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Photophosphorylation in Attached Leaves of *Helianthus annuus* at Low Water Potentials[1]

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ABSTRACT

The *in situ* response of photophosphorylation and coupling factor activity to low leaf water potential (ψ_L) was investigated using kinetic spectroscopy to measure the flash-induced electrochromic absorption change in attached sunflower (*Helianthus annuus* L. cv IS894) leaves. The electrochromic change is caused by the formation of an electric potential across the thylakoid membrane associated with proton uptake. Since depolarization of the thylakoid membrane following flash excitation is normally dominated by proton efflux through the coupling factor during ATP formation, this measurement can provide direct information about the catalytic activity of the coupling factor. Under low ψ_L conditions in which a clear nonstomatal limitation of net photosynthesis could be demonstrated, we found a strong inhibition of coupling factor activity in dark-adapted leaves which was probably caused by an increase in the energetic threshold for the activation of the enzyme at low ψ_L. While this result supported earlier *in vitro* findings, we further discovered that the light-dependent reduction of coupling factor reversed any observable effect of low ψ_L on the energetics of activation or on photophosphorylation competence. Furthermore, coupling factor was reduced, even in severely droughted sunflower, almost immediately upon illumination. Based on these measurements, we conclude that the nonstomatal limitation of photosynthesis observed by us and others in droughted plants cannot be explained by impaired coupling factor activity.

Leaves of mesophytic plants frequently exist in a somewhat dehydrated condition because the ψ_L[2] must be significantly below the soil water potential in the root zone in order to attract soil water at a rate that replaces the water inevitably lost to the atmosphere during photosynthesis. It is routinely observed (3, 26) that if ψ_L becomes too low, net photosynthesis decreases. Water deficit-induced decreases in photosynthesis occur frequently in nature and, depending on the growth conditions and species, the cause of the decrease may be stomatal closure, impaired chloroplast activity or, more commonly, both. Stomata close because guard cells fail to retain the solutes necessary to generate the osmotic potential necessary to remain open (6) which, while minimizing the further loss of water, can limit photosynthesis by constraining the rate at which CO_2 diffuses into the leaf. Despite this intuitive and well-established mechanistic connection between leaf water status and stomatal conductance, there is a broad range of experimental evidence indicating that low ψ_L may also have direct effects on biochemistry in the chloroplast. Candidates for biochemical dysfunctions include restrictions in the carbon reduction cycle (1, 10–12, 20), inhibitions of photosynthetic electron transfer (2, 4, 13, 20), and the focus of this study, impaired photophosphorylation activity (34, 35).

Younis *et al.* (34) demonstrated that thylakoids isolated from leaves with a low ψ_L were incapable of attaining rates of ATP synthesis more than 35 to 40% of controls from high ψ_L leaves. This work was subsequently extended to investigations on isolated CF which indicated that magnesium ions, concentrated in the stroma due to desiccation, caused a direct irreversible effect on CF which was, in turn, the basis of the low ψ_L inhibition of ATP synthesis observed in isolated thylakoids (35). However, it has been difficult to evaluate the relevance of these *in vitro* studies to the *in situ* inhibition of net photosynthesis at low ψ_L in attached leaves. One important consideration is distinguishing between effects that are actually part of the *in situ* inhibition and inhibitory effects induced through manipulation of tissue made labile by the stress. A second factor in evaluating the physiological relevance of the *in vitro* data is the recent recognition of the central role played by the light-dependent, thioredoxin-mediated reduction of coupling factor in the regulation of its catalytic activity (9, 15, 27, 30). Whereas the *in vitro* work mentioned above was almost exclusively with coupling factor in its oxidized state, it is now clear that it is the response of the reduced state of the enzyme to low ψ_L that is most important physiologically.

Assessing the relevance of *in vitro* investigations to the actual basis of lower net photosynthesis that occurs in leaves at low ψ_L has been further complicated by recent indications that putative nonstomatal limitations may, in some cases, have been misdiagnosed. Standard procedure has been to assess the separate stomatal and nonstomatal contribution to the overall control of photosynthesis by examining the dependence of net photosynthesis on the intercellular CO_2 level. However, evidence is beginning to emerge that inhomogeneities in stomatal conductance may exist across leaves in some

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[2] Abbreviations: ψ_L, leaf water potential; CF, coupling factor complex comprised of the extrinsic CF_1 and intrinsic CF_0 components; ΔA_{518}, flash-induced absorbance change at 518 nm; τ, time constant for relaxation of the flash-induced absorbance change at 518 nm; ΔpH, transmembrane pH difference.

situations (5, 29) including leaves with low ψ_L (19, 28). Severe nonuniformities in conductance across the leaf could invalidate the underlying assumptions used in the calculation of intercellular CO_2 levels from whole leaf measurements of photosynthesis and transpiration. The effect would be to cause an overestimation of intercellular CO_2 thereby possibly creating the illusion that nonstomatal limitations exist when none are actually present (29).

In this study we have extended the earlier *in vitro* work to the level of intact leaves by examining the effects of low ψ_L on thylakoid membrane energization and coupling factor activity *in situ*. We monitored the *in situ* activity and catalytic turnover of CF by measuring the change that occurs in the absorption spectrum of certain pigments upon the formation of the flash-induced electric potential across the thylakoid membrane (33). This spectral change, known as the electrochromic change, can provide direct information about coupling factor catalytic activity because the proton efflux through CF that drives ATP formation is normally the dominant ionic current responsible for depolarization of the membrane. Thus, proton efflux through CF is the major factor controlling the relaxation kinetics of the electrochromic change. We focused on conditions in which a clear, low ψ_L-induced, nonstomatal limitation on net photosynthesis could be demonstrated. Our *in situ* results on dark-adapted sunflower leaves corroborated the earlier *in vitro* results and, in addition, we were able to show that inhibition of coupling factor activity by low ψ_L was probably due to an increase in the energetic threshold for the activation of the enzyme. However, light-dependent reduction of CF largely, if not entirely, reversed the inhibition. Since the coupling factor pool is reduced even in severely droughted sunflower almost immediately upon illumination, it does not appear that the effect that low ψ_L has on oxidized coupling factor carries much physiological importance. Thus, we conclude that low ψ_L effects on coupling factor activity make little contribution to the nonstomatal inhibition of net photosynthesis seen in droughted sunflower plants.

MATERIALS AND METHODS

Plant Growth and Drought Treatment Conditions

Sunflower plants (*Helianthus annuus* L. cv IS894) were raised from seed in 20 cm pots containing a soil/peat/perlite (2:1:1) mixture supplemented with Osmocote fertilizer (14:14:14). The plants were grown in a controlled environment chamber that maintained an air temperature of 28/25 ± 2°C and RH of 70/85 ± 5% during the 12 h day/12 h night cycle. Illumination was supplied by cool-white fluorescent lamps and incandescent bulbs in a wattage ratio of 4:1 at a PPFD of 600 to 800 $\mu mol \cdot m^{-2} \cdot s^{-1}$.

Control plants were watered when the soil surface became dry thereby maintaining a nearly constant ψ_L (−0.2 to −0.4 MPa) during the entire growth period. Leaf water potentials as low as −2.5 MPa were induced in fully expanded leaves of 3 to 4 week old plants by withholding water from the soil for 4 to 6 d (Fig. 1). Leaf water potentials were determined by isopiestic thermocouple psychrometry as detailed elsewhere (6).

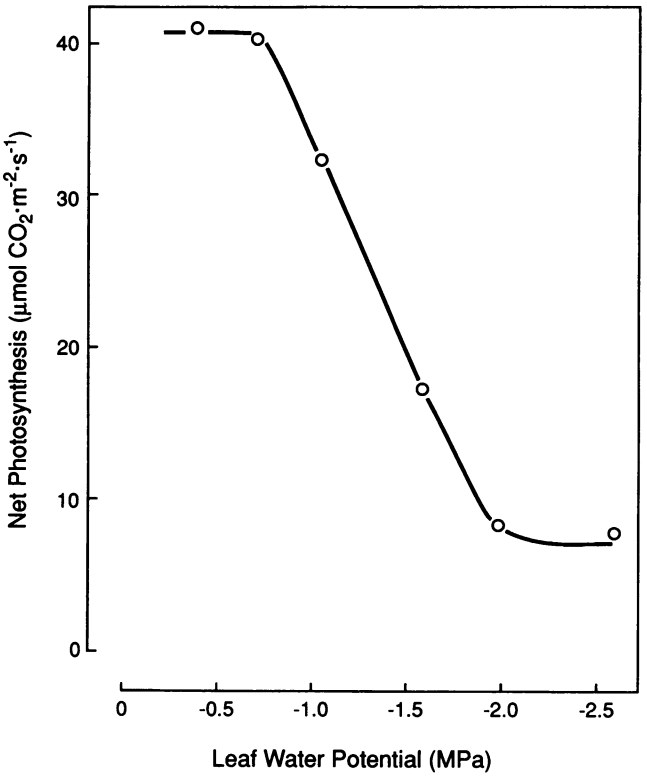

Figure 1. Dependence of net photosynthesis on water potential of attached sunflower leaves. The rate of net photosynthesis was measured at 25°C at saturating irradiance (1600 $\mu mol \cdot m^{-2} \cdot s^{-1}$) in air containing 3000 $\mu L \cdot L^{-1}$ CO_2 on the same leaf over a period of 6 d as the plant dehydrated to progressively lower ψ_L.

Measurements of Net Photosynthesis

Air containing 3000 μL $CO_2 \cdot L^{-1}$ was passed through an assimilation chamber at a constant flow rate (0.05 $L \cdot s^{-1}$). The rate of net photosynthesis was calculated from the change in CO_2 concentration measured with an infrared gas analyzer (Analytical Development Co., LTD, Model 225-MK3, Hoddesdon, U.K.). The leaf area was determined gravimetrically from a tracing of the leaf on paper.

Radiation (PPFD = 1600 $\mu mol \cdot m^{-2} \cdot s^{-1}$) was supplied by a 1000 W General Electric FSS Quartzline bulb (5200K filament) positioned above the leaf chamber. The light was filtered through a 25 × 25 cm IR reflective mirror (Optical Coatings Laboratory, Inc. Santa Rosa, CA) and through 10 cm of water before reaching the leaf. These precautions, in addition to rapid stirring within the chamber to maintain a minimal boundary layer resistance, ensured that the leaf temperature was within 1 degree of the 25°C air temperature.

Visualization of the Uniformity of Photosynthesis within Attached Leaves

Attached sunflower leaves were illuminated in an assimilation chamber at PPFD of 1600 $\mu mol \cdot m^{-2} \cdot s^{-1}$ at an ambient $[CO_2]$ of 350 $\mu L \cdot L^{-1}$ until a steady-state rate of photosynthesis was attained. Thereafter, $^{14}CO_2$ (18.6 $GBq \cdot mol^{-1}$) was pulsed into the chamber. After 60 s of illumination, the leaf was

Table I. *Effect of Rewatering on the Rate of Net Photosynthesis (P), Stomatal Conductance (g_{H_2O}), and Ψ_L*

The stomatal conductance and the rates of net photosynthesis were measured using a Li-Cor 6200 IR gas analysis system in an atmosphere containing 350 $\mu L \cdot L^{-1}$ CO_2 and at an irradiance of 1600 $\mu mol \cdot m^{-2} \cdot s^{-1}$. The range of values obtained for each treatment are reported with the sample size given in parentheses.

Treatment	Ψ_L	P	g_{H_2O}
	MPa	*$\mu mol\ CO_2 \cdot m^{-2} \cdot s^{-1}$*	*$mol \cdot m^{-2} \cdot s^{-1}$*
Well-watered (10)	−0.33 to −0.5	27–33	0.5–1.7
Droughted (8)	−1.6 to −1.8	3–5	0.03–0.07
Rewatered[a] (6)	−0.3 to −0.55	17–18	0.4–1.5

[a] Twenty-four hours after rewatering.

rapidly harvested in the dark and quickly frozen between aluminum plates at dry ice temperature. Radioactive photosynthate was detected in the leaf by autoradiography (19). The images show that very little radioactivity entered the leaf veins, indicating that little if any translocation or intercellular movement of radioactive photosynthate occurred on the short time scale of these measurements.

Flash-Induced Absorption Change in Attached Leaves

The flash-induced electrochromic absorption change was measured in attached leaves as described previously (32) using a laboratory-built single beam spectrophotometer. We have shown in earlier work (18) that the wavelength dependence of the flash-induced absorption change in attached sunflower leaves has a maximum at 518 nm and is indistinguishable from spectra we have observed for other plants and very similar to spectra observed by us (32) and others (33) in isolated thylakoid membranes. Although there were no detectable differences in the spectra taken on dehydrated leaves, we did take the additional precaution of using the reference wavelength of 540 nm to ensure that the electrochromic change was isolated from any other contributing factors such as light-induced scattering changes that might be accentuated at low ψ_L. We found that, even in severely dehydrated leaves, the correction was negligible.

The relaxation of the flash-induced absorption change at 518 nm has a biphasic decay. The initial, faster component of the decay is mechanistically associated with the phosphorylation of ADP (33) and was used in this study to monitor *in situ* photophosphorylation. Kinetic analysis of signal averaged data was accomplished by computer fitting the relaxation data to the sum of two first order exponentials using an iterative, nonlinear least squares program.

RESULTS

Low Water Potentials Cause Nonstomatal Inhibition of Photosynthesis in Sunflower Leaves

Withholding water from the soil of 3 to 4 week old potted sunflower plants caused a gradual decline in the ψ_L reaching values near −2.5 MPa after about 6 d without watering. At atmospheric levels of CO_2, autoradiographic images of leaves pulse-labeled with $^{14}CO_2$ showed a nonuniform pattern of photosynthesis at ψ_L below about −0.8 MPa (19). Figure 1 shows that the rate of net photosynthesis measured at saturating irradiance and very high ambient CO_2 levels (3000 $\mu L \cdot L^{-1}$) began to decline at ψ_L values near −0.8 MPa and eventually reached a plateau of about 90% inhibition at ψ_L values below −1.8 MPa. In this advanced state of dehydration the average stomatal conductance was quite low (Table I). Autoradiographic images showed that photosynthesis was low but relatively uniform within these leaves (Fig. 2B). Twenty-four hours after rewatering, nearly a 50% inhibition of net photosynthesis persisted even though leaf water potential and the average stomatal conductance had recovered (Table I). The uniform distribution of radioactive photosynthate in the rewatered leaves (Fig. 2C) shows that, even at normal atmospheric CO_2 levels (350 $\mu L \cdot L^{-1}$), there were few, if any, "patches" in the leaf of anomalously low conductance. Taken together these data provide convincing documentation of a dominant nonstomatal inhibition to net photosynthesis reported in Figure 1.

Illumination Causes Reversible Reduction of Coupling Factor in Sunflower Leaves

In this set of experiments we have taken advantage of the contribution that the electrical potential of accumulated protons makes to flash-driven ATP formation (8) to monitor the reduction of coupling factor in attached leaves in response to illumination. The fate of the electric potential across thylakoid membranes can be conveniently investigated, even in intact leaves, through the electrochromic absorption band shift, a red shift induced in the absorption spectrum of a specialized group of pigments in the membrane by the transmembrane

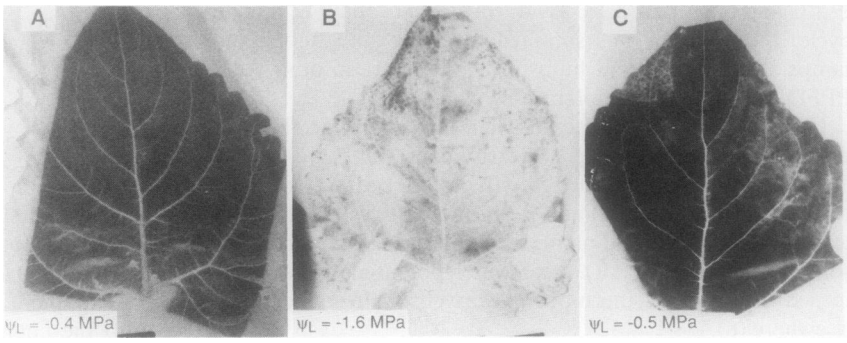

Figure 2. Autoradiograph of radioactive photoassimilate in attached sunflower leaves pulse-labeled with $^{14}CO_2$. Steady-state rates of photosynthesis were established at PPFD of 1600 $\mu mol \cdot m^{-2} \cdot s^{-1}$ at an ambient [CO_2] of 350 $\mu L \cdot L^{-1}$ before the leaf was exposed to $^{14}CO_2$ for 60 s. The autoradiographs are typical of leaves on A, well-watered plants (ψ_L = −0.3 to −0.6 MPa); B, strongly droughted plants (ψ_L = −1.6 to −2.0 MPa); and C, droughted plants 24 h after rewatering.

electric field (33). Proton efflux through the coupling factor associated with ATP formation can overwhelm all other membrane depolarizing fluxes and thereby cause up to a 10-fold faster relaxation of the flash-induced electrochromic change than would occur otherwise.

Figure 3A is a kinetic measurement of the flash-induced electrochromic change (ΔA_{518}) in sunflower leaves of adequately watered plants after 12 h of dark adaptation. The very slow decay of ΔA_{518} ($\tau > 300$ ms) indicates that no ATP synthesis was induced by the four half-saturating flashes (given at 3.5 min intervals) used to obtain the trace in Figure 3A. However, after a 90 s preillumination (PPFD = 600 μmol·m^{-2}·s^{-1}) followed by a 2 min dark interval, the ΔA_{518} decayed quite rapidly (τ = 15–30 ms, Fig. 3B) due to rapid proton efflux through activated coupling factor associated with ATP formation (15, 17, 32). It was verified previously that the 2 min dark interval was sufficient to allow the dissipation of thylakoid energization caused by the preillumination (15, 19). Thus, after the 2 min dark interval, the preilluminated leaves synthesized ATP in response to energization by the half-saturating flashes, whereas the 12 h dark-adapted leaves did not. The reduction of the γ-subunit of coupling factor, mediated *in situ* by thioredoxin *f* (14), has been shown by Hangarter *et al.* (9) to lower the energetic requirement for catalytic activation of CF. It is almost certainly the case that it is this lowering of the energetic threshold for coupling factor activation by reduction that permits, at the same level of energization, ATP formation to occur in the preilluminated but not the dark-adapted leaves (15, 32).

The time course of the reoxidation of the coupling factor was determined by monitoring the length of dark interval required for the rapid relaxation of the ΔA_{518} seen after 90 s of preillumination (*i.e.* τ = 15–30 ms) to return to the very slow decay (*i.e.* $\tau > 300$ ms) characteristic of dark-adapted leaves. Figure 4 illustrates that the half-time for reoxidation following 90 s of preillumination at an intensity close to that of the growth irradiance was about 30 min. Thus, no significant amount of CF oxidation occurred during the 2 min dark interval following preillumination.

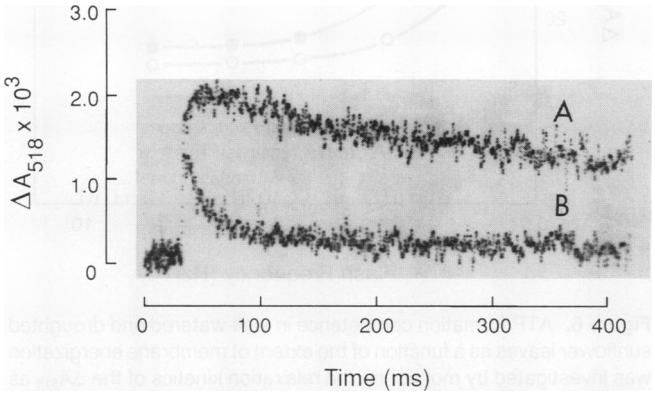

Figure 3. Effect of preillumination on the flash-induced ΔA_{518} relaxation kinetics in attached sunflower leaves. In trace A the plant had been dark adapted for 12 h. The signal averaged trace represents the sum of four half-saturating flashes given at 3.5-min intervals. In trace B a portion of the leaf was preilluminated (600 μmol·m^{-2}·s^{-1}) for 90 s then dark adapted for 2 min before the actinic flash was given. The 2 min dark interval allowed the transmembrane electrochemical potential formed during the preillumination to dissipate. This protocol was repeated for each of the four half-saturating flashes used to obtain trace B.

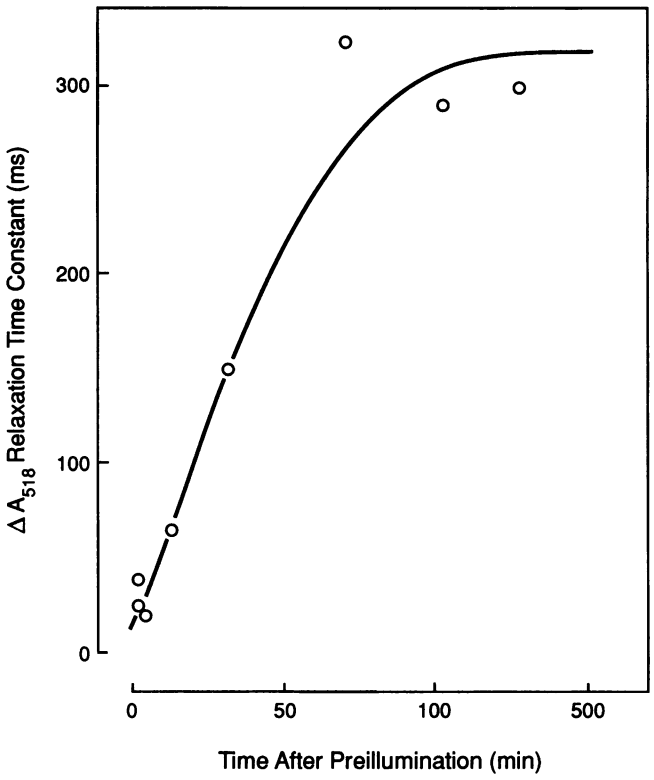

Figure 4. Time course for the reoxidation of coupling factor in attached sunflower leaves following a 90 s preillumination. A portion of an attached leaf that had been dark adapted for 12 h was preilluminated for 90 s at 600 μmol·m^{-2}·s^{-1}. The redox state of the coupling factor was then probed after various dark intervals (from 0.5–120 min) by measuring the relaxation kinetics of the ΔA_{518} induced by a half-saturating flash. The relaxation time constants presented were calculated from the signal averaged traces of the two runs.

Low Water Potential Inhibits Flash-Induced ATP Formation in Dark-Adapted Sunflower Leaves but the Inhibition is Reversed by Preillumination

When fully saturating flashes are substituted for the half-saturating pulses used in Figure 3, sufficient energization can be generated by a single flash to activate CF and induce net ATP formation even in dark-adapted leaves (*i.e.* leaves in which CF is oxidized). The relaxation kinetics of the flash-induced electrochromic change induced by a saturating flash in an attached leaf of a well-watered plant (Fig. 5B) are those expected for thylakoid membranes in which there is nearly maximal efflux of H^+ (τ = 38 ms) through CF during ATP formation. When low water potentials (−2.5 MPa in Fig. 5A) are imposed by withholding water from the soil, essentially no ATP formation is induced in a dark-adapted leaf by a saturating flash ($\tau > 300$ ms). Comparing the traces in Figure

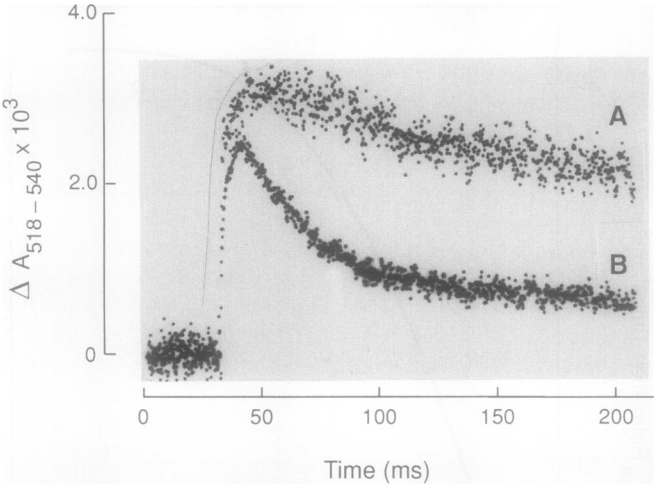

Figure 5. Low leaf water potential prevented the rapid relaxation of the $\Delta A_{518-540}$ in dark-adapted sunflower leaves. Trace A was taken on a droughted leaf (ψ_L = −2.5 MPa) and trace B is from the same leaf before water was withheld. The signal averaged traces are the difference between 16 measurements taken at 540 nm and 16 measurements taken at 518 nm. Saturating flashes were used.

5 it can be seen that the optical signal is 10 to 20% larger in amplitude at low ψ_L. Adapting the procedure of Rühle and Wild (23) we found that dehydration (−2.0 MPa) caused a 10 to 15% increase in the optical pathlength taken by the measuring beam through the leaf, thereby accounting for larger flash-induced absorbance change (19).

The long intervening dark times in Figure 5 (30 s) did not allow sequential flashes to be significantly cumulative for membrane energization. When the sequential flashes are more closely spaced they become increasingly additive resulting in the formation of a proportionally larger electrochemical potential. In well-watered leaves, distantly spaced saturating flashes were individually adequate to activate CF and induce rapid net ATP formation (Fig. 5B) so that the relaxation kinetics became only slightly faster at higher frequencies (Fig. 6A). However, the larger electrochemical potential generated by closely spaced flashes significantly accelerated ATP formation in the −2.5 MPa leaves, although even the fastest rate was barely half that of the control (Fig. 6A).

Perhaps the most physiologically important finding of our study was that, after reduction of CF by 90 s of preillumination, the inhibition of flash-induced ATP formation all but disappeared. The relaxation time constant at either high or low ψ_L was 20 to 25 ms when saturating excitation flashes were distantly spaced and was only slightly faster (15–18 ms) when the flash frequency was increased to 3 Hz (Fig. 6B). This does not necessarily mean that the inhibition has been reversed because, if the effect of low ψ_L is on the catalytic activation of coupling factor, the inhibition may only be visible when the electrochemical potential is near the threshold value for activation. Because reduction lowers the energetic requirement for CF activation (9), we attempted to compare the competence of well-watered and droughted plants to initiate ATP formation as a function of membrane energization which was varied by attenuating the flash with neutral density filters. However, because CF reduction significantly lowers the energetic threshold for activation, we were unable to make reliable kinetic measurements with flashes weak enough to approach the threshold for activation of reduced CF. Even flashes that excited less than 5% of the reaction centers induced rapid ATP formation in preilluminated leaves, and no difference could be detected between preilluminated leaves at −0.4 *versus* −2.1 MPa (data not shown).

Low Water Potential Does Not Alter the Efficiency of Coupling Factor Reduction in Sunflower Leaves

Although preillumination of sunflower leaves nearly eliminated any observable inhibitory effects of low ψ_L on photophosphorylation, the low ψ_L-induced inhibition of oxidized

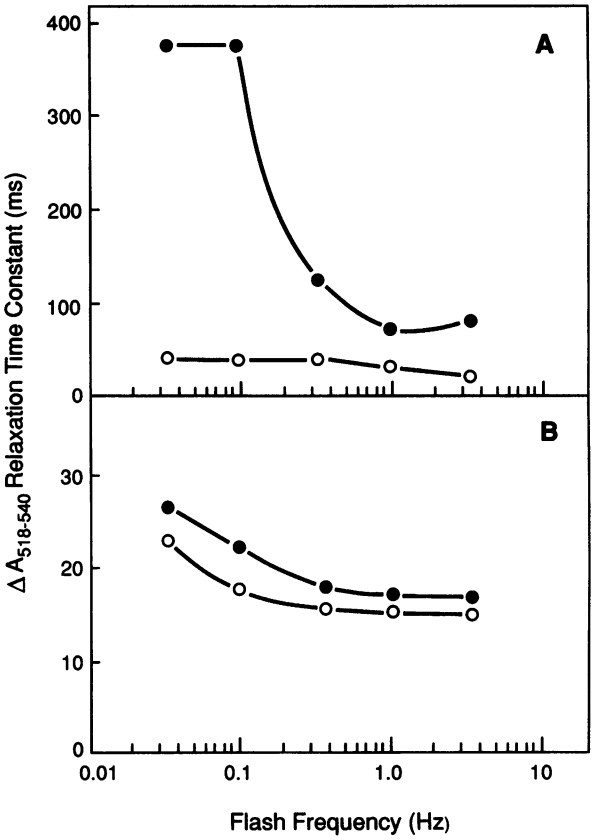

Figure 6. ATP formation competence in well-watered and droughted sunflower leaves as a function of the extent of membrane energization was investigated by monitoring the relaxation kinetics of the ΔA_{518} as a function of flash frequency. The time constant (τ) was calculated from signal averaged traces in which the average of 32 measurements taken at 540 nm was subtracted from the normalized average of 16 measurements taken at 518 nm. Fully saturating, single turnover flashes were used. The measurements were made on a single attached leaf at ψ_L values of −0.4 (○) and −2.5 MPa (●). A, Plant was dark adapted for 12 h ensuring that the coupling factor was in the oxidized state; B, dark-adapted plant was preilluminated for 90 s (600 μmol·m^{-2}·s^{-1}) to put the coupling factor into its reduced state. A 2-min dark interval separated the preillumination period and the beginning of the flash series.

CF could be physiologically meaningful if the efficiency of CF reduction is impaired in droughted plants. To investigate this possibility we looked at the light intensity dependence of *in situ* coupling factor reduction, monitoring the effect of preillumination intensity on the flash-induced ΔA_{518} relaxation kinetics in well-watered and droughted plants (Fig. 7). Even at light intensities below the light compensation point for CO_2 fixation (*e.g.* 20 $\mu mol \cdot m^{-2} \cdot s^{-1}$), preillumination of a dark-adapted leaf for 90 s resulted in a substantial reduction of the coupling factor pool in both well-watered and droughted sunflower leaves. The intensity dependence is clearly not hyperbolic which may indicate that the illumination field within the leaf was not homogeneous. In any event, 90 s of preillumination at 600 $\mu mol \cdot m^{-2} \cdot s^{-1}$ caused maximum acceleration of the decay of the absorption change at 518 nm induced by a half-saturating flash. It is likely that this indicates full reduction of the coupling factor pool in the illuminated portion of the leaf. It should be noted that longer illumination at lower intensities can also cause maximum reduction of CF (15, 19).

DISCUSSION

In this study we have been able to see in intact leaves strong effects of low ψ_L on photophosphorylation that are very likely directly related to inhibitions observed in earlier *in vitro* work with isolated thylakoid membranes (34) and purified CF (35). However, from additional experiments that were guided by recent advances in understanding the role of CF reduction in the regulation of photophosphorylation, we conclude that the nonstomatal limitation of photosynthesis seen by us and others at low ψ_L cannot be explained by impaired coupling factor activity. Preillumination is so short and irradiance so low for CF reduction that most leaves would encounter the required conditions regardless of the ψ_L during the daylight hours. Moreover, low ψ_L appears to have no direct effect on the efficiency of CF reduction, and CF would be readily maintained in the active state. Thus, after more than 10 years during which photophosphorylation has been considered a major candidate in limiting photosynthesis at low ψ_L, it is now clear that this is not the case and that attention should be focused on other factors. The results show that these factors are at the chloroplast rather than the stomatal level.

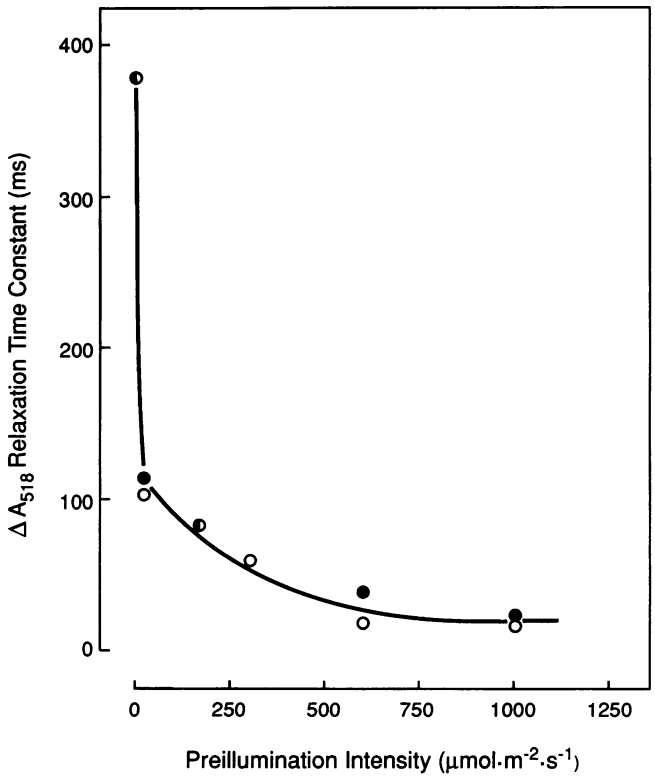

Figure 7. Dependence of coupling factor reduction on preillumination intensity in well-watered (−0.4 MPa, ○) and droughted (−2.0 MPa, ●) sunflower leaves. The leaves were preilluminated with white light of different intensities for 90 s. Thereafter the plant was dark adapted for 2 min and the relaxation kinetics of the signal-averaged ΔA_{518} traces (four half-saturating flashes, 0.1 Hz).

In some situations, dehydration can result in nonuniform photosynthesis in sunflower at ψ_L values below about −0.8 MPa (19). Sharkey and Seeman (28) observed a similar effect of mild water stress in leaves of *Phaseolus vulgaris*. At leaf water potential values below about −1.8 MPa, heterogeneity was no longer particularly evident as photosynthesis was quite low throughout the leaf (Fig. 2). It is worth noting that nonuniform photosynthesis was not seen in the leaves of sunflower grown in the field during the naturally occurring diurnal cycling of ψ_L in which values as low as −2.0 MPa were reached in the midafternoon (19, 31). While "patchy" photosynthesis is generally considered to be caused by severe inhomogeneity in stomatal conductance across the leaf, the existence of nonuniformities in photosynthesis should not be taken to mean that no biochemical limitations are involved in the control of photosynthesis or even that nonuniform stomatal conductance is the only cause of heterogeneity in photosynthesis in different parts of the leaf.

In line with our findings reported in Figure 1, Graan and Boyer (7) showed that ambient CO_2 of 3000 $\mu L \cdot L^{-1}$ was unable to fully restore the rate of net photosynthesis in sunflower leaves at ψ_L values below −1.2 MPa. In addition, they showed that further increases in the ambient CO_2 level eventually caused an inhibition of net photosynthesis in both well-watered and droughted leaves. This provides compelling evidence that CO_2 had penetrated the intercellular airspaces throughout the leaf and, therefore, that water potentials below −1.2 MPa lowered the rate of net photosynthesis in sunflower for reasons unrelated to stomatal closure. In line with the conclusions of Graan and Boyer, we found that nearly a 50% inhibition of net photosynthesis (measured at 3000 $\mu L \cdot L^{-1}$ CO_2 and saturating light) persisted after rewatering even though leaf conductance had recovered (Table I) and photosynthesis appeared uniform in autoradiographic images (Fig. 2). Thus, there is little doubt that the limitation on the rate of net photosynthesis in the low ψ_L leaves investigated in this study was dominated by nonstomatal factors.

The energetic requirement for the conversion of CF from its catalytically inactive to active state depends upon the redox state of the γ subunit. Hangarter *et al.* (9) showed that reduction of the disulfide linkage lowered the thermodynamic requirement for activation from a threshold energetically

equivalent to a ΔpH of 2.9 to a threshold of 2.6 pH units. The *in situ* manifestation of the lower activation threshold following CF reduction is seen in the effect of preillumination on the ΔA_{518} relaxation kinetics in which subsaturating flashes induced rapid decay (*i.e.* ATP formation) only after preillumination (Fig. 3). Under the thermodynamic conditions (*i.e.* phosphate group transfer potential of ATP) that prevail in the chloroplast during a light to dark transition, the energetic threshold for the activation of the coupling factor, even in its reduced form, exceeds the energy necessary to cause net phosphorylation of ADP (see ref. 9 for discussion). This fact ensures that, during a light to dark transition, coupling factor will remain inactive until net ATP formation is thermodynamically favored over net hydrolysis. In dark-adapted leaves, that is with CF oxidized, low ψ_L increased the size of the electrochemical potential required for the initiation of ATP formation, almost certainly reflecting a low ψ_L-induced increase in the threshold for CF activation. This effect of low water potential on photophosphorylation is evident in Figures 5 and 6 which show that, although individual saturating flashes induced rapid ATP formation in well-watered sunflower leaves, larger potentials generated by the cumulative energization of sequential flashes were required to induce ATP formation in droughted leaves.

The light-dependent, thioredoxin-mediated reduction of CF effectively reverses any observable inhibition by low ψ_L on photophosphorylation in attached sunflower leaves. We were unable to experimentally confirm our expectation that the low ψ_L-induced increase in the energetic threshold for CF activation may still be present after preillumination. Although coupling factor reduction lowers the threshold only by about 10% (9), the fact that the formation of the electrochemical potential is exceedingly nonlinear with proton accumulation (8, 21) means that reduction lowers the threshold flash strength more than 20-fold. While this mechanistic point remains unresolved, it is clear that if an effect of low ψ_L on photophosphorylation is present after preillumination it would manifest its inhibition only at extremely low irradiance levels. Furthermore, a mere 90 s of preillumination at an intensity near the light compensation point for net CO_2 fixation caused substantial, if not complete, reduction of the CF pool in both well-watered and dehydrated leaves (Fig. 7). It is an interesting fact that the quantum efficiency of coupling factor reduction can be remarkably high, accounting for more than 10% of initial electron transport (19). Other thioredoxin-modulated chloroplast enzymes are reported to require substantially more light for reductive activation (16, 22, 24, 25).

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