S3-018

Violaxanthin de-epoxidase: Properties of C-terminal deletions on activity, NPQ and pH-dependent lipid binding in tobacco.

AD Hieber[1], RC Bugos[1], AS Verhoeven[2], HY Yamamoto[1]

[1]*Department of Molecular Biosciences and Biosystems Engineering, University of Hawaii at Manoa, Honolulu, HI 96822.* hieber@hawaii.edu, bugos@hawaii.edu, yamamoto@hawaii.edu

[2]*Department of Biology, University of St. Thomas, St. Paul, MN 55105-1096* *asverhoeven@stthomas.edu*

Keywords: xanthophyll cycle, violaxanthin de-epoxidase, lipocalin

Introduction

The non-radiative dissipation of excess energy is a process that depends on formation of zeaxanthin and antheraxanthin in the xanthophyll cycle that is catalyzed by violaxanthin de-epoxidase (VDE) localized in the thylakoid lumen (Yamamoto et al. 1962; Hager 1969; Yamamoto et al. 1972). The xanthophyll cycle is one of the systems higher plants and green algae use for protection against the damaging effects of excess sunlight (Demmig et al. 1987; Gilmore and Yamamoto 1993).

Structural analyses of the amino-acid sequence for VDE shows that it can be divided into three domains: a cysteine-rich N-terminal region, a lipocalin barrel structure and a glutamic acid-rich C-terminal region. This glutamic acid rich C-terminal region has been proposed to be involved in binding to the thylakoid membrane (Bugos and Yamamoto 1996). VDE had previously been overexpressed in *E. coli*. Although high yields of the purified enzyme were obtained, the enzyme exhibited a low specific activity, signifying incorrect folding. The goals of this research were to overcome the irregular folding by expressing VDE in tobacco plants and to determine the functional significance of the C-terminal domain of VDE with respect to lipid binding using deletion mutants. Tobacco plants overexpressing VDE were also analyzed to determine if an increase in VDE activity would result in increased protection.

Materials and methods

Constructs and Tobacco Transformation. PCR was used to amplify the full-length *Arabidopsis* VDE (Bugos et al. 1998) including the transit peptide and an added C-terminal six-histidine tail. C-terminal deletions of the *Arabidopsis* VDE were constructed by inserting stop codons at various sites using PCR. Amplified products were put under control of a CaMV 35S-35S promoter (Datla et al. 1993) in the binary vector pBI121 (Clonetech, Palo Alto, CA). Tobacco (*Nicotiana tabacum* cv. Xanthi) was transformed using *Agrobacterium,* selected and grown as described previously (Chang et al. 2000). Plants were grown under controlled growth conditions as previously described (Chang et al. 2000).

Enzyme Activity, Protein Determination, Western-Blot Analysis. Thylakoid lumen extracts were prepared and VDE was assayed as previously described (Bugos et al. 1999). A unit (U) of VDE activity is defined as one nmol of violaxanthin de-epoxidized per minute. Western

analysis was performed as described (Bugos et al. 1999) using a polyclonal antibody against the N-terminus of lettuce VDE (Bugos and Yamamoto 1996).

pH-dependent Lipid Micelle Binding. Monogalactosyldiacylglycerol (MGDG, 270 µM in methanol) and digalactosyldiacylglycerol (DGDG, 270 µM in methanol) (Lipid Products, Surrey, UK) were mixed with violaxanthin (10 µM in methanol) in a ratio of 6:4:6 (600 µl MGDG: 400 µl DGDG: 600 µl violaxanthin), dried under a stream of N_2 and resuspended in 100 µl methanol. Micelles were formed by adding 4.9 ml of suspending medium (100 mM NaCl, 100 mM sodium acetate, pH 5.1), vortexing and incubating at room temperature in the dark for 5 min prior to use. Thylakoid-lumen extracts (up to 200 µg protein) were added to the lipid-micelle suspension and incubated at room temperature for 5 min. The micelles were pelleted at 20,000 xg for 5 min, resuspended in 1 ml of 50 mM Tris-HCl, pH 7.6 containing 100 mM NaCl to release the pH-dependent bound protein and centrifuged.

Pigment Analysis and Fluorescence Measurements. Pigments were isolated and separated as described (Chang et al. 2000). Chlorophyll-fluorescence was measured with a PAM-2000 portable fluorometer (Heinz Walz, Effeltrich, Germany) as described previously (Chang et al. 2000). Conventional fluorescence nomenclature was used and NPQ was calculated as (F_m - F_m')/F_m'.

Results and discussion

Tobacco plants transformed with an *Arabidopsis* VDE cDNA with a C-terminal histidine tail under control of the CaMV 35S-35S promoter demonstrated a mean VDE specific activity on average 18-fold greater than wild-type (Fig. 1). The use of a construct with a C-terminal histidine tail allowed for the distinguishing between the expressed VDE and native VDE by molecular size, while also allowing for the potential purification by affinity chromatography. The transformed plants did not show any noticeable phenotypic difference compared to wild-type plants grown under standard growth-chamber conditions.

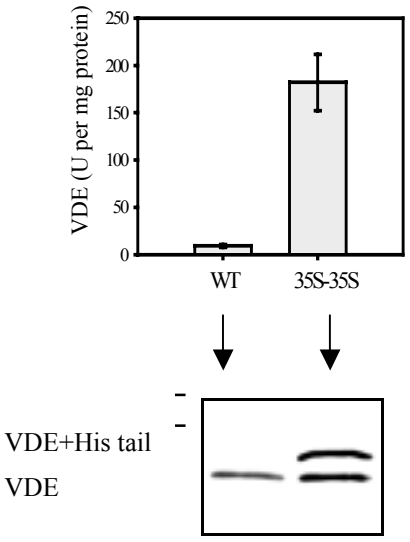

Fig. 1. Overexpression of VDE in tobacco plants. Thylakoid lumen extracts from wild-type (WT) and plants transformed with VDE + histidine tail under control of the 35S-35S promoter (35S-35S) were analyzed for VDE specific activity and by Western blotting. The error bars represent standard deviation (n=3)

The kinetics of de-epoxidation and chlorophyll fluorescence in an overexpressed plant was examined. The response to high light in the overexpressed plant showed a significantly higher initial rate of nonphotochemical quenching (NPQ) induction as compared to a wild-type plant (Fig 2B). In addition, the de-epoxidation state of the transformed plant increased more rapidly than wild-type, demonstrating that additional VDE has a significant effect on the initial rate of de-epoxidation (Fig 2A). No apparent difference in plant growth due to the increased initial rate of de-epoxidation was observed under standard growth-chamber conditions. The effect of VDE overexpression on growth of these plants will need to be evaluated under field conditions and conditions of stress.

To investigate involvement of the C-terminal region in pH-dependent binding, C-terminal deletion mutants were constructed and transformed into tobacco. Two deletion mutants were used, Cta and Ctb. Full length *Arabidopsis* VDE is 349 amino acids in length whereas Cta-VDE is reduced in length by 83 amino acids and Ctb-VDE is reduced in length by 71 amino

acids. VDE assays on thylakoid lumen extracts demonstrated high VDE activity in the Ctb plants and low activity in the Cta plants. The low VDE activity in Cta plants was equivalent to the level observed in wild-type plants.

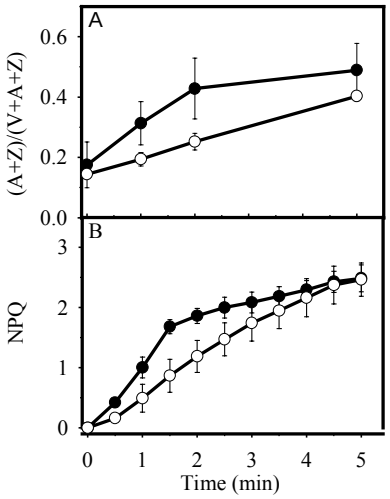

Fig. 2. Time course of de-epoxidation and NPQ in leaves of tobacco plants. A dark-adapted leaf from a wild-type (○) and an overexpressed (●) plant were exposed to 1500 μmoles photons m^{-2} s^{-1}. (A) De-epoxidation state was performed at the time points indicated. (B) NPQ was measured at 30 sec intervals. The error bars represent standard deviation (n=3).

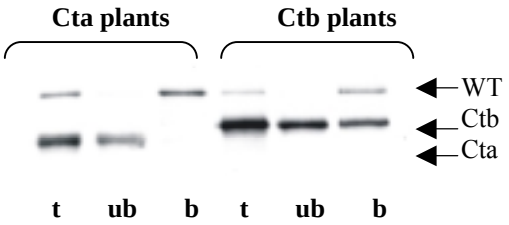

Fig. 3. Western analysis of pH-dependent micelle lipid binding. Thylakoid lumen extracts (t) from deletion mutants Cta and Ctb plants were incubated with lipid micelles at pH 5.1. Micelles were pelleted and the resulting supernatant contained unbound protein (ub). Micelles were resuspended in pH 7.5 buffer releasing any pH-dependent bound proteins (b). Samples were analyzed by Western blotting using an antibody specific to VDE. Arrows indicate wild-type VDE and deletion mutants Cta-VDE and Ctb-VDE.

As the highly charged C-terminal region had previously been hypothesized to be involved in binding to the thylakoid membrane, the lipid-binding ability of the two deletion mutants was investigated. Thylakoid-lumen extracts from the Cta and Ctb plants were incubated with lipid micelles in pH 5.1 buffer. Micelles were composed of violaxanthin with the thylakoid lipids MGDG and DGDG in a 6:4 ratio mimicking that of the thylakoid membrane. After incubation, the micelles were pelleted to remove unbound protein and subsequently resuspended in pH 7.5 buffer, releasing pH-dependent bound proteins (Fig 3). The results demonstrate that the majority of the Cta-VDE protein was found in the unbound fraction. In contrast, the endogenous wild-type VDE and Ctb-VDE proteins were both found in the bound fraction, demonstrating binding in a pH-dependent manner. These results demonstrate that *in vitro*, the low VDE activity observed in extracts from Cta plants is due to the inability of Cta-VDE to bind to the lipid micelles.

The difference between the Cta and Ctb deletion mutants is only 12 amino acids. It is possible that these 12 amino acids represent portion of a critical part of a lipid-binding domain or that removal of 12 extra amino acids from Ctb-VDE to Cta-VDE significantly changes the secondary structure of the VDE protein.

Acknowledgements

This work was supported by the U.S. Department of Energy, Division of Energy Biosciences (grant no. DE-FG03-92ER20078).

References

Bugos RC, Chang S-H, Yamamoto HY (1999) *Plant Physiology* **121,** 207-213

Bugos RC, Hieber AD, Yamamoto HY (1998) *Journal of Biological Chemistry* **273,** 15321-15324

Bugos RC, Yamamoto HY (1996) *Proceedings National Academy Science USA* **93,** 6320-6325
Chang S-H, Bugos RC, Sun W-H, Yamamoto HY (2000) *Photosynthesis Research* **64,** 95-103
Datla RSS, Bekkaoui F, Hammerlindl JK, Pilate G, Dunstan DI, Crosby WL (1993) *Plant Science* **94,** 139-149
Demmig B, Winter K, Krüger A, Czygan F-C (1987) *Plant Physiology* **84,** 218-224
Gilmore AM, Yamamoto HY (1993) *Photosynthesis Research* **35,** 67-78
Hager A (1969) *Planta* **89,** 224-243
Hieber AD, Bugos RC, Verhoeven AS, Yamamoto HY (2001) *Planta In press*
Yamamoto HY, Kamite L, Wang Y-Y (1972) *Plant Physiology* **49,** 224-228
Yamamoto HY, Nakayama TOM, Chichester CO (1962) *Archives Biochemistry Biophysics* **97,** 168-173