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FLUORESCENCE INDUCTION AND ACTIVITY OF FERREDOXIN-NADP$^+$ REDUCTASE IN *BRYOPSIS* CHLOROPLASTS *

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Effects of medium osmolarity on the rate of CO_2 fixation, the rate of the NADP$^+$-Hill reaction, and the DPS_1 transient of chlorophyll fluorescence were measured in intact *Bryopsis* chloroplasts. Upon decreasing the sorbitol concentration from 1.0 M (the isoosmotic conditions) to 0.25 M, the envelopes of the chloroplasts became leaky to small molecules, resulting in a considerable depression of the CO_2-fixation rate and a higher rate of the NADP$^+$-Hill reaction whereas the DPS_1 transient was unaffected. This DPS_1 transient of chlorophyll fluorescence is thought to be caused by the photoactivation of electron flow on the reducing side of Photosystem I at a site occurring after ferredoxin and probably before the reduction of NADP$^+$ (Satoh, K. and Katoh, S. (1980) Plant and Cell Physiol. 21, 907–916). Little effect of NADP$^+$ on the DPS_1 transient and a marked lag in NADP$^+$ photoreduction in dark-adapted (inactivated) chloroplasts support the hypothesis that the site of dark inactivation is prior to the reduction site of NADP$^+$, and therefore, that ferredoxin-NADP$^+$ reductase is inactivated in the dark and activated in the light. Moreover, at 0.25 M sorbitol, the activity of ferredoxin-NADP$^+$ reductase itself (2,6-dichlorophenolindophenol reduction by NADPH) was shown to increase according to dark-light transition of the chloroplasts. At low osmolarities (below 0.1 M sorbitol), the difference in the diaphorase activity between dark- and light-adapted chloroplasts and the lag time observed in the NADP$^+$ photoreduction were lowered. This may correspond to a less pronounced DPS_1 transient at low concentrations of sorbitol. The mechanism of the photoactivation is discussed.

Introduction

It is well known that when dark-adapted algal cells or leaves are suddenly illuminated with strong light, the yield of chlorophyll fluorescence shows several transients before it reaches a steady-state level [1–6]. The photooxidation of cytochrome *f* [7,8] and of *P*-700 also shows a similar induction phenomenon. The most pronounced transient in the induction of chlorophyll fluorescence is the DPS_1 [1,10] which occurs concomitantly with the induction of cytochrome *f* photooxidation [7] (within 5 s after the onset of illumination). The terminology of the fluorescence transient has been somewhat confused. Some authors think that the high-energy state-induced slower fluorescence quenching in higher plant chloroplasts which continues for 1–2 min is the PS decline [4,11]. But according to the terminology of Lavorel [12] and Yamagishi et al. [10], this slower decay must correspond to the MT (or M_1T) decline. The faster DPS_1 transient can be observed in leaves and intact chloroplasts but not in broken chloroplasts [13–16]. In this paper, we use the terminol-

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Abbreviations: Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonate; DCIP, 2,6-dichlorophenolindophenol; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone. DPS_1 transition, the increase and decrease in chlorophyll fluorescence which occurs from 50 ms to 5 s after the onset of illumination of chloroplasts and which is sensitive to methyl viologen.

ogy of DPS_1 to describe the transient which is complete within 5 s and has the characteristics mentioned below.

Maxwell and Biggins [9] suggested that the induction of photooxidation of *P*-700 was related to the induction of CO_2 fixation. On the other hand, Rühle and Wild [8] suggested that the transient in the photooxidation of cytochrome *f* may be related to changes in the energized state of the thylakoid membrane caused by Mg^{2+} efflux. However, KCN had no significant effect on the DPS_1 transient at the concentration where the CO_2 fixation was completely inhibited. This result suggested that the complete cycle of CO_2 fixation is not involved in the transient [7,11]. The fluorescence decline fom P to S_1 was inhibited by CCCP and nigericin whereas NH_4Cl and methylamine accelerated the transient [10,18]. Thus, the fluorescence transient cannot be related to Mg^{2+} efflux from the thylakoid membrane which is expected to be abolished by all uncouplers employed. On the other hand, methyl viologen which accepts electrons at the reducing side of Photosystem I largely decreased the DPS_1 transient [7,16,19]. Satoh et al. [7] explained the DPS_1 transient and the induction of cytochrome *f* oxidation as reflecting a photoactivation on the reducing side of Photosystem I. The proposed mechanism [7] required that during light, the inactivated site after Photosystem I inhibited electron flow to $NADP^+$. Therefore, a transient cytochrome *f* photooxidation by Photosystem I is quickly reversed due to accumulated electrons from Photosystem II. During illumination of the chloroplasts, however, photoactivation of the dark-inactivated site occurs and subsequently electrons flow freely through Photosystem I causing the reoxidation of the cytochrome. The DPS_1 transient of chlorophyll fluorescence reflects the oxidation-reduction transients of Q [20] attributed to the same phenomenon of photoactivation [7]. Recently, Satoh and Katoh [16] using intact spinach chloroplasts observed that nitrite also decreased the DPS_1 transient. Nitrite accepts electrons from reduced ferredoxin through nitrite reductase [21]. The results indicate that the dark-inactivated site is on the reducing side of ferredoxin, because nitrite reduction bypassed the inactivated site. Satoh and Katoh [16] also showed that oxalacetate and 3-phosphoglycerate which accept electrons from NADPH had little effect on the DPS_1 transient. From measurements of the activity of glyceraldehyde-3-phosphate dehydrogenase and malic dehydrogenase, they concluded that the regulation site was located before the reduction of $NADP^+$. Ferredoxin-$NADP^+$ reductase is the only enzyme occurring between ferredoxin and $NADP^+$ and, therefore, ferredoxin-$NADP^+$ reductase might be the enzyme subject to changes of activity during a dark-light transition.

In this paper, we worked with isolated intact chloroplasts from *Bryopsis* and established conditions under which the envelopes of the chloroplasts became leaky without affecting the DPS_1 transient. Under such conditions, we were able to observe the activity of the enzyme ferredoxin-$NADP^+$ reductase during the dark-light transition of the chloroplasts.

Materials and Methods

The marine green alga, *Bryopsis corticulans,* was collected at Monterey Bay, CA, U.S.A.

Preparation of intact chloroplasts was as described previously [6,18]. Chloroplasts were kept in the dark for 2 h or more before use.

Chlorophyll fluorescence was measured at right-angles to the excitation beam. The light source was a 21 V/150 W tungsten lamp. The light beam was passed through a 5 cm water layer and two Corning band-pass filters 4-96. The photomultiplier, EMI 9558B, was protected against the excitation light with a Schott cut-off filter RG-5. If not otherwise noted, the intensity of the excitation light was $2.0 \cdot 10^4$ erg/cm^2 per s.

Oxygen evolution was measured with a Clark-type oxygen electrode. The light source was a 110 V/650 W halogen lamp and the light beam was passed through a 5 cm water layer. The intensity of the actinic light was $1.7 \cdot 10^4$ lx.

Absorbance changes of DCIP and $NADP^+$ were measured with a Perkin Elmer/Hitachi 356 dual-wavelength spectrophotometer. For the measurements of the $NADP^+$-Hill reaction, a 110 V/150 W projector lamp was used as an actinic light source and the light beam was passed through a 5 cm layer of water and a Schott filter RG-2. The photomultipler was protected against the actinic light with a Balzers 340 nm interference filter. The intensity of the actinic light was $1.0 \cdot 10^4$ erg/cm^2 per s.

All reactions were carried out at room temperature. The basal reaction mixture contained, in a final volume of 2 ml, 1.0 M soribitol, 11 mM $MgCl_2$, 1 mM $MnCl_2$, 2 mM EDTA, 2 mM $NaNO_3$, 50 mM Hepes (pH 7.5) and intact *Bryopsis* chloroplasts containing the indicated amounts of chlorophyll.

Concentrations of chlorophyll were determined according to the method of Arnon [22].

Results

The difficulty in studying the cause of the DPS_1 transient was that the transient could be observed only in cells or in intact chloroplasts which had barriers to almost all ions and even small molecules. Therefore, we tried to obtain leaky chloroplasts by changing the osmolarity of the reaction medium. Fig. 1 shows the rates of CO_2 fixation (circles) and $NADP^+$-Hill reaction (triangles) at various concentrations of sorbitol in the reaction mixture. Chloroplasts were incubated in the reaction mixture for 3 min and

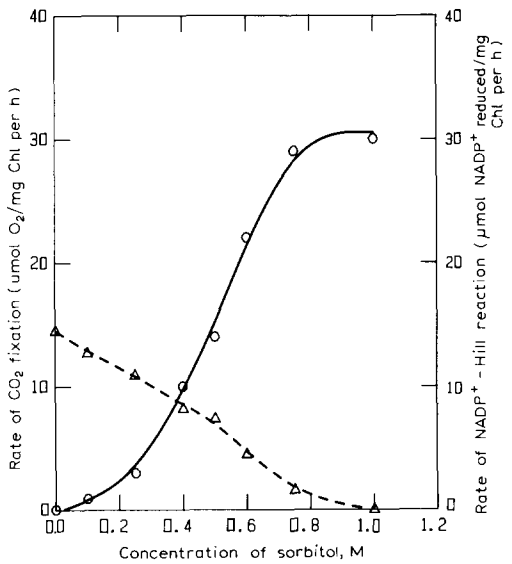

Fig. 1. Effects of concentration of sorbitol in the reaction medium on the rate of photosynthesis and the $NADP^+$-Hill reaction in *Bryopsis* chloroplasts. The reaction mixture contained in 2 ml, 50 mM Hepes (pH 7.5), 11 mM $MgCl_2$, 1 mM $MnCl_2$, 2 mM $NaNO_3$, 2 mM EDTA and various concentrations of sorbitol as indicated in the figure. Chlorophyll concentrations were 26.2 μg/ml in the photosynthesis measurements (○———○) and 10.5 μg/ml in the $NADP^+$-Hill reaction measurements (△- - - - -△).

then illuminated by the actinic light. In the case of the $NADP^+$-Hill reaction, 1 mM $NADP^+$ was added to the reaction mixture and absorbance changes at 340 nm were recorded. At 1.0 M sorbitol, *Bryopsis* chloroplasts showed a high rate of CO_2 fixation but the $NADP^+$-Hill reaction was not observed. This is because $NADP^+$ cannot penetrate into the chloroplasts in intact *Bryopsis* chloroplasts. The photoreduction of intrinsic $NADP^+$ was probably too small to be measured by our equipment. Upon lowering the concentration of sorbitol, the rate of the CO_2-fixation reaction was decreased. Concomitantly, the rate of the $NADP^+$-Hill reaction increased. At 0.25 M sorbitol, the CO_2 fixation was greatly depressed and the rate of the $NADP^+$-Hill reaction reached a relatively high level. The increase in the $NADP^+$-Hill reaction and the decrease in the CO_2 fixation show that the envelopes of the chloroplasts became leaky only to small molecules such as $NADP^+$ and that large molecules such as ferredoxin may have remained in the chloroplasts, thus explaining the high rate of the $NADP^+$-Hill reaction at low concentrations of sorbitol. This idea was supported by the reversibility test. When the sorbitol concentration was returned to 1.0 M the CO_2 fixation recovered up to 70%, indicating that most of the chloroplasts still retained their envelopes.

Fig. 2. shows the time course of chlorophyll fluorescence at various concentrations of sorbitol. The marked DPS_1 transient remained unaffected at a concentration of 0.25 M (Fig. 2b) but it became less pronounced at concentrations less than 0.1 M (Fig. 2c and d). The persistence of the DPS_1 transient despite the loss of the CO_2 fixation at low concentrations of sorbitol supports the idea that the CO_2-fixation reaction is not required for the DPS_1 transient.

Fig. 2e shows that addition of 1 mM $NADP^+$ had no significant effect (less than 6% decrease of the P level) on the DPS_1 transient at 0.25 M sorbitol, suggesting that the availability of $NADP^+$ is not a requirement for the DPS_1. Only when the sorbitol concentration was lowered below 0.1 M did the effect of $NADP^+$ become noticeable. In the absence of sorbitol, $NADP^+$ markedly decreased the extent of chlorophyll fluorescence, indicating that the functional integrity of the system may have been damaged. This may explain the decrease in the fluorescence yield upon addition of $NADP^+$.

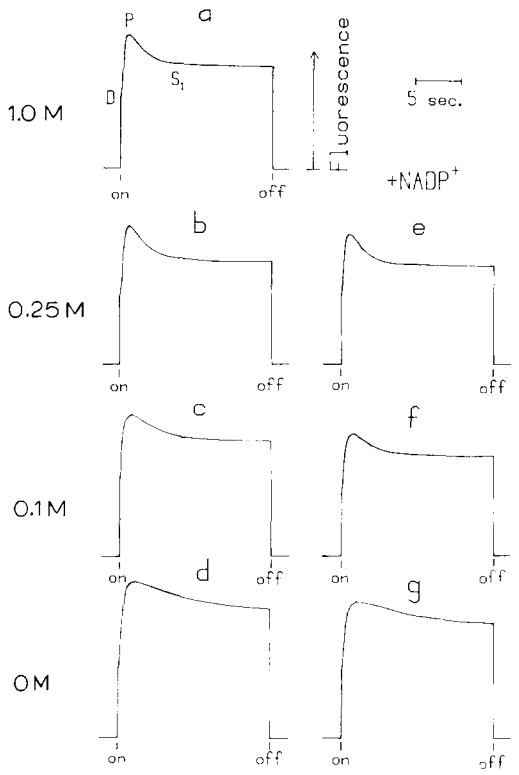

Fig. 2. Effects of concentration of sorbitol and addition of $NADP^+$ on the DPS_1 transient of chlorophyll fluorescence: (a) 1.0 M sorbitol, (b and e) 0.25 M sorbitol, (c and f) 0.1 M sorbitol, (d and g) 0 M sorbitol, 1 mM $NADP^+$ was also added in e–g. The intensity of the excitation light was $2.0 \cdot 10^4$ erg/cm^2 per s. Chlorophyll concentration was 5.41 μg/ml. Other conditions were the same as in Fig. 1.

Fig. 3 shows the time courses of $NADP^+$ photoreduction at the concentration of 0.25 M sorbitol. With dark-adapted chloroplasts, the $NADP^+$ photoreduction had a pronounced lag (3–5 s). In the preilluminated chloroplasts, the lag was shortened greatly. If we assume that ferredoxin-$NADP^+$ reductase is inactivated in the dark and that the photoactivation of the enzyme proceeds within 3 s (the time at which S_1 is observed), this lag in the $NADP^+$-photoreduction can easily be explained. With dark-adapted chloroplasts, the flavoprotein was inactivated and the photoreduction of $NADP^+$ did not occur until the enzyme was photoactivated. On the other hand, in the preilluminated chloroplasts, the enzyme was already in the activated state and the photoreduction of $NADP^+$ started immediately upon the illumination

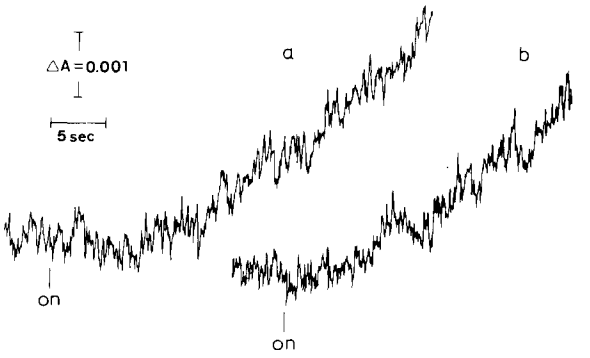

Fig. 3. Time courses of $NADP^+$ photoreduction in dark-adapted and preilluminated chloroplasts. (a) Dark-adapted chloroplasts, (b) preilluminated chloroplasts. Preillumination was done with the actinic light for 10 s, 2 min before the measurements. Sorbitol and chlorophyll concentrations were 0.25 M and 15.6 μg/ml, respectively. Other conditions were the same as in Fig. 1.

of the chloroplasts. We observed that lowering the concentration of sorbitol below the 0.1 M value eliminated the lag period (data not shown). This result correlates with the fact that addition of $NADP^+$ became effective in decreasing the fluorescence at low sorbitol concentrations only (Fig. 2).

In most higher plants and algae the dark-inactivation process occurs within a few minutes [5,9,22,24], making it difficult to observe several activities of the chloroplasts in the light-adapted state, because for measurements of the activities, we had to take some dark time due to technical reasons. However, in *Bryopsis*, the rate of the dark-inactivation process is very slow. Fig. 4 shows that the DPS_1 transient was not observed until after 10 min dark incubation following 10 s preillumination of the chloroplasts. For the complete recovery, it took about 1 h. Therefore, activity measurement within 10 min after the end of preillumination can be considered as reflecting the chloroplast function in the light-activated state. After 30 min of dark incubation of the chloroplasts at the concentration of 0.25 M sorbitol, the dark-recovery curve differed from that at 1.0 M and stayed at lower levels (Fig. 4). This may show that after prolonged exposure of the chloroplasts to low osmolarity such as 0.25 M sorbitol for more than 30 min, the structure of ferredoxin-$NADP^+$ reductase or that of the thylakoid membrane around it became further modified.

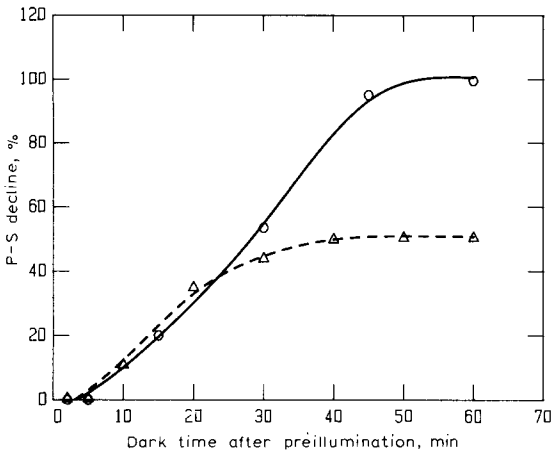

Fig. 4. Effect of dark time after preillumination of the chloroplasts on the extents of the PS_1 decline. Preillumination of the chloroplasts was done with the actinic light for 10 s and the extent of the PS_1 decline was measured after dark incubation of the chloroplasts for the periods indicated in the figure. 100% shows the full recovery to the dark-adapted chloroplasts. Chlorophyll concentration was 4.89 μg/ml. (o———o) In 1.0 M soribitol, (△- - - - -△) in 0.25 M sorbitol. Other conditions were the same as in Fig. 2.

Using this property, we measured the activity of ferredoxin-$NADP^+$ reductase in the dark- and light-adapted states. Ferredoxin-$NADP^+$ reductase is known to catalyze the reduction of DCIP by NADPH in the dark [25]. Therefore, if this enzyme changes its activity under dark and light conditions, this diaphorase activity may also change according to the dark-light transition of the chloroplasts. Fig. 5a and b shows the time course of DCIP reduction by NADPH in dark-adapted and preilluminated chloroplasts. A higher rate of diaphorase activity was observed in preilluminated chloroplasts than in dark-adapted chloroplasts. DCIP was also reduced by NADPH nonenzymatically (Fig. 5c). After subtracting this nonenzymatic reaction, the preilluminated chloroplasts were found to have a rate double that in the dark-adapted chloroplasts.

Table I documents the results from similar experiments. The diaphorase activities were measured at two sorbitol concentrations, 0.25 and 0 M. At 0.25 M, a marked difference in the rate was observed in dark- and light-adapted chloroplasts. But at 0 M sorbitol, little difference was observed.

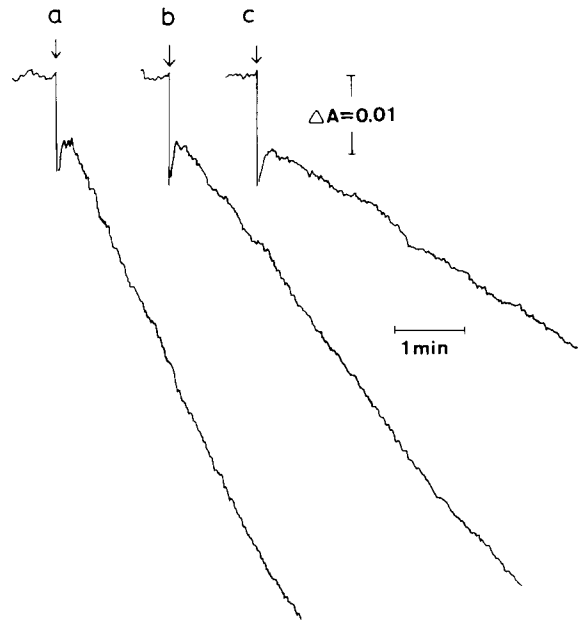

Fig. 5; Time courses of DCIP reduction by NADPH. (a) In preilluminated chloroplasts, (b) in dark-adapted chloroplasts, (c) without chloroplasts. 1 mM NADPH was added where indicated by arrows in the figure. Chloroplasts were incubated in the medium containing 0.25 M soribitol for 3 min and then illuminated with red light (greater than 650 nm, $1.7 \cdot 10^4$ erg/cm^2 per s) for 10 s in the case of a, and then 0.05 mM DCIP was added. Absorbance changes at 600 nm were observed 3 min after the addition of DCIP. Concentration of the chloroplasts was equivalent to 3.18 μg chlorophyll/ml.

Discussion

The results presented in this paper may be best explained by the idea that ferredoxin-$NADP^+$ reduc-

TABLE I

DIAPHORASE ACTIVITIES IN DARK- AND LIGHT-ADAPTED CHLOROPLASTS

Numbers in parentheses are S.D. Each value is the average of five measurements. Chlorophyll concentration was 14.8 μg/ml. Other conditions were the same as in Fig. 5.

Sorbitol concentration (M)	Rates of DCIP reduction (relative units)	
	Dark-adapted	Light-adapted
0.25	3.0 (±0.5)	5.5 (±0.9)
0	7.4 (±1.1)	7.8 (±1.0)

tase is inactivated in the dark and activated in the light, and this correlates with the DPS_1 transient in the chlorophyll fluorescence. Rühle and Wild [8] showed that the extent of the initial photooxidation of cytochrome *f* was increased by preillumination with Photosystem I light. They suggested that Photosystem I light brought the thylakoid into a state similar to State 1 and that the energized state regulated by Mg^{2+} efflux was related to the induction [8]. Their data can be explained more directly by the Photosystem I light-mediated oxidation of the plastoquinone pool, thus allowing the full extent of cytochrome *f* oxidation by a subsequent illumination. Uncouplers and ionophores such as NH_4Cl which affect the Mg^{2+} efflux apparently had no inhibitory effect on the DPS_1 transient and on the induction of cytochrome *f* photooxidation, suggesting that the high-energy state may not be involved in the transient stated [10,16]. Previously, Satoh et al. [7] reported that CCCP decreased the PS_1 decay in *Bryopsis* chloroplasts. However, the effect of CCCP was not that of an uncoupler. The rate of PS_1 decay was largely dependent upon the pH [18]. At acidic pH, the rate of PS_1 decay was very slow and at alkaline pH it was faster. The effects of uncouplers including CCCP were explained in terms of changes in the internal pH of chloroplasts which had been affected by uncoupler-induced proton transport across the outer limiting membranes and the thylakoid membranes [18].

If the hypothesis is true, the physiological meaning of the dark inactivation of ferredoxin-$NADP^+$ reductase might be as follows: under light conditions, it is necessary for the flavoprotein to be active to perform photosynthesis. But the enzyme when reduced by NADPH, also can reduce oxygen. Therefore, if it is active in the dark, it will consume NADPH and cause a loss of energy. In any case, the fact that the DPS_1 transient is observed in all algae and higher plants reported may show that it has some universal physiological application.

Unfortunately, this hypothesis has not yet been sufficiently proved. Table I shows that ferredoxin-$NADP^+$ reductase changes its diaphorase activity during a dark-light transition. But, the difference of the activity is smaller than that which would be expected. The reason for this is not clear at the moment. This may be due to contaminating flavoproteins which catalyze the DCIP reduction with NADPH or may be due to some change in the conformation of the thylakoid membrane in the vicinity of ferredoxin-$NADP^+$ reductase. However, there still remains the possibility that there is another cause of the DPS_1 transient and that the activity changes of ferredoxin-$NADP^+$ reductase observed here are only a side effect thereof. However, it is clear that the 'renal cause' is not related to high-energy states of the chloroplasts, because an uncoupler CCCP (1 μM) had little effect on the preillumination effects indicated in this paper (data not shown).

As mentioned previously, light-induced activation of the diaphorase activity of ferredoxin-$NADP^+$ reductase was not observed at lower osmolarity where the DPS_1 transient had disappeared. This corresponds well with the results that the lag of the $NADP^+$-Hill reaction was decreased and that addition of $NADP^+$ became effective in decreasing fluorescence at low sorbitol concentrations (Fig. 2). One explanation for these phenomena is that the photoactivation of ferredoxin-$NADP^+$ reductase is caused by a structural change of the enzyme itself or that of the thylakoid membrane around the enzyme and that, at the lower osmolarity, the arrangement of the conformation is already altered to some extent. Further work is now in progress in order to learn more about the mechanisms of the photoactivation of this enzyme.

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