The Rate of ATP Hydrolysis Catalyzed by Reconstituted CF_0F_1-Liposomes

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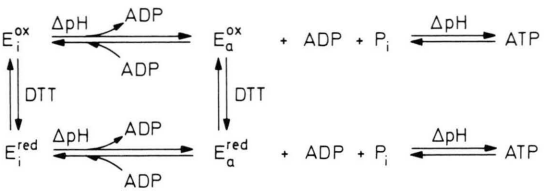
The conditions for optimal rates of ATP hydrolysis catalyzed by the chloroplast ATP-synthase (ATPase), CF_0F_1, after isolation and reconstitution into asolectin liposomes have been investigated. The rate of ATP hydrolysis was measured either after oxidation of CF_0F_1 (by incubation with iodosobenzoate) or after reduction of CF_0F_1 (by incubation with dithiothreitol). In both cases a rate of about 1–2 ATP $(CF_0F_1 \cdot s)^{-1}$ was observed under uncoupled conditions. If the proteoliposomes are first energized by an acid-base transition and a K^+/valinomycin diffusion potential, the uncoupled rate of ATP hydrolysis is about 1–2 ATP $(CF_0F_1 \cdot s)^{-1}$ for the oxidized enzyme and about 20 for the reduced species. This rate is about a factor 2 smaller than that observed in chloroplasts under the same conditions.

Introduction

The membrane-bound chloroplast ATP synthase (ATPase), CF_0F_1, catalyzes reversibly proton transport-coupled ATP synthesis and hydrolysis. For ATP synthesis a maximum turnover of about 400 ATP $(CF_1 \cdot s)^{-1}$ [1] and for ATP hydrolysis a maximum turnover of about 40 ATP $(CF_1 \cdot s)^{-1}$ [2] has been found. Recently, we have shown that CF_0F_1 reconstituted into liposomes is able to catalyze ATP synthesis with rates of about 200 ATP $(CF_0F_1 \cdot s)^{-1}$, when measured under the same experimental conditions as in chloroplasts [3]. In both cases the membrane was energized artificially by an acid-base transition and a K^+/valinomycin diffusion potential, and the rate was measured with a rapid mixing quenched flow apparatus [1]. In literature the reported rates of ATP hydrolysis catalyzed by reconstituted CF_0F_1 were between 1–3 ATP $(CF_0F_1 \cdot s)^{-1}$; *i.e.*, about five percent of that found in chloroplasts [4–9]. Therefore, we have tried to find conditions under which high rates of ATP hydrolysis can be observed in the reconstituted system.

The starting point for our experiments were the following considerations: [10–12] the chloroplast ATP synthase can exist in – at least – four different states, in an inactive oxidized state, E_i^{ox}, an inactive reduced state, E_i^{red}, and both species can be brought into an active state by energization of the membrane. The relations between the different states are depicted in the following scheme:



In isolated class-II-chloroplasts the ATP synthase is usually in the state, E_i^{ox}, energization of the membrane (*e.g.*, by illumination) leads to the release of tightly bound ADP and to the activation of the enzyme, E_a^{ox}. If dithiothreitol is present, the ATP synthase is reduced giving the species E_a^{red}. Binding of ADP to E_a^{red} then leads to the reduced, inactive form, E_i^{red}. Functionally, both inactive forms cannot catalyze ATP synthesis or hydrolysis. In the form E_i^{ox} the ΔpH to transform the enzyme into E_a^{ox} is higher than the equilibrium ΔpH for ATP synthesis (at least at low phosphate potentials) so that with the form E_a^{ox} usually no ATP hydrolysis is observed. On the other hand, only a low ΔpH is necessary to transform E_i^{red} into E_a^{red} so that with the species E_a^{red} ATP synthesis and ATP hydrolysis can be observed.

Based on this scheme, we have tried to optimize experimental conditions for measuring high rates of ATP hydrolysis in reconstituted CF_0F_1 liposomes and

Abbreviations: CF_0F_1, proton translocating ATPase from chloroplasts; IBZ, iodosobenzoate; DTT, dithiothreitol.

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have tried to clarify which enzyme form is obtained after the isolation and reconstitution procedures.

Materials and Methods

The ATP synthase, CF_0F_1, was isolated from spinach chloroplasts as described in [4]. The protein concentration after the sucrose density gradient centrifugation was between 2–4 mg/ml. This preparation was stored in liquid nitrogen until use. CF_0F_1 was reconstituted in the presence of 1 mg/ml bovine serum albumine into asolectin liposomes as described in ref. [3]. The protein concentration after reconstitution was about 1 μM based on a mole mass of 500 kD.

ATP hydrolysis was measured with $\gamma-^{32}P-ATP$ and the $^{32}P_i$ released was extracted as molybdate complex into isobutanol/toluene as in ref. [13]. The $\gamma-^{32}P-ATP$ was synthesized in a similar way as described in ref. [14].

The experimental protocol for activation of CF_0F_1 and measurement of the rate of hydrolysis was as follows: 10 μl proteoliposomes were added to 50 μl of solution I containing 30 mM sodium succinate pH 4.9, 0.5 mM KCl, 2 mM $MgCl_2$, 2 mM NaH_2PO_4 and 1 μM valinomycin. Final pH was 5.0. After 30 s incubation 50 μl of solution II containing 200 mM tricine, 120 mM KOH and 2 mM $MgCl_2$ (pH 8.7) were added. Final pH was 8.4. After 15 s 100 μl of a solution containing 20 mM NH_4Cl, 2 mM ATP and 10^4 Bq $\gamma-^{32}P-ATP$ was added. After the reaction time (between 3 s and 5 min) 50 μl of the solution were quenched in the same volume trichloroacetic acid (40 g/l). 10 μl of this solution were used for measuring the total amount of $\gamma-^{32}P-ATP$, 30 μl were used for measuring the released $^{32}P_i$. The control value was measured by adding trichloroacetic acid first and then NH_4Cl and $\gamma-^{32}P-ATP$. All experiments were carried out at room temperature (20 °C).

When ATP hydrolysis was to be measured without activation, the same procedure was used except that solution I and solution II were mixed first and then the proteoliposomes added. Reduction of CF_0F_1 was carried out after reconstitution by incubation of the proteoliposomes for 1–2 h in 50 mM DTT adjusted to pH 8 by NaOH.

Oxidation of CF_0F_1 was carried out after reconstitution by incubation of the proteoliposomes in 2 mM iodosobenzoate, IBZ, for 20 min. A solution of 160 mM IBZ in dimethylsulfoxide was prepared. From this solution an aqueous solution with 20 mM IBZ was prepared which was adjusted to pH 8.5 by NaOH.

Results

Fig. 1 shows P_i released as a function of the reaction time under different conditions. The slope of these curves gives the rate of ATP hydrolysis. The numbers give the rate in ATP $(CF_0F_1 \cdot s)^{-1}$. The rate of ATP hydrolysis before and after reconstitution is practically the same, *i.e.*, about 1.5 s^{-1}. For different preparations rates between 0 and 2 s^{-1} were found. However, if after reconstitution the proteoliposomes are first energized by an acid-base transition and a K^+/valinomycin diffusion potential, the rate of ATP hydrolysis is increased to 16 s^{-1} in this preparation.

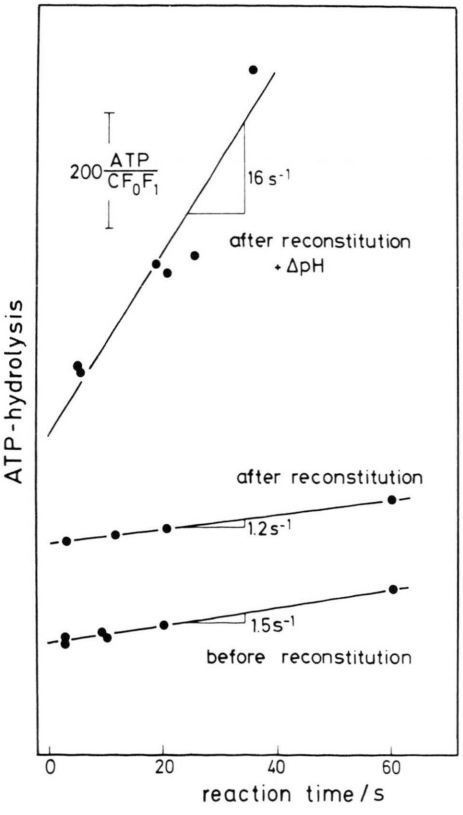

Fig. 1. P_i released as a function of reaction time before reconstitution, after reconstitution and energization. The slope of these curves gives the rate of ATP hydrolysis, the numbers give this rate in ATP $(CF_0F_1 \cdot s)^{-1}$. The curves are displaced arbitrarily from the origin. For details see Materials and Methods and text.

Fig. 2 shows the rate of ATP hydrolysis after an incubation of 2 h with DTT. Without activation by ΔpH (+DTT −ΔpH) the rate is 1 s^{-1}; *i.e.*, similar to that without DTT-incubation. For different preparations the rate is between 0–2 s^{-1}. Activation by ΔpH (+DTT +ΔpH) results in a rate of 12 s^{-1}, again similar to that without DTT. Fig. 2 also shows the rate after incubation with IBZ and a subsequent activation by ΔpH (+IBZ +ΔpH). In this case, a rate of 2 s^{-1} is observed. Practically the same rate as found by us without activation. Also, in this case the rate for different preparations was between 0–2 s^{-1}.

It is possible that IBZ inactivates the ATPase irreversibly. Therefore, the proteoliposomes were incubated for one hour with DTT after the IBZ (+IBZ +DTT +ΔpH)-incubation. In this case, after activation with a ΔpH, a rate of 14 s^{-1} was observed; practically the same as before the IBZ treatment. This implies that the oxidation by IBZ is reversible.

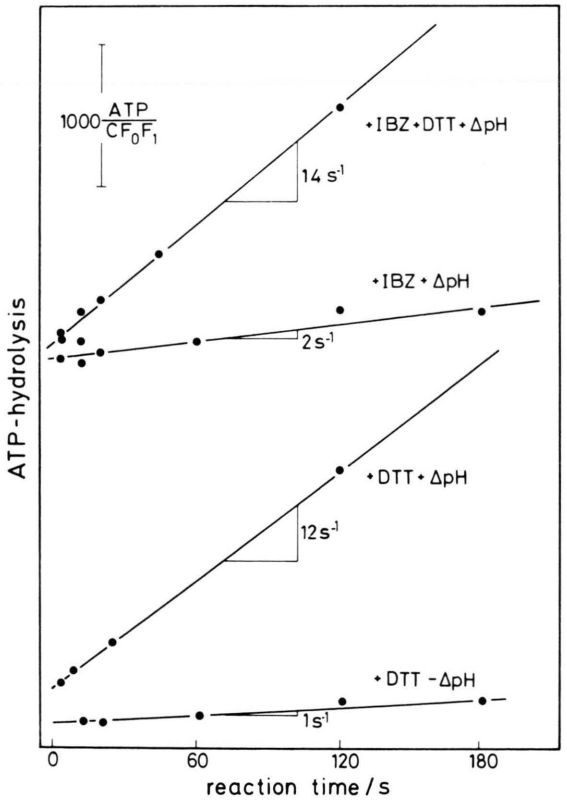

Fig. 2. P_i released as a function of reaction time under different conditions: proteoliposomes first treated with DTT, then energized (bottom); proteoliposomes first treated with DTT then with IBZ and then energized (center); proteoliposomes treated first with DTT, then with IBZ, again with DTT and energized (top). For details see Materials and Methods and text.

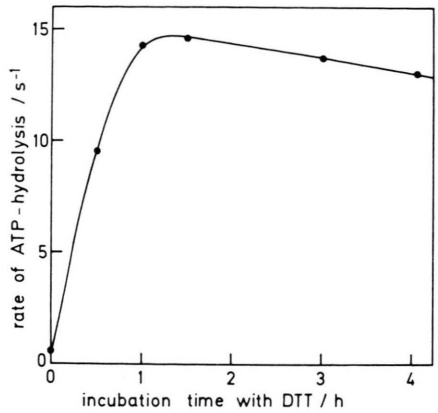

Fig. 3. Rate of ATP hydrolysis (measured as shown in Fig. 1 and 2) as a function of incubation time with DTT (50 mM, 20 °C). The proteoliposomes were first treated with IBZ and then incubated for a variable time with DTT. Before measuring the rate the proteoliposomes were energized. For details see Materials and Methods and text.

Fig. 3 shows the kinetics of the reduction of reconstituted CF_0F_1 by DTT. The degree of reduction is measured by the rate of ATP hydrolysis after activation with ΔpH. First, the enzyme was fully oxidized by IBZ and activated by a ΔpH. This gives the rate without DTT incubation which is, in this case, 0.6 ± 0.5 s^{-1}. The other control rates of this preparation were 0 ± 0.5 s^{-1} (after reconstitution, without ΔpH); 0.4 ± 0.5 s^{-1} (after IBZ treatment, without ΔpH); 0.4 ± 0.5 s^{-1} (IBZ treatment was followed by DTT treatment, without ΔpH). Upon incubation with DTT a maximum of the rate is observed after about 1–2 h and a slight decrease occurs upon prolonged incubation. Therefore, the usual incubation time with DTT was 1–2 h.

These results allow us to identify the state of CF_0F_1 after preparation and reconstitution. Without activation for the oxidized and the reduced form a rate of ATP hydrolysis between 0–2 s^{-1} is observed, with activation the oxidized form catalyzes again a rate between 0–2 s^{-1}, whereas the reduced form catalyzes a rate between 16–25 s^{-1}.

Fig. 4 shows the rate of ATP hydrolysis for four different preparations (A–D). After activation (+ΔpH) rates between 2 s^{-1} (A) and 16 s^{-1} (D) are observed. When these different preparations are first incubated with DTT and then activated by a ΔpH

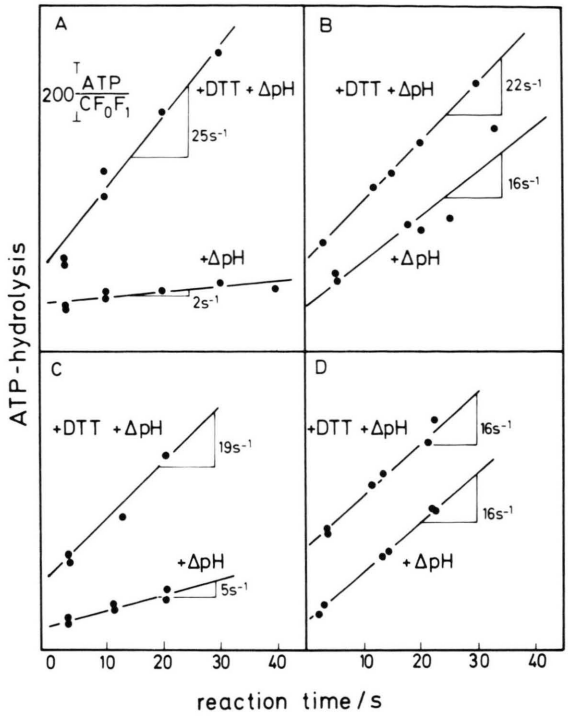

Fig. 4. P_i released as a function of reaction time for different preparations (A-D). The rate was measured either directly after reconstitution and energization (+ΔpH) or reconstitution was followed by a DTT treatment and energization (+DTT +ΔpH). For details see Materials and Methods and text.

(+DTT +ΔpH), the rate is between 16 s^{-1} and 25 s^{-1}. Our interpretation of this result is that after reduction and activation CF_0F_1 catalyzes high rates of about 20 s^{-1}. The observed rates of ATP hydrolysis when the DTT-treatment is omitted show the different fractions of CF_0F_1 in the oxidized state after the usual preparation and reconstitution procedure. This means when a small rate is observed after activation (0–2 s^{-1}), practically all CF_0F_1 is in the oxidized state (Fig. 4A), when a high rate is observed which does not change with DTT incubation practically all CF_0F_1 is in the reduced state (Fig. 4D).

When CF_0F_1 proteoliposomes are stored at 4 °C for several days, the rate of ATP hydrolysis after activation by ΔpH decreases, whereas the rate measured after DTT treatment remains practically the same at least up to 10 days. This indicates that oxygen can oxidize the reduced CF_0F_1.

Discussion

The scheme relating the different states of the ATPase (see Introduction) has been developed for the explanation of the relation between the redox reaction of the chloroplast ATPase, its activation by membrane energization and the catalytic reaction [10]. A similar scheme has been proposed for description of these relations *in vivo; i.e.*, in class-I-chloroplasts [11]. In this work it is shown that this scheme is also useful for understanding the behavior of the reconstituted CF_0F_1.

When the membrane was not energized, a rate of ATP hydrolysis between 0–2 s^{-1} was found (Fig. 1–4). This rate is practically the same, when CF_0F_1 is treated with IBZ; *i.e.*, when the ATPase is in the form E_i^{ox} or with DTT; *i.e.*, when it is in the form E_i^{red}. Also, before reconstitution the isolated CF_0F_1 catalyzes a rate of ATP hydrolysis of about 1–2 s^{-1} (Fig. 1). Similar rates have been reported repeatedly in literature [4–9]. The reason for the difference between the high rates observed with chloroplasts (approx. 40 s^{-1}) and the small rates catalyzed by CF_0F_1 proteoliposomes were not known.

It is shown in this work that an activation of reconstituted CF_0F_1 by ΔpH and ΔΨ gives – after uncoupling – a rate of 2 s^{-1} for the species E_a^{ox} and about 20 s^{-1} for the species E_a^{red}. These latter rates are only a factor 2 smaller than those found in chloroplasts. It should be mentioned that the rate of ATP synthesis catalyzed by these CF_0F_1 liposomes is also a factor 2 smaller than that observed in chloroplasts [3]. Since some denaturation of CF_0F_1 may occur during isolation and reconstitution, the rate per correctly reconstituted active ATPase is actually higher. It must also be considered that activation of the ATPase leads to a release of tightly bound ADP [15–19]. During the time between activation of the ATPase by ΔpH and the addition of ATP (15 s) some rebinding of ADP may occur, thereby leading to an inactivation of a part of the enzymes. Therefore, one might conclude that the rates of ATP hydrolysis catalyzed by CF_0F_1 in the thylakoid membrane and of CF_0F_1 reconstituted into the asolectin membrane are practically the same.

Using CF_0F_1 in its inactive form, it has been shown that a stimulation of the rate of ATP hydrolysis from 1 s^{-1} to 2 s^{-1} is observed, when CF_0F_1 is reconstituted in chloroplast lipids instead of asolectin [9]. This indicates the necessity for a special lipid-protein

interaction for optimal activity of CF_0F_1. If the same stimulation effect were to occur also with the enzyme in its active form, exactly the same rate as in chloroplasts would be obtained.

In this work the different forms of CF_0F_1 reconstituted into liposomes have been identified by their different functions with regard to activation and ATP hydrolysis. Biochemically, the different redox states of the enzyme were well characterized; it was shown that DTT treatment leads to a reduction of an −S−S-bridge to two −SH-groups in the γ-subunit and incubation with IBZ leads to an oxidation of these −SH-groups [20−23]. The difference between the inactive and the active state has not yet been biochemically characterized. However, it is known that upon activation the enzyme releases tightly bound ADP [15−19]. Also, the pK-values of the groups for proton binding in the activation process are different when the ATPase is in the oxidized and reduced form [12].

Whereas the reduced, active species always shows high ATP hydrolysis activity, the results concerning the activity of the inactive (reduced and oxidized) species are not so clear. At the beginning of our experiments we expected that − similar as in chloroplasts − the oxidized inactive enzyme would show no ATP hydrolysis. However, rates between 0−2 s^{-1} were found; most preparations showed rates of about 1 s^{-1}. This activity might be either an intrinsic property of the species E_i^{ox} or it is an artifact of the preparation procedure: (1) During the isolation of the ATPase proteases might partially digest the ATPase and this might lead to an ATP hydrolysis activity [24]. (2) Octylglucoside in the presence of DTT is used to extract the ATPase from the membrane. When part of CF_1 is disconnected from CF_0, the octylglycoside can stimulate ATP hydrolysis of CF_1 [25]. (3) CF_0F_1 is reconstituted into the liposomes by dialysis (5 h, 30 °C). Since it is known that a heat treatment (5 min, 55 °C) [26] leads to CF_1-catalyzed ATP hydrolysis, the milder heat treatment might also elucidate some hydrolysis activity.

Since several of our preparations showed no ATP hydrolysis indeed, when treated with IBZ, we assume at present that the intact, reconstituted ATPase in the form E_i^{ox} shows no ATP hydrolysis.

The situation concerning form E_i^{red} is even unclearer. For all preparations in which no ATP hydrolysis activity was found for form E_i^{ox}, we did not find hydrolysis activity after DTT treatment without energization either; *i.e.*, for the form, E_i^{red}. However, it is known that in chloroplasts in the presence of DTT and ATP a "self-activation" of the ATPase might occur [27]. This would imply that the form E_i^{red} itself has a small hydrolysis activity. On the basis of our results we have to conclude that form E_i^{red} has no ATP-hydrolysis activity different from E_i^{ox}, possibly it has none at all. The form E_a^{ox} is not stable enough to allow ATP hydrolysis measurements under the conditions of our experiment.

However, despite these open questions, this work has shown that reconstituted CF_0F_1 can catalyze high rates of ATP hydrolysis. Furthermore, for the interpretation of all experiments with CF_0F_1 it is important to characterize the redox state and the activity state of the ATPase.

Acknowledgements

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