Zeaxanthin epoxidation — an in vitro approach*

Paulina Kuczyńska1,2, Dariusz Latowski1,3, Sylwia Niczyporuk3, Monika Olchawa-Pajor1, Peter Jahns3, Wiesław I. Gruszeczki4 and Kazimierz Strzałka1

1Department of Plant Physiology and Biochemistry, Jagiellonian University, Kraków, Poland; 2Department of Plant Physiology, Institute of Biology, Pedagogical University, Kraków, Poland; 3Plant Biochemistry, Heinrich-Heine-University Düsseldorf, Düsseldorf, Germany; 4Department of Biophysics, Maria Curie-Skłodowska University, Lublin, Poland

Zeaxanthin epoxidase (ZE) is an enzyme operating in the violaxanthin cycle, which is involved in photoprotective mechanisms. In this work model systems to study zeaxanthin (Zx) epoxidation were developed. Two assay systems are presented in which epoxidation of Zx was observed. In these two models of Arabidopsis thaliana which have active only one of the two xanthophyll cycle enzymes were used. The npq1 mutant possesses an active ZE and is thus able to convert Zx to violaxanthin (Vx) but the violaxanthin de-epoxidase (VDE) is inactive, so that Vx cannot be converted to Zx. The other mutant, npq2, possesses an active VDE and can convert exogenous Vx to Zx under strong light conditions but reverse reaction is not possible. The first assay containing thylakoids from npq1 and npq2 mutants of A. thaliana gave positive results and high efficiency of epoxidation reaction was observed. The amount of Zx was reduced by 25%. To optimize high efficiency of epoxidation reaction additional factors facilitating both fusion of the two types of thylakoids and incorporation of Zx to their membranes were also studied. The second kind of assay contained npq1 mutant thylakoids of A. thaliana supplemented with exogenous Zx and monogalactosyldiacylglycerol (MGDG). Experiments with different proportions of Zx and MGDG showed that their optimal ratio is 1:60. In such system, due to epoxidation, the amount of Zx was reduced by 38% of its initial level. The in vitro systems of Zx epoxidation described in this paper enable analysis some properties of the ZE without necessity of its isolation.

Key words: zeaxanthin epoxidation, xanthophyll cycle, model system, Arabidopsis thaliana mutants npq1, npq2

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INTRODUCTION

Violaxanthin cycle is the most common of the six xanthophyll cycle types described so far in the literature. It occurs in higher plants, mosses, lichens and some groups of algae protecting them against overexcitation (Garcia-Plazaola et al., 2007; Goss et al., 2006). Two reversible reactions occur in the violaxanthin cycle. One of them is de-epoxidation of a di-epoxide xanthophyll, violaxanthin (Vx), by violaxanthin de-epoxidase (VDE). This enzyme is located in the thylakoid lumen and for activation requires ascorbate as a donor of protons, monogalactosyldiacylglycerol (MGDG) or another non-bilayer lipids such as, phosphatidylethanolamine (PE) and an acidic pH which is necessary for binding VDE to thylakoid membrane (Hager & Holocher, 1994; Latowski et al., 2004, Jahns et al., 2009). In the reverse reaction catalysed by zeaxanthin epoxidase (ZE), zeaxanthin (Zx), an epoxy-free pigment is converted to Vx, ZE, which is located on the stromal side of thylakoids, is a monooxygenase epoxidizing 3-hydroxy β-ionone rings of xanthophylls in 5, 6 position. NADPH, FAD, molecular oxygen and pH 7.5 are required for Zx epoxidation (Siefermann & Yamamoto, 1975; Büch et al., 1995). There exist model systems to study Vx de-epoxidation with the use of isolated enzyme (Yamamoto & Higashi, 1978; Latowski et al., 2002). Such approach in the case of Zx epoxidation is difficult because the respective enzyme has not been isolated and purified so far. In this paper two kinds of model systems for studies on Zx epoxidation in vitro are presented for the first time. In both systems reduction in the Zx amount was observed. In these assays two mutants of Arabidopsis thaliana which have active only one of the two xanthophyll cycle enzymes were used. The first one, npq1, produces active ZE and it converts Zx to Vx, while VDE is inactive and photoprotection by xanthophyll cycle mechanism is not observed (Havaux et al., 2000). The other mutant, npq2, possesses active VDE and it is able to convert exogenous Vx to Zx under strong light but reverse reaction is not possible. This mutant can accumulate Zx and it was used as a source of this pigment in our experiments.

MATERIALS AND METHODS

Two model systems of Zx epoxidation in vitro were tested using thylakoids isolated from Arabidopsis thaliana. Six week old plants grown in a 12 h/12 h photoperiod at 20°C, after 0.5 h dark incubation were illuminated for 30 min with 1500 µE·m−2·s−1 and homogenized 2 times by 10 s in isolation medium (330 mM Sorbitol, 20 mM Tricine, 5 mM MgCl2, 2.5 mM EDTA, pH 7.6). After centrifugation for 5 min at 4000 rpm, the supernatant was discarded and residue was resuspended in resuspension medium (300 mM Sorbitol, 20 mM Tricine, 5 mM EGTA, 5 mM EDTA, 10 mM Na2CO3, pH 7.6). After centrifugation for 5 min at 4000 rpm, the supernatant was discarded and residue was resuspended in resuspension medium (300 mM Sorbitol, 20 mM Hepes, 5 mM MgCl2, 2.5 mM EDTA, pH 7.6). At the end chlorophyll concentration in 80% acetone was measured by the method of Arnon (Arnon, 1949). The capability of Zx epoxidation in thylakoids isolated by above described method, was tested first for wild type of A. thaliana.

*e-mail: dariuszlatowski@gmail.com

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Abbreviations: Ax, antheraxanthin; MGDG, monogalactosyldiacylglycerol; PE, phosphatidylethanolamine; VDE, violaxanthin de-epoxidase; Vx, violaxanthin; ZE, zeaxanthin epoxidase; Zx, zeaxanthin.
In the case of assay using the mutants for Zx epoxidation in vitro, thylakoids from two A. thaliana mutants (npq1 and npq2) were mixed together and then sonicated. The first tested parameter to develop the optimal model system for Zx epoxidation was the time of sonication of the npq1/npq2 thylakoids mixture. Five different times of sonication were tested (1, 2, 5, 7, 10 seconds with 125 kHz and 20% amplitude) always with three repetitions. The second parameter studied in this assay system was the ratio of thylakoids from npq1 and npq2 mutants. The amount of thylakoids was determined by chlorophyll concentration. There were tested following npq1 to npq2 ratios: 1:1 (50:50 µg/ml), 1:1 (100:100 µg/ml), 3:1 (75:25 µg/ml), 3:1 (150:50 µg/ml).

In another kind of model system tested, only thylakoids from npq1 mutant were used because they retain a capability of Zx epoxidation in vivo. In this system, instead of npq2 thylakoids as the only source of Zx, the effect of different ratios of exogenous Zx and MGDG on epoxidation efficiency were studied. 20 nmol of the pigment was mixed with MGDG in different proportion (1:15, 1:30, 1:60, 1:100), using methanolic stock solutions of both compounds. After mixing, the solution was evaporated by nitrogen and then the dry residue was dissolved in ethanol. This solution was added into reaction mixture. Reaction was performed for 120 min. Ax, antheraxanthin; Chl, chlorophyll; Vx, violaxanthin; Zx, zeaxanthin.

RESULTS AND DISCUSSION

The studies on Zx epoxidation in in vitro systems were not possible because up today the enzyme involved in this reaction, zeaxanthin epoxidase, has not been isolated and no model system for analysis of kinetics or molecular mechanism of this reaction has been developed. It was also difficult to study of Zx epoxidation in isolated thylakoids although two methods for thylakoids isolation...
where ZE was working were described for plants. One of them was for lettuce (Siefermann & Yamamoto, 1974) and the other for spinach (Büch et al., 1995). The obtained results show that our method of thylakoids isolation from model plant, A. thaliana, which is presented in this work, also permits to study Zx epoxidation in isolated thylakoids (Fig. 1). In fact the presented method uses a fraction containing fragmented chloroplasts with thylakoids and stroma and therefore ZE, which is located at the stromal side of thylakoids, is also present in the final suspension. In assay system using thylakoids from npq1 and npq2 mutants the optimal time of their sonication was defined as 7 seconds with 125 kHz and 20% amplitude with 3 repeats. It was observed that after that time of sonication, epoxidation is the most efficient (Fig. 2). Probably such time of sonication allow better fusion of the two types of thylakoids and, on the other hand, it does not cause significant destruction of isolated structures. The second parameter studied in this model system was the ratio of thylakoids from npq1 and npq2. The optimal ratio was estimated as 1:1 (Fig. 3). The amount of thylakoids in reaction mixture was also important for Zx epoxidation efficiency. The optimum was defined as 100 µg of chlorophylls per 1 ml of epoxidation mixture. Higher concentration is not advantageous. In this model system with optimal proportion of the thylakoids from both mutants and optimal sonication conditions the amount of Zx was reduced by 25% (Fig. 3).

The most simplified system we studied consists of npq1 mutant thylakoids supplemented with exogenous Zx with MGDG in varying proportions. The optimal ratio of Zx to MGDG was defined as 1:60. The low amount of ethanol added to the assay mixture with Zx and MGDG did not reduce the efficiency of the reaction. In optimal conditions the amount of Zx was reduced by 38% (Fig. 4). The in vitro assay with exogenous Zx, is a very promising model system permitting studies on the Zx epoxidation. The use of exogenous substrate allows better controlling the reaction conditions and provides new possibilities to investigate molecular mechanism of epoxidation in the xanthophylls cycle.

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REFERENCES

Arnon DI (1949) Copper enzymes in isolated chloroplasts. Polyphenol oxidase in Beta vulgaris. Plant Physiol 24: 1–15.

Büch K, H. Stransky, A. Hager (1995) FAD is a further essential cofactor of the NAD(P)H and O2-dependent zeaxanthin epoxidase. FEBS Letters 376: 45–48.

Garcia-Plazaola JL, Matsubara S, Osmond CB (2007) The lutein epoxide cycle in higher plants: its relationships to other xanthophyll cycles and possible functions. Funct Plant Biol 34: 759–773.

Goss R, Lepeit B, Wilhelm C. (2006) Evidence for a rebinding of antheraxanthin to the light-harvesting complex during the epoxidation reaction of the violaxanthin cycle. J. Plant Physiol 163: 585–590.

Hager A, Holocher K (1994) Localization of the xanthophyll-cyclic enzyme violaxanthin de-epoxidase within the thylakoid lumen and abolition of its mobility by a (light-dependent) pH decrease. Planta 192: 581–589.

Havaux M, Bonfils JP, Lütz C, Niyogi KK (2000) Photodamage of the photosynthetic apparatus and its dependence on the leaf developmental stage in the npq1 Arabidopsis mutant deficient in the xanthophyll cycle enzyme violaxanthin deepoxidase. Plant Physiol 124: 273–284.

Jahns P, Latowski D, Strzałka K (2009) Mechanism and regulation of the violaxanthin cycle: the role of antenna proteins and membrane lipids. Biochim Biophys Acta 1787: 3–14.

Latowski D, Åkerlund HE, Strzałka K (2004) Violaxanthin de-epoxidase, the xanthophyll cycle enzyme, requires lipid inverted hexagonal structures for its activity. Biochemistry 43: 4417–4420.

Latowski D, Kruk J, Burda K, Skrzyniecka Jaskier M, Kostecka-Gugała A, Strzałka K (2002) Kinetics of violaxanthin de-epoxidation by violaxanthin de-epoxidase, a xanthophyll cycle enzyme, is regulated by membrane fluidity in model lipid bilayers. Eur J Biochem 269: 4656–4665.

Siefermann D, Yamamoto HY. (1975) Light-induced de-epoxidation of violaxanthin in lettuce chloroplasts IV. The effects of electron-transport conditions on violaxanthin availability. Biochim Biophys Acta 387: 149–58.

Yamamoto HY, Higashi RM (1978) Violaaxanthin de-epoxidase. Lipid composition and substrate specificity. Arch Biochem Biophys 190: 514–522.