Electronic Supporting Information

Crosstalk of the structural and zinc buffering properties of mammalian metallothionein-2

Agnieszka Drozd,[‡a] Dominika Wojewska,[‡a] Manuel David Peris-Díaz, [a] Piotr Jakimowicz,[b] Artur Krężel[a]*

Contribution from

[a] *Department of Chemical Biology, Faculty of Biotechnology, University of Wrocław, F. Joliot-Curie 14a, 50-383 Wrocław, Poland*

[b] *Department of Protein Biotechnology, Faculty of Biotechnology, University of Wrocław, F. Joliot-Curie 14a, 50-383 Wrocław, Poland*

* corresponding author, E-mail: artur.krezel@uwr.edu.pl

‡ These authors contributed equally. Current address of A. Drozd: Captor Therapeutics Ltd Wrocław, Poland; D. Wojewska: Groningen Biomolecular Science and Biotechnology Institute (GBB), University of Groningen, Groningen, Netherlands.

*corresponding author

E-mail: artur.krezel@uwr.edu.pl

‡These authors contributed equally

Materials

$ZnSO_4 \cdot 7H_2O$, $NaClO_4 \cdot H_2O$, $(NH_4)_2CO_3$, hydrochloric acid, (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), D-fructose, β-nicotinamide adenine dinucleotide (NADH)**,** Tris base, tris(2-carboxyethyl)phosphine hydrochloride (TCEP), ethylenediaminetetraacetic acid (EDTA), iodoacetamide (IAA), dipicolinic acid, trifluoroacetic acid (TFA), thioanisole, anisole, 1,2-ethanedithiol (EDT), sorbitol dehydrogenase (SDH) from ship liver were purchased from Sigma-Aldrich. Trypton, yeast extract, LB broth, agar, agarose, isopropyl-β-D-1-thiogalactopyranoside (IPTG), SDS were from Lab Empire, NaCl, NaOH, glycerol, ethyl ether (Et_2O), $KH_2PO_4 \cdot H_2O$, K_2HPO_4 from POCH (Gliwice Poland), pTYB21 vector and chitin resin from New England BioLabs, 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) from TCI Europe N.V., Chelex 100 resin from BioRad, and trypsin (mass spectrometry grade) from Promega GmbH. Fmoc-protected amino acids, piperidine, *N,N,N′,N′*-tetramethyl-*O*-(1*H*-benzotriazol-1-yl)uronium hexafluoro-phosphate (HBTU), *N,N*-dimethylformamide (DMF), dichloromethane (DCM), *N,N*-diisopropylethylamine (DIEA), 1-methyl-2-pyrrolidinone (NMP), and dithiothreitol (DTT), and acetonitrile (ACN) were purchased from Iris Biotech GmbH. All of the experiments were performed in chelexed buffers and solutions. All buffers were prepared with Milli-Q water obtained with deionizing water system (Merck Millipore, USA).

Primer sequences used in PCR reaction for the amplifaction of MT2 (MT2a) DNA fragment:

forward 5'-GGTGGTTGCTCTTCCAACATGGATCCGAACTGCAGCTGTGCGGC-3',

reverse 5'-CCACTATAGAATGCGCGTCGTCGACGTAAATAGCGAGC-3'.

Conditions for PCR reaction.

PCR reactions were performed using PHUSION polymerase, nucleoside triphosphates (dNTP), DNA 6X Loading Dye, GeneRuler 1 kb DNA Ladder, GeneRuler Low Range DNA Ladder) (Fermentas). Conditions for PCR reactions were:

Steps	Reaction	Temperature	Time
1	first denaturation	98°C	60 s
2	denaturation	98°C	10 s
3	annealing	52.6°C	30 s
4	elongation	72°C	3 s
30 cycles of steps 2-4			
5	final elongation	72°C	5 min
6	end of reaction	12°C	Inf.

Table S1. List of calculated and found *m/z* values of diversely modified, undigested MT2. Found values are averaged from all measured spectra. High measurement error due to the use of linear mode. Peaks with asterisk were found only once, so no standard deviation is given.

number of modifications	*m/z* calculated (average)	*m/z* found (average)
0	6043.3	6043.7 ± 1.3
1	6100.4	6100.3 ± 1.1
2	6157.4	6156.4 ± 0.7
3	6214.5	6212.7 ± 1.0
4	6271.5	6269.1 ± 0.7
5	6328.6	6326.9 ± 0.7
6	6385.6	6383.7 ± 0.5
7	6437.4	6440.8 ±1.1
8	6499.7	6498.0 ± 0.9
9	6556.8	6555.5 ± 0.6
10	6613.8	6611.6 ± 2.2
11	6670.9	6669.2 ± 1.3
12	6727.9	6726.0 ± 1.9
13	6785.0	6783.9 ± 1.6
14	6842.0	6841.1 ± 1.4
15	6899.1	6899.8 ± 1.3
16	6956.1	6956.9 ± 1.2
17	7013.2	7014.8 ± 0.6
18	7070.2	7073.0 ± 1.0
19	7127.3	7132.4*
20	7184.3	7188.9*

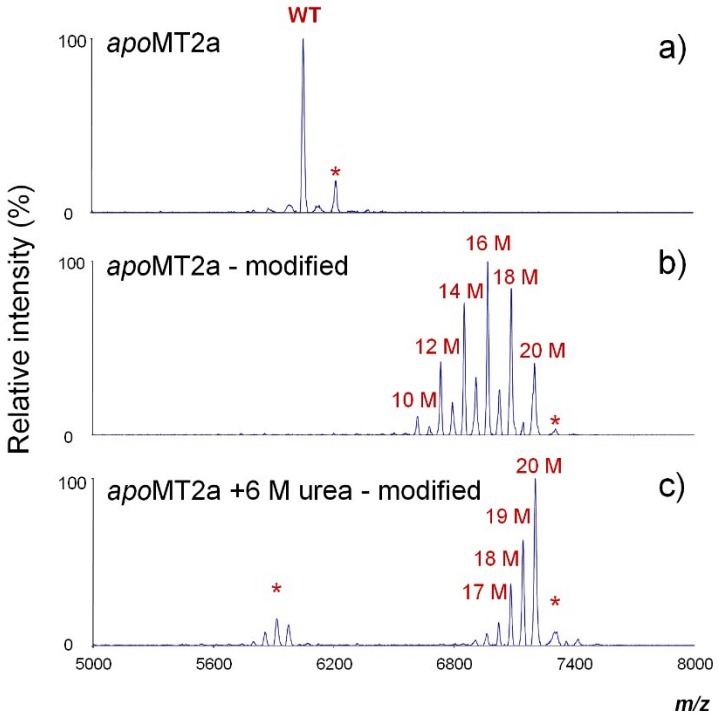

Figure S1. MALDI spectra of undigested *apo*-MT2 form. a) not modified control, b) modified with IAA, c) modified with IAA after incubation with 6 M urea. M stands for modification and the preceding number indicates the number of modified cysteines; unidentified signals are indicated with asterisk.

Table S2. All identified tryptic fragments in MS analysis for partially modified MT2, previously titrated with sequential molar equivalents of Zn(II). M refers to acetamide moiety (preceding number indicates the number of modified cysteines in the fragment); *n.d.* - not detected; ^{ox} - disulfide bridge (cysteine oxidation); ^{ox,ox} - occurrence of two disulfide bridges, $^{ox,\ ox,\ ox}$ - occurrence of three disulfide bridges. Yellow and green color demonstrate cysteine residues and cleavage site of trypsin, respectively.

apo-MT2

MDPNCSCAAGDSCTCAGSCK	CK	ECK	CTSCKK	SCCSCCPVGCAK	CAQGCICK	GASDKCSCCA
[1-20], 3M [1-20], 4M [1-20], 5M	*n.d.*	*n.d.*	[26-31], 1M	[32-43], 3M [32-43], 4M [32-43], 5M	[44-51], 2M	[52-61], 0M [52-61], 2M
		ECKCKCTSCKK				
		[23-31], 1M				

apo-MT2 + 6M urea

MDPNCSCAAGDSCTCAGSCK	CK	ECKCTSCKKSCCSCCPVGCAK	CAQGCICKGASDKCSCCA
[1-20]^{ox}, 1M [1-20]^{ox}, 2M [1-20], 2M [1-20]^{ox}, 3M [1-20], 3M [1-20], 5M	*n.d.*	[23-43], 8M	[44-61], 5M [44-61], 6M
		CTSCKKSCCSCCPVGCAK	
		[26-43], 6M [26-43], 7M	
		SCCSCCPVGCAK	
		[32-43], 4M [32-43], 5M	

apo-MT2 + 1 eq. Zn(II)

MDPNCSCAAGDSCTCAGSCK	CK	ECK	CTSCKK	SCCSCCPVGCAK	CAQGCICK	GASDKCSCCA
[1-20], 3M [1-20], 4M [1-20], 5M	*n.d.*	*n.d.*	[26-31], 2M	[32-43], 2M [32-43], 3M [32-43], 4M [32-43], 5M	[44-51], 2M	[52-61], 2M

apo-MT2 + 2 eq. Zn(II)

MDPNCSCAAGDSCTCAGSCK	CKECK	CTSCKK	SCCSCCPVGCAK	CAQGCICK	GASDKCSCCA
[1-20], 2M [1-20], 3M [1-20], 4M [1-20], 5M	*n.d.*	[26-31], 1M	[32-43], 1M [32-43], 2M [32-43], 3M [32-43], 4M [32-43], 5M	[44-51], 1M [44-51], 2M [44-51], 3M	[52-61], 1M [52-61], 2M
	CKECKCTSCKKSCCSCCPVGCAK				
	[21-43], 2M				

apo-MT2 + 3 eq. Zn(II)

MDPNCSCAAGDSCTCAGSCK	CKECK	CTSCKK	SCCSCCPVGCAK	CAQGCICK	GASDKCSCCA
[1-20], 5M	*n.d.*	[26-31], 1M	[32-43]^{OX}, 1M [32-43], 1M [32-43]^{OX}, 2M [32-43], 2M [32-43]^{OX}, 3M [32-43], 3M [32-43], 4M [32-43], 5M	[44-51]^{OX}, 1M [44-51], 1M [44-51], 2M	[52-61], 0M [52-61], 1M [52-61], 2M
	CKECKCTSCKKSCCSCCPVGCAK				
	[21-43], 1M [21-43], 2M				

apo-MT2 + 4 eq. Zn(II)

MDPNCSCAAGDSCTCAGSCK	CKECKCTSCK	SCCSCCPVGCAK	CAQGCICK	GASDKCSCCA
[1-20], 3M	*n.d.*	[32-43]$^{OX, OX}$, 0M [32-43]^{OX}, 0M [32-43]^{OX}, 1M [32-43], 1M [32-43]^{OX}, 2M [32-43], 2 M [32-43]^{OX}, 3M [32-43], 3M [32-43], 4M [32-43], 5M	[44-51]^{OX}, 0M [44-51], 0M [44-51]^{OX}, 1M [44-51], 1M [44-51], 2M	[52-61]^{OX}, 0M [51-61], 0M
		KSCCSCCPVGCAK	CAQGCICKGASDK	
		[31-43]^{ox}, 0M [31-43], 0M	[44-56]^{ox}, 1M	

	[31-43]^{ox}, 1M		
		CAQGCICKGASDKCSCCA	
		[44-61]^{ox,ox,ox}, 0M	

apo-MT2 + 5 eq. Zn(II)

MDPNCSCAAGDSCTCAGSCK	CKECK	CTSCKK	SCCSCCPVGCAK	CAQGCICK	GASDKCSCCA
[1-20]$^{ox, ox}$, 0M [1-20]^{ox,} 0M [1-20], 0M [1-20]^{ox}, 1M [1-20], 1M [1-20]^{ox}, 2M [1-20], 2M	*n.d.*	[26-31], 1M	[32-43]$^{ox, ox}$, 0M [32-43]^{ox}, 0M [32-43] , 0M [32-43]$^{ox, ox}$, 1M [32-43]^{ox}, 1M [32-43], 1M [32-43]^{ox}, 2M [32-43], 2M	[44-51]^{ox}, 0M [44-51] , 0M [44-51]^{ox}, 1M [44-51], 1M	[52-61]^{ox}, 0M [52-61] , 0M
			KSCCSCCPVGCAK	CAQGCICKGASDK	
			[31-43]$^{ox, ox}$, 0M [31-43]^{ox}, 0M	[44-56]^{ox}, 1M	
				CAQGCICKGASDKCSCCA	
				[44-61]$^{ox, ox, ox}$, 0M [44-61]$^{ox, ox}$, 0M	

apo-MT2 + 6 eq. Zn(II)

MDPNCSCAAGDSCTCAGSCK	CKECK	CTSCKKSCCSCCPVGCAK	CAQGCICKGASDKCSCCA	
[1-20]$^{ox, ox}$, 0M [1-20]^{ox}, 0M [1-20], 0M	*n.d.*	[26-43]$^{ox, ox, ox}$, 0M [26-43]$^{ox, ox}$, 0M [26-43]^{ox}, 0M [26-43], 0M	[44-61]$^{ox, ox, ox}$, 0M [44-61]$^{ox, ox}$, 0M [44-61]^{ox}, 0M [44-61], 0M	
		KSCCSCCPVGCAK	CAQGCICKGASDK	
		[31-43]$^{ox, ox}$, 0M [31-43] ^{ox}, 0M [31-43], 0M	[44-56]^{ox}, 0M	
		SCCSCCPVGCAK	CAQGCICK	GASDKCSCCA
		[32-43]$^{ox, ox}$, 0M [32-43]^{ox}, 0M [32-43], 0M	[44-51]^{ox}, 0M	[52-61]^{ox}, 0M

apo-MT2 + 7 eq. Zn(II)

MDPNCSCAAGDSCTCAGSCK	CKECKCTSCK	SCCSCCPVGCAK	CAQGCICKGASDKCSCCA

[1-20]^{ox}, 0M [1-20], 0M	*n.d.*	[32-43] $^{ox, ox}$, 0M [32-43]^{ox}, 0M [32-43], 0M	[44-61]$^{ox, ox, ox}$, 0M [44-61]$^{ox, ox}$, 0M [44-61]^{ox}, 0M
MDPNCSCAAGDSCTCAGSCKCK [1-22]^{ox}, 1M [1-22], 1M		KSCCSCCPVGCAK [31-43]$^{ox, ox}$, 0M [31-43]^{ox}, 0M	CAQGCICK [44-51]^{ox}, 0M
			CAQGCICKGASDK [44-56]^{ox}, 0M [44-56], 0M

Table S3. Full list of calculated and found *m/z* of all tryptic fragments from MT2, found in all samples. M - carbamidomethylated cysteine (preceding number indicates the number of modified cysteines in the fragment); Asterisk - peptide found only once; ^{ox} - occurrence of disulfide bridge (cysteine oxidation); ^{ox,ox} - occurrence of two disulfide bridges; ^{ox,ox,ox} - occurrence of three disulfide bridges. Yellow and green color demonstrate cysteine residues and cleavage site of trypsin, respectively.

fragment	sequence	*m/z* calculated	*m/z* found
[1-20]$^{ox, ox}$, 0M	MDPNCSCAAGDSCTCAGSCK	1919.62	1919.61 ± 0.02
[1-20]^{ox}, 0M	MDPNCSCAAGDSCTCAGSCK	1921.63	1921.63 ± 0.02
[1-20], 0M	MDPNCSCAAGDSCTCAGSCK	1923.65	1923.65 ± 0.02
[1-20]^{ox}, 1M	MDPNCSCAAGDSCTCAGSCK	1978.65	1978.65 ± 0.01
[1-20], 1M	MDPNCSCAAGDSCTCAGSCK	1980.67	1980.70*
[1-20]^{ox}, 2M	MDPNCSCAAGDSCTCAGSCK	2035.67	2035.69 ± 0.02
[1-20], 2M	MDPNCSCAAGDSCTCAGSCK	2037.69	2037.69 ± 0.02
[1-20]^{ox}, 3M	MDPNCSCAAGDSCTCAGSCK	2092.70	2092.77 ± 0.05
[1-20], 3M	MDPNCSCAAGDSCTCAGSCK	2094.71	2094.71 ± 0.04
[1-20], 4M	MDPNCSCAAGDSCTCAGSCK	2151.73	2151.74 ± 0.01
[1-20], 5M	MDPNCSCAAGDSCTCAGSCK	2208.75	2208.75 ± 0.01
[1-22]^{ox}, 0M	MDPNCSCAAGDSCTCAGSCKCK	2152.74	2152.74*
[1-22]^{ox}, 1M	MDPNCSCAAGDSCTCAGSCKCK	2209.76	2209.85 ± 0.01
[1-22], 1M	MDPNCSCAAGDSCTCAGSCKCK	2211.77	2211.85*
[21-43], 2M	CKECKCTSCKKSCCSCCPVGCAK	2052.83	2052.76 ± 0.02
[23-30]^{ox}, 0M	ECKCKCTSCK	899.34	899.35*
[23-30]^{ox}, 1M	ECKCKCTSCK	956.36	956.37*
[23-31], 1M	ECKCKCTSCKK	1086.47	1086.47*
[23-43], 8M	ECKCTSCKKSCCSCCPVGCAK	2627.01	2627.02*
[26-30], 0M	CTSCK	541.21	541.22*
[26-31], 0M	CTSCKK	669.31	669.31*
[26-31], 1M	CTSCKK	726.33	726.34 ± 0.00
[26-31], 2M	CTSCKK	783.35	783.36*
[26-43]$^{ox, ox, ox}$, 0M	CTSCKKSCCSCCPVGCAK	1804.64	1804.65 ± 0.02
[26-43]$^{ox, ox}$, 0M	CTSCKKSCCSCCPVGCAK	1806.66	1806.65 ± 0.01
[26-43]^{ox}, 0M	CTSCKKSCCSCCPVGCAK	1808.67	1808.68*

[26-43], 0M	CTSCKKSCCSCCPVGCAK	1810.69	1810.68*
[26-43], 6M	CTSCKKSCCSCCPVGCAK	2152.82	2152.84 ± 0.01
[26-43], 7M	CTSCKKSCCSCCPVGCAK	2209.84	2209.86 ± 0.01
[31-43]$^{ox, ox}$, 0M	KKSCCSCCPVGCAK	1284.47	1284.46 ± 0.02
[31-43]^{ox}, 0M	KKSCCSCCPVGCAK	1286.48	1286.47 ± 0.03
[31-43], 0M	KKSCCSCCPVGCAK	1288.50	1288.52 ± 0.02
[32-43]$^{ox, ox}$, 0M	KSCCSCCPVGCAK	1156.37	1156.37 ± 0.01
[32-43]^{ox}, 0M	KSCCSCCPVGCAK	1158.39	1158.38 ± 0.01
[32-43], 0M	KSCCSCCPVGCAK	1160.40	1160.41 ± 0.01
[32-43]^{ox}, 1M	KSCCSCCPVGCAK	1215.41	1215.41 ± 0.02
[32-43], 1M	KSCCSCCPVGCAK	1 217.42	1217.42 ± 0.02
[32-43]^{ox}, 2M	KSCCSCCPVGCAK	1272.43	1272.43 ± 0.02
[32-43], 2M	KSCCSCCPVGCAK	1274.45	1274.45 ± 0.02
[32-43]^{ox}, 3M	KSCCSCCPVGCAK	1329.45	1329.46 ± 0.02
[32-43], 3M	KSCCSCCPVGCAK	1331.47	1331.47 ± 0.01
[32-43], 4M	KSCCSCCPVGCAK	1388.49	1388.49 ± 0.01
[32-43], 5M	KSCCSCCPVGCAK	1445.51	1445.51 ± 0.02
[44-51]^{ox}, 0M	CAQGCICK	823.33	823.33 ± 0.02
[44-51], 0M	CAQGCICK	825.34	825.34 ± 0.01
[44-51]^{ox}, 1M	CAQGCICK	880.35	880.35 ± 0.02
[44-51], 1M	CAQGCICK	882.36	882.37 ± 0.01
[44-51], 2M	CAQGCICK	939.39	939.39 ± 0.01
[44-56]^{ox}, 0M	CAQGCICKGASDK	1281.54	1281.55 ± 0.01
[44-56], 0M	CAQGCICKGASDK	1283.55	1283.54*
[44-56]^{ox}, 1M	CAQGCICKGASDK	1338.56	1338.56 ± 0.02
[44-61]$^{ox, ox, ox}$, 0M	CAQGCICKGASDKCSCCA	1744.60	1744.60 ± 0.02
[44-61]$^{ox, ox}$, 0M	CAQGCICKGASDKCSCCA	1746.62	1746.61 ± 0.02
[44-61]^{ox}, 0M	CAQGCICKGASDKCSCCA	1748.64	1748.56 ± 0.13
[44-61], 0M	CAQGCICKGASDKCSCCA	1750.65	1750.64*
[44-61], 5M	CAQGCICKGASDKCSCCA	2035.76	2035.74 ± 0.00
[44-61], 6M	CAQGCICKGASDKCSCCA	2092.78	2092.77 ± 0.01
[52-61]^{ox}, 0M	GASDKCSCCA	942.31	942.31 ± 0.02
[52-61], 0M	GASDKCSCCA	944.33	944.33 ± 0.00
[52-61], 1M	GASDKCSCCA	1001.35	1001.35 ± 0.01
[52-61], 2M	GASDKCSCCA	1058.37	1058.37 ± 0.01

Table S4. The solvent-accessible surface area (SASA) for Cys residues in *apo*-MT2 obtained after 4 ns MD production. SASA was obtained from PDBePISA.

Cysteine residue number	ASA (Å^2)
5	59.49
7	12.12
13	14.52
15	53.90
19	39.88
21	67.05
24	33.29
26	99.71
29	120.33
33	19.31
34	30.86
36	80.54
37	47.85
41	31.42
44	79.25
48	87.87
50	70.32
57	13.26
59	98.33
60	95.75

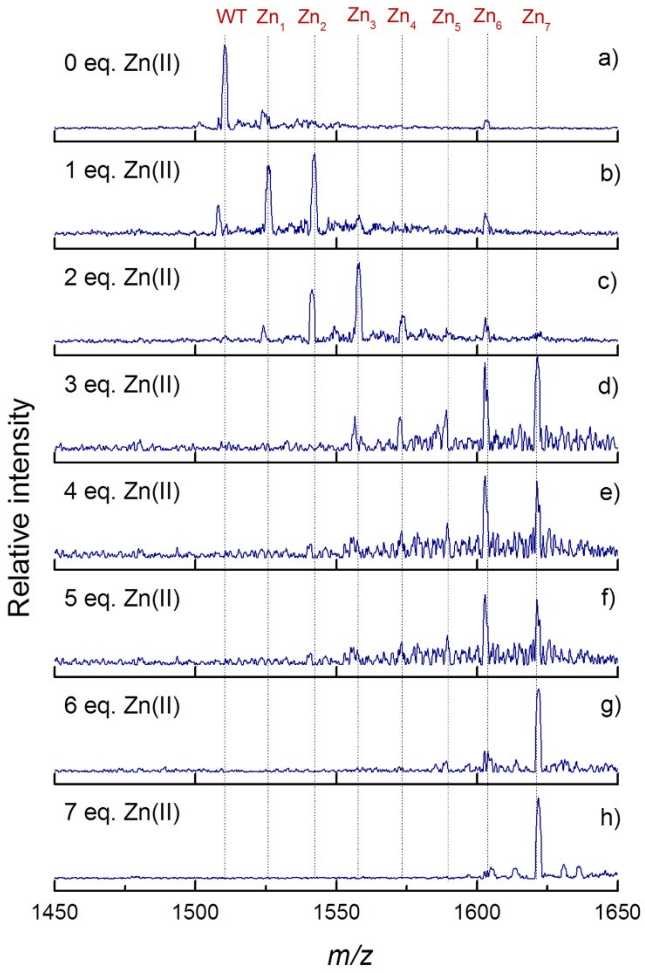

Figure S2. Comparison of ESI-MS spectra of *apo*-MT2 titrated with sequential 0-7 eq of Zn(II) (a-h). All shown signals have +4 charge. Found and calculated m/z are listed in Table S2.

Table S5. List of calculated and found *m/z* values for *apo*-MT2 (signal +4) titrated with Zn(II) ions on ESI-MS spectrometer.

number of bound Zn(II)	coordination mode used for calculation	*m/z* calculated (average)	*m/z* found
0	-	1511.6	1510.0 ± 1.7
1	ZnS_4	1526.9	1524.7 ± 1.2
2	$2 \times ZnS_4$	1542.2	1541.8 ± 0.5
3	Zn_3S_9	1558.3	1557.2 ± 1.0
4	Zn_4S_{11}	1574.2	1573.2 ± 0.6
5	Zn_5S_{15}	1589.5	1589.2 ± 0.2
6	Zn_6S_{19}	1604.1	1603.5 ± 0.9
7	Zn_7S_{20}	1621.0	1621.7 ± 0.2

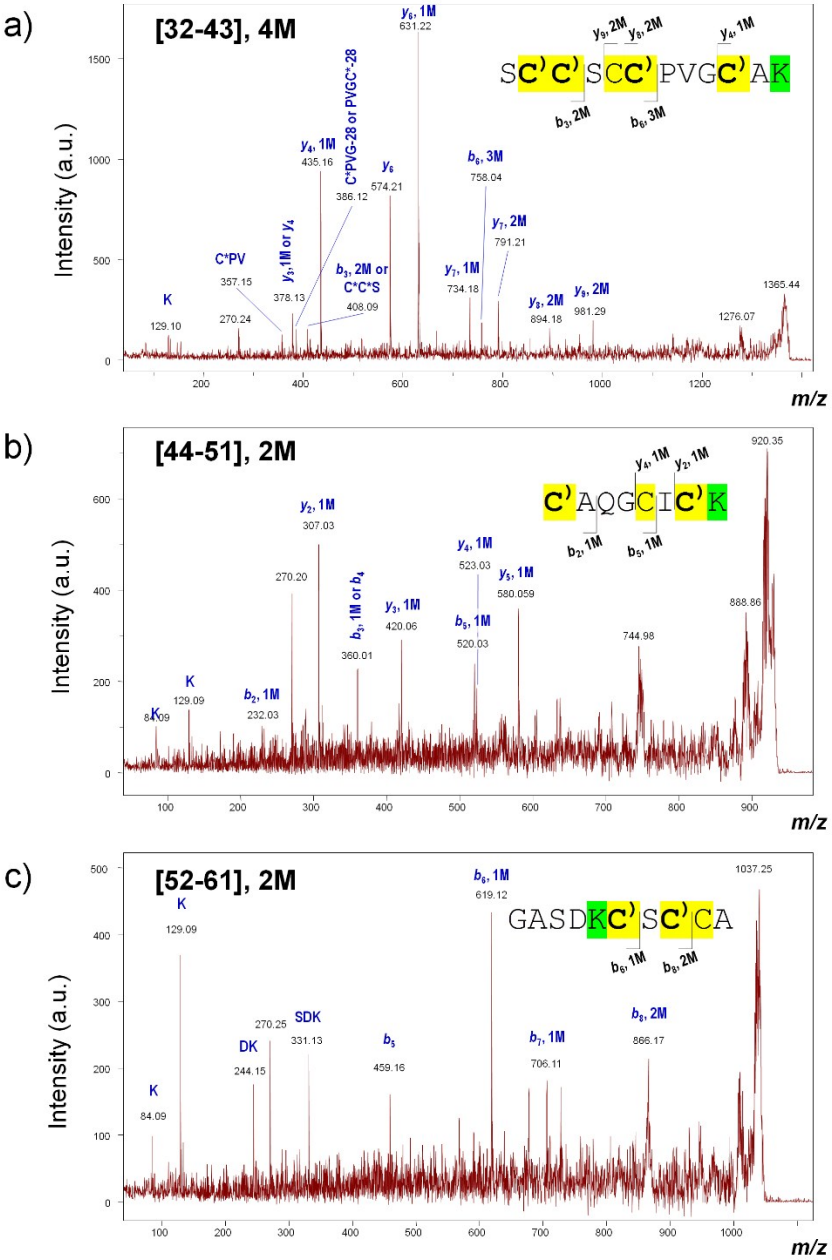

Figure S3. MS/MS spectra of selected tryptic fragments of MT2 with 1 eq. Zn(II) modified with IAA. a) MS/MS of tryptic fragment [32-43], 4M (calculated 1388.49 *m/z*, found 1388.43 *m/z*). Obtained daughter ions localize the modifications on C33, C34, C37 and C41. C36 is not modified. b) MS/MS of tryptic peptide [44-51], 2M (calculated 939.38 *m/z*, found 939.40 *m/z*). Fragment ions localize the modifications on C44 and C50, while C48 is not modified. c) MS/MS of tryptic fragment [52-61], 2M (calculated 1058.37 *m/z*, found 1058.38 *m/z*). Modified are C57 and C59, while C60 is not modified. M stands for carbamidomethylation and preceding number indicates the number of modified cysteines in given fragment. **C)** indicates carbamidomethylated cysteine, C is unmodified cysteine.

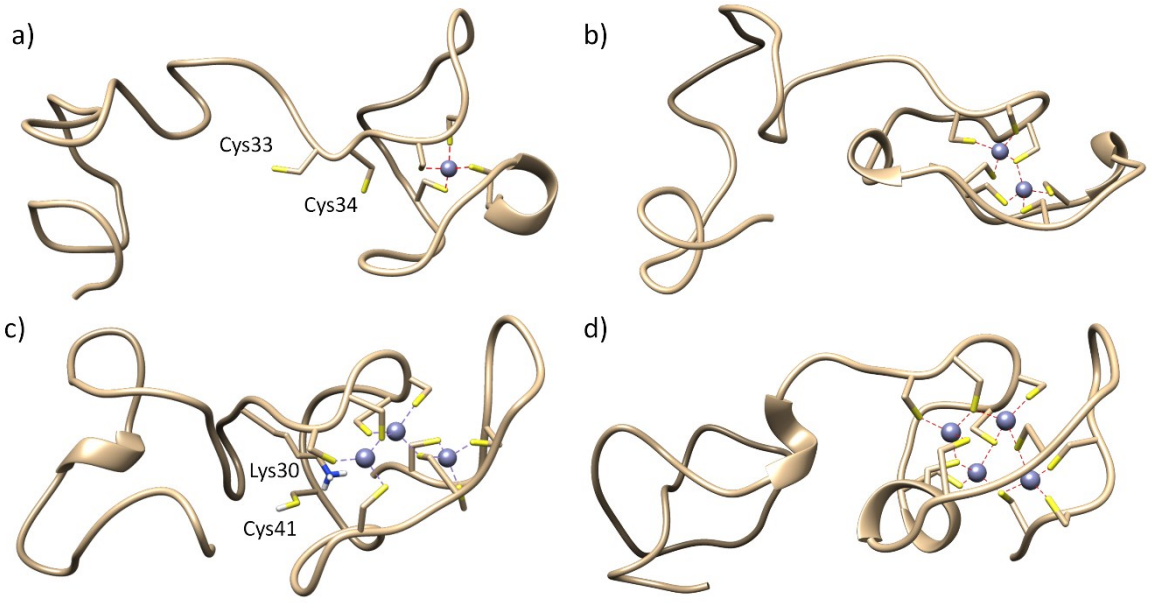

Figure S4. Model structures of $Zn_{0\text{-}4}$MT2 obtained from molecular dynamics simulations. a) Zn_1MT2, b) Zn_2MT2, c) Zn_3MT2 and d) Zn_4MT2 species.

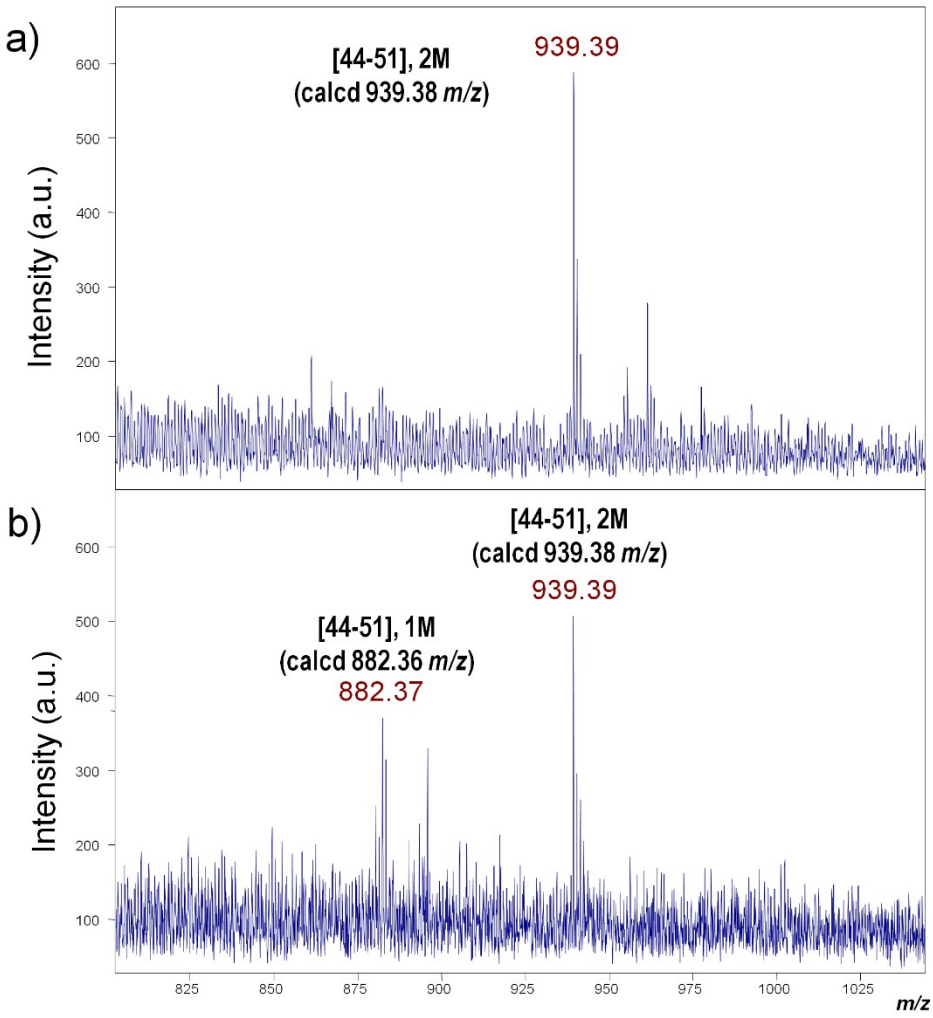

Figure S5. MALDI-MS spectra for two selected spots of nano-LC separation of tryptic digest, sample titrated with 2 eq. Zn(II). a), b) Fragment [44-51], 2M is more abundant on both spots than fragment [44-51], 1M.

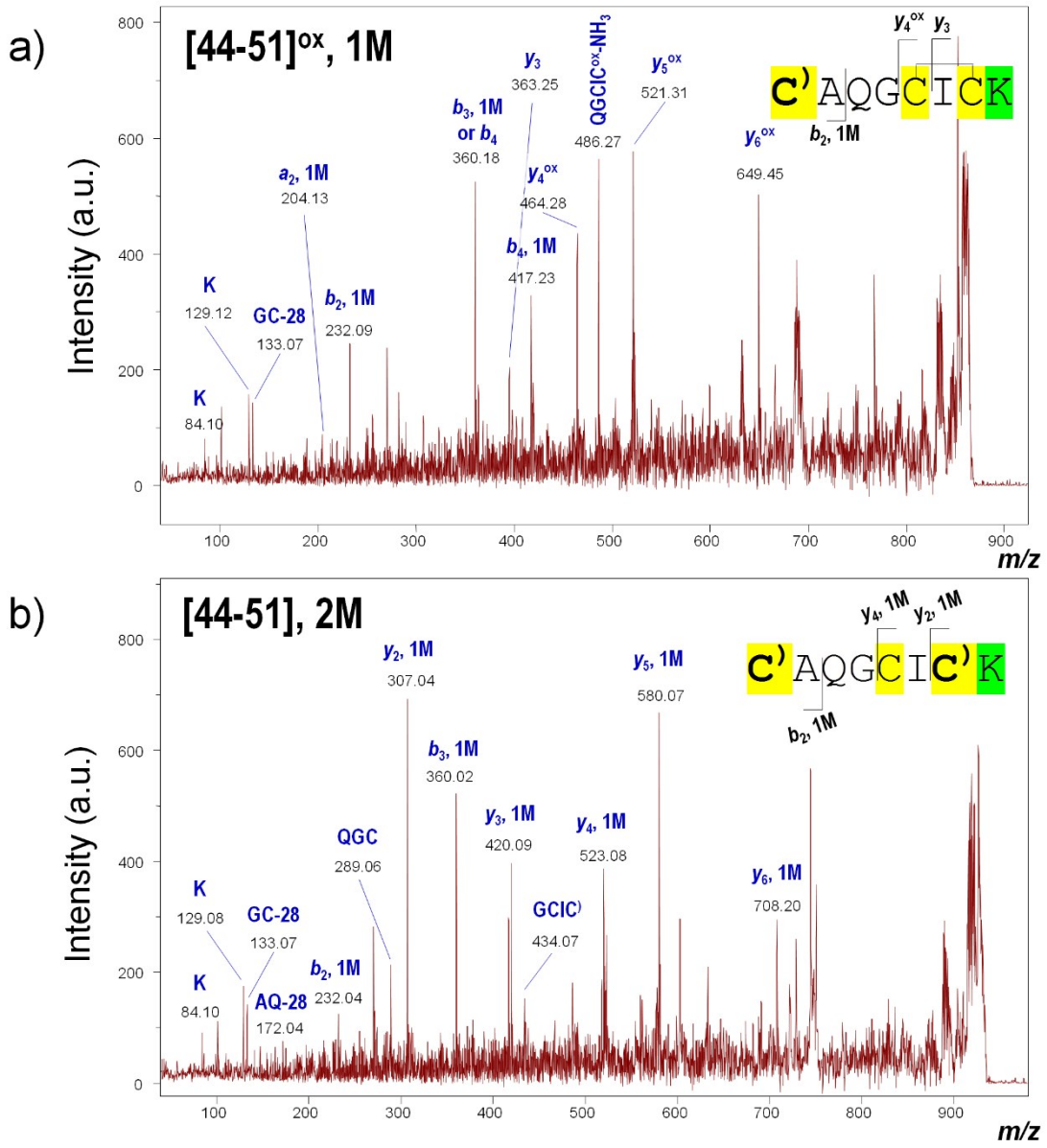

Figure S6. MS/MS spectra of [44-51] tryptic peptides of MT2a sample with 2 eq. Zn(II), modified with IAA. a) MS/MS for tryptic fragment [44-51]^{ox}, 1M (calculated 880.34 *m/z*, found 880.36 *m/z*). C44 is modified, while C48 and C50 are forming disulfide bridge. b) MS/MS for tryptic peptide [44-51], 2M (calculated 939.38 *m/z*, found 939.40 *m/z*). Fragment ions localize the modifications on C44 and C50, while C48 is not modified. M stands for acetamide moiety and preceding number indicates the number of modified cysteines in given fragment. **C)** indicates modified cysteine, C is unmodified cysteine. All spectra were recorded in LIFT mode.

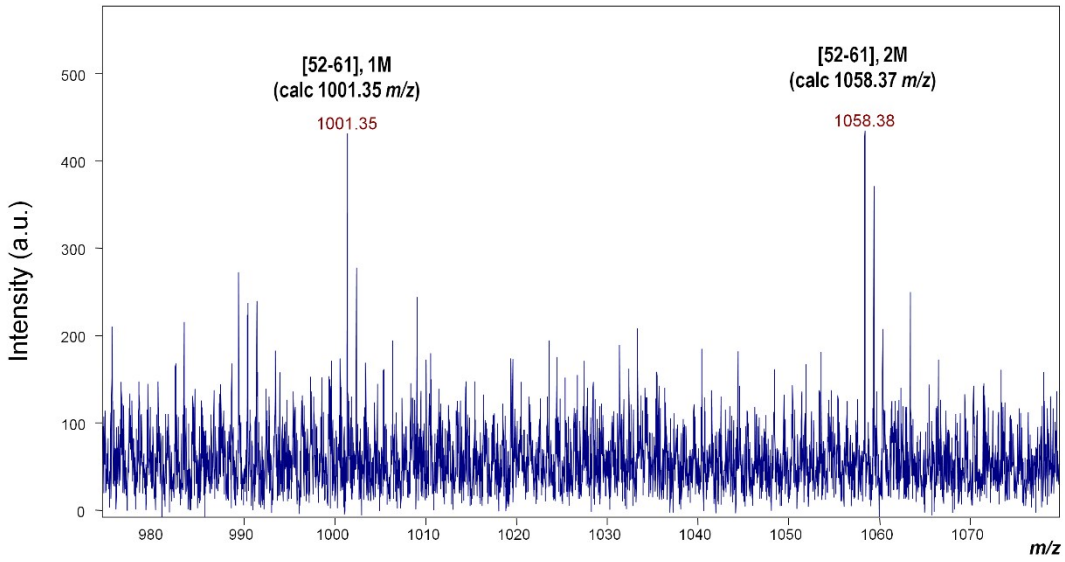

Figure S7. MALDI-MS spectrum of separated tryptic digest of MT2 with 3 eq. Zn(II), single spot in 970-1080 *m/z* range. Fragment [52-61] exists with one and two modified cysteines simultaneously.

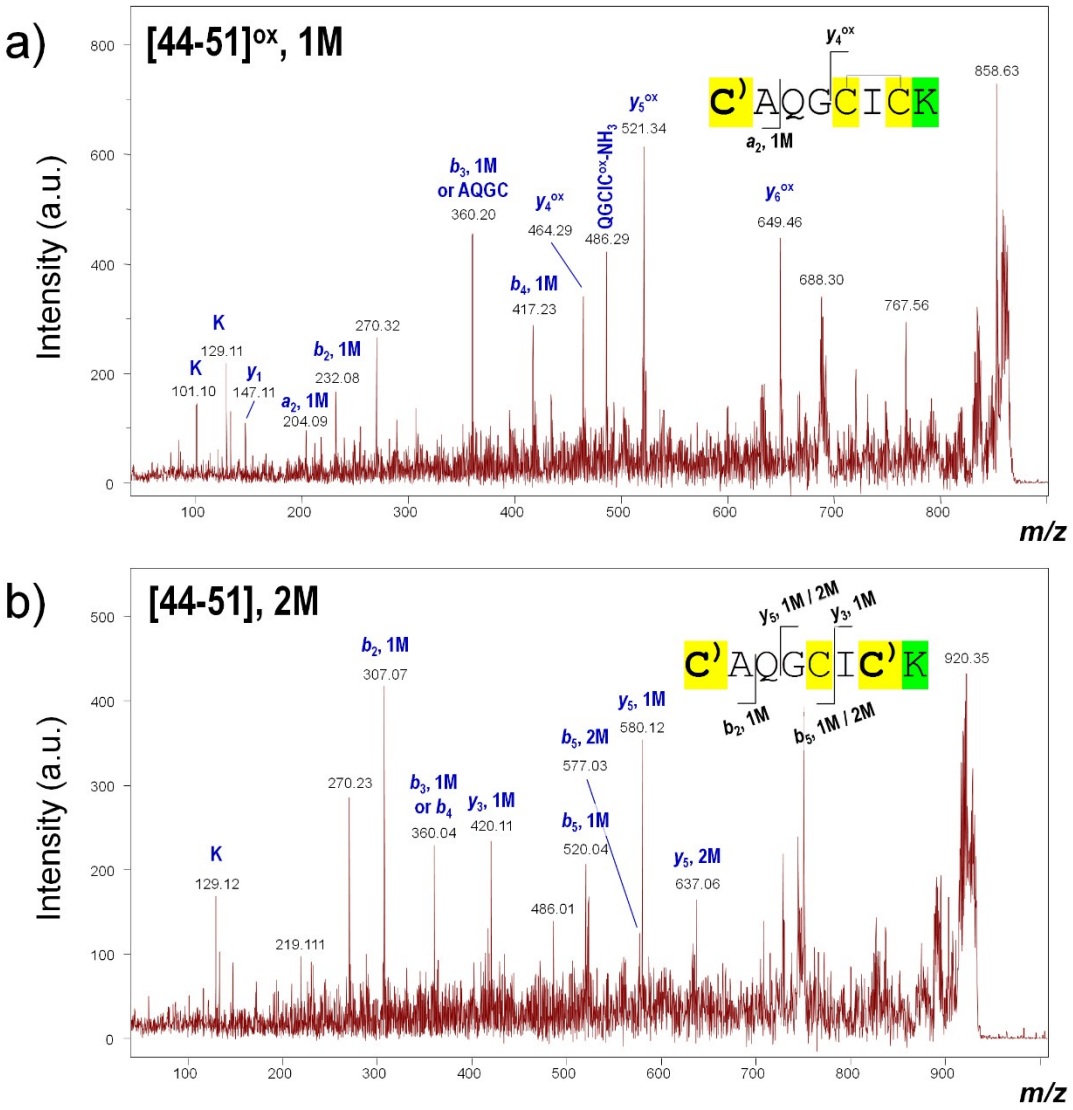

Figure S8. MS/MS spectra of [44-51] tryptic peptides of MT2 sample with 3 eq. Zn(II), modified with IAA. a) MS/MS for tryptic fragment [44-51]^{ox}, 1M (calculated 880.34 *m/z*, found 880.36 *m/z*). C44 is modified, while C48 and C50 are forming disulfide bridge (the same is true for 2 eq Zn^{2+}). b) MS/MS for tryptic peptide [44-51], 2M (calculated 939.38 *m/z*, found 939.40 *m/z*). Fragment ions localize the modifications on C44 and C50, but also on C48. It should be the result of co-existence of several isoforms. However, C48 shows a slight preference to be unmodified. M stands for acetamide moiety and preceding number indicates the number of modified cysteines in given fragment. **C)** indicates modified cysteine, C is unmodified cysteine. All spectra were recorded in LIFT mode.

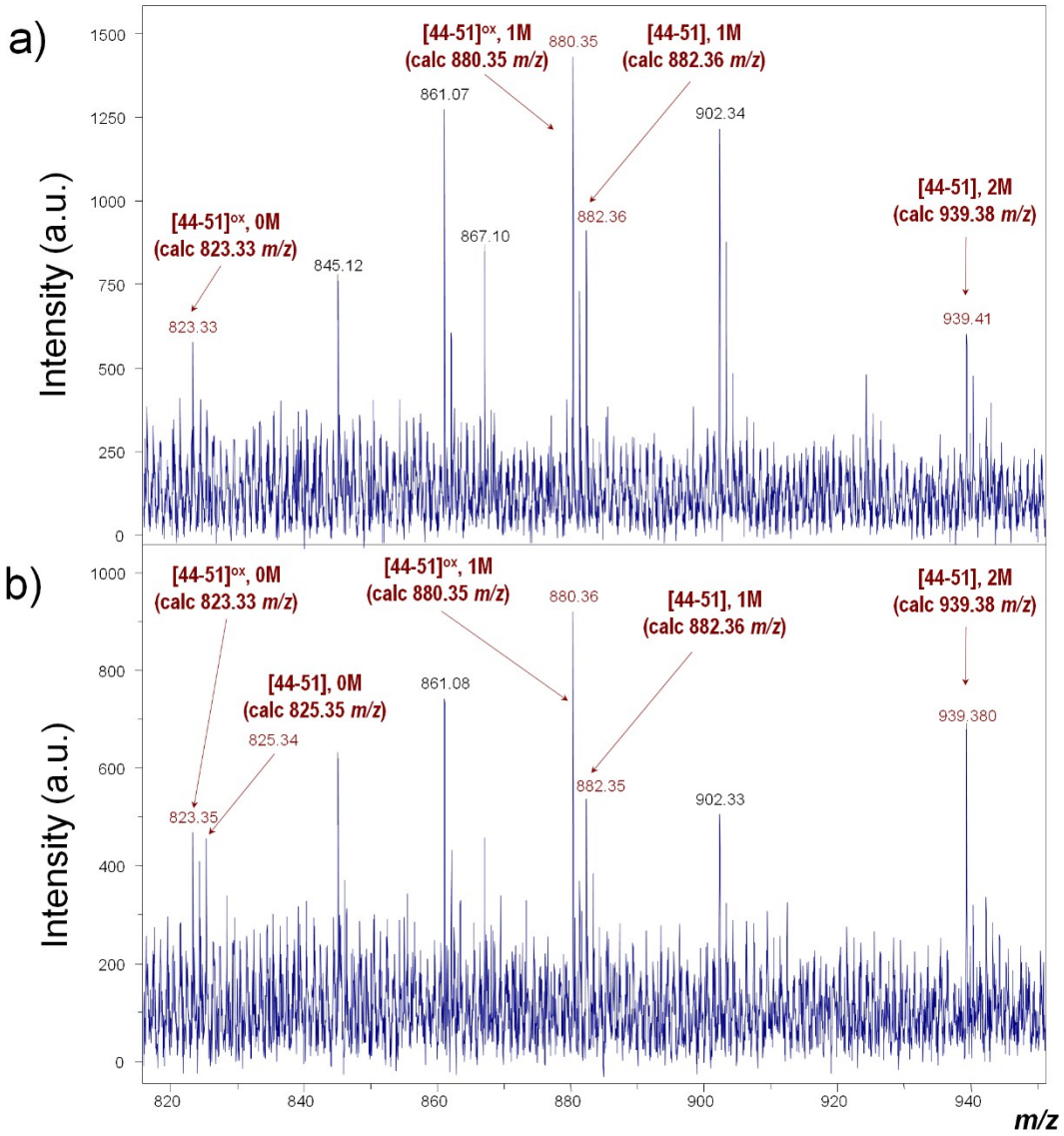

Figure S9. MALDI-MS spectra of nano-LC separated tryptic digest, sample titrated with 4 eq. Zn(II), two different spots. a) spot one, 815-950 *m/z* range; b) spot two, 815-950 *m/z* range. Tryptic fragment [44-51] is visible with zero, one and two modified cysteines at the same time.

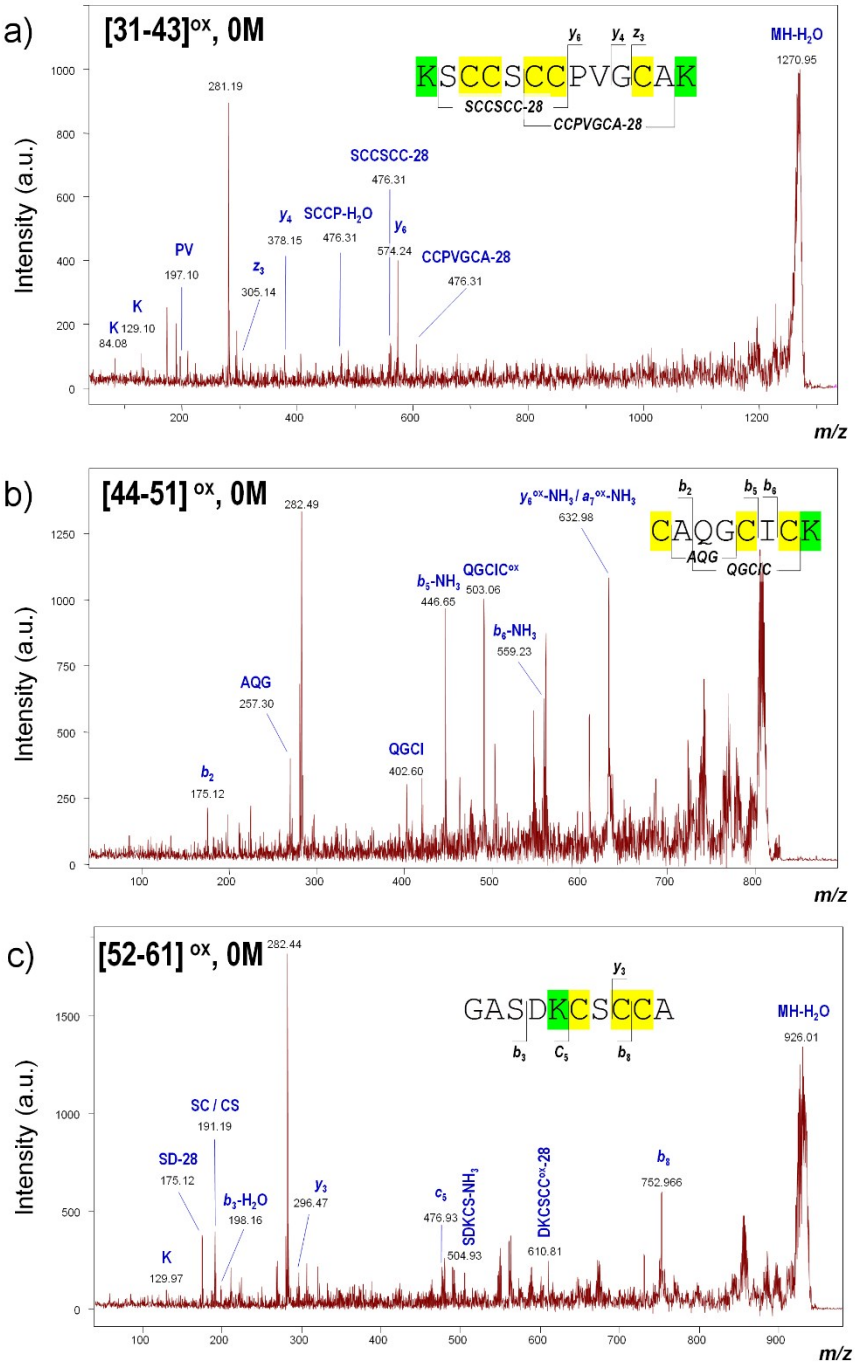

Figure S10. MS/MS spectra of MT2 sample with 6 eq. Zn(II) prove that there is no modification in amino acid region 31-61. a) MS/MS for [31-43]^{ox}, 0 M (calculated 1286.48 *m/z*, found 1286.48 *m/z*), b) MS/MS for [44-51]^{ox}, 0 M calculated 823.33 *m/z*, found 823.36 *m/z*), c) MS/MS for [52-61]^{ox}, 0 M calculated 942.31 *m/z*, found 942.29 *m/z*). All spectra were recorded in LIFT mode.

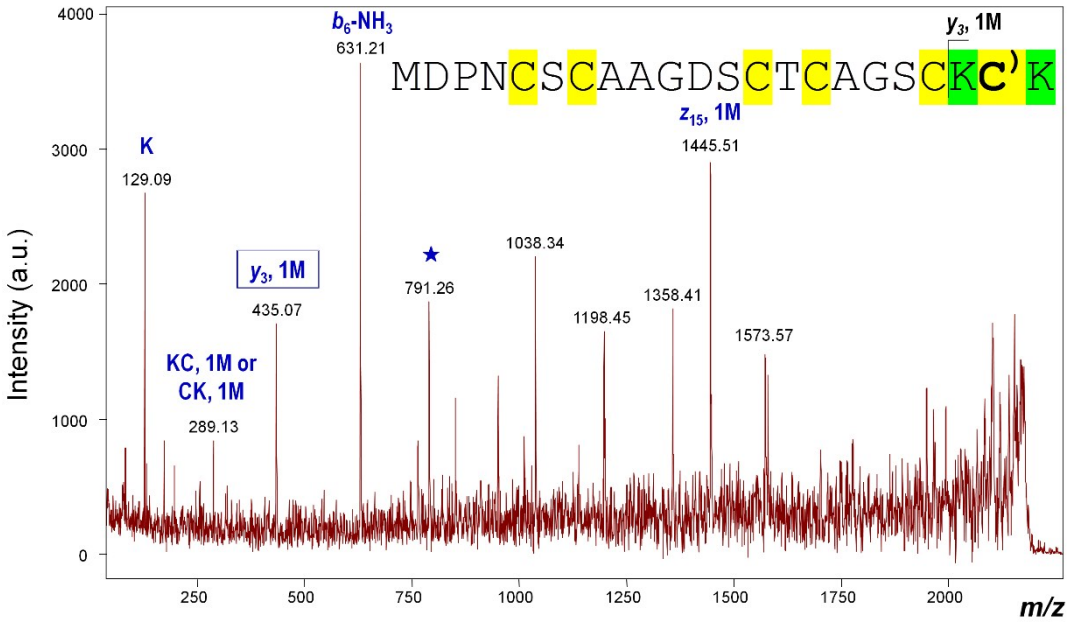

Figure S11. MS/MS spectrum of tryptic peptide [1-22]^{ox}, 1M (calculated 2209.76 *m/z*, found 2209.85 *m/z*) detected in MT2 with 7 eq. Zn(II). Found fragment ions localize the modification on C21. Asterisk indicates the fragment for which four different sequences can be attributed: DPNCSCAAG-28, PNCSCAAGD-28, TCAGSCGC')-H_2O, NCSCAAGDS-H_2O. 1 M stands for single modification. C') indicates modified cysteine, C is unmodified cysteine. Spectrum was recorded in LIFT mode.

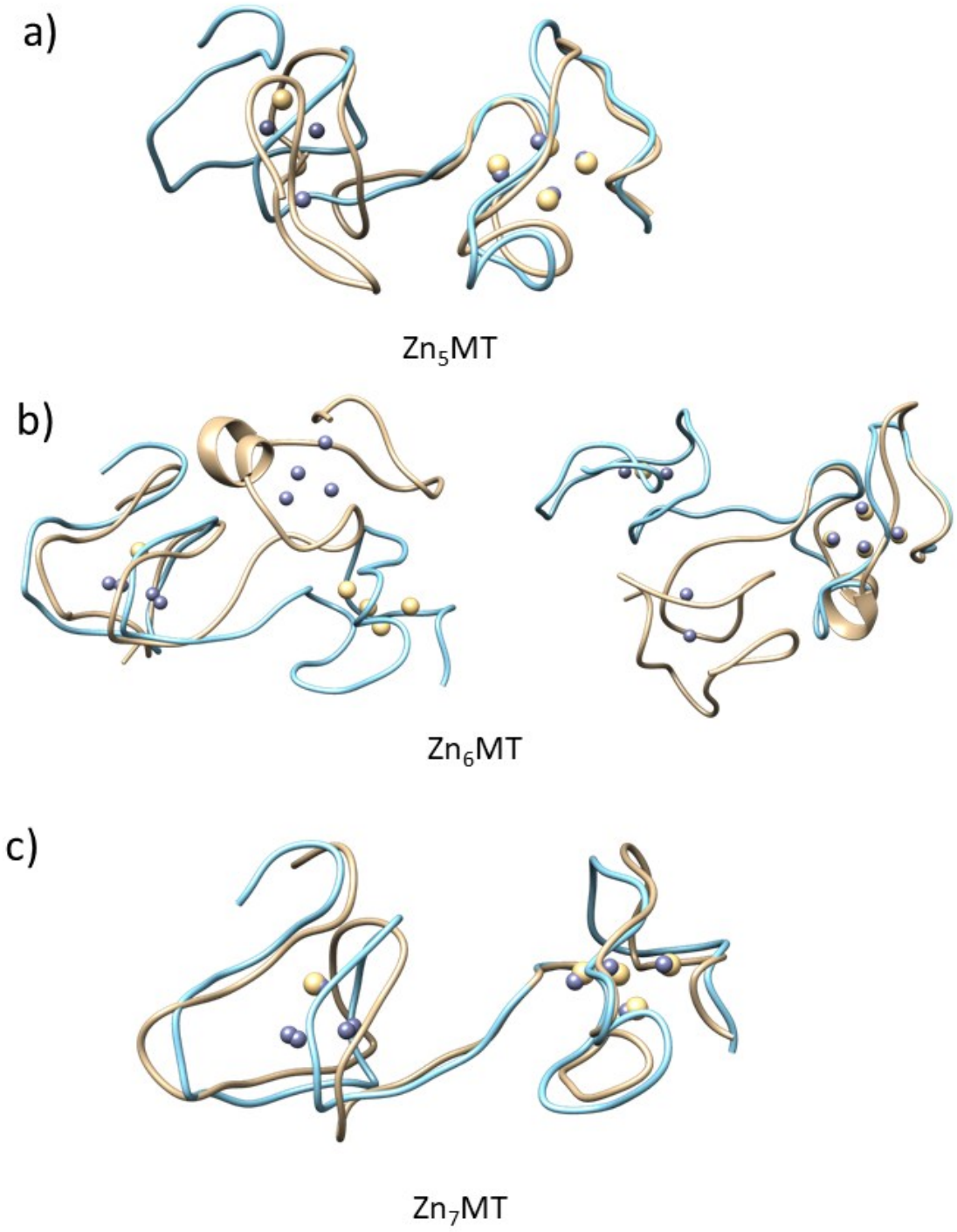

Figure S12. Comparison of MD-based and X-ray (PDB ID: 4MT2) structures. Blue ribbon represents X-ray MT2 and brown ribbon MD structure. Zn(II) is represented by grey balls, and Cd(II) by yellow balls. a) Comparison of α domain for Zn_5MT2a. b) Comparison of β- and α-domain for Zn_6MT. c) Comparison of Zn_7MT obtained through MD and X-ray structure.

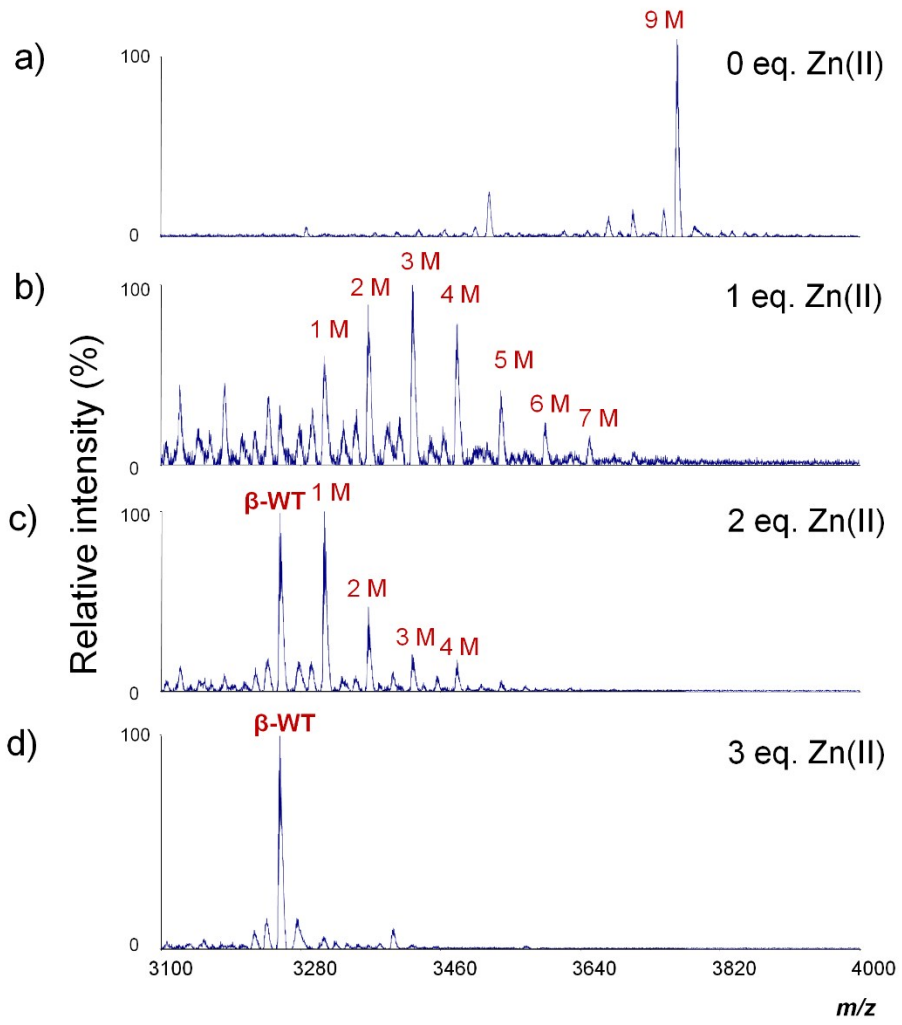

Figure S13. Comparison of MALDI-MS spectra of undigested, differentially modified samples, previously treated with 0-3 eq. of Zn(II) over *apo*βMT2a (a-d). M denotes acetamide moiety and preceding number indicates the number of modified cysteines in the fragment; β-WT – unmodified βMT2 domain. See main text for details. List of calculated and found *m/z* values can be found in Table S5.

Table S6. List of calculated and found *m/z* values for diversely modified βMT2. Found values are averaged from all measured spectra. High measurement error due to the use of linear mode. Peaks with asterisk were detected only once, so no standard deviation is given; - peak not detected.

number of modifications	*m/z* calculated (monoisotopic)	*m/z* found (monoisotopic)
0	3251.2	3251.2 ± 0.0
1	3308.3	3308.3 ± 0.0
2	3365.3	3365.3 ± 0.0
3	3422.3	3422.6 ± 0.5
4	3480.3	3479.3 ± 0.0
5	3537.3	3536.4*
6	3594.3	3596.4*
7	3651.4	3650.4*
8	3708.4	-
9	3765.4	3764.4*

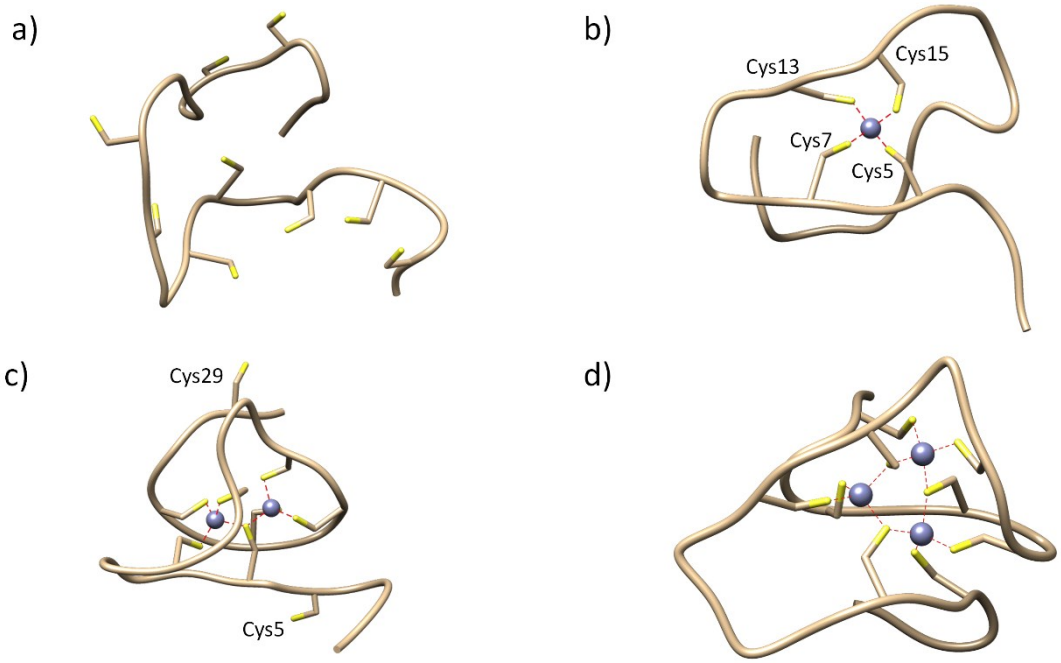

Figure S14. Model MD-based structures of isolated β-domain of MT2a with 0-3 Zn(II) ions. a) *apo*-βMT2, b) Zn_1βMT2, c) Zn_2βMT2, d) Structure for Zn_3βMT2.

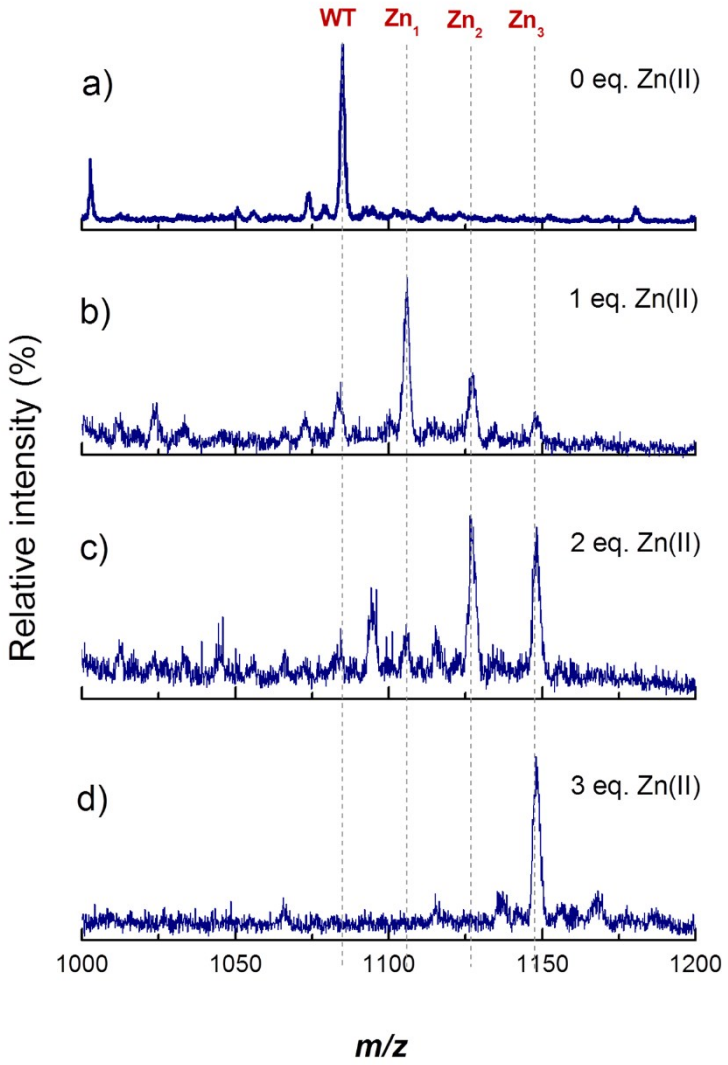

Figure S15. Comparison of ESI-MS spectra (peak +3) of *apo*-βMT2 titrated with 0-3 eq. of Zn(II) (a-d). List of calculated and found *m/z* values can be found in Table S6.

Table S7. List of calculated and found *m/z* values of βMT2a titrated with Zn(II) on ESI mass spectrometer, signal +3.

number of bound Zn(II)	coordination mode used for calculation	*m/z* calculated (average)	*m/z* found
0	-	1085.3	1084.6 ± 0.3
1	ZnS_4	1105.7	1105.8 ± 0.2
2	2 × ZnS_4	1126.2	1127.0 ± 0.5
3	Zn_3S_9	1147.7	1148.1 ± 0.3

Table S8. All identified tryptic fragments of βMT2 titrated with 0-3 eq. of Zn(II). M denotes acetamide moiety (preceding number indicates the number of modified cysteines in the fragment); * - peptide found only once; ^{ox} – occurrence of disulfide bridge (cysteine oxidation); $^{ox,\ ox}$ - occurrence of two disulfide bridges; ^{ox,ox,ox} - occurrence of three disulfide bridges, C – cysteine residue, K - cleavage site of trypsin.

fragment	Sequence	*m/z* calculated	*m/z* found
[1-20]$^{ox,\ ox}$, 0M	MDPNCSCAAGDSCTCAGSCK	1919.6	1919.5 ± 0.0
[1-20]^{ox}, 0M	MDPNCSCAAGDSCTCAGSCK	1921.6	1921.6 ± 0.0
[1-20]^{ox}, 1M	MDPNCSCAAGDSCTCAGSCK	1978.7	1978.6 ± 0.1
[1-20]^{ox}, 2M	MDPNCSCAAGDSCTCAGSCK	2035.7	2035.6 ± 0.1
[1-20], 3M	MDPNCSCAAGDSCTCAGSCK	2094.7	2094.7 ± 0.0
[1-20], 4M	MDPNCSCAAGDSCTCAGSCK	2151.7	2151.8 ± 0.0
[1-20], 5M	MDPNCSCAAGDSCTCAGSCK	2208.8	2208.6*
[1-22], 1M	MDPNCSCAAGDSCTCAGSCKCK	2211.8	2211.6*
[1-25]^{ox,ox}, 1M	MDPNCSCAAGDSCTCAGSCKCKECK	2567.9	2567.8*
[1-25]^{ox,ox}, 2M	MDPNCSCAAGDSCTCAGSCKCKECK	2624.9	2624.7*
[1-25], 3M	MDPNCSCAAGDSCTCAGSCKCKECK	2686.0	2685.8 ± 0.2
[1-25], 4M	MDPNCSCAAGDSCTCAGSCKCKECK	2743.0	2742.0*
[1-25], 5M	MDPNCSCAAGDSCTCAGSCKCKECK	2800.0	2800.6*
[21-25]^{ox}, 0M	CKECK	608.3	608.2*
[21-25], 1M	CKECK	667.3	667.3 ± 0.0
[21-25], 2M	CKECK	724.3	724.3 ± 0.0
[21-30]^{ox}, 0M	CKECKCTSCK	1130.4	1130.4*
[21-31]^{ox}, 0M	CKECKCTSCKK	1258.5	1258.5 ± 0.0
[21-31]^{ox}, 1M	CKECKCTSCKK	1315.6	1315.5 ± 0.0
[21-31]^{ox}, 2M	CKECKCTSCKK	1372.6	1372.6 ± 0.0
[21-31], 3M	CKECKCTSCKK	1431.6	1431.6 ± 0.0
[21-31], 4M	CKECKCTSCKK	1488.6	1488.6 ± 0.0
[23-31], 3M	ECKCTSCKK	1200.5	1200.5*
[26-31], 2M	CTSCKK	783.3	783.3 ± 0.0

Table S9. All identified daughter ions in MALDI-MS/MS spectrum of undigested βMT2, 5 M, detected in sample treated with 1 eq. Zn(II). These fragments prove the modification on C19, C21, C24, C26, C29 residues (highlighted verses indicate the modification sites). $^{I-V}$ - 1-5 modifications, localization not known, **C** - cysteine.

fragment	sequence	*m/z* (calculated)	*m/z* (found)
y_9+2M	CKCTSCKKS-NH_2	1 100.5	1 100.3
y_9+3M	CKCTSCKKS-NH_2	1 157.5	1 157.2
y_{10}+3M	ECKCTSCKKS-NH_2	1 286.6	1 286.4
y_{11}+2M	KECKCTSCKKS-NH_2^{II}	1 357.6	1 357.1
y_{12}+1M	CKECKCTSCKKS-NH_2^{I}	1 403.6	1 403.2
y_{11}+3M	KECKCTSCKKS-NH_2	1 414.7	1 414.3
y_{12}+2M	CKECKCTSCKKS-NH_2^{II}	1 460.6	1 460.2
y_{12}+3M	CKECKCTSCKKS-NH_2^{III}	1 517.7	1 517.3
y_{12}+4M	CKECKCTSCKKS-NH_2	1 574.7	1 574.3
y_{13}+2M	KCKECKCTSCKKS-NH_2^{II}	1 588.7	1 588.4
y_{14}+2M	CKCKECKCTSCKKS-NH_2^{II}	1 691.7	1 691.4
y_{13}+4M	KCKECKCTSCKKS-NH_2	1 702.8	1 702.4
y_{15}+1M	SCKCKECKCTSCKKS-NH_2^{I}	1 721.8	1 721.4
y_{14}+3M	CKCKECKCTSCKKS-NH_2^{III}	1 748.8	1 748.3
y_{14}+4M	CKCKECKCTSCKKS-NH_2^{IV}	1 805.8	1 805.3
y_{15}+3M // y_{16}+2M	SCKCKECKCTSCKKS-NH_2^{III} / GSCKCKECKCTSCKKS-NH_2^{II}	1 835.8	1 835.4
y_{14}+5M	CKCKECKCTSCKKS-NH_2	1 862.8	1 862.5
y_{15}+4M // y_{16}+3M	SCKCKECKCTSCKKS-NH_2 / GSCKCKECKCTSCKKS-NH_2^{III}	1 892.8	1 892.5
y_{16}+4M	GSCKCKECKCTSCKKS-NH_2^{IV}	1 949.8	1 949.4
y_{17}+3M	AGSCKCKECKCTSCKKS-NH_2^{III}	1 963.9	1 963.4
y_{16}+5M	GSCKCKECKCTSCKKS-NH_2	2 006.9	2 006.5
y_{17}+4M	AGSCKCKECKCTSCKKS-NH_2^{IV}	2 020.9	2 020.5
y_{18}+4M	CAGSCKCKECKCTSCKKS-NH_2^{IV}	2 123.9	2 123.5
y_{21}+3M	SCTCAGSCKCKECKCTSCKKS-NH_2^{III}	2 358.0	2 357.6
y_{21}+4M	SCTCAGSCKCKECKCTSCKKS-NH_2^{IV}	2 415.0	2 414.5
y_{21}+5M	SCTCAGSCKCKECKCTSCKKS-NH_2^{V}	2 472.0	2 471.6
y_{23}+3M // y_{24}+2M	DSCTCAGSCKCKECKCTSCKKS-NH_2^{III} / GDSCTCAGSCKCKECKCTSCKKS-NH_2^{II}	2 530.0	2 529.5
y_{23}+4M // y_{24}+3M	DSCTCAGSCKCKECKCTSCKKS-NH_2^{IV} / GDSCTCAGSCKCKECKCTSCKKS-NH_2^{III}	2 587.0	2 586.6
y_{24}+3M	AGDSCTCAGSCKCKECKCTSCKKS-NH_2^{III}	2 601.0	2 600.4
y_{23}+5M // y_{24}+4M	DSCTCAGSCKCKECKCTSCKKS-NH_2^{V} / GDSCTCAGSCKCKECKCTSCKKS-NH_2^{IV}	2 644.0	2 643.4

Table S10. All identified tryptic fragments obtained after MS analysis of MT2 incubated with *apo*-SDH and modified with iodoacetamide. M denotes acetamide moiety (preceding number indicates the number of modified cysteines in the fragment); *n.d.* - not detected, ^{ox} – occurrence of disulfide bridge (cysteine oxidation); ^{ox,ox} – occurrence of two disulfide bridges; ^{ox,ox,ox} – occurrence of three disulfide bridges; C – cysteine residue, K - cleavage site of trypsin.

MT2 + *apo*-SDH (5 min)

MDPNCSCAAGDSCTCAGSCK	CKECK	CTSCK	SCCSCCPVGCAK	CAQGCICKGASDKCSCCA	
[1-20]$^{ox,\ ox}$, 0M [1-20]^{ox}, 0M [1-20], 0M	*n.d.*	*n.d.*	[32-43]^{ox,ox}, 0M [32-43]^{ox}, 0M [32-43], 0M	[44-61]$^{ox,\ ox}$, 0M	
			KSCCSCCPVGCAK	CAQGCICK	GASDKCSCCA
			[31-43]$^{ox,\ ox}$, 0M	[44-51]^{ox}, 0M [44-51], 0M	[52-61]^{ox}, 0M

MT2 + *apo*-SDH (120 min)

MDPNCSCAAGDSCTCAGSCK	CKECK	CTSCK	SCCSCCPVGCAK	CAQGCICKGASDKCSCCA	
[1-20]$^{ox,\ ox}$, 0M [1-20]^{ox}, 0M [1-20], 0M	*n.d.*	[26-31], 1M	[32-43]^{ox,ox}, 0M [32-43]^{ox}, 0M [32-43], 0M	[44-61]$^{ox,\ ox,\ ox}$, 0M [44-61]$^{ox,\ ox}$, 0M [44-61]^{ox}, 0M	
			KSCCSCCPVGCAK	CAQGCICK	GASDKCSCCA
			[31-43]$^{ox,\ ox}$, 0M [31-43]^{ox}, 0M	[44-51]^{ox}, 0M [44-51], 0M	[52-61]^{ox}, 0M [52-61], 0M

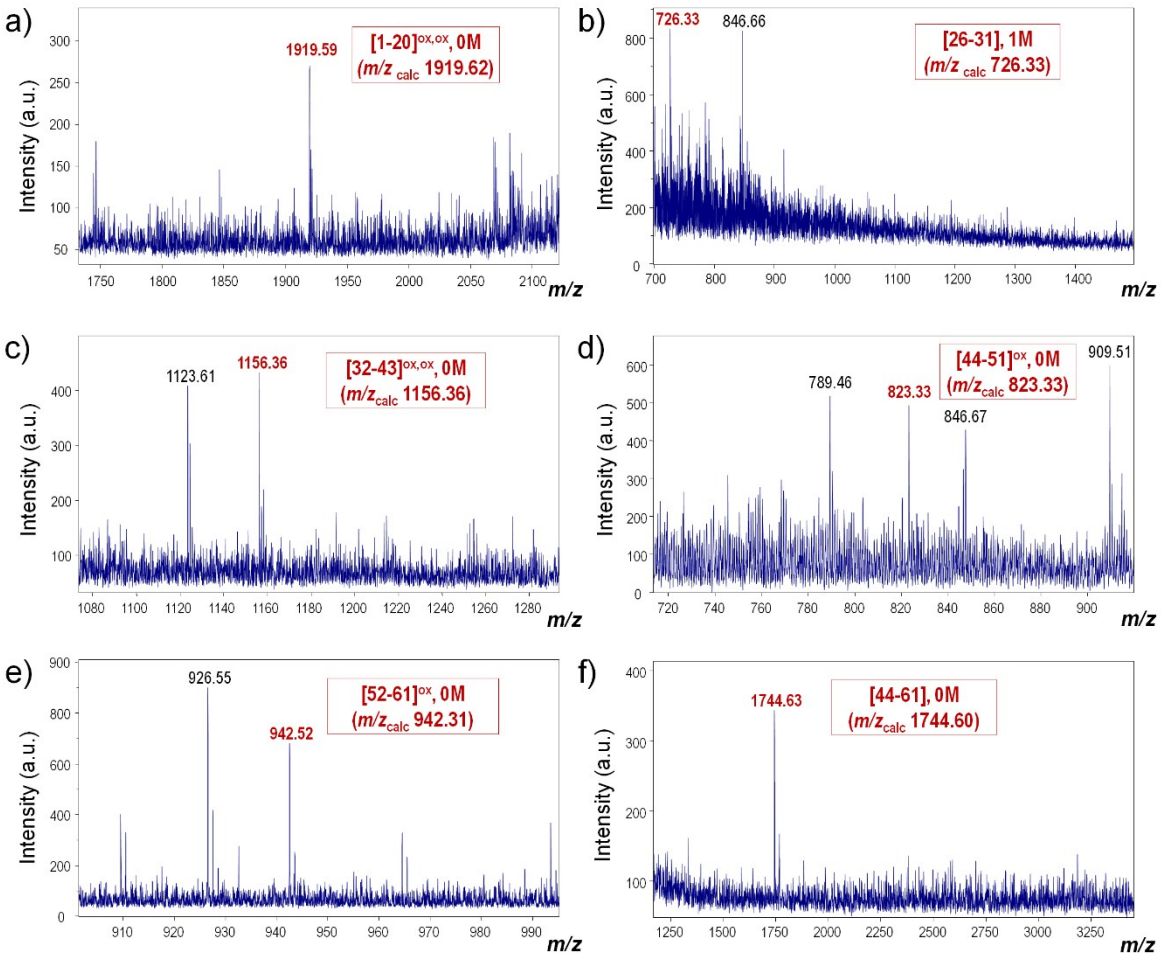

Figure S16. MALDI spectra in selected ranges from different spots of digested MT2 sample incubated with *apo*-SDH for 120 min. a) tryptic fragment [1-20]$^{ox,\ ox}$, 0 M (*m/z* calculated 1919.62, *m/z* found 1919.59). b) tryptic fragment [26-31], 1M (*m/z* calculated 726.33, *m/z* found 726.33). c) tryptic fragment [32-43]^{ox,ox}, 0 M (*m/z* calculated 1156.36, *m/z* found 156.36). d) tryptic fragment [44-51]^{ox}, 0 M (*m/z* calculated 823.33, *m/z* found 823.33). e) tryptic fragment [52-61]^{ox}, 0 M (*m/z* calculated 942.31 *m/z* found 942.52). f) tryptic fragment [44-61], 0 M (*m/z* calculated 1744.60, *m/z* found 1744.63).

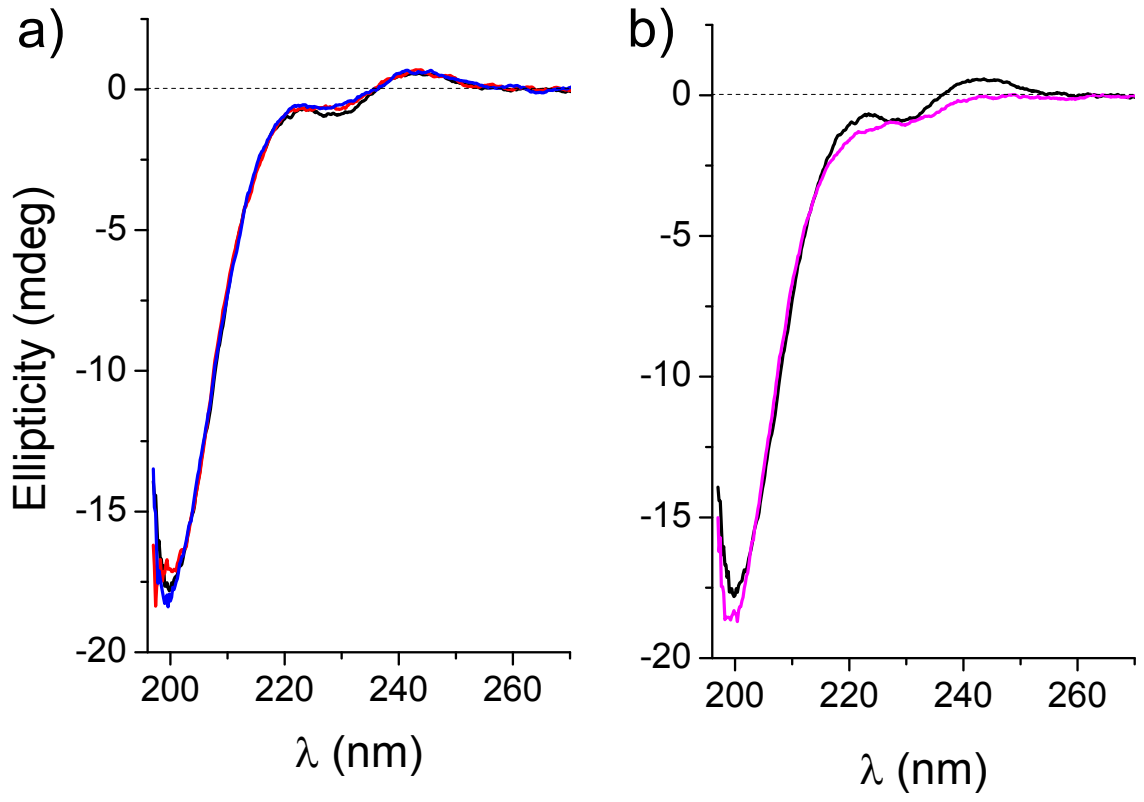

Figure S17. a) CD spectra of 10 µM fully Zn(II)-loaded MT2 (Zn_7MT2, black line) in 20 mM Tris-HCl buffer (100 mM NaCl, 200 µM TCEP pH 7.4) or Zn_6MT2 species obtained by the addition of 6 Zn(II) equivalents to *apo*-MT2 (red line) and incubation with 20 µM EGTA (blue line). b) Magenta line shows metallothionein obtained by the addition of 100 µM EDTA.