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ATP-hydrolysis in chloroplasts: uni-site catalysis and evidence for heterogeneity of catalytic sites

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ATP-hydrolysis was measured with thylakoids under uni-site conditions. The rate constant for the ATP binding is 10^6 $M^{-1} \cdot s^{-1}$, the 'equilibrium constant' of the enzyme-substrate complex, $EADPP_i/EATP$, is 0.4. The rate constants for the P_i-release and the ADP-release are 0.23 s^{-1} and 0.07 s^{-1}. This indicates that the enzyme carries out a complete turnover under uni-site conditions. The interaction of the nucleotide binding sites was investigated by first measuring the [γ-^{32}P]ATP hydrolysis under uni-site conditions. After that, 1 mM unlabeled ATP was added, so that all ATP-binding sites were occupied. The [γ-^{32}P]ATP, bound to the first site, was hydrolyzed at a rate of 0.8 ATP/(CF_0F_1 s). In a second experiment, first unlabeled ATP was hydrolyzed under uni-site conditions, and then 1 mM [γ-^{32}P]ATP was added. Under otherwise identical conditions, this allows the measurement of the rate of ATP hydrolysis catalyzed by the second (and possibly third) site. A rate of 40 ATP/(CF_0F_1 s) was found. It was concluded that there exists a heterogeneity of the ATP-hydrolyzing sites on CF_0F_1 in thylakoid membranes.

Introduction

The membrane-bound ATP-synthases of the F_0F_1-type catalyze ATP synthesis/hydrolysis coupled with a transmembrane proton transport in bacteria, mitochondria and chloroplasts. The enzymes consist of a hydrophilic part, F_1, containing the nucleotide binding sites and a hydrophobic membrane embedded part, F_0, which is assumed to act as a proton channel.

The mitochondrial F_1 can bind six nucleotides; three of the nucleotide binding sites have catalytic properties [1]. The presence of three catalytic sites raises the question of whether they act cooperatively or not [2,3]. The mechanistic investigations are easier when only one catalytic site is occupied ('single-site conditions') and only one turnover is carried out ('single turnover conditions'). Experimentally, such conditions are established when the enzyme concentration is higher than the concentration of the substrate. For MF_1, a complete set of rate constants for the uni-site reaction cycle was obtained, allowing a complete thermodynamic and kinetic description of the reaction [4,5].

The H^+-ATPase from chloroplasts, CF_0F_1, was reconstituted into asolectin liposomes and it was found that the uni-site turnover was much faster than in the soluble MF_1 [6]. Additionally, under multi-site conditions a heterogeneity of the catalytic sites was found [7]. These differences can result from the presence of the F_0 part but changes in the enzyme properties during the isolation and reconstitution procedure cannot be excluded.

Therefore, in this work we report on uni-site ATP-hydrolysis in thylakoid membranes. This is a much more complex system than the purified, reconstituted enzyme and side reactions are possible; however, the advantage is that CF_0F_1 is in the natural environment. Furthermore, the membrane can be energized by light, and the involvement of protonation/deprotonation reactions in uni-site catalysis can be investigated.

Abbreviations: CF_0F_1, H^+-translocating ATPase ('ATP-synthase') from chloroplasts; ME_1, isolated hydrophilic part of the H^+ translocating ATPase from mitochondria; Chl, chlorophyll.

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Materials and Methods

Isolation of thylakoids and determination of CF_0F_1 content. The thylakoids were isolated from spinach as described in Ref. 8. The amount of CF_0F_1 per chloro-

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phyll was determined by immune electrophoresis as described in Refs. 9 and 10. For the preparations used in this work a ratio of 730 ± 100 chlorophyll/CF_0F_1 was measured. The thylakoids were rapidly frozen and stored under liquid nitrogen in buffer 1 (10 mM Tricine-Na (pH 8.0), 100 mM sorbitol, 3 mM $MgCl_2$, 10 mM NaCl and 30% (v/v) ethylene glycol). In order to remove the ethylene glycol before each set of measurements, 500 μl of the thylakoid suspension were washed with 7 ml of buffer 2 (5 mM Tricine-Na (pH 8.0), 50 mM Sorbitol and 2 mM $MgCl_2$), and then centrifuged for 5 min at $8000 \times g$. The pellet was resuspended in 500 μl buffer 2 and could be stored on ice up to 2–3 h without loss of activity.

Determination of nucleotides. The concentration of the nucleotides (ATP and ADP) was measured by the luciferine/luciferase technique as described elsewhere [6].

The nucleotide content of the thylakoids was determined as follows: (i) Free nucleotides: 10 μl thylakoid suspension was diluted with 20 μl H_2O. 10 μl of this sample was used for the determination of $[ATP_f]$ and $[ADP_f + ATP_f]$ in two separate assays with luciferin/luciferase. The subscript f stands for free. (ii) Bound nucleotides: 10 μl thylakoid-suspension was denatured with 10 μl 0.6 M perchloric acid and, subsequently, 10 μl 1 M $KHCO_3$ was added. The denatured thylakoids were separated by centrifugation ($10000 \times g$) before 10 μl of the supernatant was used for the determination of the nucleotide content with luciferin/luciferase. The amount of bound nucleotides was determined by the difference between measurements (ii) and (i).

The concentrations of free nucleotides were $4.1 \cdot 10^{-4}$ ATP_f/Chl and $2.7 \cdot 10^{-4}$ ADP_f/Chl, the concentrations of bound nucleotides were $2.7 \cdot 10^{-3}$ ATP_b/Chl and $1.4 \cdot 10^{-3}$ ADP_b/Chl. The subscript b stands for bound. If all nucleotides are bound to CF_0F_1, the result is 2 ATP_b/CF_0F_1 and 1 ADP_b/CF_0F_1.

Reduction of CF_0F_1 in thylakoids. After washing, the thylakoids were in the oxidized, inactive state E_i^{ox}. The reduction was performed as follows: 520 μl of buffer 3 (5.67 mM Tricine-Na (pH 8.2), 8.57 mM EDTA, 2.83 mM $MgCl_2$, 2.27 mM NaH_2PO_4, 22.7 mM DTT and $2 \cdot 10^{-4}$ M benzylviologen) was added to 80 μl of the washed thylakoid suspension (final chlorophyll concentration about 400 μM) and iluminated for 3 min. After that, the enzyme was in the active, reduced state E_a^{red}. During this activation/reduction period about 1 ATP/CF_0F_1 was synthesized from ADP. After illumination, the enzyme was stored on ice in the dark for another 15 min. During this time the inactive, reduced state, E_i^{red}, was formed, and one ATP was hydrolyzed to enzyme-bound ADP. The nucleotide content was almost the same as that found for the inactive, oxidized enzyme. The enzyme can be stored on ice, for up to 3 h without loss of activity.

Reactivation of CF_0F_1 in thylakoids. The activation of inactive, reduced CF_0F_1 was carried out either by a $\Delta pH/\Delta\psi$ jump (i) or by illumination (ii).

(i) 50 μl thylakoids was incubated for 15 s with the same volume of buffer 4 (40 mM succinate-Na (pH 5.5), 2 mM $MgCl_2$, 1.2 mM KCl, 2 mM NaH_2PO_4 and freshly added 20 μM DCMU (3-(3,4-dichlorophenyl)-1,1-dimethylurea) and 5 μM valinomycin). Then 50 μl of this solution was added to 50 μl of buffer 5 (200 mM tricine-Na (pH 8.3), 2 mM $MgCl_2$, 120 mM KCl, 2 mM NaH_2PO_4 and freshly added 20 μM DCMU) inducing a $\Delta pH/\Delta\psi$ jump ($\Delta pH = 2.2$, $\Delta\psi \approx 90$ mV).

(ii) 5 μl of thylakoids was illuminated for 15 s in 100 μl of buffer 6 (one part buffer 4 and one part buffer 5, both without valinomycin and DCMU but with additionally 40 μM pyocyanin) for 15 s. The maximal rate of ATP-hydrolysis was measured after both types of energization by addition of MgATP (final concentration 1 mM) and NH_4Cl (final concentration 3 mM). In these cases when the thylakoids had been activated by light, the reaction was started directly after the end of the illumination period; in the case of thylakoids, activated by a $\Delta pH/\Delta\psi$ jump, the reaction was started 15 s after the $\Delta pH/\Delta\psi$ jump. The same rate (e.g., 90 ± 10 ATP/($CF_0F_1 \cdot s$)) was always measured for the same thylakoid preparation. Different thylakoid preparations give rates between 40–90 ATP/($CF_0F_1 \cdot s$).

Separation of free and enzyme-bound reactants. Free and enzyme-bound reactants were separated by filtration with a spin-x filter system (Tecnomara no. 8160). It consists of a 2 ml reaction vessel with an inserted filter head. The filter head contains a membrane with 0.2 μm pore width. The reaction medium containing the activated, reduced thylakoids (200 μl, 10 μM chlorophyll) and the labeled reactants (i.e., either [^{14}C]ATP or [γ-^{32}P]ATP) was added to the reaction vessel. After the reaction time t_x it was centrifuged at $13000 \times g$ for 15 s. The thylakoids with bound reactants stayed on the filter together with 3–10 μl of the reaction medium.

The weight of the filter was determined before and after centrifugation. From the difference, the remaining reaction medium was calculated. The radioactivity of the filtrate and on the filter was measured with a scintillation counter (TriCarb, Packard). The filter was put directly into 4 ml of the scintillation fluid (High Ionic Fluor, Packard). Additionally an aliquot of the filtrate was measured. Since the amount of reaction medium on the filter was known, the amount of membrane-bound radioactivity (bound to the thylakoid membranes) can be calculated. Corrections were made for quenching by chlorophyll in the different samples.

Kinetic measurements with CF_0F_1 in thylakoids. The kinetics of the reaction were measured as follows: at the end of the illumination period the reaction was started by addition of ATP (either radioactive or unlabeled) and NH_4Cl in buffer 6. The final mixture, containing 3

mM NH_4Cl as uncoupler, will be called reaction medium in the following text.

The initial nucleotide concentrations were the sum of nucleotides released by activation and added nucleotides. The resulting ADP and ATP concentrations were determined by luciferin/luciferase for every set of experiments and are called ADP^0 and ATP^0. Samples were taken from the reaction medium and subjected to different treatments to measure free and enzyme-bound reactants.

(i) The determination of the concentrations of free nucleotides: after time t_x, a sample of 100 μl of the reaction medium was immediately added to a luciferine/luciferase assay, and the free concentrations of ATP or (ATP + ADP) were determined as described above. In control experiments we separated the thylakoids by filtration (2 s) and we found the same concentrations of free nucleotides in the filtrate as without separation.

(ii) The determination of the enzyme-bound species: for these experiments the reaction medium contained either [γ-^{32}P]ATP or [^{14}C]ATP. After the reaction time, t_x, the bound and free species were separated by spin-x filters as described above. At the time t_x, the centrifuge was started.

(iii) The determination of the sum of free and enzyme-bound P_i, (acid quench): in these experiments the reaction medium cotnained [γ-^{32}P]ATP. The reaction was stopped after the reaction time, t_x, by addition of trichloroacetic acid (final concentration 2% (w/v)). Then, 1 mM of unlabeled ATP was added.

(iv) Cold chase: in these experiments the reaction medium contained [γ-^{32}P]ATP. After the reaction time, t_x, an ATP solution was added containing unlabeled ATP and $MgCl_2$ (final concentration 1 mM) in a buffer identical to the reaction medium. The reaction was stopped 10 s after the cold chase by addition of trichloroacetic acid (final concentration 2% (w/v)).

The separation of $^{32}P_i$ and [γ-^{32}P]ATP was performed as described in Ref. 6.

Results

CF_0F_1 can exist in – at least – four different states: an oxidized and reduced form, and both forms either in an inactive or active state [11–13]. The following experiments have been carried out with the active, reduced enzyme, E_a^{red}. The enzyme was brought into this state as described in Materials and Methods. Under our experimental conditions the enzyme inactivates with a half-life time of about 90 s with a first-order kinetics and without rebinding of ADP, forming the inactive, reduced state E_i^{red}. The same behavior was found for the isolated, reconstituted enzyme in Ref. 6. Therefore, the reaction time was limited to 90 s.

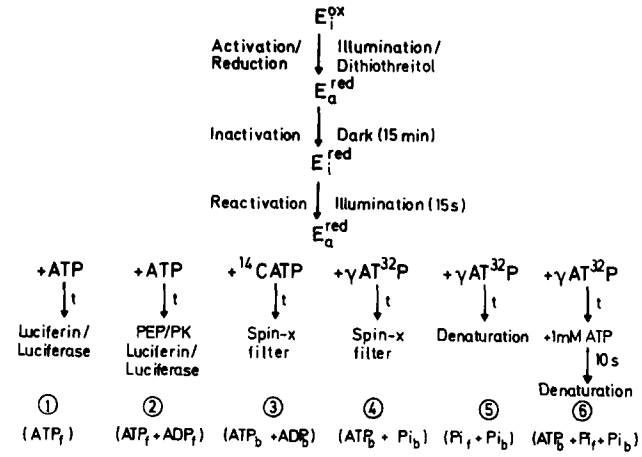

Fig. 1. Scheme of the experiments for determination of the concentrations of free and enzyme-bound species (for further explanations see text).

Fig. 1 shows the experimental protocol: CF_0F_1 was activated and reduced by illumination in the presence of dithiothreitol. The thylakoids were stored in the dark for at least 15 min, leading to an inactivation of the enzyme. Then, the enzyme was reactivated by illumination for 15 s. At the end of the illumination period the reaction was started by addition of ATP and NH_4Cl. Typical concentrations at the beginning of the experiment were: 11 μM chlorophyll containing 15 nM CF_0F_1, 3 mM NH_4Cl and 29 nM ATP (the ATP^0 resulted from 7 nM ATP added and 22 nM ATP generated during enzyme activation).

Under these conditions the different types of experiments were carried out as described in Materials and Methods:

(1) The measurement of free ATP with luciferine/luciferase.

(2) The measurement of free (ADP + ATP) with luciferine/luciferase.

(3) The measurement of enzyme-bound (ADP + ATP) with ^{14}C-labeled ATP. At the reaction time, t_x, free and enzyme-bound nucleotides are separated by spin-x filters.

(4) The measurement of enzyme-bound (ATP + P_i) with [γ-^{32}P]ATP. At the reaction time, t_x, free and enzyme-bound species were separated by spin-x filters.

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

(6) The measurement of the sum of free and enzyme-bound P_i and enzyme-bound ATP with [γ-^{32}P]ATP. At the reaction time t_x, 1 mM unlabeled ATP was added. Because of the high isotope dilution no further binding of labeled ATP takes place, but the labeled ATP already bound to the enzyme was allowed to hydrolyze for an additional 10 s. Finally, the enzyme was denatured (cold chase).

Fig. 2 shows the results of these measurements. The concentration of the different species are plotted as a function of the reaction time, t_x. The numbers indicate the type of measurement as described in Fig. 1. (1) The concentration of ATP_f decreases continuously. (2) The concentration of $(ATP_f + ADP_f)$ decreases from 29 nM to 26.5 nM. This difference can be related to the amount of enzyme-bound nucleotides (see below). (3) The concentration of $(ATP_b + ADP_b)$ is about 2.5 nM and has reached a constant level after 20 s. (4) The concentration of $(ATP_b + P_{i_b})$ is about 1 nM and has reached a constant level after 20 s. (5) The concentration of $(P_{i_f} + P_{i_b})$ increases continuously during the reaction time. (6) The concentration of $(P_{i_f} + P_{i_b} + ATP_b)$ increases continuously during the reaction time.

The concentration of free and enzyme-bound ATP, ADP and P_i are calculated from these measurements. The numbers refer to the type of measurement indicated in Fig. 1 and Fig. 2.

Free species: ATP_f was measured directly in measurement (1), $ADP_f = (2) - (1)$ and $P_{if} = (6) - (4)$; Enzyme-bound species: $ATP_b = (6) - (5)$, $ADP_b = (3) - (6) + (5)$ and $P_{ib} = (5) - (6) + (4)$. The indices stand for free (f) and enzyme-bound (b).

The results are shown in Fig. 3. The free species are depicted on the left, indicating a binding of free ATP and a release of P_i and ADP from the enzyme. Free P_i appeared with a small lag (about 5 s), free ADP could be detected after a delay of about 15 s. The enzyme-bound species are depicted on the right. Constant levels of the enzyme-bound species were established after 15 s. The concentration of enzyme-bound P_i was about 0.3 nM, that of the enzyme-bound ADP about 1.5 nM, and the concentration of enzyme-bound ATP about 0.8 nM. It should be mentioned that, under the experimental conditions, there was no rebinding of $^{32}P_i$ because the

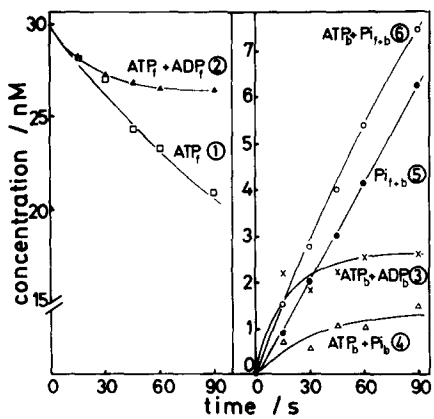

Fig. 2. Results of the different experiments described in Fig. 1. The concentration of the different species is plotted as a function of the reaction time. The indices stand for free (f) and enzyme-bound (b). The concentration of chlorophyll is 11 μM, the enzyme concentration is 15 nM and the initial ATP concentration is 29 nM.

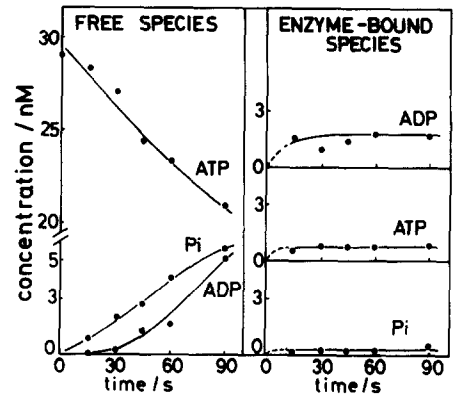

Fig. 3. Concentration of free and enzyme-bound ATP, ADP and P_i as a function of time. Data from Fig. 2; details see text.

$^{32}P_i$ was released into a medium containing 2 mM cold P_i.

The data obtained with ^{14}C-labeled ATP show that [^{14}C]ADP was released. This implies that the same adenine moiety is released that was bound to the enzyme in the previous step. This excludes that ADP from a second site (e.g., from one of the unlabeled, bound ATP) is released upon binding of ATP to the first site. The data obtained with [γ-^{32}P]ATP showed, that also the phosphate originates from the same nucleotide which was bound to the enzyme before. According to these results, the enzyme carried out a complete turnover under these conditions, regenerating the same free catalytic site at the end of the cycle.

The time resolution in these experiments is limited to reaction times longer than 15 s, because of the centrifugation through the filters. Additionally, the enzyme concentration was limited by the capacity of the filters. Therefore, we carried out 'acid quench'/'cold chase' experiments which allowed a higher time resolution and higher enzyme concentrations.

The enzyme was reactivated into the active, reduced state by a $\Delta pH/\Delta\psi$ jump and the reaction was started 15 s after the activation by addition of [γ-^{32}P]ATP and NH_4Cl. The initial concentrations were 42 μM chlorophyll containing 57 nM CF_0F_1, 3 mM NH_4Cl and 30 nM ATP. (The ATP^0 results from 14.5 nM added ATP and 15.5 nM ATP generated during the $\Delta pH/\Delta\psi$ jump).

At different reaction times (t_x = 0–20 s) the enzyme was either denatured by addition of trichloroacetic acid (acid quench, AQ)) or 1 mM unlabeled ATP was added, and 10 s later the enzyme was denatured by addition of trichloroacetic acid (cold chase, CC). In both cases, the concentration of $^{32}P_i$ was determined as a function of the reaction time. The result is shown in Fig. 4A. The concentration of $^{32}P_i$ increased with the reaction time and more $^{32}P_i$ was detected in the cold-chase samples. The isotope dilution after addition of 1 mM unlabeled ATP was so high that there was no further binding of [γ-^{32}P]ATP to the enzyme after the chase. The ad-

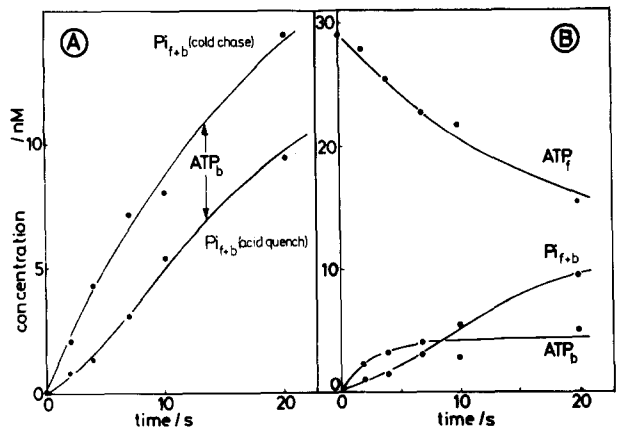

Fig. 4. Concentration of free and enzyme-bound ATP and P_i as a function of time with increasing time resolution. The concentration of chlorophyll is 42 μM, with a CF_0F_1-concentration of 57 nM; the initial concentration of ATP is 30 nM. (A) Concentration of $^{32}P_i$ as a function of the reaction time. Acid quench: the sample was denatured after the indicated reaction time. Cold chase: After the indicated reaction time 1 mM unlabeled ('cold') ATP was added and 10 s later the sample was denatured. (B) Concentrations of P_i (free plus bound), free ATP and enzyme-bound ATP resulting from measurement (A). Further details see text. The indices stand for free (f) and enzyme-bound (b).

ditional $^{32}P_i$ in the cold-chase samples must be related to hydrolysis of [γ-^{32}P]ATP, which was already bound on the enzyme before the cold chase. Therefore, the difference between cold chase and acid quench represents the concentration of enzyme-bound ATP, i.e., $[ATP_b] = [P_i(CC)] - [P_i(AQ)]$ (see Fig. 4A).

The concentration of free and bound species are calculated from these data. The concentration of $^{32}P_i$ found in the acid quench samples represents the concentration of free and bound P_i, i.e., $[P_i(AQ)] = [P_{i_f}] + [P_{i_b}]$. The concentration of P_i found in the cold chase samples additionally includes the bound ATP, i.e., $[P_i(CC)] = [P_{i_f}] + [P_{i_b}] + [ATP_b]$. All $^{32}P_i$ originates from the initial [[γ-^{32}P]ATP^0], i.e., [[γ-^{32}P]ATP^0] = $[ATP_f] + [ATP_b] + [P_{i_f}] + [P_{i_b}]$ and it results for the free ATP: $[ATP_f] = [[\gamma\text{-}^{32}P]ATP^0] - [P_i(CC)]$.

Fig. 4B shows the time course of free and enzyme-bound ATP and the sum of free and enzyme-bound P_i, calculated from the data in Fig. 4A. The concentration of enzyme-bound ATP increased and reached a constant level of 2.4 nM ATP after about 8 s. The concentration of enzyme-bound ATP is four times higher as compared to the result in Fig. 3, since the enzyme concentration is also four times higher.

From the data in Fig. 4B the 'equilibrium constant' of the enzyme substrate/product complex can be calculated: at the beginning of the reaction no $^{32}P_i$ is present. When ATP was bound to the enzyme and hydrolyzed, the species $EADPP_i$ is formed first, and then P_i and ADP are released, resulting in the species EADP as well as free enzyme, ADP and P_i. As long as there is no ADP and P_i released from the enzyme, the amount of $^{32}P_i$ in the acid quench experiments indicates only enzyme-bound $^{32}P_i$ in the enzyme species $EADPP_i$, i.e., $[P_i(AQ)] = [P_{ib}] = [EADPP_i]$. Thus, if there is an equilibrium between EATP and $EADPP_i$, the ratio of $[P_{i_b}]/[ATP_b] = [P_i(AQ)]/([P_i(CC)] - [P_i(AQ)])$ should be constant, giving the equilibrium constant $K_2 = [EADPP_i]/[EATP]$. As in later reaction steps ADP and P_i are released, the P_i in the acid quench experiment reflects the species: $[P_i(AQ)] = [EADPP_i] + [P_{i_f}]$ and then the ratio P_{i_b}/ATP_b should increase.

The data of Fig. 4 and further measurements show a constant ratio of $[P_i(AQ)]/([P_i(CC)] - [P_i(AQ)]) = 0.4$ during the first 5 s. After 5 s the ratio increases as expected. The enzyme-bound species, P_{i_b} and ATP_b, have been measured also by radioactive labeling (see Fig. 3). In this case a ratio of 0.38 is obtained. This is almost identical with the results obtained from the measurements shown in Fig. 4.

In the following experiments we investigated the kinetics on the first catalytic site when the other sites become occupied. The enzyme had been reactivated by a $\Delta pH/\Delta\psi$ jump as described in Materials and Methods and the following experiments were carried out:

In measurement (A) the rate of hydrolysis of enzyme-bound [γ-^{32}P]ATP was measured. [γ-^{32}P]ATP and NH_4Cl were added to the thylakoids after activation. The initial concentrations were 45 μM chlorophyll containing 62 nM CF_0F_1, 3 mM NH_4Cl and 21 nM ATP (8 nM added ATP and 13 nM ATP generated during activation). The enzyme was allowed to bind and to hydrolyze [γ-^{32}P]ATP for $t_x = 15$ s, and then unlabeled Mg-ATP was added (final concentration 1 mM) in a buffer identical with the reaction medium. The isotope dilution after the addition of 1 mM unlabeled ATP was so high that there was no more binding of [γ-^{32}P]ATP to the enzyme. Furthermore, all other nucleotide binding sites were filled with unlabeled ATP. At different times after addition of the unlabeled ATP ($t_y = 0.5$–15 s), the enzyme was denatured and the amount of $^{32}P_i$ was determined. For comparison, also acid quench experiments were carried out as described in Fig. 4.

Fig. 5 (A) shows the result: the dashed line indicates the formation of $^{32}P_i$ if only [γ-^{32}P]ATP was bound to the enzyme at the first site. This was identical with the 'acid quench' curve in Fig. 4A. A rate of 0.4 nM/s resulted. At the time $t_y = 0$ unlabeled ATP was added (arrow). Now, bound [γ-^{32}P]ATP was hydrolyzed with an initial rate of 1.8 nM/s. The formation of $^{32}P_i$ was due only to enzymes which have already bound [γ-^{32}P]ATP at the time of the addition of unlabeled ATP, i.e., [γ-^{32}P]ATP bound to the first site hydrolyzes at this rate, if all other nucleotide binding sites are occupied by unlabeled ATP.

In order to obtain the rate constant for ATP hydrolysis, the rate must be divided by the concentration of

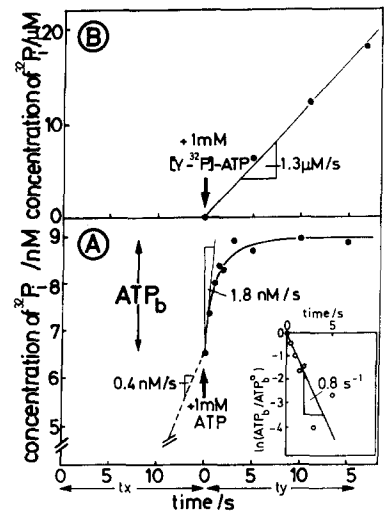

Fig. 5. Uni-site and multi-site ATP hydrolysis on thylakoid membranes: concentration of $^{32}P_i$ as a function of the reaction time. The concentration of chlorophyll is 45 μM, containing 62 nM CF_0F_1. The initial concentration of ATP is 21 nM. (A) Hydrolysis of [γ-^{32}P]ATP on the first site was measured as a function of time (dashed line). At the reaction time $t_x = 15$ s 1 mM unlabeled ATP is added as indicated by an arrow. Hydrolysis of [γ-^{32}P]ATP bound on the first site is measured for a further reaction time $t_y = 0.5$ to 15 s before denaturation. Inset: first-order plot of the data. (B) The enzyme is allowed to hydrolyze 21 nM unlabeled ATP on the first site for $t_x = 15$ s and then 1 mM [γ-^{32}P]ATP is added as indicated by an arrow. Hydrolysis of [γ-^{32}P]ATP is measured for reaction time $t_y = 5$ to 15 s before denaturation (details see text).

enzyme-bound ATP, which was 2.35 nM at $t_x = 15$ s (see Fig. 5). A rate constant of 0.76 s^{-1} resulted. Since the complete reaction cycle cannot be faster than its slowest step, this is the maximal rate of the first site when all other sites are occupied by ATP. Fig. 5 (A, inset) shows a first order plot of the data from Fig. 5 (A). An exponential decay of the concentration of enzyme-bound ATP showed again a rate constant of 0.8 s^{-1}. Since the curve extrapolated to zero, all enzyme-bound ATP was hydrolyzed with the same rate constant, excluding a rapid initial phase.

In experiment (B) the rate of ATP hydrolysis catalyzed by the other sites was measured under the same conditions as in experiment (A): the enzyme was incubated with unlabeled ATP (21 nM) for $t_x = 15$ s. During this time cold ATP was bound to the first site and was hydrolyzed. Then 1 mM of Mg-[γ-^{32}P]ATP was added ($t_y = 0$) and the enzyme was allowed to hydrolyze the labeled ATP. After reaction time $t_y = 5$–15 s, the enzyme was denatured and the amount of $^{32}P_i$ was determined.

Fig. 5 (B) shows the result: The rate of ATP-hydrolysis was 1.3 μM/s. Based on the concentration of CF_0F_1 a rate of 40 ATP/(CF_0F_1 s) was obtained.

A comparison of the experiments A and B indicates that during the first 2 s after addition of 1 mM ATP only about 50% of the ATP bound to the first site, is hydrolyzed, whereas the other sites of the enzyme perform 80 turnovers in the same time.

Discussion

Reaction scheme and calculation of the rate constants

The catalytic reaction at one site can be described in a simplified scheme (E, enzyme):

$$E + ATP \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} EATP \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} EADPP_i \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} EADP + P_i \underset{k_{-4}}{\overset{k_4}{\rightleftharpoons}} E + ADP + P_i$$

Based on this scheme some rate constants and the equilibrium constant, K_2, can be calculated from the data shown in Fig. 3 and Fig. 4: the rate constant of ATP-binding can be estimated from the initial rate of ATP binding, i.e., $d[ATP]/dt = k_1[ATP][CF_0F_1]$. With the known initial concentrations, $[ATP]^0$ and $[CF_0F_1]^0$, k_1 can be calculated. In the time range between 0 to 10 s, the release of the products and the inactivation of the enzyme can be neglected. Therefore k_1 can be determined also from a second order plot of the data.

In the time range above 10 s, free enzyme is regenerated after the reaction cycle, i.e., the concentration of free enzyme remains almost constant. In this case a pseudo-first-order reaction is expected. However, the concentration of the reduced, active enzyme is decreased by the inactivation step: $E_a^{red} \rightarrow E_i^{red}$. If the data are corrected for inactivation, the ATP binding follows pseudo-first-order kinetics and the rate constant can be calculated also from such a plot. The average from eleven measurements is $k_1 = (1.2 \pm 0.4) \cdot 10^6\ M^{-1} \cdot s^{-1}$.

The release of ADP and P_i are first order reactions, i.e., $d[ADP_f]/dt = k_4[ADP_b]$ and $d[P_{i_f}]/dt = k_3[P_{i_b}]$. The rate constants of ADP and P_i release can be calculated from the rate of ADP and P_i release at reaction times between 10 to 30 s. In this time range the concentration of enzyme-bound species was determined (see Fig. 3) and the inactivation of the enzyme could be neglected. The rate of ADP release was 0.1 $nM \cdot s^{-1}$

TABLE I

Rate constants for uni-site catalysis measured on purified, reconstituted CF_0F_1 (data from Refs. 6 and 7) and on thylakoids

Rate constants	CF_0F_1	Thylakoids
ATP binding k_1 ($M^{-1}\ s^{-1}$)	$1.1 \cdot 10^6$ [a]	$1.2 \cdot 10^6$
Equilibrium constant $K_2 = k_2/k_{-2}$	0.4	0.4
ADP release k_4 (s^{-1})	0.08	0.07
P_i release k_3 (s^{-1})	0.18	0.23
P_i formation on one site under multi-site conditions k (s^{-1})	0.5	0.8
Maximal rate of ATP-hydrolysis under multi-site conditions v_{max} (s^{-1})	40–100 [a]	40–90

[a] Calculated for the reconstituted CF_0F_1 on the basis of the concentration of active enzymes, measured on the basis of nucleotide release (see Ref. 6).

and the rate of P_i release was 0.07 nM · s^{-1}. The concentration of enzyme-bound ADP was 1.5 nM and of enzyme-bound P_i 0.3 nM. Rate constants of $k_4 = 0.07$ s^{-1} for ADP release and of $k_3 = 0.23$ s^{-1} for P_i release were obtained.

The 'equilibrium constant' of the catalytic reaction on the enzyme was calculated from the results shown in Figs. 3 and 4. In both cases $K_2 = 0.4 \pm 0.02$ was obtained in accordance with the results found with the reconstituted enzyme.

Table I shows these results together with the corresponding rate constants obtained with the isolated, reconstituted enzyme [6]. The rate constants obtained in both systems are nearly identical *. These results indicate that CF_0F_1 in the thylakoid membrane catalyzes ATP-hydrolysis under uni-site conditions. The rate constants for the individual steps of the reaction cycle are virtually the same as obtained with the isolated, reconstituted CF_0F_1.

Influence of enzyme-bound nucleotides on catalysis

Uni site catalysis on thylakoids is measured under conditions, where the enzyme contains at least 2 ATP/CF_0F_1. The reconstituted enzyme contains only 1 ATP/CF_0F_1 after activation. Therefore, we have to conclude that the presence of one or two ATP_b/CF_0F_1 does not influence the kinetics of ATP hydrolysis. The experiments with [^{14}C]ATP and [γ-^{32}P]ATP have shown that these bound ATP molecules do not participate in the reaction cycle neither on CF_0F_1 in thylakoids nor on the reconstituted CF_0F_1. Therefore, we must conclude that they are bound on non-catalytic sites. It should be mentioned that single-site catalysis on isolated MF_1 occurs in the presence of at least three nucleotides bound at non-catalytic sites [4].

Heterogeneity of catalytic sites

The next question is what happens with the ATP bound on the catalytic site characterized above, when the other catalytic nucleotide binding sites will become occupied.

This was investigated in the experiment shown in Fig. 5. [γ-^{32}P]ATP bound at this site is hydrolyzed with a rate of 0.4 nM/s. Addition of 1 mM unlabeled ATP, i.e., occupation of all nucleotide binding sites, increases the rate to 1.8 nM/s. This demonstrates that there is positive cooperativity between different ATP-hydrolyzing sites. The maximal rate observed at this site when all other sites are occupied is 0.8 s^{-1}.

If unlabeled ATP is first bound to this site and then ATP hydrolysis by the other sites is measured with 1 mM [γ-^{32}P]ATP, a rate of 40 s^{-1} results. With different thylakoid preparations rates between 40 and 90 s^{-1} are observed. This implies that within 2 s after addition of 1 mM ATP only about 50% of the ATP bound to the first site is hydrolyzed, whereas the other sites of the enzyme have performed more than 80 turnovers in the same site.

It might be argued that the heterogeneity of catalytic sites shown in Fig. 5 does not represent a heterogeneity of catalytic sites on one enzyme but a heterogeneity of different enzymes. This would imply that there is a fraction of 'sluggish' enzymes which bind and hydrolyze ATP slowly and a fraction of active enzymes, hydrolyzing ATP fast. In this case the amount of enzyme-bound ATP and P_i mainly reflects the 'sluggish' enzymes. (Since the reaction of the other enzymes is fast, they contain almost no bound intermediates.) The P_i release is mainly due to the fast enzymes. This can be excluded, because the initial rate of the decrease in free ATP is equal to the initial rate of the increase in enzyme-bound ATP (see Figs. 3 and 4).

The other possibility is that the binding and hydrolysis of ATP transforms the enzymes into 'sluggish' enzymes, whereas the other ones remain active, i.e., the presence of ADP (or MgADP) at the catalytic site leads to an inactivation. Also, this is not the case, since the ADP is released from the enzyme (see Fig. 3). Furthermore, there is no inactivation at the time between activation and the start of the reaction. This can be calculated from the half life time of inactivation under the experimental conditions (90 s, see also Ref. 12). Furthermore, the rate of ATP-hydrolysis started at the end of the illumination period is identical with that 15 s after the activation with a $\Delta pH/\Delta\psi$ jump (see Materials and Methods). This shows that there is no significant inactivation during the first 15 s and that activation of the enzyme is identical in both cases.

Lastly, we have to discuss the possibility that all catalytic sites are identical, however, the first turnover at one site is different from the following ones. The data in Fig. 5 show that the first turnover has a maximal rate of 0.8 s^{-1}, when all other sites are occupied by ATP. Thus, we expect a lag of about 1–2 s in ATP hydrolysis when ATP is added to thylakoid membranes, where the enzyme is in the state E_a^{red} with no bound nucleotides at catalytic sites. The rate of ATP-hydrolysis has been measured under these conditions with high time resolution [12]. No lag could be detected (shortest reaction time 100 ms). Therefore, we conclude that the observed heterogeneity represents a heterogeneity of catalytic sites on the enzyme.

Comparison with literature data

In Table I the rate constants for uni-site catalysis measured with purified, reconstituted CF_0F_1 are compared with those from thylakoids. These rate constants

* It should be mentioned that the amount of active enzymes was determined from the nucleotide release during the activating $\Delta pH/\Delta\psi$ jump for the reconstituted CF_0F_1 [6]. For the thylakoids we assume that all CF_0F_1 molecules are active and, therefore, the total concentration of CF_0F_1 was used for determination of k_1.

are almost identical. This leads to the following conclusions: (i) single site, single turnover ATP-hydrolysis can be measured with thylakoid membranes. There is no interference with other ATP-dependent enzymes, e.g. adenylate kinase. (ii) Other enzymes or special lipids are not necessary for the catalytic process on CF_0F_1.

Also, under multi-site conditions the rate of ATP-hydrolysis at the first site and at the other sites are nearly the same in thylakoids and in reconstituted CF_0F_1. Therefore, we conclude: (i) the heterogeneity of catalytic sites of CF_0F_1 exists in CF_0F_1 reconstituted into liposomes as well as in CF_0F_1 integrated in the thylakoid membrane, i.e., this asymmetry is not induced by the isolation and reconstitution procedure. (ii) Neither the rate constants of single-site catalysis nor the heterogeneity of the catalytic sites is influenced by the nucleotide content on non-catalytic sites (2 ATP/CF_0F_1 in thylakoids, 1 ATP/CF_0F_1 in reconstituted CF_0F_1).

Evidence for the existence of two different types of catalytic sites came also from measurements with thylakoids in Ref. 14 and from results obtained on CF_1 [15].

Single-site ATP hydrolysis has been measured with isolated F_1 from different sources [16–19]. Most data are obtained with the isolated mitochondrial F_1. In this case the ATP on the first site is hydrolyzed very slowly under uni-site conditions. The reaction is limited by the slow release of ADP with a rate constant of 10^{-3} to 10^{-4} s^{-1} [4,20,23]. Even more pronounced is the difference in cooperativity: ATP, bound on the first site is hydrolyzed with the maximal turnover when the other sites are filled with ATP [5,20,22]. Similar results have been reported for membrane-bound MF_1 in submitochondria [21]. However, other groups have reported [24,25] that under appropriate conditions the uni-site turnover is about a factor 100 higher than found in Ref. 4 and is, therefore, in the same order of magnitude as found on the reconstituted CF_0F_1 in Ref. 6 and in thylakoids in this work. Furthermore, a heterogeneity of catalytic sites has been proposed also for MF_1 [26].

The results reported in this work show that single site kinetics can be measured successfully with thylakoid membranes. Since thylakoids can be energized by light this opens now the possibility to investigate the influence of protonation/deprotonation reactions (membrane energization) on single-site kinetics.

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