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Uni-site catalysis in thylakoids

The influence of membrane energization on ATP hydrolysis and ATP-P_i exchange

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ATP-hydrolysis was measured with thylakoid membranes during continuous illumination. The concentrations of free and enzyme-bound ATP, ADP and P_i were measured using either cold ATP, [γ-^{32}P]ATP or [^{14}C]ATP. The concentration of free ATP was constant, free ADP and enzyme-bound ATP were below the detection limit. Nevertheless, [γ-^{32}P]ATP was bound, hydrolyzed and $^{32}P_i$ was released. The ADP was not released from the enzyme but cold P_i was bound from the medium, cold ATP was resynthesized and released. A quantitative analysis gave the following rate constants: ATP-binding $k_{ATP}=2\cdot10^5$ $M^{-1}s^{-1}$, ADP-release: $k_{ADP}<10^{-2}s^{-1}$, P_i-release: $k_{P_i}=0.1$ s^{-1}. These rate constants are considerably smaller than under deenergized conditions. The rate constant for the release of ATP can be estimated to be at least 0.2 s^{-1} under energized conditions. Obviously, energization of the membrane, i.e. protonation of the enzyme leads mainly to a decrease of the rate of ATP-binding, to an increase of the rate of ATP release and to a decrease of the rate of ADP-release.

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

1. INTRODUCTION

The H^+-ATPase from chloroplasts catalyzes ATP-synthesis/hydrolysis coupled with a transmembrane proton transport. The enzyme belongs to the F_0F_1-type ATPases [1] which can bind 6 nucleotides [2]. Three of the nucleotide binding sites have catalytic properties [2]. The kinetics of ATP-hydrolysis was measured when only one catalytic site was operating. Mostly, the isolated F_1 parts from different sources were used for the kinetic investigations [3-11] and a complete set of rate constants for the uni-site cycle was obtained in [3,4]. Single site ATP-hydrolysis was also measured with complete F_0F_1 under deenergized (uncoupled) conditions [12-14]. In this work we measured uni-site ATP hydrolysis with thylakoid membranes during illumination, i.e. under energized conditions. A comparison with earlier results allows the identification of those steps in the reaction cycle which are coupled with the protonation/deprotonation reactions of the enzyme, leading to the proton pumping.

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Abbreviations: CF_0F_1, H^+-translocating ATPase ('ATP-synthase') from chloroplasts; Chl, chlorophyll

2. MATERIALS AND METHODS

The thylakoids were isolated from spinach as described earlier [15]. The amount of CF_0F_1 per chlorophyll was determined by immuno-electrophoresis [15]. It resulted in 730 ± 100 chlorophyll per CF_0F_1 for the preparation used in this work. Free and bound ATP and ADP were measured with luciferin/luciferase [15]. The preparations contained the following bound nucleotides: 2 ± 0.2 ATP_b/CF_0F_1 and 1 ± 0.1 ADP_b/CF_0F_1. The reaction conditions were further characterized by measuring multi-site ATP-synthesis and ATP-hydrolysis under energized and uncoupled conditions. These measurements were carried out under the same experimental conditions (reaction vessel, light intensity, reaction medium) as single site ATP hydrolysis. The CF_0F_1 was brought into the reduced, inactive state and was reactivated by light as described in [15]. After 15 s illumination all CF_0F_1 was in the active, reduced state. At this time different additions were made. (The final reaction medium contained 100 mM tricine (titrated to pH 8.3 with NaOH), 20 mM succinate, 60 mM KOH, 2 mM NaH_2PO_4, 2 mM $MgCl_2$, 20 μM pyocyanin, 9.5 μM chlorophyll containing 13 nM CF_0F_1.)

(A) ATP-synthesis, energized conditions: during continuous illumination 300 μM ADP were added.

(B) ATP-synthesis, dark-control: without illumination and reactivation 300 μM ADP were added.

(C) ATP-hydrolysis, energized conditions: during continuous illumination 1 mM MgATP, containing 12 kBq [γ-^{32}P]ATP was added.

(D) ATP-hydrolysis, uncoupled conditions: light was switched off and simultaneously 1 mM MgATP, containing 12 kBq [γ-^{32}P]ATP, 3 mM NH_4Cl, 1 μM valinomycin (final concentrations) were added. The samples were denatured with 2% trichloroacetic acid (final concentration). (A + B) ATP was measured by luciferin/luciferase. (C + D) ATP hydrolysis was measured by release of $^{32}P_i$ [15].

Fig. 1 shows the result:

(A) The rate of ATP-synthesis was 140 $ATP/(CF_0F_1\cdot s)$.

(B) The control rate in the dark was 3 $ATP/(CF_0F_1\cdot s)$ and was due to the adenylate kinase.

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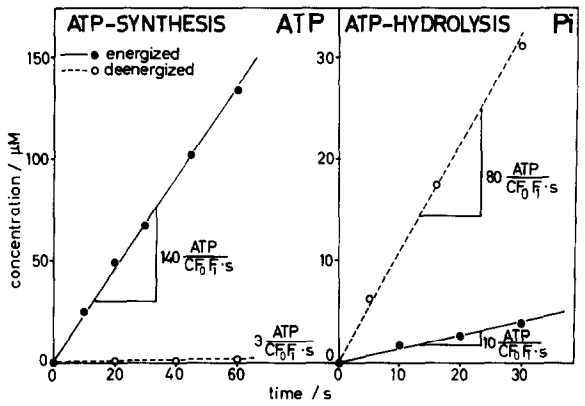

Fig. 1. Left: ATP-synthesis as a function of the reaction time (A) energized conditions during continuous illumination (full circles), and (B) deenergized conditions without illumination (open circles, dashed line). In this case adenylate kinase activity is measured. Initial concentrations are 2 mM P_i, 300 µM ADP, 9.5 µM chlorophyll containing 13 nM CF_0F_1. Right: ATP-hydrolysis as a function of the reaction time (C) energized conditions during continuous illumination (full circles), and (D) deenergized (uncoupled) conditions without illumination in the presence of uncouplers (open circles, dashed line). Initial concentrations are 2 mM P_i, 1 mM [γ-^{32}P]ATP, 9.5 µM chlorophyll containing 13 nM CF_0F_1, in (D) additionally 3 mM NH_4Cl and 1 µM valinomycin are present. For further details see text.

(C) The rate of ATP-hydrolysis was 80 ATP/(CF_0F_1 · s) under deenergized (uncoupled) conditions (maximal rate).

(D) The rate of ATP hydrolysis was 10 ATP/(CF_0F_1 · s) under energized conditions. This is about 12% of the maximal rate. The results from these measurements can be used to estimate the magnitude of the ΔpH. The ΔpH dependency of the rate of ATP hydrolysis catalyzed by the active, reduced enzyme was measured in [16]. About 12% of the maximal rate of ATP hydrolysis was found at a ΔpH between 3.1 and 3.3. ΔpH dependency of the rate of ATP synthesis was measured also in [16,17]. A rate of 200 mM ATP/(M Chl·s), i.e. 140 ATP/(CF_0F_1·s) was observed at a ΔpH of 2.8. The ΔpH of illuminated thylakoids is decreased by about 0.3 ΔpH units due to the phosphorylation coupled proton flux in the presence of 300 µM ADP as compared to non phosphorylating conditions [18,19]. Therefore, the ΔpH under uni-site conditions (where the substrate concentration is in the nM range) is presumably about 3.1. This estimation is in good agreement with that based on the rate of ATP-hydrolysis. At this ΔpH more than 90% of the enzymes are completely protonated from the inside.

Single site ATP-hydrolysis was measured as described in [15], however, thylakoids were illuminated during the whole reaction time. Free and enzyme-bound substrates were separated by spin-x filters as described in [15] and the enzyme-bound [γ-^{32}P]ATP and $^{32}P_i$ were separated as follows:

After centrifugation the filters were weighted during illumination for determination of the amount of reaction medium on the filter. Then, the thylakoids were denatured by addition of 100 µl trichloroacetic acid (final concentration 2% (w/v)). The enzyme-bound nucleotides and P_i were released and were found quantitatively in the filtrate after an additional centrifugation for 15 s at 13000 × g. No radioactivity remained on the filter. One mM ATP was added to the filtrate in order to protect hydrolysis of labelled ATP. [γ-^{32}P]ATP and $^{32}P_i$ were separated and their concentrations were determined as described before [13]. The radioactivity resulting from the free [γ-^{32}P]ATP and $^{32}P_i$, in the reaction medium on the filter was subtracted from the total radioactivity in order to obtain the concentrations of the enzyme-bound species.

3. RESULTS

The single site ATP-hydrolysis was measured under exactly the same conditions as characterized above. Different experiments were carried out in order to measure the time course of all free and enzyme-bound substrates and products. The enzymes were brought into the active, reduced state by illumination and 15 s after the beginning of the illumination ATP was added, so that the initial free ATP concentration was always 30 nM. The illumination was continued during the reaction. The following experiments were carried out (for detailed description of the methods see [15]).

(1) Addition of cold ATP and measurement of the concentration of free ATP as a function of time with luciferin/luciferase.

(2) Addition of cold ATP and measurement of the concentration of free ADP and free ATP as a function of time.

(3) Addition of [^{14}C]ADP and measurement of the sum of enzyme-bound ADP and ATP as a function of the reaction time. Free and enzyme-bound species were separated by spin-x filters.

(4a) Addition of [γ-^{32}P]ATP and measurement of enzyme-bound P_i as a function of reaction time.

(4b) Addition of [γ-^{32}P]ATP and measurement of the sum of enzyme-bound ATP and P_i as a function of reaction time.

In both cases free and enzyme-bound species were separated by spin-x filters.

(5) Addition of [γ-^{32}P]ATP and measurement of the sum of free and enzyme-bound P_i. At reaction time t_x samples were denatured by trichloroacetic acid ('acid quench').

(6) Addition of [γ-^{32}P]ATP and measurement of the sum of free and enzyme-bound P_i. At reaction time t_x 1 mM cold ATP was added. Ten seconds after the addition of the cold ATP samples were denatured by addition of trichloroacetic acid ('cold chase').

(7) Addition of [γ-^{32}P]ATP and measurement of the sum of free and enzyme-bound P_i. At reaction time t_x 1 mM cold ADP is added. Ten seconds after addition of the cold ADP samples were denatured by addition of trichloroacetic acid.

The results of these experiments are summarized in Fig. 2. The concentration of free ATP decreases from 30 nM to 29 nM within the first 15 s and remains nearly constant up to 90 s (measurement 1). The sum of free ADP and ATP gives exactly the same results (measurement 2). This indicates that no free ADP can be detected. The concentration of enzyme-bound ATP and ADP increases from zero to about 1 nM within the first 15 s and increases then slowly to 1.5 nM during reaction time up to 90 s (measurement 3). The concentration of enzyme-bound P_i increases to about 0.8 nM within the first 15 s and decrease then slowly during reaction time (measurement 4a). The sum of enzyme-bound ATP and

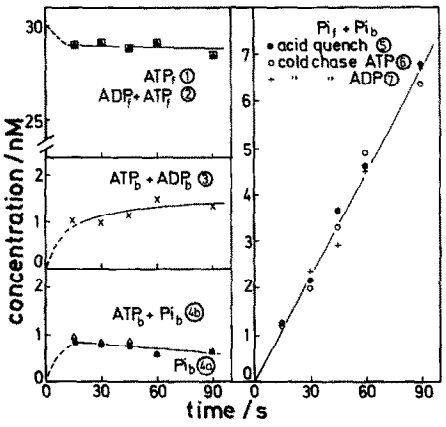

Fig. 2. Concentrations of free and enzyme-bound substrates and products as a function of reaction time. The numbers refer to the type of measurement described in the text. The indices stand for f = free and b = enzyme-bound. The concentration of chlorophyll is 9.5 μM, containing 13 nM CF_0F_1, the initial ATP concentration is 30 nM.

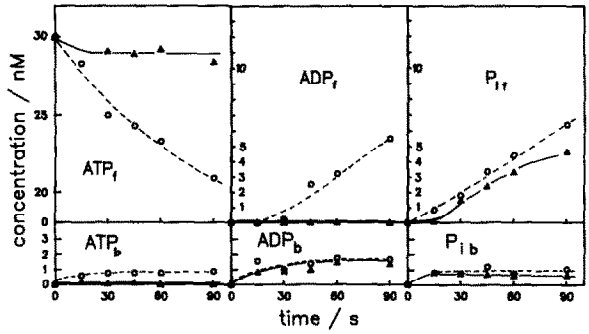

Fig. 3. Concentrations of free and enzyme-bound ATP, ADP and P_i as a function of reaction time. The data were calculated as described in the text from the results in Fig. 2 (full triangles, solid lines). For comparison results obtained under deenergized conditions are depicted (open circles, dashed lines, data from [15]).

P_i gives exactly the same result (measurement 4b). This indicates that no enzyme-bound ATP can be detected. The concentration of free and bound P_i increases to 4.7 nM during the reaction time. There is no difference in P_i concentration whether the sample is denatured (measurement 5) or whether a cold chase is carried out with ATP (measurement 6) or whether a cold chase is carried out with ADP (measurement 7). The concentration of free and enzyme-bound ATP, ADP and P_i can be calculated from these measurements:

Free nucleotides: ATP_f is measured directly in measurement (1), ADP_f = (2) − (1) and P_{if} = (5) − (4a). Enzyme-bound nucleotides: ATP_b = (4b) − (4a), ADP_b = (3) − (4b) + (4a) and P_{ib} is measured directly in measurement (4a). The indices stand for f = free and b = enzyme-bound.

The results are depicted in Fig. 3 (full triangles, solid lines). In order to show clearly the differences between ATP-hydrolysis under energized and deenergized conditions we have depicted in Fig. 3 additionally the results obtained under deenergized (uncoupled) conditions (dashed lines, open circles, data from ref. [15]). The free species are shown on the top, the enzyme-bound species are shown on the bottom (full triangles). Under energized conditions the concentration of free ATP decreases within 15 s from 30 nM to 29 nM and remains then constant. Simultaneously with the decrease of free ATP enzyme bound ADP (about 1 nM) and enzyme bound P_i (about 0.7 nM) is generated whereas no enzyme-bound ATP can be observed (detection limit about 0.1 nM). No free ADP is detected, whereas P_i is released from the enzyme after a small time lag. It should be mentioned that the P_{if} depicted in Fig. 3 top is the P_i released from the [γ-^{32}P]ATP which was bound in a previous step to the enzyme. The medium contains always 2 mM cold P_i. The high P_i concentration prevents rebinding of $^{32}P_i$ to the enzyme because of the high isotope dilution. These results lead to the following conclusions:

(1) Free [γ-^{32}P]ATP had been bound to the enzyme under energized conditions, but the concentration of enzyme-bound [γ-^{32}P]ATP was below the detection limit. Obviously, it is hydrolyzed to enzyme-bound ADP and $^{32}P_i$ and the $^{32}P_i$ is released from the enzyme.

(2) On the other hand the concentration of free ATP remains constant. Therefore, there must be a binding of cold P_i, synthesis of ATP and release of the newly synthesized (cold) ATP into the medium with the same rate as $^{32}P_i$ is released to the medium.

(3) The concentration of free ADP is below the detection limit (< 1 nM).

The differences between ATP-hydrolysis under energized and deenergized conditions are evident. The concentrations of enzyme-bound ADP and free $^{32}P_i$ are similar under both conditions. When the membrane is deenergized (uncoupled) the concentration of free ATP decreases with reaction time, enzyme-bound ATP is observed and the concentration of free ADP increases with reaction time.

Obviously, energization of the membrane leads to a change of unisite kinetics. Mechanistically, this implies that protonation of the enzyme leads to a change of the rate constants of some steps of the reaction cycle.

4. DISCUSSION

The magnitude of the following rate constants can be estimated from the data in Fig. 3.

(1) The concentration of the enzyme-bound ADP and the enzyme-bound $^{32}P_i$ are constant in the steady state (between 15–90 s). Therefore, the rate of P_i release is the same as the rate of ATP-binding, i.e.

$$\frac{d\,[^{32}P_i]}{dt} = -\frac{d\,[[\gamma\text{-}^{32}P]ATP]}{dt} = k\,[E]\,[[\gamma\text{-}^{32}P]ATP]$$

The rate of $^{32}P_i$ release is 0.08 nM s^{-1}, the concentration of free enzyme is 13 nM, the concentration of free ATP is 29 nM. It results for the rate constant k_{ATP} = $2 \cdot 10^5$ $M^{-1}s^{-1}$ with these data.

(2) The $^{32}P_i$ release is a first order reaction i.e.,

$$\frac{d[^{32}P_{if}]}{dt} = k\,[^{32}P_{i\ bound}]$$

The rate of $^{32}P_i$ release is 0.08 nM s^{-1}, the concentration of $^{32}P_{i\ bound}$ is 0.8 nM. With these data then kP_i = 0.1 s^{-1}.

(3) The release of ADP is a first order reaction:

$$\frac{d\,[ADPf]}{dt} = k_{ADP}\,[ADP_{bound}]$$

No free ADP is detected. Therefore, we can estimate only the upper limit of the rate constant. The detection limit of free ADP is 1 nM, the maximal reaction time is 90 s, i.e. the smallest detectable rate is 0.01 nM s^{-1}. The concentration of enzyme-bound ADP is 1.2 nM. It results for the rate constant of ADP release k_{ADP} < 0.01 s^{-1}. This is the upper limit for the rate constant since the true rate of ADP-release can be much smaller.

(4) The lower limit for the rate constant for ATP-release can be estimated also from these data. The concentration of free ATP remained constant between 15 and 90 s, the concentration of free $^{32}P_i$ increases indicating hydrolysis of [γ-^{32}P]ATP. Therefore, cold ATP must be synthesized on the enzyme and released into the medium with the same rate as [γ-^{32}P]ATP is hydrolyzed and $^{32}P_i$ is released.

If ([ATP] + [[γ-^{32}P]ATP]]) is constant (see Fig. 2) then

$$-\frac{d[[\gamma\text{-}^{32}P]ATP}{dt} = \frac{d\,[ATP]}{dt}$$

With $-\dfrac{d\,[[\gamma\text{-}^{32}P]ATP]}{dt} = \dfrac{d\,[^{32}P_{if}]}{dt}$ it is finally obtained

$$\frac{d[ATP]}{dt} = \frac{d\,[^{32}P_{if}]}{dt} = k\,[ATP_{bound}]$$

The rate of $^{32}P_i$ release was 0.08 nM s^{-1}. The concentration of enzyme-bound ATP can be estimated as follows. In measurement (3) (see Fig. 2) the total amount of ^{14}C-labelled nucleotides was measured to be about 1.2 nM. Because no ^{14}C-ADP was released this includes all enzyme-bound nucleotides. The concentration of enzyme-bound ^{32}Pi was determined to be about 0.8 nM and this represents only the enzyme species EADP $^{32}P_i$. Therefore at least 0.8 nM of the total amount of 1.2 nM ^{14}C nucleotides are ^{14}C-ADP. The concentration of ^{14}C-ATP can be maximally 0.4 nM. With these data the rate constant for ATP release can be estimated to be $k_{ATP\ off}$ >0.2 s^{-1}.

This is in good agreement with [20], where a rapid energy dependent dissociation of ATP from submitochondrial particles was found. Under deenergized conditions the corresponding rate constants were k_{ATP} = 10^6 $M^{-1}s^{-1}$, k_{Pi} = 0.2 s^{-1} and k_{ADP} = 0.1 s^{-1} [15]. Release of ATP was not observed under deenergized conditions. The ratio between enzyme-bound ATP (i.e., the enzyme species E-ATP) and enzyme-bound P_i (i.e., the enzyme species E-ADP-P_i) was 0.4 ('equilibrium constant').

When the membrane was energized no enzyme-bound ATP was found. This behaviour can be explained in two ways:

(1) The equilibrium is shifted to E-ADP-P_i under energized conditions, i.e. in the opposite direction as expected.

(2) The rate constant for ATP release is increased so that it is higher than that for the synthesis of E-ATP. The second explanation implies that there is no equilibrium on the enzyme under energized conditions. When the membrane is energized about 90% of the enzyme are protonated under our experimental conditions (see Materials and Methods). We assume that the calculated rate constants refer to the completely protonated enzymes. Nevertheless it is possible that the observed ATP-hydrolysis is due to the 10% of the enzyme, which are not completely protonated. In this case the rate constant of ATP release was underestimated and the rate constants for ATP binding, ADP release and P_i release were overestimated, i.e. the change of the rate constants under energized conditions would be even higher.

The comparison of the results under energized and deenergized conditions shows that the rate constants for substrate binding and product release in direction of hydrolysis are decreased under energized conditions, whereas the release of ATP is increased. The change of the rate constants supports the binding change mechanism proposed by Boyer [21], i.e. protonation/deprotonation of the enzyme leads to a change of the binding affinity substrates and products.

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