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Research paper

Probing selected structural regions in the secreted phospholipase A_2 from *Arabidopsis thaliana* for their impact on stability and activity

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ABSTRACT

In contrast to the well characterized secreted phospholipases A_2 ($sPLA_2$) from animals, their homologues from plants have been less explored. Their production in purified form is more difficult, and no data on their stability are known. In the present paper, different variants of the $sPLA_2$ isoform α from *Arabidopsis thaliana* ($AtPLA_2\alpha$) were designed using a new homology model with the aim to probe the impact of regions that are assumed to be important for stability and catalysis. Moreover tryptophan residues were introduced in critical regions to enable stability studies by fluorescence spectroscopy. The variants were expressed in *Escherichia coli* and the purified enzymes were analyzed to get first insights into the peculiarities of structure stability and structure activity relationships in plant $sPLA_2$s in comparison with the well-characterized homologous enzymes from bee venom and porcine pancreas. Stability data of the $AtPLA_2$ variants obtained by fluorescence or CD measurements of the reversible unfolding by guanidine hydrochloride and urea showed that all enzyme variants are less stable than the enzymes from animal sources although a similar tertiary core structure can be assumed based on molecular modeling. More extended loop structures at the N-terminus in $AtPLA_2\alpha$ are suggested to be the main reasons for the much lower thermodynamic stabilities and cooperativities of the transition curves. Modifications in the N-terminal region (insertion, deletion, substitution by a Trp residue) exhibited a strong positive effect on activity whereas amino acid exchanges in other regions of the protein such as the Ca^{2+}-binding loop and the loop connecting the two central helices were deleterious with respect to activity.

1. Introduction

Secreted phospholipases A_2 ($sPLA_2$) (E.C. 3.1.1.4) are small enzymes (14–18 kDa), which catalyze the stereospecific cleavage of the acyl ester bond in *sn-2* position of glycero-phospholipids in a calcium-dependent manner producing the corresponding lysophospholipids and free fatty acids. They are ubiquitously distributed in all living organisms and fulfill different physiological functions [1]. All $sPLA_2$s are characterized by a compact, highly conserved core structure (Fig. S1) harboring the active site residues and a Ca^{2+}-binding loop [2], containing one essential Ca^{2+} ion. Between 5 and 8 disulfide bonds make significant contributions to enzyme stability. In the well-studied animal $sPLA_2$s, a hydrogen-bonding network, which supports the His–Asp dyad and contributes to conformational stability [3], connects the interfacial binding site and the catalytic site with the N-terminus [4]. The interfacial binding site, an important feature of $sPLA_2$s, represents a flat surface region that is important for binding to the membrane [5]. It is linked with the active site via a hydrophobic channel. Binding of the enzyme to the phospholipid membrane and phospholipid conversion are two kinetically distinct events [5].

Despite several highly conserved features $sPLA_2$s from different organisms differ significantly in terms of stability and activity, the reasons for this are hitherto poorly understood. Most information on the structure and function of $sPLA_2$s originates from animal enzymes such as from porcine pancreas ($ppPLA_2$), bovine pancreas ($bpPLA_2$), snake venom or bee venom ($bvPLA_2$), whereas much less

Abbreviations: $AtPLA_2\alpha$, $sPLA_2\alpha$ from *Arabidopsis thaliana*; $bpPLA_2$, $sPLA_2$ from bovine pancreas; $bvPLA_2$, $sPLA_2$ from bee venom; DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; GdnHCl, guanidine hydrochloride; GdnSCN, guanidine thiocyanate; $OsPLA_2$, $sPLA_2$ from rice (*Oryza sativa*); $ppPLA_2$, $sPLA_2$ from porcine pancreas; pWT, pseudo-wild type of $AtPLA_2\alpha$; $sPLA_2$, secreted phospholipase A_2; WT, wild-type of $AtPLA_2\alpha$.

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is known about $sPLA_2$s from plants. In *Arabidopsis thaliana* the existence of four $sPLA_2$s ($AtPLA_2\alpha$-δ) was predicted from the DNA sequence (At2g06925, At2g19690, At4g29460, At4g29470). Even though considerable progress in the knowledge of their biochemical properties and cellular functions has been made within the last decade [6,7], no data on the stability of these enzymes are currently available. Also, only one crystal structure of a $sPLA_2$ from plant origin (*Oryza sativa*) ($OsPLA_2$) has been elucidated to date [8]. In contrast, more than 250 structures of $sPLA_2$s from animal sources have been determined. The crystal structure of $OsPLA_2$ shows the unique character of plant $sPLA_2$s in comparison to $sPLA_2$s from animals. Striking features of the plant enzyme are a very long C-terminal helix, an N-terminus with extended loops, a concave interfacial binding site, a different disulfide pattern, low conservation of amino acid residues involved in the hydrogen-bonding network and substitution of the conserved Asp residue in the catalytic His-Asp dyad by an Asn (Ser in $AtsPLA_2\alpha$) residue.

The main reason for the paucity of information on plant $sPLA_2$s is found in their low expression yields in plants and the strong propensity of the recombinant enzymes to aggregate [9]. Moreover, most of plant $sPLA_2$s do not contain any Trp residues, rendering stability studies of these enzymes by fluorescence measurements difficult.

In this paper, we present for the first time data on the thermodynamic stability of plant $sPLA_2$s and compare them with stabilities of $sPLA_2$s from animal sources. The enzymes are variants of $AtPLA_2\alpha$, which were produced in *Escherichia coli* as inclusion bodies and refolded after solubilization by using a redox-shuffling system [9]. Making use of a new homology model basing on the only crystal structure of a plant $sPLA_2$ [8] the variants of $AtPLA_2\alpha$ were designed to probe regions that were shown to be critical for the stability and activity in animal $sPLA_2$s such as amino acids of the N-terminus, of the Ca^{2+}-binding loop, and the loop between the two central helices. To be able to apply fluorescence spectroscopy for stability studies, Trp residues were introduced into these regions. The results yield first insights into the particularities of the structure–stability and structure–activity relationships in plant $sPLA_2$s.

2. Materials and methods

2.1. Materials

1,2-Dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) was a gift of Lipoid GmbH (Ludwigshafen, Germany). The Low Molecular Weight protein markers (LMW) were supplied by Amersham Biosciences (Freiburg, Germany). Oligonucleotides were prepared by MWG Biotech GmbH (Ebersberg, Germany). Restriction endonucleases were purchased from New England Biolabs (Frankfurt/Main, Germany). 1,2-Dilauroyl-*sn*-glycero-3-phosphocholine and all other reagents and buffer substances were from Sigma (Taufkirchen, Germany). The BCA protein assay kit was from Pierce (Bonn, Germany). The NEFA C Kit was obtained from WAKO Chemicals (Neuss, Germany). All other reagents were of the purest quality available.

2.2. Homology modeling of $AtPLA_2\alpha$

The crystal structure of $OsPLA_2$ (2wg8) [8] was used as a template to model the structure of $AtPLA_2\alpha$ whose primary structure is 56.1% identical to that of $OsPLA_2$ by MOE (Molecular Operating Environment, Chemical Computing Group Inc., Montreal, Canada). Energy-minimization of the structure, proof of the resulting stereochemical quality of the structural model [10], and evaluation of native folding were performed as described in Ref. [11]. In the model, 87.5% of all residues were found in the most favored areas of the Ramachandran plot and only one outlier appeared. All other criteria such as peptide bond planarity, bad contacts, H-bond energies were inside the allowed regions for a virtual X-ray resolution of 2 Å. The graphical analysis of the energy plot of PROSA II [12] showed all amino acid residues in the negative energy range. The resulting combined energy *z* score (−6.42 for 110 amino acids) is in the expected range for natively folded proteins [12]. The structure has been accepted at the Protein Model DataBase (PMDB) (http://mi.caspur.it/PMDB/main.php) [13] and received the PMDB id: PM0079084 for free download.

2.3. Construction and purification of the enzyme variants

The substitutions V5W, L41W and the deletion Δ1-13 were introduced into the $AtPLA_2\alpha$ wild-type (WT) gene in the pET-26b(+) vector [9] by using the standard protocol of the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, USA). The following oligonucleotides were used:

5′-GCTTAACGTCGGT**TGG**CAGCTCATACATC-3′ (V5W-fw),

5′-TGGGAAGTACTGCGGA**TGG**CTTTACAGTGGATG-3′ (L41W-fw),

5′-GGAGATATACATATG↓TTGACTAAAGAATGTAG-3′ (Δ1-13-fw).

The exchanged bases are highlighted by bold letters and the point of deletion is marked by an arrow.

The pseudo-wild type (pWT) gene optimized in codons for expression in *E. coli* (GeneArt, Regensburg, Germany) and containing the codon for an additional Gly residue at the N-terminus was cloned into the Nde I and Hind III sites of the vector pET-26b(+). The substitutions Y35W and Y73F were introduced into the pWT gene by the standard QuikChange site-directed mutagenesis protocol (Stratagene, La Jolla, USA) and the following oligonucleotides: 5′-CCTCCGTTTCTGCGT**TGG**GGTAAATATTGCGGTC-3′ (Y35W-fw) and 5′-GAGCAAAAATAATGAC**TTT**CTGAGCCAGGAATGC-3′ (Y73F-fw).

The authenticity of the sequences was confirmed by sequencing (Seqlab, Göttingen, Germany or MWG Biotech, Ebersberg, Germany).

Production of all variants in *E. coli* BL21(DE3) cells (Stratagene, La Jolla, CA, USA), isolation and refolding were performed as described previously [9]. The refolded proteins were purified to homogeneity by two consecutive runs of cation-exchange chromatography on a SOURCE 15S (4.6/100 mm) column (GE Healthcare, Freiburg, Germany) or a POROS HS 20 (4.6/100 mm) column (Life Technologies, Darmstadt, Germany) using a linear NaCl gradient (0–1 M) in 50 mM sodium acetate buffer, pH 5.0, 10 mM $CaCl_2$.

2.4. Protein determination

Protein concentrations were determined by the BCA protein assay kit with bovine serum albumin as standard according to the instructions of the supplier or by UV-spectroscopy using the extinction coefficients of the variants at 280 nm calculated by ProtParam of the ExPASy Proteomics Server (http://www.expasy.org/tools/protparam.html).

2.5. Spectroscopic measurements

Fluorescence spectra were recorded at a protein concentration of 40–100 μg mL^{-1} in 50 mM sodium acetate buffer, pH 5.0, 10 mM $CaCl_2$ at 20 °C using a FluoroMax-3 spectrometer (Jobin Yvon, Bensheim, Germany). The excitation wavelength was 280 nm. The slit width was 5 nm for excitation and emission, and time of integration was 0.2 s. Emission spectra were recorded between 290 and