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The deactivation process of the proton-ATPase in isolated chloroplasts and whole leaves *

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Using rapid micromethods for chloroplast isolation and ATPase solubilization from preilluminated leaves, the deactivation of the proton-ATPase after different treatments was compared. The rate of decay of the 'in vivo' light-activated membrane-bound Mg^{2+}-ATPase was highly dependent on temperature. However, the soluble Ca^{2+}-ATPase, extracted from the temperature-inactivated membrane-bound ATPase, was active. Coupling factor 1 with a manifest and stable Ca^{2+}-ATPase activity was also solubilized from chloroplasts activated by light in whole leaves and deactivated after chloroplast isolation with gramicidin D. Deactivation of the proton-ATPase in isolated chloroplasts was only associated with the dissipation of the proton gradient. Reaction of the accessible sulfhydryl groups of the membrane-bound proton-ATPase with iodoacetamide prevent inactivation of the enzyme by oxidants. However, the iodoacetamide treatment had not effect on the temperature-dependent decay. The rate of deactivation of the proton-ATPase in whole leaves was similar for both membrane-bound and soluble ATPases. Thus, the oxidation process may play an important role in physiological conditions.

Introduction

Synthesis of ATP by chloroplasts, mitochondria and bacteria is catalyzed by a reversible and highly regulated proton-ATPase, using energy derived from the proton-gradient formed across an energy-transducing membrane [1]. This proton-ATPase consists of two readily separable parts, an oligomeric, hydrophilic protein extrinsic to the membrane, called coupling factor 1 and a hydrophobic protein intrinsic to the membrane, denoted F_0.

The chloroplast enzyme which catalyzed photophosphorylation (F_0-CF_1) can exist in several states of different activities depending on the experimental conditions. The activation of the proton-ATPase complex which is required for ATP hydrolysis occurs when an electrochemical potential is developed across the thylakoids in the presence of a reducing agent and may involve conformational changes [2,3], redox changes of sulfhydryl groups of CF_1 subunits [4,5] and release of adenine nucleotides [6–8]. CF_1, which may be easily detached from the membrane and purified [2], contains five different kinds of subunits denoted α, β, γ, δ and ε with a probably stoichiometry of 3 : 3 : 1 : 1 : 1 [9].

In dark-adapted chloroplasts the proton-ATPase is catalytically inactive and the soluble CF_1 isolated from them is a latent Ca^{2+}-ATPase that may be activated by different treatments

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Abbreviations: P_i, inorganic phosphate; Chl, chlorophyll.

[10,11]. Illumination of broken chloroplasts in the presence of thiol reducing agents leads to a modulation of the enzyme which is manifested by its capacity to hydrolyze ATP [12]. It has been suggested that thioredoxin is involved in the light triggering of the proton-ATPase in intact chloroplasts [13] and in whole leaves [14]. CF_1 with a manifest and stable Ca^{2+}-ATPase activity was solubilized from chloroplasts activated by light 'in vivo' [14].

The γ subunit of CF_1 has four cysteinyl residues [15,16] and two of them are reduced in association with the ATPase activation 'in vitro' by incubation of soluble CF_1 with dithiothreitol [17] or 'in vivo' by illumination of whole leaves [14]. On the other hand the thiol modulation process can be reversed, in isolated chloroplasts, by oxidants such as *o*-iodosobenzoate, dithiobis 2-nitropyridine, ferricyanide, oxidized thioredoxin or H_2O_2 [18,21]. Moreover, when chloroplasts or isolated CF_1 were treated with *o*-iodosobenzoate, enzyme inactivation was associated with the formation of a disulfide bridge in γ subunit [5,22]. It has been suggested that the γ subunit is the 'gate' that allows proton flux through the ATPase complex under phosphorylating conditions [17]. Thus, the flow of protons through the enzyme may be controlled by the redox potential.

In this paper we studied the participation of the proton gradient and the thiol redox changes in the deactivation mechanism of the chloroplast proton-ATPase. The kinetics of deactivation under physiological conditions are also reported.

Experimental procedures

Chloroplasts isolation

Isolated chloroplasts were prepared by a rapid procedure described earlier [14], from 8–11-week-old spinach plants (*Spinacia olearacea* L.) grown in a green-house at 20°C. Dark adapted chloroplasts were obtained from both isolated intact leaves or plants, kept in the dark for 3 h. Light-adapted chloroplasts were obtained from both isolated intact leaves or plants preilluminated 1 min by 150 W tungsten lamp through 2.5 cm of 0.1% $CuSO_4$ as heat absorbing filter (1200 $\mu E/m^2$ seg). Chloroplasts were suspended at 0°C in a medium containing 10 mM NaCl, 5 mM $MgCl_2$, 0.5 mM EDTA and 20 mM Tricine-NaOH (pH 8) at a final concentration of 0.4–1.0 mg of chlorophyll/ml.

Solubilization of CF_1

Chloroplast suspensions containing 40 mg of chlorophyll, were added to 0.75 mM EDTA (pH 8) to a final volume of 0.5 ml and incubated during 5 min at 20°C. Then they were centrifugated at 12000 rpm in an Eppendorf microcentrifuge during 4 min. Supernatants (0.4 ml) were employed for activity assay.

Analytical methods

ATPases activities were assay as previously described [14]. Chlorophyll concentration was determined according to Ref. 22. Protein was measured as in Ref. 24 with bovine serum albumin as standard and CF_1 concentration in the EDTA extracts were estimated by Laurell-rocket immunoelectrophoresis [25].

Results and Discussion

Dark decay 'in vitro' of the chloroplast proton-ATPase

The membrane-bound Mg^{2+} dependent proton-ATPase (Mg^{2+}-ATPase) activity of chloroplasts isolated from preilluminated leaves ('light' chloroplasts) was stable at 0°C for 60 min at least, while gramicidin D added to a final concentration of 50 nM induced a very rapid inactivation of the enzyme (Table I), in agreement with our previous results [14]. However, the soluble Ca^{2+}-dependent

TABLE I

EFFECT OF GRAMICIDIN D ON IN VIVO ACTIVATED CHLOROPLAST PROTON-ATPase

Chloroplasts (0.5 mg chlorophyll per ml) isolated from preilluminated spinach leaves were incubated at 0°C during 3 min, with the addition stated. The activities were the same even after 60 min of incubation.

Addition	Membrane-bound Mg^{2+}-ATPase (μmol P_i per mg Chl per h)	Soluble Ca^{2+}-ATPase (μmol P_i per mg protein per min)
None	523	12.8
Gramicidin 50 nM	88	12.4

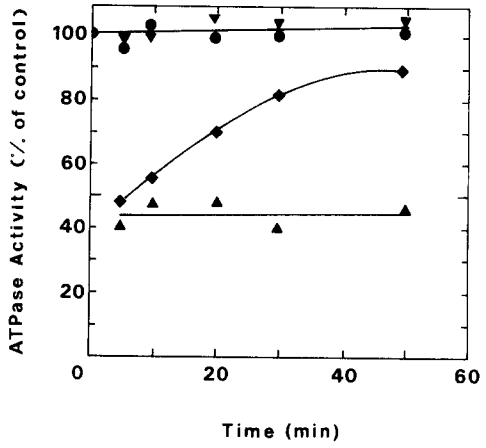

Fig. 1. Prevention of the *o*-iodosobenzoate inactivation of chloroplast ATPase activity by iodoacetamide treatment. Chloroplasts (0.3 mg/ml of chlorophyll) isolated from preilluminated spinach leaves were incubated in darkness at 0°C in the absence (●, ▲) or in the presence (▼, ◆) of iodoacetamide (10 mM). At the time stated, aliquots were incubated 2 min at 0°C in the absence (●, ▼) or in the presence (▲, ◆) of 5 mM *o*-iodosobenzoate and then Mg^{2+}-ATPase was measured. The activity of the control was 673 μmol P_i/mg Chl per h.

ATPase (soluble ATPase) isolated from gramicidin-treated chloroplasts was as active as the soluble enzyme isolated from control 'light' chloroplasts (Table I). Thus, we were able to obtain an active soluble CF_1 from gramicidin deactivated membrane bound proton-ATPase.

The reagent *o*-iodosobenzoate has been successfully employed as dithiol oxidant of both membrane-bound and soluble chloroplast ATPases [17,21,22,26], leading to inhibition of their activities. On the other hand, incubation of 'light' chloroplasts with iodoacetamide, an alquilant sulfhydryl reagent, had no effect on the Mg^{2+}-ATPase activity (Fig. 1). Treatment of 'light' chloroplasts with 2 mM *o*-iodosobenzoate during 2 min at 0°C, induced a fast inactivation of the enzyme [14]. However, this inactivation was prevented by iodoacetamide pretreatment (Fig. 1). The residual Mg^{2+}-ATPase activity increased with the preincubation time and represents the kinetics of the enzyme modification by iodoacetamide. A slow reaction rate of sulfhydryl residues with 10 mM iodoacetamide was observed at 0°C and at least 40 min of incubation were needed for obtaining a good protection against the oxidant. It is noteworthy that while *N*-ethylmaleimide, like *o*-iodosobenzoate, induced a fast inactivation of the Mg^{2+}-ATPase of 'light' chloroplasts [14], iodoacetamide did not (Fig. 1). This different behavior may be a consequence of distinct alquilating groups. Similarly to the results of Fig. 1, Arana and Vallejos [17] previously reported that iodoacetamide did not affect the ATPase activity of soluble CF_1, but effectively prevented inactivation by *o*-iodosobenzoate. These results are in agreement with the postulated correlation between the redox state of the disulfide bond of the γ subunit and the activation-deactivation process of the chloroplast ATPase [14,17,27].

In previous reports [19,21] ferricyanide was used as oxidant for inhibiting the Mg^{2+}-ATPase activated by illumination of intact chloroplasts. Table II shows the inhibition of both Mg^{2+}-ATPase and soluble ATPase of 'light chloroplasts' by ferricyanide treatment. At variance with the results obtained with uncouplers, the Mg^{2+}-ATPase and soluble ATPase were similarly inactivated. Inactivation by ferricyanide was also

TABLE II

PREVENTION OF THE ATPase INACTIVATION BY IODOACETAMIDE

Chloroplasts (0.2 mg chlorophyll per ml) isolated from preilluminated leaves were incubated twice for 40 min at 0°C with the additions stated. ATPases activities were measured as described in the text.

Additions		Membrane-bound Mg^{2+}-ATPase (μmol P_i per mg Chl per h)	Soluble Ca^{2+}-ATPase (μmol P_i per mg protein per min)
first incubation	second incubation		
None	none	750	19.2
None	ferricyanide (5 mM)	325	8.5
Iodoacetamide (10 mM)	none	716	18.9
Iodoacetamide (10 mM)	ferricyanide (5 mM)	550	19.4

prevented by iodoacetamide pretreatment, which presumably affects only the accessible cysteinyl residues. Their modification afforded a total protection to the soluble ATPase and a partial protection to the Mg^{2+}-ATPase activity (Table II). These results suggested that the action of ferricyanide is at the level of accessible sulfhydryl groups of the enzyme. The partial protection observed for the Mg^{2+}-ATPase may be due to some uncoupling effect of the ferricyanide treatment.

Morita et al. [28] reported the dark decay of the in vivo activated membrane-bound proton-ATPase and Vallejos et al. [14] shows the strong dependence of this process with temperature. The activated state of the proton-ATPase requires maintaining both an electrochemical potential and a redox change of the sulfhydryl groups of CF_1, according with the observed effects of uncouplers and oxidant reagent [14].

As is evident from Fig. 2, the rate of decay at 20°C of the Mg^{2+}-ATPase activity was similar in both, control chloroplasts or iodoacetamide-treated chloroplasts. Similar results were obtained at 15 of 30°C (data not shown). On the other hand, an active soluble ATPase was isolated from both control and iodoacetamide treated chloroplasts after incubation at 20°C for the stated times (Fig. 2). These results suggest that the temperature-dependent decay of in vivo activated proton-ATPase is only due to the dissipation of the electrochemical proton gradient.

The activity of chloroplast membrane bound proton-ATPase was usually measured like Mg^{2+}-dependent although it may also exhibited a Ca^{2+}-dependent activity. For this reason we study the behavior of membrane bound ATPase activities with both cations under different treatments. The

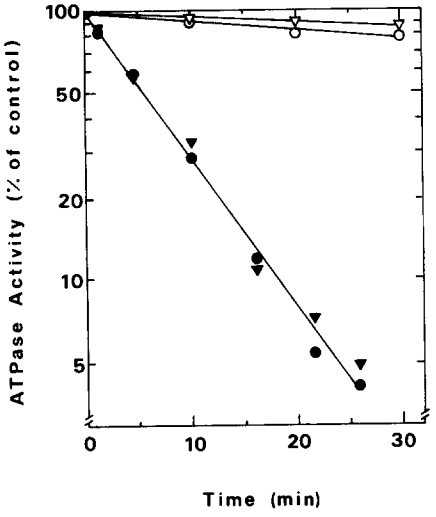

Fig. 2. Effect of temperature on membrane bound Mg^{2+}-ATPase and soluble Ca^{2+}-ATPase. Chloroplasts (0.3 mg/ml of chlorophyll) isolated from preilluminated spinach leaves were treated 30 min at 0°C in darkness, in the absence (○, ●) or in the presence (▽, ▼) of 10 mM iodoacetamide. The samples were incubated at 20°C for the time stated, and membrane bound Mg^{2+}-ATPase (●, ▼) and Ca^{2+}-ATPase solubilized thereof (○, ▽) were measured in aliquots as described in the text. The activities of the controls were 570 μmol P_i/mg Chl per h for the Mg^{2+}-ATPase and 13.2 μmol P_i/mg protein per min for the Ca^{2+}-ATPase.

TABLE III

EFFECT OF GRAMICIDIN, Cu^{2+} AND TEMPERATURE ON THE CHLOROPLAST PROTON-ATPase

Chloroplast (0.4 mg/chlorophyll per ml) prepared from preilluminated leaves (light chloroplasts) or from dark-adapted leaves (dark chloroplasts) were incubated 30 min with the additions stated. ATPase activities were measured as described in the text.

Treatments	Membrane bound ATPase (μmol P_i/mg Chl per h)		Soluble Ca^{2+}-ATPase (μmol P_i/mg protein per min)
	Mg^{2+}-dependent	Ca^{2+}-dependent	
Light chloroplasts			
None, 0°C	602	216	17.0
Gramicidin 0.1 nM, 0°C	58	36	16.2
$CuCl_2$ 0.1 μM, 0°C	200	45	6.7
None, 25°C	39	25	15.5
Dark chloroplasts			
None, 0°C	40	21	3.5

Mg^{2+}-dependent activity was three times higher than the Ca^{2+}-dependent (Table III). However, both activities of this enzyme respond similarly to uncouplers, oxidants and temperature treatments. On the other hand, the soluble ATPase was only inhibited by $CuCl_2$.

Dark decay in vivo of the chloroplast proton-ATPase

Fig. 3 shows the decay of both membrane bound and soluble proton-ATPase activities when intact leaves were incubated for different times in darkness at 25°C, after preillumination.

It is striking that this result is clearly different from that shown in Fig. 2. Thus, while both membrane-bound and soluble ATPases decreased similarly in vivo, only the membrane-bound activity diminished when the dark incubation was carried out with isolated chloroplasts.

Morita et al. [28] reported that the dark inactivation of the membrane bound proton-ATPase in vivo at 11°C was slower than in vitro and showed a sigmoideal curve when the results were plotted semilogarithmically. Fig. 4 shows the decay of Mg^{2+}-ATPase in leaves after preillumination of intact leaves or plants. As is evident, the lag period was observed when the dark adaptation

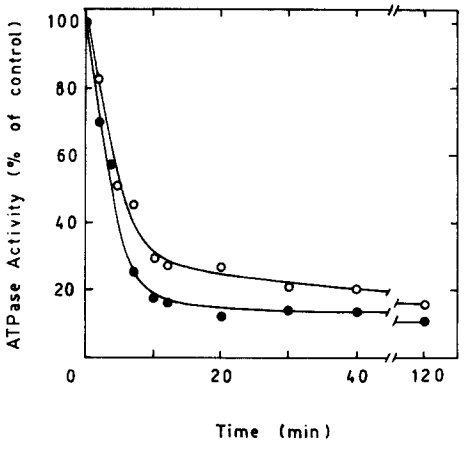

Fig. 3. Physiological deactivation of the in vivo activated proton-ATPase. Leaves, obtained from preilluminated plants, were kept at 25°C in darkness/ At the time stated, chloroplasts were isolated and membrane bound Mg^{2+}-ATPase (●——●) and soluble Ca^{2+}-ATPase (○——○) were measured. The activities of the controls were 747 μmol P_i/mg Chl per h for the Mg^{2+}-ATPase and 14.4 μmol P_i per mg protein per min for the Ca^{2+}-ATPase.

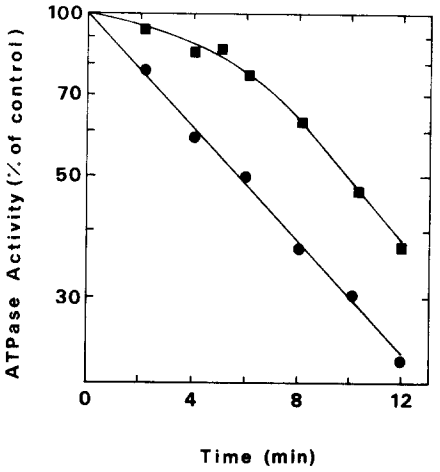

Fig. 4. Deactivation kinetics of the proton-ATPase activated in vivo. Spinach plants or isolated spinach leaves were dark-adapted for 3 h. Then they were illuminated for 1 min at 20°C and kept in darkness. At the time stated, chloroplasts were isolated and Mg^{2+}-ATPase activity measured as described in the text. The activities of the controls were: 584 μmol P_i/mg Chl per h for preilluminated plants (●——●); and 472 μmol P_i/mg Chl per h for preilluminated leaves (■——■).

and subsequent preillumination was carried out with isolated leaves, while the experiment performed using whole plants shows a linear plot. Thus, deactivation of the Mg^{2+}-ATPase was faster in whole plants than in isolated leaves and the half-time (about 5–6 min) was similar to that reported for in vitro inactivation [14].

The data presented here led us to suggest that the deactivation process of the proton-ATPase in isolated chloroplasts is mainly associated with the dissipation of the proton-gradient, since we were able to isolate CF_1 with a high ATPase activity from the inactive membrane bound enzyme. On the other hand, deactivation in vivo seems to involve both, the decay of the proton gradient and a chemical change in CF_1, which probably is oxidation of the vicinal dithiol of the γ subunit [14]. This interpretation is supported by the fact that CF_1 isolated from in vivo deactivated leaves has lost its ATPase activity. The physiological oxidant that affects CF_1 during in vivo deactivation seems to be absent in isolated thylakoids.

Why is it possible to obtain an active soluble ATPase from in vitro deactivated thylakoids? It is known that the energy state of thylakoids correlates with conformational changes of CF_1 as shown

by Ryrie and Jagendorf [29]. Since membrane-bound CF_1c is inactive in spite of remaining 'reduced' (i.e., its γ subunit dithiol is reduced) deactivation of thylakoids must have affect the conformation of CF_1. Thus, during solubilization 'reduction' switched back CF_1 to an active conformation similar to the one induced on the membrane by energization, free from the restriction probably imposed by its interaction with CF_0.

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