FEBS Letters 583 (2009) 2727–2733

journal homepage: www.FEBSLetters.org

Binding of divalent cations is essential for the activity of the organellar peptidasome in *Arabidopsis thaliana*, *At*PreP

Hans G. Bäckman, João Pessoa, Therese Eneqvist, Elzbieta Glaser *

Department of Biochemistry and Biophysics, Stockholm University, SE-106 91 Stockholm, Sweden

ARTICLE INFO

Article history:
Received 3 July 2009
Revised 21 July 2009
Accepted 22 July 2009
Available online 28 July 2009

Edited by Miguel De la Rosa

Keywords:
PreP
Protease
Peptidasome
Metal binding
Mitochondria
Chloroplast

ABSTRACT

The dual-targeted mitochondrial and chloroplastic zinc metallooligopeptidase from Arabidopsis, *At*PreP, functions as a peptidasome that degrades targeting peptides and other small unstructured peptides. In addition to Zn located in the catalytic site, *At*PreP also contains two Mg-binding sites. We have investigated the role of Mg-binding using *At*PreP variants, in which one or both sites were rendered unable to bind Mg^{2+}. Our results show that metal binding besides that of the active site is crucial for *At*PreP proteolysis, particularly the inner site appears essential for normal proteolytic function. This is also supported by its evolutionary conservation among all plant species of PreP.

Structured summary:
MINT-7231937, MINT-7232017, MINT-7232035, MINT-7232051, MINT-7232070, MINT-7232090:*AtPreP1* (uniprotkb:Q9LJL3) *enzymaticly reacts* (MI:0414) *pF1 beta* (uniprotkb:P17614) by *protease assay* (MI:0435)
MINT-7232132:*AtPreP1* (uniprotkb:Q9LJL3) *enzymaticly reacts* (MI:0414) *galanin* (uniprotkb:P22466) by *protease assay* (MI:0435)
MINT-7232175:*AtPreP1* (uniprotkb:Q9LJL3) *enzymaticly reacts* (MI:0414) *Cecropin A* (uniprotkb:P14954) by *protease assay* (MI:0435)
MINT-7232163:*AtPreP1* (uniprotkb:Q9LJL3) *enzymaticly reacts* (MI:0414) *hPrPss* (uniprotkb:P04156) by *protease assay* (MI:0435)

1. Introduction

Proteolysis plays a vital role in the cell. Proteins and peptides are continuously degraded to sustain the cell homeostasis. In addition, the majority of mitochondrial and chloroplastic proteins are encoded in the nucleus and then directed to their correct intracellular destination by an N-terminal peptide extension called presequence for mitochondrial proteins and transit peptide for chloroplastic proteins (for reviews see [1–5]). After import to the respective organelle the targeting peptide is cleaved off in mitochondria by the mitochondrial processing peptidase (MPP) [6,7], and in chloroplasts by the stromal processing peptidase (SPP) [8]. Targeting peptides can be harmful to cells due to their membrane penetrating properties, which can lead to uncoupling of the membrane potential and disruption of the cellular compartmentalization [9,10].

Presequence protease from *Arabidopsis thaliana*, *At*PreP, is a protease that degrades targeting sequences and other unstructured peptides of 10–65 amino acids in length in both mitochondria and chloroplasts [11–14]. *At*PreP is a 110 kDa zinc metallooligopeptidase that belongs to the pitrilysin M16C family of peptidases (MEROPS database) [15]. The M16 peptidases are often referred to as inverzincins, since they comprise an inverted zinc-binding motif, HXXEH, at the active site [16]. In *A. thaliana* there are two isoforms of PreP, referred to as *At*PreP1 and *At*PreP2, with high sequence conservation (89% identity). *At*PreP1 and *At*PreP2 are dually targeted to both the mitochondrial matrix and chloroplast stroma [14]. The yeast homolog of *At*PreP, called MOP112 or CYM1p, appears to be located in the mitochondrial intermembrane space [17] whereas the human homolog (hPreP or MP1) is localized to the mitochondrial matrix and is also known to degrade amyloid-beta peptide (Aβ) [18], the toxic agent that accumulates in mitochondria in Alzheimer's disease [19].

The crystal structure of *At*PreP1 with a bound peptide in the active site was determined at 2.1 Å [20]. The structure showed that the 995-residue polypeptide folds into four topologically similar domains, which form two bowl-shaped halves connected via a V-shaped hinge region that protrudes from the protease (Fig. 1). The inverted zinc-binding motif of the active site is situated in the N-terminal portion of the enzyme. However, Arg-848 and

* Corresponding author. Fax: +46 8153679.
E-mail address: e_glaser@dbb.su.se (E. Glaser).

0014-5793/$36.00 © 2009 Federation of European Biochemical Societies. Published by Elsevier B.V. All rights reserved.
doi:10.1016/j.febslet.2009.07.040

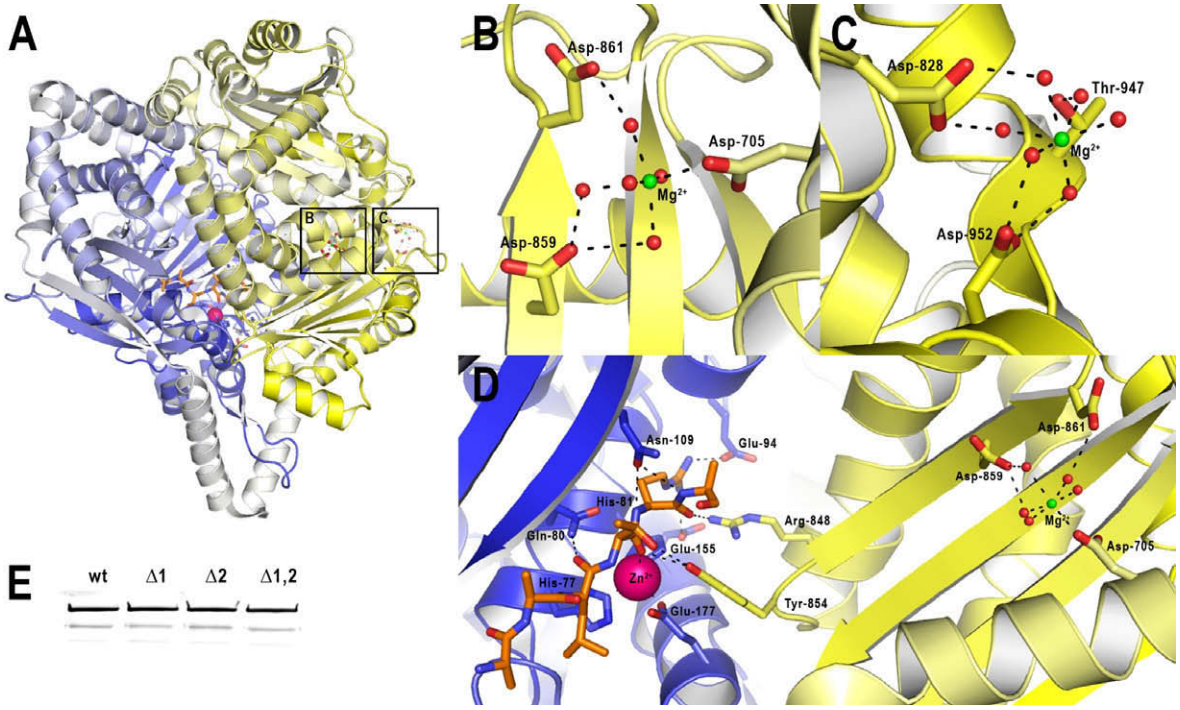

Fig. 1. (A) Crystallographic structure of *At*PreP1 colored from blue (N-terminal) to yellow (C-terminal). The substrate peptide bound to the crystallized protein is shown as orange sticks, the active site zinc as a purple sphere, the two Mg^{2+} ions as green spheres and waters coordinated to Mg^{2+} as red spheres. (B) The inner Mg^{2+}-binding site. (C) The outer Mg^{2+}-binding site. (D) The active site and inner Mg^{2+}-binding site located within the proteolytic chamber. (E) SDS–PAGE of the overexpressed and purified wildtype *At*PreP1 and *At*PreP1 variants, Δ1, Δ2, and Δ1,2.

Tyr-854 located at in the C-terminal portion, at a distance of about 800 residues in sequence from the zinc-binding motif, contribute to the catalytic site. The structure of *At*PreP1 revealed a unique proteolytic chamber of more than 10 000 Å^3, in which the active site resides and to which the substrate peptide binds. A novel proteolytic mechanism was proposed, involving hinge bending to open and close the two enzyme halves, allowing the C-terminal residues Arg-848 and Tyr-854 to interact with N-terminal residues to form a functional active site [20].

Magnesium ions were needed to facilitate the crystallization of *At*PreP1. Interestingly, the structure also revealed two hydrated magnesium ions coordinated by acidic residues [20]. One magnesium-binding site is located inside the proteolytic chamber (the inner site), whereas the second site is located on the surface of the enzyme (the outer site). In the present work we have investigated the importance of these non-catalytic metal binding sites with respect to the enzymatic activity of *At*PreP1. We produced *At*PreP1 variants, in which the inner site (Δ1), outer site (Δ2) or both metal binding sites (Δ1,2) were replaced with neutral residues unable to bind metal ions. We have investigated the effect of magnesium, calcium and zinc ions on peptide degradation using several unstructured peptides of different lengths and amino acid compositions as substrates. We also studied the kinetics of degradation by means of a fluorogenic peptide substrate (Substrate V). Our results point to the conclusion that non-catalytic metal binding, especially to the inner metal binding site, is essential for *At*PreP1 activity.

2. Materials and methods

2.1. Multiple sequence alignment

Sequences were found using BLAST [21] and conveniently managed with the Biology Workbench available from San Diego Supercomputer Center at http://workbench.sdsc.edu. In most cases default parameters were applied. The amino acid sequence of *At*PreP1 was used to scan protein sequences (blastp), using the BLOSUM62 substitution matrix with a gap-opening penalty of 11 and a gap extension cost of 1. The multiple sequence alignment was constructed with ClustalW [22], using the Gonnet weight matrix with gap-opening and gap extension penalties of 10.0 and 0.10, respectively.

2.2. Generation of AtPreP1 variants

The mutations D705N, D859N, D861N for the inner metal binding site and D828N, T947V, D952N for the outer metal binding site were introduced to a pGEX-6P-2 plasmid (Amersham Biosciences) containing wildtype *At*PreP1 using the Quick-Change Site Directed Mutagenesis Kit (Stratagene). The constructs were verified by DNA sequencing utilizing the DYEnamic Sequencing Kit (Amersham Biosciences). All primers are listed in Supplementary Table 1.

2.3. Expression and purification of AtPreP1 and its variants

The pGEX-6P-2 vector containing wildtype and the three *At*PreP1 variants N-terminally fused to Glutathione S-Transferase (GST) were used to transform *E. coli* BL21 (DE3). Colonies were grown at 37 °C in 500 ml LB medium containing 100 μg/ml ampicillin, induced with 1 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG) at OD_{600} of 0.5 and incubated at 30 °C to OD_{600} of 2. The cells were harvested by centrifugation and stored at −80 °C. Cells were resuspended in phosphate-buffered saline (PBS) buffer (140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 1.8 mM KH_2PO_4, pH 7.4) and cell lysis was induced by the addition of 0.5 mg/ml lysozyme followed by sonication. The cell lysate was centrifuged for 30 min at 20 000×*g* to remove cell debris and the supernatant was filtered through a 0.20 μm membrane. The filtered lysate was then incubated with Glutathione Sepharose® beads (Amersham Biosciences) equilibrated with PBS buffer and