

De-epoxidases of xanthophyll cycles require non-bilayer lipids for their activity

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Using an in vitro system to study lipid dependence of the xanthophyll cycle de-epoxidase activity, such as violaxanthin de-epoxidase (VDE) and diadinoxanthin de-epoxidase (DDE), we found that replacement of monogalactosyldiacylglycerol (MGDG) with digalactosyldiacylglycerol (DGDG) or phosphatidylcholine (PC) in the assay medium resulted in a strong inhibition of xanthophyll cycle pigments, violaxanthin (Vx) and diadinoxanthin (Dx), de-epoxidation. On the other hand, replacement of MGDG with phosphatidylethanolamine (PE) sustained a high activity of these enzymes, in spite of the very different chemical character of these two lipids. We have also found that Ddx and Vx solubility in lipids depends on the lipid species used. Using phosphorus NMR measurements, we detected the existence of the inverted hexagonal phase (H_{II}) in a binary (MGDG/PC) lipid mixture as well as in thylakoid membranes. The obtained results clearly indicate that only H_{II} forming lipids (MGDG and PE) may effectively support Vx or Ddx de-epoxidation, whereas bilayer-forming lipids (DGDG and PC) are not effective in this process. Hence, a conclusion is drawn that VDE and DDE activity depends not on the chemical character of lipids but on the kind of structure they form in water environment. VDE and DDE constitute a new family of enzymes whose activity depends on the presence of inverted hexagonal structures of lipids. We propose a molecular model of Vx and Dx de-epoxidation in which H_{II} forming lipids play a crucial role.

Key words: de-epoxidation, diadinoxanthin, inverted hexagonal phase, violaxanthin, xanthophyll cycle

INTRODUCTION

One of the most important photoprotective mechanisms associated with plant thylakoid membranes is the xanthophyll cycle. So far, three different xanthophyll cycles have been described: the violaxanthin (Vx) cycle of vascular plants and green and brown algae [1–3], the diadinoxanthin (Ddx) cycle of the algal classes Bacillariophyceae, Chrysophyceae, Xanthophyceae, Haptophyceae and Dinophyceae [4], and the lutein epoxide

cycle detected in a limited set of vascular plants [5, 6]. In all these xanthophyll cycles engaged are two types of enzymes belonging to the lipocalin family [7]: de-epoxidases, catalyzing conversion of epoxy-xanthophylls into non-epoxy pigments under strong light conditions, and epoxidases, carrying out the backward reactions of the cycles in low light or darkness. The de-epoxidising enzymes are located in the thylakoid lumen and are activated by a decreasing luminal pH due to light-driven photosynthetic electron transport, whereas location of the epoxidising enzymes is postulated on the stromal side of the thylakoid membrane and their pH optimum is 7.5 [8]. The Vx cycle consists of two de-epoxidation steps which transform the di-epoxy-xanthophyll Vx into the epoxy-free zeaxanthin (Zx) via the intermediate mono-epoxy-xanthophyll, antheraxanthin (Ax). These reactions are catalysed by the enzyme

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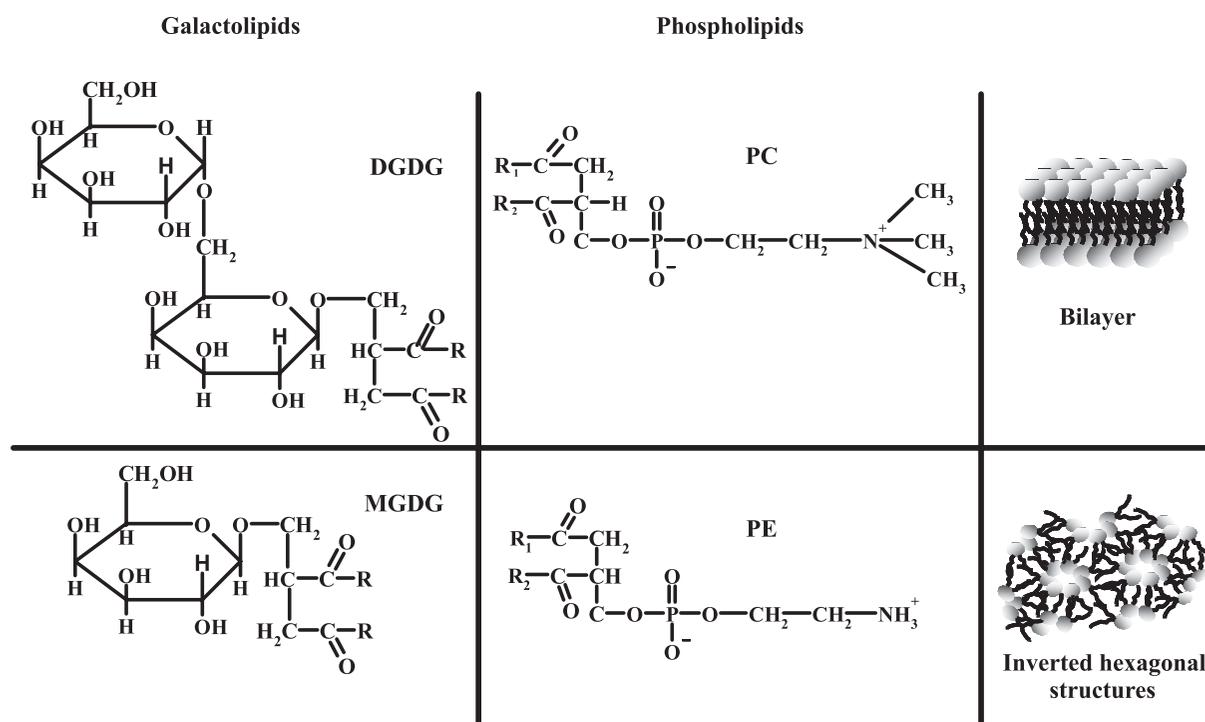


Fig. 1. Classification of lipids according to their chemical structure and the type of structures formed in water

Vx de-epoxidase (VDE). The Ddx cycle comprises only one step from the mono-epoxy xanthophyll Ddx to the epoxy-free diatoxanthin (Dtx), and this reaction is carried out by another enzyme, Ddx de-epoxidase (DDE). The de-epoxidation in the third type of xanthophyll cycle is also a one-step reaction, and 5,6-epoxylutein is directly converted into epoxide-free lutein.

DDE is optimized in various ways with respect to its function compared with VDE. DDE activation can be observed at almost neutral pH values [9, 10], whereas for VDE activation more acidic pH values are needed [11, 12]. Furthermore, DDE exhibits a significantly lower K_M value for its co-substrate ascorbate compared with the VDE [10].

Despite these differences, both enzymes require the presence of the major thylakoid membrane lipid, monogalactosyldiacylglycerol (MGDG), for their activity [13–16]. The role of this lipid in activation of the de-epoxidases was unclear until 2003. In the present paper, we present a review of our earlier works which give an answer to the question why MGDG is necessary for VDE and DDE activity.

MATERIALS AND METHODS

MGDG and digalactosyldiacylglycerol (DGDG) were purchased from Lipid Products (England). Phosphatidylcholine (PC) was obtained from Sigma (P2772) (England), phosphatidylethanolamine (PE) from Fluka (60647) (England), Vx was isolated from daffodil petals as described in [17] and saponified [18]. Ddx and DDE were isolated from dark-adapted diatoms *Cyclotella meneghiniana* (strain 1020-1a), which was obtained from Sammlung von Algenkulturen Göttingen (SAG, Germany) and grown as a batch culture in silica-enriched ASP medium according to Provasoli et al. [19] with the modifications introduced by

Lohr and Wilhelm [20]. The preparation of DDE and pigments was as described in [21].

VDE was isolated and purified from 7-day-old wheat leaves according to the method described by Hager and Holocher [22], with a minor modification [14]:

1. The modifications of the method of Hager and Holocher [22] were as follows:

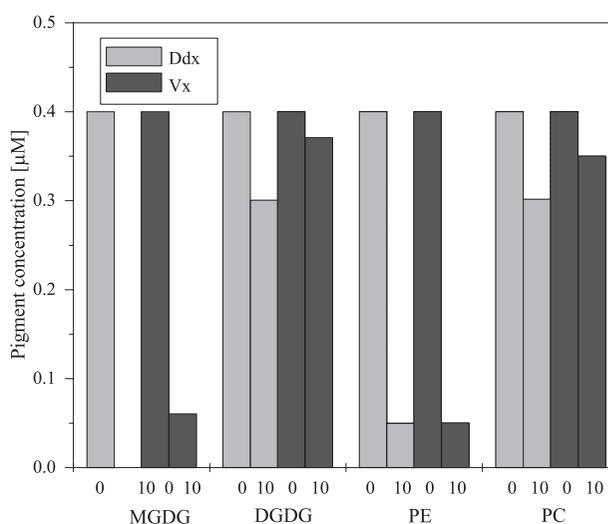


Fig. 2. Effect of lipids on xanthophyll de-epoxidation at the beginning and after 10 min of reaction in the assay mixture. The reaction mixture contained 0.4 μM Vx or Ddx; 12.9 μM lipid (one of the following: MGDG, DGDG, PC, PE), VDE and 30 mM ascorbic acid. All reagents were suspended in 0.1 M sodium citrate buffer, pH 5.2. The de-epoxidation reaction was initiated by addition of ascorbic acid. Concentrations of diadinoxanthin and violaxanthin at the beginning (0) and after 10 min (10) of the reaction. The bars represent concentrations of individual pigments, not of their mixture

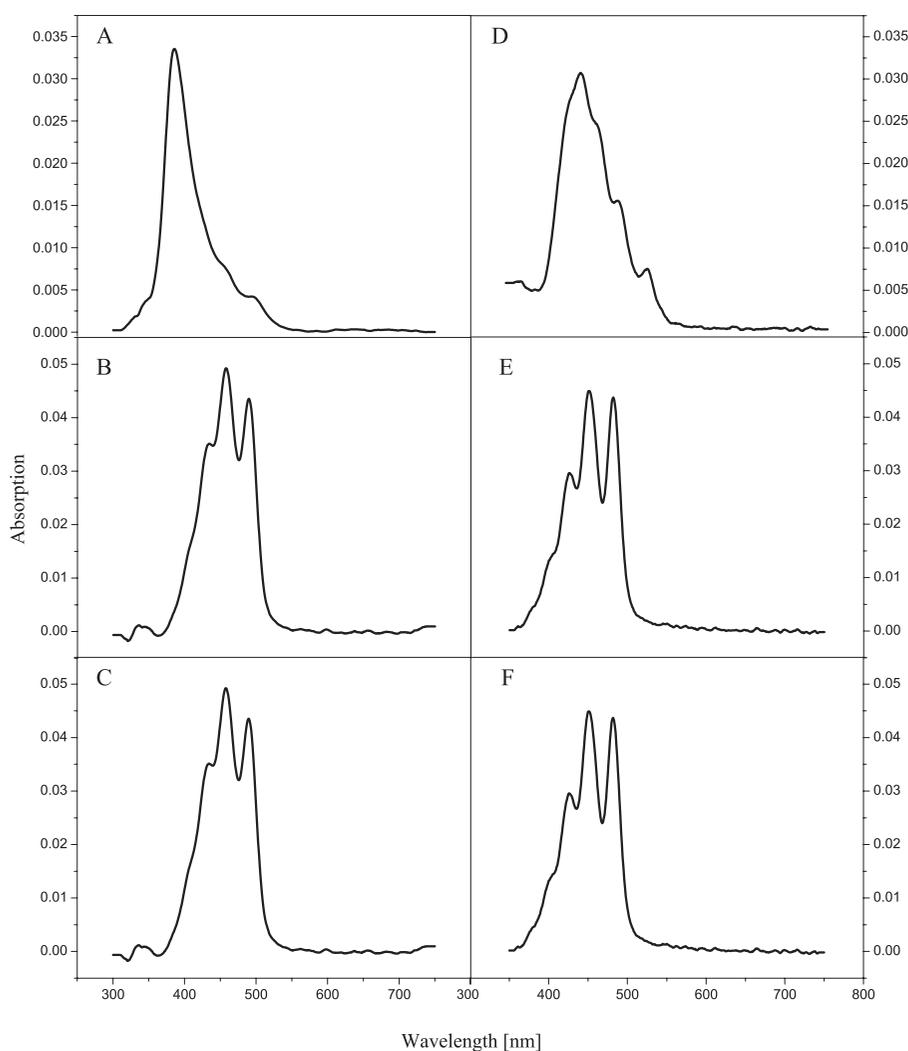


Fig. 3. Absorption spectra of Ddx in reaction medium at pH 5.2 without lipids in aqueous medium (A), with optimal concentration of lipids (B), in ethanol (C). Absorption spectra of Vx in reaction medium at pH 5.2 without lipids (D), with optimal concentration of lipids (E), in ethanol (F). Final volume of assay mixture was 3 ml

- chloroplasts were isolated from wheat instead of spinach
- leaves were kept in the dark for 1 hour before chloroplast isolation

- phosphate buffer was used instead of HEPES-NaOH buffer.

2. Figure 2 shows the concentrations of diadinoxanthin and violaxanthin at the beginning (0) and after 10 min (10) of the reaction. The bars represent the concentrations of individual pigments, not their mixture.

3. The final volume of the assay mixture was 3 ml.

The enzyme activity was determined by dual-wavelength measurements (502 minus 540 nm) using a DW-2000 SLM Aminco spectrophotometer at 25 °C and by HPLC as described in [15, 21]. Xanthophyll solubility in lipids was determined employing absorption spectroscopy [16, 21].

Table. Lipid concentration at which total solubilization of xanthophylls was achieved. Pigment concentration was fixed at 0.4 μM in reaction medium at pH 5.2

| Xanthophyll type | MGDG (μM) | DGDG (μM) | PE (μM) | PC (μM) |
|------------------|------------------------|------------------------|----------------------|----------------------|
| Ddx | 2.0 | 38.7 | 2.0 | 38.7 |
| Vx | 11.6 | 38.7 | 11.6 | 38.7 |

RESULTS

The lipids used in the studies on xanthophyll de-epoxidation by VDE and DDE were selected according to two criteria. One of them was the chemical composition of the lipid molecules, and the other concerned the type of structures formed by the lipids in water environment [23] (Fig. 1). With respect to the chemical composition, two groups of lipids were chosen: neutral lipids containing a sugar moiety in their molecules (galactolipids) and charged lipids without sugar residues (phospholipids) (Fig. 1). Among galactolipids and phospholipids, those producing bilayers and those forming inverted hexagonal structures were additionally selected (Fig. 1). A comparison of the effect of these lipids on Vx and Ddx de-epoxidation is shown in Fig. 2. In the presence of MGDG and PE in the assay mixture all Vx and Ddx were de-epoxidised, whereas only a very weak de-epoxidation, if any, occurred when MGDG had been replaced by its homologue, DGDG or another phospholipid, PC.

We also performed studies on the solubility of Vx and Ddx in the lipids exhibiting the molecular specifications described above. Both xanthophylls studied aggregate in an aqueous medium in the absence of lipids. This manifests in a characteristic absorption spectrum without any visible fine structure (Fig. 3 A, D). A gradual increase in lipid concentration in the medium results in

a progressive desaggregation of the pigments, and this process may be monitored by changes in the absorption spectrum of the xanthophylls. After a complete desaggregation, the spectra of the pigments dissolved in the lipids added (Fig. 3 B, E,) resemble those in organic solvents (Fig. 3 C, F). Non-bilayer lipids were found to be much more effective than bilayer lipids in dissolving both Vx and Ddx. Complete solubilization for Vx was achieved at the lipid: pigment ratio of 29. For the total solubilization of this pigment in bilayer-forming lipids, about 3.5 times higher concentration of the lipid was necessary (lipid : pigment ratio of about 100, Table). In the case of Ddx the effect of the lipid type on solubility was even more pronounced (Table). Complete solubilization of this xanthophyll was achieved at the non-bilayer lipid : pigment ratio of 5, whereas in the case of bilayer lipids this ratio was almost 100.

The effect of lipid concentration on the Vx and Ddx de-epoxidation rate was also studied. Ddx de-epoxidation by DDE was saturated at an MGDG or PE concentration of 2 μM . This corresponds well with the complete solubilization of Ddx. Further increases in lipid concentration did not enhance the Ddx de-epoxidation rate. On the contrary, at an MGDG concentration of 11.6 μM a slight reduction in the Ddx de-epoxidation rate was observed. On the other hand, 11.6 μM of MGDG or PE is the optimal concentration for Vx de-epoxidation by VDE. Quite a different situation was observed for the bilayer lipids. Ddx and Vx de-epoxidation was strongly suppressed even in the presence of high concentrations of PC and DGDG (38.7 μM) at which both xanthophyll cycle pigments are completely solubilized.

DISCUSSION

The results presented in this paper clearly show that the xanthophyll cycle de-epoxidases are active when the non-bilayer lipids MGDG or PE are present in the assay mixture, but no or very weak de-epoxidation occurs when these lipids are replaced by the bilayer lipids PC or DGDG (Fig. 2). This means that it is not the chemical character of the lipid molecule but rather the type of structure formed by the lipids that plays an important role in sustaining the activity of VDE and DDE (Fig. 1). Although MGDG and DGDG, both containing galactose residues in their molecules, are very similar in their chemical character, activity of VDE and DDE was observed only when MGDG was present in the assay mixture. The kind of structures formed in water by MGDG and DGDG are completely different [23, 24]. DGDG is characterized by a high hydration level (about 50 water molecules per lipid) [23] and its critical packing parameter value is between 0.5–1 [24]. These features are responsible for bilayer formation by DGDG (Fig. 1). PC, which also forms lipid bilayers, has the critical packing parameter and hydration level similar to DGDG [23, 24]; neither VDE nor DDE activity is observed when MGDG or PE are replaced by PC.

MGDG molecules form the H_{II} phase in water due to their much lower hydration level (only 5 water molecules per lipid) [23] and the critical packing parameter value greater than one (Fig. 1) [24]. Replacement of MGDG by PE ensured a high VDE and DDE activity, although PE does not contain a sugar group in the molecule (Fig. 1). However, despite the difference in the head group structure, PE shares similar features with MGDG and may form inverted hexagonal structures [23, 25, 26].

MGDG and PE, the hexagonal structure-forming lipids, exhibited almost the same efficiency in pigment solubilization. Even at low lipid concentrations Ddx and Vx were solubilized completely, also saturating the de-epoxidase reaction. Bilayer forming lipids, such as DGDG and PC, were also able to solubilize Ddx and Vx, but at significantly higher concentrations. However, these lipids were not able to activate xanthophyll de-epoxidation by DDE and VDE. This indicates that xanthophyll solubilization is not the only factor that controls de-epoxidation, although it may play an important role to provide the monomer forms of Ddx and Vx as the substrates for VDE and DDE. We propose that in the thylakoid membrane Vx or Ddx, after their detachment from the LHC complex, reach by diffusion the MGDG-rich domains which are the binding sites of de-epoxidases. Due to the H_{II} phase existing in such an MGDG domain, Ax or Ddx can easily turn in the membrane, performing a flip-flop type movement. This allows the enzymes to reach all epoxy groups located at the ionone rings of Ddx, Vx and Ax, regardless of their original orientation in the thylakoid membrane.

Using phosphorus NMR measurements, we detected the existence of the H_{II} phase in a binary (MGDG / PC) lipid mixture as well as in thylakoid membranes [14, 27].

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