S3-051

Cloning, expression and functional studies of violaxanthin de-epoxidase from *Spinacia oleracea*

A Emanuelsson, M Eskling and H-E Åkerlund

Department of Plant Biochemistry, Lund University, POB 117, S-221 00 Lund, Sweden
Fax +46462224116, Anna.Emanuelsson@plantbio.lu.se

Key words: xanthophyll, cloning, enzyme activity, point mutation

Introduction

Violaxanthin de-epoxidase (VDE) is a key enzyme of the xanthophyll cycle of all higher plants. It catalyzes the conversion of violaxanthin to zeaxanthin upon high light, resulting in photoprotection of the plant by energy dissipation (Gilmore 1997, Eskling et al. 2001). VDE has been purified from spinach thylakoids in low amount (Arvidsson et al. 1996). To be able to obtain recombinant VDE, the gene was cloned by screening a spinach cDNA library. The gene was sequenced and overexpression in *E. coli,* resulted in highly active recombinant protein.

Comparisons of the VDE amino acid sequence from spinach to other species reveal a number of highly conserved regions within the polypeptide (fig 1). The N-terminal consists of a cysteine-rich region, containing 11 of the 13 cysteines in the protein. The enzyme also contains a lipocalin signature (Bugos et al., 1998, Hieber 2000) which is a possible binding site for the thylakoid membrane lipid MGDG and for the substrate violaxanthin. Finally, the enzyme contains a highly charged C-terminus. Four histidine residues, marked in figure 1, are also conserved in most of the known sequences. We were particularly interested in these residues, since spinach VDE has been determined to bind to the thylakoid membrane with a cooperativity of 4, with respect to protons, and an inflexion point of pH 6.7 (Bratt et al., 1995). We speculated that binding might require protonation of histidine side chain, which occurs around this pH interval (6,0). Chemical modification of the histidines with DEPC, which carbethoxylates histidine residues, destroyed the enzyme activity, while remodification with hydroxylamine reversed the inhibition and the enzyme regained 28**%** of the initial activity. Point mutation of the histidines, where these residues were replaced with alanine, resulted in complete loss of VDE activity.

Materials and methods

Cloning and sequencing of spinach VDE. VDE isolated from spinach was cleaved with cyanogenbromide and the fragments were N-terminally sequenced, which provided information to synthesize degenerated DNA primers used to make a DNA probe. A cDNA library from spinach was screened twice with the DNA probe. A resulting clone was sequenced, and the sequence for the signal peptide, which was truncated in the 5'-end was obtained by SMART$_{TM}$ RACE PCR. The nucleotide and corresponding amino

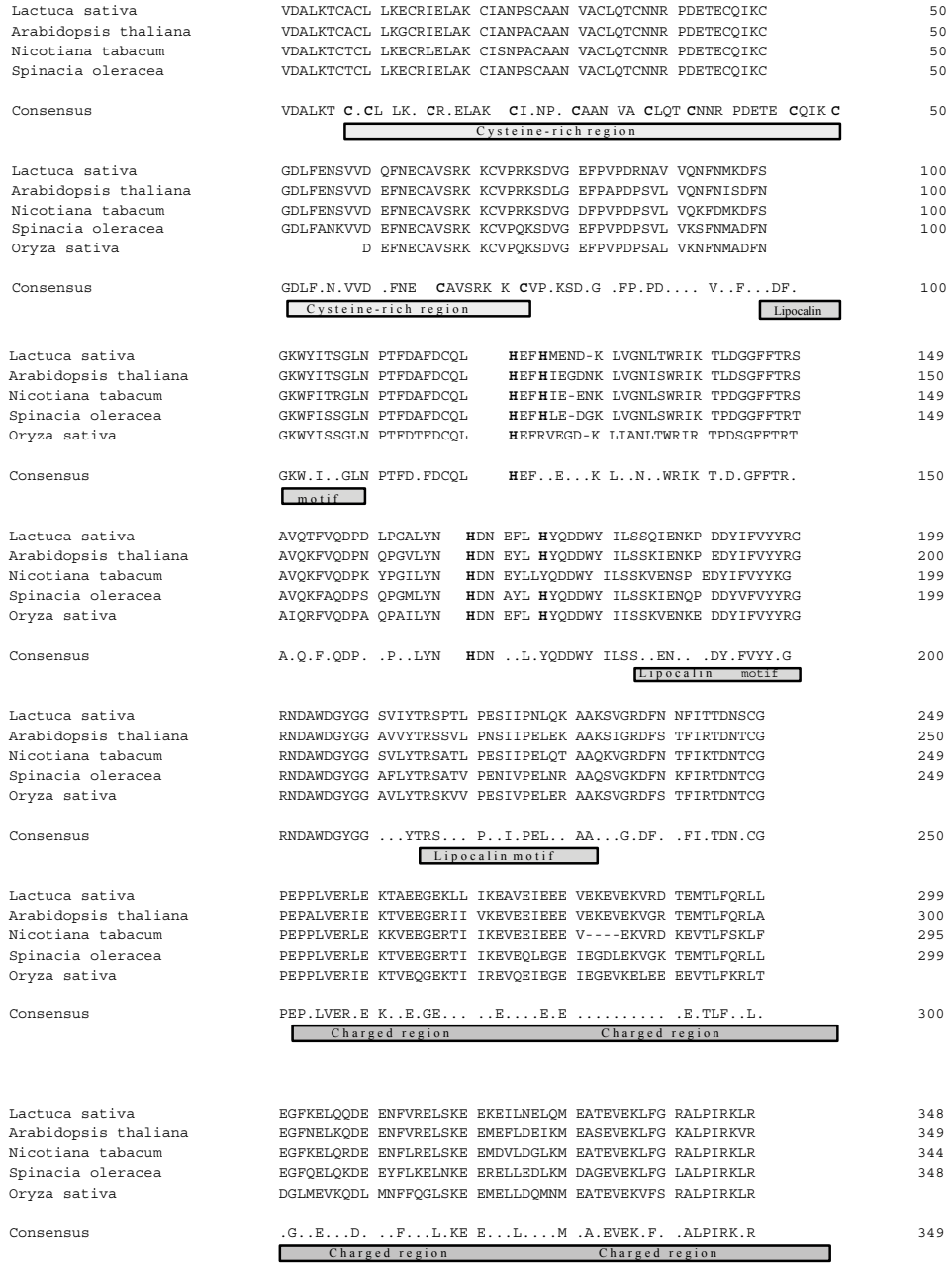

Fig. 1. Alignment of known VDE sequences from different species. Specific regions within the enzyme and the histidines investigated are marked in the figure.

acid sequence, including the signal peptide necessary for import of the nuclear encoded VDE into the thylakoid lumen, was submitted to data base (GenBank AJ 250433).

Overexpression of recombinant VDE in E. coli. The isolated gene was subcloned into the pET 16b vector and transformed into the E. coli strain BL21(DE3)pLysS. Transformed bacteria were selected on ampicillin/chloramphenichol LB plates follow by growth at 30°C in LB medium supplemented with 0,2 mg/ml ampicillin. At $OD_{600} \approx 0,6$ expression of VDE was induced by addition of IPTG to a final concentration of 0,5 mM.

Growth was continued for 4-12 h. Cells were collected at 6 000 g for 10 min, 4°C, resuspended in lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 1 mM PMSF) with 1 mg lysozyme/ml buffer, agitated for 1 h. at 4°C and sonicated for 1 min. (pulsed 5 s.) at 0°C. Cell debris was spun down at 10 000 g for 30 min.

Electrophoresis and Western blot Samples were run on homogenous 12 % SDS-PAGE in Bio-Rad Mini-protean cells with buffer system according to Laemmli (1970). Gels were run at 160 V for 10 min and 200 V for 35-45 min and stained with Coomassie brilliant blue R-250. Alternatively, protein was transferred from SDS-PAGE to PVDF membranes at 100 V for 1 h, using wet blot according to BioRad. PVDF membranes were blocked with 10% (w/w) non-fat dry milk and 0.05% Tween 20 in PBS (10 mM Na_2HPO_4, 3 mMKH_2PO_4 and 140 mM NaCl) over night and rinsed with PBS before adding primary antibodies in antibody buffer (PBS, 5% BSA) for 2 h at room temperature. Membranes were washed three times with wash buffer (PBS, 0.05% Tween 20) and secondary antibodies in antibody buffer were added at room temperature for 1 h . Membranes were washed three times in wash buffer and three times in PBS and color reaction was developed by adding developer solution (100 mM Tris-HCl, pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$, BCIP and NBT).

Chemical modification of histidine residues. 2,5 µg recombinant VDE, in 1 ml 50 mM phosphate buffer pH 6.0, was carbethoxylated by adding a 0,4 M diethylpyrocarbonate (DEPC) solution in acetonitrile to a final concentration of 6 mM. The reaction mixture was incubated on ice and activity assays were run after 1, 2, 5 and 10 minutes. Histidine residues were remodified by incubation of the DEPC-treated enzyme with 20 mM hydroxylamine over night at 4°C.

Point mutations of histidine residues. Point mutations were inserted into the VDE gene using the QuickChange$_{TM}$ procedure. Mutant clones were selected by sequencing and point mutated enzyme was grown as described above.

Activity assay. The assay mixture contained enzyme of varying concentrations, 0.33 µM violaxanthin, 9 µM MGDG and 0.1 M citrate-phosphate buffer, pH 5.1. The assay was started by adding Na-ascorbate to a concentration of 30 mM and the VDE activity was determined by dual-wavelength measurements (502-540 nm) on a Shimadzu UV-3000 spectrophotometer at 26°C. (Bratt et al. 1995, Yamamoto and Higashi 1978).

Results and discussion

Overexpression of recombinant VDE in E. coli The soluble part of bacterial lysate was run on SDS-PAGE and transferred to Western blot. Specific VDE antibody confirmed the presence of recombinant VDE by binding to a single protein bond at 43 kDa (results not shown here).

Chemical modification of histidine residues. Already after 5 minutes of incubation with 6 mM DEPC, VDE activity was fully inhibited. However, the reaction is not totally specific for histidines, but can also modify tyrosines and lysines. The modification of histidines can be reversed by treatment with hydroxylamine, while modification of tyrosines and lysines cannot. This reaction went very slowly, but after incubating the chemically modified VDE with 20 mM hydroxylamine over night, 28% of the catalytic activity was regained (table 1).

Point mutations of histidine residues. All four histidine residues in spinach VDE were substituted by alanine. The point mutated enzyme was expressed in E, coli in the same

way as the non-mutated recombinant protein, but showed no activity at all when run in the enzymatic assay, as described above.

Table 1. Relative enzymatic activity of VDE after chemical treatment and point mutations, compared to non-treated recombinant VDE.

Type of modification	Relative activity
Non-modified VDE	100%
2 min DEPC	59%
5 min DEPC	0%
5 min DEPC + hydroxylamine	28%
Point mutated VDE	0%

These results strongly suggest that histidines are important for VDE activity. The four histidines are located in pairs within the region containing the lipocalin motifs. Since lipocalins are known to bind small, hydrophobic molecules, this could be the site where VDE binds MGDG, hence docking the enzyme to the thylakoid membrane at low pH, followed by violaxanthin de-epoxidation and energy dissipation. When histidine is modified, chemically or by point mutation, binding to MGDG is inhibited and activity is lost.

Acknowledgments

This work was supported by grants from the Swedish Natural Sciences Research Council and the Carl Trygger Foundation.

References

Arvidsson P-O, Bratt CE, Carlsson M and Åkerlund H-E (1996) *Photosynthesis Research* 49: 119-129

Bratt CE, Arvidsson P-O, Carlsson M and Åkerlund H-E (1995) *Photosynthesis Resarch* 45: 169-175

Bugos RB, Hieber AD and Yamamoto HY (1998) *Journal of biological chemistry* 273 (25): 15321-15324

Eskling M, Emanuelsson A and Åkerlund H-E (2001) In: *Regulatory Aspects of Photosynthesis* (B Andersson and E-M Aro, eds) Kluwer Academic Publishers.

Gilmore AM (1997) *Physiologia Plantarum* 99: 197-209

Hieber AD, Bugos RC and Yamamoto HY (2000) *Biochimica Biophysica Acta* 1482: 84-91

Laemmli UK (1970) *Nature* 227: 680-685

Yamamoto HY and Higashi RM (1978) *Archives of Biochemistry and Biophysics* 190 (2): 514-522