VU Research Portal

Controlling the conformational constraint of peptides to modulate their target affinity

Müller, C.

2019

document version
Publisher's PDF, also known as Version of record

Link to publication in VU Research Portal

citation for published version (APA)
Müller, C. (2019). *Controlling the conformational constraint of peptides to modulate their target affinity*.

Chapter 4

Plant cysteine oxidases are dioxygenases that directly enable arginyl transferase-catalysed arginylation of N-end rule targets

Author contributions

M.D.W. performed the PCO1/4 activity assays and MALDI/LC–/MS/MS analyses. M.K. performed and established arginylation reactions on peptides coupled to biotin pulldown and scintillation measurements and purified ATE1 protein. R.J.H. performed the NMR assays with E.F., D.A.W. prepared the pDEST17-PCO1 and four plasmids. **C.M. synthesized the biotinylated peptides**, T.N.G. supervised and designed the synthesis and C.N. cloned and established purification and activity assays for ATE1. R.O. conducted LC–MS to analyse +12 Da mass shifts. J.W. performed LC–MS analysis. J.Y. and J.C.B.-B. prepared samples for micro-PIXE analysis, and J.C.B.-B. and E.F.G. collected and analysed micro-PIXE data. E.F. performed the PCO1 and PCO4 protein purification and selected activity assays. E.F., M.D.W., M.K. and N.D. designed the study. E.F. and N.D. wrote the manuscript. M.D.W., M.K., N.D. and E.F. designed the figures. All authors read and approved the final version of this manuscript.

Introduction

Aerobic organisms need homeostatic mechanisms to assure that oxygen demand and supply are balanced. If oxygen supply is reduced (hypoxia), decreased demand or improved supply is needed to keep this balance. In plants, hypoxia is a repercussion of reduced oxygen dispersion under an environment of submergence or waterlogging. Plants can survive temporary periods of hypoxia but it has a negative impact on plant growth and can result in plant damage or death.[1] Under normal oxygen conditions with levels of 10-21 %, the N-terminal cysteine of group VII ethylene response factors (ERF VII) is oxidized by plant cysteine oxidases (PCOs) via the N-end rule pathway. The oxidation is followed by arginylation, catalyzed by the arginyl transfer RNA transferase (ATE1), leading to subsequent proteasomal degradation (figure 1).[2,3] ATE1 is responsible for arginylation of Asp, Glu and Cys-sulfonic acid.[4,5] Besides Cys-sulfonic acid, Cys-sulfinic acid is also repeatedly reported as another potential arginylation substrate of ATE1, due to its sequence homology to the acidic residues mentioned above.[2,3,6] So far, detailed evidence has only been presented for arginylation of Cys-sulfonic acid.[4,5] In case of abnormal conditions, e.g. submergence-induced hypoxia (oxygen levels around 1 - 5 %), ERF VII are stabilized, leading to enhanced cell survival. Stabilization of ERF VII in hypoxia presumably arises from reduced PCO activity. PCOs and ATE1 may be viable intervention targets to stabilize N-end rule substrates, including ERF-VIIs to enhance tolerance to hypoxia. Therefore studies and detailed knowledge of the product of PCOs dioxygenase activity are desirable, as well as molecular evidence of the N-terminal Cys oxidation product Cys-sulfinic acid and its suitability as a substrate of ATE1.
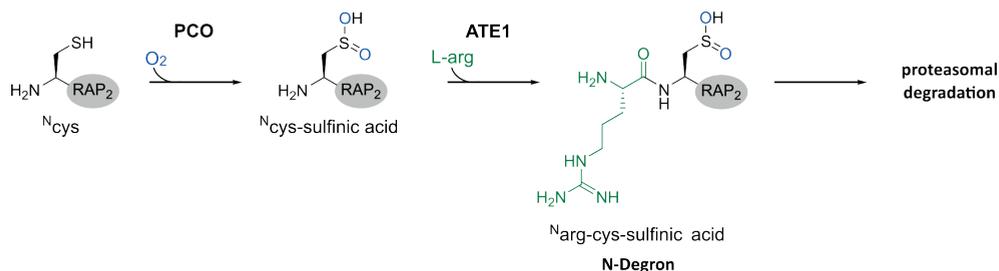
Figure 1. Scheme of plant cysteine oxidase (PCO)- and arginyl transfer RNA transferase (ATE1)-catalyzed reactions of N-terminal cys of RAP2 protein (grey) leading to proteasomal degradation.

Results

In this study, the catalysis of N-terminal cysteine oxidation of a synthetic 10-mer of the ERF VII peptide RAP2 (RAP2$_{2-11}$ H$_2$N-^{N}CGGAIISDFI^C-OH) by *Arabidopsis* PCO1 and PCO4 was investigated. PCO activity assays under aerobic and anaerobic conditions performed by our collaborators revealed O$_2$ necessity and dependence. Amino-terminal oxidation of cysteine to Cys-sulfinic acid of the synthetic ERF VII peptide was verified via matrix-assisted laser desorption/ ionization mass spectrometry (MALDI-MS), liquid chromatography tandem MS (LC-MS/MS) and ^{1}H-nuclear magnetic resonance (NMR) (experiments performed by Marc D. White, Richard J. Hopkinson and Emily Flashman (Chemistry Research Laboratory, University of Oxford, UK). Taken together, these experiments provide confirmation that *Arabidopsis* PCOs 1 and PCO4 act as plant cysteinyl dioxygenases and catalyze the incorporation of O$_2$ into N-terminal cysteine residues on RAP2 to form Cys-sulfinic acid.

Next, it is of interest whether the PCO-catalyzed oxidation is sufficient for ATE1-catalyzed arginylation and thus leading to an N-degron, as proposed by the Arg/Cys branch of the N-end rule pathway. Addressing this question, four 12-mer peptides of RAP2 consisting of the amino acids 2 - 13 were synthesized on solid support introducing different N-terminal residues (H$_2$N – *X*GGAIISDFIPP-Peg$_2$-K(biotin) - NH$_2$, X-RAP2$_{2-13}$). Amino-terminal residues are Gly, Asp, Cys and Cys-sulfonic acid ((O$_3$)-Cys). All four peptides harbor a biotin attached via ε-amino group of an additional C-terminal lysine, necessary for the arginylation assay (table 1). Christin Naumann produced and purified recombinant hexahistidine-tagged *Arabidopsis* ATE1 (Leibniz Institute for Plan Chemistry, Halle, Germany). Subsequently, she performed an arginylation assay of all four peptides in presence and absence of PCO1 and PCO4. During this assay, the incorporation of ^{14}C-Arg was detected by scintillation counting (figure 2). G-RAP2$_{2-13}$ served as a negative control and did not show ^{14}C-arginine incorporation under any condition.

Table 1. List of all peptides; peptide names and sequences (Peg_2 = 8-amino-3,6-dioxaoctanoyl, all peptides bear a C-terminal amide), molecular weight (MW).

Peptide	Sequence	MW / $g \cdot mol^{-1}$
C-RAP2$_{2-13}$	H_2N - C GGAIISDFIPP-Peg_2-K(Biotin) - NH_2	1688,04
CO$_3$-RAP2$_{2-13}$	H_2N - CO3 GGAIISDFIPP-Peg_2-K(Biotin) - NH_2	1736,03
G-RAP2$_{2-13}$	H_2N - G GGAIISDFIPP-Peg_2-K(Biotin) - NH_2	1641,95
D-RAP2$_{2-13}$	H_2N – D GGAIISDFIPP-Peg_2-K(Biotin) - NH_2	1699,99

The Asp-harboring peptide D-RAP2$_{2-13}$ showed incorporated radio-labelled arginine as a result of arginine transfer through ATE1, as described before.[4,5] The same result was observed for fully oxidized (O_3)-C-RAP2$_{2-13}$. Both arginyl transferase reactions were independent of PCO1/4. Peptide C-RAP2$_{2-13}$ showed no incorporation of ^{14}C-Arg in the absence of PCO1/4. However, arginyl transferase activity increased in the presence of PCO1 and PCO4, proposing a strong dependence on the dioxygenase activity of PCO before arginylation. To further confirm these results, the experiment was repeated using non-radiolabelled arginine and LC-MS as readout, performed by James Wickens (Chemistry Research Laboratory, University of Oxford, UK). Incubation of C-RAP2$_{2-13}$ with PCO revealed a mass increase of +32 Da corresponding to Cys-sulfinic acid, while incubation with PCO4 and ATE1 led to a mass increase of +188 Da, corresponding to arginylated N-terminal Cys-sulfinic acid (figure 3). MS/MS experiments verified the oxidized N-terminal Cys-sulfinic acid (+32 Da) and the oxidized and arginylated N-terminus (+188 Da) (Mark D. White, Chemistry Research Laboratory, University of Oxford, UK).
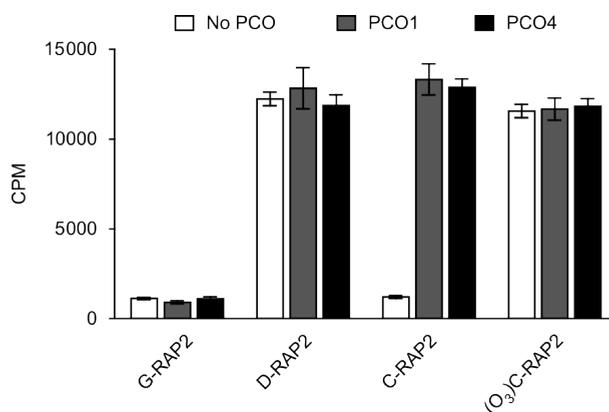

Figure 2. Results of liquid scintillation counting of ^{14}C-Arg containing immobilized biotinylated RAP2$_{2-13}$ peptides; ^{14}C-Arg incorporation by ATE1 into 12-mer RAP2$_{2-13}$ peptides, unreacted ^{14}C-Arg was removed after the arginylation reaction (n = 3, errors stand for s.e.m).

The control measurement of C-RAP2$_{2-13}$ under reaction conditions in absence of PCO and ATE1 revealed a mass increase of +12 Da. Incubation in water did not show a mass difference. These observations were addressed with time-dependent investigations of C-RAP2$_{2-15}$ incubation in 50 mM HEPES and 1 mM DTT buffer. This experiment, performed by Rebecca O'Neill (Chemistry Research Laboratory, University of Oxford, UK), suggests modifications of the N-terminal cysteine through formaldehyde-induced thiazolidine formation.[7] In summary, PCO-catalyzed oxidation of cysteine-harboring ERF VII peptide is sufficient for arginylation by ATE1.

Within this study, it was shown that arginine transfer is mediated by plant ATE and that arginylation depends on the N-terminal residue. Amino-terminal Cys-sulfinic and Cys-sulfonic acids serve as substrates for ATE1. PCO-catalyzed oxidation and subsequent arginylation of Cys-RAP2$_{2-13}$ supports the assumption that N-terminal residues sterically and electrostatically resampling Asp or Glu can serve as arginine acceptors in reactions catalyzed by ATE.[5] Hence it was shown that PCOs are important connectors between ERF VII stability and O$_2$ availability.
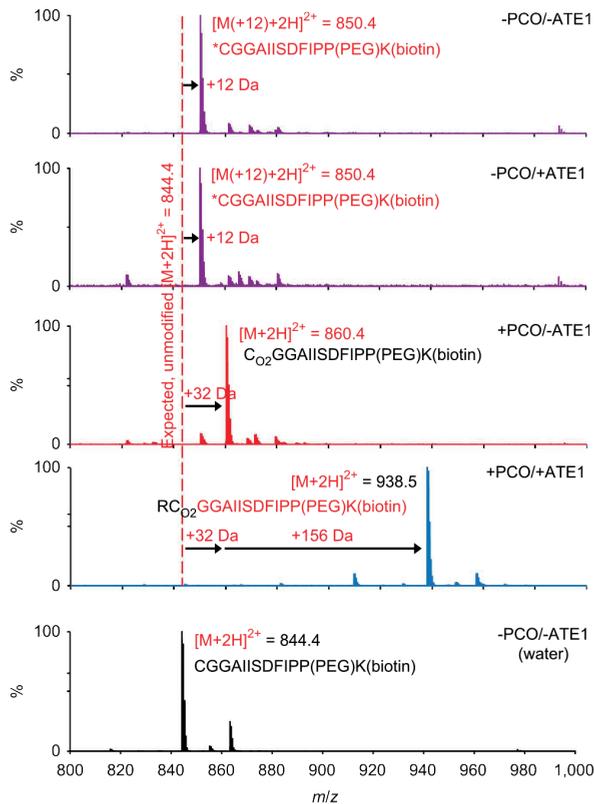

Figure 3. LC-MS spectra of PCO activity and arginylation assay with C-RAP2$_{2-13}$ (using non-radiolabelled Arg).

Acknowledgements

Methods

Peptide synthesis

The 12-mer RAP2$_{2–13}$ peptides used in the coupled oxidation-arginylation assay (H$_2$N-X-GGAIISDFIPP-Peg$_2$-K(biotin)-NH$_2$) were synthesized by Fmoc-based solid-phase peptide synthesis on NovaSyn TGR resin (with X = C, CO$_3$, G and D). Fmoc-protected amino acids were coupled using 4 equivalents (eq) of the amino acid according to the initial loading of the resin. 4 eq amino acid were mixed with 4 eq O-(6-chlorobenzotriazol-1-yl)-N,N,N0,N0-tetra-methyluronium hexafluorophosphate (HCTU) and 8 eq N,N-diisopropyl-ethylamine (DIPEA) and added to the resin for 1 h. In a second coupling, the resin was treated with 4 eq of the Fmoc-protected amino acid mixed with 4 eq benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluoro-phosphate and 8 eq 4-methylmorpholine for 1 h. After double coupling, a capping step to block free amines was performed using acetanhydride and DIPEA in N-methyl-2-pyrrolidinone (NMP) (1:1:10) for 5min. The C-terminal Fmoc-Lys(biotin)-OH, 8-(9-fluorenylmethyloxycarbonyl-amino)-3.6-dioxa-octanoic acid (Peg$_2$) linker and the different Fmoc protected N-terminal amino acids were coupled manually. The remaining peptide sequence was assembled using an automated synthesizer (Syro II, MultiSynTech GmbH). Fmoc deprotection was performed using 20 % piperidine in dimethylformamide (DMF) for 5min, twice. After each step, the resin was washed five times with DMF, methylene chloride (DCM) and DMF, respectively. Final cleavage was performed with 94 % trifluoroacetic acid (TFA), 2.5 % 1,2-ethanedithiole, 2.5 % H$_2$O and 1 % triisopropylsilane for 2 h, twice. The cleavage solutions were combined and peptides were precipitated with diethyl ether (Et$_2$O) at -20 °C for 30 min. Peptides were dissolved in water/acetonitrile (ACN) 7:3 and purified by reversed phase HPLC (Nucleodur C18 column; 10×125 mm, 110 Å, 5 mm particle size; Macherey-Nagel) using a flow rate of 6 mL·min^{-1} (A: ACN with 0.1 % TFA, B: water with 0.1 % TFA). Obtained pure fractions were pooled and lyophilized. Peptide characterization was performed by analytical HPLC (1260 Infinity, Agilent Technology; flow rate of 1 mL·min^{-1}, A: ACN with 0.1 % TFA, B: water with 0.1 % TFA) coupled with a mass spectrometer (6120 Quadrupole LC–MS, Agilent Technology) using electrospray ionization (Agilent Eclipse XDB-C18 column, 4.6 × 150 mm, 5 mm particle size). Analytical HPLC chromatograms were recorded at 210 nm. Quantification was performed by HPLC-based comparison (chromatogram at 210 nm) with a reference peptide.

Arginylation assay

experiments performed by Maria Klecker and Christin Naumann (Independent Junior Research Group of Protein Recognition and Degradation, Leibniz Institute of Plant Biochemistry; ScienceCampus Halle Plant – based Bioeconomy; Prof. Dr. N. Dissmeyer)

The conditions for arginylation of the 12-mer peptide substrates were modified from ref. 43. In detail, ATE1 was incubated at 10 µM in the reaction mixture containing 50 mM HEPES pH 7.5, 25 mM KCl, 15 mM MgCl$_2$, 1 mM DTT, 2.5 mM ATP; 0.6 mg·mL^{-1} *E. coli* tRNA (R1753, Sigma), 0.04 mg·mL^{-1} *E. coli* aminoacyl-tRNA synthetase (A3646, Sigma), 80 µM (4 nCi·µL^{-1}) ^{14}C-arginine (MC1243, Hartmann Analytic), 50 µM C-terminally biotinylated 12-mer peptide substrate and, where indicated, 1 µM purified recombinant PCO1 or PCO4 in a total reaction volume of 50 µL. The reaction was conducted at 30 °C for 16–40 h. After incubation, each 50 µL of avidin agarose bead slurry (20219, Pierce) equilibrated in PBSN (PBS-Nonidet; 100 mM NaH$_2$PO$_4$, 150 mM NaCl; 0.1 % Nonidet-P40) was added to the samples and mixed with an additional 350 µL of PBSN. After 2 h of rotation at room temperature, the beads were washed four times in PBSN, resuspended in 4 mL of FilterSafe scintillation solution (Zinsser Analytic) and scintillation counting was performed using a Beckmann Coulter LS 6500 Multi-Purpose scintillation counter.

Appendix

Literature

1.   Bailey-Serres, J. *et al.* Making sense of low oxygen sensing. *Trends in Plant Sci.* **17,** 129–138 (2012).

2.   Varshavsky, A. The N-end rule pathway and regulation by proteolysis. *Protein Sci.* **20,** 1298–1345 (2011).

3.   Tasaki, T., Sriram, S. M., Park, K. S. & Kwon, Y. T. The N-End Rule Pathway. *Annu. Rev. Biochem.* **81,** 261–289 (2012).

4.   Hu, R. G. *et al.* The N-end rule pathway as a nitric oxide sensor controlling the levels of multiple regulators. *Nature* **437,** 981–986 (2005).

5.   Kwon, Y. T. An Essential Role of N-Terminal Arginylation in Cardiovascular Development. *Science* **297,** 96–99 (2002).

6.   Gibbs, D. J., Bacardit, J., Bachmair, A. & Holdsworth, M. J. The eukaryotic N-end rule pathway: Conserved mechanisms and diverse functions. *Trends Cell Biol.* **24,** 603–611 (2014).

7.   Kallen, R. G. The Mechanism of Reactions Involving Schiff Base Intermediates. Thiazolidine Formation from L-Cysteine and Formaldehyde. *J. Am. Chem. Soc.* **93,** 6236–6248 (1971).
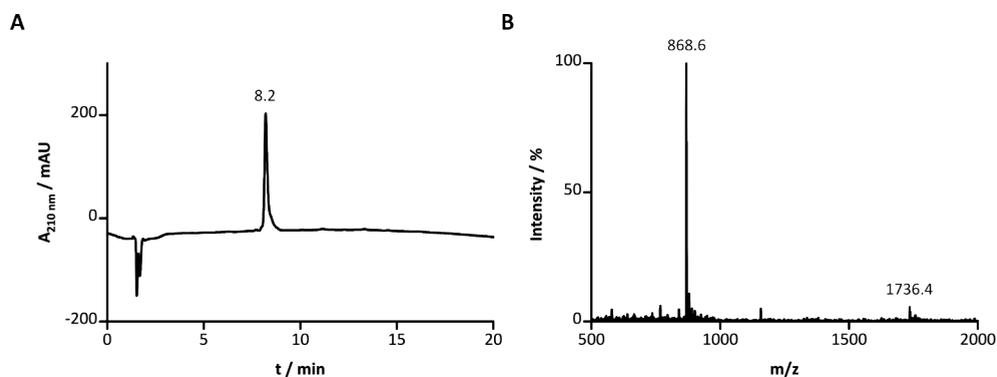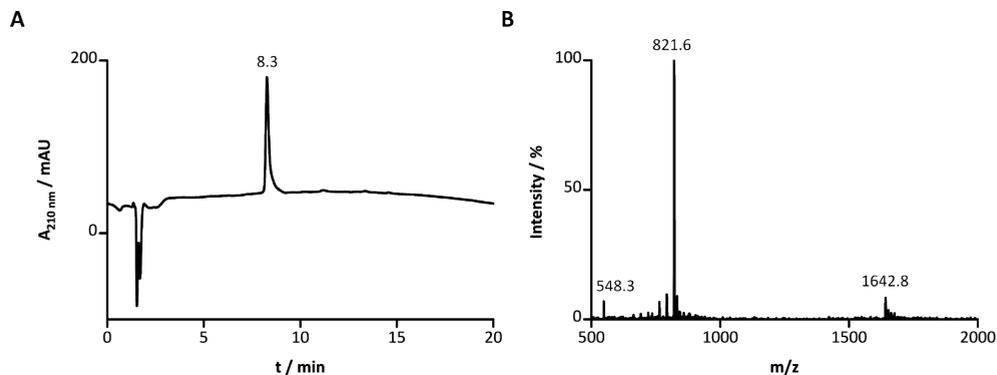
Analytics

C-RAP2

A

B
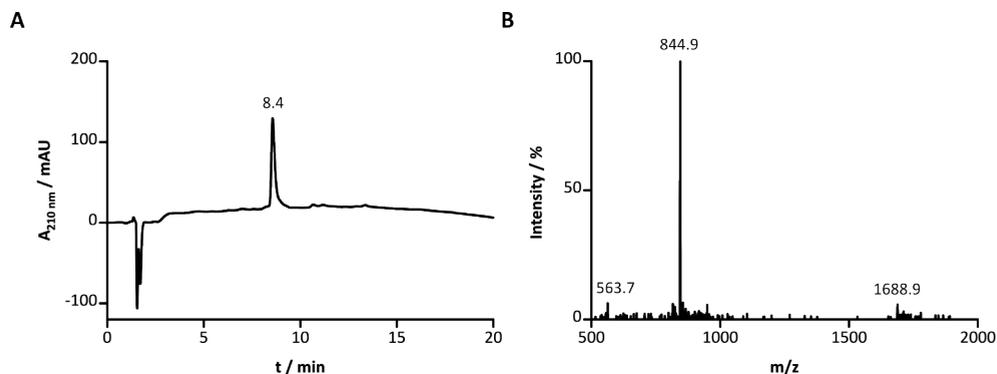
A: HPLC chromatogram at λ = 210 nm, peak retention time: 8.4 min, gradient: 20–60 % ACN in 20 min; **B**: MS spectrum, MW: 1688.04 g·mol^{-1}, calc. m/z: 1687.9 / 844.5 / 563.3.

(O$_3$)-C-RAP2

A

B

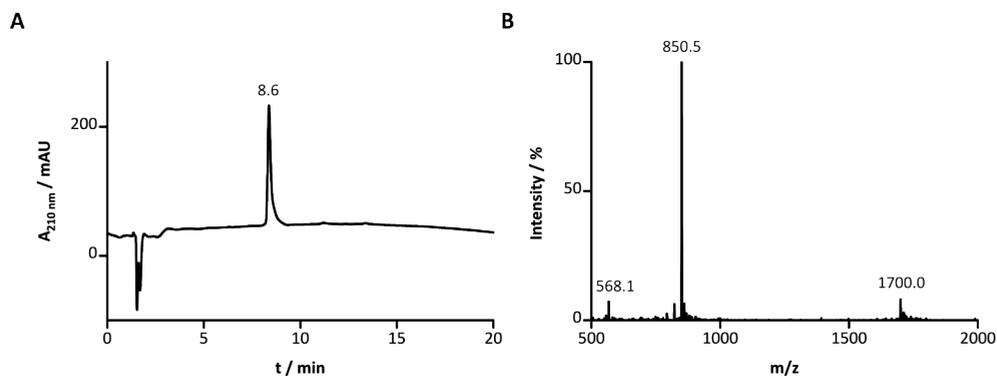**A**: HPLC chromatogram at λ = 210 nm, peak retention time: 8.2 min, gradient: 20–60 % ACN in 20 min; **B**: MS spectrum, MW: 1736.0 g·mol^{-1}, calc. m/z: 1735.8 / 868.4.

G-RAP2

A

B

A: HPLC chromatogram at λ = 210 nm, peak retention time: 8.3 min, gradient: 20–60 % ACN in 20 min; **B**: MS spectrum, MW: 1641.95 g·mol^{-1}, calc. m/z: 1641.9.3 / 821.4 / 548.0.

D-RAP2

A

B

A: HPLC chromatogram at λ = 210 nm, peak retention time: 8.6 min, gradient: 20–60 % ACN in 20 min; **B**: MS spectrum, MW: 1699.99 g·mol^{-1}, calc. m/z: 1699.9 / 850.4 / 567.3.