Supplementary Material

to

METAL ION RELEASE FROM METALLOTHIONEINS:
PROTEOLYSIS AS AN ALTERNATIVE TO OXIDATION

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Fitting of Zn\textsuperscript{II}-release data in Origin with equations for exponential association kinetics

Depending on the plot form obtained from the Zn\textsuperscript{II}-MT / PAR metal ion exchange reaction two different equations were used to extrapolate the data to equilibrium conditions, if necessary:

1) two-phase exponential association

\[ y = y_0 + A_1 \cdot (1 - e^{-\frac{x}{w_1}}) + A_2 \cdot (1 - e^{-\frac{x}{w_2}}) \]

2) three-phase exponential association

\[ y = y_0 + A_1 \cdot (1 - e^{-\frac{x}{w_1}}) + A_2 \cdot (1 - e^{-\frac{x}{w_2}}) + A_3 \cdot (1 - e^{-\frac{x}{w_3}}) \]

All fittings were performed with Origin\textsuperscript® 7.
Calculation of apparent binding constants, $K_{app}$, from PAR data

$$K_{app,MT} = \frac{[\text{ZnBS}] [\text{PAR}]^2}{[\text{BS}] [\text{Zn(PAR)}]^2} \cdot 10^{-6} \cdot K_{app,Zn(PAR)}$$

$K_{app,Zn(PAR)}$ (pH 7.4) = $10^{13.49}$ M$^{-2}$

1) All Zn$^{II}$ binding sites (ZnBS) in the respective MT are treated as non-interacting with equal $K_{app}$

   Example: Zn$_6$Ec-1 (control, 1$^{st}$ measurement)

   $$A_{500nm} = 0.11954$$
   $$[\text{Zn(PAR)}] = A_{500nm} / \varepsilon_{Zn(PAR)} = 0.11954 / 0.065 \mu M^{-1} \text{ cm}^{-1} = 1.8391 \mu M$$
   $$[\text{PAR}] = [\text{PAR}]_o - 2*[\text{Zn(PAR)}] = 100 \mu M - 2*1.8391 \mu M = 96.3218 \mu M$$
   $$[\text{ZnBS}] = [\text{ZnBS}]_o - [\text{Zn(PAR)}] = 9 \mu M - 1.8391 \mu M = 7.1609 \mu M$$
   $$[\text{BS}] = [\text{Zn(PAR)}] = 1.8391 \mu M$$

   $\Rightarrow K_{app,MT} = 10^{11.78}$ M$^{-1}$

2) All ZnBS of (partially) released Zn$^{II}$ ions have the same $K_{app}$

   All other ZnBS have much higher $K_{app}$ and are not affected

   Example: Zn$_6$Ec$^{-1}$: 1.14 eq Zn$^{II}$ are released, hence only 2 of the 6 ZnBS are affected by PAR

   $$[\text{ZnBS}] = [\text{ZnBS}]_o - [\text{Zn(PAR)}] = \frac{9 \mu M}{6} * 2 - 1.8391 \mu M = 1.1609 \mu M$$

   $\Rightarrow K_{app,MT} = 10^{10.99}$ M$^{-1}$
3) If more than 1 eq Zn$^{II}$ is released by PAR:

This Zn$^{II}$ is much weaker bound and not considered

a) All remaining Zn$^{II}$ binding sites (ZnBS) in the respective MT have the same $K_{app}$

Example: Zn$_6$E$_c$$^-$1: 1.21 eq Zn$^{II}$ are released, hence only 5 of the 6 ZnBS are considered for the calculation, i.e. a Zn$_5$E$_c$$^-$1 species

\[
1 \text{ eq Zn}^{II} \equiv 9 \ \mu M / 6 = 1.5 \ \mu M \ Zn^{II}
\]

\[
1.5 \ \mu M \ Zn^{II} \equiv A_{500nm} = 1.5 \times 0.065 = 0.0975
\]

\[
A_{500nm} = 0.11954 - 0.0975 = 0.02204
\]

\[
[Zn(PAR)_{2}] = A_{500nm} / \varepsilon_{Zn(PAR)_{2}} = 0.02204 / 0.065 \ \mu M^{-1} \ cm^{-1} = 0.3391 \ \mu M
\]

\[
[PAR] = [PAR]_o - 2[Zn(PAR)_{2}] = 100 \ \mu M - 2 \times 1.5 \ \mu M - 2 \times 0.3391 \ \mu M = 96.3218 \ \mu M
\]

\[
[ZnBS] = [ZnBS]_o - [Zn(PAR)_{2}] = 9 \ \mu M - 1.5 \ \mu M - 0.3391 \ \mu M = 7.1609 \ \mu M
\]

\[
[BS] = [Zn(PAR)_{2}] = 0.3391 \ \mu M
\]

\[\Rightarrow K_{app,MT} = 10^{13.25} \ M^{-1}\]

b) Only $K_{app}$ of the ZnBS of the remaining partially released Zn$^{II}$ ion is calculated

All other filled ZnBS have much higher $K_{app}$ and are not affected

Example: the Zn$_5$E$_c$$^-$1 species from a), hence only 1 of the 5 remaining ZnBS is affected by PAR

\[
[ZnBS] = [ZnBS]_o - [Zn(PAR)_{2}] = 9 \ \mu M / 6 \times 1 - 0.3391 \ \mu M = 1.1609 \ \mu M
\]

\[\Rightarrow K_{app,MT} = 10^{12.46} \ M^{-1}\]
**Calculation of first-order rate constant k of Zn\(^{II}\) release**

Integrated law of first-order reaction:

\[
\ln \left( \frac{[\text{ZnBS}]}{[\text{ZnBS}]_0} \right) = -k \cdot t
\]

Formation of the Zn(PAR)\(_2\) complex is followed with UV/vis spectroscopy, and hence the observed absorption increase is a measure for the Zn\(^{II}\) release from the Zn\(^{II}\) binding sites (ZnBS) in the respective MT:

\[
[\text{ZnBS}]_0 \sim A_{\text{max}} - A_0 \quad \text{and} \quad [\text{ZnBS}]_t \sim A_{\text{max}} - A_t
\]

(in our measurements, \(A_0\) equals 0).

Hence:

\[
\ln \frac{A_{\text{max}} - A_t}{A_{\text{max}} - A_0} = -k \cdot t
\]

For the determination of the first-order rate constant, \(\ln \frac{A_{\text{max}} - A_t}{A_{\text{max}} - A_0}\) (or \(\ln(A_{\text{max}} - A_t)\)) is plotted against time resulting in a straight line with the slope \(-k\).

In the experiments presented here, straight lines were generally obtained for the data points between 2.5 and 100 min. The first faster Zn\(^{II}\) release step cannot be monitored with the UV/vis instrument available due to the limited number of data points (0, 0.5, 2.5 min, etc). Fittings were performed with Origin\(^\circledR\) 7.
**SUPPLEMENTARY FIGURES**

Figure S1. Competition experiment of PAR with musMT3 (A) and E_c-1 (B) followed by UV/vis spectroscopy at 500 nm under the different conditions indicated. All solutions, except for the control experiment, contain additionally 1 mM GSH. Data fitting (lines) to obtain the equilibrium absorption values $A_{\text{max}}$ were performed with equations for exponential association kinetics as described in detail above in the Supplementary Material.
**Figure S2.** Amino acid sequences of the different MTs studied here. Cys-rich regions are highlighted with black boxes and potential proteinase K cleavage sites indicated by red vertical bars.

**cicMT1**

MSGCNSSCNCQCKKKSGLSYVEAGETETTVLVGPTKIHFEQAEEMVAAEDGECXCGSSCTDCFCXK

**cicMT2**

MSCGGNCSCQCCSKQGQGKYMPSYTEQTTSETLVMVGASQKQFQAGEQGATQGCAENQXCGSNCNTCTCK

**musMT3**

MSCTCNCDCYXKQVKKGNYGLIDIVETEKSYVDEVIVAAEAEDGECXCGAAGACTTCKSN

**E-1**

MCDDKCGAVFEGGTCECRFTRSGAAAGHNTTGGCGHCYCNFCADREGTSPGRANNRANCSCGAACNCASCASSATA

**huMT2**

MDVAOGANGUCSCQCKCQCKCQCTSLQKXGCYVCAQCTQCTCKGASJWRSKCXCA
**Figure S3.** Competition experiment of PAR with E_c-1, γ-E_c-1, and β_E-E_c-1 followed by UV/vis spectroscopy at 500 nm under control conditions. Evidently, a 1:1 mixture of γ-E_c-1 and β_E-E_c-1 results in the same absorption values (meas.) as the sum of the values obtained with the individual domains (calc.).
Figure S4. Percentage and equivalents of Zn\textsuperscript{II} ions released per individual domain (fitted equilibrium data) based on two Zn\textsuperscript{II} ions in $\gamma$-Ec-1 and 4 Zn\textsuperscript{II} ions in $\beta$-Ec-1 for the different conditions tested. Compare also Fig. 7 in the main text.
Figure S5. Summary of Zn\textsuperscript{II} release data for E\textsubscript{c}-1, \(\gamma\)-E\textsubscript{c}-1, and \(\beta\text{E}-E\text{c}-1\) at equilibrium under control and oxidizing conditions (1 mM GSH/4.5 mM GSSG) as well as upon proteolytic cleavage with trypsin. To allow better comparison with the full-length E\textsubscript{c}-1 protein, the sum of Zn\textsuperscript{II} release values of the individual domains is also displayed.