Influence of the redox state and the activation of the chloroplast ATP synthase on proton-transport-coupled ATP synthesis / hydrolysis *

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The membrane-bound ATP synthase from chloroplasts can occur in different redox and activation states. In the absence of reductants the enzyme usually is oxidized and inactive, E_i^{ox}. Illumination in the presence of dithiothreitol leads to an active, reduced enzyme, E_a^{red}. If this form is stored in the dark in the presence of dithiothreitol an inactive, reduced enzyme E_i^{red} is formed. The rates of ATP synthesis and ATP hydrolysis catalyzed by the different enzyme species are measured as a function of ΔpH ($\Delta\psi = 0$ mV). The ΔpH was generated with an acid-base transition using a rapid-mixing quenched flow apparatus. The following results were obtained. (1) The oxidized ATP synthase catalyzes high rates of ATP synthesis, $v_{max}^{ox} = 400$ ATP per CF_0F_1 per s. The half-maximal rate is obtained at $\Delta pH = 3.4$. (2) The active, reduced ATP synthase catalyzes high rates of ATP synthesis, $v_{max}^{red} = 400$ ATP per CF_0F_1 per s. The half-maximal rate is obtained at $\Delta pH = 2.7$. It catalyzes also high rates of ATP hydrolysis $v_{max}^{red} = -90$ ATP per CF_0F per s at $\Delta pH = 0$. (3) The inactive species (both oxidized and reduced) catalyze neither ATP synthesis nor ATP hydrolysis. The activation / inactivation of the reduced enzyme is completely reversible. (4) The activation of the reduced, inactive enzyme is measured as a function of ΔpH by measuring the rate of ATP hydrolysis catalyzed by the active species. Half-maximal activation is observed at $\Delta pH = 2.2$. (5) On the basis of these results a reaction scheme is proposed relating the redox reaction, the activation and the catalytic reaction of the chloroplast ATP synthase.

Introduction

The membrane-bound ATP synthase, CF_0F_1, from chloroplasts catalyzes ATP synthesis/hydrolysis coupled with a transmembrane proton transport [1]. Besides this catalytic reaction, the ATP synthase carries out also two other reactions: a redox reaction where an —S–S-group on the γ-subunit becomes reduced or oxidized [2–4] and a protolytic reaction where the ATPase is converted from an inactive into an active state [5–9].

The relations between the different states are depicted in a simplified way in the reaction scheme (Scheme I) [10]. In class-II chloroplasts, i.e., chloroplasts without envelope membranes, the ATP synthase is usually in the oxidized state, E_i^{ox}. When the chloroplasts are illuminated in the presence of dithiothreitol, the ATP synthase becomes activated and reduced, E_a^{red}. In state E_a^{red} the ATP

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Abbreviations: Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl) ethyl]glycine; CF_0F_1, proton-translocating ATP synthase from chloroplasts; P_i, inorganic phosphate; Chl, chlorophyll; DCMU, 3-(3,4-dichlorophenyl)-1,1 dimethylurea.

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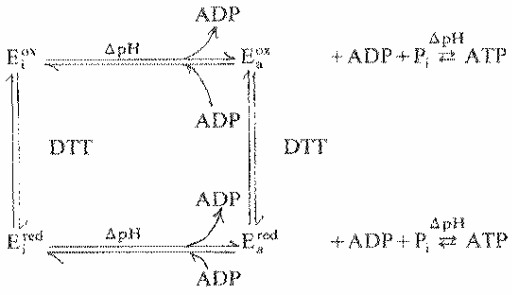


Scheme I

DTT, dithiothreitol.

synthase is able to catalyze high rates of ATP hydrolysis and ATP synthesis [7–13]. The capability of CF_0F_1 for hydrolyzing ATP declines with time. The presence of ADP accelerates this decline, whereas the presence of phosphate stabilizes the ATP hydrolysis activity [14–16].

When the ATP synthase is in its form E_i^{ox}, energization of the membrane leads to an active state, E_a^{ox}. From parallel measurements of the activation and ATP synthesis as a function of the energization it was concluded that the activation (of the oxidized form) requires a higher energization than ATP synthesis itself – at least at low phosphate potentials [6,8,17]. Correspondingly, ATP hydrolysis is hardly observed in class-II chloroplasts, since at low energization the ATP synthase is inactive and at high energization ATP synthesis occurs.

According to this scheme four different reactions of the ATP synthase respond to the membrane energization, ΔpH (and $\Delta\psi$): the activation of the oxidized and of the reduced enzyme and the catalytic reaction catalyzed by the oxidized and the reduced enzyme.

In this work we investigate the functional dependence of the activation of the reduced enzyme on ΔpH and compare it with the dependence of the activation of the oxidized enzyme and with that of the catalytic reaction.

Materials and Methods

Class-II chloroplasts were prepared from spinach as described elsewhere [18]. They were either stored on ice and used within 4 h after preparation or they were rapidly frozen and stored in liquid nitrogen in a medium containing 100 mM sorbitol, 30% ethyleneglycol (v/v), 2 mM $MgCl_2$, 25 mM NaCl, 20 mM tricine/NaOH (pH = 7.4) and 2–5 mM chlorophyll [19]. After thawing the chloroplasts were washed once with a solution containing: 2 mM $MgCl_2$, 5 mM NaCl, 2 mM tricine/NaOH (pH = 8.0) and then resuspended to give a stock solution containing: 50 mM sorbitol, 2 mM $MgCl_2$, 5 mM Tricine/NaOH (pH = 8.0) and 1.5–2 mM chlorophyll. This stock solution can be stored up to 4 h on ice without loss of activity, i.e., fresh and thawed chloroplasts show, under otherwise identical conditions, the same rates of ATP synthesis and ATP hydrolysis. The amount of CF_1 per chlorophyll was determined by rocket immune electrophoresis [20,21].

Dithiothreitol reduction. Chloroplasts were illuminated with white light (100 mW/cm^2) in a water bath at 15–18°C for 5 min. The dithiothreitol, reduction medium contains: 6 mM Tricine/NaOH (pH = 8.2), 2.5 mM $MgCl_2$, 0.5 mM Na_2EDTA, 20 mM dithiothreitol 200 μM methyl viologen, 300 μM chlorophyll and – when indicated – 2 mM NaH_2PO_4. The light was filtered through 10 cm 1% $CuSO_4$ solution. After the illumination the chloroplasts were either used immediately or stored at room temperature up to 1.5 h without loss of activity.

Energization of the membrane. The thylakoid membrane was energized by an acid-base transition in the following way.

(a) Acidic stage: 250 μl dithiothreitol-reduced chloroplasts (in the dithiothreitol reduction medium) were mixed with the same volume of medium A containing 40 mM succinic acid, 10 mM Tricine, 2 mM $MgCl_2$, 20 μM 3-(3,4-dichlorophenyl)-1,1-dimethylurea, 5 μM valinomycin, x mM KOH for pH control ($30 \le x \le 90$), $200 - x$ mM KCl and – when no P_i was present in the reduction medium – 2 mM NaH_2PO_4. The chloroplasts were incubated for 30–40 s in this medium so that internal and external phase are equilibrated.

(b) Basic stage: 100 μl of the chloroplasts incubated in the acidic-stage medium were mixed with the same volume of medium B containing 200 mM Tricine, 100 mM KOH, 2 mM $MgCl_2$, 1

mM NaH_2PO_4, 10 μM DCMU and y mM NaOH ($20 \leq y \leq 50$) so that the pH after mixing was 8.20 ± 0.05.

Assay of ATP hydrolysis. Usually, 5 s after the acid–base transition ATP hydrolysis was started by addition of 100 μl chloroplasts to the same volume of the following assay medium: 100 mM Tricine, 50 mM KOH, 50 mM KCl, 200 mM LiCl, 2 mM $MgCl_2$, 1 mM NaH_2PO_4, 6 mM NH_4Cl and 2 mM ATP containing [γ-^{32}P]ATP ($2 \cdot 10^5$ Bq/ml) and NaOH (13 mM) to adjust the final pH of assay to 8.20 ± 0.05. After different time intervals samples were taken, denatured with 0.6 M perchloric acid and analyzed for ^{32}P content similar as described in Ref. 23. The [γ-^{32}P]ATP was synthesized according to ref. 24.

When no preceding acid-base transition was performed, 50 μl medium A and 100 μl medium B were mixed before 50 μl chloroplasts (in the dithiothreitol reduction medium) were added. The experimental procedure for reduction, activation, inactivation, reactivation and assay of ATP hydrolysis is given in Scheme II. The ion concentrations in the activation step and during the assay of ATP hydrolysis are summarized in Table I.

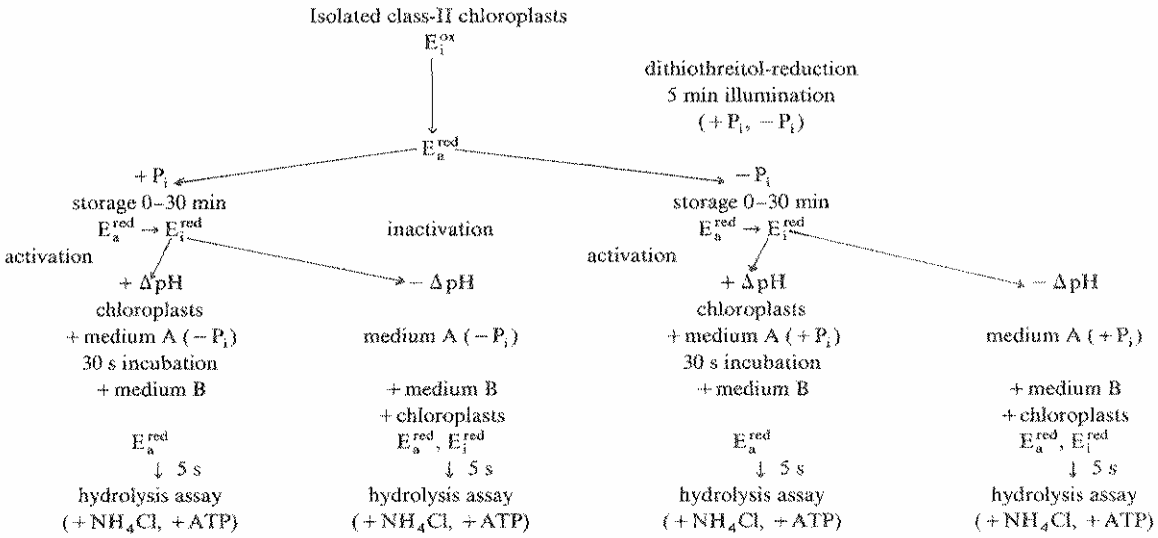
In the experiments where the coupled and uncoupled rates of ATP hydrolysis are compared under the same conditions, hydrolysis was started by mixing with medium B which contains additionally 100 mM LiCl, 2 mM ATP and [γ-^{32}P]ATP with 10^5 Bq/ml and 6 mM NH_4Cl for the measurement of the uncoupled rate of ATP hydrolysis. High time resolution (ms range) was obtained by performing the mixing with [γ-^{32}P]ATP in a rapid mixing quenched flow system [22].

TABLE I

ION CONCENTRATIONS IN THE ACTIVATION STEP AND DURING ASSAY OF ATP HYDROLYSIS

	Inside (mM)	/	Outside (mM)	Assay medium (mM)
K^+	100	/	100	100
Na^+	5	/	28–1	21–14
Li^+	–	/	–	100
Cl^-	89–64	/	46–34	153–147
NH_4^+	–	/	–	3
ATP	–	/	–	1
Mg^{2+}	2	/	2	2
P_i	1	/	1	1
Tricine	8	/	104	102
Succinic acid	20	/	10	5

Assay of ATP synthesis. The rate of ATP synthesis after an acid-base transition was measured with a rapid mixing quenched flow system as described earlier [22]. Combination of an acid-base transition with a change in K^+ concentration



(in the presence of valinomycin) leads to the generation of ΔpH and $\Delta\psi$ which can be varied independently from each other [22,25]. In this work we always used the condition $\Delta\psi \approx 0$ mV. The chloroplasts were subjected either before (enzyme in the oxidized, inactive form) or immediately after reduction by dithiothreitol (enzyme in the reduced, activated form) to an acid-base transition using the same reaction media as described above ('energization of the membrane'), except that the P_i concentration was 5 mM in all media and solution B contains 200 μM ADP and no added ATP.

Results

When the activation of the reduced ATP synthase is to be measured, the reduced, inactive form, E_i^{red}, must be generated first. This has been done as follows: class-II chloroplasts were illuminated in the presence of dithiothreitol, giving the species E_a^{red}. The kinetics of the inactivation process after illumination in the dark to the species E_i^{red} was investigated by measuring the uncoupled rate of ATP hydrolysis at different times.

Fig. 1 shows the result. At the top, the P_i released as a function of the reaction time is shown, the parameter at the curves is the storage time after the illumination. The slopes of these curves are the rates of ATP hydrolysis. On the left, results are shown when no phosphate is present in the dithiothreitol reduction medium; on the right, results in the presence of 2 mM phosphate are shown. It can be seen that the rate of ATP hydrolysis decreases with increasing time after illumination. In the absence of phosphate after 10 min no ATP hydrolysis is observed. In the presence of phosphate, the decline of the hydrolysis activity is much slower in accordance with earlier observations [14–16]. Fig. 1, bottom, shows the rate of ATP hydrolysis; i.e., the slope of the curves from Fig. 1, top, as a function of time after illumination. Without phosphate the rate declines to its half-maximal value within 30 s, in the presence of 2 mM P_i within about 130 s.

One might now ask whether this decline is reversible or not; i.e., whether the initial activity can be restored by energization of the membrane. This experiment has been carried out as follows:

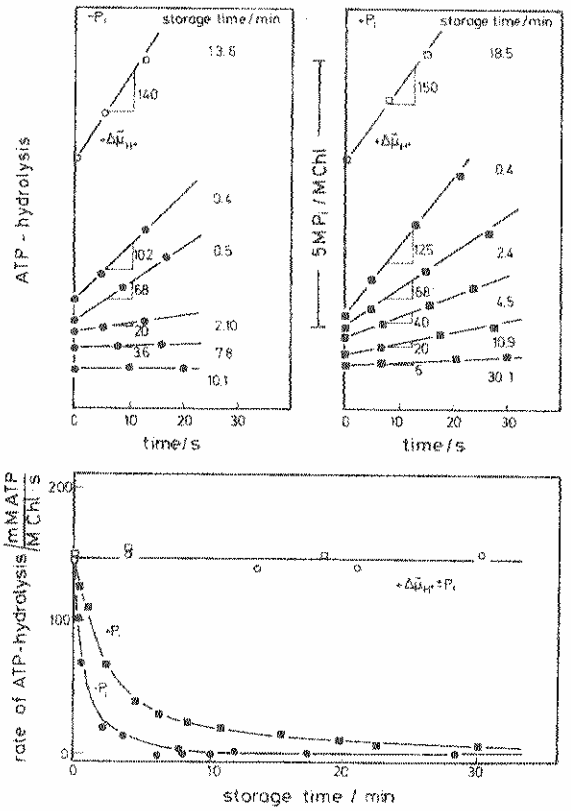


Fig. 1. ATP hydrolysis in chloroplasts preilluminated in the presence of dithiothreitol. Top left: ATP hydrolyzed as a function of reaction time. The slopes of the curves are the rates of ATP hydrolysis. The numbers give the rates in mM ATP per M Chl per s. These curves are measured at different storage times at 20 °C after illumination. The curve labeled ($+\Delta\tilde{\mu}_{H^+}$) was measured 13.6 min after illumination and after a preceding membrane energization. The curves are displaced arbitrarily from the origin for a clearer presentation. Actually, the zero point (representing the $^{32}P_i$ content of the [γ-^{32}P]ATP) is the same for all curves. Illumination and storage was carried out in the absence of P_i, chlorophyll concentration was 450 μM. Top right: same as top left, but illumination and storage were carried out in the presence of 2 mM P_i. Bottom: rate of ATP hydrolysis as a function of storage time in the presence and absence of P_i. Open symbols ($+\Delta\tilde{\mu}_{H^+}$): the membrane was energized after different storage times.

first the chloroplasts are stored until the ATP hydrolysis activity has declined completely (after 13.6 min in the absence of P_i). Then the membrane is first energized by an acid-base transition ($\Delta pH = 3.2$) supplemented by a K^+/valinomycin diffusion potential ($\Delta\phi \approx 85$ mV) [22] and after 5 s uncoupled ATP hydrolysis is started as described in Materials and Methods. The result is shown in

Fig. 1, top ($+\Delta\tilde{\mu}_{H^+}$). In the absence as well as in the presence of phosphate in the dithiothreitol reduction medium a rate of about 150 mM ATP per M Chl per s is found. This rate is identical with the rate measured directly after pre-illumination, i.e., before inactivation has occurred. Fig. 1, bottom ($+\Delta\tilde{\mu}_{H^+}$), shows that this rate can be restored at any time after preillumination. This implies that the activation/inactivation process is completely reversible.

We tried different uncouplers in order to have an optimal uncoupling. It turned out that with most uncouplers, e.g. gramicidin D, there exists an optimal concentration: at lower concentrations the rate of ATP hydrolysis decreases (incomplete uncoupling) and also at higher concentrations the rate decreases. (Possibly, there is a direct interaction between uncoupler and ATPase which inactivates the enzyme.) Unfortunately, the optimal concentration also varies from preparation to preparation. Therefore, we finally decided to use NH_4Cl which requires rather high concentrations (more than 1 mM) but increased concentrations (10 mM) do not inhibit ATP hydrolysis.

It may now be asked whether the uncoupling is complete. This has been tested as follows. First, we observed that the different uncouplers used give the same maximal rate of ATP hydrolysis. Second, we have measured ATP hydrolysis under coupled and uncoupled conditions. This is shown in Fig. 2. The rate of ATP hydrolysis, i.e., the slopes of the curves in Fig. 2 are 150 mM ATP per M Chl per s for the uncoupled and 20 mM ATP per M Chl per s for the coupled rate, i.e., uncoupling stimulates the rate by a factor 7.5.

The curve in the presence of NH_4Cl extrapolates to zero, whereas in the absence of NH_4Cl a burst can be observed indicating a fast phase of ATP hydrolysis shortly after initiating the reaction. With the rapid-mixing quenched-flow technique the initial phase has been investigated (Fig. 2, inset). It can be seen that in the first 300 ms the rates of ATP hydrolysis in the presence and absence of NH_4Cl are identical and give a rate of 150 mM ATP per M Chl per s. In the presence of NH_4Cl this rate is constant up to 20 s; whereas, in the absence of NH_4Cl after 300 ms the rate decreases and after 1 s the rate of 20 mM ATP per M Chl per s is observed which is constant for at least 30 s. This result shows that during the first 300 ms the internal proton concentration is so low that the rate of ATP hydrolysis is not limited by the deprotonation reaction at the inside. Due to the hydrolysis-coupled influx of H^+, the proton concentration increases and after about 1 s the proton concentration is so high that

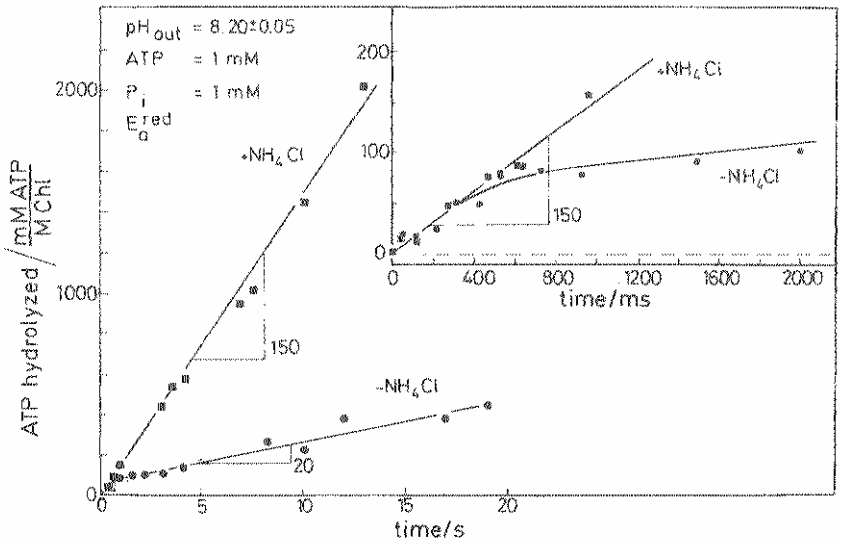


Fig. 2. Coupled ($-NH_4Cl$) and uncoupled ($+NH_4Cl$) ATP hydrolysis as a function of reaction time measured 40 s after illumination in the presence of dithiothreitol. The numbers give the rate in mM ATP per M Chl per s. Inset: ATP hydrolysis with high time resolution measured with a rapid-mixing quenched flow apparatus under coupled and uncoupled conditions.

the rate is limited by the deprotonation. Since the same rate is observed under uncoupled conditions and in the first 300 ms under coupled conditions, it can be concluded that the NH_4Cl concentration is high enough so that ATP hydrolysis is not limited by the deprotonation inside and that, on the other hand, the NH_4Cl concentration is low enough not to decrease the activity of the enzyme. We conclude, therefore, that the observed rate 150 mM ATP per M Chl per s is the maximal rate of the enzyme under these conditions (pH = 8.2, 1 mM ATP, Mg^{2+} = 2 mM, P_i = 1 mM, 20 °C). The amount of chlorophyll per CF_1 as determined by rocket immune electrophoresis was 600 M Chl per M CF_1. This gives a turnover of 90 ATP per CF_1 per s. The finding that the rate of ATP hydrolysis is not limited by the deprotonation reaction inside does not imply that under uncoupled conditions there exists no ΔpH across the membrane. By measuring the rate of ATP hydrolysis in the first 300 ms with the rapid mixing technique as a function of ΔpH it has been shown that the rate is not influenced up to ΔpH = 1.2 (at pH_{out} = 8.2) [26].

Based on these results the procedure for measuring the dependence of activation on ΔpH was as follows: class-II chloroplasts were preilluminated in the presence of dithiothreitol without phosphate. After pre-illumination they were stored in the dark at room temperature. Under these conditions, after 15 min no hydrolysis activity is observed as shown in Fig. 1, bottom. With these chloroplasts acid-base transitions with different ΔpH are carried out so that the pH_{out} is always 8.2 and $\Delta\psi \approx 0$ mV. 5 s after the acid-base jump, NH_4Cl and [γ-^{32}P]ATP is added and the $^{32}P_i$ released is measured as a function of the reaction time (see Materials and Methods).

Fig. 3 shows the amount of ATP hydrolyzed as a function of reaction time with different ΔpH in the activating step. When the ΔpH is low in the activating step (0.13) no ATP hydrolysis is observed. With increasing ΔpH in the activating step the rate of ATP hydrolysis increases.

Fig. 4 shows the uncoupled rate of ATP hydrolysis as a function of ΔpH in the activating step ($\Delta\psi$ = 0 mV). Data are from Fig. 3 and additional measurements. A sigmoidal dependence can be seen with a maximum rate of 180 mM ATP per M

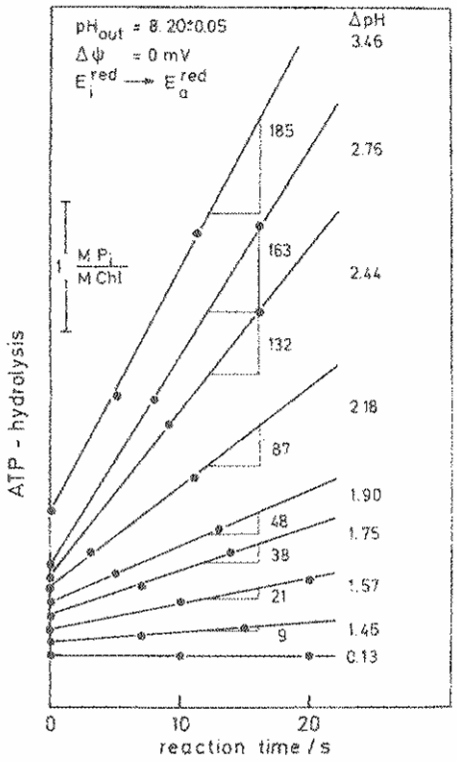


Fig. 3. ATP hydrolysis as a function of reaction time. Chloroplasts were illuminated in the presence of dithiothreitol and stored for 15 min at 20 °C. They show no ATP hydrolysis activity. These chloroplasts are energized by different ΔpH given as parameter of the curves and then the uncoupled ATP hydrolysis is measured. The numbers at the slope give the rate of ATP hydrolysis in mM ATP per M Chl per s. All curves start at the same zero point. They are arbitrarily displaced for clearer presentation.

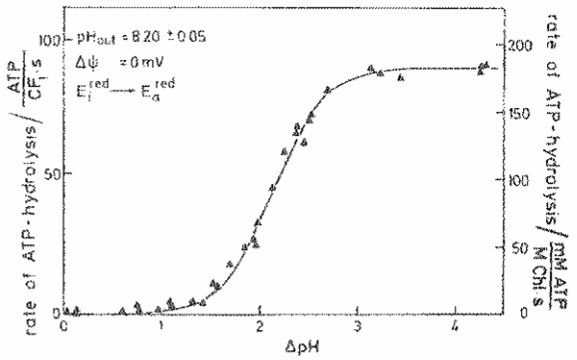


Fig. 4. Uncoupled rate of ATP hydrolysis as a function of ΔpH in the preceding activation step. Data from Fig. 3 and similar measurements. The scale on the left side gives the rate in ATP per CF_1 per s.

Chl per s, scale on the right. The left scale gives the rate in ATP per CF_1 per s. The half-maximal rate is obtained at $\Delta pH = 2.2$. Provided that during pre-illumination in the presence of dithiothreitol all ATP synthases have been reduced and activated, it results that $E_a^{red} = E_t$, where E_a^{red} is the concentration of the active, reduced enzyme and E_t is the total enzyme concentration. It has been verified that neither an increase of dithiothreitol concentration nor an increase of the illumination time increased the rate of ATP hydrolysis. Additionally, it has been observed that there is no enzyme-bound ADP at the end of the pre-illumination period. This indicates that practically all CF_0F_1 has been activated with a concomitant release of tightly-bound ADP [6,15,16,28]. Since the rate is proportional to the enzyme concentration, it results for the relative rate:

$$\frac{v_{ATP}(hydr)}{v_{ATP}^{max}(hydr)} = \frac{E_a^{red}}{E_t} \qquad (1)$$

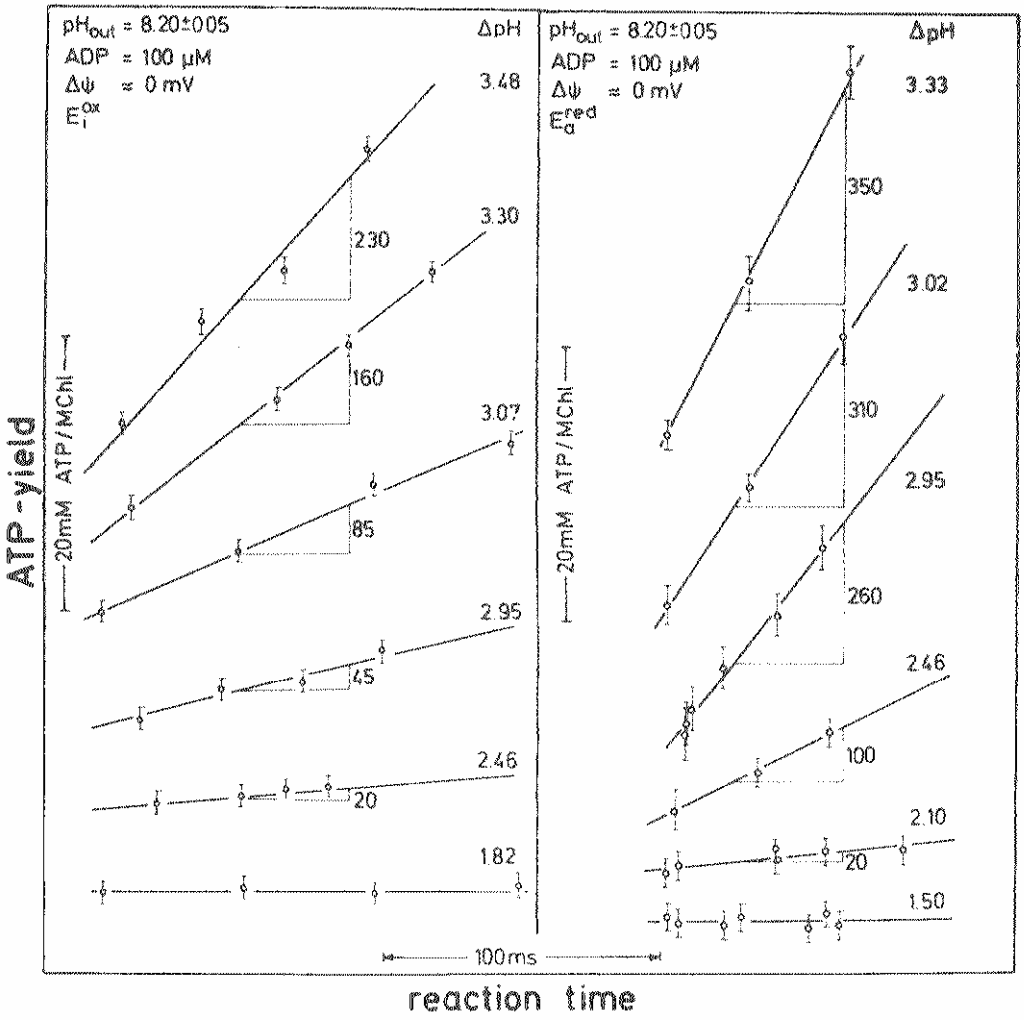


Fig. 5. ATP synthesis as a function of the reaction time measured with a rapid-mixing quenched flow apparatus. The slopes of the curves are the rates of ATP synthesis; numbers give the rates in mM ATP per M Chl per s. The curves are displaced arbitrarily from the origin for a clearer presentation. Actually, the zero point is the same for all curves. Different ΔpH have been generated by acid-base transitions. $\Delta\psi$ was zero in all cases. Details see Materials and Methods. Left: experiments are carried out with untreated class-II chloroplasts, i.e., CF_0F_1 was at reaction time $t = 0$ in the state E_I^{ox}. Right: experiments are carried out with class-II chloroplasts which have been preilluminated in the presence of dithiothreitol, i.e., CF_0F_1 was at reaction time $t = 0$ in the state E_a^{red}.

i.e., the relative rate of ATP hydrolysis directly gives the fraction of reduced, active ATP synthases.

Fig. 5 shows ATP synthesis at different ΔpH ($\Delta\psi \approx 0$ mV) measured with a rapid-mixing quenched flow apparatus. On the left, results are shown when chloroplasts are used before dithiothreitol treatment, i.e., when CF_0F_1 is in the form E_i^{ox}. On the right, results with dithiothreitol-treated chloroplasts are shown, i.e., when CF_0F_1 is in the form E_a^{red}. At the same ΔpH the rate catalyzed by E_a^{red} is much higher than that catalyzed by E_i^{ox} in accordance with earlier results [9].

In Fig. 6 the rates of ATP synthesis, i.e., the slopes of the curves in Fig. 5 and similar measurements are plotted as function of ΔpH for both forms of the enzyme. Measurements of the rate of ATP synthesis as a function of ΔpH immediately after generation of E_a^{red} are not kinetically controlled by a preceding activation process and reflect, therefore, the dependence of the catalytic reaction on ΔpH (see Discussion). Starting with the oxidized, inactive enzyme, E_i^{ox}, the relative rate of ATP synthesis gives according to Eqn. 3 the fraction of the concentrations of the activated, oxidized enzymes, E_a^{ox}/E_t, as a function of ΔpH. The measurement of the activation of the reduced, inactive enzyme is more complicated: in a two-step procedure first the inactive, reduced enzyme, E_i^{red}, is generated and then E_i^{red} is transformed into E_a^{red}. The fraction of the concentrations of the active, reduced enzymes, E_a^{red}/E_t, is then given (see Eqn. 1) by the relative uncoupled rate of ATP hydrolysis. Therefore, the data from Fig. 4 are replotted in Fig. 6, giving the fraction of active, reduced enzymes as a function of ΔpH. It can be

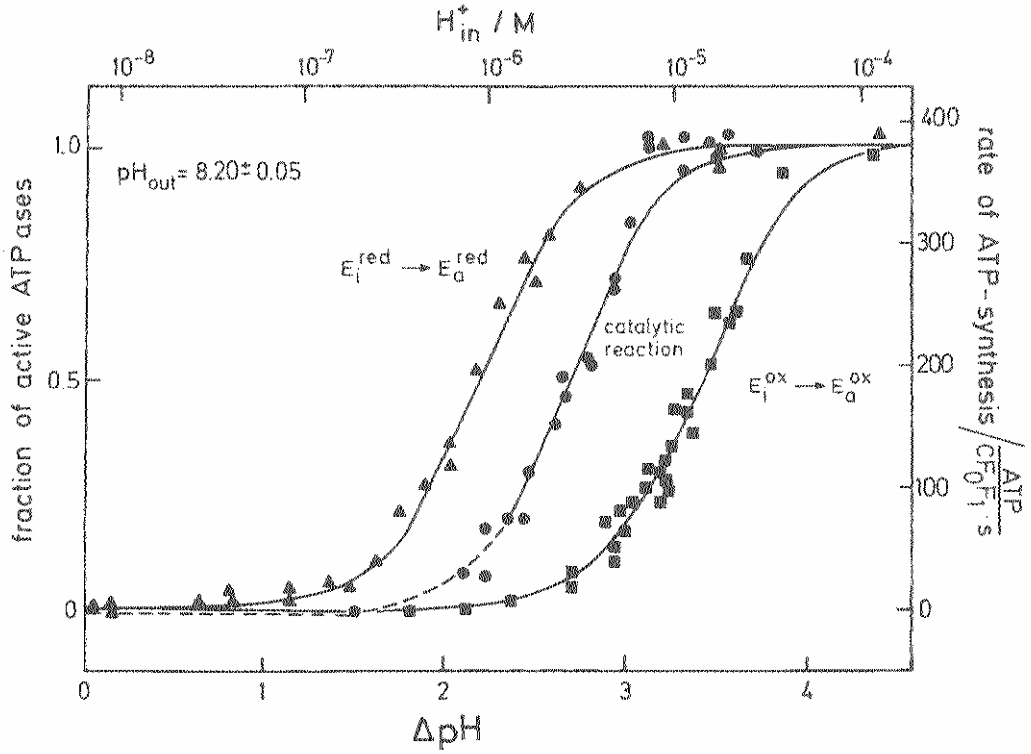


Fig. 6. Rate of ATP synthesis (scale on the right) catalyzed by untreated class-II chloroplasts, E_i^{ox}, and by reduced, activated class-II chloroplasts, E_a^{red}, as a function of ΔpH. Data from Fig. 5 and similar sets of experiments. Relative rate of ATP hydrolysis catalyzed by reduced, inactive class-II chloroplasts, E_i^{red}, as a function of ΔpH in the activation step. Data from Fig. 4. The relative rate of ATP hydrolysis represents, according to Eqn. 1, the fraction of reduced, active ATP synthases, E_a^{red}; the relative rate of ATP synthesis catalyzed by untreated chloroplasts represents, according to Eqn. 3, the fraction of oxidized, active ATP synthases, E_a^{ox} (scale on the left).

seen from Fig. 6 that for each of the three different processes a sigmoidal dependence on ΔpH is found. Half maximal activation is achieved for the oxidized enzyme at $\Delta pH = 3.4$, for the reduced enzyme at $\Delta pH = 2.2$. For the catalytic reaction a half-maximal rate of ATP synthesis is obtained at $\Delta pH = 2.7$. The maximal rate of ATP synthesis (400 ATP per CF_0F_1 per s) is the same for the oxidized and reduced enzyme.

Discussion

Reproducibility of the data

About 40–50 different chloroplast preparations have been used to collect the data presented in this work. If rates of ATP synthesis/hydrolysis are measured per chlorophyll, differences of about a factor of 3 are observed between different preparations under otherwise identical conditions. Therefore, the amount of CF_1 per chlorophyll was determined by rocket immune electrophoresis for each preparation. If rates are expressed per CF_1, the difference between different preparations was maximally a factor of 1.3; i.e., the main differences between the preparations was the CF_1-to-chlorophyll ratio. Based on a molecular mass of 400 kDa for CF_1, this ratio varies between 450 and 1200 chlorophyll per CF_1 in accordance with earlier results [21].

The measurement of the transmembrane ΔpH is as exact as the pH can be measured with glass electrodes using standard buffers for calibration of the electrode response. The underlying assumption is that during incubation in the acidic medium there is a complete equilibration between internal and external pH. This complete equilibration has been demonstrated in our earlier work by measuring the rate as a function of the incubation time [22]. With this method all vesicles have at the beginning of the reaction the same internal pH independent of their size. In small vesicles the ΔpH decreases faster during the reaction due to their smaller amount of protons stored inside. The second assumption that the ΔpH is constant in all vesicles during the rate measurement is controlled in each measurement, since only the linear parts of the ATP yield vs. reaction time curves are used for the determination of the rate.

Also the other reaction conditions are exactly known: all membrane potentials (diffusion potential, surface potential difference, Donnan potential) are vanishingly small because of the high KCl concentration and the presence of valinomycin; the substrate concentrations remain at their initial value because of the short reaction time; the product concentration is measured. Therefore, the data in Fig. 6 refer to precisely defined reaction conditions with a high degree of reproducibility. They can, therefore, serve as standard or reference curves for other measurements; e.g., if the rate of ATP synthesis and the state of the enzyme is known, the ΔpH can be taken from Fig. 6. This is especially useful, since our ΔpH measurement does not require the knowledge of the internal volume and the use of ΔpH-indicating probes (e.g., amines).

The activation of the reduced ATP synthase

The results shown in Fig. 1 indicate that the inactivation of the ATP hydrolysis occurring after dithiothreitol treatment can be completely reversed by membrane energization. In our earlier experiments only a partial reversibility was observed [10]. We have found two experimental conditions under which a complete reversibility can be achieved.

(1) The dithiothreitol treatment is carried out as described earlier [10] and subsequently the chloroplasts are treated with *N*-ethylmaleinimide [3,27]. This leads to alkylation of –SH groups and prevents the reoxidation of the –SH groups in the γ-subunit which had been reduced during the dithiothreitol treatment [3,27]. The decay of ATP hydrolysis activity is slower in the case of maleinimid-treated chloroplasts than with non-treated samples. The maleinimid-treated chloroplasts can be completely reactivated by energization; with the non-treated chloroplasts only a partial reactivation is observed [10].

(2) If EDTA is included in the dithiothreitol-reduction medium (see Materials and Methods), also a complete reactivation of the ATP hydrolysis activity by energization is observed. The reason for this effect is not completely clear.

The observed complete reversibility facilitates the interpretation of the results as well as the experiments. It is clear that as far as CF_0F_1 remains in the reduced state there exists a reversible

activation-deactivation process which depends on ΔpH. Increasing the ΔpH increases the fraction of active, reduced enzymes until at $\Delta pH \geq 3.2$ all CF_0F_1 is in the form E_a^{red}. We assume that there is a protolytic equilibrium between the internal proton concentration and proton accepting groups on the enzyme. If these are fully protonated from the inside while other groups which are in contact with the external phase are fully deprotonated, all enzymes are active [8,17]. The two-step procedure used here (firstly, a reduction by dithiothreitol under illumination and then inactivation while keeping the enzyme in the reduced state, and secondly, an activation of the reduced enzyme by ΔpH) allows a quantitative measurement (Eqn. 1) of the fraction of active, reduced enzymes as a function of ΔpH. The highest rate of ATP hydrolysis observed under our conditions was 180 mM ATP per M Chl per s corresponding to a turnover of about 90 ATP per CF_0F_1 per s. This is about a factor 2 higher than observed earlier [11].

The activation of the oxidized ATP synthase

The rate of ATP synthesis/hydrolysis catalyzed by the oxidized enzyme is given by

$$v_{ATP}^{ox} = w_{ATP}^{ox}(\Delta pH, \Delta\psi)\frac{E_a^{ox}}{E_t}(\Delta pH, \Delta\psi) \qquad (2)$$

(For simplicity it is assumed that the concentration of substrates, products and cofactors is constant.) E_a^{ox}/E_t is the fraction of active, oxidized ATP synthases and w_{ATP}^{ox} is the rate of the catalytic reaction per active, oxidized ATP synthase.

If the functional dependence of the catalytic reaction does not depend on the redox state of the enzyme, i.e., if $w_{ATP}^{ox} = w_{ATP}^{red}$, it can be seen that the measured rate, v_{ATP}^{ox}, reflects indeed mainly the activation of the oxidized enzyme. At $\Delta pH = 3.0$ the catalytic reaction reached practically the maximal turnover, whereas v_{ATP}^{ox} has reached only 20% of its maximal rate. This implies that a further increase of ΔpH does not increase the rate of the catalytic reaction, only the fraction of active CF_0F_1 increases. Under these conditions we obtain from Eqn. 2

$$\frac{v_{ATP}^{ox}(synth)}{v_{ATP}^{ox}(max.synth)} \approx \frac{E_a^{ox}}{E_t} \qquad (3)$$

Therefore, in Fig. 6 the scale on the left side indicates the fraction of active ATP synthases. The fraction of active, reduced CF_0F_1 has been measured in a two-step procedure by the relative rate of ATP hydrolysis catalyzed by E_a^{red} (Eqn. 1). The fraction of active, oxidized CF_0F_1 has been measured by the relative rate of ATP synthesis catalyzed by the oxidized form (Eqn. 3). Comparison of both results shows that a $\Delta pH = 3.4$ is necessary to activate half of the oxidized species; whereas a $\Delta pH = 2.2$ is necessary to activate half of the reduced species. This implies that dithiothreitol treatment of chloroplasts, i.e., the reduction of an –SS-group in the γ-subunit leads to a decrease of the ΔpH for activation. This conclusion is in agreement with earlier results obtained by completely different methods [9].

The catalytic reaction

If an acid-base transition is carried out immediately after dithiothreitol treatment; i.e., when the enzyme is in the state E_a^{red} the rate of the catalytic reaction can be measured without the involvement of the activation process. The time between dithiothreitol treatment and the measurement of the rate of ATP synthesis is 30 s (due to the incubation in the acidic medium). According to the results shown in Fig. 1 approx. 20% of E_a^{red} is converted into E_i^{red} in this time (in the presence of 2 mM P_i and at a chlorophyll concentration of 450 μM). The decay kinetics of hydrolytic activity is slowed down by decreasing chlorophyll concentration (because then also the concentration of free ADP decreases). Dithiothreitol reduction for the measurement of ATP synthesis was carried out in the presence of 5 mM P_i and 150 μM chlorophyll and the chloroplasts were diluted with the medium A immediately after illumination. Under these conditions a maximum of 5% of the activated enzymes is inactivated during incubation. Therefore, the maximal systematic error in the measurement could be that all rates are 5% to small. However, since the ΔpH jump applied for the catalytic reaction is higher than that necessary for activation, this leads to a reactivation of the 5% of inactive enzymes. Therefore, we think that the curve labeled 'catalytic reaction' represents the true dependence of the rate of ATP synthesis on ΔpH without involvement of the activation. Anal-

ogously, as for the oxidized form, the rate of ATP synthesis/hydrolysis for the reduced form is given by

$$v_{ATP}^{red} = w_{ATP}^{red}(\Delta pH, \Delta\psi)\frac{E_a^{red}}{E_t}(\Delta pH, \Delta\psi) \quad (4)$$

For the reduced enzyme Fig. 6 shows both dependencies, w_{ATP}^{red} on ΔpH and E_a^{red}/E_t on ΔpH. If, e.g., the enzyme is in the state E_i^{red} and we apply a ΔpH of 2.5, we obtain from Fig. 6 $w_{ATP}^{red} = 150\ s^{-1}$ and $E_a^{red}/E_t \approx 0.8$; i.e., the measured rate should be $v_{ATP}^{red} = 120\ s^{-1}$ according to Eqn. 4.

Unfortunately, the catalytic rate catalyzed by the oxidized enzyme, w_{ATP}^{ox}, cannot be measured in a similar way as described for the reduced enzyme, because the form E_a^{ox} is so unstable that upon deenergization it reacts rapidly to E_i^{ox}. Presently, we have no method to measure w_{ATP}^{ox}. The simplest assumption in this respect is that the redox state of the enzyme only influences the activation process but not the catalytic reaction. In this case it is $w_{ATP}^{ox} = w_{ATP}^{red}$ and the curve labeled catalytic reaction in Fig. 6 represents the ΔpH dependence of the rate for the oxidized and the reduced ATP synthase.

The rate of ATP synthesis as a function of ΔpH using class-II chloroplasts has been measured by different groups [29–31,53] in all cases v_{ATP}^{ox} was measured which reflects – according to the results presented here – at low phosphate potentials the activation E_a^{ox}/E_t as a function of ΔpH. In our interpretation all measurements where the enzyme is in the oxidized state give no direct information on the catalytic reaction but on the activation process and the data (e.g., H^+/ATP, K_M values, etc.) require therefore a reinterpretation.

Data for the catalytic reaction without involvement of the activation can be obtained only when the enzyme is in state E_a^{red} during the measurement or – at least – when the fraction of enzymes in the different states is exactly known and this fraction does not change during the experiment. In all other cases – we think – a mechanistic interpretation of the data in terms of a coupling between proton transport and ATP synthesis is practically impossible.

The regulation of the ATP synthase

The activity of CF_0F_1 is strongly regulated by the redox state of HS-groups in the γ-subunit. This regulation is not universal among F_0F_1 ATP synthases, e.g., the γ-subunit of the ATP synthase from the thermophilic bacterium PS3 does not contain a HS-group [52]. Additionally, like other ATP synthases the activity of CF_0F_1 is controlled by the energization of the membrane and the binding of substrates and cofactors. Presumably, this strong regulation is necessary to avoid ATP hydrolysis in vivo at the end of an illumination period.

The regulation by binding of ADP and P_i can be neglected in vivo, since the P_i concentration is about 10 mM [33] and the ADP concentration as well as the ATP concentration is in the 1 mM range [34,35]. This implies that compared to the K_M values of the regulatory ADP and P_i binding [15,16,36] their absolute concentrations are too high to play a significant role in regulation. On the other hand, the membrane energization and the redox potential, i.e., the NADPH/$NADP^+$ ratio, change strongly. It has been shown that in vivo CF_0F_1 can be reduced by thioredoxin [37,38] so that after illumination of leaves in the class-I chloroplasts (with intact envelope membranes) high rates of ATP hydrolysis are observed [37–42]. The rate of ATP hydrolysis and ATP synthesis is stimulated regardless whether reduction is carried out in vivo by thioredoxin or in vitro by dithiothreitol treatment of class-II chloroplasts [4].

The redox state of thioredoxin is coupled via ferredoxin to the redox state of NADPH. The redox state of NADPH/$NADP^+$ is controlled by the rate of its reduction due to the electron transport and the rate of its oxidation in the Calvin cycle. On the basis of the results shown in Fig. 6 and the reaction Scheme I the following phenomena are expected in vivo. In dark-adapted leaves as well as in class-I and class-II chloroplasts CF_0F_1 is usually in the oxidized state, E^{ox}.

According to Fig. 6 at, e.g., $\Delta pH \approx 2.7$ a part of CF_0F_1 is transformed into the state E_a^{ox} and a rate of about 30 s^{-1} will be observed. If the CO_2 fixation is limited by the ATP supply, NADPH will be accumulated and this will lead via thioredoxin to a reduction of CF_0F_1. If all CF_0F_1 is reduced, this would lead at constant ΔpH to an

increase of the rate up to 300 s^{-1}. However, we must take into account that possibly not all CF_0F_1 will be reduced, and that due to the increased phosphorylation-coupled proton efflux the ΔpH will decrease. If, e.g., ΔpH decreases by about 0.3 units, the rate will increase only from 30 s^{-1} to 60 s^{-1}; i.e., we can expect that during decrease of ΔpH the rate of ATP synthesis is increased. In fact such a behavior has been observed: the rate of CO_2 fixation in class-I chloroplasts is increased upon addition of NH_4Cl which decreases the ΔpH [43,44]. This has been interpreted as a stimulation of reactions in the Calvin cycle [43]. In our scheme this would be interpreted as follows: addition of NH_4Cl leads to a decreased ΔpH and therefore to an increased rate of electron transport and consequently to an increased NADPH concentration. This gives rise to reduction of CF_0F_1 via thioredoxin and thereby the rate of ATP synthesis can be increased according to the results in Fig. 6, although the ΔpH was lower than before NH_4Cl addition. It is evident from our results that this effect can be expected only if CF_0F_1 is at the beginning of the experiment in the oxidized state. If all CF_0F_1 is in the reduced state, this effect should not be observed. In chloroplasts where the envelope membrane has been made permeable for ADP an uncoupler stimulated ATP synthesis has been observed [45]. Although phenomenologically similar to the effects in vivo (and class-I chloroplasts) the origin of the phenomenon seems to be different [45].

It has been shown that in dark-adapted class-I chloroplasts there exists a rather high ATP concentration which is obviously not hydrolyzed [35,45]. When these chloroplasts are illuminated with a series of single turnover flashes, first ATP hydrolysis and, after some flashes, ATP synthesis is observed. According to the reaction scheme I this is interpreted as follows: at the beginning of the experiment CF_0F_1 is in the state E_i^{red} (no ATP hydrolysis). A few single turnover flashes (even two flashes can be sufficient (Schreiber, U., unpublished results) energize the membrane to such an extent that part of the enzyme is activated (according to Fig. 6 this energization corresponds to a $\Delta pH \geqslant 1.5$) and correspondingly ATP hydrolysis is observed. When in a series of flashes ΔpH and $\Delta\psi$ is increased so that the energization is higher than $\Delta pH \geqslant 2.2$ ATP synthesis is observed. It is clear from our results that these phenomena can only be observed if the enzyme is in state E_i^{red}. If it were in state E_i^{ox}, the energization for the activation of the ATP synthesis would be approx. $\Delta pH \geqslant 3.0$. In this case no ATP hydrolysis can be observed at lower ΔpH, since no active enzyme is present. However, when $\Delta pH \geqslant 3.0$ is reached, this high energization leads automatically to ATP synthesis.

The increased rate of ATP synthesis in the range of $2.0 \leqslant \Delta pH \leqslant 3.5$ catalyzed by E_a^{red} in comparison to the oxidized enzyme explains also why addition of dithiothreitol to intact chloroplasts reduces the quantum requirement for CO_2-reduction [34]. Obviously, at low light intensities CO_2-reduction is limited by the ATP supply and the rate of ATP synthesis catalyzed by the oxidized enzyme is too low to allow efficient CO_2-reduction.

The data presented in this work allow the quantitative interpretation of the redox reaction, the activation reaction, and the catalytic reaction of the ATP synthase in class-II chloroplasts provided the relevant parameters are known. Actually, the data have been used to simulate successfully the time-course of the membrane potential [46]. We think that the proposed scheme also describes the phenomena observed in class-I chloroplasts and in vivo; however, in vivo the relevant parameters (redox state of the enzyme, membrane energization) usually are not known exactly. Therefore, this behavior can be described only qualitatively.

Comparison with literature data

It is well established in class-II chloroplasts that illumination in the presence of dithiothreitol elicits proton-transport coupled ATP hydrolysis in the following dark period [11–14].

Also, pre-illumination of leaves or intact chloroplasts leads to ATP hydrolysis in the subsequent dark period [37–42]. In class-II chloroplasts the stimulation of ATP synthesis after dithiothreitol treatment has been shown by different groups [4,7,9,47,48]. The initiation of ATP synthesis, ATP hydrolysis and [^{14}C]ADP release was studied when the CF_0F_1 was in the form E_i^{ox} and E_i^{red} [49,50] (the release of tightly bound [^{14}C]ADP reflects the

activation of CF_0F_1 [6,28]). The number of single turnover flashes required to initiate the different activities was measured under conditions where the membrane potential was abolished. Therefore, these experiments reflect the minimal ΔpH required for the CF_0F_1 activity under consideration. In qualitative agreement with our results six flashes are required for the activation $E_i^{red} \rightarrow E_a^{red}$, 12 flashes for $E_i^{ox} \rightarrow E_a^{ox}$. The initiation of ATP synthesis and ATP hydrolysis catalyzed by E^{red} required about ten flashes, ATP synthesis by E^{ox} about 15 flashes. Additionally, it was demonstrated that both activation processes do not depend on the phosphate potential [49,50].

The rate of ATP synthesis and ATP hydrolysis has been measured as a function of ΔpH energizing the membrane by light and measuring the ΔpH by the distribution of imidazol [9]. Three different sigmoidal dependencies were found for the activation of the oxidized enzyme, of the reduced enzyme, and of the catalytic reaction catalyzed by the reduced enzyme. The ΔpH for the half-maximal activation and of the half-maximal catalytic rate were (in brackets are given the data from our work): w_{ATP}^{red}: $\Delta pH = 2.75$ (2.7); E_a^{ox}/E_t: $\Delta pH = 3.2$ (3.4); E_a^{red}/E_t: $\Delta pH = 2.35$ (2.2). In view of the fact that the measurement of the rate of ATP synthesis/hydrolysis and that of ΔpH was completely different, the agreement of these data is surprisingly good. On the basis of completely different experiments carried out with intact chloroplasts a reaction scheme has been proposed for the activation and the redox reaction of CF_0F_1 which is quite similar to our reaction scheme (Scheme I) [42].

On the basis of these data obtained by different groups the relation between the redox reaction of CF_0F_1 (also called 'thiol-modulation'), the activation of CF_0F_1 and the catalytic reaction seems to us to be clarified now. However, this does not mean that the regulation of this enzyme is completely understood. It is well known that the activity is also regulated by the absolute concentrations of ADP, ATP, P_i and Mg^{2+} [51], so that a final scheme of the regulation of CF_0F_1 is more complicated than the simplified scheme proposed here. For further investigations it is important that the state of the enzyme is well defined and does not change during the experiment.

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