.5. Experimental conditions as in Materials and Methods. NDP concentrations were: (a) 500 μM ADP; (b) 1000 μM GDP; (c) 500 μM GDP. Valinomycin was omitted in (c).

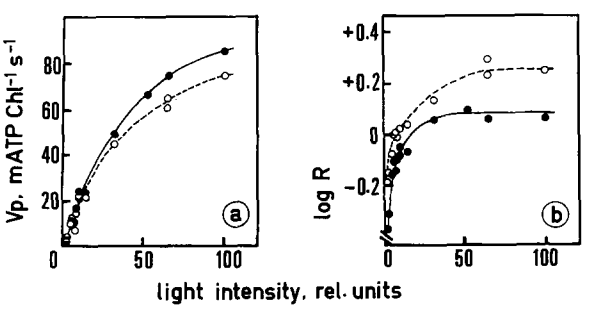

Fig. 3. (a) Rate of photophosphorylation (V_p) and (b) 9-aminoacridine fluorescence quenching (log R) as functions of the light intensity for lettuce chloroplasts. Conditions and symbols as in Fig. 2a. ●———●, pH 8.0; O- - - - -O, pH 8.5.

light intensity, the 9-aminoacridine fluorescence quenching is always higher at pH 8.5 than at pH 8.0 (Fig. 3(b). Therefore, the simplest way to explain all of the data is that: (i) the maximum turnover rate does not depend on the external pH, (ii) under limiting light the ΔpH magnitude is the same at pH 8.0 and pH 8.5, and (iii) its value is systematically overestimated by 0.15 at pH 8.5 with respect to pH 8.0. If this shift were due to a variation in the internal space for 9-aminoacridine with pH, it would correspond to a lumenal volume 40% higher at pH 8.5 than at pH 8.0 (see Discussion). Thus, after the adequate correction, the curves of Fig. 2 would become exactly the same at pH 8.0 and 8.5, suggesting that the coupling factor activation depends on ΔpH, regardless of the absolute value of external and internal pH.

Rate of photophosphorylation and 9-aminoacridine fluorescence quenching as a function of external pH at low light intensity

While in low light, the rate of phosphorylation is the same at pH 8.0 and 8.5, in strong light, it was found to be lower, equal or higher at pH 8.0 than at pH 8.5, depending on the chloroplast preparation (data not shown). This variable result could be interpreted as arising from the great complexity of the regulation of ΔpH and ATP synthesis in strong light (see Discussion). This makes the results strongly dependent upon proton leaks, abundance of PS I in the membrane, etc. In low light, however, all the parameters coming from thermal steps of the electron transfer or from its regulation by internal pH disappear. In this case the effect of the external pH on steady-state ΔpH reflects only its effect on membrane leaks and not on proton input. Consequently, the constancy of ΔpH and phosphorylation at pH 8.0 and 8.5 would mean that proton leaks are controlled by ΔpH, regardless of the internal and external pH. To verify these results over a larger pH range, we have measured the light-induced quenching of 9-aminoacridine and the rate of phosphorylation at a low light intensity between pH 7.7 and 8.7. The results are depicted in Figs. 4 and 5. Fig. 4 shows that with or without hexylamine, a progressive shift of the fluorescence quenching occurs when external pH is varied. But only in the presence of hexylamine is a 0.15 difference on the ΔpH scale found between pH 8.5 and 8.0. Without hexylamine, a higher difference is found, probably because a small increase of ΔpH with external pH is superimposed upon the change of 9-aminoacridine response. Accordingly, the rate of ATP synthesis is constant from pH 7.8 to pH 8.6 in the presence of hexylamine (Fig. 5a), but slightly increases with pH if this buffer is omitted (Fig. 5b). This weak difference of behaviour with and without hexylamine could be due to the fact that in the latter case, the ΔpH is higher and thence the electron transfer begins to be sensitive to internal pH. However, an alternative explanation will be considered in the Discussion. Whatever the cause, a significant dependence of the rate of ATP synthesis on external pH appears in strong light, which confirms the complexity of the regulation in this case: see Fig. 6.

Nature of the effect of external pH on the 9-aminoacridine response

To obtain more precise information about the supposed effect of external pH on the 9-aminoacridine response, we have also measured ΔpH by the increase of light-induced proton uptake upon imidazole addition [29]. 9-Aminoacridine fluorescence and proton uptake were monitored on the same sample, in non-phosphorylating

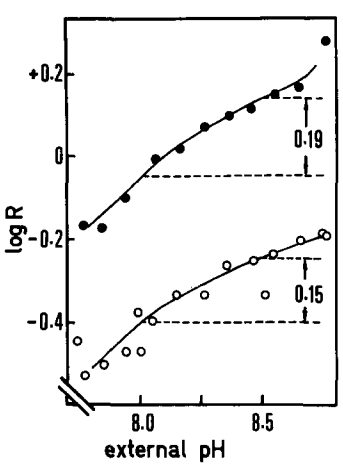

Fig. 4. Light-induced quenching of 9-aminoacridine fluorescence (log R) versus external pH for lettuce chloroplasts, with 200 μM hexylamine (○) and without hexylamine (●). Conditions as in Materials and Methods. The medium contained 500 μM ADP. Light intensity was around 20 $W \cdot m^{-2}$ (2.5% of the control).

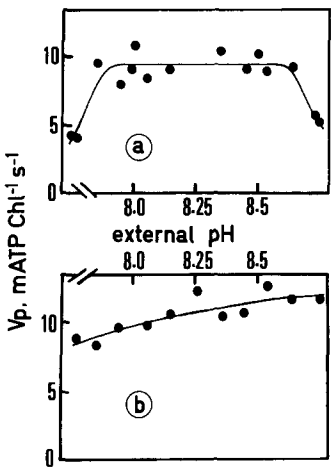

Fig. 5. Rate of ATP synthesis (V_p) versus external pH at low light intensity. Same experiment as in Fig. 4. (a) 200 μM hexylamine present; (b) no hexylamine.

conditions, at pH 8.0 and 8.5; the ΔpH was varied by light intensity. The results, displayed in Fig. 7, show that: (i) 9-aminoacridine and imidazole techniques give different ΔpH values; (ii) the correlation between these two ΔpH values does not depend on the external pH. This mean that, although the two probes differ in their absolute responses, their sensitivity to the external pH is exactly the same. Therefore, a change in the internal volume does seem to be the most likely interpretation for the pH-dependent shift in the 9-aminoacridine response, observed in Fig. 2. Indeed, this is the only nonspecific effect, expected

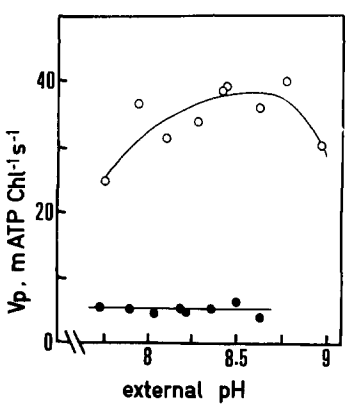

Fig. 6. Rate of ATP synthesis (V_p) versus external pH at two light intensities: 20 (●) and 200 (○) W·m^{-2}. Conditions as in Materials and Methods. Lettuce chloroplasts were assayed with 500 μM ADP.

to have the same weight on all probe partition techniques.

By centrifugation experiments, the internal

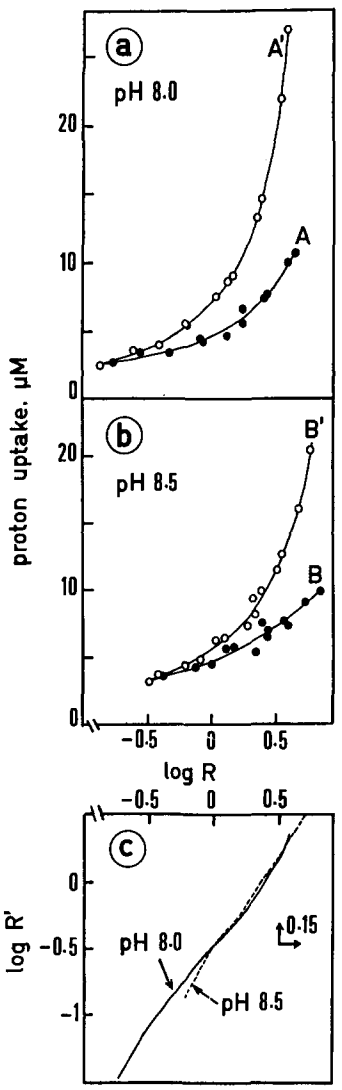

Fig. 7. Simultaneous measurement of 9-aminoacridine fluorescence and additional proton uptake due to imidazole addition. Conditions as in Materials and Methods. Lettuce chloroplasts were assayed at pH 8.0 (a,c) and pH 8.5 (b,c). (a,b) Proton uptake as a function of log R without (●———●) or with (○———○) imidazole. (c) log R' (imidazole uptake) as a function of log R (9-aminoacridine uptake), at pH 8.0 (———) or 8.5 (— — —). The imidazole-dependent proton uptake used to calculate log R' was obtained by subtracting curve A from A' (a, pH 8.0) and curve B from B' (b, pH 8.5). In (c), the slight divergence of the two curves for log $R < 0$ is not significant. The maximum divergence should correspond to a 25% error in the proton uptake at pH 8.5, a precision hard to obtain in this range.

volume of thylakoids was estimated at pH 8.0 and 8.5. The aqueous external space was probed by the non-permeant dye, FITC-Dextran, and the total aqueous content was estimated from the amount of water lost during desiccation of the preparation. Typical results are shown in Table I. It appears that: (i) the lumenal volume increases with the external pH; (ii) the volumetric ratio found between pH 8.5 and 8.0 is much higher than expected from Figs. 2–4. Maybe this estimation of the internal aqueous volume is not strictly identical to the operational space for the ΔpH probes. It is also possible that the internal volumetric ratio between pH 8.5 and 8.0, estimated in darkness, is greater than that under energized conditions. Finally, the important feature is that the results of Table I are qualitatively consistent with an increase of the lumenal volume with external pH.

We have also tried to calibrate the 9-aminoacridine response by acid-base jumps, as in Ref. 28, with the final pH being 8.0 or 8.5. Unfortunately, a dramatic decrease of the pH-jump-dependent 9-aminoacridine fluorescence quenching occurred for acidic stages below pH 6.5, making calibrations impossible for significant ΔpH ranges. Thus, this method, which gave satisfactory results with bacterial chromatophores [28], does not seem to apply to thylakoids.

9-Aminoacridine response and its sensitivity to external pH with lettuce and spinach thylakoids

The above results can hardly explain all of the discrepancies found in the literature [17–21] on the relationship between the rate of ATP synthesis, the electrochemical proton gradient and the external pH. Indeed, at variance with our present data, a flow–force relationship independent of the external pH was reported, without correction for 9-aminoacridine response or thylakoid internal volume changes [17]. We do not think that the experimental conditions used in this early report (flashing light and investigation of very low activities only) may account for the difference in the probe behaviour. We rather suspect that the use of spinach [17] or lettuce chloroplasts (this work) might be responsible for these conflicting results. To verify this point, we have repeated the experiment shown in Fig. 2a, but with spinach instead of lettuce chloroplasts. The result is displayed in Fig. 8. Although the flow–force curves are not exactly the same at pH 8.0 and pH 8.5, the interval between them is clearly diminished in comparison with lettuce chloroplasts: it went from 0.15 to about 0.07 on the ΔpH scale, which would represent a 17% instead of 40% difference in osmotic volume between pH 8.5 and 8.0. This smaller interval may fall into the limits of the errors encountered in Ref. 17. The following conclusion is that the probe behaviour with respect to the medium pH depends on the species from which the chloroplasts are extracted. Assuming that the pH-dependency of the probe response results from an internal volume variation, we have compared the osmotic response of 9-aminoacridine with lettuce and spinach chloroplasts. The results are plotted in Fig. 9. At pH 8.0, the light-induced fluorescence quenching (basal conditions) increases with sorbitol concentration up to 150–200 mM.

TABLE I

ESTIMATION OF THE THYLAKOID VOLUME IN DARKNESS AT EXTERNAL pH 8.0 AND 8.5

Three representative experiments carried out with different lettuce chloroplast preparations are presented. Total chloroplast and membrane volumes were estimated as described in the Materials and Methods. The membrane volumes, assumed to be independent of the external pH, was found to be more reproducible than in the total thylakoid volume. The value for the membrane volume used here, 12.4 l/mol Chl, was subtracted from the total volume to give the lumenal volume. The last column indicates the shift in ΔpH estimation, which should occur between pH 8.5 and 8.0 with such internal volume variations.

Experiment	Total chloroplast (l/mol Chl)		Lumen (l/mol Chl)		Lumen ratio $\frac{\text{pH 8.5}}{\text{pH8.0}}$	Shift in apparent ΔpH
	pH 8.0	pH 8.5	pH 8.0	pH 8.5		
I	28.6	63.8	16.2	51.4	3.17	+0.501
II	20.5	30.2	8.1	17.8	2.20	+0.342
III	20.5	73.2	8.1	60.8	7.51	+0.875

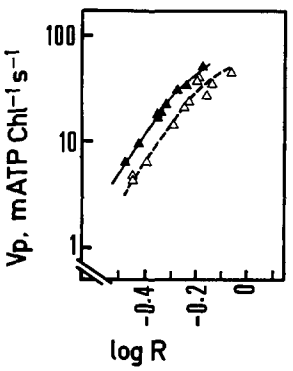

Fig. 8. Rate of ATP synthesis (V_p) as a function of 9-aminoacridine fluorescence quenching (log R) at two external pH for spinach chloroplasts: ▲, pH 8.0; △, pH 8.5. Conditions as in Fig. 2a, except that spinach chloroplasts were assayed.

This has previously been observed [40,41] and means that a good membrane energization is not possible when thylakoids are too swollen, whatever the origin of chloroplasts. For higher osmolarities however, the behaviour of spinach and lettuce thylakoids differs. Whereas the light-induced 9-aminoacridine fluorescence quenching by the former decreases at high sorbitol concentration, as expected from thylakoid shrinkage, this is not the case for the latter, which is insensitive to high osmolarity. At pH 6.5, normal behaviour is found for lettuce thylakoids, with the additional feature that a good membrane energization occurs, even at low osmolarities. Whatever the reasons, discussed in the following section, the experiments at pH 8.0 clearly confirm that 9-aminoacridine, and maybe other ΔpH probes, have different osmotic responses in lettuce and spinach chloroplasts.

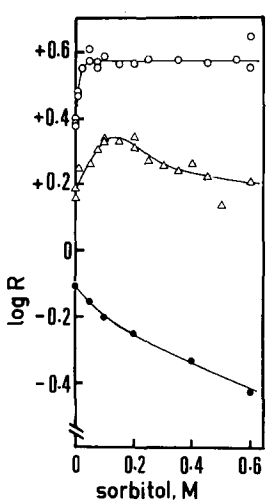

Fig. 9. Light-induced quenching of 9-aminoacridine fluorescence (log R) as a function of the medium osmolarity for chloroplasts from two different species. At pH 8.0, lettuce (○) and spinach (△); at pH 6.5, lettuce (●). Basal (= non-phosphorylating) conditions as described in Materials and Methods.

Discussion

Order of the reaction of phosphorylation with respect to ΔpH

The dependence of the rate of ATP synthesis on substrate concentrations (ΔpH being considered as a substrate) has been widely investigated. Very scattered values of the K_m for ADP have been reported (for a review, see Ref. 4). A related problem is that the apparent affinity of ADP for ATP synthesis was found to decrease when ΔpH was increased by accelerating the proton input [18,33,42,43]. When ΔpH was adjusted with a protonophore, the K_m for ADP was found to be slightly or not sensitive to the magnitude of the proton gradient, depending on the uncoupler concentration range [18,43].

These apparent discrepancies may be overcome, considering as in Refs. 4, 18 that an accurate determination of the rate/substrate concentration relationship should be carried out at a constant ΔpH, a condition realized only if artificial proton leaks prevent the decrease of ΔpH upon ADP addition. As a precise control of the ΔpH amplitude with light intensity is in practice difficult, it seems better to measure ΔpH for every value of the phosphorylation rate to obtain correlations, as in Fig. 1. Even though we have used only two GDP concentrations, the constancy of the rate ratio at each ΔpH clearly indicates that the apparent affinity of GDP for phosphorylation does not depend upon the ΔpH magnitude. We have considered whether some bireactant systems could account for such a behaviour. Among simple mechanisms involving two substrates (here NDP and ΔpH, phosphate being considered as saturating), we have found only two cases where it could be theoretically possible: on the one hand, the so-called 'ordered bireactant system' [44], but

only for small values of ΔpH and phosphorylation rates, whilst we have investigated a large range of ΔpH (Fig. 1); on the other hand, the 'random bireactant system' [44], but provided H^+ translocation through the ATP synthase complex does not affect ADP binding, and vice versa. This second possibility can be ruled out, in view of the now prevailing idea, experimentally well supported, of binding changes for substrates and products as energetic processes of phosphorylation [45–47].

If simple mechanisms cannot account for a constant ratio of the rates of phosphorylation at two NDP concentrations over a large ΔpH range, it seems doubtful that more complex ones would succeed. So the more likely interpretation remains that the phosphorylation reaction becomes zero order towards ΔpH as soon as a detectable rate of phosphorylation is measured. Thus, in accordance with Gräber and co-workers' view [4,19], but at variance with the interpretation of McCarty's group [18,35], we think that the increase of the rate of phosphorylation only reflects the variation of the number of coupling factors activated by ΔpH, each of them running at the maximum turnover rate.

Activation of the coupling factor by $\Delta\tilde{\mu}_{H^+}$

We consider that, after an adequate correction for the ΔpH probe response, made necessary by a probable effect of external pH on the thylakoid volume, the rate of ATP (or GTP) synthesis depends only on ΔpH, irrespective of the external pH, as in Refs. 17, 18. Although this was not indicated in the reports mentioned, we suppose that such a correction for the pH-dependency of the internal volume was made in Ref. 18, but not in Refs. 20 and 21, which would explain the conflicting results. In addition, it is likely that such a pH effect was minimized in the former case [18], as spinach thylakoids were used (see Results and compare Figs. 2 and 8). It is interesting to note (Fig. 2) that the lack of the dependency of the enzyme activation on the external pH was obtained with ADP as well as with GDP. This shows that a shift in equilibrium between active and inactive coupling factors due to tight binding of ADP does not depend on the pH.

The electrical and chemical components of the proton gradient seem to be equivalent to trigger phosphorylating activity [48,49]. It has been proposed that a transmembrane proton well (maybe F_0?) in thermodynamic equilibrium with the high potential compartment actually converts $\Delta\psi$ to ΔpH, which allows the basic process of activation to be reduced to acid–base reactions [11]: activation could depend on the protonation of some internal groups and/or deprotonation of external functions [4,11,16]. In such a scheme, the control of the activity by ΔpH and not by external and internal pH would mean that both internal protonation and external deprotonation are required for the activity, at variance with the scheme proposed in Ref. 16. In addition, these two events should be strictly coupled [4]. In other words, the pK of one pole of the enzyme would greatly depend on the protonation state of the other pole [11]. A simple electrostatic effect could be responsible for these pK shifts, provided that the internal and external groups are separated by a very short distance, or by a medium of very low dielectric constant. Otherwise, conformational changes should be invoked.

An alternative hypothesis for the primary processes of activation could be that the proton well does not convert $\Delta\psi$ to ΔpH, but ΔpH to $\Delta\psi$. In fact, the sense of the conversion might be determined by the position of charged buffering groups in this proton well. It is thus possible to imagine that switching the enzyme from the inactive to the active form basically depends on $\Delta\psi$, due to the orientation of some polar groups in the transmembrane electrical field [50,4]. Such a mechanism, though to operate in many other voltage-dependent channels, would easily explain the lack of an external pH effect.

pH-dependency of the 9-aminocridine response

We have shown that the quenching of 9-aminoacridine fluorescence increases with the external pH, especially when lettuce chloroplasts are used. By a comparison with the imidazole technique, we have demonstrated that this change in response is not a specific behaviour of this probe, at least at a high ionic strength as in Figs. 1–8. Thus, problems arising from the fluorescence properties of 9-aminoacridine or specific interactions with the membrane may be discarded. It

seems reasonable to interpret the variations of the probe response as an increase in thylakoid volume with pH, which should have the same effect on all of the measurements of ΔpH based on the distribution of amines. For a given ΔpH, the higher the external pH, the higher the internal pH, and the greater is the internal negative surface charge density. Thus the increase of external pH would lead to a swelling of the lumen through the increase of repulsive forces between opposite internal faces of the thylakoids [51]. This is qualitatively consistent with the data of Table I. However, a troublesome point is that the pH dependency of the 9-aminoacridine response does not depend on the amplitude of ΔpH: the internal volume ratio between pH 8.5 and 8.0 would be around 1.40 for lettuce thylakoids (Fig. 2) and 1.17 for spinach thylakoids (Fig. 8) for all of the ΔpH range investigated. This is rather unexpected, because the changes in internal surface charges and resulting repulsive forces may be a complex function of the internal pH.

In this context of internal volume changes, the different behaviour of spinach and lettuce thylakoids emphasized in Figs. 8 and 9 would appear to be due to different osmotic properties. This could be due to an interspecific variability of the internal surface charge density or of the dielectric constant of the lumen. Thus, the lack of lettuce thylakoid shrinkage at high sorbitol concentration and at pH 8.0 (Fig. 9) could be due to strong internal repulsive forces. Such an effect would be minimized at external pH 6.5, due to the protonation of the internal groups. But it is also possible, with the low ionic strength used in Fig. 9, that the unvaried 9-aminoacridine fluorescence quenching at high sorbitol concentration and pH 8.0 reflects trapping of the probe at the interfaces of lettuce thylakoids rather than in the internal bulk. Indeed, it has been shown [27] that negative surface potential should increase the contribution of surface probe trapping upon energization.

An increase [52] as well as a decrease [53] in thylakoid volume with external pH have been reported. Discrepancies may come from the different techniques used (light scattering [52] and labeling with tritiated water [53]). In our hands, an increase was always observed. Lastly, it is not certain that the actual operational volume for 9-aminoacridine (and other probes) can be measured [28]. Whatever it may be, the very different behaviour of lettuce and spinach thylakoids is an important parameter to consider when one compares data from the literature. It is probably as important as the nature of the probe or the physicochemical properties of the medium.

Constant ΔpH and phosphorylation rate at a low light intensity and variable external pH

The extent of the steady-state proton gradient depends on redox-driven proton influx and on H^+ leaks, which are back-controlled by the electrochemical proton gradient, but maybe also by internal and/or external pH. In limiting light, the weight of the pH sensitive thermal steps of the electron flow is minimized; moreover, the back-pressure effect of ΔpH on the redox chain, which might be dependent on the internal pH, is negligible. Thus the proton input may be considered as independent of the external (and internal) pH. The constancy of the rate of phosphorylation, and probably also ΔpH, over an external pH range of almost one unit (Figs. 4–6) should mean that the proton leaks controlling the ΔpH amplitude depend on ΔpH only and not, for example, on the internal pH. This is not surprising for the coupling factors, since we suppose that they are strictly controlled by ΔpH. But in this low ΔpH condition, since the phosphorylation yield is very low [35,55], the activated coupling factors are not the only leaks to take into consideration. Therefore, non-productive leaks must be also under the strict control of ΔpH. The fact that we observed this strict control only in the presence of hexylamine 200 μM (Fig. 5), at variance with Ref. 18, might be explained by a different ΔpH range, as mentioned in the Results section. It may also mean that, unless this permeant buffer is added, basal and phosphorylating proton leaks are controlled by slightly different local proton gradients in lettuce thylakoids.

The $\Delta\tilde{\mu}_{H^+}$ dependency of both productive and not productive leaks may be related to an interesting observation by Hangarter and Ort [56], who have shown that the threshold for ATP synthesis under flashing light does not depend on the flash frequency, but only on the number of protons translocated. They concluded that phosphorylat-

ing and basal H^+ efflux begin in the same $\Delta\tilde{\mu}_{H^+}$ range. ΔpH-dependent basal pores could hardly consist of CF_0 completely free of CF_1, because it was recently suggested [57] that such a channel is sufficient to uncouple the thylakoid fully. But the possibility cannot be excluded that basal leaks represent CF_0 where some CF_1 subunits remain bound, or more simply damaged coupling factors. However, some other voltage-dependent pores may naturally exist. Also, hexylamine, of which the internal concentration is ΔpH-dependent, must significantly contribute to proton leakage.

Acknowledgements

We are indebted to Françoise de Kouchkovsky for her excellent technical work, to Dr. Yaroslav de Kouchkovsky for helpful suggestions, and to Dr. Michael Hodges for reading the manuscript.

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