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Investigating the interaction between the violaxanthin cycle enzyme zeaxanthin epoxidase and the thylakoid membrane

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ABSTRACT

In the present study the interaction between the violaxanthin cycle enzyme zeaxanthin epoxidase (ZEP) and the thylakoid membrane was investigated. Isolated, active thylakoid membranes of spinach (*Spinacia oleracea* L.) were subjected to different salt and detergent treatments that are generally used to isolate peripheral and integral membrane proteins. These salt and detergent treatments included the use of the salts NaBr, Na_2CO_3 and Tris and the detergents octylglucoside (OG) and dodecylmaltoside (DM). After the treatments the activity of the ZEP was determined in washed thylakoid membranes. To obtain additional information about the mode of ZEP binding to the membrane a hydrophobicity plot based on the amino acid sequence of the protein was constructed. The plot was then compared to a diagram obtained for the photosystem II antenna Lhcb1 protein whose integration into the thylakoid membrane is known. The results of the salt and detergent treatments of the thylakoid membrane suggest that the ZEP is a peripheral, rather weakly bound membrane protein. Results from the hydrophobicity plots indicate the existence of specialized protein domains which may realize the partial integration and binding of the ZEP to the thylakoid membrane.

1. Introduction

The violaxanthin (Vx) cycle plays a major role in the photoprotection mechanisms of higher plants. The cycle consists of a forward reaction, in which the di-epoxy xanthophyll Vx is de-epoxidized to antheraxanthin (Ax) and zeaxanthin (Zx), and a back reaction, which comprises the epoxidation of Zx to Ax and Vx [1,2]. The de-epoxidation reaction of the Vx cycle is catalyzed by the enzyme violaxanthin de-epoxidase (VDE), which is activated by the decrease of the pH of the thylakoid lumen caused by the light-driven photosynthetic proton gradient [2,3]. The back reaction of the Vx cycle is usually observed under low light conditions or in darkness, but also takes place during high light illumination of plants [4–6]. It is catalyzed by the enzyme zeaxanthin epoxidase (ZEP). The epoxidation rates of the ZEP are 5–10 times lower than the de-epoxidation rates of the VDE [7] which means that the constitutive activity of the ZEP only slightly affects the accumulation of Zx during periods of high light illumination.

The ZEP has a pH-optimum of pH 7.5 [4,8] and utilizes oxygen and NADPH as cosubstrates [9]. The pH-optimum and the cofactor requirements, in conjunction with the impermeability of the thylakoid membrane for NADPH, make it likely that the ZEP is located at the stromal side of the thylakoid membrane. Based on the results of [9] the electrons from NADPH are transferred to FAD which then acts as electron donor for the reduction that is needed for the formation of the epoxy group. This is agreement with the findings of [10] who described the presence of an FAD-binding domain within the sequence of the ZEP. The binding of FAD by the ZEP makes the enzyme sensitive against the inhibitor diphenyleneiodoniumchloride (DPI) which blocks the reoxidation of FAD in flavin-containing enzymes [9]. Further studies have shown that ZEP activity can also be inhibited by cadmium and amino sugars [11–13]. Until today the isolation of an active ZEP by conventional protein purification methods has not been reported. This means that *in vitro* assays with the isolated enzyme are not possible and that the investigation of Zx epoxidation is constricted to the study of pigment conversions in isolated active thylakoid membranes.

Although the protein has not been purified so far various ZEP amino acid sequences have been published, including those for the higher plants *Nicotiana plumbaginifolia* [14], *Capsicum annuum* [10], *Lycopersicon esculentum* [15], *Oryza sativa* [16] and the green alga *Chlamydomonas reinhardtii* [17]. The amino acid sequences indicate that the mature ZEP protein consists of around 600 amino acids and has a molecular mass of 67 kDA. Based on the analysis of the ZEP amino acid sequence the ZEP belongs to the lipocalin family of proteins as it has been reported for the VDE [18,19]. Lipocalins are a diverse group of proteins which are characterized by a central motif consisting of a barrel-like structure. This structure also contains the substrate binding site of the lipocalins which in

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general interact with small hydrophobic molecules. Since both the VDE and the ZEP are able to bind and convert the intermediate reaction product of the Vx cycle, Ax, it has been suggested that the catalytic site of both enzymes has a comparable structure [18]. Interestingly, the lipocalin domain of the ZEP contains additional amino acids in comparison to the VDE and it has been proposed that these form a loop-like structure which is important for the association of the ZEP with the thylakoid membrane [18,19]. Besides the lipocalin domain and the FAD binding site the ZEP contains a second putative FAD binding site, an ADP binding site which probably interacts with the ADP part of the NADPH molecule and various PEST sequences which are target sites for proteases during the ZEP degradation [10].

Since information on the interaction of the ZEP with the thylakoid membrane is extremely sparse it was the intention of the present study to analyze this aspect of the Vx cycle in greater detail. We have treated isolated thylakoid membranes with different salts and detergents, which are generally used to isolate peripheral and integral membrane proteins, to obtain information on the strength of the ZEP binding to the membrane. Freeze-thaw cycles of thylakoids were used to compare the membrane association of the VDE and ZEP at different pH-values. Finally, we have constructed hydrophobicity plots of the ZEP based on the published amino acid sequences to find protein regions which might enable the interaction of the ZEP and the thylakoid membrane.

2. Materials and methods

2.1. Plant material and thylakoid preparation

Fresh spinach (*Spinacia oleracea* L.) leaves were obtained from the local market. Thylakoid membranes of spinach were isolated according to standard protocols based on the method by [20]. In addition to this protocol thylakoids were also prepared with isolation media where bovine serum albumin (BSA) was omitted.

2.2. Vx de-epoxidation and Zx epoxidation assays

All de-epoxidation and epoxidation assays as well as the salt and detergent treatments were performed with freshly isolated thylakoids within a few hours after the preparation.

Vx de-epoxidation assays were performed with isolated thylakoid membranes in reaction medium pH 5.2 (330 mM sorbitol, 10 mM NaCl, 5 mM $MgCl_2$, 40 mM MES) in the presence of 30 mM ascorbate in the dark according to [21].

The Zx epoxidation assays were carried out with thylakoid membranes which contained high concentrations of Zx. A high level of Zx was obtained by a 20 min incubation of thylakoid membranes at pH 5.2 in the dark in the presence of 30 mM ascorbate as described for the Vx de-epoxidation assays above. After the incubation the de-epoxidation reaction was terminated by a centrifugation of the thylakoids at 17.000 g and 4 °C for 10 min (Allegra 64R, Beckman Coulter, Germany) followed by two washing steps with reaction medium pH 7.5 (330 mM sorbitol, 10 mM NaCl, 5 mM $MgCl_2$, 40 mM HEPES). Washing of the thylakoid membranes had no influence on the ZEP activity (data not shown) but ensured that the cofactor of the VDE, ascorbate, was eliminated from the sample.

All the treatments with salts, detergents, ultra sound and freeze-thaw-cycles described below were performed with the Zx-enriched and washed thylakoid membranes. For the Zx epoxidation assays (see also [9]) the Zx-enriched and washed thylakoid membranes were incubated in reaction medium pH 7.5 in the dark. The cofactors of the ZEP, NADH and FAD, were added at concentrations of 1 mM and 5 μM, respectively. For both the Vx de-epoxidation and the Zx epoxidation assay the thylakoids were used with a chlorophyll (Chl) concentration of 20 μg mL^{-1} and the assays were performed at 20 °C. To determine the kinetics of Vx de-epoxidation and Zx epoxidation and the activity of the VDE and ZEP samples were collected at various time points of the assays (see Section 3 and the figures for further information). After sample collection the de-epoxidation and epoxidation reactions were immediately stopped by the direct extraction of the pigments from the samples (see [22]).

2.3. Pigment analysis

The extracted pigments were then analyzed and quantified by HPLC according to [21].

2.4. Salt and detergent treatment of thylakoid membranes

To study the strength of ZEP binding to the thylakoid membrane isolated thylakoids were treated with different salts and detergents.

For the treatment with NaBr isolated thylakoids containing preformed Zx were diluted in reaction medium pH 7.5 to yield a final Chl content of 20 μg mL^{-1} and NaBr was added with a concentration of 2 M. After the incubation time of 30 min on ice the thylakoids were centrifuged at 30.000 g and 4 °C for 10 min (Allegra 64R, Beckman Coulter, Germany) to remove the NaBr containing supernatant. The thylakoids were resuspended in reaction medium pH 7.5 and the Zx epoxidation assays were started as described above. In a second treatment the NaBr incubation was performed twice before the thylakoids were washed and used for the assays.

Incubation of the thylakoid membranes with Na_2CO_3 was done as described for NaBr with the exception that different concentrations of Na_2CO_3 were used for the treatments (see Section 3 and Fig. 3 for actual salt concentrations).

Thylakoids were also treated with 0.8 M Tris/HCl pH 8.5. In this case the incubation was performed on ice for 15 min before the thylakoids were centrifuged and resuspended in reaction medium pH 7.5 for the epoxidation assays. For the Tris experiments control thylakoids were incubated with a reaction medium without Tris, buffered to pH 8.5 to exclude negative effects of the basic pH on the activity of the ZEP.

The mild detergents octylglucoside (OG) and dodecylmaltoside (DM) were used to treat the thylakoid membranes. OG was added with a concentration of 30 mM to isolated thylakoids (1 mg Chl mL^{-1}) in reaction medium pH 7.5. The detergent incubation was performed for 30 min on ice in the dark. The thylakoids were then centrifuged at 140.000 g and 4 °C for 60 min (J2-MC, Beckman Coulter, Germany) and the ZEP activity was determined in the resulting pellet and supernatant.

For the incubation with the detergent DM different concentrations were used (see Section 3 and Fig. 3). The isolated thylakoids were diluted in reaction medium pH 7.5 to a Chl concentration of 20 μg mL^{-1}. After addition of different concentrations of DM the membranes were incubated at 20 °C in the dark for 30 min. The treated thylakoids were centrifuged at 17.000 g and 4 °C for 10 min (Allegra 64R, Beckman Coulter, Germany). The activity of ZEP was analyzed in the resuspended pellet in reaction medium pH 7.5. Additional activity determinations were carried out with solubilized thylakoid membranes directly after the incubation with DM.

2.5. Ultrasound treatment and freeze-thaw cycles of thylakoid membranes

To determine the strength of ZEP binding to the thylakoid membrane thylakoids were also subjected to ultrasound and freeze-thaw cycles.

Thylakoids in reaction medium pH 7.5 with a Chl concentration of 20 μg mL^{-1} were slowly stirred on ice for 30 min. After 0, 15 and