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**Drought and Cold Damage to Plants in
Relation to the Structure and Function of
the ATP Synthase from Chloroplasts**

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(2) Abstract

The major aim of this research was to find drought-resistant plants in which the CF_o - CF_1 ATP synthase is relatively stable to inactivation with the hope to develop in the future drought-resistant crop plants by hybridization. This aim has not been achieved since none of the CF_o - CF_1 preparations which were isolated from different plant or algal species exhibited a higher resistance to inactivation. However, this research enabled us to isolate and characterize CF_o - CF_1 from a large variety of plants and one alga, to show that drought-inactivation of CF_o - CF_1 is a general phenomenon which can be mimicked in CF_o - CF_1 proteoliposomes by high salt or high Mg concentrations, to develop methods for detection of conformational changes in CF_1 (by ANS fluorescence changes) and for chemical modification of the ATP-binding sites of the enzyme (by FITC) and to clarify a basic problem in the regulation of ATPase activity by energization and by thiol-modulation. The most important outcome of this research was the discovery that the detergent octylglucoside (OG) activates a Mg-specific-ATPase in CF_1 . We have shown that OG micelles bind to soluble CF_1 and induce in it a conformational change which mimicks to effects of light-energization in the membrane bound-enzyme. This observation turned out to be very important for elucidation of the mechanism of ATPase activation and deactivation and for the understanding of the phenomena of "allotopy" in CF_1 .

(3) Research Results

(a) Distribution of the Phenomenon of Drought-Inactivation in Different Plant Species

In order to find out whether the drought-inactivation of photophosphorylation and of chloroplast CF_1 -ATPase activity, which were previously demonstrated in spinach, is a general phenomenon we have compared the effect of drought on different plant species and the results are summarized in Table I.

These results demonstrate that the inactivation of photophosphorylation by exposure to drought is not limited to spinach.

Table I: Comparison of the effect of drought on photophosphorylation in different plant species

Plant Species	Rate of Photophosphorylation		
	μ mol ATP formed/mg Chl./ h	% of control	
	control	dehydrated	
Spinach	1350	570	42
Tobacco	1050	440	42
Pea	830	220	27
Radish	620	380	61
Wheat	320	165	52
Corn	540	180	33

All plants were germinated and grown in a controlled, artificially illuminated (10h light/14 h dark cycles) room as previously described (1). Irrigation was provided daily with tap water. After 21 (pea, radish) 30 (corn, wheat) or 40 (spinach, tobacco) days, the plants were divided into two groups, and irrigation was either stopped (dehydrated) or continued (control) for 6 - 10 days, until leaf water potential of the dehydrated group declined to -20 bars. Chloroplasts were prepared by published procedures (2) but with the presence of a protease inhibitor (10^{-4} M p-chloromercuribenzensulphonate) during the grinding (3). Cyclic photophosphorylation, in the presence of 30 μ M PMS was measured as previously described (4).

Table II demonstrates the effects of dehydration on different activities of CF_1 in thylakoids, CF_1 , and CF_0 - CF_1 proteoliposomes from spinach.

Table II: Analysis of the effect of drought on different partial reactions of CF_1

Preparation		Control	Dehydrated	% of control
chloroplast thylakoids ^a	cyclic photophosphorylation	1430	429	30
	light-activated Mg-ATPase	115	29	25
	trypsin-activated Mg-ATPase	55	36	65
	latent Ca-ATPase	1.20	0.26	22
	trypsin activated Ca-ATPase	14	10	70
	OG-activated Mg-ATPase	28	26	92
CF_1 ^b				
CF_0 - CF_1 proteoliposomes ^c	P_i -ATP exchange	110	60.5	55
	Mg-ATPase	185	111	60

Spinach was grown and dehydrated as described in Table I. Chloroplast thylakoids (2), CF_1 (5) and CF_0 - CF_1 (3) preparations and assays of photophosphorylation (4), light-activated Mg-ATPase (6), trypsin activated Mg ATPase (7), OG-activated ATPase (8) and P_i -ATP exchange (3) were measured as previously described. Activities are expressed in μ mole/mg Chl/h^a, μ mol/mg parotein/min^b or in n mol/mg protein/min.

The data in Table II demonstrate that extrinsic activation of CF_1 in either chloroplasts (trypsin) or in the soluble CF_1 (by trypsin or OG) partially relieves the inhibition in agreement with Younis et al. (9). These data may suggest that the mechanism of inhibition is in preventing the activation of CF_1 by energization of the chloroplasts rather than by affecting the catalytic site of the enzyme.

(b) Development of procedures to follow conformational changes in CF_1

One of our aims in this project was to develop non-destructive procedures to follow conformational changes in CF_1 which would enable to follow ATPase activation and inactivation. We have used two fluorescent labels for this purpose, Anilino-naphthalenesulphonate (ANS), which reversibly binds to CF_1 , and turned out to be an excellent sensor of detection of conformational changes, and fluresceiniso-thiocyanate (FITC) which modifies ATP binding sites of cation-translocating ATPase (10,11) and has been used also in order to detect conformation changes (10,12,13).

ANS

Only the major features of the use of ANS for studies of conformational changes in CF_1 are summarized below and the data relevant for the characterization of drought-inactivation. The data are described in detail in manuscript No. 1.

1. Chloroplast coupling factor 1 (CF_1) contains a high-affinity binding site for 8-anilino-1-naphthalene sulphonate (ANS, $K_d = 5 - 6 \mu M$). The binding of ANS to the enzyme is associated with a fluorescence enhancement and a blue-shift in the emission spectrum. ANS only slightly inhibits ATP hydrolysis by CF_1 .
2. Adenine nucleotides and inorganic phosphate induce a fast ANS fluorescence quenching of about 50% which is due to a decrease in the affinity of the enzyme for ANS (K_d increases from $6 \mu M$ to $22 \mu M$) and in the fluorescence quantum yield of the bound probe (by 33%) but not in the number of ANS sites ($n = 1$). Conversely, Mg and Ca ions induce a fluorescence enhancement of bound ANS.
3. Inactivation of CF_1 -ATPase by denaturing procedures such as high temperature, extensive trypsin digestion or incubation with the detergent octyl glucoside in the absence of ATP (section e) further enhance ANS fluorescence, eliminate the response to adenine nucleotides and inorganic phosphate, but increases the response to divalent metals.

Exposure of spinach leaves to low water potential also leads to an enhancement of ANS fluorescence upon association with CF_1 and to a partial inhibition of the ATP - induced fluorescence quenching (Fig. 1).

Fig. 1: The effect of drought inactivation on CF_1 -induced ANS fluorescence enhancement and on ATP-induced ANS fluorescence quenching. CF_1 was isolated by chloroform extraction from control or dehydrated spinach as in Table I. Measurements were carried out as described in the attached manuscript No. 1, Fig. 3.

4. The affinity of latent CF_1 for ADP ($K_d = 12 \mu M$) is considerably higher than for ATP ($K_d = 95 \mu M$) in buffer containing EDTA. The K_d for inorganic phosphate is $140 \mu M$. Mg increases the apparent affinity for ATP ($K_d = 28 \mu M$) but not for ADP or P_i .
5. Binding of ATP to the tight-sites does not inhibit the ADP or P_i -induced fluorescence quenching but decreases the affinity for ADP ($K_d = 34 \mu M$) and for inorganic phosphate ($K_d = 320 \mu M$). These results suggest that the ADP and phosphate binding sites are different but not independent from the tight sites.
6. Activation of a Mg-specific ATPase in CF_1 by octyl glucoside (section e) decreases the affinity for ADP and inorganic phosphate by about three fold but increases the affinity for ATP. ATPase activation of CF_1 also increases the K_i for ADP inhibition of ATP hydrolysis.

It is suggested, therefore, that the adenine nucleotide and P_i binding sites responsible for the ANS fluorescence quenching are the catalytic sites of CF_1 and that ATP are activation or inactivation involves a modification of these sites.

7. ATPase activation also influences the ANS responses to Ca and Mg. Ca-ATPase activation increases the fluorescence enhancement and the apparent affinity for Ca whereas Mg-ATPase activation specifically increases the Mg-induced fluorescence enhancement.
8. The fluorescence of CF_1 -bound ANS is enhanced by Dio-9 and quenched by phloridzin, quercetin, NBD-Cl and FITC. NBD-Cl and FITC completely inhibit the ADP-induced fluorescence quenching whereas Dio-9 inhibits the Mg-induced fluorescence enhancement. ANS does not relieve the quercetin or phloaridzin inhibition of ATP hydrolysis indicating that these inhibitors do not compete with ANS for a common binding site.

ANS may be used, therefore, as a sensitive probe to detect conformational changes in CF_1 in response to activation or inactivation and to binding of substrates and of inhibitors.

FITC

We (11) and others (10) have previously shown that FITC selectively modifies lysine residues involved in ATP binding in different cation transporting ATPases. Conformational changes could be detected in the fluorescein labeled enzymes in response to the binding of ligands by following fluorescein fluorescence changes (10,12,13). We have tried, therefore, to modify CF_1 with FITC and found that similar to other different cation ATPases the activity of lettuce CF_1 is inhibited by FITC, that the inhibition is prevented by adenine nucleotides and is associated with incorporation of fluorescein into the α and β subunits of CF_1 (manuscript 2).

In more recent studies we found that FITC also similarly inhibits CF_1 from spinach and from the halotolerant algae Dunaliella bardawil but in these enzymes **the inhibition was accompanied by an exclusive incorporation of fluorescein into the α subunit.** These results suggest the existence of a catalytic ATP binding site in α or at the interface between α and β subunits of CF_1 and also that FITC acts as an affinity probe for different ATP binding sites. We were not able, however, to follow conformational changes in CF_1 in response to different ligands, to activation, or inactivation of CF_1 .

(c) Attempts to simulate the Effects of Drought Inactivation of CF_1 and of CF_0-CF_1 by Mg or by high NaCl concentrations

Younis, Boyer and Govinjee (9) have previously reported that preincubation of CF_1 with Mg or sucrose inhibits CF_1 -ATPase activity and suggested that the effect simulates the effect of drought on the membrane bound enzyme, which causes a local increase of the Mg ion concentration in the chloroplasts. In order to test this hypothesis we have exposed chloroplast thylakoids, CF_1 and CF_0-CF_1 proteoliposomes to 10 mM Mg or to 300 mM NaCl and tested the effect on their activities.

Table III: Effect of preincubation of thylakoids, CF_1 and CF_0-CF_1 with Mg or NaCl on their activities

Preparation	Activity	% of control activity following 30 min incubation with:	
		10 mM $MgCl_2$	300 mM NaCl
Chloroplast thylakoids	ATP synthesis	75	92
CF_1 (latent)	Ca-ATPase	65	54
CF_1 (heat activated)	Ca-ATPase	26	18
CF_1 (+ 25 mM OG)	Mg-ATPase	60	72
CF_0-CF_1 proteoliposomes	P_i -ATP exchange	85	93

Chloroplast thylakoids (2), CF_1 (5) and CF_0-CF_1 (3) were prepared from spinach leaves as previously described. The enzymes were incubated 30 min at 4° (thylakoids, CF_0-CF_1) or at 23° (CF_1) in the presence of 50 mM Na tricine (pH 8), 0.1 mM EDTA and the indicated additions. The enzyme concentrations were 0.1 mg Chl/ml (chloroplasts), 0.3 mg protein/ml (CF_1) and 1 mg protein/ml (CF_0-CF_1 proteoliposomes). Excess Mg or NaCl was removed either by Sephadex centrifugation (CF_1 and CF_0-CF_1 proteoliposomes, ref. 14) or by pelleting the chloroplasts by centrifugation. Activities were tested as in Table II.

These results confirm the previous results of Younis et al. (9) but they also indicate large differences in the degree of sensitivity to Mg and NaCl not only between CF_1 and CF_0-CF_1 but also between different states of CF_1 . The heat activated preparation seems particularly sensitive to both Mg and salt. This may be partly due to sensitization of CF_1 to inactivation following the heat

treatment which I have also observed before (Pick, unpublished results). Nevertheless there is a significant, although partial inhibition of the activity in all the enzyme preparations, which in the case of CF_1 is also associated with an increase in ANS fluorescence of CF_1 (section b). It is concluded therefore that the effect of Mg on CF_1 may indeed simulate the effect of low water potential.

(d) Comparisons of Drought-resistant with Drought-Sensitive Plant Species with respect to the Sensitivity of CF_1 to low water Potentials

In an attempt to find out whether resistance to water stress involves a modification in CF_0 - CF_1 we have compared drought-resistant with drought-sensitive plant species with respect to the sensitivity of the enzyme to low water potentials. Several families which are of economical importance (Lacuca, Pisum, Triticum) as well as others were selected, and for each family a species which is used as a crop plant was compared under identical conditions to the wild types, collected in arid or salty environments. The leaves were dehydrated down to a water potential of -20 bars, CF_1 and CF_0 - CF_1 were isolated and their activities were compared with those of control plants. A representative experiment summarizing the results with two families is demonstrated in Table IV.

Fresh leaves of control or wild species were collected in different locations (Negev, Judean desert, sea-shore) during April, 1982 and kept on ice. Exposure to low water potential was performed within 24 h by partial drying of the leaves in an illuminated chamber at 25° for 2 - 5 hours during which samples were continuously removed for measurement of the leaf water potentials. CF_1 (5) and CF_0 - CF_1 (3) were isolated from control and dehydrated plants, and assayed as previously described. CF_1 -Ca-ATPase was not activated by any treatment.

Table IV

			CF ₁ -Ca-ATPase		CF ₀ -CF ₁ P _i -ATP exchange			
			(μmol/mg/min)		(μmol/mg/min)			
			control	dehydrated %	control	dehydrated %	control	dehydrated %
Lactuca	sativa	(control)	2.5	1.0	40	115	70	61
	undulata	(desert)	3.4	1.5	44	65	30	46
	orientalis	(desert)	1.7	0.9	53	40	25	62
Mesembryanthemum	roseum	(control)	3.5	1.5	43	85	35	41
	nodiflorum	(desert)	2.2	0.7	32	45	20	44
	forskahelei	(salty)	1.5	0.5	33	70	30	43
	crystallinum	(sea shore)	4.8	2.2	46	120	55	46

Similar measurements were carried out also with several species of wheat grown from seeds and dried as described in Table I (T. aestivum, T. longissimum, T. kotschgi. Mayoral and Pick, unpublished results).

We have noticed, in agreement with previous reports, that the time required to reach the same level of dehydration is always longer in the desert-species in comparison to the control (data not shown). However, at similar degrees of dehydration, similar degrees of inactivation of CF₁ and of CF₀-CF₁ were obtained in the controls and in the desert species. These results indicate, therefore, that in these species the mechanism of resistance to drought does not involve a modification of CF₀-CF₁.

(e) Activation of a Mg-specific ATPase in Chloroplast CF₁ by Octylglucoside

Two characteristic properties of CF₁ are (a) it is a latent ATPase, like the CF₀-CF₁ in dark adapted thylakoids, (b) the detachment from the membrane changes the structural and catalytic properties of CF₁ (Allotopy). These changes include lability to cold and to high ionic strength, alteration in the Me²⁺ ion-specificity for ATP hydrolysis by heat-activated or trypsin-activated CF₁ and lack of ATPase deactivation by ADP.

In the course of our studies we made an observation that may shed light on both of these anomalous properties, namely, that the non-ionic detergent octyl-glucoside (OG) reversibly activates a Mg-specific ATPase in CF₁ and converts the soluble enzyme to a state which closely resembles the state of the enzyme in activated (illuminated) chloroplasts. Since these results are summarized in detail in the four enclosed manuscripts (Nos. 3 - 6), I will only briefly summarize here the major results and their impact on the mechanisms of activation and allotopy of CF₁.

1. OG above its critical micellar concentration (CMC) reversibly induces a high Mg-specific ATPase activity in CF_1 . Similar activations were obtained with CF_1 preparations from spinach, lettuce, petunia, radish, tobacco, pea and the alga Chlamidomonas reinhardi. OG also reversibly stimulates Mg-ATPase and inhibits Ca-ATPase activity of heat-activated or trypsin activated CF_1 . The results indicate that binding of OG micelles to CF_1 induce a reversible change in CF_1 which is associated with Mg-ATPase activation.
2. Of the detergents tested, only two classes-bile salts and saturated fatty acids containing 8 - 12 carbon chains slightly activate CF_1 -Mg ATPase above their CMC. The results indicate a relatively small size of the OG binding site, and a specific effect of the polar (sugar) portion of the detergent on the enzyme.
3. Extensive tryptic digestion of CF_1 which destroys the δ , ϵ and γ subunits does not abolish Mg-ATPase activation by OG. These results suggest that the OG binding site is on the α or β subunit of CF_1 .
4. The OG-dependent Mg-ATPase of CF_1 is insensitive to a high-ionic strength unlike the Ca-ATPase of heat-activated CF_1 . It appears, therefore, that OG stabilizes CF_1 against inactivation by a high-ionic strength.
5. A kinetic analysis of CF_1 -Mg-ATPase in the presence of OG demonstrated that Mg acts as a low-affinity ($K_i = 1$ mM) non competitive inhibitor with respect to the substrate, Mg-ATP ($K_m = 100$ μ M). These results indicate that the apparent stimulation of Mg-ATPase activity by OG in comparison to heat-activated Ca-ATPase is due to alteration of the interaction and to lowering of the affinity for free Mg inhibition and also to an increase of the affinity for Mg-ATP (manuscript 4). Also the ANS studies (section b) indicated that OG increases the affinity of CF_1 for the substrate (ATP) and decreases its affinity for the products (ADP, P_i) of ATP hydrolysis.
6. OG remarkably stimulates the exchange of bound ADP (but not ATP) at the tight sites similar to the effects of light on CF_0 - CF_1 in thylakoids (ref. 15). OG also modifies the interactions between low affinity adenine nucleotide binding sites and the tight sites in CF_1 . The result suggest that Mg-ATPase activation by OG is associated with a manifestation of the tight sites and by modulation of the cooperative interactions between the tight-sites and the catalytic sites.

7. We suggested that detachment of CF_1 from the chloroplast thylakoid membrane exposes a hydrophobic site which distorts the conformation and alters the properties of CF_1 . OG micells specifically fit into the site and convert the enzyme back to its native conformational state in light-activated thylakoids. Since the major lipid constituents of the thylakoid membranes are galactolipids which may resemble an octylglucoside micell (sugary interface, hydrophobic core) we suggested that activation of CF_1 in illuminated thylakoids involves association of the OG-binding site with the membrane interface. Recently we obtained evidence for specific activation of CF_0 - CF_1 by galactolipids from chloroplasts which strongly supports this hypothesis (Pick, Gounaris, Admon and Barber, manuscript in preparation).

(f) The Interrelations between Energization and Thiol Modulation in the Regulation of ATP Hydrolysis by Soluble and Membrane-Bound CF_1 .

The capacity of chloroplast thylakoids to hydrolyze ATP in the dark depends on prior reduction of SH groups in the γ subunit of CF_1 during illumination (thiol-modulation). Yet, the capacity of the enzyme to synthesize ATP in the dark is independent on thiol modulation. For this reason it has been suggested by some workers that the mechanism of ATP synthesis differs from the mechanism of ATP hydrolysis (reviewed by Shavit, ref. 16). We have reevaluated this discrepancy by testing the effect of thiol modulation on ATP hydrolysis in chloroplast thylakoids and in CF_1 under energized condition. We observed that Mg-ATPase activity of chloroplasts is hardly affected by thiol modulation when measured during illumination, although the presence of thiol reducing agents in the light stimulates ATPase activity in the subsequent dark period. Similarly energization of CF_1 either by OG micelles or at 64°C reversibly stimulated a Mg-ATPase activity which is insensitive to thiol-modulation. Detergent dilution or cooling to 37° converts the enzyme to a modulation sensitive Ca-specific ATPase. It is suggested, therefore, that the enzyme may exist in two different catalytically active states: An energized state, insensitive to thiol modulation and an deenergized state which is stimulated by thiol modulation (manuscript 7).

(g) A Time Lag in the Onset of ATP-P_i Exchange Catalyzed by Purified ATP-Synthase (CF₀-CF₁) Proteoliposomes and Chloroplasts

The time course of ATP-P_i exchange which is catalyzed by the isolated chloroplast ATP synthase in phospholipid vesicles was studied. The following observations were made (i) The onset of ³²P_i incorporation into ATP lags behind ATP hydrolysis. The lag lasts for 2 min at 37°C and is followed by a steady-state rate which is constant for more than 30 min. Under the same experimental conditions, ATP hydrolysis shows an initial burst followed by a constant, slower rate. (ii) The initial lag is independent of Mg-ATP concentration in the range 0.2-5 mM and of the presence of ADP. In contrast, the steady-state rate of ATP-P_i exchange has an apparent K_m of 0.3 mM for Mg-ATP and is stimulated by ADP. (iii) Increasing the temperature from 30 to 45°C decreases the lag from 6 min to zero. The steady-state rate of ATP-P_i exchange is affected to a much smaller extent by the temperature in this range. (iv) The lag is insensitive to valinomycin or tetraphenylboron, while the steady-state rate is partially inhibited. Nigericin and protonophores affect both the lag and steady-state rate. (v) ATP-induced membrane potential formation, as followed by oxonol VI, does not correlate with the lag in its kinetics and temperature dependence. ATP-induced pH gradient formation could not be detected in the proteoliposome system. (vi) Light-triggered ATP-P_i exchange in chloroplasts shows essentially the same time course as the proteoliposome system, but the lag lasts for about 20 s at room temperature and is unaffected by a preexisting proton gradient.

These results suggest that the initial lag in ATP-P_i exchange does not reflect the time required for the buildup of a protomotive force ($\Delta\bar{\mu}_{H^+}$) nor the time required to produce ADP. It is suggested, therefore, that the lag reflects an internal autocatalytic conformational change in the ATP-synthase complex which is initiated by ATP hydrolysis and which converts the enzyme from an "exclusive ATPase state" to a "reversible ATP-synthase state". This slow transition is not directly coupled to a transmembrane pH or potential gradient.

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Cooperation

Throughout the studies during 1980-83 we kept close contact by correspondence and by meetings in Urbana. The cooperation concentrated on updating the principle investigator (UP) in methods to measure water potentials in plants and in discussions of the problems encountered during the research.