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New Concepts

Violaxanthin De-Epoxidase, the Xanthophyll Cycle Enzyme, Requires Lipid Inverted Hexagonal Structures for Its Activity†

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ABSTRACT: Bilayer-forming lipids were shown to be ineffective in sustaining the enzymatic activity of violaxanthin de-epoxidase. On the other hand, non-bilayer-forming lipids, regardless of their different chemical character, ensured high activity of violaxanthin de-epoxidase, resulting in conversion of violaxanthin to zeaxanthin. Our data indicates that the presence of lipids forming reversed hexagonal structures is necessary for violaxanthin de-epoxidase activity and this activity is dependent on the degree of unsaturation of the fatty acids. The significance of the reversed hexagonal phase domains in the conversion of violaxanthin into zeaxanthin in model systems and in the native thylakoid membranes is discussed.

The xanthophyll cycle is a thylakoid membrane associated protective mechanism functioning in all higher plants, ferns, mosses, and several algal groups (*1*). Two enzymes are engaged in this process: violaxanthin de-epoxidase (VDE),[1] converting violaxanthin into zeaxanthin upon strong illumination, and zeaxanthin epoxidase, carrying out the reverse reaction of epoxidation of zeaxanthin to violaxanthin in low light or darkness. In both reactions, antheraxanthin is formed as an intermediate product (*2*).

VDE is a water-soluble enzyme located in the thylakoid lumen (*3*). It was shown that at pH below 6.5 VDE becomes tightly bound to the membrane and this binding is an important factor regulating VDE activity (*4*). The enzyme is activated when the thylakoid lumen pH reaches a value of about 5.0 and when a suitable reducing agent (ascorbate) is available (*3*). VDE for its activity specifically requires the major thylakoid lipid, MGDG (*5*, *6*). It is also known that MGDG is four times more efficient in precipitating VDE compared to the second most common lipid in thylakoids, DGDG, and up to 38 times more efficient than other thylakoid lipids (*7*). The role of MGDG as necessary lipid component of liposomes in the xanthophyll cycle studies was reported earlier (*8*, *9*). In this work, we have investigated which specific features of the MGDG molecule are important for sustaining the VDE activity.

MATERIALS AND METHODS

Chemicals. MGDG and DGDG were purchased from Lipid Products. PC was obtained from Sigma (P2772), ePE from

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[1] Abbreviations: DGDG, digalactosyldiacylglycerol; ePE, egg yolk phosphatidylethanolamine; HPLC, high-performance liquid chromatography; MGDG, monogalactosyldiacylglycerol; PC, egg yolk phosphatidylcholine; PE, phosphatidylethanolamine; PE1, 1,2-dioleoyl-*sn*-glycero-3-phosphatidylethanolamine; PE2, 1,2-dilinoleoyl-*sn*-glycero-3-phosphatidylethanolamine; VDE, violaxanthin de-epoxidase.

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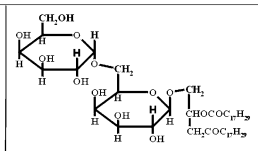
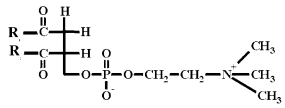
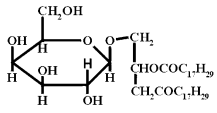
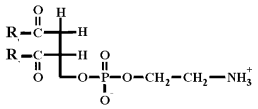
Table 1: Division of the Lipids According to Their Chemical Character and Kind of Structures Created in Water

	Galactolipids	Phospholipids
Bilayers	 Digalactosyldiacylglycerol	 Phosphatidylcholine
Inverted hexagonal structures	 Monogalactosyldiacylglycerol	 Phosphatidylethanolamine

Fluka (60647), and synthetic phosphatidylethanoloamines, PE1 and PE2, from Larodan Fine Chemicals (37-1821, 37-1822). Violaxanthin was isolated from daffodil petals as described in ref *10* and saponified (*11*). VDE was isolated and purified from 7-day-old wheat leaves according to the method described by Hager and Holocher (*4*) with a minor modification (*8*).

Measurement of the VDE Activity. The enzyme activity was determined by dual-wavelength measurements (502 minus 540 nm) using a DW-2000 SLM Aminco spectrophotometer at 25 °C. The reaction mixture contained 0.33 μM violaxanthin, 12.9 μM lipid (one of the following: MGDG, DGDG, PC, or one of the three kinds of PE, ePE, PE1, or PE2) in methanol, VDE, and 30 mM ascorbic acid. All reagents were suspended in 0.1 M sodium citrate buffer, pH 5.1. The de-epoxidation reaction was initiated by addition of ascorbic acid (*12*). Kinetics of violaxanthin de-epoxidation was investigated both in the system of inverted hexagonal structures and in unilamellar liposomes at 25 °C, and the de-epoxidation products were analyzed by HPLC as described earlier (*8*).

Preparation of Unilamellar Liposomes. The liposomes composed of violaxanthin, PC, and one of the lipids MGDG, DGDG, PC, or ePE were preparation as described in ref *8*.

Thin-Layer Chromatography of Lipids. The thin-layer chromatography of phosphatidylcholine (Sigma, Type IV-S from soybean, P 3644) was performed as described in ref *13*.

RESULTS

Lipids used in our studies on the VDE activity were selected according to two criteria: (i) chemical composition of the lipid molecules and (ii) kind of structures created by the lipids (*14*) (Table 1). In the view of the chemical composition, two groups of lipids were chosen; neutral lipids containing sugar moiety in their molecules (galactolipids) and charged lipids without sugar residues (phospholipids) (Table 1). Among galactolipids and phospholipids, those creating bilayer and inverted hexagonal structures were additionally selected (Table 1).

A comparison of the effect of MGDG and DGDG on VDE activity is shown in Figure 1. In the presence of MGDG in the assay mixture, typical VDE activity was observed, but no activity occurred when MGDG was replaced by its homologue, DGDG. The de-epoxidation of violaxanthin was also observed when ePE instead of MGDG was present in the assay mixture (Figure 2). However, no VDE activity could be detected when MGDG was replaced by PC (Figure 2). The rates of violaxanthin de-epoxidation by VDE in the presence of MGDG and ePE were similar and amounted to 20.3 ± 3.0 nmol/min/mL for MGDG and 22.6 ± 2.1 nmol/min/mL for ePE (Figure 3).

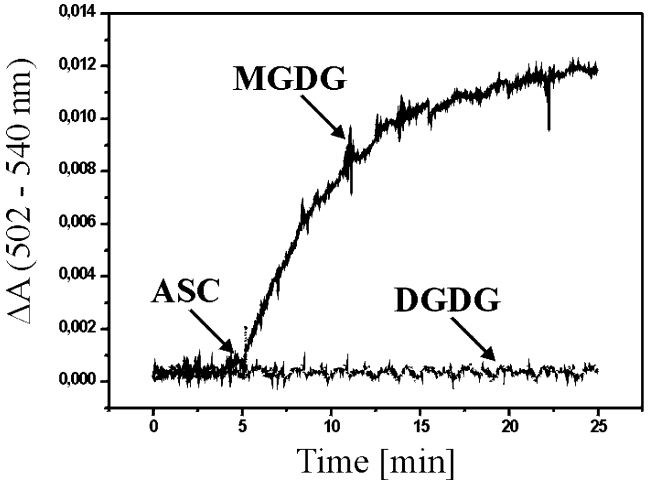


FIGURE 1: Violaxanthin de-epoxidation by VDE in the presence of MGDG or DGDG in the assay mixture measured as absorption changes at 502 minus 540 nm.

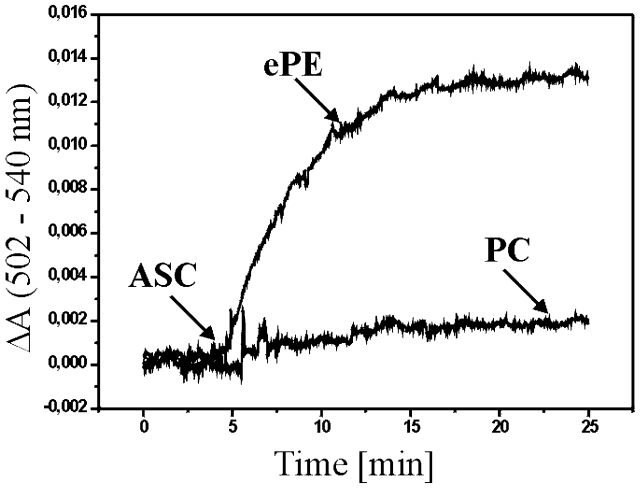


FIGURE 2: Violaxanthin de-epoxidation by VDE in the presence of PE or PC in the assay mixture measured as absorption changes at 502 minus 540 nm.

The effect of the fatty acid composition of PE on VDE activity was also tested. Two kinds of synthetic PEs having their fatty acids of the same length but differing in the degree of unsaturation were used. In the case of PE2, VDE activity was similar to that with MGDG and ePE (25.2 ± 1.3 nmol/min/mL (Figure 3)). Interestingly, the highest VDE activity was obtained when PE1 was applied instead of the MGDG. In this case, the enzyme activity reached the level of 40.3 ± 4.2 nmol/min/mL, which was 2 times higher than the enzyme activity in the presence MGDG. This is a new finding because, up until now, MGDG was considered to be the optimal lipid for maximal activity of VDE.

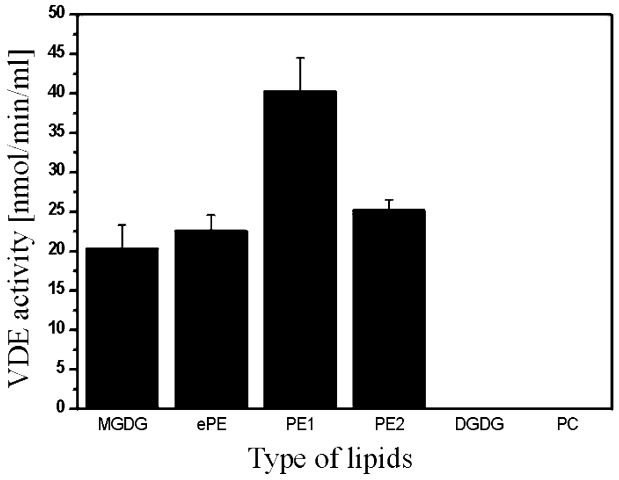


FIGURE 3: Effect of the different lipids on VDE activity measured as a drop of violaxanthin level.

The kinetics of violaxanthin de-epoxidation was also investigated in the system of unilamellar liposomes. When violaxanthin-containing PC liposomes were enriched with MGDG or ePE the de-epoxidation reactions were observed, and the kinetics of these reactions were similar for both kinds of binary lipid mixtures. On the other hand, violaxanthin located in liposomes composed of only PC or of PC enriched with DGDG was not de-epoxidated by VDE.

4. DISCUSSION

The results presented in this paper clearly show that the enzyme VDE is active in de-epoxidation of violaxanthin when MGDG or PE are present in the assay mixture but no de-epoxidation occurs when MGDG is replaced by PC or DGDG (Figures 1 and 2). This means that it is not the chemical character of the lipid molecule but rather the kind of structures formed by the lipids that plays an important role for VDE activity (Table 1). Although both MGDG and DGDG contain sugar residues in their molecules, VDE activity is observed only when MGDG is present in the assay mixture. However, the kind of structures created by MGDG and DGDG are completely different (*14*,*15*). DGDG is characterized by a high hydration level (about 50 water molecules per lipid) (*14*) and its critical packing parameter value is between 0.5 and 1 (*15*). These features are responsible for bilayer formation by DGDG. PC, which also forms bilayer, has a critical packing parameter and hydratation level similar to DGDG (*14*, *15*), and VDE activity was also not observed when MGDG was replaced by PC. On the other hand, MGDG forms inverted hexagonal structures in water due to its much lower hydratation level (only 5 water molecules per lipid) (*14*) and a critical packing parameter value that is greater than one (*15*). When MGDG was replaced by PE, VDE activity was observed, although PE does not contain a sugar group in the molecule. Despite the difference in headgroup structure, PE has similar features to MGDG and may form inverted hexagonal structures (*14*, *16*, *17*).

In our experiments, the highest activity of the VDE was observed when PE1 was present in the assay mixture (Figure 3). In the case of PE2, which had a higher degree of unsaturation than PE1, VDE activity was lower and more comparable to that obtained with MGDG. This suggests that subtle structural differences in the inverted hexagonal structures formed may significantly influence the VDE activity.

The key role of inverted hexagonal structures in molecular mechanism of violaxanthin de-epoxidation is supported by the fact that some commercial MGDG preparations, which are hydrogenated for their stability, are unsuitable for VDE activity assay (*12*). It is known that the formation of inverted hexagonal structures by MGDG at 20 °C was significantly reduced by decreasing the average number of double bounds per MGDG molecule. More complete saturation promoted the formation of a laterally segregated lamellar gel phase (*16*, *18*) instead of the inverted hexagonal structures. In conclusion, MGDG seems to be necessary for VDE activity not as galactolipid but as non-bilayer-prone lipid forming reversed hexagonal structures. Such structures, created by both MGDG and PE are effective in sustaining VDE enzymatic activity.

An apparently contradictory finding was published recently by Grotz et al. (*19*) who demonstrated VDE activity in the presence of soybean phosphatidylcholine. However, the reason for this activity is probably the contamination of the applied soybean phosphatidylcholine (Sigma, Type IV-S from soybean, P 3644, 40% purity) with other lipids. TLC analysis of this lipid preparation revealed that it contains a significant amount of MGDG and other lipid species (data not shown).

The conclusion about involvement of reversed hexagonal structures in de-epoxidation of violaxanthin by VDE in model systems can be extended for native thylakoid membranes. It has been demonstrated by use of various techniques that such structures of unknown function may exist either within the photosynthetic membranes (*20*−*23*) or are closely associated with them (*24*). We postulate that one of the possible functions of the reversed hexagonal phase domains in the thylakoid membranes is their involvement in the molecular mechanism of the xanthophyll cycle. It is possible that the inverted hexagonal structures are required by some enzymes for their activity in vivo.

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