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Transport protons do not participate in ATP synthesis/hydrolysis at the nucleotide binding site of the H^+-ATPase from chloroplasts

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The H^+-ATPase from chloroplasts, CF_0F_1, was brought into the active, reduced state by illumination of thylakoids in the presence of thioredoxin and dithiothreitol. Uni-site ATP synthesis was initiated by the addition of 20 nM [α-^{32}P]ADP, and enzyme-bound and free nucleotides were separated by a pressure column. The ratio of enzyme-bound ADP to ATP was 0.55 ± 0.05. In a second experiment, uni-site ATP hydrolysis under energized conditions was initiated by the addition of 36 nM [α-^{32}P]ATP; enzyme-bound and free nucleotides were separated by a pressure column. Both procedures were carried out under continuous illumination. The ratio of enzyme-bound ADP to ATP was 0.46 ± 0.04. In a third experiment, uni-site ATP hydrolysis under de-energized conditions was initiated by the addition of 39 nM [α-^{32}P]ATP and NH_4Cl/valinomycin in the absence of illumination. Free and enzyme-bound nucleotides were separated also by a pressure column. The ratio of enzyme-bound ADP to ATP was 0.43 ± 0.02. This ratio was always the same irrespective of whether the reaction runs in the synthesis or the hydrolysis direction. Furthermore, the ratio does not depend on the membrane energization. We conclude, therefore, that the protons are not directly involved in the reaction at the catalytic site.

Chloroplast; H^+-ATPase, Enzyme kinetics; Binding change mechanism

1. INTRODUCTION

The H^+-ATPase from chloroplasts catalyzes proton transport coupled ATP-synthesis and ATP-hydrolysis [1,2]. The enzyme is a F-type ATPase [3,4], i.e. it has a hydrophobic membrane integrated part, F_0, involved in transmembrane proton transport and a hydrophilic part, F_1, which contains the nucleotide binding sites. Both parts are connected by a narrow stalk. The mechanism of the coupling between the proton translocation through the enzyme and the chemical reaction is not yet known. Currently, two hypotheses are discussed: (i) The protons translocated through the enzyme are involved directly in the chemical reaction ('direct coupling' [5]). In this case protons from the internal aqueous phase can reach the catalytic site leading to a three-fold protonation of the bound phosphate. A nucleophilic attack of the bound ADP (which is not protonated at the catalytic site) leads via a pentavalent transition state of the phosphorous atom to the elimination of water and thereby bound ATP is formed. This mechanism predicts a steric inversion at the phosphorous atom during the reaction and this has been observed [6,7]. (ii) The protons translocated through the F_0-part lead to conformational changes which are transmitted to the catalytic site in the F_1-part and this leads to a change of the binding affinities of the nucleotides ('indirect coupling' [8,9]). In a simplified way this mechanism works as follows: ADP and phosphate are bound to an open binding site. This is followed by a conformational change leading to a closing of the binding site, so that nucleotides and phosphate cannot exchange with the medium. Under these conditions ATP is formed spontaneously and it remains tightly bound at the catalytic site. Protonation of the enzyme from the inside gives rise to a conformational change, the catalytic site is opened again and ATP is released. The existence of an equilibrium between tightly bound ADP and ATP has been demonstrated by ^{18}O-exchange measurements [10,11].

In this work, we wanted to distinguish between both types of coupling. One consequence of the direct coupling mechanism is that the ratio between bound ADP and bound ATP must depend strongly on the membrane energisation: increasing the internal proton concentration will directly lead to an increase of bound protonated phosphate and consequently to an increase of bound ATP. Therefore, we measured the bound ADP and bound ATP under energized and de-energized conditions. In order to avoid complication which might arise from the cooperativity between different nucleotide binding sites, all measurements were carried out under uni-site conditions.

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2. MATERIAL AND METHODS

2.1. *Isolation and reduction of CF_oF_1 in thylakoids*

Thylakoid membranes were prepared as described earlier and stored under liquid nitrogen [12]. The chlorophyll-to-CF_oF_1 ratio was 730 ± 100 as determined by immunoelectrophoresis [13,14] The refrigeration medium was removed and the enzyme was reduced in the presence of thioredoxin, pyocyanin and dithiothreitol under continuous illumination as described earlier [15] After this treatment the thylakoid suspension contained about 300 μM chlorophyll, 3 μM thioredoxin, 20 μM pyocyanin, 1 mM dithiothreitol, 5 mM Na-tricine, 0.5 mM EDTA and 2.5 mM $MgCl_2$ (buffer 1). The thylakoids were stored on ice in the dark between 15 min up to 4 h The inactive, reduced enzyme contained two bound ATP/CF_oF_1 and one bound ADP/CF_oF_1 In order to reactivate the enzyme 42 μl of the thylakoid suspension was added to 182 μl buffer 2 (100 mM Na-Tricine, 60 mM KOH, 20 mM succinate, 2 mM $MgCl_2$, 2 mM NaH_2PO_4, titrated to pH 8.05 with NaOH, freshly added 43 μM pyocyanin) and illuminated for 15–30 s with saturating white light. ATP synthesis or ATP hydrolysis was then started by addition of the corresponding substrate.

2.2. *Preparation of [α-^{32}P]ADP*

Highly labelled [α-^{32}P]ADP was prepared as follows. Twenty microliters [α-^{32}P]ATP (PB. 10200, Amersham Buchler) was mixed with 1 μl each of 100 mM $MgCl_2$, 20 mM D-glucose, 10 mM EDTA and 1 g/l hexokinase. After 60 min at room temperature, ATP hydrolysis was complete and the hexokinase was denatured by adding 2.2 μl trichloroacetic acid (24%, w/v). The solution was neutralized with 1 N NaOH, diluted with water up to 300 μl and stored at −20°C. Product and educt were analyzed for ATP, AMP, ADP and P_i. The concentration of ADP and ATP was determined with luciferin/luciferase [16]. The separation of $^{32}P_i$ and [α-^{32}P]ATP was performed by extracting inorganic phosphate as phosphoammonium molybdate complex (see [16]). [α-^{32}P]ATP, [α-^{32}P]ADP and [α-^{32}P]AMP were separated by thin-layer chromatography as described below. It resulted in [α-^{32}P]ATP: 120 TBq/mmol, 393 MBq/ml, 3.2 μM (97.5%), 53 nM ADP (1.6%), 27 nM AMP (1%); P_i-background: 1.4 MBq/ml (0.4%). The product [α-^{32}P]ADP had a specific activity of 114.2 TBq/mmol, a total radioactivity of 25 MBq/ml, 218 nM ADP (97.3%), 2 nM ATP (1%), 3.7 nM AMP (1.7%). The use of these highly labelled nucleotides increased the detection limit considerably because the specific activity was improved by a factor of 5,700 compared to [U-^{14}C]ADP used earlier [15].

2.3 *Separation of labelled [α-^{32}P]nucleotides*

The samples were mixed with cold ADP, AMP and ATP (final concentrations 1 mM in each case) in order to localize the spots under a UV lamp (λ = 254 nm) after running the thin-layer chromatography. Between 20 and 100 μl of the neutralized samples were applied on a polyethyleneimine-coated cellulose plate (Polygram CEL 300, PEI/UV_{254}, Macherey-Nagel). For the thin-layer chromatography 1.2 M LiCl was used as eluant. The spots were cut out and directly counted in 4 ml scintillation fluid (Hionic Fluor, Canberra-Packard, Counter: TriCarb 1600 CA, Canberra Packard) 10–100 μl of the samples were directly counted in a scintillation counter giving the total amount of adeninnucleotides (AdN). Corrections were made for quenching by solid particles. A control experiment with D-[6-^{3}H]glucose (see below) indicated that glucose was running with the solvent front.

2.4 *Separation of free and enzyme-bound reactants*

Free reactants were separated from reactants bound to thylakoid membrane by a modification of centrifugation columns introduced by Penefsky [17] A syringe (1 ml) was closed by a cylindrical plate (diameter 5 mm, pore width 35 μm, polyethylene 6900, Reichelt Chemie) and the tip of the syringe was cut to diminish the dead volume. 1.5 g Sephadex-G 50-fine (Pharmacia) was swollen in 60 ml water for 24 h at room temperature. Then, 60 ml double concentrated buffer 2 was added and the gel suspension was filled into the syringe 20 μM pyocyanin was added when the separation was carried out during continuous illumination. The columns were centrifuged for 5 min at 4,600 × g. They were then filled after centrifugation exactly up to 0.35 ml. Filling and centrifugation was repeated twice, so that the columns were filled up to 0.7 ml after the second and 0.85 ml after the third centrifugation. Usually 310 μl of suspension was put on the column and the thylakoids were pressed through by pushing the piston to the 1 ml mark (starting point of separation) The separation process was stopped 45 s later. The volume of the eluate was determined by weighing (usually between 120 and 200 μl). Partial adsorption of thylakoids onto the gel particles leads to a loss of chlorophyll in the eluate. The chlorophyll concentration was measured for each sample by mixing 20 μl eluate with 80 μl 100% aceton using ultramicrocuvettes (No. 105.202-QS, Hellma) The chlorophyll concentration in the eluate was usually between 25 and 40% of the initial concentration.

The separation factor was determined as follows. D-[6-^{3}H]glucose (50 kBq) was added to the thylakoid suspension. The glucose concentrations before and after the column were measured The ratio between these was about 1,500 and this represents the separation factor The same separation factor was measured when cold ATP (1 mM) was added to buffer 2. The concentrations of free AMP, ADP and ATP after the column were calculated from their known concentrations before the column and the separation factor. This factor was determined in each experiment. The enzyme-bound nucleotides were calculated from the total nucleotides by subtracting the free nucleotides. A quench correction for ^{3}H was carried out and also the overlapping of the ^{32}P and ^{3}H spectra was corrected. When enzyme-bound nucleotides were determined under energized conditions, the columns were illuminated from three sides and the top by saturating white light. The rate of ATP synthesis was measured (buffer 2 + 300 μM ADP) before and after the column A decrease of the rate by a factor of 2 was found (from 110 ± 20 mM ATP/(MChl.s) to (50 ± 5) mM ATP/(MChl.s). The same decrease was found independently whether the separation was performed in the dark or during illumination.

2.5. *Kinetic measurements with CF_oF_1 from thylakoids*

For all experiments the enzyme was brought into the active, reduced state as described above. After 15 s illumination the reaction was started (reaction time t_r = 0) by addition of 176 μl thylakoids in buffer 2 to 165 μl of buffer 2 containing additional [α-^{32}P]ATP or [α-^{32}P]ADP (in each case 50 kBq) and D-[6-^{3}H]glucose (50 kBq, sp. act.: 932.4 GBq/mmol, Amersham). The resulting mixture is referred to as reaction medium in the following text. From this solution an aliquot of 310 μl was put on the column at the reaction time t_x during continuous illumination. The free and enzyme-bound species were separated with the pressure column. The reaction time t_r was the time when the piston of the syringe stopped at the 1 ml mark, i.e. the time when the separation was started. The eluate dropped directly into 20 μl 24% trichloroacetic acid. After 45 s the separation was finished. The labelled nucleotides [α-^{32}P]AMP, [α-^{32}P]ADP and [α-^{32}P]ATP were separated by thin-layer chromatography. When ATP hydrolysis under de-energized conditions was measured, the preillumination time was 30 s. After this time the light was switched off and the reaction was started by addition of 165 μl buffer 2 containing additionally [α-^{32}P]ATP, 6 mM NH_4Cl and 3 μM valinomycin.

3. RESULTS

CF_oF_1 was brought in the active, reduced state by continuous illumination in the presence of thioredoxin, pyocyanin and dithiothreitol (see Section 2). The thylakoids were illuminated for 15 s and ATP-synthesis was started by adding [α-^{32}P]ADP (initial concentrations: $[ADP]_0$ = 20 nM; $[CF_oF_1]_o$ = 40 nM and 20 nM cold ATP synthesized during preillumination). Five seconds before the reaction time t_x the suspension was put on the column and enzyme-bound and free nucleotides were separated in the pressure column as described in

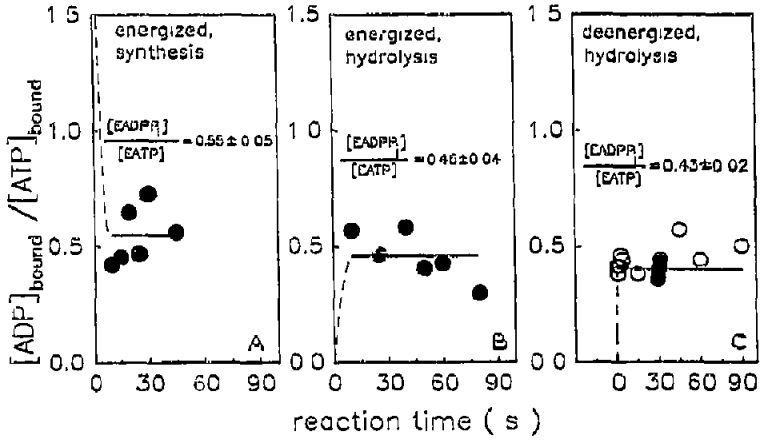

Fig. 1. Ratio between enzyme-bound ADP and enzyme-bound ATP. The H^+-ATPase was brought into the active, reduced state. Radioactive nucleotides were added and after different reaction times free and enzyme-bound nucleotides were separated using a pressure column. The eluate was denatured by trichloroacetic acid and analyzed by thin-layer chromatography for the bound nucleotides. (A) Energized conditions: ATP-synthesis was initiated by the addition of 20 nM [α-^{32}P]ADP. The thylakoids were illuminated during the reaction and during the separation on the column (B) Energized conditions: ATP-hydrolysis was initiated by the addition of 36 nM [α-^{32}P]ATP. The thylakoids were illuminated during the reaction and during the separation on the column (C) De-energized conditions. ATP-hydrolysis was initiated by the addition of 39 nM [α-^{32}P]ATP, 6 mM NH_4Cl and 3 μM valinomycin. Then, the light was switched off (open circles, see [18]).

Section 2. The reaction time t_x is the time between addition of ADP and the start of the separation. During the whole procedure the thylakoids were illuminated. The eluate dropped directly into 24% TCA and was analyzed by thin-layer chromatography for enzyme-bound AMP, ADP and ATP as described in Section 2.

In Fig. 1A the ratio between enzyme-bound ADP and ATP was plotted against the reaction time t_x. The ratio remains constant up to 50 s after an initial phase which is not resolved (dashed line). The ratio between enzyme-bound ADP and enzyme-bound ATP was 0.55 ± 0.05.

In a second experiment, the ratio between enzyme-bound ADP and ATP was measured when ATP was hydrolyzed under energized conditions. In this case, [α-^{32}P]ATP was added after 15 s preillumination (initial concentrations: $[ATP]_0$ = 36 nM including the ATP synthesized during preillumination, $[CF_oF_1]_o$ = 32 nM). After different reaction times t_x the separation of free and bound nucleotides was started as described above. The concentration of the bound nucleotides was determined by thin-layer chromatography as described above. In Fig. 1B the ratio between enzyme-bound ADP and enzyme-bound ATP is shown. The ratio is constant after an initial fast phase which is not resolved here (dashed line). The ratio is 0.46 ± 0.04.

In a third experiment, the ratio between enzyme-bound ADP and ATP was measured when ATP was hydrolyzed under de-energized conditions. In this case, [α-^{32}P]ATP together with 6 mM NH_4Cl and 3 μM valinomycin was added after 30 s preillumination (initial concentrations: $[ATP]_0$ = 39 nM including the ATP synthesized during preillumination), $[CF_oF_1]_o$ = 34 nM). ATP was hydrolyzed for 30 s, then free and enzyme-bound nucleotides were separated and the enzyme-bound nucleotides were analyzed by thin-layer chromatography. The ratio between the enzyme-bound ADP and enzyme-bound ATP is shown in Fig. 1C (filled circles). This ratio was measured earlier using Spin-X filters for the separation of bound and free substrates (t_x = 15–90 s), and it was also determined by acid quench/cold chase experiments (t_x = 0.5–5 s) [18]. These data are depicted by open circles in Fig. 1C. The ratio 0.43 ± 0.02 is constant after an initial fast phase which is not resolved here (dashed line). All three methods give the same result which is an indication of the equivalence of these three methods.

4. DISCUSSION

When protons participate directly in the reaction at the catalytic site the equilibrium constant at this site is given by:

$$E\ ATP + nH^+_{out} \rightleftarrows E\ ADP\ P_i + nH^+_{in}$$

$$K = \frac{[E\ ADP\ Pi]\ [H^+_{in}]^n}{[E\ ATP]\ [H^+_{out}]^n}$$

From de-energized (ΔpH = 0) to energized (ΔpH = 3) conditions the ratio $(H_{in}/H_{out})^n$ changes from 1 to $(10^3)^n$, i.e. for n = 3 by a factor 10^9. Correspondingly, the ratio [E ADP P_i]/[E ATP] should change by 10^{-9} in order to maintain the same equilibrium constant. The data in Fig. 1 show that the ratio $[ADP]_{bound}/[ATP]_{bound}$ does not change. In the synthesis direction the enzyme is always saturated with P_i, i.e. [EADP] $\ll$ [EADP P_i]. In the hydrolysis direction (de-energized) the same result [EADP P_i]/[EATP] was obtained when [α-^{32}P]ATP (full circles, Fig. 1) and when [γ-^{32}P]ATP was used (ref. [18], open circles, Fig. 1). This implies that [EADP] and [EP$_i$]

are small compared to [EADP P_i]. Therefore, $[ADP]_{bound}$ represents nearly exclusively [EADP P_i].

We conclude, therefore, that the transported protons do not participate directly in the reaction at the catalytic nucleotide binding site. This is in accordance with the proton-induced binding change mechanism proposed by Boyer [8,9] where bound ATP is formed spontaneously on the enzyme and the energy derived from the transmembrane electrochemical potential difference of protons is used for dissociation and binding of substrates and products.

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