<u>Corresponding author</u>

Emily Flashman

Department of Chemistry, Department of Biology,

University of Oxford

emily.flashman@chem.ox.ac.uk

1 # Kinetic Measurement investigating the Oxygen-Sensing

2 # Properties of the Plant Cysteine Oxidases

3 Anna Dirr[1], Dona M. Gunawardana[1], Emily Flashman[1, 2]

4

5 **Affiliations**

6 1 Department of Chemistry, University of Oxford, Mansfield Road, OX1 3TA.

7 2 Department of Biology, University of Oxford, South Parks Road, OX1 3RB.

8

9 ## Abstract

10 Enzymatic O_2-sensors transduce the availability of O_2 within the cell into a 11 physiological, typically adaptive response. One such O_2-sensing enzymatic family are 12 the N-terminal cysteine dioxygenases in plants (plant cysteine oxidases, PCOs). 13 Kinetic *in vitro* studies have determined the O_2-sensing capacity of PCOs. Here we 14 describe the rationale and experimental protocol for an assay with which the O_2-15 sensitivity of *Arabidopsis thaliana* PCOs (AtPCOs) can be measured. We explain each 16 step from recombinant protein synthesis of AtPCOs to the assays for steady-state 17 kinetics of AtPCOs for peptides and O_2. The same techniques can be applied to other 18 N-terminal cysteine thiol dioxygenases, e.g. 2-aminoethanethiol dioxygenase (ADO), 19 and similar principles can be applied to determine kinetic constants of other 20 oxygenase enzymes towards O_2.

21

Key words

oxygen sensor, thiol dioxygenase, steady-state, plant cysteine oxidase, hypoxia, Cys/Arg-N-degron, enzyme kinetics

1 Introduction

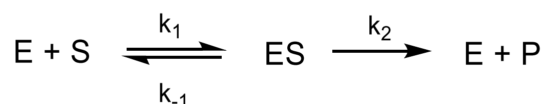
Enzymatic O_2-sensors respond to O_2-levels within the cell and transduce a physiological and, in most cases, adaptive response to O_2-availability. Prolyl hydroxylase domain (PHD) enzymes found in metazoans, plant cysteine oxidases (PCOs) and their mammalian homologue, 2-aminoethanethiol dioxygenase (also known as cysteamine dioxygenase, ADO), are all able to act as O_2-sensors.[1–4] This chapter describes techniques which can be used to measure the O_2-sensitvity of the five *Arabidopsis thaliana* (*A. thaliana*) PCOs (AtPCO1-5); similar approaches have been used to measure O_2-sensitivity of the PHDs and ADO [5, 6] as well as other enzymes which have O_2-sensing capacity [5, 7].

PCOs are thiol dioxygenases that facilitate cellular responses to O_2-availability by regulating the stability of their substrates: PCOs utilise molecular O_2 to oxidise the N-terminal cysteine of their substrates (Figure 1) which results in subsequent proteasomal degradation of the oxidised substrate via the N-degron pathway.[8–10] Substrates of the *A. thaliana* PCOs include the Group-VII ethylene response factors (ERF-VIIs).[8] ERF-VIIs are transcription factors which up-regulate genes that enable an adaptive hypoxic (low oxygen) response in plants.[11–13] During plant hypoxia,

42 the activity of the AtPCOs is reduced due to lack of O_2 and, as a result, ERF-VIIs are
43 stabilised.[8, 10, 14] Under normoxia (atmospheric oxygen concentrations), however,
44 AtPCOs can oxidise the ERF-VIIs and thereby prevent their accumulation.[8, 10, 14]
45 Nitric oxide (NO) may also regulate the stability of ERF-VIIs via the N-degron
46 pathway [15–17].
47 PCOs are considered good O_2-sensors because their rate of activity varies depending
48 on cellular O_2-levels, as defined by their $K_M(O_2)$ values.[14, 18] $K_M(O_2)$ is the Michaelis-
49 Menten constant which describes the O_2-concentration at which the enzyme catalyses
50 substrate oxidation at half-maximal velocity; a $K_M(O_2)$ value at or above physiological
51 O_2-concentrations indicates that enzyme activity will decrease if O_2 levels drop [14,
52 18]. Michaelis-Menten constants are determined in enzyme activity assays conducted
53 under steady-state conditions, in which substrate(s) are available in excess compared
54 to the enzyme.[19] To achieve steady-state conditions, the initial rate of product
55 formation is measured before the rate starts to slow as equilibrium is reached
56 (Figure 2). The dependence of this initial rate (velocity) on substrate concentration is
57 then used to calculate the steady-state kinetic constants.[19] Scheme 1 & Equation 1
58 show that the K_M value also defines the stability of an enzyme-substrate [ES] complex,
59 which depends on the velocity of the [ES] complex formation (k_1), as well as its decay,
60 which includes both separation back into enzyme [E] + substrate [S] (k_{-1}) and catalysis
61 to form enzyme [E] + product [P] (k_2).[20] More information about enzymes and the
62 underlying principles of enzyme kinetics can be found in textbooks (e.g. Biochemistry
63 textbooks [19–22]).

64

65 **Scheme 1. Michaelis-Menten model.**



66

67 **Equation 1. Michaelis-Menten constant.**

68 $$K_M = \frac{k_{-1} + k_2}{k_1}$$

69

70 The O_2-sensitivity of the PCOs is determined by analysing reactions between
71 recombinant enzymes and peptides (typically 14 residues (14-mers)) which represent
72 the Cys-initiating substrates:[14] (i) Enzyme is mixed with substrate peptide and other
73 cofactors and (ii) steady-state kinetic assays are conducted at varying concentrations
74 of O_2. (iii) Reactions are quenched at given time points, then (iv) the oxidation of
75 substrate peptide (sulfinylation at the N-terminal Cys), which adds 32 Dalton (Da) to
76 the peptide, is measured using liquid chromatography-coupled mass spectrometry
77 (LC-MS). Over time, a decrease in un-oxidised substrate peptide (free substrate) and
78 an increase in oxidised peptide (product) can be observed, allowing rate calculations.
79 It is important when measuring steady-state kinetic constants for O_2 that experiments
80 are conducted under conditions where enzyme activity is only limited by O_2-
81 availability.[14, 23] In case of the PCOs, it is therefore first necessary to consider the
82 optimal concentration of Fe(II) cofactor and primary (peptidic) substrate (Figure 1).
83 As PCOs are non-haem metallo-enzymes,[24] the maximal amount of active enzyme

84 is achieved when each PCO active site binds Fe(II); supplemental Fe(II) can therefore
85 often (though not always[23]) increase PCO activity.[14] Supplemented Fe(II) can
86 readily oxidise to Fe(III) in solution and therefore *in vitro* assays usually contain a
87 reducing agent such as ascorbate to ensure reduced iron remains in solution; both
88 (iron and ascorbate) must be in excess to maintain continuous substrate turnover.
89 Similarly, the primary substrate of the PCOs should be in excess to secure continuous
90 turnover. The amount of primary substrate, cofactors and enzyme needed for PCOs
91 to achieve maximal enzymatic activity (max. velocity, V_{max}, Figure 2) must be pre-
92 determined in order to attain steady-state O_2-assays.

93 Here we report our protocols used to measure the O_2-sensitivity of the AtPCOs. We
94 describe the procedures for recombinant AtPCO production, initial activity assays,
95 optimisation of assay conditions and measurement of steady-state kinetics for both
96 substrate peptide RELATED TO AP2.12$_{2\text{-}15}$ (RAP2.12$_{2\text{-}15}$, one of the Arabidopsis ERF-
97 VIIs)[14] and O_2[14]. We use AtPCO4 as an example throughout, however all methods
98 are equally applicable to other PCOs and the principles are applicable to other thiol
99 dioxygenases and O_2-sensitive enzymes.

100

2 Materials

101 Store and prepare all reagents and buffers at room temperature between 17 - 25 °C if
102 not indicated otherwise. Use ultrapure water (ddH_2O) to prepare buffers.

103 **2.1 Medium and supplements for protein expression**

104 Conduct steps under sterile conditions in a laminar flow hood or in close proximity
105 to a flame.

106 1. *Escherichia coli* (*E. coli*) BL21(DE3) competent cells transformed with the
107 plasmid pET-28a(+)-His-TEV-AtPCO4 (Addgene)
108 2. Autoclaved 2-YT medium: dissolve the relevant weight/volume (w/v) in
109 deionised water according to the instructions (Note 1). Prepare 100 mL 2-YT
110 medium in a 250 mL flask and 6 - 12 x 600 mL 2-YT medium in 2 L flasks. Close
111 the flasks with aluminium foil and autoclave using a liquid cycle setting during
112 which the load temperature reaches 121 °C for 15 minutes (min).
113 3. 0.5 M isopropyl β-d-1-thiogalactopyranoside (IPTG, = 1000x stock, 10 mL):
114 filter sterilise the IPTG stock solution through a 0.2 μm sterile syringe filter.
115 This can be prepared in advance and stored at -20 °C.
116 4. 40 mg/mL kanamycin (= 1000x stock, 10 mL): filter sterilise the antibiotic stock
117 solution through a 0.2 μm sterile syringe filter. This can be prepared in advance
118 and stored at -20 °C.
119 5. 50 mM iron (= 1000x stock, 10 mL): make fresh on the day. Weigh out a known
120 quantity (approximately 2 - 10 mg) of di-ammonium iron(II) sulphate
121 hexahydrate ($(NH_4)_2Fe(SO_4)_2.6H_2O$), hereafter referred to as Fe(II). Dissolve the
122 Fe(II) in the correct volume of 20 mM HCl to achieve a 0.5 M Fe(II) stock. Prior
123 to use, dilute the 0.5 M Fe(II) stock with ddH_2O to 50 mM Fe(II). Filter sterilise
124 the 50 mM Fe(II) stock through a 0.2 μm sterile syringe filter.

125 6. 10 mL 4 x LB buffer (laemmli / loading buffer): 1 mL 1 M Tris-HCl, pH 6.8,
126 4 mL 10% (w/v) sodium dodecyl sulphate (SDS) solution, 2 mL glycerol, 2.5 mL
127 β-mercaptoethanol, 0.5 mL of 1% (w/v) Brilliant Blue R.

128 **2.2 Buffers, SDS-PAGE and materials used during protein purification**

129 1. Purification buffers: for the affinity purification two buffers are required, the
130 resuspension / binding buffer, **HisTrap™ buffer A**, and the elution buffer,
131 **HisTrap™ buffer B**. To make 1 L of **HisTrap™ buffer A**, dissolve 50 mM
132 Trizma, 400 mM NaCl and 20 mM imidazole in 800 mL ddH_2O. For 0.5 L of
133 **HisTrap™ buffer B**, dissolve 50 mM Trizma, 400 mM NaCl and 1 M imidazole
134 in 400 mL ddH_2O. For the size exclusion chromatography (SEC) make 2 L of
135 **SEC buffer** by preparing 50 mM Trizma and 400 mM NaCl in 1.8 L ddH_2O.
136 Adjust all buffers to pH 7.5 at the temperature at which the purification will be
137 performed (4 – 8 °C) and fill the buffers to the intended volume with ddH_2O.
138 Subsequently, filter and degas each buffer as follows: Load each buffer into a
139 filtration unit comprising funnel and bottle of sufficient volume (containing a
140 magnetic stir bar), in which the funnel is fitted with filter paper (grade 601
141 70 mm). Connect the filtration unit to a vacuum pump and start the pump to
142 assist the filtration. Once the buffer is filtered into the bottle, (i) stop the vacuum
143 pump, (ii) close the filtration system to ensure it is air-tight, (iii) put it onto a
144 magnetic stirrer plate, (iv) re-apply the vacuum and (v) increase the stir speed
145 until a vortex is formed. Bubbles should be visible. Degas the buffer in this way
146 for approximately 30 min until there are no bubbles rising to the surface.

147 2. Degassed ddH_2O: filter and degas 2 L of ddH_2O as described above.

148 3. 20% (volume/volume, v/v) ethanol: filter and degas 800 mL of ddH_2O as
149 described above. Slowly add 200 mL ethanol to the degassed ddH_2O to achieve
150 a final solution of 20% (v/v) ethanol.

151 4. Protease inhibitor cocktail (ethylenediaminetetraacetic acid (EDTA)-free)

152 5. DNase I with commercially supplied DNase I buffer

153 6. Protease to cleave His-tag: Tobacco Etch Virus (TEV) protease (His-**TEV**-
154 AtPCOs) or thrombin protease (His-**thrombin**-AtPCOs), depending on the
155 vector

156 7. Syringes: 5 mL, 10 mL, 60 mL

157 8. Sterile syringe filters: 0.22 μm and 0.45 μm

158 9. Fast protein liquid chromatography (FPLC) system equipped with ultraviolet
159 (UV) absorbance monitor

160 10. Affinity column: 5 mL HisTrap™ HP

161 11. Size exclusion column: HiLoad 26/600 Superdex 75 pg

162 12. Buffer exchange column: PD-10 column

163 13. 10 kDa cut-off centrifugal filter units: Ultra-15 Centrifugal Filter Unit

164 14. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) chamber and power
165 supplier

166 15. 12% polyacrylamide gels

167 16. Prestained protein ladder

168 17. 10x running buffer (for SDS-PAGE): 144 g glycine, 30 g Trizma, 10 g SDS,

169 dissolve and fill to 1 L with ddH_2O.

170 18. Staining solution: Quick protein stain (commercially available) or Coomassie
171 staining solution: 500 mL methanol, 100 mL acetic acid, a spatula tip of
172 Coomassie Brilliant Blue R-250, dissolve and fill to 1 L with ddH_2O.

173 19. De-staining solution: 400 mL methanol, 100 mL acetic acid, fill to 1 L with
174 ddH_2O.

175 **2.3 Buffers, stocks and equipment used for the initial activity assay**

176 Prepare all stocks fresh unless indicated otherwise. Weigh out a known quantity
177 (approximately 0.5 - 2 mg) of peptide, tris(2-carboxyethyl)phosphine hydrochloride
178 (TCEP), (+)-sodium L-ascorbate and Fe(II), then calculate the buffer volume to be used
179 for stock preparation. Prepare all solutions at room temperature as the reactions will
180 be performed at 25 °C. Reaction and enzyme storage buffers can be stored at 4 °C.

181 1. Reaction buffer, pH 8.0: dissolve 50 mM bis-tris propane (1,3-
182 bis[tris(hydroxymethyl)methylamino]propane) and 50 mM NaCl in LC/MS
183 grade water.

184 2. AtPCO4 storage buffer = SEC buffer

185 3. 0.5 M TCEP stock, pH 8.0: dissolve TCEP (solid) to 0.5 M in reaction buffer.
186 Adjust pH to 8.0 using NaOH. Prepare 50 µL aliquots and store at -80 °C for up
187 to three months.

188 4. 0.1 M L-ascorbate stock: dissolve (+)-sodium L-ascorbate (solid), hereafter
189 referred to as L-ascorbate, to 0.1 M in reaction buffer.

190 5. 0.5 mM iron stock: dissolve Fe(II) (solid) in 20 mM HCl to 0.1 M. Dilute it

191 further to 0.5 mM in LC/MS grade water (Note 6).

192 6. Peptide substrate stock (example used here is RAP2.12$_{2-15}$, CGGAIISDFIPPPR):

193 dissolve RAP2.12$_{2-15}$ solid peptide to 1 mM in reaction buffer. Store at -20 °C.

194 7. AtPCO4 stock: dilute enzyme to 4 µM in SEC buffer.

195 8. Substrate and enzyme master-mixes (MMs) are described in section 3.3.

196 9. Assay plate: 96-well PCR plate

197 10. Thermocycler or temperature-controlled block with 96-well block

198 11. Multichannel pipettes (8- or 12-channels): 10 µL and 100 µL / 200 µL

199 12. Reagent reservoir for multichannel pipettes

200 13. Quench solution: 15% (v/v) formic acid (LC/MS grade) in LC/MS grade water,

201 diluted to 5% (v/v) formic acid in final quench well (Note 7).

202 14. Quench plate: 96-well plate for MS analysis

203 15. Self-adhesive aluminium seals for PCR plates

204 16. Plate-spinning centrifuge

205 17. Column for peptide separation depending on the system, e.g. a C-4 solid-

206 phase extraction cartridge or a C-18 column

207 18. Acetonitrile (LC/MS grade)

208 19. High-throughput or ultra performance liquid chromatography (UPLC)

209 system coupled to a quadruple time of flight (Q-TOF) mass spectrometer.

210 20. MS data analysis software, e.g. Agilent's MassHunter and RapidFire

211 Integrator.

212 ## 2.4 Buffers and equipment for O_2-dependent kinetic assays

213 Prepare stocks as explained in 2.3 except for following modifications and additions:

214 1. 0.4 M L-ascorbate stock (Note 15): dissolve in reaction buffer and store on ice.
215 2. 8 mM iron stock (Note 15): dissolve Fe(II) in 20 mM HCl to 0.1 M, then dilute
216 to 8 mM in LC/MS grade water and store on ice (Note 6).
217 3. 10 mM peptide substrate stock: dissolve solid peptide (e.g. RAP2.12$_{2\text{-}15}$) in
218 reaction buffer.
219 4. X μM enzyme stock: dilute the enzyme in storage/SEC buffer so that the
220 optimal enzyme concentration (determined in section 3.4) is achieved when
221 0.5 μL enzyme stock are added to the reaction solution (total reaction volume:
222 100 μL, e.g. a 20 μM enzyme stock results in 0.1 μM enzyme in the final reaction
223 solution.) Store on ice.
224 5. Prepare enzyme and substrate MM as described in section 3.7.2.
225 6. Quench solution: 10% (v/v) formic acid in LC/MS grade water.
226 7. Gas-tight Hamilton syringes: 10 μL and 1000 μL
227 8. Needles: e.g. BD Microlance 3, 21G x 2” (0.8 x 50 mm)
228 9. Glass vial caps for O_2-exposure: must be resealing, e.g. Sulpeco certified
229 polypropylene bonded caps with septa, 9 mm cap, PTFE/red rubber septum.
230 10. Glass vials for O_2-exposure / reaction vials: e.g. 12x32 mm glass screw neck vial,
231 Quick Thread, Maximum Recovery
232 11. Glass vial caps with septa for MS instrument: must contain slit, e.g. Screw Caps,
233 Blue, 9-425mm, Pre-Slit PTFE/Silicone Septa.

234 12. Glass vials for diluted samples for MS system: e.g. SureSTART™ 0.3 mL Glass
235 Screw Top Microvials for < 2 mL Samples, Level 3 High Performance
236 Applications.

237 13. Water bath or equivalent to keep reaction vial at 25 °C.

238 14. Gas cylinders: O_2 and N_2 gas cylinders with appropriate regulators.

239 15. At least two elastomer-sealed pressure controllers capable of flow rates
240 0 - 100 mL/min.

241 16. Program and system to regulate O_2-concentrations: e.g. SmartInterface.

242 17. C-18 column for peptide separation: e.g. Chromolith Performance RP-18e 100
243 2-mm column.

244 18. UPLC system coupled to a Q-TOF mass spectrometer.

3 Methods

245 Perform all procedures at room temperature except where indicated otherwise.

246 3.1 Expression of AtPCO4

247 Perform all procedures in sterile conditions until harvesting the cells. After use,
248 decontaminate glass and plasticware which has been in contact with bacteria using
249 Virkon or by autoclaving. Surfaces can be cleaned with 70% (v/v) ethanol.

250 1. Prepare a pre-culture as follows: Add 0.1 mL of the 40 mg/mL kanamycin stock
251 to the autoclaved 100 mL 2-YT medium. Inoculate with one BL21(DE3) *E. coli*
252 colony containing the plasmid pET-28a(+)-His-TEV-AtPCO4 (Addgene) which
253 encodes His-TEV-AtPCO4 and contains a kanamycin resistance gene. Incubate

254 the pre-culture at 37 °C and 120 – 180 rpm in a shaking incubator overnight (≤
255 16 hours (h)).

256 2. Prepare the bacterial cultures as follows: Add 600 µL of the 40 mg/mL
257 kanamycin stock to each autoclaved 2 L flask containing 600 mL 2-YT medium.
258 Swirl the flasks to mix the supplemented media. Add 2 mL of the pre-culture
259 bacteria suspension to each flask.

260 3. Incubate the bacterial cultures at 37 °C shaking at 120 – 180 rpm until the
261 cultures reach an optical density at 600 nm (OD_{600}) of 0.4. Reduce the
262 temperature to 20 °C.

263 4. Once the cultures are at OD_{600} of 0.6 - 0.8, add 600 µL of the 50 mM Fe(II) stock
264 and induce protein expression by adding 600 µL of the 0.5 M IPTG stock to each
265 flask (Note 2). Incubate the induced cultures at 20 °C and shake at
266 120 – 180 rpm for 16 - 18 h.

267 5. Harvest the cells by centrifugation at 4 – 8 °C and 15,970 xg (times gravity) for
268 10 min, collect the cell pellet in a plastic bag, weigh it and store at -80 °C.
269 Sterilise and discard the supernatant.

270 **3.2 Purification of AtPCO4**

271 Perform the purification at 4 – 8 °C using an FPLC system. Keep the cells and protein
272 on ice whenever possible to maintain protein structural integrity. Before purifying
273 AtPCO4 on a large scale for the first time (as described below), purify a small amount
274 of protein (using ~3 g cell pellet) to test the cell lysis efficiency, the affinity of the His-
275 tagged protein to nickel and the cleavage efficiency of the respective protease (Note 3).

276 1. Resuspend ~20 g cell pellet in 140 mL of HisTrap™ buffer A supplemented
277 with one tablet of protease inhibitor cocktail (EDTA-free), 20 µL DNase I and
278 1x DNase I buffer at 4 – 8 °C.
279 2. Lyse the cells by sonication with a 1 cm sonicator tip at 60% amplitude (of 20
280 kHz) for 6 cycles of 2 min (10 s on, 10 s off) with 2 min breaks (Note 4).
281 3. Centrifuge the lysate at 48,380 xg and 4 – 8 °C for 30 min. Filter the supernatant
282 which contains the soluble protein fraction through a 0.45 µm syringe filter to
283 remove remaining cell debris particles. Discard the pellet into biohazardous
284 waste.
285 4. In the meantime, connect a 5 mL HisTrap™ HP column to the FPLC system.
286 Wash and equilibrate the column for 5 – 10 column volumes (CV) at
287 1 – 3 mL/min with filtered ddH_2O and then HisTrap™ buffer A.
288 5. Load the filtered lysate onto the equilibrated HisTrap™ column at
289 1 – 1.5 mL/min using a buffer inlet primed with HisTrap™ buffer A.
290 6. Remove non-binding proteins with 5 – 10 CV HisTrap™ buffer A until the UV
291 absorbance is < 100 mAU (milli-Absorbance Units), then elute further
292 impurities with 2% HisTrap™ buffer B for 2 CV.
293 7. Elute AtPCO4 at 1 mL/min with a 2% - 100% gradient of HisTrap™ Buffer B
294 (1 M imidazole) over 2 CV, followed by 100% HisTrap™ Buffer B for 2 CV.
295 Collect 2 mL fractions of eluted protein over the duration of the elution stage.
296 Take 10 µL of eluted fractions with the highest UV absorption (indicating
297 presence of protein), add 1x loading buffer to each sample and analyse them

298 via SDS-PAGE (section 3.2, 16) to assess the protein content in each fraction.

299 8. Select the fractions which contain a high proportion of AtPCO4, but no other
300 proteins with similar masses (Note 5), and concentrate the protein to a volume
301 of 2.5 mL using a 10 kDa cut-off centrifugal filter unit. This may require several
302 centrifugation steps (each 15 - 20 min, 2,727 xg, 4 °C); to prevent viscosity of
303 high protein concentration causing the filter to block, disperse the protein in
304 between each centrifugation step using a pipette.

305 9. Remove the imidazole from the concentrated protein using a PD-10 column
306 equilibrated with SEC buffer. Load the 2.5 mL protein onto the column and
307 elute with 3.5 mL SEC buffer.

308 10. Measure the protein concentration at 280 nm using a UV/Vis-spectrophometer.
309 The extinction coefficient of AtPCO4 (Table 1) can be computed using the
310 online tool ExPASy [25]. Add the TEV protease (Note 3, TEV protease for His-
311 TEV-AtPCO constructs; other proteases may be required for other PCO
312 constructs depending on the tag cleavage site) and incubate at 4 °C for 16 h on
313 a rotating mixer to cleave the His-tag.

314 11. Prepare the SEC column by washing it at 0.8 – 1 mL/min with 1.5 CV ddH_2O
315 then equilibrating the column with 1.5 CV SEC buffer. This can be performed
316 overnight concurrently with His-tag cleavage.

317 12. If using a protease which has an intrinsic His-tag itself (e.g. TEV protease),
318 remove the protease and any un-cleaved His-AtPCO from the cleaved AtPCO
319 by reverse affinity purification. Use a 5 mL sample loop to load the products of

320 the cleavage reaction onto a 5 mL HisTrap™ HP column which has been
321 previously washed and equilibrated with HisTrap™ buffer A; collect the flow-
322 through, which contains the cleaved AtPCO4, at 1 mL/min. Wash the column
323 with HisTrap™ buffer A for 3 CV or until UV < 30 mAU; collect the wash
324 eluant, which will also contain residual cleaved AtPCO4. Take samples from
325 the eluted fractions to check cleaved AtPCO4 is present and to determine its
326 purity and quantity by SDS-PAGE (section 3.2, 16). Concentrate the fractions
327 containing AtPCO4 until the volume reaches < 2 mL (section 3.2, 8).

328 13. Load the concentrated AtPCO4 at 1 mL/min onto the pre-equilibrated SEC
329 column using a syringe and a 2 mL sample loop.

330 14. Elute the protein with SEC buffer at 1 mL/min and collect fractions when UV
331 absorbance > 80 mAU. AtPCO4 will elute after ~150 - 160 mL. Take samples
332 from the fractions containing protein (as identified by UV absorbance) and
333 determine the protein purity and quantity by SDS-PAGE (section 3.2, 16).

334 15. Concentrate the fractions containing AtPCO4, if necessary, and aliquot at
335 concentrations that are useful for subsequent use, e.g. 15 μM. Shock-freeze the
336 aliquots in liquid nitrogen and store at -80 °C.

337 16. SDS-PAGE: 12% polyacrylamide gels are inserted into a gel electrophoresis
338 chamber which is subsequently filled with 1x running buffer. Denature protein
339 samples in loading buffer at 95 °C for 5 min, then transfer 10 – 15 μL denatured
340 protein samples into the loading wells. Run the gel at 85 V for 10 min followed
341 by 180 V for 45 min - 1 h (until the protein dye from the loading buffer has

342 reached the bottom of the gel). Remove the gels from the chamber and stain
343 with a Coomassie solution or a quick protein stain rocking on an orbital shaker.
344 When the staining is adequate (minimum 30 min), drain the staining solution.
345 Cover the gel in de-staining solution and leave rocking on an orbital shaker
346 until protein bands appear. Change the de-staining solution whenever the
347 solution becomes saturated with the staining dye.

348 **3.3 Initial activity assay**

349 To ensure the purified AtPCO4 is active, perform an initial activity assay by
350 incubating AtPCO4 with substrate and comparing the amount of un-oxidized to
351 oxidised substrate peptide (e.g. RAP2.12$_{2-15}$ and O_2S-RAP2.12$_{2-15}$, respectively) over a
352 10 min time course (e.g. 0, 0.5, 1, 1.5, 3, 5, 10 min).

353 *Tip: You can include a 30 min time point to ensure that low enzymatic activity is detected as*
354 *well.*

355 Initiate the reaction by mixing the substrate and enzyme master-mixes (MMs, see
356 below). Subsequently, acid-quench small volumes of the reaction mixture at the
357 respective time points. Analyse the quenched reaction mixture using an LC-MS
358 system. Perform all reactions in triplicate.

359 1. Assays are carried out in 96-well PCR plates (assay plate). Plan a layout for the
360 assay and the quench plate; an example template for an assay comparing
361 AtPCO4 activity with a 'no enzyme control' is shown in Figure 3B.

362 *Tip: Highlighting the respective rows on the assay and quench plates will prevent*
363 *mixing up samples / wells during the assay. It also helps to have a tick-box system for*

364 *the quench plate to know which are the next wells to add reaction mixture to at each*

365 *respective time point.*

366 2. Prepare all buffers and stocks as described in section 2.3.

367 3. Pre-temper the empty assay plate to 25 °C on the thermocycler or the

368 temperature-controlled block.

369 4. Prepare the quench plate by adding 95 μL 5% (v/v) formic acid into each well

370 needed (Note 7) using a multichannel pipette.

371 5. Prepare the substrate MM by diluting the RAP2.12$_{2-15}$ stock to 400 μM and the

372 0.5 M TCEP stock to 10 mM in reaction buffer (Note 8). Mix by pipetting and

373 inverting the tube.

374 6. Prepare the 'no enzyme control' by diluting the ascorbate and iron stocks to

375 2 mM L-ascorbate and 40 μM Fe(II) in reaction buffer (Note 8).

376 7. Prepare the enzyme MM by combining L-ascorbate, iron and AtPCO4 stocks to

377 achieve final concentrations of 2 mM L-ascorbate, 40 μM Fe(II) and 0.8 μM

378 AtPCO4 in reaction buffer (Note 8). Mix by pipetting without introducing

379 bubbles.

380 *Tip: Prepare the enzyme MM just before use to prevent any loss of enzymatic activity.*

381 8. Add 27.5 μL substrate MM into all the appropriate wells of the assay plate and

382 incubate for 5 min at 25 °C (Figure 3).

383 9. Transfer 30 μL enzyme MM and 'no enzyme control' into the respective wells

384 of the assay plate.

385 10. For the 0 min time point, transfer 2.5 μL of the incubating enzyme/'no enzyme

386 control' mix to the correct wells of the quench plate (Figure 3). Then, add 2.5 µL
387 of the incubating substrate mix into the same well of the quench plate. Mix
388 thoroughly by pipetting up and down without introducing bubbles.
389 *Tip: Use a multichannel pipette.*
390 11. Start the reactions by transferring 25 µL of the incubated enzyme / control mix
391 into the incubating substrate mix using a multichannel pipette (Figure 3). Mix
392 5 - 8 times by pipetting.
393 *Tip: Using a multichannel pipette allows multiple reactions to be started at the same*
394 *time and thereby decreases inter-assay variations.*
395 12. At each time point take 5 µL of the reaction solution and quench in 5% (v/v)
396 formic acid in the respective wells of the quench plate using a multichannel
397 pipette.
398 13. When the time course is complete, seal the quench plate with an aluminium
399 seal and store the plate at -20 °C (Note 9).
400 14. For analysis, thaw and spin the quench plate. Remove the aluminium seal,
401 inject the samples into a high-throughput (Note 10) or UPLC system (Note 11)
402 coupled to a Q-TOF mass spectrometer operated in positive ion/electrospray
403 mode. Optimise the source conditions for minimal fragmentation and maximal
404 sensitivity.
405 15. Identify the chromatogram peaks which correspond to substrate $RAP2.12_{2-15}$
406 (1442.7 Da) and the oxidised product (+32 Da, Note 12) using their molecular
407 masses. Integrate the area under the chromatogram peaks of the substrate and

408 product.

409 16. Calculate the proportion of the total peptide in the quench well that has been
410 oxidised (Equation 2), then use this to determine the amount of oxidised
411 peptide in moles (Equation 3) and subsequently the turnover of the peptide per
412 mass of AtPCO4 in the quench well (Equation 4). Calculate the mean of each
413 triplicate set of assays and the standard deviation (SD).

414

415 **Equation 2. Proportion of the total peptide that is oxidised.**

416
$$\%\ oxidised = \frac{A_{product}}{A_{substrate} + A_{product}}$$

417 A: integrated area under the peak

418

419 **Equation 3. Moles of oxidised peptide.**

420
$$\mu mol\ oxidised\ peptide = \%\ oxidised * \ \mu mol\ total\ peptide\ in\ quench\ well$$

421

422 **Equation 4. Turnover.**

423
$$turnover\ \left(\frac{\mu mol\ oxidised\ peptide}{mg\ enzyme}\right) = \frac{\mu mol\ oxidised\ peptide\ in\ quench\ well}{mg\ enzyme\ in\ quench\ well}$$

424

425 17. Display the means with SD error bars in a graph (x-axis: time, y-axis: turnover).
426 18. Use graphical software to fit linear regression to the steady-state portion
427 (before equilibrium) of the turnover means.

428 **3.4 Optimisation of enzyme concentration**

429 Optimise the enzyme concentration to ensure that at the highest anticipated substrate
430 concentration (e.g. 2 mM), the rate of turnover falls within the steady-state over 2 - 3
431 min before reaching equilibrium.

432 1. Repeat the initial activity assay from section 3.3 steps 1 - 18 with the following
433 modifications:
434   - Adapt the time points to shorter periods, e.g. 0 - 3 min.
435   - Make four enzyme MMs which result in four different enzyme
436 concentrations in the final assay (e.g. between 0.1 - 2 μM AtPCO4,
437 Note 8). Mix by pipetting without introducing bubbles.

438 19. Determine the optimal enzyme concentration to use for subsequent steady-
439 state kinetic analysis; product formation should increase linearly with time
440 over at least 1 - 3 min and should be sufficient to be measured adequately (Note
441 13).

442 **3.5 Optimisation of iron and ascorbate concentrations**

443 AtPCOs co-purify with a certain amount of iron in their active site.[14] However,
444 adding Fe(II) and L-ascorbate (which keeps the Fe(II) in the reduced state necessary
445 for AtPCO activity) can increase enzymatic activity *in vitro*.[14] Therefore, iron and
446 ascorbate concentrations should be optimised for maximal enzymatic activity.

3.5.1 Optimisation of the iron concentration

447 Determine the Fe(II) concentration at which AtPCO4 displays maximal activity. For

448 this, measure AtPCO4 activity at varying iron titrations.

449 1. Repeat the initial assay from section 3.3 steps 1 - 18 with the following
450 modifications:
451 • Use the optimal enzyme concentration determined in section 3.4.
452 • Prepare different enzyme MMs (optimal [AtPCO4] and 2 mM L-ascorbate,
453 Note 8) with varying Fe(II) concentrations (e.g. 0, 5, 10, 20 μM) in reaction
454 buffer. Mix by pipetting without introducing bubbles.
455 • Determine the activity during a time course up to 10 min (e.g. 0, 1, 3, 10 min)
456 19. Identify the Fe(II) concentration which gives the optimal AtPCO4 activity
457 through comparison of initial turnover rates, and use this Fe(II) concentration
458 subsequently for all future assays with this batch of AtPCO4 (Note 14).

3.5.2 Optimisation of the ascorbate concentration

459 To verify that L-ascorbate is necessary and high L-ascorbate concentrations are not
460 detrimental for AtPCO4 activity, determine the AtPCO4 activity at optimal iron
461 concentration varying the L-ascorbate concentrations (e.g. 0 - 2 mM).

462 1. Repeat the initial assay from section 3.3 steps 1 - 18 with the following
463 modifications:
464 • Use the optimal enzyme and iron concentration determined in sections 3.4
465 and 3.5.1, respectively.
466 • Prepare different enzyme MMs (optimal [AtPCO4], X μM L-ascorbate and
467 optimal [Fe(II)] in reaction buffer, Note 8) using a range of L-ascorbate
468 concentrations (e.g. between 0 - 2 mM)

469 • Determine AtPCO4 activity with a time course up to 10 min (e.g. 0, 1, 3,
470 10 min)

471 19. Identify the L-ascorbate concentration which gives maximal AtPCO4 activity
472 by comparing the initial turnover rates at the different ascorbate
473 concentrations. Use the optimal ascorbate concentration in all future assays.
474 Ensure that, with the optimal Fe(II) and ascorbate concentrations, the rate of
475 turnover at the chosen enzyme concentration is still in the steady-state range.

476 **3.6 Steady-state kinetic assays with substrate peptide**

477 Once all assay components are optimised (3.4 and 3.5), determine the rate of enzyme
478 activity as a function of substrate concentration under steady-state conditions.

479 1. Follow the assay procedure as described in section 3.3 steps 1 - 18 with the
480 following modifications:
481 • Prepare substrate MMs at a range of concentrations (e.g. nine substrate
482 concentrations between 0 - 4000 μM, Note 8).
483 *Tip: For better accuracy, use serial dilutions to make the substrate MMs.*
484 • Enzyme MM (Note 8):
485 o Use the optimal enzyme concentration determined in section 3.4.
486 o Use the optimal Fe(II) and L-ascorbate concentrations determined in
487 section 3.5.
488 • Adjust formic acid concentrations in the quench wells as appropriate
489 (Note 7).

490 19. Conduct steps 19 - 21 using graphical software. Plot product formation against

491 time and display regression lines of best fit to calculate the initial rates (velocity,

492 υ, μmol/mg/min) of AtPCO4 activity (μmol/mg) for each RAP2.12$_{2-15}$

493 concentration.

494 20. Plot the initial rate (υ) of AtPCO4 activity against RAP2.12$_{2-15}$ concentration and

495 fit the data to the Michaelis-Menten equation (Equation 5).

496 21. Calculate the Michaelis-Menten kinetic parameters for AtPCO4 with respect to

497 RAP2.12$_{2-15}$ (Equation 5): the Michaelis-Menten constant K_M (μM, substrate

498 concentration required to achieve half-maximal AtPCO4 velocity), the maximal

499 enzyme velocity for AtPCO4 (V_{max}, μmol/mg/min) and the turnover number

500 per AtPCO4 enzyme k_{cat} (1/min, amount of substrate oxidised by one active site

501 per time). More details on Michaelis-Menten kinetic parameter derivations can

502 be found in textbooks (e.g. [19–22]).

503

504 **Equation 5. Michaelis-Menten equation using RAP2.12$_{2-15}$ peptide as**

505 **substrate.**

506 $$\mathbf{v_{observed}} = \frac{\mathbf{V_{max} * [RAP2.12_{2-15}]}}{\mathbf{K_M + [RAP2.12_{2-15}]}}$$

507 $$\mathbf{K_M = [RAP2.12_{2-15}]\ at\ \frac{V_{max}}{2}}$$

508 $$k_{cat} = \frac{V_{max}}{[E_t]}$$

509 $v_{observed}$: enzyme velocity; oxidation rate at a specific substrate concentration

510 E_t: total enzyme

511

512 **3.7 O_2-dependent kinetic assays**

513 Determine the O_2-sensitivity of AtPCO4 by assessing its rate of activity at different O_2-
514 concentrations while other reaction components are non-limiting. Use the optimal
515 enzyme concentration for steady-state kinetics obtained in section 3.4 as well as the L-
516 ascorbate, iron and substrate concentrations which resulted in the highest enzymatic
517 activity (sections 3.5 and 3.6).

3.7.1 Setup of the O_2-exposure experiment

518 1. Connect the O_2 and N_2 gas cylinders, with appropriate regulators, to the
519 respective gas pressure controllers (Figure 4A) via a tube which can be opened
520 and closed.
521 2. Regulate the O_2 and N_2 flow through computer software-linked gas pressure
522 controllers. Adjustment of the gas flow will achieve the final O_2/N_2-mixture
523 produced (Figure 4A).
524 3. Connect the inert tube to a needle to enable exposure of the reaction buffer to
525 different O_2-concentrations in a glass vial (Figure 4B).

3.7.2 Steady-state kinetic assays with O_2

526 The nature of the apparatus and the volumes of assay mix required for measurement
527 at multiple O_2-concentrations mean, it is unfeasible to conduct time course
528 experiments. Instead, enzyme turnover is measured at a single time point that is
529 already known to fall into the steady-state region of enzyme activity, e.g. 1 min
530 (sections 3.4 - 3.6): Product to total peptide ratios are determined for each O_2-
531 concentration at this defined time point. Consistency is essential at every step of this

532 experiment.

533 1. Prepare all stocks as described in section 2.4.

534 2. Prepare 2,614 μL substrate MM for eight different O_2-concentrations (Note 16).

535 Dilute the substrate stock to a final concentration which matches the optimal

536 RAP2.12$_{2\text{-}15}$ concentration determined in section 3.6 in reaction buffer with

537 5 mM TCEP (Note 17).

538 3. Transfer 99 μL substrate MM into each glass vial for O_2-exposure and close

539 with a resealable cap.

540 4. Open the gas cylinders and the pressure controllers, and set the O_2 and N_2 flow

541 rates to 20 mL/min O_2 and 80 mL/min N_2 which equals 20% O_2.

542 *Tip: To get familiar with the assay, start with near-atmospheric O_2-concentrations*

543 *(20% O_2, 80% N_2) and compare to equivalent aerobic assays conducted without gas*

544 *regulation.*

545 5. Check the gas flow by holding the needle connected to the gas mixture tube

546 (needle B, Figure 4B) into water; bubbles should be visible.

547 6. Take a glass vial containing the substrate MM and perforate the resealable cap

548 with needle A (Figure 4B). This will prevent overpressure in the vial when

549 equilibrating the buffer with the respective O_2/N_2 gas mixtures.

550 *Tip: Mark on the glass where the needle tip ends. Make an equivalent mark on the other*

551 *glass vials for consistency.*

552 7. Perforate the lid with needle B and submerge the needle in the substrate buffer

553 for 10 min to equilibrate the mixture with 20% O_2 (Notes 18 and 19).

554 8. Prepare a 1000 μL gas-tight Hamilton syringe, hereafter called quench syringe,
555 with 200 μL quench solution (10% (v/v) formic acid).
556 9. Prepare the enzyme MM and start the reaction as follows:
557 a. While the substrate MM is equilibrating with the respective O_2-
558 concentration (after ~8 mins equilibration), prepare the enzyme MM:
559 Mix 1 μL iron stock and 1 μL ascorbate stock, with 2 μL enzyme stock
560 just before taking up 3 μL of this enzyme MM (Note 20) with a 10 μL
561 gas-tight Hamilton syringe, herein called enzyme syringe.
562 b. When the 10 min substrate MM O_2 equilibration is complete,
563 immediately, remove needle A, then needle B from the glass vial to
564 establish overpressure in the glass vial.
565 c. Transfer 1 μL enzyme MM into the equilibrated buffer by perforating
566 the sealing cap with the enzyme syringe (Note 20).
567 d. Remove the enzyme syringe and seal the lid with parafilm.
568 e. Mix the reaction components by gently flicking the tube from opposite
569 sites (5-times each).
570 10. Incubate the reaction at 25 °C for 1 min (within the initial activity rate of
571 AtPCO4).
572 11. Add 100 μL quench solution to the assay vial by perforating the sealing cap
573 with the quench syringe. Remove the quench syringe and mix by flicking from
574 opposite sites (5-times each).
575 12. Seal the lid with parafilm and store the vial at -20 °C.

576 13. Repeat the procedure in triplicate for each O_2-concentration (suggested range
577 of O_2-concentrations: 60 - 0 O_2%, e.g. 60, 40, 20, 10, 5, 2.5, 1.25, 0 O_2%). Take care
578 to be consistent across assays in the time intervals between O_2/N_2 equilibration
579 and enzyme MM injection.
580 22. For analysis, transfer the quenched reaction mixture into a glass vial with a pre-
581 slit cap for MS systems, adjust peptide and formic acid concentrations (Note 7)
582 and inject samples into the UPLC-MS system (Note 11).
583 23. Analyse as described in section 3.6 referring to O_2 as a substrate.
584 24. Calculate the Michaelis-Menten parameters for AtPCO4 with respect to O_2, as
585 described in section 3.6.
586

4 Notes

587 ## 4.1 Notes for protein expression

588 1. 2-YT medium granules can be bought pre-mixed. The granular form prevents
589 generating lots of dust when weighing out and therefore reduces health risks.
590 Alternatively, mix each component separately (10 g/L of yeast extract, 16 g/L of
591 tryptone and 5 g/L of NaCl).
592 2. To check the expression level of the protein, take 1 mL samples of the bacteria
593 culture (i) before induction with IPTG and (ii) after induction with IPTG before
594 cell harvest. Centrifuge at 3,396 xg for 10 min, remove supernatant and
595 resuspend the pellet in 1x LB according to the OD_{600} (e.g. add 54 μL 1x LB for

596 an OD_{600} of 0.54). Samples can be stored at -20 °C. Visualise protein expression
597 levels by SDS-PAGE (section 3.2, 16).

598 **4.2 Notes for protein purification**

599 3. The protease:protein ratio depends on the cleavage efficiency of the protease.
600 Thus, determine the molar ratio of protease to His-AtPCO in a small test trial
601 (e.g. 1:10, 1:20, 1:50, 1:100) beforehand. If necessary, the His-tag can be left on
602 AtPCO mitigating the tag cleavage step. However, the potential influence of
603 the His-tag on AtPCO activity and selectivity is not established.
604 4. When sonicating to lyse the bacterial cells, swirl the cell suspension during the
605 breaks and extend the breaks on ice, if the cells warm up to prevent degradation
606 of target proteins.
607 5. Only proteins of different mass to AtPCO4 can be separated from AtPCO4 by
608 SEC which is the last step of the purification.

609 **4.3 Notes for activity assays**

610 6. Dilute the iron stock in water just prior to use for each reaction to prevent
611 Fe(II) from oxidising to Fe(III). If the solution turns yellow, prepare a fresh
612 dilution.
613 7. Adjust the quench solution for assays with high peptide concentration (e.g.
614 above 100 µM) to achieve a final concentration of 5% (v/v) formic acid in the
615 quench well and a peptide concentration in the detection range of the MS
616 instrument. Further peptide dilutions using 5% (v/v) formic acid might be

617 necessary to prevent saturation of the MS system which can lead to inaccurate
618 measurements.

619 8. To initiate the enzymatic reaction, substrate and enzyme MM are added 1:1 in
620 the assay plate. Thus, all components of both MMs will have half the
621 concentration in the final assay. Account for this by preparing 2x concentrated
622 substrate and enzyme MMs, e.g. the final $RAP2.12_{2-15}$ concentration in the initial
623 activity assay (section 3.3) will be 200 μM, following preparation of MM with
624 $RAP2.12_{2-15}$ at 400 μM.

625 9. If the assay is going to be analysed on the same day, store the quench plate at
626 4 °C.

627 10. When using a high throughput MS system, elute the peptides with
628 1.25 - 1.5 mL/min flow rate in 85% (v/v) acetonitrile supplemented with 0.1%
629 (v/v) formic acid using a C-4 solid-phase extraction cartridge at room
630 temperature which has been equilibrated with deionised water and 0.1% (v/v)
631 formic acid.

632 11. When using a UPLC MS system, elute the peptides with 0.3 mL/min flow rate
633 during a gradient of 95% (v/v) deionised water with 0.1% (v/v) formic acid to
634 95% (v/v) acetonitrile using a C-18 column which is heated to 40 °C.

635 12. AtPCO4 oxidises the Nt-cysteine of its substrates to cysteine sulfinic acid ($-SO_2^-$
636).[10] Some MS systems can lead to further oxidation to cysteine sulfonic acid
637 ($-SO_3^{2-}$).[10] Therefore, treat both cysteine sulfinic (+32 Da) and sulfonic acid
638 (+48 Da) as oxidised products of $RAP2.12_{2-15}$.

639 **4.4 Notes for optimising the enzyme concentration**

640 13. Enzyme concentration can be altered if either the product formation (i) does
641 not increase linearly in time over at least 1 - 3 min or (ii) is insufficient to be
642 measured, and neither can be compensated for by comparison of rates in
643 moles/mg enzyme/min.

644 **4.5 Notes for optimising the iron concentration**

645 14. Repeat the assay at higher iron concentrations (e.g. 20 - 200 μM Fe(II)) if the
646 AtPCO4 activity did not plateau at 10 – 20 μM Fe(II).

647 **4.6 Notes for O_2 kinetics**

648 15. Ascorbate and iron stocks differ from previous stocks. Prepare both stocks, so
649 that only 0.25 μL of each need to be added to the reaction solution for a final
650 reaction concentration as determined in section 3.5. The concentrations
651 provided are for a final Fe(II) concentration of 20 μM and a final L-ascorbate
652 concentration of 1 mM; adjust if necessary.

653 16. Prepare 2,614 μL substrate MM which will be split into 8 x 99 ul aliquots (in
654 glass vials) to which 1 μL enzyme MM will be added to start the reaction.
655 Preparation of 2,614 uL accounts for pipetting error:
656 8 (reactions) x 3 (triplicates) x 99 μL (volume/reaction) x 1.1 (pipetting error).

657 17. Usually, the final substrate concentration is 5 x K_M(substrate) to ensure
658 saturation of the enzyme with the respective substrate. However, the $RAP2.12_{2-}$
659 $_{15}$ peptide has previously shown inhibitory effects at concentrations

660 > 1 mM.[14]

661 18. When repeating the experiment, be consistent with placing of the needle.

662 19. It has been shown previously that equilibrating 99 µL buffer in a 12x32 mm
663 glass screw neck vial with quick thread with an O_2/N_2-mixture for 10 min is
664 sufficient to reach the desired O_2-concentration in the buffer.[14] The dissolved
665 O_2-concentration in the buffer can also be estimated using an oxygen converter
666 tool, e.g. https://www.loligosystems.com/convert-oxygen-units.

667 20. Taking up 3 µL of enzyme MM into the 10 µL Hamilton syringe and only
668 dispensing 1 µL into the substrate buffer prevents the introduction of air into
669 the reaction mixture.

670

671 ## Acknowledgements

672 The authors are grateful for funding from the European Research Council (ERC)
673 under the European Union's Horizon 2020 research and innovation program
674 (PCOMOD project, Grant Agreement 864888 AD, DMG and EF) and the UK
675 Engineering and Physical Sciences Research Council (EPSRC) research programme
676 (Grant reference EP/S019901/1, EF). We thank Dr W. D. Figg, Jr. for thoroughly
677 reading the manuscript. Figures were created with BioRender.com.

678

679 **References**

680 1. Schofield CJ, Ratcliffe PJ (2004) Oxygen sensing by HIF hydroxylases. Nat Rev

681 Mol Cell Biol 5:343–354. https://doi.org/10.1038/nrm1366
682 2. Hammarlund EU, Flashman E, Mohlin S, Licausi F (2020) Oxygen-sensing
683 mechanisms across eukaryotic kingdoms and their roles in complex
684 multicellularity. Science 370:1–11. https://doi.org/10.1126/science.aba3512
685 3. Gibbs DJ, Holdsworth MJ (2020) Every Breath You Take: New Insights into
686 Plant and Animal Oxygen Sensing. Cell 180:22–24.
687 https://doi.org/10.1016/j.cell.2019.10.043
688 4. Holdsworth MJ, Gibbs DJ (2020) Comparative Biology of Oxygen Sensing in
689 Plants and Animals. Curr Biol 30:R362–R369.
690 https://doi.org/10.1016/j.cub.2020.03.021
691 5. Tarhonskaya H, Hardy AP, Howe EA, et al (2015) Kinetic Investigations of the
692 Role of Factor Inhibiting Hypoxia-inducible Factor (FIH) as an Oxygen Sensor.
693 J Biol Chem 290:19726–19742. https://doi.org/10.1074/jbc.M115.653014
694 6. Masson N, Keeley TP, Giuntoli B, et al (2019) Conserved N-terminal cysteine
695 dioxygenases transduce responses to hypoxia in animals and plants. Science
696 364:65–69. https://doi.org/10.1126/science.aaw0112
697 7. Hancock RL, Masson N, Dunne K, et al (2017) The Activity of JmjC Histone
698 Lysine Demethylase KDM4A is Highly Sensitive to Oxygen Concentrations.
699 ACS Chem Biol 12:1011–1019. https://doi.org/10.1021/acschembio.6b00958
700 8. Weits DA, Giuntoli B, Kosmacz M, et al (2014) Plant cysteine oxidases control
701 the oxygen-dependent branch of the N-end-rule pathway. Nat Commun 5:1–
702 10. https://doi.org/10.1038/ncomms4425

703 9. van Dongen JT, Licausi F (2015) Oxygen sensing and signaling. Annu Rev
704 Plant Biol 66:345–367. https://doi.org/10.1146/annurev-arplant-043014-114813
705 10. White MD, Klecker M, Hopkinson RJ, et al (2017) Plant cysteine oxidases are
706 dioxygenases that directly enable arginyl transferase-catalysed arginylation of
707 N-end rule targets. Nat Commun 8:1–9. https://doi.org/10.1038/ncomms14690
708 11. Bailey-Serres J, Fukao T, Gibbs DJ, et al (2012) Making sense of low oxygen
709 sensing. Trends Plant Sci 17:129–138.
710 https://doi.org/10.1016/j.tplants.2011.12.004
711 12. Gibbs DJ, Lee SC, Md Isa N, et al (2011) Homeostatic response to hypoxia is
712 regulated by the N-end rule pathway in plants. Nature 479:415–418.
713 https://doi.org/10.1038/nature10534
714 13. Licausi F, Kosmacz M, Weits DA, et al (2011) Oxygen sensing in plants is
715 mediated by an N-end rule pathway for protein destabilization. Nature
716 479:419–422. https://doi.org/10.1038/nature10536
717 14. White MD, Kamps JJAG, East S, et al (2018) The plant cysteine oxidases from
718 Arabidopsis thaliana are kinetically tailored to act as oxygen sensors. J Biol
719 Chem 293:11786–11795. https://doi.org/10.1074/jbc.RA118.003496
720 15. Gibbs DJ, Md Isa N, Movahedi M, et al (2014) Nitric Oxide Sensing in Plants Is
721 Mediated by Proteolytic Control of Group VII ERF Transcription Factors. Mol
722 Cell 53:369–379. https://doi.org/10.1016/j.molcel.2013.12.020
723 16. Hartman S, Liu Z, van Veen H, et al (2019) Ethylene-mediated nitric oxide
724 depletion pre-adapts plants to hypoxia stress. Nat Commun 10:1–9.

725 https://doi.org/10.1038/s41467-019-12045-4
726 17. Jagadis Gupta K, Mur LAJ, Wany A, et al (2020) The role of nitrite and nitric
727 oxide under low oxygen conditions in plants. New Phytol 225:1143–1151.
728 https://doi.org/10.1111/nph.15969
729 18. Taylor-Kearney LJ, Flashman E (2022) Targeting plant cysteine oxidase activity
730 for improved submergence tolerance. Plant J 109:779–788.
731 https://doi.org/10.1111/tpj.15605
732 19. Lorsch JR (2014) Chapter One - Practical Steady-State Enzyme Kinetics. In:
733 Lorsch JR (ed) Laboratory Methods in Enzymology: Protein Part A. Academic
734 Press, pp 3–15
735 20. Voet D, Voet JG (2011) Chapter 14 - Rates of Enzymatic Reactions. In:
736 Biochemistry, 4th ed. John Wiley & Sons, Inc., Hoboken, N.J., pp 482–505
737 21. Berg JM, Stryer L, Tymoczko JL (2019) Chapter 8 - Enzymes: Basic Concepts
738 and Kinetics. In: Biochemistry, 9th ed. Macmillan Learning, New York, pp
739 531–608
740 22. Bugg T (2012) Introduction to Enzyme and Coenzyme Chemistry, 3rd ed. John
741 Wiley & Sons, Ltd
742 23. Taylor-Kearney LJ, Madden S, Wilson J, et al (2022) Plant Cysteine Oxidase
743 Oxygen-Sensing Function Is Conserved in Early Land Plants and Algae. ACS
744 Bio Med Chem Au 0: https://doi.org/10.1021/acsbiomedchemau.2c00032
745 24. White MD, Dalle Carbonare L, Lavilla Puerta M, et al (2020) Structures of
746 Arabidopsis thaliana oxygen-sensing plant cysteine oxidases 4 and 5 enable

747 targeted manipulation of their activity. Proc Natl Acad Sci U S A 117:23140–
748 23147. https://doi.org/10.1073/pnas.2000206117
749 25. Gasteiger E, Hoogland C, Gattiker A, et al (2005) Protein Identification and
750 Analysis Tools on the ExPASy Server. In: Walker JM (ed) The Proteomics
751 Protocols Handbook. Humana Press Inc., Totowa, NJ, pp 571–608
752 26. Consortium TU (2021) The UniProt Consortium, UniProt: the universal protein
753 knowledgebase in 2021. Nucleic Acids Res 49:D480–D489.
754 https://doi.org/10.1093/nar/gkaa1100
755
756 **Figure captions**
757 **Figure 1. AtPCO catalysed oxidation of proteinic N-terminal cysteine to cysteine**
758 **sulfinic acid**. The reaction is promoted by an Fe(II) cofactor. The oxidation state of
759 Fe(II) is maintained *in vitro* by ascorbate (Asc).
760
761 **Figure 2. Graphical illustration of principles of Michaelis-Menten steady-state**
762 **enzyme kinetics. (A)** Enzyme activity (turnover, defined as µmol product formed/mg
763 enzyme) is plotted against time and used to determine initial velocity (v) at different
764 substrate concentrations (S_1 - S_9). **(B)** Initial velocities are plotted against substrate
765 concentrations (equivalent colours for substrate concentrations in **A** and **B**) and the
766 resulting data points can be fitted to the Michaelis-Menten equation (Equation 5 in
767 main text). The maximal enzymatic velocity (V_{max}, dotted line) is reached when the
768 turnover rate remains unchanged whilst the substrate concentration [S] is increased

769 (brown [S_7], orange [S_8] and pink [S_9] dots). The substrate concentration at half-

770 maximal velocity ($V_{max}/2$) is indicated as the Michaelis-Menten constant (K_M, M).

771

772 **Figure 3. Graphical illustration of the procedure and the layout of an activity assay.**

773 **(A)** Flow of an assay procedure starting with (1.1) pre-tempering the empty 96-well

774 assay plate, followed by (1.2) incubating the substrate master-mix (MM) and (1.3) the

775 enzyme MM / 'no enzyme control' (see B) in the wells. After preparing 0 min samples,

776 the reaction is started (2) by transferring 25 µL enzyme/control MM into the wells

777 which contain substrate MM. (3) At each time point, 5 µL reaction solution is removed

778 and quenched in 5% (v/v) formic acid in the 96-well quench plate and (4) analysed

779 using mass spectrometry. **(B)** Example layout of an assay (top) and a quench plate

780 (bottom). The assay plate contains enzyme MM (blue, A1-3) and 'no enzyme control'

781 (purple, A4-6) in row A. Six wells adjacent to the enzyme/control mix contain the

782 substrate MM (yellow, B1-6). The quench plate contains 5% (v/v) formic acid solution

783 (green) in all columns in which enzyme (light green) and control reaction mixes (dark

784 green) are quenched at different time points (here: 0 - 30 min).

785

786 **Figure 4. Schematic of the O_2-experiment setup. (A)** Scheme of the N_2 and O_2 gas

787 cylinders connected to flow regulators that are controlled via an interposed

788 communication system and associated computer software. The flow-regulated gas

789 mixture is directed into a glass vial **(B)** with a cone-shaped bottom via an inert tube

790 and needle B. Needle A prevents overpressure in the vial while the substrate mix

791 (blue) is equilibrated with the respective O_2/N_2 gas mixture.

792

793 **Tables**

794 **Table 1. Protein parameters of recombinant *Arabidopsis thaliana* PCOs from pET-**
795 **28a(+)-His-AtPCO.** Of constructs available at the time of publication, His-AtPCO1 - 3
796 contain a thrombin cleavage site and His-AtPCO4 - 5 a TEV protease cleavage site.
797 AtPCO3 and 4 exist in two isoforms which differ by one amino acid and are produced
798 by alternative splicing. The isoforms 1 (AtPCO3-1 and AtPCO4-1) are referred to as
799 canonical sequences.[26] Abbreviations: ε – extinction coefficient, Abs 0.1% - absorbance
800 of a 1 mg/mL protein solution at 280 nm (pathlength 10 mm), Da - Dalton, MW - molecular
801 mass.

	MW (Da)	Oxidising conditions		Reducing conditions	
		ε	Abs 0.1%	ε	Abs 0.1%
His-AtPCO1	35,177	28920	0.822	28420	0.808
AtPCO1	33,295	28920	0.869	28420	0.854
His-AtPCO2	32,984	27890	0.846	27390	0.830
AtPCO2	31,102	27390	0.897	27390	0.881
His-AtPCO3-1	33,454	34880	1.043	34880	1.028
AtPCO3-1	31,572	34880	1.105	34880	1.089
His-AtPCO3-2	33,383	34880	1.045	35870	1.030
AtPCO3-2	31,501	34880	1.107	34880	1.091
His-AtPCO4-1	30,152	26275	0.871	25900	0.859
AtPCO4-1	27,455	24785	0.903	24410	0.889
His-AtPCO4-2	30,281	26275	0.868	25900	0.855
AtPCO4-2	27,584	24785	0.899	24410	0.885
His-AtPCO5	30,192	37275	1.235	36900	1.222
AtPCO5	27,495	35785	1.301	35410	1.288

802

803 **Running head**

804 Oxygen Kinetics of Plant Cysteine Oxidases