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The rate of ATP synthesis as a function of ΔpH in normal and dithiothreitol-modified chloroplasts

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The rate of ATP synthesis catalyzed by normal and by dithiothreitol-modified ATPases is investigated as a function of ΔpH in spinach chloroplasts at constant pH_{out}. The transmembrane ΔpH was generated by an acid-base transition and the reaction time was limited to 150 ms by using a rapidly mixing quenched-flow apparatus. The result was that the functional dependence of the rate on ΔpH is shifted to lower ΔpH values and that the shape of this curve is altered after dithiothreitol modification. The maximal rate (400 ATP / CF_1 per s) is the same under both conditions.

Introduction

Membrane-bound ATPases, F_0F_1, catalyze reversibly the proton-transport-coupled ATP synthesis/hydrolysis in different biological membranes. However, in chloroplasts under non-energized conditions, i.e., in the absence of ΔpH and/or $\Delta\psi$, no significant ATP hydrolysis is observed, although this reaction is thermodynamically possible [1]. Obviously, the reaction is kinetically inhibited, i.e., the enzyme is inactive under non-energized conditions. When the membrane is energized, two processes occur: the ATPase is activated, and the catalytic reaction takes place. From measurements of the activation and ATP synthesis as a function of the energization it has been concluded that the activation requires a higher energization than ATP synthesis at low phosphate potentials [2,3]. Correspondingly, ATP hydrolysis is hardly observed in class II chloroplasts, since at low energization the ATPase is inactive and at high energization ATP synthesis occurs.

However, preillumination of class II chloroplasts in the presence of dithiothreitol leads to preparations which show high rates of proton-transport-coupled ATP hydrolysis [4–8]. Under these conditions the inactive ATPase is converted into an active state by preillumination (i.e., by membrane energization), and in the active state the ATPase is modified by dithiothreitol in such a way that the active state has a much longer lifetime than before modification.

Investigations of ATP hydrolysis have been carried out with the dithiothreitol-modified ATPase, whereas ATP synthesis was mainly measured using non-modified ATPases (for reviews, see Refs. 9–12). In this work we compare the functional dependence of the rate of ATP synthesis on ΔpH obtained with dithiothreitol-modified and non-modified chloroplasts.

Materials and Methods

Class II chloroplasts were prepared as described elsewhere [13]; the rapid mixing experiments have been described in ref. 14. Dithiothreitol-modification was carried out as follows. 5 ml chloroplasts

Abbreviations: DCMU, 3-(3′,4′-dichlorophenyl)-1,1-dimethylurea; Chl, chlorophyll.

in a solution containing 5 mM Tricine, 9 mM KOH (pH 8.2), 51 mM KCl, 5 mM NaH_2PO_4, 2 mM $MgCl_2$, 10 mM dithiothreitol, 100 μM methyl viologen, 120 μM Chl were illuminated with white light for 6 min (light intensity, 100 mW/cm^2) at room temperature. The light was filtered through 10 cm 1% $CuSO_4$ solution and through a KG 3 (4 mm Schott) IR-absorbing glass. After modification, the chloroplasts were either used immediately or stored on ice. The modified chloroplasts were mixed with a solution (ratio, 1 : 2) containing 30 mM succinic acid, $(120 - x)$ mM KCl, x mM KOH for pH control (x varies from 20 to 65), 5 mM NaH_2PO_4, 2 mM $MgCl_2$, 15 μM DCMU, 1 μM valinomycin. The chloroplasts were incubated for 30 s in this acidic solution and then rapidly mixed with a basic solution (ratio 1 : 1), containing 200 mM Tricine, 100 mM KOH, 20–30 mM NaOH (pH 8.3–8.4), 5 mM NaH_2PO_4, 2 mM $MgCl_2$, 200 μM ADP, 10 μM DCMU. The pH was adjusted in such a way that after mixing with the acidified chloroplasts the final pH was 8.2 $\pm$ 0.05. The reaction was terminated by mixing with 4% trichloroacetic acid (ratio, 1 : 1). ATP was measured with luciferin-luciferase [14]. The luciferase signal was calibrated for each sample with a standard amount of ATP.

Non-modified chloroplasts were diluted in the same media as modified chloroplasts and treated in two ways: (a) no preillumination was given, or (b) instead of dithiothreitol in the modification media, 10 mM ascorbate was present and the chloroplasts were illuminated for 6 min. Both treatments for non-modified chloroplasts give, at the same ΔpH, the same rate of ATP synthesis.

Hydrolysis was measured by the release of ^{32}P from [γ-^{32}P]ATP. [γ-^{32}P]ATP was synthesized according to Ref. 15, in the same reaction medium as for ATP synthesis. Uncoupled hydrolysis was started by addition of [γ-^{32}P]ATP (final concentration, 1 mM) together with NH_4Cl (final concentration, 1 mM). Samples were taken and denatured with perchloric acid (final concentration, 0.5 M). ^{32}P was determined as described in Ref. 16. All measurements were carried out at room temperature (23°C).

Results

When ATP synthesis is measured in an acid base transition, the chloroplasts must be incubated in an acidic medium (e.g., at pH 5.0 for 30 s). This acid incubation might change the activity of the modified ATPases. Since the rate of ATP synthesis is to be measured under conditions where all ATPases are fully active, we investigated the stability of the modified active conformation of the ATPase under the following two conditions.

(A) Modified chloroplasts were added to the reaction medium either directly after modification or after storage on ice before initiating ATP hydrolysis (Fig. 1A, top).

(B) Modified chloroplasts were incubated for 30 s at pH 5.0 either directly after modification or after different storage times on ice. Then they were mixed with the basic solution, and 10 s after the pH jump, ATP hydrolysis was started (Fig. 1B, top).

A linear increase of the amount of P_i released is observed, i.e., the rate of ATP hydrolysis is constant at least up to 50 s. Fig. 1 (bottom) shows the rate of ATP hydrolysis (i.e., the slope of the curves from Fig. 1 (top) and additional sets of experiments) as a function of the storage time on ice. It can be seen that under both conditions immediately after modification practically the same rate is observed (48 mM ATP/M Chl per s); i.e., the acidic incubation for 30 s at pH values of at least 5.0 does not significantly affect the rate of ATP-hydrolysis. Under both conditions the rate of hydrolysis decays with storage time. However, when hydrolysis is measured after the ΔpH jump the decay is slower. This shows that the ΔpH partly reverses the inactivation.

In order to observe in which way the decreasing ATP hydrolysis activity (see Fig. 1) influences the results obtained on ATP synthesis with the rapid-mixing apparatus, the experiments were carried out as follows (Fig. 2). The ATP yield was measured at different reaction times, using chloroplasts either directly after preillumination and modification or after storing these chloroplasts on ice up to 10 min. For the same reaction time (e.g., $t_r = 76$ ms) the ATP yield is constant during the first 5 min after modification (see Fig. 2, left-hand side). After this time, the ATP yield decays. The

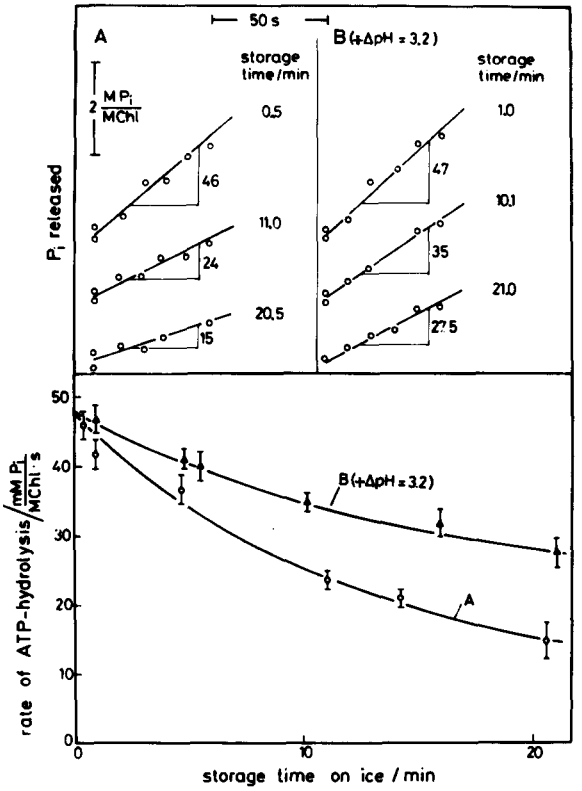


Fig. 1. Amount of P_i released as a function of the reaction time measured at different storage times: (A) after dithiothreitol-modification; (B) after dithiothreitol-modification and an additional ΔpH jump before the measurement. The slopes give the rate of ATP hydrolysis, the numbers give the rate in mM ATP/M Chl per s. Bottom: rate of ATP hydrolysis as a function of storage time on ice. Data from panels A and B and additional sets of experiments. Curve A, rate after dithiothreitol modification; curve B, rate after dithiothreitol modification and an additional pH jump 10 s before starting ATP hydrolysis.

time range after modification where a constant ATP yield was observed varied for different chloroplast preparations. The mean values of the ATP yields (in the constant range) are replotted as a function of the reaction time and the slope of this curve gives the rate of ATP synthesis (see Fig. 2, right-hand side).

Fig. 3 shows the rate of ATP synthesis as a function of ΔpH (at $\Delta\psi = 0$) with modified chloroplasts (data from Fig. 2 and similar experiments). For a comparison, the rate of ATP synthesis as a function of ΔpH is shown using the same chloro-

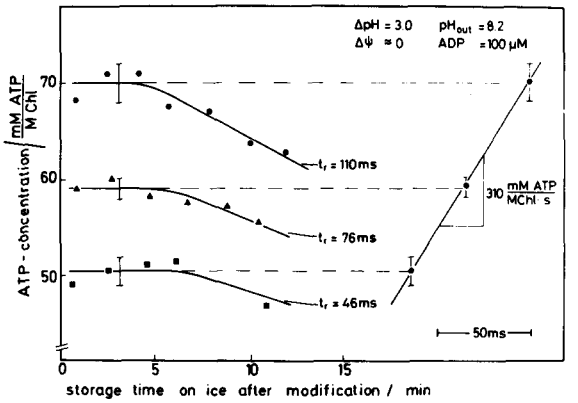


Fig. 2. Left-hand side: amount of ATP generated in an acid-base transition during reaction times of 46, 76 and 110 ms, when the chloroplasts are stored for different times on ice after dithiothreitol-modification. Right-hand side: amount of ATP as a function of the reaction time. The slope gives the rate of ATP synthesis.

plast preparation but without modification. For these control measurements three different treatments of the chloroplasts were used: (a) the chloroplasts were used directly after preparation; (b) the chloroplasts were preilluminated as described above (however, the reaction medium contained 10

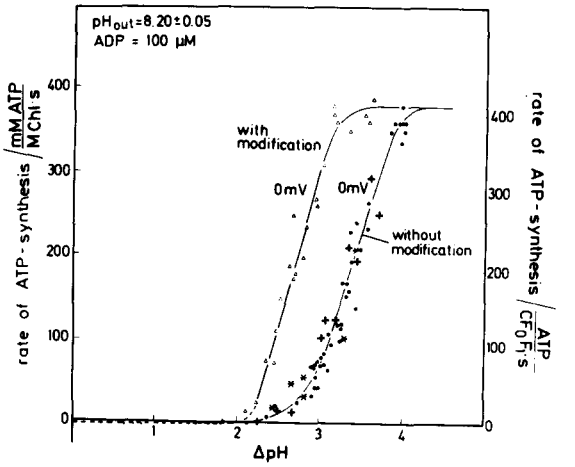


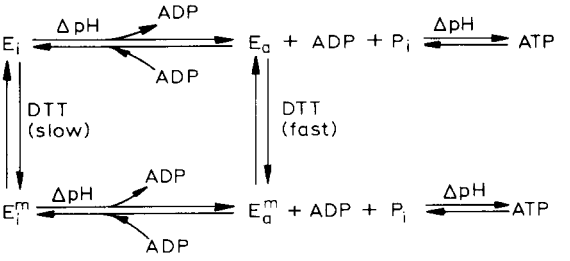
Fig. 3. Rate of ATP synthesis as a function of ΔpH at $\Delta\psi \approx 0$ with modified chloroplasts (Δ) and with non-modified chloroplasts. Non-modified chloroplasts were treated in three different ways: they were incubated for 6 min in the modification medium without dithiothreitol and without illumination (●), without dithiothreitol and with illumination (*), with dithiothreitol and without illumination (+). The dashed line shows the expected shape at low rates.

mM ascorbate instead of dithiothreitol), and (c) the chloroplasts were suspended in the modification medium for 6 min but no preillumination was given. All three treatments gave the same functional dependence of the rate of ATP synthesis on ΔpH, as can be seen in Fig. 3. Preillumination in the presence of dithiothreitol increases the rate significantly at constant ΔpH; for example, at ΔpH 3.0 from about 50 mM ATP/M Chl per s to about 310 mM ATP/M Chl per s. The same maximal rate of ATP-synthesis is found with or without dithiothreitol modification. The curve after modification is shifted to lower ΔpH values and the slope is steeper. This difference in the slopes is especially large at low rates. In principle, it is expected that the curve (with dithiothreitol modification) extends continuously through the zero-rate point into the hydrolysis range, reaching for small ΔpH values a maximal rate of ATP hydrolysis. However, under our measuring conditions, the phosphate potential was very low. With [ADP] = $1 \cdot 10^{-4}$ M, [P_i] = $5 \cdot 10^{-3}$ M, [ATP] = $(8.4 \pm 2) \cdot 10^{-7}$ M and $\Delta G_p^{0'} = 33.6 \pm 0.5$ kJ/mol [17] it results in $\overline{\Delta G}_p = (34.9 \pm 1.0)$ kJ/mol. Therefore, one would expect a very low rate of hydrolysis at ΔpH = 0. For an estimation of this value we used the expression for a competitive inhibition. With K_m(ATP) = 34 μM, K_i(ADP) = 7 μM [18] and $V_{hydr}^{max}(\Delta pH \approx 0) = 100$ mM ATP/M Chl per s [19] it results $V_{hydr} = 0.16$ mM/M Chl per s. Therefore, we expect for the curve with dithiothreitol modification at rates lower than 20 mM ATP/M Chl per s a shape as indicated by the dashed line in Fig. 3.

According to the chemiosmotic theory [20], at equilibrium (zero-rate) the value of $\overline{\Delta G}_p = 34.9 \pm 1.0$ kJ/mol corresponds with $n = 3$ to $\Delta pH_{eq} = 2.06 \pm 0.06$. (Linear extrapolation to zero-rate gives $\Delta pH_{eq} = 2.18$ and with $\overline{\Delta G}_p = 34.9 \pm 1.0$ kJ/mol, it results in $n = 2.84 \pm 0.1$.) At this point it is expected that with a small displacement to lower ΔpH, ATP hydrolysis occurs. This has not been measured yet, because with our measuring technique we have to measure the coupled rate of ATP hydrolysis over a very short time range. However, using different techniques, it has been shown [19] that at high phosphate potentials the curve extends continuously from the synthesis to the hydrolysis range.

Discussion

It is shown in this work that the functional dependence of the rate of ATP synthesis on ΔpH is different for dithiothreitol-modified and nonmodified ATPases. This is in accordance with results from measurements of the ATP yield in acid-base transitions [21] and from measurements of the rate of photophosphorylation with dithiothreitol-modified and nonmodified ATPases [19,22,23], and extends our earlier measurements [37]. Our results can be explained in the following scheme (where DTT is dithiothreitol):



Scheme I

The inactive ATPase, E_i, is transformed in an active form, E_a, by energization (ΔpH and/or $\Delta\psi$). This event is accompanied by the release of tightly bound ADP [2], two H^+ are necessary for activation and the pK values of the protonizable groups is 5.9 [14]. In the active form, dithiothreitol can react with the ATPase, forming a 'modified' active ATPase within 2–5 min. Also, when the membrane is not energized the ATPase can be modified by dithiothreitol; however, it then takes about 1–3 h. It is not yet known whether E_i can be directly modified to E_i^m as suggested in the scheme. Since under deenergized conditions a small fraction of ATPases (10^{-6} [14]) is always in the E_a state, it is possible that this small fraction is modified to E_a^m which then reacts to E_i^m. In this way all E_i may be successively modified. Functionally, E_i and E_i^m cannot catalyze ATP synthesis/hydrolysis. In the form E_i, a rather high ΔpH is necessary to transform the ATPase to E_a (the pK value for the H^+-binding sites from inside for activation is about 5.9 [14]). This ΔpH is – at least at low phosphate potentials – higher than the equilibrium ΔpH for ATP synthesis/hydrolysis, so that under

these conditions no ATP hydrolysis is observed.

In the form E_i^m, a low ΔpH is necessary to transform the ATPase to E_a^m (the pK value for the H^+-binding sites from the inside is higher than 5.9). It has been shown that the ΔpH for half-maximal activation is shifted by about 0.9 units to lower values [19].

Under these conditions ATP hydrolysis can be observed and, additionally, the ΔpH generated by ATP hydrolysis is high enough to maintain the ATPase in the active modified form, E_a^m. The rate of the back reaction $E_a^m \rightarrow E_i^m$ (and presumably also that of $E_a \rightarrow E_i$) depends on different parameters, such as ADP and P_i concentration [24–28].

ATP hydrolysis has usually been measured with ATPase in the form E_i^m and E_a^m; ATP synthesis has been measured with ATPase in the form E_i and E_a. In our earlier work [2,3,12,14] it was shown that the observed rate of ATP synthesis/hydrolysis at constant ATP, ADP and P_i is given by

$$V_{ATP} = W_{ATP}(\Delta pH, \Delta\psi)\frac{E_a}{E_t}(\Delta pH, \Delta\psi)$$

where E_a/E_t is the fraction of active ATPases and W_{ATP} is the rate of the catalytic reaction (ATP synthesis/hydrolysis) per active ATPase. For the modified form we obtain correspondingly:

$$V'_{ATP} = W_{ATP}(\Delta pH, \Delta\psi)\frac{E_a^m}{E_t^m}(\Delta pH, \Delta\psi)$$

Two extreme cases can now be discussed. In non-modified ATPases the proton binding sites for activation leading to E_a have lower pK values than the proton binding sites for the catalytic reaction W_{ATP}. This implies that the observed rate V_{ATP} reflects practically only the dependence of E_a/E_t on ΔpH and $\Delta\psi$. If the ATPase is modified with dithiothreitol, the pK value of the proton-binding sites for activation is increased to values higher than that for the catalytic reaction. This implies that V'_{ATP} reflects mainly the dependence of W_{ATP} on ΔpH and $\Delta\psi$.

Our interpretation of the results in Fig. 3 is therefore that the curve obtained with non-modified ATPases reflects the activation E_a/E_t, whereas that obtained with modified ATPase reflects the catalytic reaction W_{ATP}.

The dependence of the rate of ATP synthesis on ΔpH has been measured in earlier photophosphorylation experiments with non-modified chloroplasts. The rate depends exponentially on ΔpH up to a rate of approx. 100 mM ATP/M Chl per s [29–33]. This is in accordance with the results (without modification) shown in Fig. 3. If the ΔpH is varied up to about $\Delta pH \approx 4.0$ – such a high ΔpH has not been obtained in the photophosphorylation experiments – a sigmoidal dependence on ΔpH is found (see also Fig. 12 in Ref. 14); i.e., the exponential dependence is found only at low ΔpH. The slope in the exponential range has been used for estimating H^+/ATP [29]. In our interpretation, this slope reflects the number of protons involved in the activation process (for details see Ref. 14).

In this work the different states of the ATPase have been identified by their different functions with regard to ATP synthesis/hydrolysis. Biochemically it has been shown [23,34,35,36] that dithiothreitol treatment leads to a reduction of an -S-S group to -S-H groups in the γ-subunit. Furthermore, the release of tightly bound ADP is coupled with the activation of the ATPase [2] and the rebinding is coupled with the inactivation [24–28].

Based on these results, the following may be concluded: E_i contains a tightly bound ADP and an -S-S group in the γ-subunit. The groups to be protonized for activation have a pK of about 5.9. In E_a these groups are protonized and the tightly bound ADP is released. E_i^m contains a tightly bound ADP and the -S-S group is reduced, the groups to be protonized for activation have a pK of more than 5.9; in E_a^m these groups are protonized, the -S-S group in the γ-subunit is reduced and the tightly bound ADP is released.

The modulation of the ATPase by the redox state of the -S-S group in the γ-subunit (coupled to the redox state of thioredoxin) also plays an important role for the regulation of the ATPase activity in vivo [38–41]. It was reported that, when the ATPase was activated in vivo, K_m and V_{max} for ATP synthesis increase; i.e., K_m increases from 11–15 μM [39,41]. Since our measurements were carried out at an ADP concentration of 100 μM, no effect on the rate is expected. Moreover, since K_m increases after modification, a decrease

of the rate is expected at constant ADP concentration; whereas, we observed an increase at any ΔpH. The increase of V_{max} after modification can be explained by the data shown in Fig. 3: energization by light is not sufficient to reach the maximal rate of ATP synthesis in non-modified chloroplasts. Since modification results in a shift of the curve to lower ΔpH values, the observed rate increases.

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