Uni-site ATP synthesis in thylakoids

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Uni-site ATP synthesis was measured with thylakoids. The membrane-bound ATP-synthase, CF_0F_1, was brought into the active, reduced state by illumination in the presence of thioredoxin, dithiothreitol and phosphate. This enzyme contains two tightly bound ATP per CF_0F_1. ATP was released from the enzyme when ADP was added in substoichiometric amounts during illumination. Experiments with [^{14}C]ADP indicated that after binding the same nucleotide was phosphorylated and released as [^{14}C]ATP, i.e. only one site is involved in ATP-synthesis ('uni-site ATP-synthesis'). The two tightly bound ATP are not involved in the catalytic turnover. The rate constant for ADP binding was $(4 \pm 2) \times 10^6$ $M^{-1}s^{-1}$. Compared to deenergized conditions the rate constant for ADP binding and that for ATP-release were drastically increased, i.e. membrane energization increased the rate constants for the ATP-synthesis direction.

Chloroplast; ATPase H^+; CF_0F_1; Uni-site catalysis; ATP synthesis

1. INTRODUCTION

Membrane-bound ATP synthases of the F_0F_1-type catalyze ATP synthesis/hydrolysis coupled with a transmembrane proton transport. These ATP-synthases have a hydrophilic part, F_1, which contains the nucleotide binding sites and a hydrophobic part, F_0, which is inserted into the membrane and is supposed to act as a proton channel.

The F_1 part of the enzyme is able to bind a maximum of six nucleotides [1,2]; three of them have catalytic properties [3,4]. The cooperativity of the three catalytic sites impedes the detailed investigation of the mechanism of the catalytic reaction. Therefore, kinetics of ATP hydrolysis was measured with substoichiometric amounts of ATP, so that only one catalytic site was operating ('uni-site conditions'). Such kinetic investigations were carried out with the isolated F_1 from different sources [5–16]. Nevertheless, all these investigations have the disadvantage that the mechanism of the catalytic reaction, i.e., the coupling of the proton flux with the ATP-synthesis/hydrolysis cannot be investigated, because the F_0-part is missing. Single-site ATP hydrolysis with the complete enzyme was reported for submitochondrial particles [17,18] for the isolated, reconstituted F_0F_1 from chloroplasts [19,20] and for thylakoids [21,22]. In this work we investigate ATP synthesis with thylakoid membranes using substoichiometric amounts of ADP.

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Abbreviation: CF_0F_1, H^+-translocating ATPase ('ATP-synthase') from chloroplasts

2. MATERIALS AND METHODS

2.1. *The isolation of thylakoids and characterization of CF_0F_1*

The thylakoids were isolated from spinach chloroplasts as described before [23]. The CF_0F_1 content was 730 ± 100 chlorophyll per CF_0F_1 as determined by immunoelectrophoresis [24,25]. The concentration of free and enzyme-bound nucleotides (ATP and ADP) was measured by the luciferin/luciferase-technique as described elsewhere [19]. Two bound ATP and about one bound ADP per CF_0F_1 were found. The concentration of free nucleotides was about 0.04 ATP per CF_0F_1 and 0.4 ADP per CF_0F_1.

2.2. *The reduction of CF_0F_1 in thylakoids*

CF_0F_1 was in the oxidized, inactive state after preparation of the thylakoids. The enzyme was reduced by thioredoxin and dithiothreitol as follows. The thylakoids (75 μl) were suspended in 425 μl of buffer 1 (final concentrations: about 300 μM chlorophyll, 3 μM thioredoxin, 20 μM pyocyanin, 1 mM dithiothreitol, 5 mM Na-Tricine, pH 8.0, 0.5 mM EDTA and 2.5 mM $MgCl_2$) and illuminated for 1 min. After this illumination the enzyme was in the active, reduced state. The thylakoids were stored on ice in the dark for at least 15 min. During this time the inactive, reduced state of the enzyme is formed. The nucleotide content after this treatment is again 2 ATP_{bound} per CF_0F_1 and 1 ADP_{bound} per CF_0F_1.

2.3. *Kinetic measurements*

Before each experiment the inactive, reduced CF_0F_1 was reactivated by illumination: 25 μl thylakoids were illuminated in 250 μl of buffer 2 (100 mM Tricine, 10 mM succinate, 60 mM KOH, 2 mM Na_2HPO_4, 20 μM pyocyanin, 2 mM $MgCl_2$; the solution was finally titrated with NaOH to pH 8.3) for 15 s. During that time all free ADP was phosphorylated to ATP, so that only free ATP was observed. After 15 s illumination ($t_x = 0$) 250 μl of buffer 3 were added, this being the same as buffer 2 but containing additionally 5 to 8 nM [U-^{14}C]ADP (specific activity 20 GBq/mmol). The illumination was continued and after different reaction times (t_x = 0–90 s) free and enzyme-bound nucleotides were separated by filtration under con-

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tinuous illumination as follows: A Dyna Gard filter tip (Tecnomara no. 24060) with a pore width of 0.2 μM and an area of 5.5 cm^2 was set on a 5 ml syringe. The tip was put into the reaction solution and the piston of the syringe was immediately pulled. The filtration was finished in less than 2 s. The reaction time was the time after which the separation was finished.

The filtrate was divided into three parts and the free concentrations of the nucleotides were measured:

(1) 75 μl of the filtrate were used to determine the concentration of the sum of free (ATP + ADP) with luciferin/luciferase and pyruvate kinase/phospho*enol*pyruvate [19].

(2) 75 μl of the filtrate were used to determine the concentration of free ATP, with luciferin/luciferase [19].

(3) 100 μl of the filtrate were mixed with 4 ml of the scintillator Hionic Fluor (Canberra-Packard), and the ^{14}C activity was determined in a liquid scintillation counter.

The total ^{14}C activity was determined as follows: 25 μl of the buffer 1 were added to 250 μl of the buffer 2 instead of the chloroplast suspension and all the further steps were performed as described before. 100 μl of the filtrate were used to determine the total ^{14}C activity.

3. RESULTS AND DISCUSSION

In order to characterize the thylakoid membranes and the reaction conditions, the rate of ATP synthesis and the rate of ATP hydrolysis (coupled and uncoupled) were measured as described before [22]. The same reaction conditions as for single site ATP synthesis were used except that 300 μM ADP were present (for ATP synthesis) or 1 mM ATP, labeled with 12 kBq [γ-^{32}P]ATP (for ATP hydrolysis), or 1 mM ATP, labeled with 12 kBq [γ-^{32}P]ATP, 6 mM NH_4Cl and 1 μM valinomycin (for ATP hydrolysis under uncoupled conditions). For ATP synthesis (110 ± 30) ATP/(CF_0F_1 s) were found; for coupled ATP hydrolysis (10 ± 3) ATP/(CF_0F_1 s) and for uncoupled ATP hydrolysis (70 ± 30) ATP/(CF_0F_1 s).

In order to measure the uni-site ATP synthesis, 5.5 nM ADP were added after 15 s of illumination (reaction time t_x = 0). The enzyme concentration was 13 nM. The free and enzyme-bound nucleotides were separated after different reaction times (t_x = 10–60 s) as described in section 2 and the concentrations of free ATP and ADP were measured. In the control experiment the same procedure was carried out, but no ADP was added.

Fig. 1 shows the result of these measurements. The concentrations of the different nucleotides are plotted as a function of the reaction time. The concentration of free (ATP + ADP) is constant, whereas the concentration of free ATP increases. The concentration of free ADP is calculated from these two measurements and decreases during reaction time. In the control experiment the concentration of ATP remains constant.

The binding of ADP is a second-order process, i.e.

$$-\frac{d[ADP]}{dt} = k[ADP][E] \quad (1)$$

The initial rate of ADP binding is 0.32 $nM \cdot s^{-1}$ (see

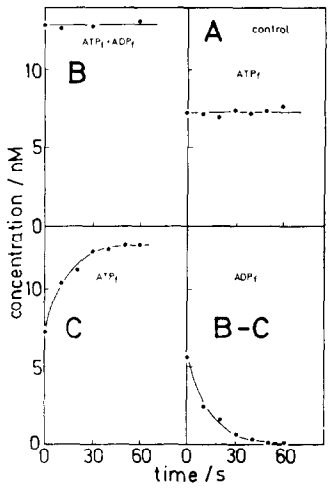

Fig. 1. The concentration of free nucleotides as a function of time during illumination of the thylakoid membranes. At zero reaction time 5.5 nM ADP was added. In the control experiment no ADP was added (A). The concentration of free ATP and ADP (B) and of free ATP (C) was measured by luciferin/luciferase. The concentration of ADP (B–C) was determined by the difference between these two measurements.

Fig. 1). The initial concentrations are $[ADP]_0$ = 5.5 nM and $[E]_0$ = 13 nM. The rate constant for ADP-binding is calculated from Eqn. 1 and was found to be $k = 4.5 \times 10^6\ M^{-1} \cdot s^{-1}$.

According to Fig. 2 the concentration of enzyme-bound nucleotides is constant during the reaction. This implies that also the concentration of free enzyme is constant. Therefore, ADP-binding can be described by first-order kinetics. From an average of 10 different experiments, $k = (4 \pm 2) \times 10^6\ M^{-1} \cdot s^{-1}$. This result is almost identical with the rate constant for ADP binding measured with the rapid mixing quenched flow technique [26].

The ATP is released from the enzyme with a similar rate as the ADP is bound (see Fig. 1). However, since the enzyme contains at the beginning of the experiment tightly bound ATP (see section 2), it may be asked whether the released ATP is really newly synthesized or whether ADP binding to one site is followed by ATP release from another site. The latter possibility is expected, when a binding change mechanism is operating [27–30].

Therefore, a similar experiment was carried out as described in Fig. 1, except that 7.6 nM [^{14}C]ADP were added instead of unlabelled ADP. The total amount of labelled nucleotides was measured in the filtrate. The two mechanisms discussed above will give different results.

If [^{14}C]ADP is bound to one site, phosphorylated and then [^{14}C]ATP is released from the enzyme it is expected that the total amount of radioactive nucleotides in the filtrate remains constant during reaction time. Only initially, a decrease of total radioactivity is ex-

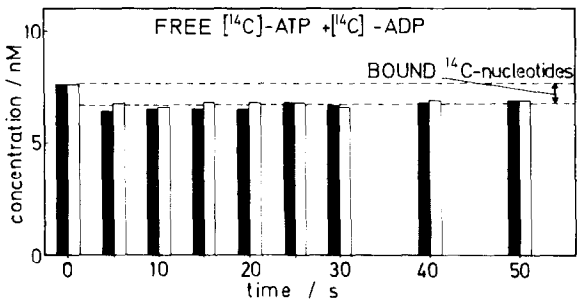

Fig. 2. The total concentration of free [^{14}C]nucleotides as a function of reaction time. The separation was carried out by filtration as described in the text. At zero reaction time 7.6 nM ADP was added. The black and white columns show two different experiments.

pected, since enzyme-substrate and enzyme-product intermediates are formed.

If [^{14}C]ADP is bound to one site and unlabelled ATP is released from a second site, the total radioactivity in the filtrate should decrease to zero with the same kinetics as found for ADP binding (see Fig. 1).

The total amount of free [^{14}C]nucleotides in the filtrate was measured as described in section 2. The result is shown in Fig. 2. The total amount of nucleotides in the filtrate remains constant during the reaction time. Only the zero-time control is about 0.9 nM higher. This difference indicates the concentration of enzyme-bound radioactively labelled nucleotides. In order to demonstrate the reproducibility, two different experiments are shown in Fig. 2. These results unequivocally show that [^{14}C]ATP is released from the enzyme, i.e. ADP-binding, phosphorylation and ATP release occurs on one site (uni-site ATP-synthesis). Therefore, the two tightly bound ATP are not involved in the catalytic turnover.

Based on these results, a lower limit of the rate constant for ATP release can be estimated. The rate of ATP release is given by

$$-\frac{d[ATP]}{dt} = k[ATP_{bound}] \quad (2)$$

The rate of ATP release is 0.32 nM·s^{-1}. The concentration of enzyme-bound ATP is maximally 0.9 nM (see Fig. 2). With these data, Eqn. 2 gave for the rate constant $k > 0.36$ s^{-1}. This is the lower limit, i.e. it is assumed that all the enzyme-bound [^{14}C]nucleotides are present as [^{14}C]ATP. If only a small fraction of the enzyme-bound nucleotides is ATP, the true rate constant is much higher.

The rate constant for ATP release under deenergized conditions was so low, that it was not possible to measure it with CF_0F_1 [19,20]. Obviously, energization of the membrane leads to a large increase of the rate constant for ATP release.

The rate constant for ADP binding under deenergized conditions was 10^5 M^{-1}·s^{-1} [19]. In this work we observed 4×10^6 M^{-1}·s^{-1}, when the membrane is energized, i.e. energization leads to a large increase of the rate constant for ADP binding.

Both effects strongly favour the ATP synthesis direction of the catalytic reaction when the membrane is energized. This is in accordance with the model of Boyer, where a ΔpH-induced conformational change leads to the release of ATP [31].

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