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ADP binding and ATP synthesis by reconstituted H^+-ATPase from chloroplasts

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Abstract

The H^+-ATPase from chloroplasts, CF_0F_1, was isolated, purified and reconstituted into asolectin liposomes. The enzyme was brought either into the oxidized state or into the reduced state, and the rate of ATP synthesis was measured after energisation of the proteoliposomes with an acid–base transition ΔpH (pH_{in} = 5.0, pH_{out} = 8.5) and a K^+/valinomycin diffusion potential, $\Delta\phi$ (K^+_{in} = 0.6 mM, K^+_{out} = 60 mM). A rate of 250 s^{-1} was observed with the reduced enzyme (85 s^{-1} in the absence of $\Delta\phi$). A rate of 50 s^{-1} was observed with the oxidized enzyme under the same conditions (15 s^{-1} in the absence of $\Delta\phi$). The reconstituted enzyme contained 2 ATP_{bound} per CF_0F_1 and 1 ADP_{bound} per CF_0F_1. Upon energisation the enzyme was activated and 0.9 ADP per CF_0F_1 was released. Binding of ADP to the active reduced enzyme was observed under different conditions. In the absence of phosphate the rate constant for ADP binding was 10^5 $M^{-1} \cdot s^{-1}$ under energized and de-energized conditions. In the presence of phosphate the rate of ADP binding drastically increased under energized conditions, and strongly decreased under de-energized conditions.

Key words: Chloroplast; H^+-ATPase; Liposome

1. Introduction

The H^+-ATPase from chloroplasts, CF_0F_1, couples a transmembrane proton transport with ATP synthesis and ATP hydrolysis. The enzyme was isolated, reconstituted into asolectin liposomes and high rates of ATP synthesis and ATP hydrolysis were measured after an acid–base transition using rapid mixing quenched flow techniques [1–3]. Uni-site ATP hydrolysis and activation/inactivation was also measured with CF_0F_1 proteoliposomes [4]. However, in the latter case ATP synthesis was low and only approximately 20–30% of the enzymes were active as estimated from the release of tightly bound nucleotides in a ΔpH/$\Delta\phi$ jump.

In this work we have investigated ATP synthesis with CF_0F_1 in different redox states using simple mixing with a magnetic stirrer. Additionally, nucleotide release and binding to the enzyme was measured under different conditions.

2. Materials and methods

The H^+-ATPase from chloroplasts was isolated and purified as described earlier [5,6] and stored in liquid nitrogen. The CF_0F_1 was reconstituted by detergent dialysis into asolectin liposomes as described similarly in [2] using different lipid-to-protein ratios. The proteoliposomes contained finally approximately 0.2 μM CF_0F_1, 50 g/l asolectin, 10 mM Na-tricine, pH 8.0, 0.2 mM EDTA, 2.5 mM $MgCl_2$ and 0.25 mM dithiothreitol.

The proteoliposomes were either used directly, E (non-treated), or the enzyme was oxidized, E^{ox}, or reduced, E^{red}. For reduction the proteoliposomes were incubated for 2 h in 50 mM dithiothreitol, pH 8, at room temperature. For oxidation the proteoliposomes were incubated 10 min with 2 mM iodosobenzoate, pH 8, at room temperature.

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ATP and ADP concentrations were measured with luciferin/luciferase and phosphoenolpyruvate and pyruvate kinase as described in [4] except that the volume of the samples varied up to 100 μl.

2.1. ATP synthesis in CF_0F_1 liposomes

The proteoliposomes were energized by an acid–base transition (pH jump) as follows.

Acidic stage: 10 μl of the proteoliposome suspension was incubated in 50 μl of acidic medium for 2 min. The acidic medium (LI) contained 20 mM succinate, 5 mM NaH_2PO_4, 2.5 mM $MgCl_2$, 0.6 mM KCl, and was titrated to pH 4.9 with NaOH. The final pH after addition of the proteoliposomes was 5.0. Valinomycin (2 μM final concentration) was freshly added.

Basic stage: after the acidic stage the proteoliposomes were mixed with an equal volume of the basic medium. The basic medium (LII) contained 5 mM NaH_2PO_4, 2.5 mM $MgCl_2$, 200 μM ADP, 200 mM Na-tricine (pH 8.9) and 120 mM KCl. The final pH after mixing was 8.5. The reaction was stopped after different reaction times between 0.5 and 30 s by adding 100 μl of trichloroacetic acid (40 g/l). The proteoliposomes in LI (60 μl) were stirred continuously with a magnetic stirrer. The reaction was initiated by the injection of LII (60 μl) with one pipette (t = 0). After the reaction time (e.g. t = 0.5 s) trichloroacetic acid (100 μl) was injected with a second pipette, and this stopped the reaction. All further steps (neutralization of the sample, assay for ATP) were carried out separately. The ATP background was determined in the same way except that LI and LII were mixed before the addition of the proteoliposomes.

2.2. Nucleotide release and rebinding after a ΔpH/$\Delta\phi$ jump

The acid–base transition was carried out as described for ATP synthesis except that no ADP and no P_i were present in buffers LI and LII. The reaction medium (1.2 ml) was continuously stirred with a magnetic stirrer. At different reaction times (between 15 s–22 min) after the acid–base transition, 100 μl samples were taken and added to the luciferin/luciferase assay and ATP was determined. In a separate experiment, ADP was measured. The background was determined in the same way except that LI and LII were mixed before the addition of proteoliposomes.

When rebinding of ADP was to be measured under different reaction conditions, the acid–base transition was carried out as described above. 15 s after the pH jump either 5 mM P_i or 10 mM NH_4Cl or both (final concentrations) were added. The nucleotides were assayed as described above.

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3. Results and discussion

After reconstitution of CF_0F_1 the proteoliposomes were characterized by measuring free and bound nucleotides. The average from six different reconstitutions was: ADP_{free} = 0.15 ± 0.1 ADP/mol CF_0F_1, ATP_{free} = 0.1 ± 0.02 mol ATP/mol CF_0F_1, ADP_{bound} = 1.1 ± 0.2 mol ADP/mol CF_0F_1 and ATP_{bound} = 1.9 ± 0.3 mol ATP/mol CF_0F_1.

The proteoliposomes were energized by an acid–base transition and the ATP concentration was measured as a function of reaction time between 0.5 s and 20 s. The ATP yield increased rapidly and reached a maximal value after about 5 s.

The time course can be described by the following function [7]:

$$ATP = ATP_{max}(1-\exp(-kt)) + ATP_{background}$$

where ATP is the ATP concentration at reaction time t and ATP_{max} is the maximal concentration of newly synthesized ATP. The data were fitted by non-linear regression with ATP_{max} and k as free parameters (see solid lines in Fig. 1). The initial rate (t = 0) is then calculated from ATP_{max} and k and the result is indicated in the figure.

When the liposomes were energized by ΔpH and $\Delta\phi$ the rates were 250 mol ATP/(mol $CF_0F_1 \cdot s$), i.e. 250 s^{-1}, for the non-treated and the reduced enzyme; for E^{ox} a rate of 50 s^{-1} was observed. In the absence of $\Delta\phi$ the rates are about a factor of 3–4 smaller. Obviously, the non-treated enzyme is in the reduced state. However, depending on the preparation and storage conditions the non-treated enzyme is sometimes in the oxidized form. The dependence of the rate on the redox state of CF_0F_1 has not yet been reported for the reconstituted enzyme. This effect is well-known in thylakoid membranes [8].

Nucleotide release and rebinding was measured with these proteoliposomes. Fig. 2 (top) shows an experiment where the proteoliposomes are energized in the presence of phosphate, i.e. LI and LII contain 5 mM phosphate. At 15 s after the pH jump only free ATP is found which is subsequently hydrolyzed to free ADP. Based on earlier results these data are interpreted as follows: after energisation, ADP is released in the presence as well as in the absence of phosphate [9,10]. In the presence of phosphate the ADP rebinds rapidly to the enzyme (second order rate constant for ADP binding is $k = 4 \cdot 10^6$ $M^{-1} \cdot s^{-1}$ [11]), it is phosphorylated to ATP, and the ATP is released. After 15 s (hatched part in Fig. 2) only free ATP is found. About 15 s after the pH jump the membrane energisation is nearly dissipated. Since the enzyme is still in its active reduced state ATP is bound, hydrolyzed and ADP is released. Only the last two steps are observed in Fig. 2 (top).

Fig. 2 (center) shows the same type of experiment, however, the pH jump is carried out in the absence of phosphate. In this case 15 s after the jump only ADP is found and the ADP rebinds to the enzyme after dissipation of the membrane energisation (control). Rate constants between 0.4–1 · 10^5 $M^{-1} \cdot s^{-1}$ are determined for different experiments from second order plots. Extrapolation of the kinetics to t = 0 (the time of the pH jump) gives 0.9 ADP/CF_0F_1. This is the amount of ADP released initially in the ΔpH jump. Since the CF_0F_1 contains 1.05 ADP/CF_0F_1 nearly 90% of the bound ADP is released upon energisation.

It is important to note that there is no evidence for a fast phase of ADP rebinding during the first 15 s when the membrane is energized. If ADP binding would be as

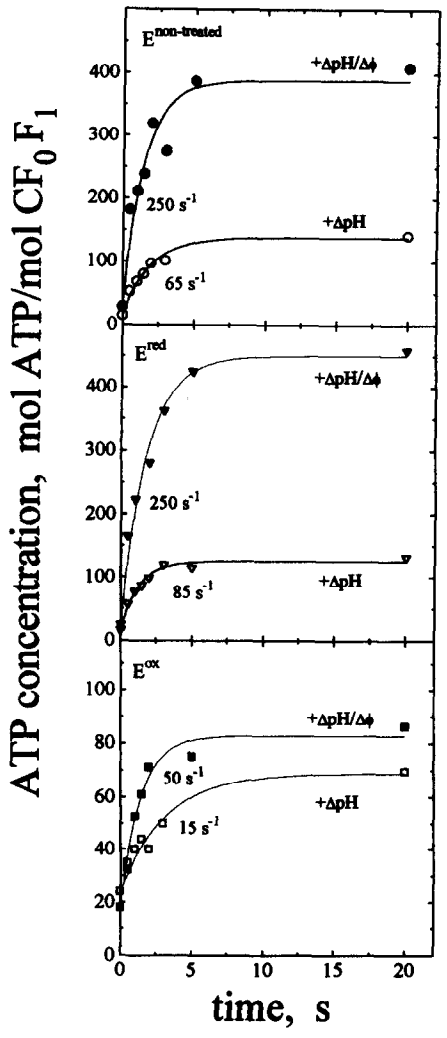

Fig. 1. ATP yield (mol ATP per mol CF_0F_1) as a function of reaction time after an acid–base transition. CF_0F_1 was reconstituted into asolectin liposomes and subjected to an acid–base transition (pH_{in} = 5.0, pH_{out} = 8.5) either in the presence of a K^+/valinomycin diffusion potential (K^+_{in} = 0.6 mM, K^+_{out} = 60 mM) or in its absence (K^+_{in} = 120 mM, K^+_{out} = 120 mM). The rates for the initial conditions (t = 0) are indicated. The curves start at about 20 ATP/CF_0F_1. This results from the background of ATP in the ADP. The enzyme was either in the oxidized state, E^{ox} (bottom panel), in the reduced state, E^{red} (center panel) or in a not defined state, $E^{non\text{-}treated}$ (top panel).

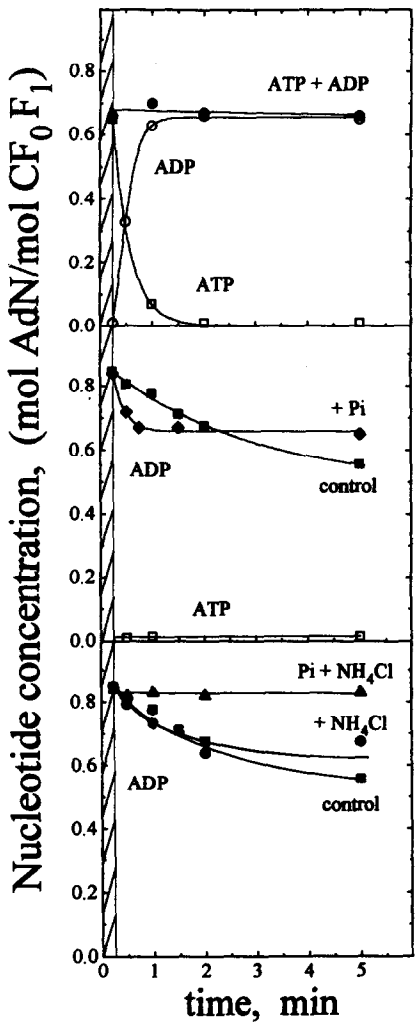

Fig. 2. Nucleotide concentrations as a function of reaction time after an acid–base transition without exogenous nucleotides. The proteoliposomes (60 nM CF_0F_1) were subjected to an acid base transition (pH_{in} = 5.0, pH_{out} = 8.5) and a diffusion potential (K^+_{in} = 0.6 mM, K^+_{out} = 60 mM). The first 15 s after the pH jump are dashed. The background ADP (0.07 mol ADP/mol CF_0F_1) and background ATP (0.1 mol ATP/mol CF_0F_1) were subtracted. (Top panel) Phosphate (5 mM) was present during the pH jump (in LI and LII). (Center panel) Phosphate (5 mM final concentration) was added 15 s after the pH jump. (Bottom panel) NH_4Cl (10 mM final concentration) and NH_4Cl (10 mM) + phosphate (5 mM) were added 15 s after the pH jump. For details see text.

fast as in the presence of phosphate, nearly all the free ADP must bind in this time. Obviously, the absence of phosphate leads to a decrease in the rate of ADP binding under energized conditions. Additonally, the kinetics of ADP binding does not respond to the changing energization after the pH jump in the absence of phosphate. This is in accordance with earlier results from thylakoid membranes where ADP rebinding does not change upon addition of uncouplers [12].

What happens when phosphate is added after the pH jump? This is also shown in Fig. 2 (center, + P_i): the pH jump is carried out in the absence of phosphate and 15 s later the concentration of free ADP is measured. Immediately after taking the sample for nucleotide analysis (about 1 s later) 5 mM phosphate (final concentration) is added. In this case ADP rebinding is biphasic: after addition of P_i a fast rebinding is followed by a very slow ADP rebinding. The amplitude of the fast phase (0.1 ADP/CF_0F_1 in Fig. 2, center) varies between 0.03 and 0.15 for different preparations. Our interpretation of the biphasic binding is as follows: after 15 s a small part of the liposomes is still energized, and after addition of P_i the energized enzymes bind ADP very rapidly (as in Fig. 2, top). In most liposomes the membrane energisation is dissipated after 15 s. They should bind ADP with a similar rate as in the control. However, ADP binding is much slower. Obviously, these de-energized enzymes also bind P_i since in its presence no ADP binding (or a very slow ADP binding) is observed.

If this interpretation is true de-energisation of the liposomes before addition of phosphate should completely abolish the fast phase. The corresponding experiment is shown in Fig. 2 (bottom). The pH jump is carried out in the absence of phosphate. The control curve again shows rebinding of ADP in the absence of phosphate. In the second experiment after 15 s 10 mM NH_4Cl and 5 mM P_i (final concentrations) are added. In this case the fast phase is almost completely abolished and only the very slow ADP rebinding is observed. When only NH_4Cl is added the rebinding of ADP in the absence of phosphate is not significantly influenced for 1–2 min after its addition in accordance with earlier results from thylakoids [12].

The rebinding of ADP in the presence of NH_4Cl and phosphate was measured with a different reconstituted CF_0F_1 preparation and its kinetics were analyzed. The second order rate constant for binding was calculated either from the initial rate or from a second order plot. We obtained rate constants between $0.4–2 \cdot 10^3$ $M^{-1} \cdot s^{-1}$ (average $1.2 \cdot 10^3$ $M^{-1} \cdot s^{-1}$).

These data show that the properties of purified reconstituted CF_0F_1 are similar to those observed in thylakoid membranes. (i) the rate of ATP synthesis is between 200–300 s^{-1} for different preparations; (ii) at suboptimal $\Delta pH/\Delta\phi$ the rate catalyzed by the reduced enzyme is much higher than that by the oxidized enzyme; (iii) at suboptimal ΔpH the rate is stimulated by $\Delta\phi$ with the reduced and the oxidized enzyme; (iv) almost all bound ADP is released upon energisation of the membrane.

The improvement of the enzyme activity (rate of ATP synthesis and nucleotide release) as compared to earlier results [1–4] is presumably due to two effects. The enzyme contains 2 ATP/CF_0F_1 and 1 ADP/CF_0F_1 whereas 1 ATP/CF_0F_1 and 1 ADP/CF_0F_1 was found in [1–4]. Furthermore, the enzyme-to-lipid ratio was decreased. This results in a lower number of enzymes per liposome and in a prolonged energisation of the membrane. Our

data show that the kinetics of ADP binding by the active reduced CF_0F_1 is strongly influenced by phosphate.

(i) In the absence of phosphate the rate constant for ADP binding is $0.4–1 \cdot 10^5$ $M^{-1} \cdot s^{-1}$ and this rate constant is not changed significantly when the membrane is energized. Both observations are in accordance with earlier results from thylakoid membranes [12]. With a higher time resolution an increased rate of ADP binding was observed [13].

(ii) In the presence of phosphate the rate constant for ADP binding is drastically increased when the membrane is energized. The rate constant for ADP binding under these phosphorylating conditions is between $4–10 \cdot 10^6$ $M^{-1} \cdot s^{-1}$ [11,14].

(iii) In the presence of phosphate the rate constant for ADP binding is drastically decreased when the membrane is de-energized. The rate constant under these conditions is $0.4–2 \cdot 10^3$ $M^{-1} \cdot s^{-1}$. The inhibition of ADP binding by phosphate has been observed earlier in thylakoids [13,15]. In the reconstituted system this effect is much more pronounced. A reason might be that in thylakoid membranes small concentrations of phosphate are always present, whereas in liposomes the presence of phosphate can be avoided.

Obviously, there is an interplay between energisation of the membrane, phosphate binding and ADP binding: ADP binding is not influenced by energisation in the absence of phosphate. However, in the presence of phosphate ADP binding is drastically increased; it is nearly abolished when the membrane is de-energized.

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