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ATPase state and activity in thylakoids from normal and water-stressed lupin

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To compare with the case of phosphorylation, ATP hydrolysis was investigated in thylakoids from plants submitted to drought. For medium stresses the relationship between hydrolysis rate and deactivation constant of the de-energized enzyme when ΔpH varies is unchanged, hence the basic mechanisms are preserved; (PSI-driven) energization then is neither affected, at any light intensity. As drought intensifies, hydrolysis and monogalactolipids fall somewhat in parallel, whereas the deactivation constant changes with digalactolipids and phosphatidic acid content. This alteration of the F_0 lipid environment must be transduced to F_1, the subunits of which, however, are preserved, as shown by LDS-PAGE. ATP synthesis (thiol-oxidized enzyme) and hydrolysis (thiol-reduced enzyme) show an identical decrease with stress. Thus the latter impairs a common early step, probably the activation of the oxidized enzyme.

F_0F_1 H^+-ATPase; Proton gradient; Lipid; Enzyme activation; Thylakoid; *Lupinus albus*

1. INTRODUCTION

The minimum scheme of the F_0F_1 ATPase activation involves a cyclic series of transitions (Scheme 1) [1,2]. Membrane-bound ATPase of isolated thylakoids kept in a de-energized state (dark) is in an oxidized and inactive (E_o^i) form. Energization by high $\Delta\tilde{\mu}_{H^+}$ activates the enzyme in parallel with the release of a tightly bound ADP [3]. In this new conformation (E_o^a) ATPase can serve for phosphorylation. Thiol-modulation, by thioredoxin in vivo [4] or by dithiothreitol in vitro [5], reduces a disulfide bond in the γ-subunit of the F_1 moiety of ATPase [6]. A lower $\Delta\tilde{\mu}_{H^+}$ is then required to reach the same fraction of activated enzymes which are now in a reduced state (E_r^a). Thiol-modulation is a slow process unless ATPase is already activated by $\Delta\tilde{\mu}_{H^+}$. The active and reduced conformation (E_r^a) lives for several tens of seconds (instead of milliseconds for E_o^a) which then allows a measurement of ATP hydrolysis in darkness without any sustained $\Delta\tilde{\mu}_{H^+}$ [7,8].

Drought is known to impair photophosphorylation in thylakoids. Younis et al. [9] reported a conformational change of ATPase which then is unable to bind nucleotides and synthesize ATP. On the other hand Ortiz-Lopez et al. [10] recently concluded from in vivo analysis of flash-induced electrochromic changes in leaves that water stress decreases ATPase activation. Our previous observations [11] showed that drought, at a given ΔpH, slows down ATP synthesis by oxidized and active ATPase (E_o^a) so we aimed to investigate if this is true for the other form (E_r^a), that is, if ATP hydrolysis is also impaired. The question then is to determine whether a common step in the presented scheme (Scheme 1) is affected by drought.

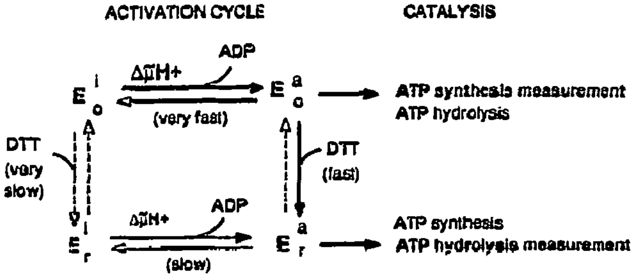
2. MATERIALS AND METHODS

2.1. *Plant culture*

Two genotypes of *Lupinus albus* L., resistant and susceptible to drought, were cultivated in a controlled chamber as already described [11]. They were submitted to drought by not watering them for 6–15 days. The plant status was expressed as percent of water of fresh leaf.

2.2. *Thiol-modulated thylakoids*

Thylakoids were isolated from pre-modulated chloroplasts by modifying the procedure of Biaudet et al. [8]. After extraction, chloroplasts were submitted to a hypotonic shock and thylakoids (40 μM chlorophyll, Chl) were suspended in buffer, pH 8.3, containing 100 mM



Scheme 1.

Abbreviations: $\Delta\tilde{\mu}_{H^+}$, transmembrane proton electrochemical potential difference or proton gradient (expressed here as ΔpH); Chl, chlorophyll; F_0F_1 or E, ATPase (synthase–hydrolase); kd, deactivation constant; MGDG, DGDG, mono- and digalactosyldiacylglycerol.

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sorbitol, 10 mM tricine, 10 mM NaCl, 4 mM K_2HPO_4, 0.5 mM $MgCl_2$ and 20 mM dithiothreitol. They were strongly illuminated (1.5 $kW \cdot m^{-2}$, infra-red-free) for 5 min at 20°C, in the presence of 50 μM phenazine methosulfate to generate the high proton gradient needed for a large enzyme activation (cyclic electron flow around photosystem 1). The suspension was diluted twofold in 100 mM sorbitol, 30 mM NaCl, 6 mM K_2HPO_4, 10 mM MES (pH 6.5) and centrifuged for 10 min at 1,000 × *g* (4°C). The pellet of thiol-modulated thylakoids was finally suspended in a small volume of the previous medium and stored on ice in darkness.

ΔpH and hydrolysis rate were measured with thiol-modulated thylakoids (20 μM Chl) at 20°C in 100 mM sorbitol, 50 mM KCl, 5 mM $MgCl_2$, 0.5 mM K_2HPO_4, 1 mM Tricine, pH 8.2, with 50 μM pyocyanine, an analogue of phenazine methosulfate devoid of optical interferences, to generate the proton gradient.

2.3. *Proton gradient*

The transmembrane pH difference was estimated by light-induced quenching of 9-aminoacridine fluorescence [12]. The probe signal was calibrated by the steady-state method of Strotmann and Lohse [13]. As previously discussed [11], this method actually expresses the whole proton gradient ($\Delta\tilde{\mu}_{H^+} = F\Delta\Psi - 2.3\ RT\ \Delta pH$) in terms of ΔpH and any unrelated $\Delta\Psi$ is cancelled by 50 nM valinomycin.

2.4. *ATP hydrolysis*

ATP hydrolysis was measured with a fast and sensitive glass electrode [8]. The suspension of modulated thylakoids was first illuminated for 1 min to reactivate ATPases, then 0.5 μM nigericin and 500 μM ATP were simultaneously injected as the light was switched off. Nigericin abolishes the back-pressure of ΔpH on the reverse H^+ flux due to ATP hydrolysis. The hydrolysis rate (V_h) was measured by medium acidification due to scalar proton production: $MgATP^{2-} + H_2O \rightarrow MgADP^- + P_i^{2-} + H^+$ [14]. The deactivation constant of the reaction $E_r^{ii} \rightarrow E_r^{i}$ was calculated by the first order decay of this kinetics [15]. Light intensity was adjusted with neutral filters.

2.5. *Biochemical analysis*

Lipids of a thylakoid pellet were isolated and analysed by thin-layer and gas chromatography as previously described [11].

Thylakoid polypeptides were separated by LDS-PAGE [16]. The membranes were solubilized in 20 mM PIPES-NaOH (pH 6.6), 50 mM dithiothreitol, 100 mM sucrose and 2% LDS for 15 min at 4°C (mass ratio LDS/protein = 10/1). Electrophoresis was run for about 18 h at 4°C on a 12–24% resolving gel with a stacking gel at 11% acrylamide.

To extract F_1, thylakoids were first treated with 10 mM pyrophosphate, which removes ribulose diphosphate carboxylase, and then with a 0.3 M sucrose solution of low ionic strength, which releases F_1 [17]. The 30,000 × *g* supernatants of these extracts were essentially free of contaminating proteins.

3. RESULTS AND DISCUSSION

3.1. *Enzyme functional studies*

A medium drought, decreasing the leaf water content from 84 to 75%, did not affect the ΔpH generated during the light activation period (Fig. 1A) but strongly decreased the hydrolysis rate (Fig. 1B). The flow–force relationship, $V_h = f(\Delta pH)$ was below that of the control, so that for the same energization, the hydrolysis rate was cut by about 50% (Fig. 1C). A similar behaviour was observed in resistant lupin (not shown). Therefore, as previously observed [11], there is no uncoupling but rather an impairment or loss of ATPases.

For medium stresses at least, drought did not change either the relationship [15] between the ATPase deactivation constant, K_d, and the hydrolysis rate V_h (Fig. 2). This curve was traced by decreasing the activity through a decreased ΔpH-dependent enzyme reactivation (light reduction) and is similar to that obtained with reversible ATPase inhibitors [15]. Thus, even if there were fewer active enzymes they behaved in a similar manner as their controls. It is possible that drought indirectly modifies the enzyme conformation to retain ADP more tightly, a situation known to cause inactivation [18].

3.2. *Effect of an increasing drought*

As drought intensified the hydrolysis rate declined (Fig. 3A), whereas the deactivation constant first dropped then recovered (Fig. 3B). Several reports have shown a key role for lipids on ATPase activity [18–20] so it is interesting to note here that, qualitatively at least, V_h decreased along with monogalactosyldiacylglycerol (MGDG), whereas kd changed with lipids in general (to be precise, digalactosydiacylglycerol (DGDG) and phosphatidic acid). Both resistant and susceptible genotypes behaved similarly.

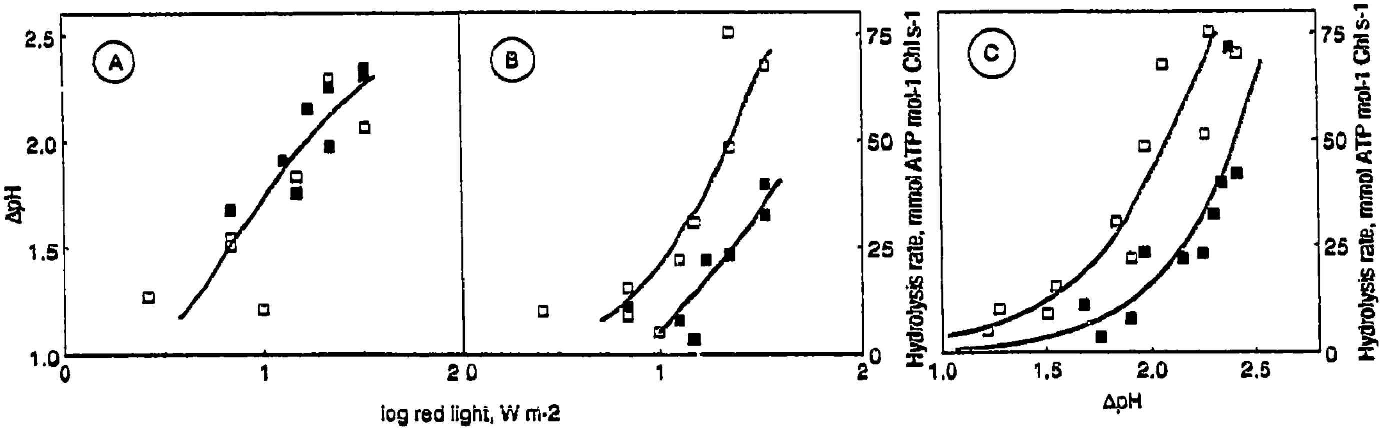

Fig. 1. Light-intensity curves of proton gradient, ΔpH (A), subsequent ATP hydrolysis uncoupled rate, V_h (B), and flow (V_h)-force (ΔpH) relationship (C) of thylakoids from control (□) and stressed susceptible lupin (■). Leaf water content = 84% in control and 75% in stressed plant. See text for details.

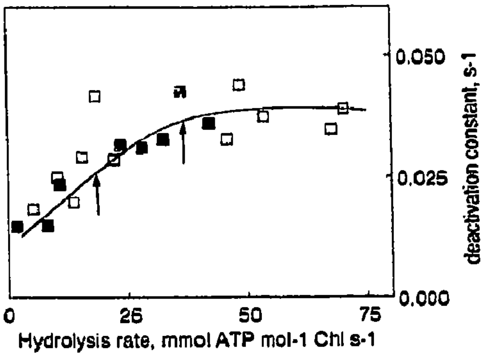

Fig. 2. Relationship between ATPase deactivation constant, K_d, and ATP hydrolysis rate, V_h. Same conditions as Fig. 1, control (□), stressed (■). Curves were traced from right to left by decreasing light intensity, which lowers reactivating ΔpH. Arrows indicate data for ΔpH = 2.3 (left, stressed; right, unstressed).

Another important point is that ATP synthesis and hydrolysis were identically affected by drought (Fig. 4). This was true independent of ΔpH (not shown). Hence, it is highly probable that the target of water stress is a common step of both catalytic activities. Since thiol-modulation starts with activation of the oxidized ATPase, this step may be the reaction $E_o^i \rightarrow E_o^a$ (top, scheme 1) or even a shift from an *inactive* to an *inactivatable* state ($E_o^i \rightarrow E_o^{i\prime}$). Ortiz-Lopez et al. [10] suggested that loss of activity may reflect a higher energy threshold to activate the enzyme; since to some extent a lower energization may be compensated for by a longer modulatio, we tried to increase this time but noticed no effect (not shown).

3.3. *ATPase biochemical state*

Fig. 5 shows that drought did not affect the α, β, γ, δ and perhaps ε subunits of F_1. A similar picture was observed in susceptible plant and for stronger stresses (water content down to ca. 60%).

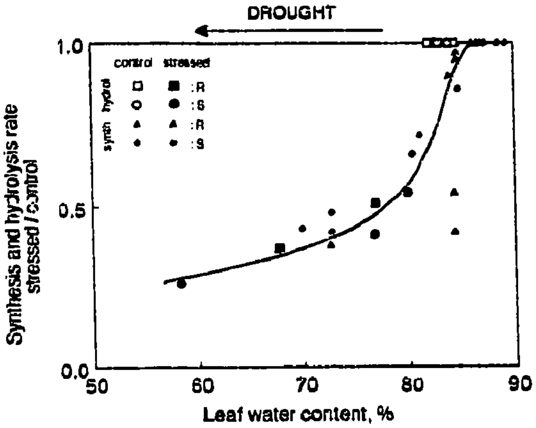

Fig. 4. Drought effect on ATP synthesis (△▲,◇◆) and hydrolysis (□■,○●) rates as a function of leaf water content. Resistant (△▲,□■) and susceptible (◇◆,○●), control (△◇,□○) and stressed (▲◆,■●) lupin. Hydrolysis was measured at ΔpH = 2.3 with photosystem 1 (pyocyanine) and synthesis at ΔpH = 3.0 with photosystem 1 (pyocyanine) or photosystem 2 (dimethylquinone) chains.

4. CONCLUSION

Since enzyme destruction seems to be excluded by the above results the cause of the drop in ATPase activity must reside within the enzyme. This may concern F_1 [9] but indirect data point also to F_0 [19]. Indeed, the deficit in MGDG may decrease the lateral packing pressure applied on F_0 [20] and MGDG was found to control hydrolysis activity [21]. Here we have shown that two processes are affected. The first is the rate of ATP hydrolysis (and synthesis) which probably reflects the number of active enzymes and hence its correlation with MGDG. The second is the lifetime of the active enzyme, which seems to involve other – or additional – factors and is apparently correlated with DGDG and phosphatidic acid (not excluding other lipids, which increase when recovery occurs [11]). Since activation/de-

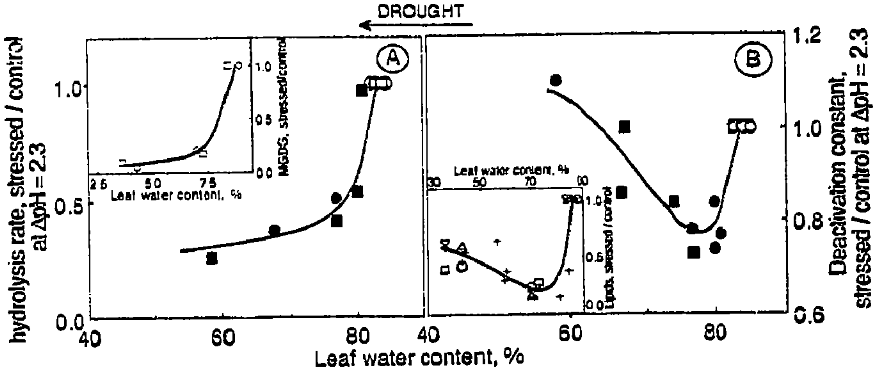

Fig. 3. Effect of an increasing drought on hydrolysis rate, V_h, (A) and on the deactivation constant K_d, (B), both measured at the same ATPase reactivating ΔpH = 2.3. Resistant (○,●) and susceptible (□,■) lupin, control (□,○) and stressed (■,●) lupin. (Inset A) Change in thylakoid MGDG in resistant (□) and susceptible (○) plants. (Inset B) Change in DGDG (□,○) and, probably, phosphatidic acid (▽,△) in resistant (□,▽) and susceptible lupin (○,△), and general change of lipids in both resistant and susceptible lupin (+).

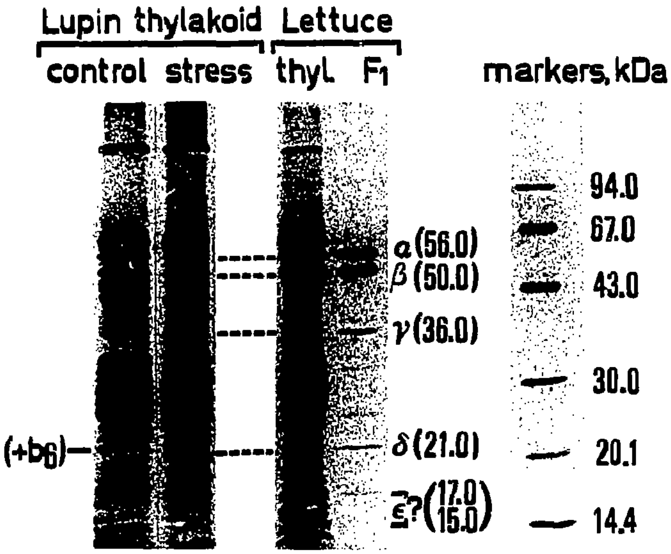

Fig. 5. Gel electrophoresis pattern, revealed by Coomassie brilliant blue, of whole thylakoid proteins and of F_1. Thylakoids equivalent to 20 μg Chl were present in each lane. Resistant genotype: control (water content 81%) and 11-days stressed (water content 72%); for comparison, lettuce thylakoids from which F_1 was extracted have been included.

activation and, by definition, synthesis/hydrolysis have their seat in the catalytic moiety of ATPase, F_0 environment alterations must be transduced to F_1, perhaps through the minor subunits of the enzyme. Further investigations are needed to uncover these mechanisms.

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REFERENCES

[1] Bakker-Grunwald, T. and Van Dam, K. (1974) Biochim. Biophys. Acta 347, 290–298.

[2] Junesch, U. and Gräber, P. (1985) Biochim. Biophys. Acta 809, 429–434.

[3] Gräber, P., Schlodder, E. and Witt, H.T. (1977) Biochim. Biophys. Acta 461, 426–440.

[4] Mills, J.D. and Hind, G. (1979) Biochim. Biophys. Acta 547, 455–462.

[5] Mills, J.D. and Mitchell, P. (1984) Biochim. Biophys. Acta 764, 93–104.

[6] Ketchám, S.R., Davenport, J.W., Warncke, K. and McCarty, R.E. (1984) J. Biol. Chem. 259, 7286–7293.

[7] Rumberg, B. and Becher, U. (1984) in: ATPases, Structure, Function, Biogenesis (Papa, S., Altendorf, L., Ernster, L. and Packer, L., ed.) pp. 421–430, Adriatica Editrice, Bari.

[8] Biaudet, P., de Kouchkovsky, F. and Haraux, F. (1988) Biochim. Biophys. Acta 933, 487–500.

[9] Younis, H.M., Boyer, J.S. and Govindjee (1979) Biochim. Biophys. Acta 548, 328–340.

[10] Ortiz-Lopez, A., Ort, D. and Boyer, J.S. (1991) Plant Physiol. 96, 1018–1025.

[11] Meyer, S., Phung Nhu Hung, S., Trémolières, A. and de Kouchkovsky, Y. (1992) Photosynth. Res., 32, 95–107.

[12] Schuldiner, S., Rottenberg, H. and Avron, M. (1972) Eur. J. Biochem. 25, 64–70.

[13] Strotmann, H. and Lohse, D. (1988) FEBS Lett. 229, 308–312.

[14] Nishimura, M., Ito, T. and Chance, B. (1962) Biochim. Biophys. Acta 59, 177–182.

[15] Valerio, M., Haraux, F. and de Kouchkovsky, Y. (1992) Biochemistry, 31, 4239–4247.

[16] Delepelaire, P. and Chua, N.H. (1979) Proc. Natl. Acad. Sci. USA 76, 111–115.

[17] Strotmann, H., Hesse, H. and Edelmann, K. (1973) Biochim. Biophys. Acta 314, 202–210.

[18] Lohse, D., Thelen, R. and Strotmann, H. (1989) Biochim. Biophys. Acta 976, 85–93.

[19] Pick, U. (1989) Biochemistry 27, 8284–8290.

[20] Rawyler, A. and Siegenthaler, P.A. (1989) Biochim. Biophys. Acta 975, 283–292.

[21] Pick, U., Gounaris, K., Admon, A. and Barber, J. (1984) Biochim. Biophys. Acta 765, 12–20.