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BIOCHEMISTRY OF THE VIOLAXANTHIN CYCLE IN HIGHER PLANTS

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Abstract - The biochemistry of the violaxanthin cycle in relationship to photosynthesis is reviewed. The cycle is a component of the thylakoid and consists of a reaction sequence in which violaxanthin is converted to zeaxanthin (de-epoxidation) and then regenerated (epoxidation) through separate reaction mechanisms. The arrangement of the cycle in the thylakoid is transmembranous with the de-epoxidation system situated on the loculus side and epoxidation on the outer side of the membrane.

Photosynthetic activities affect turnover of the cycle but the cycle itself consists entirely of dark reactions. Light has at least two roles in de-epoxidation. It establishes through the proton pump the acidic pH in the loculus that is required for de-epoxidase activity and it induces a presumed conformational change in the inner membrane surface which determines the fraction of violaxanthin in the membrane that enters the cycle. De-epoxidation, which requires ascorbate, is presumed to proceed by a reductive-dehydration mechanism. Non-cyclic electron transport can provide the required reducing potential through the dehydroascorbate-ascorbate couple. Whether ascorbate reduces the de-epoxidase system directly or through an intermediate has not been settled. Epoxidation requires NADPH and O_2 which suggests a reductive mechanism. In contrast with de-epoxidation, it has a pH optimum near neutrality. The coupling of photosynthetically generated NADPH to epoxidation has been shown. Turnover of the cycle under optimal conditions is estimated to be about two orders of magnitude below optimal electron transport rates. This low rate appears to exclude a direct role of the cycle in photosynthesis or a role in significantly affecting photosynthate levels in a back reaction. The fact that the cycle is sensitive to events both before and after Photosystem I suggests a regulatory role, possibly through effects on membrane properties. A model showing the various relationships of the cycle to photosynthesis is presented. The contrasting view that the cycle can participate directly in photosynthesis, such as in oxygen evolution, is discussed.

Violaxanthin de-epoxidase has been purified. It is a lipoprotein which contains monogalactosyldiglyceride (MG) exclusively. The enzyme is a mono-de-epoxidase which is specific for 3-OH, 5-6-epoxy carotenoids that are in a 3R, 5S, 6R configuration. In addition, the polyene chain must be all-*trans*. A model has been presented which depicts enzymic MG in a receptor role and the stereospecific active center situated in a narrow well-like depression that can accommodate only the all-*trans* structure.

INTRODUCTION

Following Sapozhnikov's *et al*. (1) first report that the level of violaxanthin in leaves could be changed reversibly by light and dark treatments, there have been numerous studies to elucidate the mechanism and function for these changes, now commonly known as the violaxanthin cycle. The cycle consists of a de-epoxidation sequence (forward reaction) in which violaxanthin is converted to zeaxanthin stepwise through the intermediate antheraxanthin, and an epoxidation sequence (back reaction) which regenerates violaxanthin, again through antheraxanthin (Fig. 1) (2). The pathway is cyclical and not an equilibrium since de-epoxidation and epoxidation mechanisms differ.

In leaves, violaxanthin cycle activity is observed readily. Typically, when exposed to high light intensity, violaxanthin decreases, antheraxanthin increases transiently, and zeaxanthin increases, all in stoichiometric relationship; reversal and recovery of violaxanthin occurs in the dark or under reduced illumination (3, 4). De-epoxidation is generally more rapid than epoxidation and under optimal conditions the entire sequence can be completed

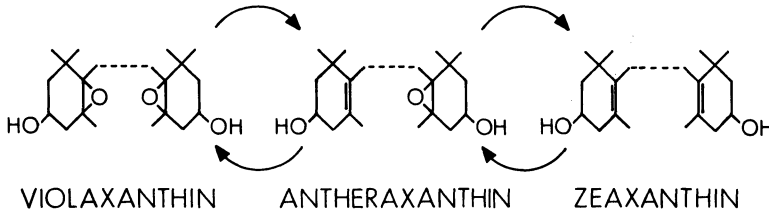

Fig. 1. Violaxanthin cycle pathway.

within an hour. Anaerobiosis enhances de-epoxidation whereas it inhibits epoxidation (1, 2, 5). The cycle generally shows a threshold response to light intensity, namely, a minimum intensity is required to induce a net change in the level of violaxanthin following a period of illumination (6). The threshold level varies with the plant and the temperature of illumination (7). Although the overall phenomenon of the cycle in higher plants is well established, there has been no general agreement regarding its mechanism or significance. Sapozhnikov (8) reviewed the violaxanthin cycle at the Third International Symposium on Carotenoids and in his final remarks noted that the available evidence did not allow a conclusive choice among the various hypotheses on function that have been proposed. The difficulty stems in part from the fact that the results of studies with leaves and algae are prone to varying and at time conflicting interpretations because of the possibility of multiple effects (4, 6, 9-11). Among the functions that have been proposed are roles in oxygen evolution (9, 10), photophosphorylation (12), back reaction to balance ATP:NADPH ratio (13) and regulation (14).

The study of the cycle has progressed in recent years to where it is now possible to observe the complete cycle in isolated chloroplast suspensions (13, 15). In the case of de-epoxidation, the enzyme has been purified (16) and characterized (17, 18). The results of these studies have contributed to a greater understanding of the biochemistry of the cycle and, while not enabling a firm conclusion regarding function, appear to have limited the possibilities. The purpose of this paper is to review the properties of the cycle in isolated chloroplast and purified enzyme systems, to discuss relationships to photosynthesis and implications regarding function, and to identify areas in need of further clarification.

VIOLAXANTHIN CYCLE - A TRANSMEMBRANE SYSTEM OF CHLOROPLASTS

Since light absorbed by chlorophyll induces violaxanthin cycle activity (19), it is reasonable to suggest that the cycle in some way must be involved in or be a consequence of photosynthetic activity. Not surprisingly then the development of the current understanding of the biochemistry of the cycle has entailed elucidating relationships to not only the photochemical processes but also to the structure of the photosynthetic apparatus.

In higher-plant chloroplasts, an outer envelope membrane surrounds the clear stroma and thylakoid membranes, the latter consisting of regions with stacks (grana) of disk-like membranes (grana thylakoid) that are seemingly interconnected by membranes (stroma thylakoid) which extend through the stroma (20). Pigments of the violaxanthin cycle are present in all membranes of the chloroplast, although the contribution to the total carotenoids by the envelope is small compared to the thylakoids (21). Chlorophyll is confined to the thylakoids as are, obviously, the light reactions of photosynthesis.

The assembly of photosynthetic components in the thylakoid is heterogeneous. Photosystem II is thought to be generally situated on the loculus side of the membrane whereas Photosystem I appears to be located on the opposite side (22). During photosynthesis transmembrane movement of electrons and protons occurs, the latter leading to acidification of the loculus and development of a pH gradient across the membrane (23). The pigment compositions of the stroma and grana thylakoids are similar but stroma thylakoids are lacking or low in Photosystem II activity (20).

The current concept of the organization of the violaxanthin cycle in thylakoids is shown in Fig. 2 (14). The cycle is yet another transmembrane system in thylakoids. The forward and back-reactions are localized on opposite sides of the membrane with the de-epoxidation system situated on the loculus side and the epoxidation system on the opposite side. Since the enzymic components are separated physically and the structure of violaxanthin is symmetric, a transmembrane migration of the pigments appears to be required. The evidence which led to this transmembrane concept and relationships of the system to photosynthetic activities are discussed in the following sections.

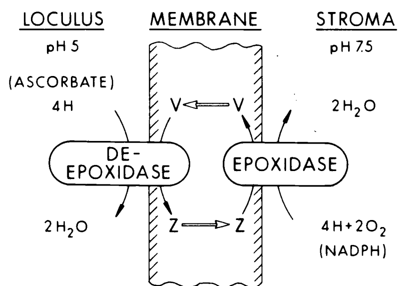

Fig. 2. Transmembrane model of the violaxanthin cycle in thylakoids. (From Ref. 14.)

DE-EPOXIDATION IN ISOLATED CHLOROPLASTS

The role of light. As first shown by Hager (24) isolated chloroplasts in the presence of ascorbate carry out a relatively rapid de-epoxidation of violaxanthin which is light-induced at pH 7.5 but not at pH 5. In addition to the effect of pH, the fact that uncouplers can inhibit the light-induced reaction is conclusive evidence that the de-epoxidation system is situated in the loculus of the thylakoid. The role of light in de-epoxidation is to establish, through the proton pump, the pH necessary for activity. Accordingly de-epoxidation can be inhibited by DCMU (3-(3'4'-dichlorophenyl)-1, 1-dimethylurea) and the activity subsequently restored with an electron carrier such as PMS (phenazine methosulfate) that can restore the proton pump (24, 25). Fig. 3 shows a demonstration of these effects in a typical preparation of lettuce chloroplasts, including the light-independent

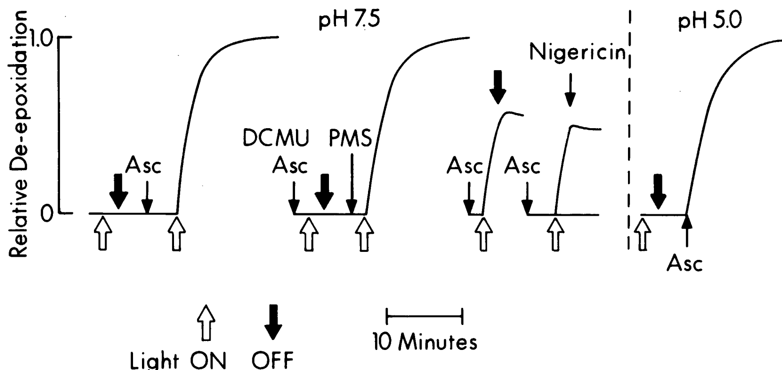

Fig. 3. Violaxanthin de-epoxidation in isolated lettuce chloroplasts. The results are normalized to a chloroplast concentration of 12 μg chlorophyll per ml. Experimental conditions are as described in Ref. 25 and 26.

de-epoxidation at pH 5. It should be noted that in this paper violaxanthin cycle activities will be illustrated for the most part with results obtained through the use of the 505 nm change. The 505 nm change which has been used extensively in our laboratory, has been shown to be a precise and accurate measure of both de-epoxidation and epoxidation (14, 26). Importantly, the 505 nm change gives a rapid and continuous indication of both the rate and extent of these activities and can show the effects of sequential treatments. As shown in Fig. 3, light-induced de-epoxidation continue for a few seconds after the light is turned off, indicating that the pH gradient decays slowly. In contrast, inhibition of de-epoxidation by the ionophore, nigericin, is rapid (25). The maximum extent of de-epoxidation shown represents about 67% of the total violaxanthin in the chloroplasts and is comparable to the extent of de-epoxidation obtained in leaves. The maximum extent of de-epoxidation in light-induced and dark de-epoxidation are comparable and only one mechanism for de-epoxidation is indicated for both conditions.

Light has a second role in violaxanthin de-epoxidation. In lettuce chloroplasts light

intensity influences the fraction of the violaxanthin that can be de-epoxidized up to saturation (26). Fig. 4 shows recorder tracings of this effect. Under low intensity illu-

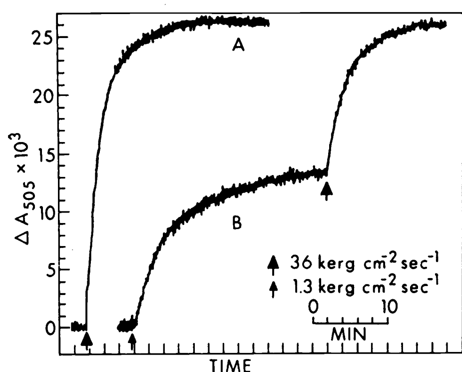

Fig. 4. The effects of light intensity on the extent of de-epoxidation in lettuce chloroplasts. (From Ref. 26.)

mination, de-epoxidation plateaus at an extent below that of the high intensity control but when the intensity is increased, attains the same extent as the control. Utilizing the first-order rate constant as a measure of activity, it has been possible to resolve the rate (activity) from the extent of de-epoxidation and to determine the factors affecting them (27). Fig. 5 shows that corresponding electron-transport rates induced under 670 and

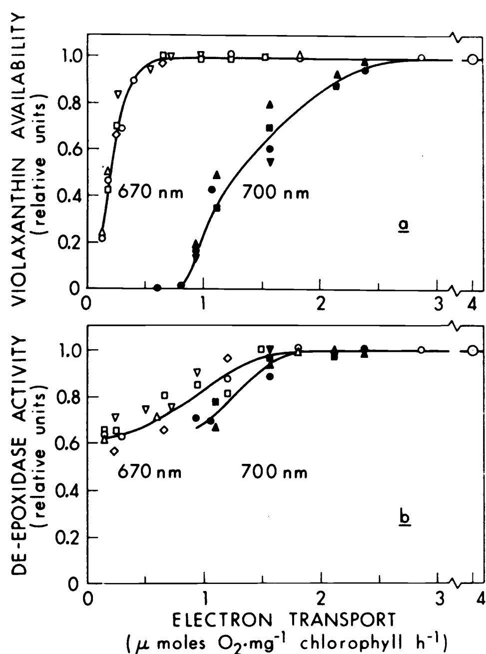

Fig. 5. The effect of 670 nm and 700 nm actinic light on the activity and extent of de-epoxidation in isolated lettuce chloroplasts. (From Ref. 27.)

700 nm actinic light affect de-epoxidation activity and extent differently. The extent of de-epoxidation is saturated considerably earlier under 670 nm actinic light than under 700 nm actinic light. In contrast, activity saturated at similar electron transport rates under both actinic lights. The correlation between electron transport and activity is consistent with the view that activity is a function of loculus pH which is in turn determined by the electron-transport dependent proton pump. In contrast the differential effect of 670 and 700 nm light suggest that the state of an electron transport carrier rather than

electron transport rate influences availability. The rate limiting step of photosynthetic electron transport is thought to be somewhere between plastoquinone and cytochrome _f_. Electron transport carriers before the rate limiting step tend to be reduced by 670 nm actinic light whereas carriers after the rate limiting step tend to be oxidized by 700 nm actinic light (28). The effects of actinic light shown and the effect of electron transport inhibitors and mediators on availability suggest that the state of reduction of an electron transport carrier between the DCMU block and plastoquinone or plastoquinone itself is involved. In view of the location of the de-epoxidation system, it has been proposed that this variable violaxanthin availability could be due to conformational changes on the internal surface of the thylakoid membrane which exposes variable amounts of violaxanthin in the membrane to de-epoxidation as a result of the state of the electron transport carrier (27).

The role of ascorbate. The fact that isolated chloroplasts require supplementation with ascorbate raises the question of the nature of the _in vivo_ reductant. Several lines of evidence support the view that ascorbate is the endogenous reductant or serves as a link to reducing pools. The presence of ascorbate has been reported in spinach chloroplasts (29); the ability of dehydroascorbate to couple de-epoxidation to non-cyclic electron transport has been reported (30); finally, the coupling of de-epoxidation to reduced pyridine nucleotide by dehydroascorbate-glutathione has been shown (13). Hence ascorbate is probably the endogenous electron donor and supplementation of isolated chloroplasts with ascorbate may be necessary because the envelope in most chloroplast preparations is damaged, at least in part. In a study utilizing chloroplasts which were more than 60% intact, Sokolove and Marsho (31) observed an ascorbate-independent (non supplemented) de-epoxidation. Ascorbate could also be the hypothetical reductor in _Lemna_ whose level, according to the model by Siefermann (6), appeared to account for the changes of violaxanthin under various light intensities.

The requirement for ascorbate suggests that the mechanism for de-epoxidation could be reduction followed by dehydration. Although ascorbate may be the endogenous electron donor, there is evidence which suggests reduction may occur through some, as yet, unidentified intermediate. Fig. 6 shows that the light-initiated reaction (ascorbate added before

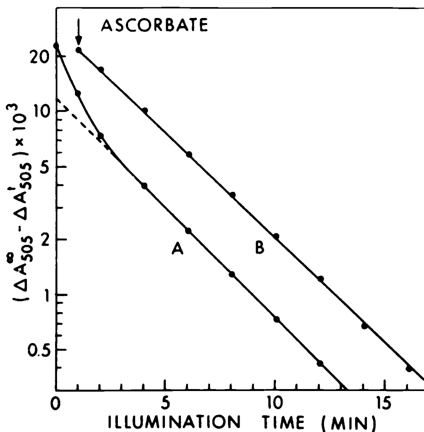

Fig. 6. Kinetics of light-induced (A) and ascorbate-induced (B) de-epoxidation in lettuce chloroplasts. (From Ref. 26.)

illumination) is faster for about half the course of the reaction than the ascorbate-initiated reaction (26). A possible interpretation is that reduction of some intermediate occurred in the dark and that accumulation of the reduced intermediate induces the higher initial rate. The beginning rate of the light-initiated reaction is comparable to the ascorbate-independent de-epoxidation reported for unamended chloroplasts (31). An alternate possibility is that diffusion rates for ascorbate into the thylakoid under light and dark differ. The permeability of the thylakoid to ascorbate has not been determined. In the case of liposomes ascorbate is impermeable (32). If thylakoids are impermeable to ascorbate, the existence of an intermediate reductant, one that is probably membrane bound, is indicated.

VIOLAXANTHIN DE-EPOXIDASE

Properties. Violaxanthin de-epoxidase has been isolated from spinach (16) and lettuce chloroplasts (17). Activity requires the presence of ascorbate and is optimal at pH 5. Endogenous violaxanthin in subchloroplast particles and purified violaxanthin are suitable

substrates but in the case of the latter optimal activity requires the presence of MG (monogalactosyldiglyceride) (17), the major lipid of chloroplasts (33). Recently, violaxanthin de-epoxidase itself was shown to contain MG and hence may be classed a lipoprotein. Enzymic MG appears to be required for activity; activity is lost on extraction of the lipid and restored when added back (18). Violaxanthin de-epoxidase appears to have a molecular weight of around 60,000 (16, 18) and contains a disulfide group that is necessary for activity (34).

Substrate specificity. Violaxanthin de-epoxidase has been tested against the substrates listed in Table 1 (18). Violaxanthin de-epoxidase was active against all naturally occurring 3-OH, 5-6-epoxy carotenoids that also were all-*trans* in the polyene chain. The absolute configuration of several of these have been determined to be *3S*, *5R*, *6S*. Semisynthetic substrates with the opposite configurations (*3S*, *5S*, *6R*) were inactive. Hence violaxanthin de-epoxidase appears to be a mono-de-epoxidase that is stereospecific for the *3S*, *5R*, *6S* configuration. Interestingly the ratio of the de-epoxidation rate for violaxanthin:antheraxanthin was about 1:5, which is in close agreement with the 1:6 ratio obtained by Siefermann (6) in the kinetic model of *Lemna*.

TABLE 1. Activity of violaxanthin de-epoxidase against natural and semisynthetic epoxy carotenoids.

Carotenoid	Configuration of 3-OH, 5-6 epoxide	Polyene chain	Activity
Natural:			
Violaxanthin	*3S*, *5R*, *3S*	all-*trans*	+
Violeoxanthin	*3S*, *5R*, *3S*	9-*cis*	-
Antheraxanthin	?	all-*trans*	+
Neoxanthin	*3S*, *5R*, *3S*	all-*trans*	+
9-*cis* neoxanthin	*3S*, *5R*, *3S*	9-*cis*	-
Diadinoxanthin	?	all-*trans*	+
Cryptoxanthin epoxide	?	all-*trans*	+
Lutein epoxide	*3S*, *5R*, *6S*	all-*trans*	+
Synthetic:			
Antheraxanthin-A	*3S*, *5S*, *6R*	all-*trans*	-
Lutein epoxide	*3S*, *5S*, *6R*	all-*trans*	-
β-carotene epoxide	-	all-*trans*	-
β-carotene diepoxide	-	all-*trans*	-

(Adapted from Ref. 18.)

Although ascorbate is effective in supporting de-epoxidation in the purified system, we have recently observed that the activity can be enhanced with menadione and partially inhibited with DBMIB (2,5-dibromo-3-methyl-isopropyl-p-benzoquinone) (35). These results give support to the view for the existence of an electron carrier after ascorbate and raise the possibility that plastoquinone may be the ultimate electron donor or even a component of the enzyme itself.

Fig. 7 shows a model incorporating several of the known properties of violaxanthin de-epoxidase (18). The stereospecific active center is situated in a well-like depression which can be accommodated only by pigments that are all-*trans* in the polyene chain. Enzymic MG may serve a receptor site role, accepting micelles of MG-containing substrate. This is not to suggest, of course, that violaxanthin in thylakoids are present in the loculus suspended in such micelles. Violaxanthin de-epoxidase is probably embedded in the inner thylakoid surface. The fact that MG micelles do function so effectively in meeting de-epoxidase requirements suggest violaxanthin in thylakoids may be in an MG-rich environment.

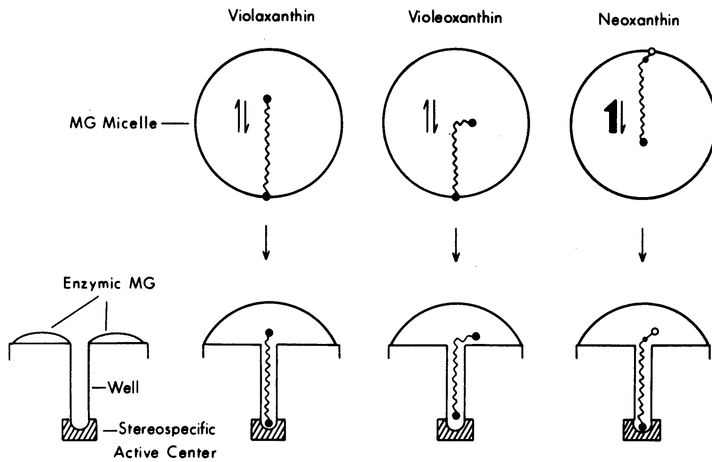

Fig. 7. A model of the active center of violaxanthin de-epoxidase and the complexing of MG-micelle-substrate. The solid circles on the carotenoid structures represent epoxide end groups. The half-arrows in the micelle are to indicate the relative oscillation of the respective carotenoids within the micelle. (From Ref. 18.)

EPOXIDATION IN ISOLATED CHLOROPLASTS

Much less is known about the properties of epoxidation than de-epoxidation. The conclusion that the epoxidase system is located on the stroma side of the membrane is based on the observation that epoxidation is optimal near pH 7.5 (13, 15) and, most importantly, that it is not inhibited under conditions where the proton pump is functional (14, 31). As is the case with de-epoxidation, epoxidation is a dark reaction. The presence of reduced pyridine nucleotide and O_2 is required (13, 15). Fig. 8 shows a complete de-epoxidation and

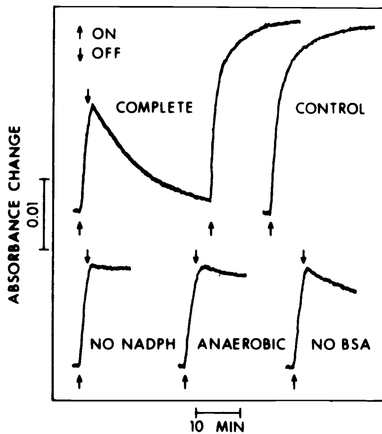

Fig. 8. Reversible violaxanthin cycle activity in isolated lettuce chloroplasts. The complete reaction mixture contained NADPH and ascorbate. The control contained only ascorbate. Bovine serum albumin (BSA) absorbs inhibitory fatty acids. (From Ref. 15.)

epoxidation sequence in isolated chloroplasts. Recovery following de-epoxidation was rapid and complete. Chloroplasts which had undergone a complete cycle were capable of undergoing de-epoxidation again and at a rate comparable to the control. The cofactor requirements indicate that epoxidation is a reductive reaction and class the epoxidase as an "external monooxygenase" according to the nomenclature of Hayaishi (36). The cofactor requirements are similar to rat liver squalene epoxidase which converts squalene to 2,3-oxidosqualene (37). The violaxanthin epoxidase appears to be tightly bound to the membrane and its purification has not yet been reported.

VIOLAXANTHIN CHANGES IN THE CHLOROPLAST ENVELOPE

The envelope of chloroplasts contain violaxanthin which appears to undergo light-induced

changes in concentration similar to changes found in thylakoids (38). Since no photosynthetic activity is known to occur in the envelope, this finding was puzzling. Recently Siefermann-Harms et al. (39) investigated this phenomenon and concluded that the envelope changes are due to an active exchange of pigments between the envelope and the thylakoid and not due to a separate de-epoxidation system in the envelope. Interestingly, when de-epoxidation is active in the thylakoid, depletion of violaxanthin in the envelope occurs without a corresponding increase in zeaxanthin. Neither the mechanism or significance of this exchange is apparent.

HYPOTHESES ON FUNCTION

Numerous links between the violaxanthin cycle and photosynthetic processes have been identified. The relationships are illustrated in Fig. 9. In summary these include, for

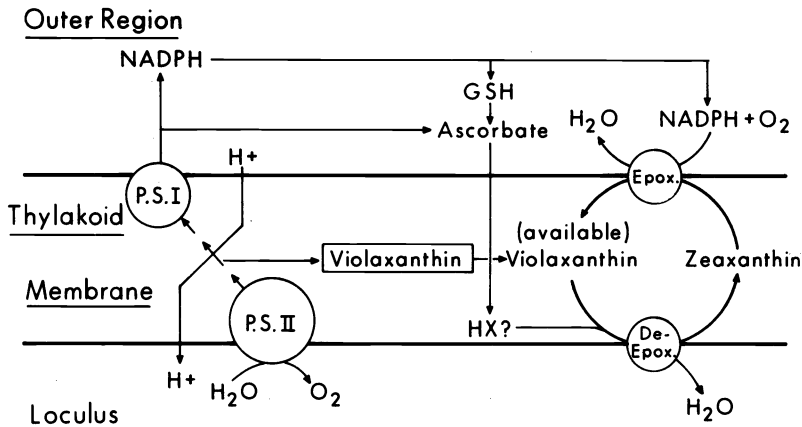

Fig. 9. Model of the arrangement of the violaxanthin cycle in relationship to photosynthetic electron transport.

de-epoxidation, loculus pH as established by the proton pump, violaxanthin availability which appears to be related to the redox state of an electron transport carrier and possibly represents a conformational change of the inner membrane surface, and, finally, reducing potential which can be derived from non-cyclic electron transport through the dehydroascorbate-ascorbate couple. The links between photosynthetic activities and epoxidation that have been found appear less direct. Although NADPH and O_2 are required for epoxidation and are products of photosynthesis, atmospheric O_2 and alternate pathways available for generation of reduced pyridine nucleotide in the chloroplast may be sufficient to maintain epoxidation at saturation. De-epoxidation therefore appears to be the major factor governing turnover of the cycle.

Although turnover of the violaxanthin cycle is light-induced, it is apparent that both the forward and back reactions of the cycle are dark reactions. This fact appears to exclude a direct role of the cycle in strictly light-dependent processes, such as photoprotection.

Another light-dependent process is O_2 evolution. The biochemical evidence does not support a role of the cycle in this process. In addition to the fact that epoxidation is a dark reaction, the requirement for O_2 and NADPH suggests a mechanism in which one atom of oxygen is incorporated as the epoxide and the other is reduced to water. The incorporation of ^{18}O from molecular oxygen is consistent with this view (11). Light-induced incorporation of ^{18}O from water that has been reported may be due to incorporation of photosynthetically evolved oxygen (cf. Sapozhnikov et al. Ref. 8).

The turnover rate of the cycle is critical to the question of function. If the cycle has a direct role in photosynthesis, such as in phosphorylation or oxygen evolution, the turnover rate of the cycle must be consistent with the rates of those processes. The turnover rate of any system is determined by the rate-limiting step. In the case of the violaxanthin cycle either de-epoxidation or epoxidation can be rate-limiting depending on conditions. Potentially any of the factors shown in Fig. 9 can influence turnover of the cycle. Under high light intensity, following an initial increase in zeaxanthin due to de-epoxidation, the turnover rate would be determined by the ability of the system to regenerate violaxanthin by epoxidation. Under low light intensity, assuming the epoxidation system is active, de-epoxidation can be expected to determine the turnover rate. Whether the cycle

does in fact turn over under low light intensity is controversial (6, 40).

The turnover rate of the violaxanthin cycle has not been measured directly since it has not been possible to distinguish the products of turnover (NADP or dehydroascorbate) from similar products that can be derived through other pathways. Only the rates of de-epoxidation and epoxidation themselves have been determined utilizing conditions favoring one or the other reaction. The turnover rates which can be inferred from these results probably reflect maximal rates since they are obtained under saturating conditions. The results with isolated chloroplasts are consistent in showing rates that are around two orders of magnitude below photosynthetic rates (41). In fact the rates would appear to be too low even for a role in a back reaction to maintain an improved ATP:NADPH ratio (13), even though the cycle does consume NADPH.

Recently it was reported that salicylaldoxime stimulates a rapid light-induced epoxidation in isolated chloroplasts (42). This result has not been confirmed. Salicylaldoxime is known to inhibit epoxidation in whole cell systems (43). In isolated lettuce chloroplasts, salicylaldoxime inhibits dark epoxidation as well as light-induced de-epoxidation and so far no evidence of a fast light-induced epoxidation has been observed (35). Inhibition of light-induced de-epoxidation is probably due to the inhibition of electron transport. Assuming that the presence of two epoxidation mechanisms (fast, light induced and slow, dark) is confirmed, it would be necessary to prove the existence of a corresponding fast de-epoxidation mechanism if a direct role in photosynthesis is to be considered. The turnover considerations discussed earlier would apply here as well. The claim that there could be an alternate oxidative pathway in which de-epoxidation occurs through a light-induced release of molecular oxygen (8) has not been supported by direct evidence.

Epoxidation and de-epoxidation are similar in that both require the presence of a hydrophilic reductant and both appear to be irreversible. Because of irreversibility, the steady-state ratio of violaxanthin to zeaxanthin reflects changes which affect their activity independently without dampening. Since the cycle's activity is affected by events both before and after Photosystem I, it follows that the violaxanthin:zeaxanthin ratio reflects or "monitors" changes in these events. It has been suggested therefore that the violaxanthin cycle could be part of a regulatory system for photosynthesis which function at the membrane level, perhaps by altering membrane properties (14). The slow turnover rate would be consistent with a regulatory role. If regulation is the function, it is apparently not in affecting the energy distribution between the photosystems (33). The fact that carotenoids can affect membrane properties has been shown with liposomes (44) and with black-lipid membranes (45). In the latter case, the presence of carotenoids has been shown to be essential for photoconductivity. Further studies with such model systems can be anticipated to contribute to a better understanding of the role of carotenoids in photosynthesis.

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