Biochimica et Biophysica Acta 933 (1988) 487–500
Elsevier

BBA 42772

ΔpH-activation of the thiol-modified chloroplast ATP hydrolase. Nucleotide binding effects

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(Received 9 November 1987)
(Revised manuscript received 2 February 1988)

Key words: ATP hydrolase; Thiol modulation; Proton gradient; Nucleotide binding; Coupling factor; (Chloroplast)

The activation of the membrane-bound ATP hydrolase by the electrochemical proton gradient $\Delta\tilde{\mu}_{H^+}$ was studied with dithiothreitol-treated, dark-adapted lettuce thylakoids. After reactivation, carried out at different light intensities, hydrolysis of ATP or GTP was measured upon fast membrane deenergization and substrate addition. The following results were obtained. (1) The initial rate of ATP hydrolysis depends on both the ΔpH attained during the light-reactivation stage and on the external pH (ΔpH was measured by the 9-aminoacridine technique, ΔΨ being collapsed by valinomycin). (2) When hexylamine is present the initial rate of ATP hydrolysis, as that of GTP hydrolysis measured in the presence of 50 μM GDP, only depends on the ΔpH. (3) ADP addition is able to inhibit a fraction of the ATPases within less than a few seconds. The affinity for the inhibitor is increased if ADP is added 10 s after complete membrane deenergization. From the results obtained, with either ATP or GTP in delocalized conditions (hexylamine present), it is proposed that the number of active thiol-reduced ATPases is a simple function of the ΔpH, independent of the external pH. The results without hexylamine are interpreted as being due to an incomplete delocalization of the proton gradient. The mechanistic implications of this ΔpH-activation are discussed. The rapid deactivation of only a fraction of active ATPases by ADP binding, and more especially the decrease in affinity for this inhibitor by ΔpH, suggests the existence of different active states which are discriminated by their deactivation and not their catalytic properties.

Introduction

When thylakoids isolated from dark-adapted leaves are illuminated, the membrane-bound chloroplast ATPase (CF_0-CF_1) catalyses ATP synthesis as predicted by the chemiosmotic theory [1]. However, only slight [2–4] or no net ATP hydrolysis can be observed unless the membranes have been incubated in the presence of thiol reductants [5–7] or products such as trypsin [8] or methanol [9].

To explain this apparent irreversibility of the enzyme, a working hypothesis has been developed

Abbreviations: CF, chloroplast coupling factor; CF_0 and CF_1, membrane sector and catalytic moiety of the chloroplast coupling factor; $\Delta\tilde{\mu}_{H^+}$, transmembrane difference of electrochemical potential of H^+; ΔpH, transmembrane pH difference; ΔΨ, transmembrane electrical potential difference; NDP, nucleotide diphosphate; NTP, nucleotide triphosphate; DTT, dithiothreitol; PS I, Photosystem I; HPLC, high-performance liquid chromatography; Chl, chlorophyll; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.

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[10,11] which invokes activation processes: the electrochemical potential difference of protons ($\Delta\tilde{\mu}_{H^+}$) generated across the membrane by light-driven electron transport or pH jumps is thought to play two roles in ATPase functioning. Besides its driving-force effect, providing the coupling factor with the free energy required for net ATP synthesis, the $\Delta\tilde{\mu}_{H^+}$ is also needed to convert the inactive form, which exists under deenergized conditions, into an active one.

Consequently, the simplest way to account for the lack of observed ATP hydrolysis can be summarized as follows: a high $\Delta\tilde{\mu}_{H^+}$ which enables ATP hydrolase activation, thermodynamically favours ATP synthesis rather than hydrolysis, while a lower $\Delta\tilde{\mu}_{H^+}$ is not sufficient to maintain the enzyme in an activated state [7,11].

It is now widely accepted and experimentally demonstrated, that the indispensable presence of thiol reactants during light triggering of ATPases is explained by the fact that the reduction of certain disulfide bonds of the γ-subunit [12–14] lowers the magnitude of the $\Delta\tilde{\mu}_{H^+}$ required for activation [15,16]. This thiol modification of the enzyme seems to mimic the physiological system which prevents in vivo ATP hydrolysis by CF_0-CF_1 in the dark [17,18].

Moreover, the nucleotides which are tightly bound to the coupling factor and exchanged only upon energization [19–21] are also thought to be involved in the regulation process (for reviews, see Refs. 22 and 23). On the one hand, the conversion of a latent form to an active enzyme was shown to be linked to the release of bound ADP [2,24], related to conformational changes [24]. On the other hand, correlations have been established between the rebinding of ADP to the 'tight sites' and the dark decay of the light-triggered CF_1 activity [25–27].

Regardless of the redox state of the enzyme, the primary processes of ΔpH-activation, although disputed, have been proposed to involve acid-base reactions on the two poles of the membrane-bound enzyme [11,28,29]. More precisely, the activation mechanism would imply protonation of the internal pole of the enzyme. Whether deprotonation of the low potential pole is necessary [11,28] or not [29], is still disputed. In the case where the protonation and deprotonation of the opposite poles are necessary and strictly coupled events, as proposed in Ref. 11 and suggested for a particular case in Ref. 28, the fraction of active ATPases would depend only on ΔpH, regardless of the external pH. With non-thiol-modulated thylakoids it has been recently shown [30], as already suggested [31], that the rate of ATP synthesis directly represents the extent of coupling factor activation by ΔpH. Furthermore, arguments have been developed to clarify discrepancies in the literature with regards to the involvement of the external pH in ATPase activation [32–36]. We have confirmed [30] the previous reports [32–34] that the amount of active ATP synthases is ΔpH dependent, irrespective of the external pH, in accordance with a strictly coupled model.

This study concerns the ATP hydrolase activation using thiol-treated thylakoids. Correlations between the hydrolysis rate and ΔpH have been established, as for ATP synthases, in order to check the concerted nature of the acid-base reactions invoked by theoretical schemes. Experiments were carried out either with the natural substrate ATP or with GTP [37] in order to avoid inhibition of the enzyme by newly synthesized ADP (GDP being known to be a poor inhibitor [38]). Furthermore, in some cases, ΔpH was artificially delocalized by hexylamine. We have also studied the effects of ADP inhibition on the initial rates of hydrolysis and on the decay of ATPase activity.

Materials and Methods

Thylakoids were prepared as in Ref. 39 and stored on ice and in darkness in a medium containing 2 mM Tricine, 50 mM KCl, 0.5 mM K_2HPO_4 (pH 8.2) at 500 μM or 1000 μM chlorophyll. NTP hydrolysis and ΔpH were simultaneously assayed in a set-up described elsewhere [40].

Estimation of ΔpH. ΔpH was estimated by the light-induced quenching of the 9-aminoacridine fluorescence using the classical model [41]:

$$\Delta pH = \log\left(\frac{F^\bullet}{F^\circ} - 1\right) + \log(1 + 10^{pH - pK}) + \log\frac{V_e}{V_i} \qquad (1)$$

where $F^\bullet$ is the fluorescence of the 9-aminoacridine in the dark (non-energized conditions), F°

the fluorescence level under illumination, V_e the external volume and V_i the internal volume of the thylakoids. pK is that of 9-aminoacridine (= 10) and pH refers to the external medium.

Since the determination of the internal volume V_i is practically difficult, the ΔpH values are qualitative and allow only comparisons at identical ΔpH. Therefore, the volume contribution was suppressed and the following simplified expression was used, where log R is linearly related to ΔpH unless V_i is varied:

$$\log R = \log\left(\frac{F^{\bullet}}{F^{\circ}} - 1\right) + \log(1 + 10^{pH-pK}) \quad (2)$$

We have recently shown with untreated thylakoids [30] that the 9-aminoacridine response was modified by the external pH due to changes in the osmotic volume. In order to account for this effect, a corrective factor must be subtracted from the log R values calculated at pH 8.5. This is also required for thiol-modulated material, where the factor differs only slightly with respect to the non-thiol-modulated system; the corrections are discussed in the Results section.

Hydrolysis-rate measurements. The NTP hydrolysis rates were deduced from the scalar proton production, measured with a glass electrode as in Ref. 42. Unless otherwise indicated, light reactivation and hydrolysis steps were carried out at 20 °C with thiol-modulated thylakoids at 30 μM chlorophyll, in the same medium containing 1 or 2 mM Tricine, 50 mM KCl, 5 mM $MgCl_2$, 0.5 mM K_2HPO_4, 50 nM valinomycin (to cancel $\Delta\Psi$), 50 μM pyocyanine, and 4 μM 9-aminoacridine. In each type of experiment the sample was adjusted to the required pH a few minutes before the measurement.

After different light-activation times, the back-pressure effect of the ΔpH was suppressed by switching off the light and complete discharging of the membrane with 2 μM nigericin, the rates of NTP hydrolysis were measured immediately afterwards. More precisely, as the scalar proton production can be fitted by first-order kinetics, the initial rate was extrapolated, because of the disturbing of the pH monitoring by the vectorial proton release and mixing during the first five seconds. The time response of the glass electrode, estimated by HCl injections, was 2 s for external pH variations equivalent to those found during hydrolysis assays.

ATP (500 μM) or GTP (1 mM) was injected concomitantly with nigericin at light extinction unless otherwise indicated. When added, ADP and GDP were mixed with the corresponding triphosphate nucleotide. In all cases the nucleotides were adjusted to the required pH before injection in order to minimize problems linked to external pH shifts. However, in certain cases such artefacts could not be fully avoided and they were subtracted from the scalar proton production recording.

In order to prevent spontaneous hydrolysis of GTP, the GTP + GDP mixture was frozen after pH adjustment and thawed a few minutes before addition of nigericin and injection into the cuvette.

Modulated chloroplast preparation. In the initial experiments, chloroplasts were illuminated for 5 min in strong light in the assay medium (pH 8.3) to which 20 mM DTT was added. After 4 min of darkness the pH was shifted to 8.0 or 8.5, 9-aminoacridine was added at 4 μM and the experiment carried out.

Premodulated chloroplasts were prepared according to a protocol adapted from Ref. 43. Thylakoids (60 μM chlorophyll) in 10 mM Tricine-KOH, 50 mM KCl, 5 mM $MgCl_2$, 1 mM K_2HPO_4, 50 μM pyocyanine, 20 mM DTT (pH 8.3) were illuminated using strong light for 5 min. After turning off the light, they were diluted fivefold in the same medium except that $MgCl_2$ and DTT were omitted. The thylakoids were diluted to 1000 μM Chl in 1 mM Tricine-KOH, 50 mM KCl, 0.5 mM K_2HPO_4, 4 mM DTT (pH 8.3) and stored on ice and in darkness. Chlorophyll was assayed by the method of Arnon [44].

ADP (sodium salt, ref A 2754) and ATP (disodium salt, ref A 5394) were purchased from Sigma; GTP (sodium salt, ref. 2431) was obtained from Serva and GDP (dilithium salt) from Boehringer.

Results

Characterization of premodulated thylakoids

It is now well accepted [15,16] that thiol modulation lowers the ΔpH requirement for ATP hy-

drolase activation. A combination of transitions induced by ΔpH and DTT leads to the following four forms of the enzyme as reported in Refs. 43 and 45.

$$E^i_{ox} \overset{\Delta pH}{\rightleftharpoons} E^a_{ox} \overset{DTT}{\rightleftharpoons} E^a_{red} \overset{\Delta pH}{\leftrightharpoons} E^i_{red} \quad (3)$$

where ox and red refer to the oxidized and reduced states, respectively; a and i to active and inactive conformations. The transition between E^i_{ox} and E^i_{red} is very slow [45] and is not indicated here.

Simple consideration of this model emphasizes the complexity of such a system in the establishment of relationships between the amount of active ATPase and ΔpH. The ATP hydrolase needs uncoupled conditions to work at a maximal turnover rate, while the oxidized state E^a_{ox} decays rapidly to E^i_{ox} in the absence of energization [11]. Therefore, only the slow equilibrium between reduced forms occurring in tens of seconds [29,40] must be considered to study ΔpH activation of ATP hydrolases.

In a first set of experiments, thiol modulation and ATP hydrolysis were carried out in the presence of DTT. The two-step procedure used to obtain a population of reduced, deactivated, light reactivable ATPase presented two difficulties: firstly, varying the ΔpH amplitude during light reactivation to build 'flow-force curves' was likely to modify the total amount of reduced enzyme,

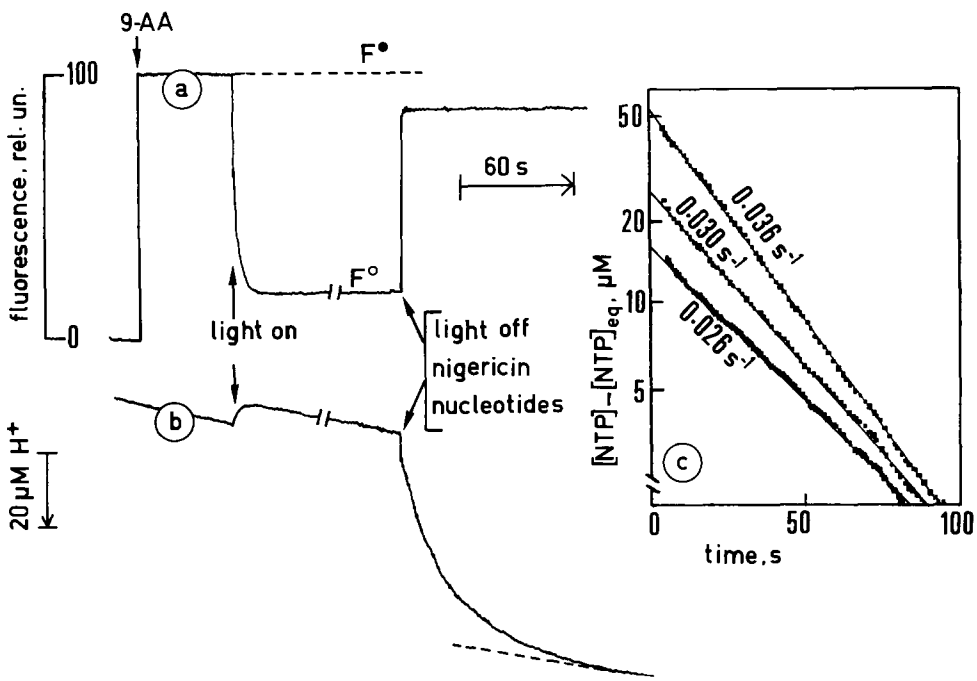

Fig. 1. Typical recording of 9-aminoacridine fluorescence and external pH changes and their significance. Conditions as in Materials and Methods. Reactivation times between 30 and 420 s. (a) Fluorescence of 9-aminoacridine. $F^\bullet$, F°: dark and light fluorescence levels used in calculations (Eqns. 1 and 2). The incomplete recovery of the dark fluorescence level upon deenergization is due to nucleotide quenching. (b) External pH change converted to proton uptake or release according to the buffer power of the suspension. The rapid proton uptake at the dark-to-light transition is a vectorial proton pumping. An equivalent proton release is observed when the light is turned off and nigericin added. The following acidification is due to NTP hydrolysis. The corresponding part of the curve was digitized, then fitted with an exponential decay superimposed on a linear drift, using a least-squares iterative method. The slope of the computed asymptote (dashed line) accounts for the slow drift of the glass electrode signal. This baseline was subtracted from the total signal to obtain the semi-log plots in (c). pH changes due to nucleotide injection were measured without chloroplasts and subtracted if necessary before any calculation. (c) Three semi-log plots of NTP consumption after 120 s reactivation at different light intensities. [NTP], instantaneous concentration of nucleotide triphosphate. $[NTP]_{eq}$, 'equilibrium' concentration of nucleotide triphosphate, i.e., at the end of the ATPase decay. Kinetic constants are indicated on the plots. The initial amplitudes give the amount of NTP hydrolysed during the total decay of activity. The initial rate is the product of the amplitude by the kinetic constant.

and secondly, noisy recordings were obtained due to a DTT effect on the glass electrode.

Therefore, premodulated thylakoids were prepared from an adapted protocol of Bakker-Grunwald and Van Dam [43]. With this type of thiol-pretreated material, hydrolysis of nucleotide triphosphates could be carried out in the absence of DTT. However, the latter was necessary in the storage medium at a low concentration to minimize the reoxidation of the reduced deactivated enzymes ($E^i_{red} \rightarrow E^i_{ox}$) during the 4–5 hours required to carry out an experiment. A pH of 8.3 was chosen for storage conditions in accordance with the results presented in Ref. 40.

The residual DTT concentration in the cuvette during the reactivation step and ATP hydrolysis measurements was only 240 μM. Thus, the system was reduced to:

$$E^a_{ox} \leftarrow E^a_{red} \overset{\Delta pH}{\leftrightharpoons} E^i_{red} \quad (4)$$

Fig. 1 shows a typical recording which was obtained under these conditions. After reactivation, the light was turned off and a mixture of ATP and nigericin was added. Dark-deactivation of the reduced enzyme was monitored by the decay of the scalar H^+ production. The fitting by first-order kinetics allowed the extrapolation of the initial rate. When thylakoids were modulated in the cuvette, the hydrolysis assay was exactly the same, except for the presence of DTT. Fig. 1c shows the results of three measurements made at different light intensities.

Fig. 2 shows initial rates of ATP hydrolysis measured as in Fig. 1, except that the time of reactivation in strong light was varied between 30 and 420 s. At pH 8.0 there was only a slow loss of activity while there was no loss at pH 8.5. This means that the light-reoxidation of the active reduced state (E^a_{red} to E^a_{ox}) can be neglected in our conditions. Thus we measure only the transition between inactive and active reduced states:

$$E^a_{red} \overset{\Delta pH}{\leftrightharpoons} E^i_{red} \quad (5)$$

The lack of any significant reoxidation allows us to choose reactivation times longer than those generally used in the literature [16,29] and thus a

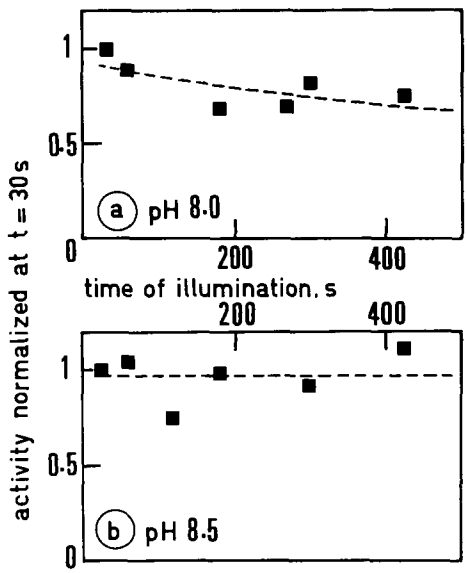

Fig. 2. Initial rate of uncoupled ATP hydrolysis as a function of the light-reactivation time in strong light. Thiol-treated chloroplasts were assayed in a DTT-free medium. No hexylamine was present. The activities were normalized to those obtained after 30 s of illumination (red light, 800 $W \cdot m^{-2}$). (a) pH 8.0; (b) pH 8.5. Maximal activities at pH 8.0 are approx. 122 mATP per Chl per s.

real equilibrium may be attained, even for a low ΔpH, between the active and inactive reduced forms (E^a_{red} and E^i_{red}). Indeed, Fig. 3 presents two types of experiment which show that the equilibrium is reached for short times of light activation even in the presence of a very low ΔpH, i.e., for a log R much smaller than these used in the following figures. Fig. 3a and b deal with the reactivation of the ATPase activity with thylakoids thiol-modulated in the cuvette at pH 8.2. After a complete dark deactivation in uncoupled conditions (see Fig. 1), checked by a nil hydrolysis rate, the thylakoids were reilluminated in strong light. The external pH changes (a) and the instantaneous rate of H^+ appearance (b), plotted versus time, show that a steady-state rate of hydrolysis was reached during the first 30 s, while log R was less than -1.6.

Since the ATPase activation may depend on the presence of ATP [46] and also whether ΔpH was lowered by light intensity or uncoupler concentration [39,47] we have also studied the ATP hydrolase activation at a very low light intensity. Fig. 3c

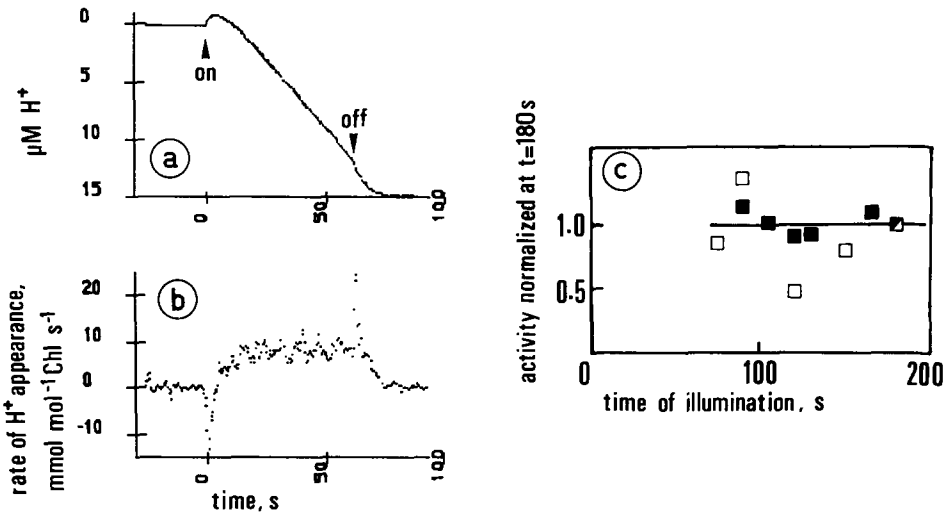

Fig. 3. Rate of ATP hydrolase activation by a very low ΔpH. (a) External proton uptake and production as a function of the reactivation time after a complete deactivation in the absence of hexylamine. Conditions as in Fig. 1, except that thylakoids were thiol-modulated in strong light, in the cuvette, at pH 8.2. The H^+ uptake or release when the light is turned on (↑) or off (↓) reflects transmembrane proton flow due to very low ΔpH. The slope corresponds to ATP hydrolysis. (b) Instantaneous rate of H^+ appearance as a function of reactivation time, obtained from the first derivative of curve (a). (c) Initial rate of uncoupled ATP hydrolysis as a function of the activation time in low light. Conditions as in Fig. 2, except that hexylamine was present at 200 μM and light intensity was approx. 2 $W \cdot m^{-2}$. (□) pH 8.0; (■) pH 8.5. Activity normalized at those obtained at 180 s (pH 8.0: approx. 2.5 mATP per Chl per s; pH 8.5: approx. 4 mATP per Chl per s).

shows the evolution of initial rate of ATP hydrolysis as in Fig. 2, except that reactivation was performed in low light. At pH 8.5, it is obvious that the maximum rate (around 4 mATP per Chl per s) was attained in less than 100 s, while log R was around −1.4. At pH 8.0, despite the scattering of the data due to very low activities (around 2.5 mATP per Chl per s, close to the limit of resolution), this result seems to be confirmed. Thus 120 s is a long enough activation time to reach the E^i_{red} to E^a_{red} equilibrium and is used in the following experiments.

ATP hydrolysis rate as a function of Δ pH amplitude and external pH

In order to investigate the respective role of ΔpH and external pH in the activation processes of NTP hydrolases, the same type of experiments were performed as for NTP synthases: the ATP hydrolase activation, i.e., changes in the percentage of active enzymes, was studied at two external pH's through the evolution of ATP hydrolysis rates as a function of ΔpH.

Such a comparison between the flow-force curves at two external pH's required a correction of the 9-aminoacridine response, due to an external pH effect. We have previously shown [30] that with lettuce chloroplasts at pH 8.5, the ΔpH was

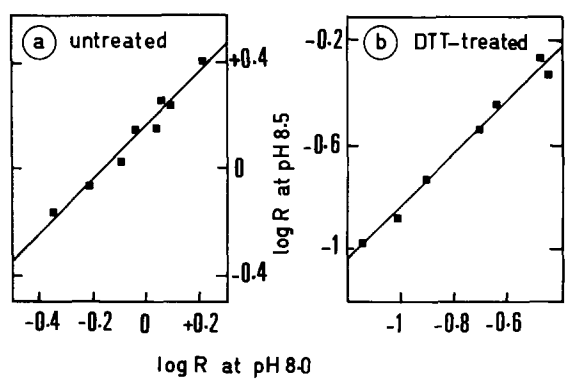

Fig. 4. Light-induced 9-aminoacridine fluorescence quenching at pH 8.5 versus quenching at pH 8.0. The quenching is expressed by log R as defined in Eqn. 2. ΔpH was varied by light intensity. Each point represents two measurements made at pH 8.0 and 8.5, but at the same light intensity. Straight lines are those obtained by linear least-squares regression. (a) Untreated thylakoids. Slope: 1.01, intercept: +0.15. (b) DTT-treated thylakoids. Slope: 1.01, intercept: +0.18.

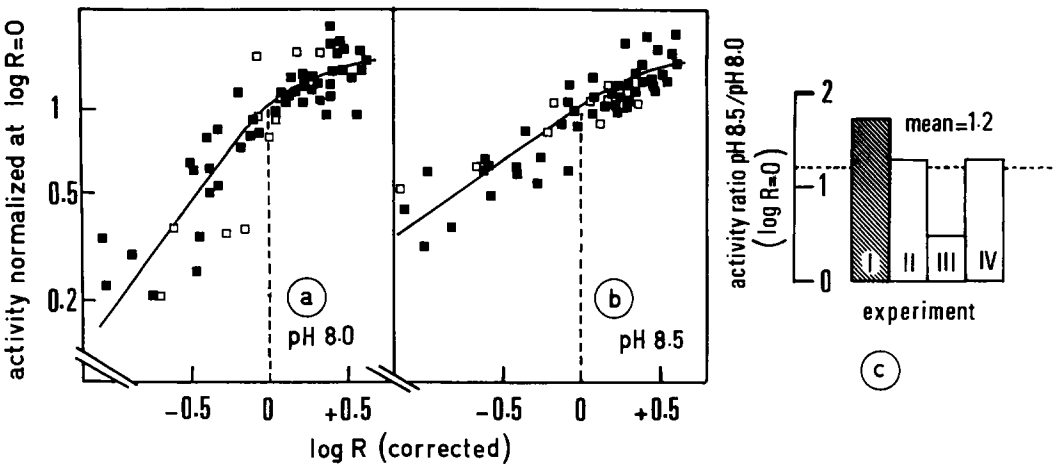

Fig. 5. Uncoupled rate of ATP hydrolysis as a function of ΔpH during the reactivation stage and at two external pH's. Conditions indicated in Materials and Methods. No hexylamine. Time of reactivation: 120 s. ΔpH, varied by light intensity, is expressed by log R, from which 0.18 was subtracted for values obtained at pH 8.5. (a) pH 8.0; (b) pH 8.5. Data were collected from four experiments carried out on different chloroplast preparations and normalized as follows: for each of the experiments, the relationship between ATPase activity and log R was smoothed by a polynome to obtain a value of hydrolysis at log $R = 0$. Then, the initial experimental rates were divided by the interpolated value. Closed symbols (■): DTT-pretreated material assayed in DTT-free medium. Open symbols (□): material reduced by DTT in the cuvette. (c) Ratio between the rates of ATP hydrolysis at pH 8.5 and 8.0. The rates are interpolated at log $R = 0$ for both pH's. Experiment 1 (hatched rectangle) was carried out with material reduced by DTT in the assay medium, others with DTT-premodulated material. Absolute activities at log $R = 0$ between 40 and 80 mATP per Chl per s at pH 8.0.

overestimated by approx. + 0.15 with respect to pH 8.0, probably due to an increase in the thylakoid volume. Since steady-state ΔpH does not depend on external pH [48], at least for low light intensities [30,32], it is easy to compare the 9-aminoacridine response at identical ΔpH's but at different external pH's. These results are presented in Fig. 4a, and confirm that with non-treated thylakoids, the ΔpH is indeed overestimated by +0.15 at pH 8.5 with respect to pH 8.0. With thiol-modulated material the correction factor becomes + 0.18 (Fig. 4b) and was found reproducible for different lettuce chloroplast preparations. Therefore, the parameter log R was corrected by subtracting 0.18 at pH 8.5 in all of the following figures, thus providing a direct comparison between activation levels at pH 8.0 and 8.5 at a given ΔpH.

Fig. 5 presents flow-force curves performed by measuring initial hydrolysis rates as a function of ΔpH at two external pH's (8.0 and 8.5). As emphasized recently by Junesh and Gräber [49], differences between activities measured per chlorophyll were observed, depending on thylakoid preparation, probably due to variations in CF_1/chlorophyll ratio. Therefore, the initial extrapolated rates collected from four separate experiments were normalized to an arbitrary ΔpH fixed at log $R = 0$. From Fig. 5a and b, it is obvious that the activation process is less sensitive to a ΔpH decrease at pH 8.5 than 8.0, at least for log R values lower than zero. This observation is in close agreement with Refs. 40 and 50. Incidently, this qualitative result is not modified by the presence of DTT in the assay medium.

The hydrolysis rate measured at a given ΔpH and external pH represents the amount of active CF_1 multiplied by their turnover rate, the latter depending only on the external pH due to the uncoupled conditions. Since the rate ratio between pH 8.0 and 8.5 is not constant over all the ΔpH range investigated, we initially concluded that the acid-base events leading to activation depended upon both ΔpH and external pH. Moreover, the merged aspect of the two curves beyond log $R = 0$ suggests that in this ΔpH range, the number of active enzymes is identical at the two pH's. Thus, an apparent ratio of the respective turnover rates

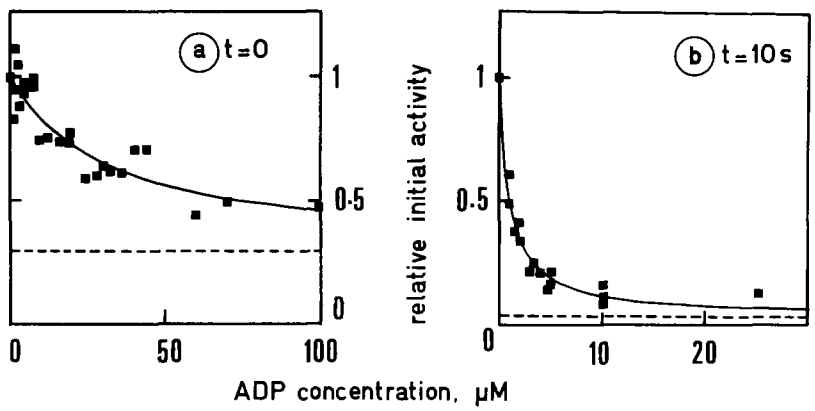

Fig. 6. Uncoupled rate of ATP hydrolysis as a function of ADP addition. Thiol-pretreated thylakoids reactivated in strong light; time of reactivation: 90 s. No hexylamine was present. Hydrolysis rates were normalized to that obtained at [ADP] = 0. ADP and ATP injected concomitantly with nigericin at light extinction (a); 10 s after uncoupling (b). The data fitted by an hyperbolic law, gave (a): K_i = 30 μM, asymptote = 0.29; (b): K_i = 1 μM, asymptote = 0.034 (asymptotes are indicated with dashed lines). Absolute activities without added ADP: (a) 75–95 m ATP per Chl per s; (b) 90–120 m ATP per Chl per s. Different preparations in (a) and (b).

between pH 8.5 and pH 8.0 can be calculated from the reaction rates ratio $r = V_{8.5}/V_{8.0}$ at log $R = 0$. The results are depicted in Fig. 5c. The average ratio calculated from the four experiments is 1.2. Given the scattering of the values, it would be unwise to consider that the ratio obtained in the presence of DTT, is significantly higher than in the absence of DTT.

At variance with the data of Fig. 5 concerning thiol-reduced ATP hydrolases, it has been previously shown that the primary processes of the ΔpH-activation of oxidized ATP synthases are only ΔpH dependent [30,32–34]. Therefore, we wondered whether an eventual effect of newly synthesized ADP or an incomplete delocalization of the ΔpH could have affected the results. ADP being well known for its inhibitor effect on the ATP hydrolase, this point was the first to be checked.

Fast binding of externally added ADP

Since newly synthesized ADP is one of the hydrolysis products, it was suspected of inhibiting a given amount of active enzymes. This inhibition is believed to arise from ADP binding onto a so-called regulator site on oxidized [26,27,51,52] or reduced [2,3,53] ATPases. These processes have K_i values in the micromolar range [25,26,51,52]. Therefore, we have checked this inhibition at low ADP concentrations in the presence of ATP under uncoupled conditions. The results are presented in Fig. 6.

It can be seen in Fig. 6a, that the initial extrapolated rate of ATP hydrolysis decreases with increasing concentrations of ADP injected in the cuvette when the light was turned off. The fitting by a hyperbolic regression gives an apparent inhibition constant (K_i of 30 μM and a residual activity of 29%, i.e., maximal inhibition 71% at infinite inhibitor concentration). This inhibitory effect is more pronounced if the ADP + ATP mixture is injected 10 s after deenergization (Fig. 6b). In this case, the apparent K_i decreases from 30 μM to 1 μM and the residual activity is reduced from 30% to 3.4% (i.e., maximal inhibition more than 95%). Furthermore, it is found that beyond 50 μM ADP, the inhibition increases only slightly with further ADP addition (Fig. 6a and b).

These results show that ADP added to the external medium can inhibit the enzyme in a time range which is beyond our resolution (i.e., a few seconds). Similarly, ADP newly synthesized or present in ATP probably inhibits some active enzymes, even if this effect is limited in the absence of prior deenergization (Fig. 6a). We have thus tried to remove ADP by using the ATP-regenerating system: pyruvate kinase, lacticodehydrogenase + phospho*enol*pyruvate and NADH, the reaction being monitored by the disappearance of NADH instead of pH metric measurements. This

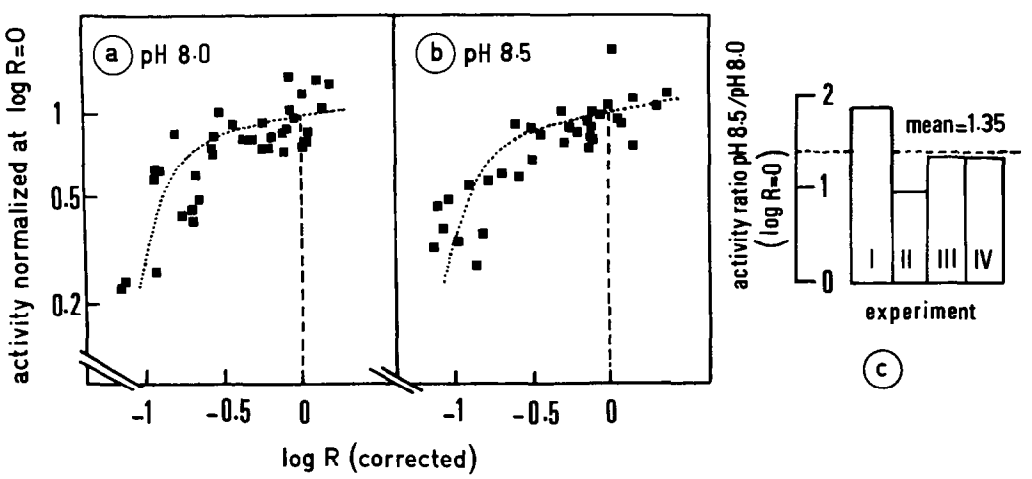

Fig. 7. Uncoupled rate of GTP hydrolysis as a function of ΔpH during the reactivation stage at two external pH's with hexylamine. Conditions as in Fig. 5, except that GTP was injected instead of ATP with nigericin and 50 μM GDP and in the presence of 200 μM hexylamine. Data were also collected from four experiments and normalized as in Fig. 4. (a): pH 8.0; (b): pH 8.5. (c): ratio between the rates of GTP hydrolysis at pH 8.5 and 8.0, each interpolated at log $R = 0$. Absolute activities at log $R = 0$ between 30 and 60 mGTP per Chl per s at pH 8.0.

did not work for two main reasons. (1) The spectroscopic detection of NADH (fluorescence or absorbance) greatly interfered with that of 9-aminoacridine. (2) This coupled enzymatic system, which was kinetically limiting even at high enzymes concentrations, did not allow extrapolation of initial activities. Indeed, in similar experimental conditions, the rates generally measured with this technique represented only 10% of our maximal activities [54].

GTP hydrolysis rates as a function of ΔpH and external pH with hexylamine

In order to avoid the disruptive effect of endogenous or newly synthesized ADP in the establishment of flow-force curves, we have used GTP as

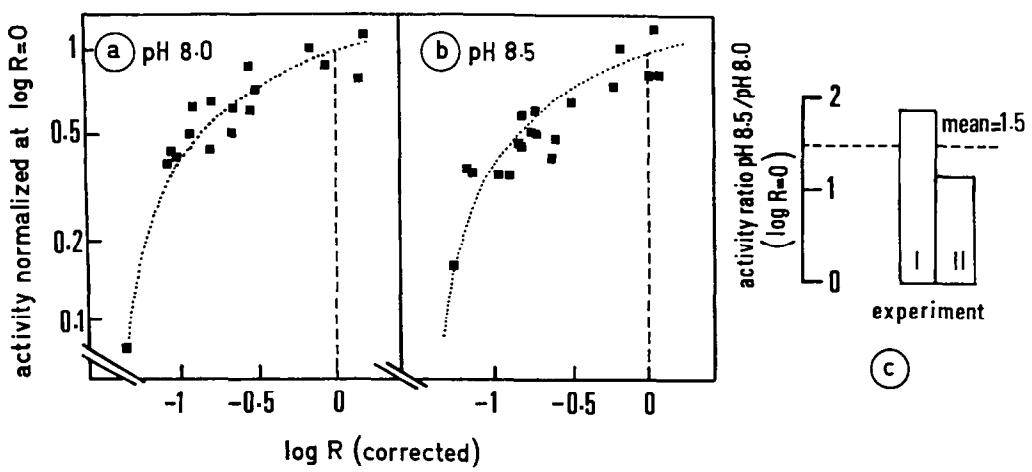

Fig. 8. Uncoupled rate of ATP hydrolysis as a function of ΔpH during the reactivation stage at two external pH's in the presence of 200 μM hexylamine. Conditions as in Fig 5, except hexylamine in the medium. Data were collected from two experiments and normalized as in Fig. 4. (a) pH 8.0; (b) pH 8.5. Absolute activities at log $R = 0$ around 40 mATP per Chl per s at pH 8.0.

the substrate, since GDP is known to be a poor inhibitor [38]. First of all, the effects of the nucleotide diphosphate on hydrolysis rate were checked by experiments similar to those presented in Fig. 6a for ADP. When GTP hydrolysis was measured at different concentrations of GDP, added at the time of uncoupling, similar phenomena were observed, except that beyond 50 μM GDP the inhibition level was much lower (20–30%) when compared with beyond 50 μM ADP. This inhibition by GDP, which is more important than previously reported [38], cannot be due to ADP contamination, since the ADP content of the GDP solutions was estimated, by HPLC, to be 0.5% at a maximum. Therefore, correlations between the amount of active GTPases and ΔpH were performed in the presence of 50 μM GDP, a concentration beyond which the residual activity remains almost constant. Further precautions were taken by delocalizing the proton gradient by the addition of 200 μM hexylamine [47]. The results are presented in Fig. 7. Normalization of hydrolysis rates was carried out at $\log R = 0$ with data collected from four experiments. At variance with the results of Fig. 5, the amount of active ATPase appeared to follow the same law whether the external pH was 8.0 or 8.5. The rate ratios calculated at $\log R = 0$ (see Fig. 7c) were found to be constant over all the ΔpH range investigated. The mean value of 1.35 is not really different from that found with the natural substrate ATP in the high ΔpH range.

If the activation processes of reduced NTP hydrolases are in fact, as well as those of oxidized NTP synthases, only dependent on ΔpH regardless of external pH, one may assume that the results of Fig. 5 have been affected by (1) the disruptive effect of nucleotide binding, or (2) an incomplete delocalization of the ΔpH.

ATP hydrolysis rates as a function of ΔpH and external pH with hexylamine

To discriminate between these two hypothesis, an experiment as in Fig. 5 was carried out in the presence of 200 μM hexylamine. The result, presented in Fig. 8, shows that in this case the ATP hydrolase activation seems indeed to depend only on ΔpH, as in Fig. 7. Thus, the apparent dependence on external pH observed in the absence of hexylamine was probably related to local proton gradients which could affect the apparent relationships between forces and fluxes.

Discussion

Complexity of the deactivation processes of thiol-modified ATP hydrolases

To study the primary mechanisms of ATP hydrolase activation by ΔpH, one is confronted by the complexity of the deactivation processes occurring under deenergized conditions. In fact, it is possible to discriminate between several types of deactivation as a result of the work presented here.

The first deactivation process is rapid (less than our resolution time) and accounts for the increasing disappearance of active enzymes upon the addition of increasing ADP concentrations to the external medium. In view of the limited inhibition, even at high levels of added ADP (Fig. 6a), it seems that only a fraction of the ATPases is available for this type of deactivation. Furthermore, given the variation of the K_i with deenergization of the system, the existence of several ATP hydrolase active states is suggested in accordance with Schumann and Strotmann [3] and with B. Huchzermeyer (personal communication) on reduced ATPases and with Strotmann et al. on oxidized ATPases [51,52].

The second deactivation mechanism occurs more slowly. Kinetic constants can be calculated when hydrolysis rates are measured as in Fig. 1. In the absence of added ADP, these constants vary from 0.023 to 0.069 s^{-1} depending on thylakoid preparation. Moreover, they were found to increase with NTPase activity when the light intensity was varied, as shown in Fig. 1c.

In this second type of deactivation, newly synthesized ADP plays only a minor role, since the kinetic constants are not very sensitive to ADP addition in the 2–100 μM range (data not shown). Moreover, even when product inhibition is minimized (GTP hydrolysis with 50 μM GDP, data not shown), the kinetic constants continue to increase with initial activities as in Fig. 1c. This probably reflects substrate exhaustion at high reaction rates, rather than an accelerated decay of

active states. Unfortunately, concentrations higher than 500 μM ATP or 1000 μM GTP, which would be saturating, cannot be used because they lead to important artifactual pH shifts upon injection.

A third type of deactivation process which is even slower, has been measured in the absence of newly synthesized or added nucleotides, by initiating the hydrolysis reaction by ATP injection, at variable dark times after uncoupling. The 'slow deactivation constants' calculated from the decrease in initial rate as a function of deenergization time are very scattered (not shown) ranging from 0.003 s^{-1} to 0.03 s^{-1}, depending on thylakoid preparation. Since this third type of deactivation takes place without added nucleotides during the dark-deactivation stage, it probably corresponds to the second type of deactivation process which has been slowed down by the absence of added ADP. With our experimental conditions, assuming a chlorophyll/CF_1 ratio of 860 [56] and 1 ADP per CF_1 [57], the concentration of endogenous ADP released during light reactivation would be approx. 35 nM. This is an overestimation, since ADP release during the thiol modulation and washing procedures is neglected.

If the ADP effect on the slow deactivation constants occurs for very low concentrations, the observed dispersion of these slow constants (type-3 deactivation), could be due to the variable concentration of endogenous nucleotides. The ADP content of ATP solutions, estimated by HPLC, was less than 0.5%. This means that when deactivation is measured under hydrolysing conditions (type 2), ADP was initially less than 2.5 μM in the cuvette. This low concentration may be, however, sufficient to reach the maximum rate of deactivation. This would explain the lack of any significant effect of added or newly synthesized ADP on the slow ATPase deactivation measured as in Fig. 1c.

In any case, even if the complex pattern of ADP-induced deactivation suggests a multiplicity of active states, the presence of a slow step, which prevents a fast equilibrium between all forms, is sufficient to justify the use of long reactivation times, especially for low activities. This should be considered when using the pH jump method, which appears to be the best way to carry out quantitative studies [49], apart from the constraint of short reactivation times which could lead to an underestimation of the reaction rates for small ΔpH's.

Nucleotide binding: disruptive effects

When ATP hydrolysis is measured after uncoupling by nigericin and light extinction, an inhibition occurs within a very short time after ADP addition. This is still observed whatever the dark deactivation time (0 to 10 s) before the injection of the ATP + ADP mixtures. On the other hand, when the enzyme deactivates for 10 s in the absence of both exogenous nucleotides and ΔpH, the affinity for the inhibition site increases when compared to the affinity found when ADP is added concomitantly with uncoupling. This suggests that one of the effects of the ΔpH is to convert the ADP-exchangeable site from a high-affinity to a low-affinity site. This dark time of 10 s is generally used in nucleotide binding studies before addition of NTP or NDP, but in these cases deactivation takes place in the absence of uncoupler [3,51,53].

Furthermore, one may assume that ATP injected at the time of uncoupling, without avoiding the inactivation process resulting from the disappearance of the ΔpH, prevents this affinity change. Indeed, under ATP hydrolysis conditions, for a given concentration of ADP, we have noticed that the instantaneous activity remaining after 10 s of deactivation is much more important than that measured at $t = 0$ upon ADP + ATP addition, 10 s after uncoupling.

These results should be discussed within the framework of complex nucleotide binding studies. In particular, the immediate inhibitory effect of ADP added in the assay medium would justify the use of the 'quenching technique' developed by Strotmann et al. to measure tightly bound ADP on oxidized ATPases [51]. This method is based on chasing the radioactivity-labelled nucleotides bound on the loose sites by cold ADP before their conversion into non-exchangeable ones. However, the fast binding of external ADP required by this type of method questions the estimation of the kinetic constants reported in this paper [51], which support the idea that the conversion of the loose complex to tightly bound ADP is very fast when compared to the limiting step of ADP binding on the loose site.

Our results could be compared with the more recent work of Strotmann et al. [58], who have shown a rapid inhibition of ATPase activity by ADP addition. However, they measured hydrolysis rates in the presence of an ATP-induced ΔpH, due to the absence of uncoupler. Especially, in one case, the inhibition effect should be considered to be only qualitative, since the maximal turnover rates (representative of the amount of active enzyme), cannot be obtained in the absence of uncoupler. Apart from this last remark, the results of Strotmann's group, which show that the K_i measured in coupled conditions is higher than the one estimated after deenergization by nigericin, would be in accordance with our work which suggests that one of the roles of the ΔpH is to convert the high-affinity site to a low affinity one.

Finally, a protection by ATP (also reported by B. Huchzermeyer, personal communication) has already been studied [3], since ATP added before ADP reduced the apparent affinity of the inhibitor site for ADP. However, as an uncoupler was not present in the assay medium, one cannot discriminate between the effect of ATP itself and that of an ATP-maintained ΔpH; for the same reason, quantitative determinations of K_i are also questionable in this work.

Moreover, it is suggested here that GDP is a better inhibitor than previously reported [38], inasmuch as those experiments were performed in coupled conditions which minimizes the differences between the measured activities due to the ΔpH back-pressure effect.

ΔpH-activation process

Finally, keeping in mind the disruptive effect of nucleotide binding, and especially the problems linked to local proton gradients which can lead to a misinterpretation of flow-force curves, our results (Figs. 7 and 8) suggest a strict dependence of the activation processes towards ΔpH regardless of external pH. Furthermore, since in Fig. 7 the residual activity measured in the presence of 50 μM GDP did not depend on the external pH, the kinetic constants or equilibrium linked to the GDP binding/release are also assumed to be independent of external pH.

The primary processes of $\Delta\tilde{\mu}_{H^+}$ activation were proposed to involve protonation and deprotonation of some buffering groups of the enzyme located in the high- and low-potential compartments, respectively. Although the structural basis of the switching device is unknown, it is usually thought to consist of a given number of identical couples of two monoprotic functions sensitive to internal and external pH [11,28,29].

Some authors assume that activation only requires the protonation of internal groups [29], while others consider the enzyme to be active [11] or convertible to the active form [28] only when all of the couples are internally protonated and externally deprotonated. One of the disputed points of the latter case deals with the concerted character of the acid-base events for each unit of the switching device, i.e., does the protonation of the high-potential pole necessarily imply the deprotonation of the low-potential pole? In the case of strictly coupled acid-base reactions, the amount of active ATPase is expected to be only dependent on the absolute value of the ΔpH, regardless of the internal and external pH [11,28].

Among the authors who state that the enzyme activation implies both internal and external protonation, Mills and Mitchell have developed a quantitative model [28] to calculate the fraction of active ATPases as a function of ΔpH, internal and external pH, and especially the pK's of the monoprotic groups that make up the units of the 'switching device'. The simplified version which considers only active and inactive conformations can explain within each unit the control exerted by the protonation state of a given pole on the pK of the opposite group. The formula used can be expressed by:

$$\frac{1}{\alpha} = \left(p \cdot 10^{pH_i - pK_i^1} + K \cdot 10^{pK_i^1 - pH_e} + K \cdot 10^{-\Delta pH} + 1 \right)^n \quad (6)$$

where α is the fraction of active ATPases; n, the number of units constituting the switching device; pH and pK have their usual significance. Subscripts i and e refer to internal and external sides, respectively. Superscripts 1 and 0 for pK of one group indicate whether the opposite group is protonated (1) or unprotonated (0).

$$p = 10^{pK_i^1 - pK_i^0} = 10^{pK_e^1 - pK_e^0} \text{ and } K = 10^{pK_e^1 - pK_i^1} = 10^{pK_e^0 - pK_i^0}.$$

To account for a strict ΔpH dependence of the

active states, the first two additive terms of Eqn. 6 must be negligible. (For more details, see Ref. 28.) By theoretically varying the parameters p, K and pK to a large degree, we have noticed that an external pH independence may occur only for $p \leq 10^{-6}$, i.e., if the protonation of one group decreases the pK of the opposite one by at least 6 pH units. Application of simple Coulombic laws shows that electrostatic effects could lead to such a change only if the two acid-base functions, exchanging protons with opposite compartments, are at the maximum 2 nm apart (assuming a dielectric constant of 2 for the membrane [59]). This distance is rather small given the membrane thickness. Therefore, the internal group could be located at the far end of the CF_0 in the vicinity of the coupling factor CF_1, so that it senses both the internal pH and the electrostatic effects due to the protonation state of the opposite one. Although this first hypothesis cannot be ruled out, another way to explain the independence of the ΔpH-activation towards external pH, would be to suppose a conversion of ΔpH into $\Delta\Psi$ across the coupling factor. In this case no external pH effects are expected, since the primary mechanisms would only correspond to the reorientation of dipoles in an electric field as proposed [10].

Whatever the precise nature of these mechanisms, we propose that, as for oxidized ATP synthases [30], the activation of thiol-reduced ATP hydrolases depends only on the size of the $\Delta\tilde{\mu}_{H^+}$. In contrast with the known difference of ΔpH requirement in activation of oxidized and reduced ATPases (i.e., lower ΔpH values for the latter), it is not yet known whether the catalytic properties of ATP hydrolases are modified by the thiol treatment. For ATP synthases at least, previous results strongly suggest that both redox forms catalyse phosphorylation at the same rate [40]. Similarly, among the thiol-reduced hydrolases, it would be interesting to know if the different active states, discriminated by their deactivation behaviours, have different catalytic properties.

Acknowledgements

We are full indebted to Dr. M. Hill for HPLC measurements of nucleotides. We wish also thank Dr. Y. de Kouchkovsky for helpful discussions and Dr. M. Hodges for reading the manuscript.

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