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The influence of phase transitions in phosphatidylethanolamine models on the activity of violaxanthin de-epoxidase

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Abstract

In the present study, the influence of the phospholipid phase state on the activity of the xanthophyll cycle enzyme violaxanthin de-epoxidase (VDE) was analyzed using different phosphatidylethanolamine species as model lipids. By using ^{31}P NMR spectroscopy, differential scanning calorimetry and temperature dependent enzyme assays, VDE activity could directly be related to the lipid structures the protein is associated with. Our results show that the gel (L_β) to liquid-crystalline (L_α) phase transition in these single lipid component systems strongly enhances both the solubilization of the xanthophyll cycle pigment violaxanthin in the membrane and the activity of the VDE. This phase transition has a significantly stronger impact on VDE activity than the transition from the L_α to the inverted hexagonal (H_{II}) phase. Especially at higher temperatures we found increased VDE reaction rates in the presence of the L_α phase compared to those in the presence of H_{II} phase forming lipids. Our data furthermore imply that the H_{II} phase is better suited to maintain high VDE activities at lower temperatures.

Keywords: Phospholipid structures; ^{31}P NMR; Inverted hexagonal phase; Arrhenius; De-epoxidation; Monogalactosyldiacylglycerol

1. Introduction

In the plant kingdom, the xanthophyll cycle is an important mechanism to protect the photosynthetic apparatus from damage caused by excess radiation [1]. It is performed by two independent enzymes, the violaxanthin de-epoxidase (VDE) in the lumen of the thylakoids and the zeaxanthin epoxidase probably being attached to the stromal surface of the thylakoids [2,3]. VDE is activated by the light driven acidification of the lumen, which induces conformational changes of the enzyme [4,5] and thereby enables its reversible and direct binding to the thylakoid membrane, where the de-epoxidation reaction takes place.

The substrate violaxanthin (Vx) is released from the photosynthetic light-harvesting complexes into the surrounding lipid phase to become accessible for the enzyme VDE [4,6]. Furthermore, it has been shown that, in addition to binding of VDE [7], its activity [8] and also the solubilization of its substrate [9,10] is strongly promoted by monogalactosyldiacylglycerol (MGDG), the major thylakoid lipid. Interestingly, MGDG by itself does not form lamellar bilayer membranes in an aqueous environment. Instead, inverted hexagonal (H_{II}) structures are formed [11] and this morphology has been proposed to be a major factor for the enhancement of the de-

Abbreviations: Ax, Antheraxanthin; DOPE, dioleoylphosphatidylethanolamine; DSC, differential scanning calorimetry; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetate; eggPE, phosphatidylethanolamine from egg yolk; GdmHCl, guanidinium hydrochloride; GSH, reduced glutathione; GSSG, oxidized glutathione; H_{II}, inverted hexagonal phase; IPTG, isopropylthiogalactoside; L_α, liquid-crystalline phase; L_β, gel phase; MGDG, monogalactosyldiacylglycerol; POPE, palmitoyloleoylphosphatidylethanolamine; VDE, violaxanthin de-epoxidase; Vx, Violaxanthin; Zx, Zeaxanthin

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epoxidation rates [12]. However, recent data [9] have questioned the importance of the non-bilayer lipid phase for the de-epoxidation.

To address the question which lipid phase is required to promote VDE activity and how the different phases affect Vx de-epoxidation, we applied a phosphatidylethanolamine (PE) model system. PE shows a very similar phase polymorphism compared to MGDG. The advantage of using PE instead of MGDG is that a detailed structural characterization of the phase polymorphism of the underlying lipid structures can be carried out by ^{31}P NMR spectroscopy. Furthermore, various synthetic or isolated PE species are commercially available, forming either lamellar or inverted hexagonal structures in aqueous environment at physiological temperatures.

2. Material and methods

2.1. Materials

L-α-phosphatidylethanolamine from egg yolk (eggPE) and synthetic 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine (POPE) were purchased from Avanti Polar Lipids, Inc. (Alabaster, Al, USA), 1,2-dioleyl-*sn*-glycero-3-phosphoethanolamine (DOPE) from Fluka (Buchs, Switzerland) and monogalactosyldiacylglycerol (MGDG) from Lipid Products (Nutfield, UK).

All solvents and chemicals were purchased from Carl Roth AG (Karlsruhe, Germany) or Sigma Aldrich (Seelze, Germany), the spinach and daffodils were obtained from local markets.

2.2. Enzyme preparation and assays

Preparation of crude VDE extracts from spinach thylakoids was performed following the standard method [3] with modifications described in [13]. Thylakoids were broken by ultrasound treatment and for our present experiments the heterogeneity of the aqueous thylakoid extract was reduced by introducing an additional washing step at pH 5.4 to remove all lumenal proteins that remain soluble at this pH. With a second ultrasound treatment at pH 7.4 the VDE containing protein fraction, which was reversibly bound to the thylakoid membranes under acidic conditions, was released. Each preparation was tested for activity in preliminary experiments with MGDG at 30 °C before using it for the in vitro assays of the present study.

Violaxanthin was isolated from daffodil petals according to [13].

In vitro VDE assays were performed with pigment to lipid ratios of 1/29 (mol/mol) following established procedures [10] in VDE reaction buffer (pH 5.2, 10 mM KCl, 5 mM $MgCl_2$, 40 mM MES).

The open reading frame encoding the mature AtVDE was ligated into pET 44a (Novagen, Madison, WI, USA) between the NdeI and XhoI restriction sites adding a C-terminal His_6-tag and over-expressed in *E. coli* BL21(DE3) cells as inclusion bodies using high density fermentation [14]. The cells were cultivated in a 6 l fed batch process in complex media. At an OD_{600} of 45, the protein expression was induced with 1 mM isopropylthiogalactoside (IPTG) and the cells were cultivated for additional 4 h at a feed restricted growth rate. More than 1 kg of wet biomass was harvested by centrifugation. Cell disruption and isolation of the inclusion bodies were preformed as described in [15] with a yield of over 20 wt% inclusion bodies per wet biomass.

The inclusion bodies were solubilized in 6 M guanidinium hydrochloride (GdmHCl), 100 mM Tris, 1 mM ethylenediaminetetraacetate (EDTA), 50 mM dithiothreitol (DTT), pH 8.0 for 2 h at room temperature. After the removal of insoluble parts by centrifugation, the DTT was removed by gel filtration using a buffer containing 4 M GdmHCl, 1 mM EDTA, 50 mM Na_2HPO_4, pH 8.0 with a HiPrep™26/10 column from Amersham Biosciences (Buckinghamshire, UK). The AtVDE was separated from the other solubilized *E. coli* proteins by metal affinity chromatography with a HisPrep™FF16/10 column (Amersham Biosciences). By using 4 M GdmHCl, 300 mM imidazole, 50 mM Na_2HPO_4, pH 8.0 the target protein was eluted from the column in a high purity as confirmed by SDS page, and with a yield of around 300 mg protein per litre medium.

For in vitro folding of the AtVDE the solubilized protein was diluted into a buffer containing 1 M L-arginine, 100 mM Tris/HCl pH 8.5, 1 mM EDTA, 1 mM oxidized glutathione (GSSG) and 5 mM reduced glutathione (GSH) with four pulses at a temperature of 4 °C. Each pulse was set in a time lag of at least 6 h [16]. After the last pulse, the folding solution was incubated for a minimum of 48 h with a final protein concentration of 200 μg/ml. Then, the folding solution was dialyzed against 40 mM HEPES, 5 mM $MgCl_2$, 10 mM KCl, pH 7.4 for several days to remove the GSSG/GSH and avoid oxidation of the cystein residues. Finally, the AtVDE was concentrated using cross-flow filtration to a maximum concentration of 2.6 mg/ml. The loss of protein during folding, dialyses and concentration was less than 30%.

AtVDE was tested for activity in 1000 fold dilution, the specific activity of the obtained enzyme was 376 U/μg protein with 1 U defined as nmol/min. Recombinant VDE was exclusively used in the NMR studies.

2.3. ^{31}P NMR spectroscopy

For the ^{31}P NMR measurements the respective lipid powder was suspended in VDE reaction buffer (pH 7.4, 10 mM KCl, 5 mM $MgCl_2$, 40 mM HEPES) and homogenized by several freeze–thaw cycles. Recombinant VDE was added at a protein to lipid molar ratio of 1/400, followed by additional freeze–thaw cycles. The buffer was exchanged overnight by dialysis against reaction buffer at pH 5.2 (see above). After a gentle centrifugation the pellet was used for NMR measurements. Usually, the samples contained about 15 to 20 mg of lipid.

Static ^{31}P NMR spectra were acquired on a Bruker DRX 600 (Bruker, Biospin GmbH, Rheinstetten, Germany) spectrometer operating at a resonance frequency of 242.8 MHz for ^{31}P using a Hahn-echo pulse sequence. Typical ^{31}P 90° pulse lengths were 8.5 μs, using a Hahn-echo delay of 50 μs, a spectral width of 100 kHz, and a recycle delay of 3 s. Continuous-wave proton decoupling was applied during signal acquisition.

2.4. Differential Scanning Calorimetry (DSC)

For DSC lipids were suspended in VDE reaction buffer (pH 5.2) to a final concentration of 1.16 mM. Measurements were performed in a VP-DSC, Microcal Inc. (Northampton, MA, USA) at a heating rate of 90 K/h.

2.5. Absorption spectroscopy

Samples for absorption spectroscopy were prepared in the same way as for the enzyme assays, but without addition of VDE. Spectra were recorded on a Hitachi U-2000 (Wokingham, UK) double beam spectrophotometer with a temperature controlled multi-sampler in a wavelength range from 350 to 600 nm with a bandpass setting of 1 nm. III/II values were calculated according to [17] with III/II-value = $(E_{483}-E_{469})/(E_{453}-E_{469})$, where E_{453} represents the 2nd and E_{483} the 3rd absorption maximum and E_{469} the valley between the respective maxima. The values were then normalized with respect to the highest absorption achieved for the lipid under examination.

3. Results and discussion

3.1. Selection of lipids and characterization of their phase behavior

As a prerequisite for the present study three types of PE with varying thermotrophic phase properties were required. The first should adopt inverse hexagonal H_{II} structures in the temperature range from 15 °C to 45 °C, the second should form a lamellar bilayer (L_α) phase at these temperatures, and the third should perform a transition from L_α to H_{II} phase within the respective temperature interval. POPE, DOPE and eggPE were identified as potential candidates, respectively. DOPE forms H_{II} phases when

suspended in excess of aqueous buffers, even at temperatures close to 0 °C [18], and was thus chosen as reference for an H_{II} phase forming PE. POPE has been described to provide lamellar bilayer structures in the temperature interval used for the present experiments, but undergoes a phase transition from the rigid gel phase (L_β) to the liquid-crystalline phase (L_α) at 25.5 °C [19]. DSC measurements confirmed this phase transition temperature of POPE in VDE reaction buffer (pH 5.2) (Fig. 1D). However, in the VDE enzyme assays the buffer contained 2% (v/v) EtOH/MetOH (1/1, v/v) from the Vx and lipid stock solutions and the lipid phase contained 3.4 mol% Vx. With respect to Vx, literature suggests that the influence of xanthophylls on the phase transition temperature can be neglected [20]. According to previous studies, the influence of small amounts of EtOH on the lipid phase transition is also marginal [21], while no literature is available describing the effects of MetOH. Nevertheless, repeating the DSC experiment in the presence of 2% (v/v) EtOH/MetOH (1/1, v/v) we measured a comparable phase transition temperature of POPE at 24.9 °C (Fig. 1D).

With respect to the L_α–H_{II} phase transition of eggPE contradictory results have been published and the reported transition temperature varies considerably in a range between 28 and 46 °C [22,23]. These differences could be due to differences in the acyl chain composition and degrees of chain unsaturation of these natural extracts and to the different buffer conditions applied. To overcome these uncertainties we analyzed the phase behavior of the eggPE lot used in our experiments by ^{31}P NMR. This method can quantitatively distinguish the different lipid phase states by characteristic line shapes [24,25]. The experiments were carried out in excess of water (reaction buffer pH 5.2). In our system the L_α–H_{II} phase transition started at temperatures below 30 °C and was completed at 36 °C (Fig. 1A–C). Subsequent cooling of the sample (data not shown) revealed an almost complete

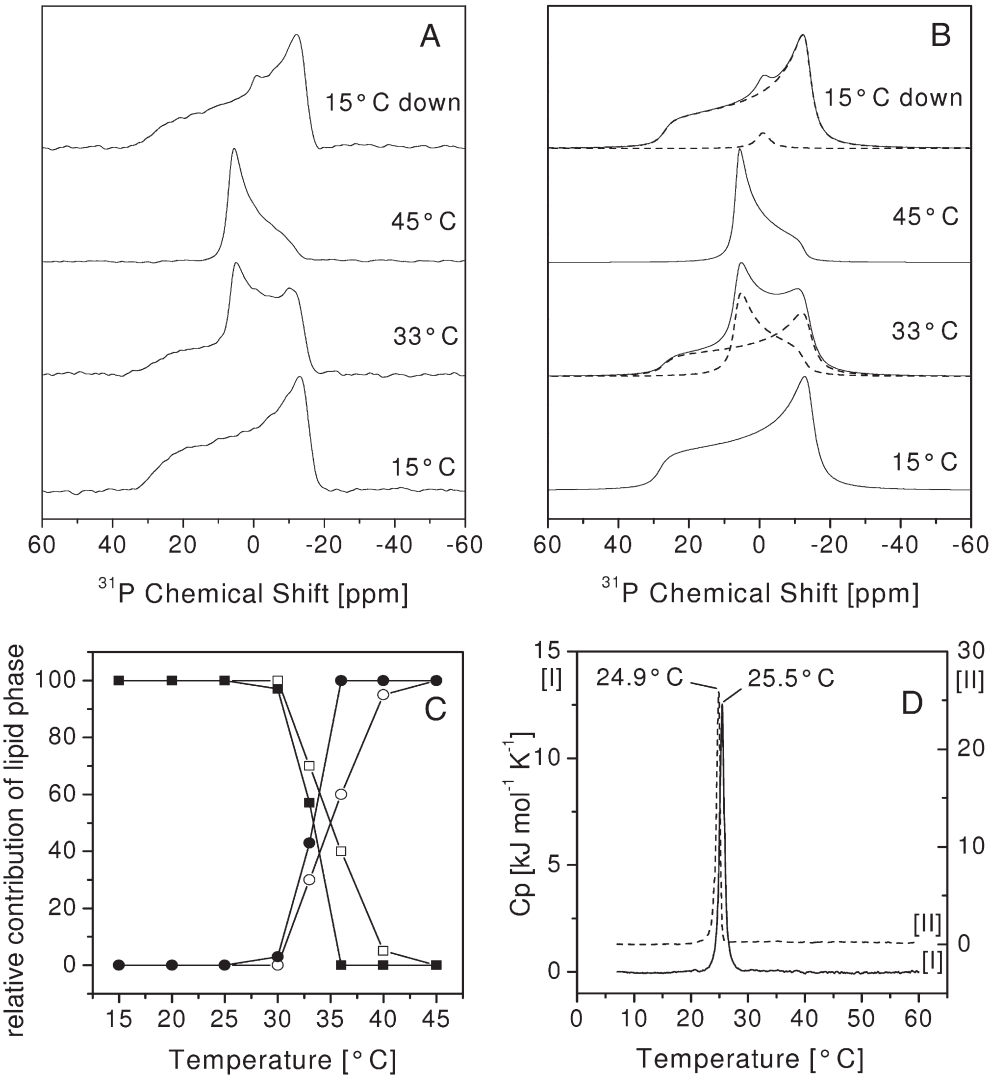

Fig. 1. ^{31}P NMR spectra (A) and their corresponding lineshape simulations (B) of eggPE in VDE reaction buffer (pH 5.2). The first measurement was performed at 15 °C, after stepwise heating the sample was gradually cooled down again to 15 °C. Contributions to the spectra from different lipid phases are shown as dashed lines. (C) Relative contributions of lamellar (squares) and hexagonal (circles) lipid phases of eggPE as obtained from ^{31}P NMR during stepwise heating in presence of AtVDE (1/400 mol/mol VDE/lipid) (open symbols) or absence of AtVDE (closed symbols). (D) DSC thermogram of POPE in VDE reaction buffer at pH 5.2 in the presence (dotted line) or absence (solid line) of 2% MetOH/EtOH (1/1 v/v).

reversibility of the phase transition with a hysteresis of approximately 1.4 °C and a small proportion of isotropic lipid phase left (less than 5% at 15 °C). To evaluate if the lipid binding of the VDE itself has an impact on the phase transition of the lipid phase, we repeated the ^{31}P NMR measurements in the presence of recombinant VDE from *Arabidopsis thaliana*, (AtVDE, sequence identity to spinach VDE = 0.79; similarity = 0.92 (BLOSUM62)). The influence of membrane inserted α-helical model peptides on the phase transition of PE has been described earlier [26] to potentially shift the L_α–H_{II} phase transition towards lower temperatures. In our present experiments, however, the phase transition in the presence of bound VDE was slightly broadened, whereas a significant shift in the phase transition temperature could not be detected (Fig. 1C). The same held true for the L_β–L_α phase transition in POPE after addition of AtVDE (data not shown).

According to these results, eggPE represents a suitable model lipid for the determination of the role of the H_{II} phase on VDE activity, as it undergoes a complete phase transition from the liquid crystalline phase to the inverted hexagonal phase within the temperature range assessed in our experiments.

Finally, MGDG was used as a further reference lipid, because it represents the main lipid of the native thylakoid membrane. MGDG forms an H_{II} phase at all temperatures investigated [27].

3.2. Influence of temperature and lipid phase on Vx solubilization

Former studies have shown that insufficient solubilization of Vx in the lipid phase limits VDE activity [10]. In aqueous environment, the hydrophobic carotenoids aggregate and the fine structure of their absorbance spectrum with three defined peaks in the wavelength range of 400 to 500 nm is substituted by a single peak in the blue wavelength region [28]. Therefore, we used the ratio of the 3rd to the 2nd absorption maximum (III/II-value) to estimate the degree of solubilization of Vx in the different lipid structures depending on the temperature (Fig. 2).

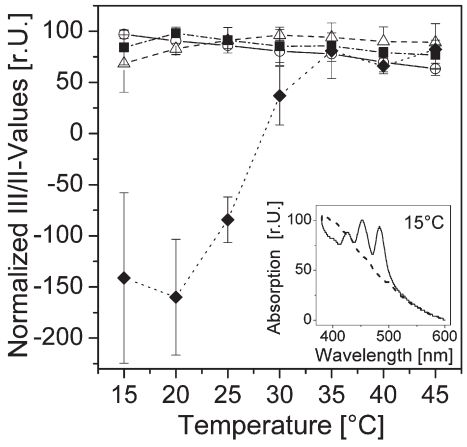

Fig. 2. Averaged III/II-values and standard deviations of Vx suspended in MGDG (open circle), DOPE (open triangle), eggPE (black square) and POPE (black diamond). III/II-values of Vx in the single lipids are normalized to their respective maximum. For details see 2.5. The inset shows typical absorption spectra of Vx in eggPE (solid line) and POPE (dotted line) at 15 °C.

As the absolute absorption values depend on the lipid species, the III/II-values were normalized to their maximum. Our data show a significant limitation of pigment solubilization only in POPE at temperatures below 30 °C. Solubilization of Vx in POPE starts at temperatures above 20 °C and is completed at 30 °C. This correlates with the phase transition of POPE from the gel phase to the liquid-crystalline phase, which was shown to occur at about 25 °C (Fig. 1D). We conclude that the L_β phase inhibits the solubilization of Vx. In all other lipids examined, Vx was completely solubilized in the whole temperature range and, therefore, the substrate accessibility would not influence the reaction rates of the de-epoxidation.

3.3. Temperature and lipid dependence of violaxanthin de-epoxidation

Significant VDE activities were obtained with all lipids applied in this study, with the exception of POPE at 15 °C, where VDE activity was extremely low or even undetectable (Fig. 3). At this temperature the two non-bilayer lipids MGDG and DOPE reached a de-epoxidation state of almost 40% (DOPE) or higher (MGDG) after a reaction time of 20 min. The de-epoxidation state in the presence of eggPE was found to reach values around 25%, which is in between those of POPE and those of MGDG and DOPE. Regarding the overall shape of the temperature curve, assays with DOPE and MGDG displayed a similar behavior. Yet, the DOPE curve was slightly shifted towards higher temperatures leading to increased de-epoxidation states at 40 °C and 45 °C compared to MGDG, although both lipids provide an H_{II} phase over the whole temperature range used in the present study. These observed differences likely result from the altered fatty acid composition and head group architecture of the two lipids, leading to different molecular geometries and phase properties. These findings are in agreement with previously published results [29]. The importance of the fatty acid composition and the nature of the head group is also indicated by the significantly different phase transition temperatures from the lamellar to the inverted hexagonal phase, which in the case of MGDG takes place at about −15 °C [27] and for DOPE is observed in the range from 2–5 °C [18].

In the presence of eggPE limited activities at 15 and 20 °C were observed. At higher temperatures this restriction slowly disappeared and at 45 °C de-epoxidation was similar or even faster and more complete than in the presence of MGDG and DOPE. However, as the L_α to H_{II} phase transition of eggPE had been shown to start at 30 °C and to be complete at about 40 °C (Fig. 1A–C), the enhanced VDE activity could not be correlated with this phase transition.

In the case of POPE, the temperature curve started with virtually undetectable VDE activity at 15 °C. This was followed by a steep increase in de-epoxidation at temperatures above 25 °C and an almost complete de-epoxidation at the highest temperatures, as observed for the other lipids used in the present study. Yet, in the temperature interval from 20 °C to 30 °C high standard errors occurred, masking the true curve progression. Remarkably, this represented the temperature interval, where

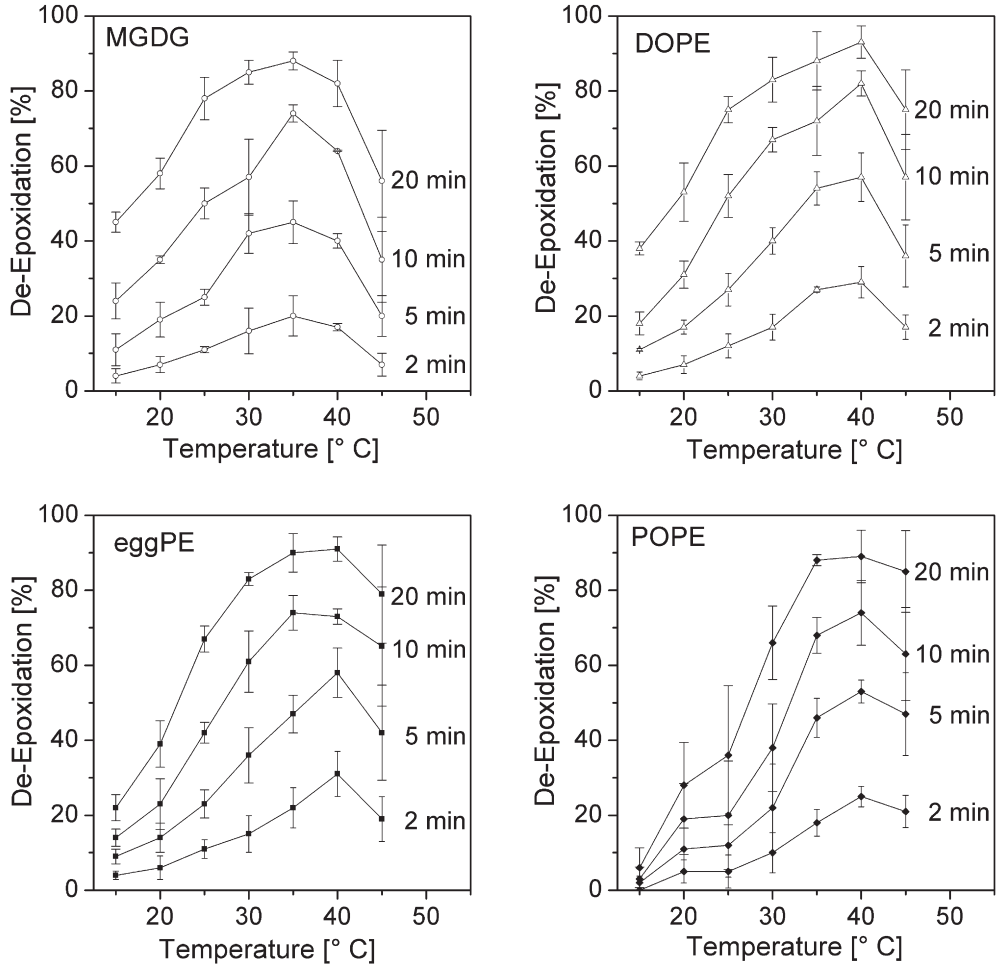

Fig. 3. De-epoxidation [%] of Vx by spinach VDE after reaction times of 2, 5, 10 and 20 min in the different lipids shown as arithmetic means with standard errors. Values were derived from 2 (MGDG), 3 (DOPE, POPE) or 4 (eggPE) independent sets of data.

the L_β–L_α phase transition of POPE occurred in our present experiments (Fig. 1D).

A better understanding of the lipid dependence of Vx de-epoxidation could be achieved by fitting the data to the Arrhenius equation and plotting them as the natural logarithm of the reaction rates versus the reciprocal reaction temperatures (Fig. 4). Thus a linear correlation can be achieved, where the temperature is the only variable influencing the changing reaction rates. For the Arrhenius plots depicted in Fig. 4 we calculated the de-epoxidation rates (in nmol pigment per min) from the de-epoxidation states reached after 5 min of the respective enzyme assays. This allowed a more reliable determination of the reaction rates compared to a fit of the complete de-epoxidation kinetics, which, in the case of low reaction rates, yielded unreliable results. For MGDG, DOPE and eggPE, the Arrhenius plots showed a reasonable linear correlation up to the temperatures of maximum VDE activity. Considering, that the L_α to H_{II} phase transition of eggPE took place within this temperature range, this phase transition did not seem to have a significant impact on the reaction rates of VDE. In contrast, in the presence of POPE two separate though overlapping linear correlations between the reaction rates and the temperature could be observed. The first covered the temperature range from about 20 °C–40 °C, including the temperature optimum of VDE activity. The second linear correlation could be observed at lower temperatures as a parallel shift of the first correlation curve with 30 °C as the maximum temperature. Interestingly, the transition between the two correlation curves took place in a temperature range between 20 °C and 30 °C. This represents the temperature range of the main phase transition of POPE (Fig. 1D) and also marks the temperature where Vx underwent complete solubilization in this lipid (Fig. 2). It is clear, that these two factors, apart from the low temperature, had a strong impact on VDE activity, thereby causing the deviation from the Arrhenius equation. Our data do not allow to unequivocally decide whether the gel phase structure prevents VDE activity directly or indirectly via its reduced solubilization capacity for the substrate Vx. However, it is likely that the limited diffusion rates of Vx are the main cause for the decreased de-epoxidation rates. Vx diffusion is supposed to be strongly restricted in the gel phase due to the aggregation of the pigment itself and due to the rigidity of the lipid gel phase.

The present findings of the temperature and lipid phase dependence of Vx de-epoxidation in the presence of eggPE, which undergoes an L_α to H_{II} phase transition, imply that the

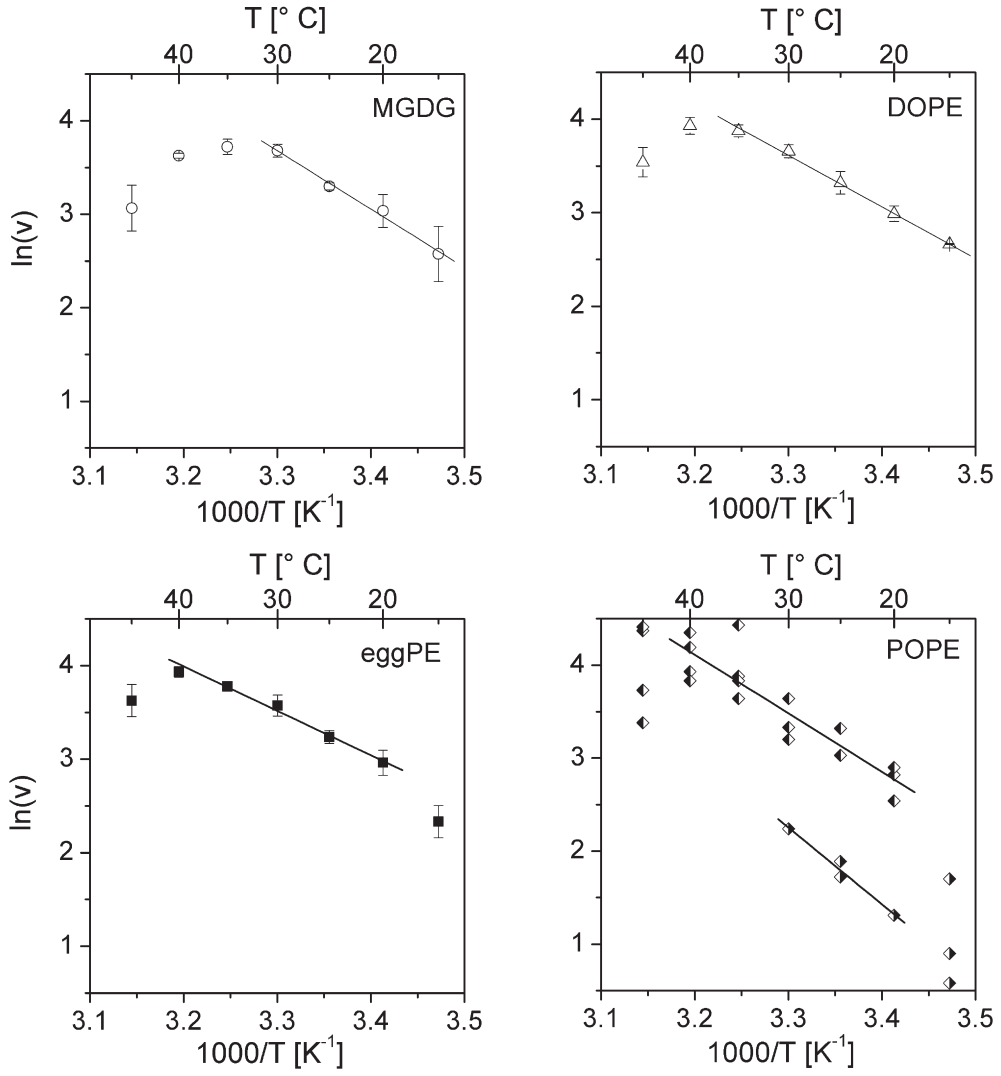

Fig. 4. Arrhenius plots of spinach VDE activities calculated from the de-epoxidation after 5 min assay time in MGDG, DOPE, eggPE and POPE. Mean values are shown except for POPE, where original data are displayed, bars indicate standard errors. Values were derived from 2 (MGDG), 3 (DOPE) or 4 (eggPE, POPE) independent sets of data.

inverted hexagonal lipid phase is not the only three-dimensional lipid structure enabling efficient Vx de-epoxidation. Even in the presence of bilayer forming POPE high de-epoxidation rates could be observed, as soon as the temperatures exceeded the temperature of the main phase transition from the gel to the liquid–crystalline phase, i.e. from the L_β to the L_α phase.

These observations in single lipid model systems, however, raise the question which general parameters are decisive for high VDE activities. According to our present results one important factor is the presence of a fluid lipid phase, providing sufficient lateral diffusion rates and as well as lipid and substrate mobility. This can not be achieved in the gel phase state. Also, as stated earlier [10], these parameters could include the formation of "hydrophobic insertion sites" at the lipid–water interface due to an altered lateral pressure profile of lipid bilayers as suggested in the literature [30,31] for a peripheral membrane protein of *E. coli.* This assumption is also supported by our former experiments in different binary lipid mixtures of bilayer and non-bilayer lipids [32], which revealed increasing de-epoxidation rates with increasing molar proportions of non-bilayer lipids. In these experiments, we used eggPE as a non-bilayer phospholipid and reaction temperatures of 30 °C. Due to the lack of detailed structural information on the phase behavior of eggPE, we assumed that eggPE formed inverted hexagonal structures in these liposome systems and came to the conclusion that H_{II} phases are required for high VDE activities. Integrating our present findings into these earlier results, it becomes clear that these lipid mixtures more likely provide an overall lamellar phase, but with altered order parameters and packing density across the membrane. This might lead to a modified surface hydrophobicity in the headgroup region of the mixed phospholipid bilayers, facilitating the insertion of the enzyme into the hydrophobic interior of the membrane. The relevant factors deciding about high VDE activities may be adjusted by any conical lipid, regardless if the overall structures of the lipid phase are lamellar or inverted hexagonal. These factors could in a lamellar system include, as very recently suggested by Szilagyi et al. [33], a negative curvature stress of the bilayer. In

our opinion, a similar interaction of VDE with the hydrophobic region of the lipid phase can also be realized in the H_{II} phase as provided by MGDG and DOPE. From our present study we conclude the following requirements regarding the lipid environment: First, the lipid matrix should provide a sufficient packing density and motility of the lipid molecules to ensure substrate solubilization and diffusion as well as binding of VDE, thereby enabling rapid de-epoxidation. These requirements are fulfilled by a fluid lipid phase, i.e. the L_α or H_{II} phase. Our data indicate that the H_{II} phase is better suited to meet these requirements at low temperatures compared to the L_α phase. Second, the lipid environment should provide sufficient stability with respect to the formation of lipid–enzyme–substrate complexes, especially at higher temperatures. Our present data imply that the L_α phase might be better suited to provide this stability as compared to the inverted hexagonal phase.

3.4. Conclusions

Our study for the first time combines data on the lipid dependence of VDE activity with a detailed examination of the structure of the respective lipid phases. We demonstrate that the L_β–L_α phase transition strongly enhances both the solubilization of Vx and the activity of VDE. According to our present results this phase transition is more important for Vx de-epoxidation than the transition from the L_α to the H_{II} phase. Especially at higher temperatures the L_α phase seems to be perfectly suited for high enzyme activities, probably due to a higher stability of the lipid–protein–substrate complexes in this lipid phase. The H_{II} phase, on the other hand, seems to be favorable for high VDE activities at lower temperatures. Consequently, the highest de-epoxidation rates at low temperatures were found in the H_{II} forming lipids MGDG and DOPE. Future measurements will have to show, in how far our present results obtained in model systems employing only single lipids are transferable to the native thylakoid membrane, which is in fact far more complex with regard to its lipid composition and structure.

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