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LIGHT-INDUCED DE-EPOXIDATION OF VIOLAXANTHIN IN LETTUCE CHLOROPLASTS

IV. THE EFFECTS OF ELECTRON-TRANSPORT CONDITIONS ON VIOLAXANTHIN AVAILABILITY*

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SUMMARY

1. In isolated chloroplasts of *Lactuca sativa* var. Manoa, the size of the violaxanthin fraction which is available for de-epoxidation is not directly dependent on electron transport but rather related to the reduced level of some electron carrier between the photosystems. This is concluded from the effects of various electron-transport conditions on violaxanthin availability: Under conditions of electron transport through both photosystems, availability was saturated at a lower electron-transport rate with actinic light at 670 than at 700 nm. Under conditions of electron transport through Photosystem I, availability was smaller for linear electron flow from reduced *N*-methylphenazonium methosulfate via methylviologen to oxygen than for cyclic electron flow mediated by either *N*-methylphenazonium methosulfate or 2,6-dichlorophenolindophenol; in addition for linea r flow from reduced *N*-methylphenazonium methosulfate via methylviologen to oxygen, availability increased with decreasing light intensity.

2. The postulated carrier whose reduced level is related to availability seems to be some carrier between plastoquinone and the primary acceptor of Photosystem II or plastoquinone itself. This conclusion follows from the fact that availability increased with increasing light intensity under conditions of electron flow through both photosystems and that 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone ($\leqslant 1\ \mu M$) had no effect on availability, whereas low levels of 3,3-(3′,4′-dichlorophenyl)-1,1-dimethylurea resulted in decreased availability (50 % decrease at 1 μM). Furthermore, availability in 3,3-(3′,4′-dichlorophenyl)-1,1-dimethylurea-poisoned chloroplasts was fully restored by 2-methyl-1,4-naphtoquinone (menadione) which mediates cyclic electron flow through plastoquinone.

3. Violaxanthin availability was zero in the dark and increased in the light to a maximum of 67 % of the total violaxanthin in chloroplasts. It is proposed that this

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Abbreviations: DBMIB, 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone; DCMU, 3,3-(3′,4′-dichlorophenyl)-1,1-dimethylurea; DPIP, 2,6-dichlorophenolindophenol; HEPES, *N*-2-hydroxethylpiperazine-*N*′,2-ethanesulfonic acid; PMS, *N*-methylphenazonium methosulfate.

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variable violaxanthin availability reflects conformational changes on the internal surface of the thylakoid membrane which result in variable exposure of violaxanthin to the de-epoxidase. The fact that not all of the violaxanthin was available for de-epoxidation may indicate a heterogenous distribution of violaxanthin in the membrane.

INTRODUCTION

When isolated chloroplasts are illuminated in the presence of ascorbate, violaxanthin is converted to zeaxanthin via the intermediate antheraxanthin [1, 2]. This pathway is a partial reaction of the wellknown violaxanthin cycle [3] which occurs in green algae as well as in higher plants [4–6].

Hager [7] showed that in spinach chloroplasts the de-epoxidase which is inactive at neutral pH is activated by light-induced acidification of the thylakoid loculus. In lettuce chloroplasts, we recently reported that light has at least two effects on de-epoxidation, namely, it not only establishes the thylakoid pH which activates the enzyme but also affects the fraction of violaxanthin in the thylakoid membrane which can be de-epoxidized [8]. The fact that violaxanthin is located in the lipophilic part of the thylakoid membrane and that lipid domains of the membrane undergo changes in organization upon illumination [9, 10] raises the possibility that the variable availability of violaxanthin could be a reflection of changes in membrane conformation.

The two effects of light on de-epoxidation can be easily distinguished. De-epoxidase activity is evident in the first-order rate constant of de-epoxidation whereas availability is seen in the final extent of de-epoxidation [8]. Since de-epoxidase activity and violaxanthin availability react to light differently, with the former reaching saturation at a much higher intensity than the latter [8], violaxanthin availability may not be influenced by thylakoid pH as is de-epoxidase activity but by some other photosynthetic factor. In the present study the effects of various electron-transport conditions on violaxanthin availability were investigated. The results support the view that violaxanthin availability is related to the reduced level of some electron carrier located before the rate-limiting step between Photosystems I and II.

MATERIAL AND METHODS

Washed, whole chloroplasts were isolated from *Lactuca sativa* var. Manoa as described previously [8]. Reactions (in a final volume of 3 ml) were run at 26 °C under actinic light from a Unitron Model LKR illuminator which was filtered through 670- or 700-nm interference filters (Baird Atomic Type I, halfband width 10 nm). Actinic light intensity was measured with a YSI Model 65 radiometer.

Stock solutions of 3,3-(3′,4′-dichlorophenyl)-1,1-dimethylurea (DCMU) or 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone (DBMIB) were prepared in methanol or in ethanol ethylene glycol (1 : 1, v/v) and diluted so that the final concentration of the organic solvent in the reaction mixture never exceeded 1 %.

The rate of electron transport mediated by methylviologen was measured as oxygen uptake in an oxygen electrode cell (Rank Brothers Bottisham). Violaxanthin de-epoxidation was monitored as the absorbance increase at 505 nm [11] in a Perkin-

Elmer Model 356 Two Wavelength Double-beam Spectrophotometer as reported previously [8]. Chlorophyll concentration was determined according to Vernon [12].

RESULTS

De-epoxidation under conditions of linear electron flow through Photosystems I and II

The effect of electron transport under 670 and 700 nm actinic light. De-epoxidation and electron transport of isolated chloroplasts in the presence of methylviologen were determined under actinic light of varying intensities. Fig. 1 shows the two parameters which characterize de-epoxidation, substrate availability (Fig. 1a) and de-epoxidase activity (Fig. 1b), plotted against electron transport.

The effects of electron transport on violaxanthin availability under 670- and 700-nm light were different. Although violaxanthin availability increased with increasing electron transport in both cases, it was maximal at a considerably lower electron-

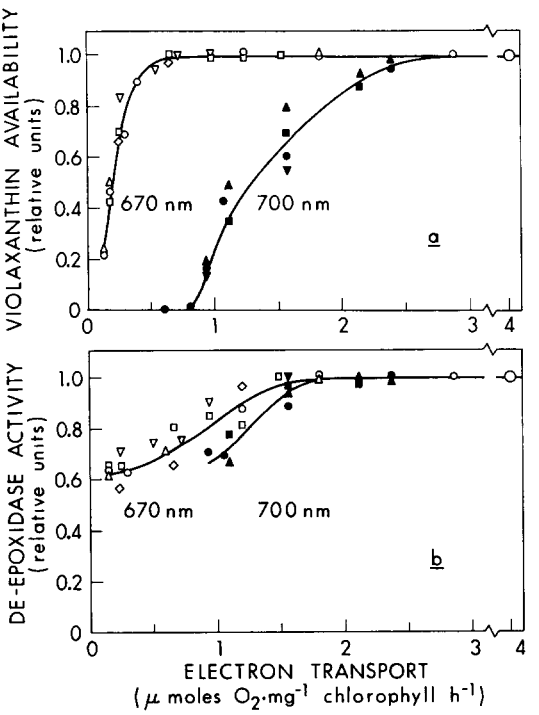

Fig. 1. Effect of electron transport on de-epoxidation in lettuce chloroplasts under 670- and 700-nm actinic light; (a) effect on violaxanthin availability, (b) effect on de-epoxidase activity. The various symbols represent different chloroplast preparations. For each preparation the amount of available violaxanthin and the de-epoxidase activity are shown relative to the control values in 670-nm light at 36 kerg · cm^{-2} · s^{-1} which were about 7 mol violaxanthin per 100 mol chlorophyll *a* and about 0.29 min^{-1} respectively. The reaction mixtures contained 400 mM sorbitol, 10 mM NaCl, 50 mM HEPES/NaOH buffer (pH 7.0), 16 mM sodium ascorbate, 100 μM NaN_3, 50 μM methylviologen and isolated chloroplasts equivalent to 14–16 μg chlorophyll per ml. An electron-transport rate of 1 μmol oxygen uptake per mg chlorophyl per h was equivalent to 6.6 kerg · cm^{-2} · s^{-1} for 670 nm light and 11.4 kerg · cm^{-2} · s^{-1} for 700-nm light.

transport rate under 670- than under 700-nm light. In contrast, the effects of electron transport on de-epoxidase activity under 670- and 700-nm light were similar with activity reaching saturation at an electron transport rate of about 2 μmol oxygen uptake per mg chlorophyll per h. The small difference in activity observed below saturation was considered to be within experimental error.

No de-epoxidation was observed in 700-nm light at electron-transport rates below 0.8 μmol oxygen uptake per mg chlorophyll per h. The fact that de-epoxidation did occur in 670-nm light at electron-transport rates below this value indicates that such rates were sufficient to establish the low thylakoid pH that is needed for de-epoxidase activity. Therefore lack of de-epoxidation in 700-nm light was not caused by too high a thylakoid pH but by the absence or "zero availability" of substrate. It follows that violaxanthin availability is also zero in the dark.

Maximal violaxanthin availability as well as maximal de-epoxidase activity were attained well within the intensity range where electron transport increases linearly with light intensity. It is also noted that even at saturation the electron-transport rate was low (about 12 μmol oxygen uptake per mg chlorophyll per h) owing to the pH of the medium (7.0) which was optimal for de-epoxidation but not

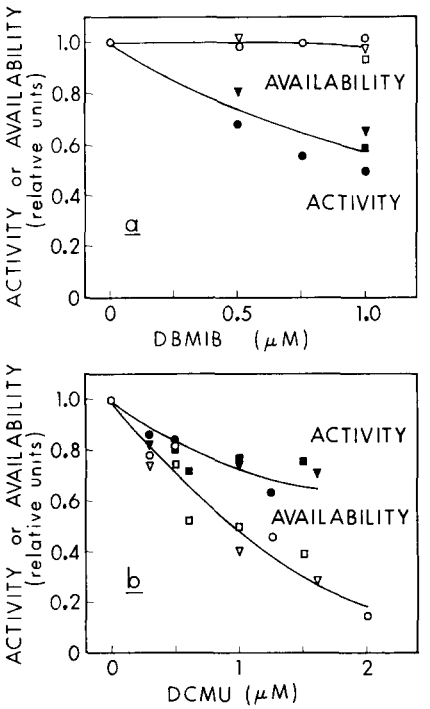

Fig. 2. Effect of electron-transport inhibitors DBMIB (a) and DCMU (b) on violaxanthin availability and de-epoxidase activity in lettuce chloroplasts. The various symbols represent different chloroplast preparations. For each preparation the amount of available violaxanthin and the de-epoxidase activity are shown relative to the control values without inhibitor. The reaction mixture contained 400 mM sorbitol, 10 mM NaCl, 50 mM HEPES/NaOH buffer (pH 7.0), 16 mM sodium ascorbate, isolated chloroplasts equivalent to 14–16 μg chlorophyll per ml and the inhibitors at indicated concentrations. Actinic light of 670 nm at 36 kerg · cm^{-2} · s^{-1} was used.

for electron transport. At pH 8.0, a condition more favorable for high electron-transport rates [13], these chloroplast preparations showed electron-transport rates of about 200 μmol oxygen uptake per mg chlorophyll per h in the absence of ascorbate.

The effects of DBMIB and DCMU. Fig. 2 shows the effects of DBMIB and DCMU on violaxanthin availability and de-epoxidase activity. Whereas addition of 1 μM DBMIB lowered de-epoxidase activity by about 40 %, availability remained unchanged. Higher DBMIB concentrations also decreased availability and completely inhibited de-epoxidation at about 4 μM DBMIB. Contrary to DBMIB, low levels of DCMU affected availability to a larger degree than activity. For example, at 1 μM DCMU availability was half maximal while activity was decreased by only about 25 %. Complete inhibition of de-epoxidation was also reached at about 4 μM DCMU. The concentrations required for full inhibition were relatively high as compared with inhibition of electron transport [14, 15] which may be owing to the fact that de-epoxidation is saturated at low electron-transport rates (Fig. 1).

De-epoxidation under conditions of electron flow through Photosystem I.

The effect of mediators of cyclic electron flow. Although it is known that 2,6-dichlorophenolindophenol (DPIP) and *N*-methylphenazonium methosulfate (PMS) can overcome the inhibition of de-epoxidation by DCMU [2, 11], the effect of these electron-transport mediators on availability has not been studied. Table I shows the

TABLE I

EFFECT OF MEDIATORS OF CYCLIC ELECTRON FLOW ON DE-EPOXIDATION IN DCMU-POISONED CHLOROPLASTS

Chloroplast suspensions containing 12–14 μg chlorophyll per ml were illuminated with actinic light of 670 nm at 36 kerg · cm^{-2} · s^{-1}. When DPIP or PMS was present, the reaction mixture contained 400 mM sorbitol, 10 mM NaCl, 50 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES)/NaOH buffer (pH 7.0), 16 mM sodium ascorbate, 6 μM DCMU and DPIP or PMS at the indicated concentrations; de-epoxidation was initiated by light. For the menadione experiment, the reaction mixture contained 350 mM sorbitol, 50 mM glucose, 10 mM NaCl, 50 mM HEPES/NaOH buffer (pH 7.0), a glucose oxidase/catalase mixture (DeeO, Miles Chemical Company) to achieve anaerobic conditions and 40 μM menadione: after 1 min of illumination 6 μM DCMU was added and de-epoxidation was initiated 1 min later with 16 mM sodium ascorbate. De-epoxidase activity and violaxanthin availability are expressed relative to the control which contained 400 mM sorbitol, 10 mM NaCl, 50 mM HEPES/NaOH buffer (pH 7.0) and 16 mM sodium ascorbate.

Addition	μM	De-epoxidase activity (relative units)	Violaxanthin availability (relative units)
DPIP	2	0.21	0.45
	9	0.21	0.89
	15	0.23	1.01
	30	0.26	0.98
PMS	2	0.90	0.99
	10	0.93	0.98
	25	0.80	1.03
	50	0.68	1.02
Menadione	40	0.63	0.96

effect of varying amounts of DPIP and PMS on the relative availability of violaxanthin as well as on the relative de-epoxidase activity in DCMU-poisoned chloroplasts. Increasing concentrations of DPIP increased the relative availability, reaching control levels at 15 μM DPIP. In contrast, relative activity was restored to only approx. 20 % of the control value at all DPIP concentrations tested. The latter would appear to be consistent with the known effects of DPIP which only partially restores proton pumping and phosphorylation [16].

In contrast with the effect of DPIP, PMS not only restored availability completely but also nearly fully restored de-epoxidase activity. At high PMS concentrations ($>$ 25 μM) relative activity was less than at lower PMS concentrations probably owing to the mild uncoupling effect at high concentrations [17].

Table I also shows that de-epoxidation occurred in DCMU-poisoned chloroplasts when cyclic electron flow was mediated by menadione (2-methyl-1,4-naphtoquinone) under anaerobic conditions. Menadione completely restored availability but not de-epoxidase activity. It should be noted that although DBMIB (2 μM) completely inhibited de-epoxidation in the menadione system, it had little effect under conditions of PMS-mediated electron flow. This observation is consistent with the fact that the plastoquinone antagonist DBMIB blocks menadione-mediated cyclic electron flow through plastoquinone [18] but has no influence on cyclic systems that bypass plastoquinone, as with PMS [18].

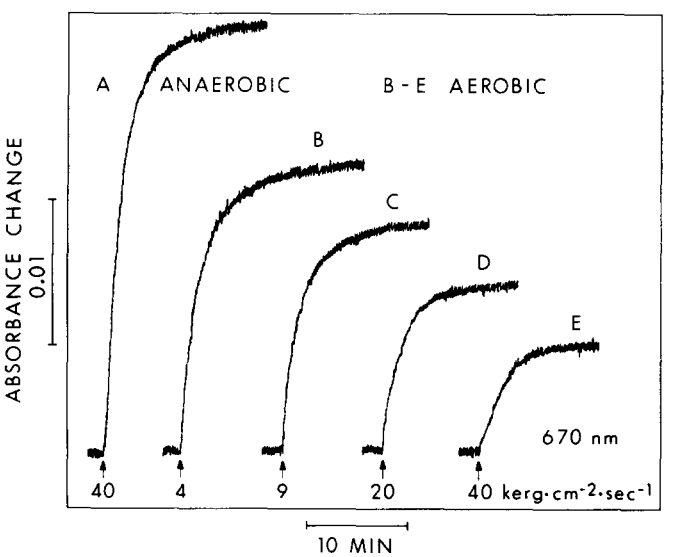

Fig. 3. Effect of light intensity on de-epoxidation under conditions of linear electron transport from reduced PMS to oxygen. De-epoxidation of violaxanthin is shown as the absorbance increase at 505 nm with 540 nm as reference. Curve A, de-epoxidation under anaerobic conditions; Curves B–E, de-epoxidation under aerobic conditions. The reaction mixture contained 350 mM sorbitol. 50 mM glucose, 10 mM NaCl, 50 mM HEPES/NaOH buffer (pH 7.0), 16 mM sodium ascorbate, 50 μM methylviologen, 2 μM PMS and chloroplasts equivalent to 13.0 μg chlorophyll per ml. Anaerobic conditions were achieved by adding a glucose oxidase/catalase mixture (DeeO, Miles Chemical Company), aerobic conditions by flushing oxygen on the surface of the reaction mixture. Actinic light of 670 nm was used.

The effect of light intensity under conditions of linear electron flow. Fig. 3 shows that violaxanthin availability under conditions of linear electron flow from reduced PMS via methylviologen to oxygen (Curves B–E) was considerably smaller than under PMS-mediated cyclic electron flow (Curve A). Under conditions of linear electron flow, availability increased as light intensity decreased. The difference in violaxanthin availability under high and low intensity illumination was sensitive to PMS concentration and was most evident under low PMS concentration.

DISCUSSION

Detailed kinetic studies on violaxanthin de-epoxidation in chloroplasts have revealed that this reaction is determined by two light-dependent processes, namely, the activation of the enzyme and the establishment of a defined amount of available substrate [8]. Earlier to this finding, the amount of violaxanthin which was de-epoxidized within a given illumination period was used to measure de-epoxidase activity [7, 11]. Although it is now evident that such data reflect a mixture of enzyme activity and substrate availability, they have served to establish a relationship between thylakoid pH and de-epoxidase activity [7]. Using the first-order rate constant of de-epoxidation as a more correct measure of de-epoxidase activity, our data (Fig. 1b) show that de-epoxidase activity is dependent on electron transport or some closely linked process which is not influenced by actinic wavelengths. This finding together with the previously reported fact that de-epoxidase activity and proton pumping are saturated at similar light intensities [8] confirm that light influences activation of the enzyme by lowering the thylakoid pH through proton pumping.

Relationship between violaxanthin availability and photosynthetic processes

Our results show that violaxanthin availability does not depend directly on electron transport or some closely linked process like proton pumping. This conclusion follows from the fact that violaxanthin availability for a given electron-transport rate was different depending on whether chloroplasts were illuminated with 670- or 700-nm light (Fig. 1a). It is well-known that the electron carriers between the two photosystems are more reduced in 670-nm light whereas they are more oxidized in 700-nm light. Since violaxanthin availability was greater in the former than in the latter for all electron-transport rates below 2.5 μmol oxygen uptake per mg chlorophyll per h (Fig. 1a) it appears that violaxanthin availability is related to the reduced level of some electron carrier between the two photosystems.

The above view is supported by the manner in which violaxanthin availability in DCMU-poisoned chloroplasts is affected by various electron transport conditions. Under conditions of DPIP-mediated cyclic electron flow the addition of higher amounts of reduced DPIP, which is known to increase the reduced level of the endogenous carriers [19], increased availability of violaxanthin (Table I). Under conditions of linear electron flow from reduced PMS via methylviologen to oxygen, electron carriers before Photosystem I are expected to become more oxidized with increasing light intensity, and indeed violaxanthin availability became smaller (Fig. 3). Whereas availability did not reach control levels when PMS-mediated electron flow was linear, maximal availability was achieved with PMS-mediated cyclic electron flow (Fig. 3, Curve A) where the electron carriers can be expected to be in more reduced state. All

of these data are consistent with the hypothesis that violaxanthin availability is related to the reduced level of some electron carrier before Photosystem I.

The rate-limiting step for electron transport between the photosystems is known to be located between plastoquinone and cytochrome *f* [20]. It is further known that redox carriers located before this rate-limiting step become more reduced with increasing light intensity whereas carriers located after this step become more oxidized [21, 22]. Since the reduced level of some carrier seems to determine violaxanthin availability, and availability increases with increasing intensity for both 670- and 700-nm light (Fig. 1a), it is reasonable to conclude that the carrier is located before the rate-limiting step.

The effect of electron-transport inhibitors on violaxanthin availability not only supports this conclusion but allows further characterization of the postulated carrier. Low levels of DBMIB which are known to inhibit electron flow on the reducing side of plastoquinone [23] did not affect violaxanthin availability (Fig. 2a). This result limits the variety of possible carriers to plastoquinone or preceding carriers in the electron-transport chain, all of which are reduced in the presence of DBMIB. Contrary to DBMIB, low levels of DCMU did result in decreased availability (Fig. 2b). Since DCMU inhibits oxidation of the primary acceptor Q of Photosystem II [24] and all carriers after Q are oxidized via Photosystem I, the postulated carrier is located after the primary acceptor Q. The postulated carrier which affects availability would be plastoquinone itself if plastoquinone accepted electrons directly from Q but this does not seem to be the case. The low potential form of cytochrome *b* 559 has been located between Q and plastoquinone [25] and further carriers may yet be discovered in this region. The fact that menadione restores availability in DCMU-poisoned chloroplasts points to plastoquinone as hypothetical carrier but does not exclude carriers between Q and plastoquinone since they might also be reduced even without being involved in electron flow.

Availability in DCMU-poisoned chloroplasts can be fully restored upon addition of PMS or DPIP, although cyclic electron flow with these mediators is known to bypass plastoquinone [18]. However, there is evidence that plastoquinone can accept electrons from reduced PMS [26] and from DPIP in the presence of excess ascorbate [27] and that ascorbate itself can reduce the low potential form of cytochrome *b* 559 [28]. Thus the ability of PMS and DPIP to restore availability is consistent with our hypothesis.

Violaxanthin availability as possible indicator of conformational membrane changes

Whereas we previously reported [8] that only about 67 % of the total violaxanthin in the thylakoid is available for de-epoxidation under saturating light, we now provide evidence that availability of violaxanthin is zero in the dark. An obvious explanation for variable availability would be that light induces conformational changes in the thylakoid membrane which influence violaxanthin availability. One might draw the following picture: In the dark the lipid layer of the membrane is covered with proteins which completely mask lipophilic violaxanthin and make it inaccessible to the de-epoxidase. Since the arrangement of proteins on the lipid layer is changed upon illumination [10] such a change may allow the de-epoxidase to approach the layer surface. It is actually the internal surface of the lipid layer which is approached by the de-epoxidase since this enzyme is located in the loculus of the

thylakoid. The fact that the amount of violaxanthin which can be de-epoxidized does not exceed 67 % of the total pigment even under very different experimental conditions (compare Fig. 1 and Table I) may further indicate an heterogenous distribution of violaxanthin in the membrane in that two thirds of the total violaxanthin can be exposed to the enzyme by changes in the arrangement of proteins on the lipid layer whereas one third remains inaccessible. For example, this would be achieved if two thirds of the violaxanthin were arranged near the internal and one third near the external surface of the lipid layer.

While violaxanthin availability seems to reflect light-induced changes of the protein arrangement on the internal surface of the thylakoid membrane, the binding between *p*-diazonium benzenesulfonate and membrane proteins has been used by Giaquinta et al. [29] to study changes on its external surface. Both assays have served to demonstrate some relationship between conformational changes of the thylakoid membrane and photosynthetic events, and it is interesting that these events are located in similar regions of the electron-transport chain. Namely, the internal membrane-surface change seems to be related to the redox state of plastoquinone or a carrier before plastoquinone but after the DCMU block, and the external membrane-surface change has been associated with electron flow through one of these carriers but not plastoquinone [30]. The fact that the internal conformational change occurs under conditions of menadione-mediated cyclic electron flow (Table I) whereas the external conformational change does not [30], indicates that different photosynthetic events are involved in the control of both changes.

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