Correlation between the ATP Synthetic Active State and the ATP Hydrolytic Active State in Chloroplast ATP Synthase-ATPase Complex CFo·CF1*

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The proton-translocating ATP synthase-ATPase of chloroplasts (CFo·CF1) is composed of a membrane-bound proton-translocating complex (CF1) and a membrane-extrinsic catalytic complex (CFo) (1). CF1, well characterized and is composed of five subunits, α, β, γ, δ, and ε, in order of decreasing molecular weight with a stoichiometry of αβγδε (2, 3). The CFo complex is less well characterized and is believed to be composed of three subunits, I, II, and III (4, 5). The active site of the enzyme resides on the β subunits or on the interface between α and β subunits (1, 6). The γ subunit has been suggested to play a role in the gating of protons (7–10) and also in the activation of CF1 (11–13). The γ subunit of CF1 contains one disulfide bond and two thiol (12–14). One thiol group reacts readily with alkylating reagents and appears to be unessential for either ATP synthesis or ATP hydrolysis activities (15). The other thiol group reacts with alkylating reagents only when the thylakoids are energized. Alkylation of this group inhibits both ATP synthesis and ATP hydrolysis (15–17). Reduction of the disulfide bond of the γ subunit by dithiothreitol activates soluble CF1 to catalyze ATP hydrolysis (11, 12, 18) and stimulates ATP synthesis of CFo·CF1 in thylakoids (13, 19–22). The ε subunit has been proposed to be an ATPase inhibitor (23). The removal of ε subunit from CF1 results in a permanent activation of the Ca2+-ATPase and the recombinant of the ε subunit-deficient CF1 with the isolated ε subunit, returns CF1 to the latent Ca2+-ATPase (24). The γ and ε subunits have been suggested to interact closely (25).

CFo·CF1 in thylakoids exhibits its ability to hydrolize ATP only after activation by illumination in the presence or absence of a thiol compound (26, 27) or by acid-base transition in the presence of a thiol compound (28). Addition of both dithiothreitol and P, in the postillumination dark also activates CFo·CF1 to hydrolyze ATP (29). ATP hydrolysis activated by illumination in the presence of dithiothreitol, by illumination in the absence of a thiol compound, or by addition of dithiothreitol and P, in the postillumination dark, is relatively insensitive to pH and sensitive to stimulation by an uncoupler (NH4Cl), relatively sensitive to pH, and highly sensitive to pH and insensitive to stimulation by NH4Cl, respectively (27, 29). The pH dependence of the light/dithiothreitol-activated ATP hydrolysis approaches that of the dithiothreitol/P, -activated ATP hydrolysis in the dark. The pH dependence of the ATP hydrolysis activated by illumination in the absence of a thiol compound is quite different from that of the dithiothreitol/P, -activated ATP hydrolysis. From these results, three distinct active states (E,*, E,*, E,*) have been suggested (27, 29). In the postillumination dark, the light/dithiothreitol-activated CFo·CF1 converts its state from E,*, which is sensitive to an uncoupler and relatively insensitive to ρH, to E,*, which is insensitive to an uncoupler and highly sensitive to pH. The presence of both dithiothreitol and P, in the postillumination dark brings about the activation of CFo·CF1 to E,*. Light activation of thylakoids in the absence of a thiol compound converts inactive CFo·CF1 to E,*, which is relatively sensitive to pH.

Chloroplast CFo·CF1, activated by illumination catalyzes both ATP synthesis and ATP hydrolysis as mitochondrial and bacterial proton-translocating ATP synthase-ATPase. In anaerobic bacteria, its ATP hydrolyzing activity plays an important role in the translocation of ions and organic substances (30). In chloroplasts, mitochondria, and aerobic bacteria, they work primarily in the direction of ATP synthesis. How the appearance of these activities is regulated is of great importance. In this communication, the correlation between the ATP synthetic active state and the ATP hydrolytic active state, as well as the time course of the ATP hydrolysis, is described.

The abbreviations used are: CFo·CF1, chloroplast ATP synthase-ATPase complex; CF1, chloroplast coupling factor 1; CFo, the hydrophobic part of the chloroplast ATP synthase-ATPase complex; ApA, P, P, (adenosine-5')-pentaphosphate; Tricine, N-[2-hydroxyethyl]glycine; NEM, N-ethylmaleimide.
state of $CF_0\cdot CF_1$, in the absence of a thiol compound has been studied.

**MATERIALS AND METHODS**

Chloroplast thylakoids were prepared from spinach leaves (29) with a medium containing 0.4 M sucrose, 20 mM KCl, and 25 mM Tris-HCl (pH 7.8) and washed four times in a medium containing 0.1 M KCl and 2 mM Tricine-KOH (pH 7.6).

N-Ethylmaleimide (NEM)-treated thylakoids were prepared as follows. 4.0 ml of reaction mixture containing KTM (50 mM KCl, 5 mM MgCl$_2$, and 25 mM Tricine-KOH at pH 8.3), 0.1 mM methyl viologen, 10 mM NEM, and thylakoids (0.25 mg of chlorophyll/ml) were illuminated for 60 s with heat-filtered white light (3.6 x 10$^2$ ergs/cm$^2$/s), chilled on ice, and centrifuged for 5 min at 20,000 x g. The pellet was suspended in 5 ml of a washing medium containing 0.1 M KCl and 2 mM Tricine-KOH (pH 7.6) and centrifuged. The washing procedure was repeated twice. The washed thylakoids were suspended in the washing medium at a concentration equivalent to 4 mg of chlorophyll/ml. Control treatment of thylakoids was performed similarly with the reaction mixture lacking NEM.

ATP synthesis was measured at 25 °C as follows. The reaction mixture contained 1.0 ml, KTM, 0.1 mM methyl viologen, 10 mM glucose, 6 units of hexokinase, and thylakoids (80–100 µg of chlorophyll) simultaneously with illumination, in the light or simultaneously with reillumination in the postillumination dark. ATP synthesis initiated by the addition of ADP and Pi, or by adding the substrates simultaneously with illumination, was terminated by the addition of 0.25 ml of 14% perchloric acid. To the mixtures, 8 mM dithiothreitol was added, and the mixtures were chilled on ice. After 20–30 min, the mixtures were centrifuged, and the supernatant solution of 1 ml was neutralized with 0.1 ml of 0.5 M Tris-KOH and centrifuged. The neutralized supernatant solution of 0.1–0.2 ml was diluted to 2 ml with a KTM medium. To the mixture, 10 µl of 20 mM NADP$^+$ was added, and the increase in the absorbance at 340 nm induced by the addition of glucose 6-phosphate dehydrogenase (0.7 unit) was measured with a modified Hitachi 100-50 spectrophotometer. The content of glucose 6-phosphate was calculated from the absorbance change (31).

ATP hydrolysis was measured as follows: 1.0 ml of the reaction mixture containing KTM, 0.1 mM methyl viologen, 10 units of pyruvate kinase, and thylakoids (0.2 mg of chlorophyll) was illuminated for 40 s and simultaneously with turning the light off, 0.5 mM ATP, 10 µM AP$_A$, and 5 mM P$_i$ was added. After 200 s, the reaction was terminated by the addition of 0.25 ml of 14% perchloric acid. The content of pyruvate plus ADP was measured as described (27).

ATP, ADP, AP$_A$, NADH, NADP, phosphoenolpyruvate, pyruvate kinase, lactate dehydrogenase, glucose-6-phosphate dehydrogenase, and hexokinase were purchased from Boehringer Mannheim, and NEM, from Wako. Hexokinase was solubilized with distilled water, and P$_i$ contained in the enzyme preparation was eliminated through an Amicon CF25 Centriflo before use.

**RESULTS**

**Decrease in ATP Synthesis Activity in the Light**—ATP synthesis initiated by illumination in the presence of ADP and P$_i$, or by adding the substrates simultaneously with illumination proceeded with a linear time course. ATP synthesis initiated by adding the substrates in the light after a 40-s preillumination, however, exhibited a curvilinear time course with a low initial rate (Fig. 1). With increasing reaction time, the rate of the latter approached the rate of the former. The amount of ATP formed in a 2-s reaction decreased with increasing illumination time before the addition of ADP and P$_i$ with a half-time of about 2 s (Fig. 2). In the light, an activation of ATP hydrolysis takes place. The time course of the activation of ATP hydrolysis coincided well with that of the decrease in the initial activity of ATP synthesis (Fig. 2).

**Increase in ATP Synthesis Activity in the Postillumination Dark**—ATP synthesis initiated by adding ADP and P$_i$ in the postillumination dark simultaneously with reillumination exhibited a less curvilinear time course than the ATP synthesis initiated by adding the substrates in the light (Fig. 3). By experiencing a dark period, the initial rate of ATP synthesis increased. The initial activity of ATP synthesis increased with an increasing time interval between the end of illumination and the addition of the substrates with the simultaneous reillumination, with a half-time of about 3 s (Fig. 4). In the postillumination dark, inactivation of ATP hydrolysis takes place. The time course of the inactivation of ATP hydrolysis coincided well with that of the increase in the initial activity of ATP synthesis (Fig. 4).

**Effect of NEM Pretreatment**—Different from thylakoids without pretreatment, thylakoids which underwent illumination in the absence (control-treated thylakoids) or in the presence (NEM-treated thylakoids) of NEM, and repeated washing procedure, catalyzed ATP synthesis which exhibited a curvilinear time course even if ADP and P$_i$ were added.
Active States of $CF_0,CF_1$

FIG. 3. Effect of dark period on the time course of ATP synthesis. ATP synthesis was initiated by adding substrates after a 40-s preillumination in the light ($\Delta$), or simultaneously with reillumination after a 40-s preillumination which was followed by a 10-s incubation in the dark (●).

FIG. 4. Dependence of the initial ATP synthetic activity and of the ATP hydrolytic activity on the postillumination dark time. ATP synthesis (●, △) was initiated by adding substrates simultaneously with reillumination after a 40-s preillumination which was followed by a 0–20-s incubation in the dark. The reaction was terminated after a 2-s incubation in the light. ATP hydrolysis (●, △) was measured as described under "Materials and Methods" except that substrates were added at 0–20 s after turning the light off. The results from two independent experiments (●, △) are shown. The rates at 1.0 value were 211 nmol/mg of chlorophyll/2 s (●), 134 nmol/mg of chlorophyll/2 s (△), 4.4 nmol/mg of chlorophyll/s (●), and 4.3 nmol/mg of chlorophyll/s (△).

simultaneously with illumination (Fig. 5). In these thylakoids, preillumination caused an increase in the initial rate of ATP synthesis contrary to thylakoids without pretreatment. It is likely that in the control-treated, or NEM-treated, thylakoids, it takes a long time to activate ATP synthesis fully because of the damage by pretreatment. In control-treated thylakoids, the initial rate of ATP synthesis increased further by experiencing a dark period between the end of preillumination and the addition of ADP and $P_i$ with simultaneous reillumination (Figs. 5 and 6). Such an increase in the initial rate of ATP synthesis was not observed in the NEM-treated thylakoids (Figs. 5 and 6).

DISCUSSION

Illumination of thylakoids activates $CF_0,CF_1$ to catalyze both ATP synthesis and ATP hydrolysis. In the absence of a thiol compound, the illumination time required for the full activation of ATP synthesis is less than 10 ms (32, 33) and for the full activation of ATP hydrolysis, more than 10 s (27). This suggests rapid formation of ATP synthetically active, but ATP hydrolytically inactive state, and slow formation of ATP hydrolytically active state in the light.

The time course of ATP synthesis changes depending on the condition of initiation (Figs. 1 and 3): the ATP synthesis initiated by illumination in the presence of ADP and $P_i$, or
by adding the substrates simultaneously with illumination, exhibits a linear time course. Preillumination of thylakoids before the addition of ADP and Pi changes the time course to be curvilinear with a low initial rate. Incubation of thylakoids in the dark after preillumination restores the initial rate and the time course is less curvilinear. This suggests the inactivation of ATP synthesis in the light in the absence of ADP and Pi, and its reactivation in the postillumination dark. The rate at the steady state does not depend on the condition of initiation. Therefore, the presence of ADP and Pi in the light seems to cause the activation of once inactivated CFo-CF1. Coincident with the inactivation of ATP synthesis, activation of ATP hydrolysis takes place in the light (Fig. 2). In the dark, inactivation of ATP hydrolysis takes place coinciding with the reactivation of ATP synthesis (Fig. 4). These facts suggest strongly that, in the absence of ADP and Pi, the rapidly formed ATP synthetically active, but ATP hydrolytically inactive state (Ei) converts to the ATP hydrolytically active, but ATP synthetically inactive (or less active) state (Ea) slowly (half-time of about 2 s) in the light. Addition of ADP and Pi in the light induces the conversion of Ea to Ei. In such a way, ADP and Pi regulate the state of CFo-CF1 in the direction of ATP synthesis. In the dark after illumination, conversion from Ea to Ei takes place.

Recently, it has been shown that in the absence of a thiol compound, the activation of CFo-CF1 in the light and its inactivation in the dark proceeds as follows: E ⇌ Ẽ ⇌ Ẽ* (29). In this model, E, Ẽ, and Ẽ* are nonactivated state, activated but ATP hydrolytically inactive state, and ATP hydrolytically active state, respectively. It is reasonable to identify E, as Ẽ*, E may be identical with Ei. As has been reported (29), addition of dithiothreitol and Pi in the postillumination dark brings about the conversion of CFo-CF1 from the inactive state (Ea) to the ATP hydrolytically active state (Ea*). Addition of dithiothreitol and Pi in the dark without preillumination does not bring about such an activation. To bring about the activation of ATP hydrolysis by dithiothreitol and Pi, preillumination for 1 s is enough. Therefore, the formation of Ei from Ẽ seems to occur rapidly. This favors the possibility that Ei is identical with Ẽ.

In NEM-treated thylakoids, the above-mentioned reactivation of ATP synthesis in the postillumination dark does not take place (Figs. 5 and 6). This suggests that NEM treatment inhibits the conversion of Ei to Ẽ in the light, or the conversion of Ẽ to Ei in the postillumination dark. In NEM-treated thylakoids, ATP synthesis is inhibited less than 50%, but ATP hydrolysis is inhibited almost completely as has been reported previously (15, 17). Therefore, the NEM treatment seems to inhibit the conversion from Ei to Ẽ in the light. The NEM treatment causes alkylation of thiol groups in the γ subunit of CF1 (14–16). The γ subunit has been suggested to interact closely with the ϵ subunit (25). The ϵ subunit regulates the Ca**-dependent ATP hydrolysis in CF1 as an ATPase inhibitor (23, 24). The interconversion between Ẽ and Ei may be associated with the behavior of the γ and ϵ subunits.

In NEM-treated thylakoids, the electron transport is stimulated (15) and is insensitive to stimulation by an uncoupler, and the transmembrane proton gradient is smaller than control-treated thylakoids. From this, NEM treatment seems to cause some kind of uncoupling, which results in a partial inhibition of ATP synthesis. Such an uncoupling effect of NEM has been observed in bovine heart mitochondria (34).

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