Electronic Supplementary Material (ESI) for Chemical Communications

Supporting Information

Femtomolar Zn(II) affinity of minimal zinc hook peptides – a promising small tag for protein engineering

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1. Abbreviations

ACN, acetonitrile

Ac_2O, acetic anhydride;

DMF, *N*,*N*-dimethylformamide;

DCM, dichloromethane;

DIEA, *N*,*N*-diisopropylethylamine;

DTT, dithiothreitol;

EDTA, Ethylenediaminetetraacetic acid;

Et_2O, diethyl ether;

Fmoc, 9-fluorenylmethyloxycarbonyl;

LB, Luria-Bertani broth medium;

OD, optical density;

TFA, trifluoroacetic acid;

TIS, triisopropylsilane;

Tris, tris(hydroxymethyl)aminomethane;

GdmCl, guanidinium chloride;

HEPES, 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid;

HBTU, O-(Benzotriazol-1-yl)-N,N,N′,N′-tetramethyluronium hexafluorophosphate;

TCEP, tris(2-carboxyethyl)phosphine;

TPEN, N,N,N’,N’-tetrakis(2-pyridylmethyl)ethylenediamine;

NMP, 1-methyl-2-pyrrolidinone;

PAR, 4-(2-Pyridylazo)resorcinol;

SDS, sodium dodecyl sulfate;

Dns, 5-(dimethylamino)naphthalene-1-sulfonyl (dansyl moiety);

IPTG, β-D-thiogalactopyranoside;

2. Materials

$ZnSO_4 \cdot 7H_2O$, $Na_2B_4O_7 \cdot 10H_2O$, H_3BO_3, $MgSO_4$, HEPES, TIS, TFA, DIEA, Ac_2O, TPEN, CNBr, EDTA (disodium salt dihydrate), DTT, TCEP (hydrochloride) dansyl chloride, PAR, imidazole, Chelex 100, Tris base, sinapic acid were purchased from Sigma-Aldrich. $NaClO_4 \cdot H_2O$, ACN and Rink Amide AM resin (200-400 mesh, substitution 0.62 mmol/g) were obtained from Merck. NaCl and Et_2O were purchased from POCH (Gliwice, Poland). DMF, DCM, NMP, HBTU, piperidine and proteced aminoacids: Fmoc-Ala-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Asp(OtBu)-OH, Fmoc-Cys(Trt)-OH, Fmoc-Gln(Trt)-OH, Fmoc-Glu(OtBu)-OH, Fmoc-Gly-OH, Fmoc-Leu-OH, Fmoc-Lys(Boc)-OH, Fmoc-Pro-OH, Fmoc-Thr(tBu)-OH, Fmoc-Trp(Boc)-OH, Fmoc-Val-OH were obtained from Iris Biotech GmbH (Marktredwitz, Germany). Restriction enzymes and T4 DNA ligase were from Thermo Scientific. The exact concentrations of metal salts were obtained from a representative series of ICP-MS measurements. All pH buffers used in this studies were treated with Chelex resin to eliminate contaminated heavy metal ions.

3. Peptide synthesis

Peptides were synthesized manually on Rink Amide AM resin (200-400 mesh, substitution 0.62 mmol/g) using Fmoc strategy.[1] Couplings of amino acids were performed with 3 eq. of N-α-Fmoc-protected amino acid, HBTU (3 eq.) and DIEA (5 eq.) in the mixture of DMF:NMP (1:1 v/v) over a period of 60 min. Fmoc protection groups were removed by 20% piperidine (v/v) in DMF (7 + 15 min). Peptides were terminated either by acetylation with Ac_2O or fluorescent modification of the N-terminal amino group with dansyl chloride. For that purpose resin was mixed with 4 eq. of Ac_2O or dansyl chloride, 4 eq. of DIEA in DMF for 4 h followed by multiple washing with DMF and DCM. Peptide cleavage was effected using a mixture of 88% of TFA, 5% DTT, 2% TIS and 5% water over a period of 1.5 h, followed by precipitation in cold (-20°C) Et_2O. Crude peptide pellets were collected by centrifugation, dried and then purified with HPLC (Dionex Ultimate 3000) on Vydac C18 or Phenomenex C18 columns using 0.1% TFA with a gradient of ACN. The identity of the peptides was finally confirmed using mass spectrometry, using an API 2000 instrument (Applied Biosystems) with electrospray ionization using a mixture of 50% MeOH, 49% H_2O and 1% HCOOH. The calculated and found m/z values of the peptides are presented in Table

S1. The purity of the peptides was determined using analytical HPLC chromatography using Phenomenex Jupiter C18 chromatography column and linear gradient performed using 0.1% TFA and ACN (Figure S1).

Table S1. The calculated and found m/z values of the synthesized peptides.

Peptide	Peptide Sequence[a]	*m/z* value found	*m/z* value calculated
HkL	Ac-AKGKCPVCGRELTD-NH_2	1517.3	1516.8
Hkwt	Dns-AKGKCPVCGRELTDW-NH_2	1894.4	1893.9
$HkK^{441}A$	Dns-AAGKCPVCGRELTDW-NH_2	1838.6	1838.2
$HkK^{443}A$	Dns-AKGACPVCGRELTDW-NH_2	1651.1	1650.7
$HkP^{445}A$	Dns-AKGKCAVCGRELTDW-NH_2	1866.8	1865.8
$HkV^{446}A$	Dns-AKGKCPACGRELTDW-NH_2	1866.8	1865.8
$HkR^{449}A$	Dns-AKGKCPVCGAELTDW-NH_2	1809.4	1808.8
$HkE^{450}A$	Dns-AKGKCPVCGRALTDW-NH_2	1650.0	1649.8
$HkL^{451}A$	Dns-AKGKCPVCGREATDW-NH_2	1852.9	1851.8
$HkT^{452}A$	Dns-AKGKCPVCGRELADW-NH_2	1865.1	1864.9
$HkD^{453}A$	Dns-AKGKCPVCGRELTAW-NH_2	1864.4	1663.8
$HkV^{446}A/L^{451}A$	Dns-AKGKCPACGREATDW-NH_2	1824.3	1823.8

[a] Ac, Dns denote acetyl or dansyl moiety, respectively.

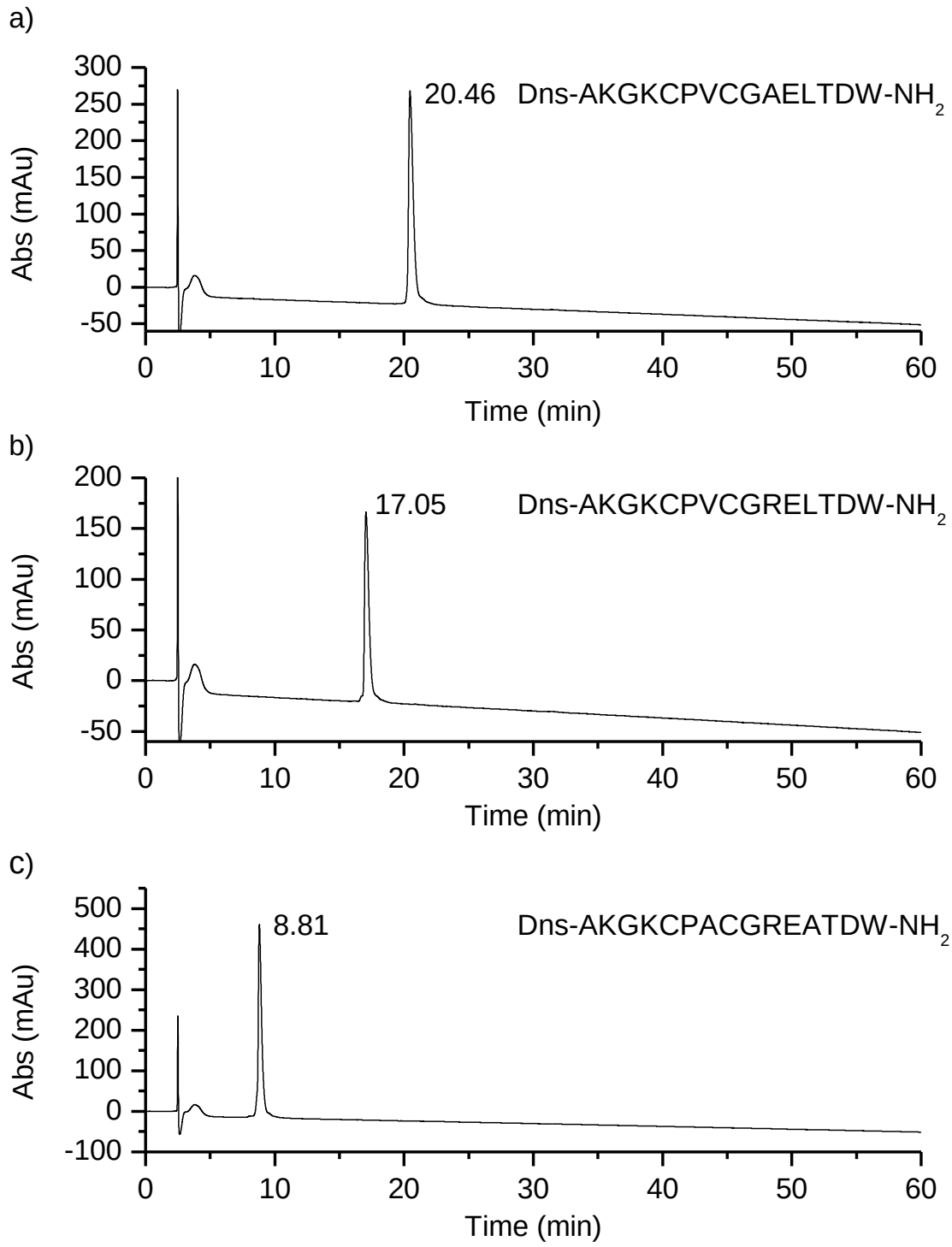


Figure S1. RP-HPLC chromatograms of purified performed on Phenomenex Jupiter C18 column (250 mm × 4.6 mm, 5.0 μm). a) Dns-AKGKCPVCGAELTDW-NH_2, retention time 20.46 min b) Dns-AKGKCPVCGRELTDW-NH_2, retention time 17.05 min c) Dns-AKGKCPVCGRELTDW-NH_2, retention time 8.81 min. The parameters of the method were as follows: linear gradient from 10% to 20% acetonitrile/water with 0.1% TFA in 1 min, then linear gradient from 20% to 45% acetonitrile/water with 0.1% TFA in 60 min, flow rate 1.5 ml/min.

4. Zn(II) complexation of the zinc hook peptides

The binding properties of zinc hook peptides to Zn(II) were examined using electronic absorption spectroscopy and circular dichroism (CD). The electronic absorption spectra were recorded on a Carry 300 spectrophotometer (Varian) at 25°C in the wavelength range of 200-260 nm, typical for ligand-to-metal charge transfer (*LMCT*) transitions.[2, 3] 15 μM peptide HkL in 50 mM borate buffer (pH 7.4, I = 0.1 M from $NaClO_4$) was titrated with small aliquots of concentrated $ZnSO_4$ in a molar ratio from 0 to 1 over HkL. After each addition of $ZnSO_4$, samples were equilibrated for 5 min and the spectra were collected (Figure S2).

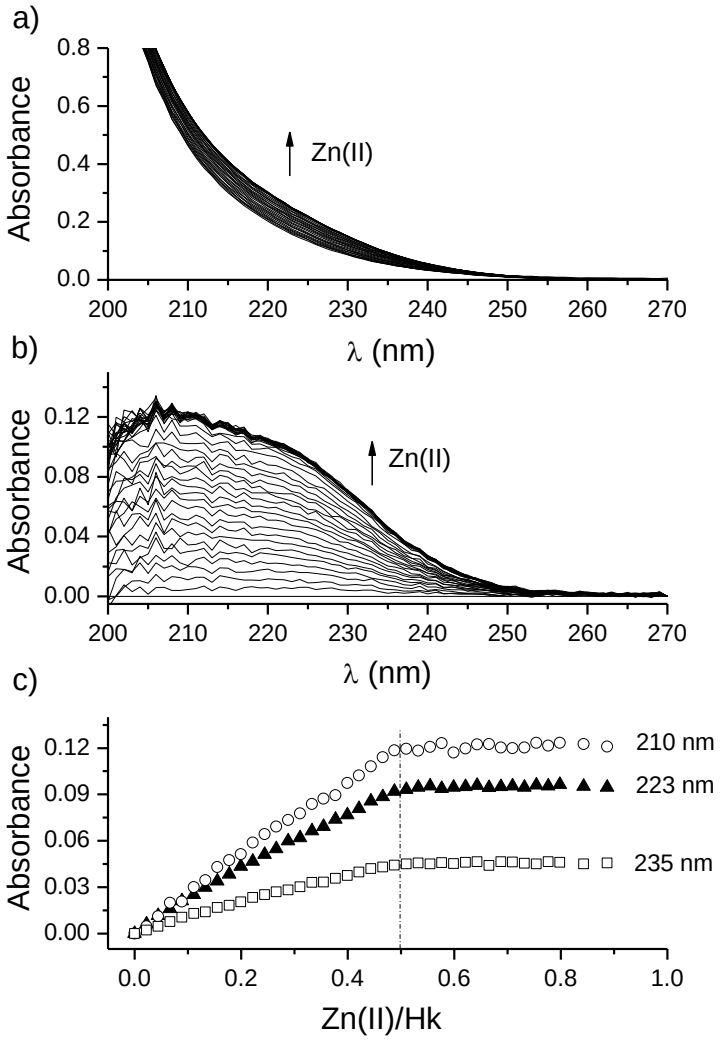


Figure S2. Spectrophotometric titration of 15 μM Hk with Zn(II) in 50 mM borate buffer, pH 7.4. a) Increase of *LMCT* transition band intensities upon Zn(II) binding to HkL peptide. b) UV-difference spectra obtained by subtracting the absorbance of Hk from spectra obtained after subsequent addition of Zn(II). c) Changes in absorbance at 210, 223 and 235 nm as a function of Zn(II)/HkL ratio. The arrow indicates the direction of absorbance change during Zn(II) titration.

CD spectra of 100 μM HkL peptide were recorded in a 1 mm quartz cuvette in the range of 194-260 nm on a Jasco J-715 spectropolarimeter at 25°C. A 100 μM solution of peptide was prepared in deoxygenated 50 mM phosphate buffer (pH 7.4, I = 0.1 M from $NaClO_4$), with 400 μM TCEP and titrated with $ZnSO_4$ solution in a molar ratio from 0 to 1 over Hk. The spectra were recorded after 5 min equilibration after each addition of $ZnSO_4$.

5. Zinc hook peptide and PAR competition

The competition of HkL peptide and PAR for Zn(II) was performed spectrophotometrically at 500 nm using a Carry 300 spectrophotometer (Varian). PAR binds Zn(II) (ε_{500} = 65 000 M^{-1} cm^{-1}) with stoichiometry 2 : 1 when present in excess over the metal ion. PAR is widely used for preliminary determination of stability constants of peptides and proteins. The dissociation constant of $Zn(PAR)_2$ complex is -logK_d = 12.3.[2-5] The solution of 100 μM PAR was partially saturated with $ZnSO_4$ to a final Zn(II) concentration of 10 μM (A_{500} = 0.65). Zinc hook peptide (Hk) was added in 26 steps up to 3.5 molar excess over Zn(II) (35 μM) (Figure S3). The linearity of absorbance decrease at 500 nm indicates high Hk affinity for Zn(II), that cannot be determined by simple competition with PAR.

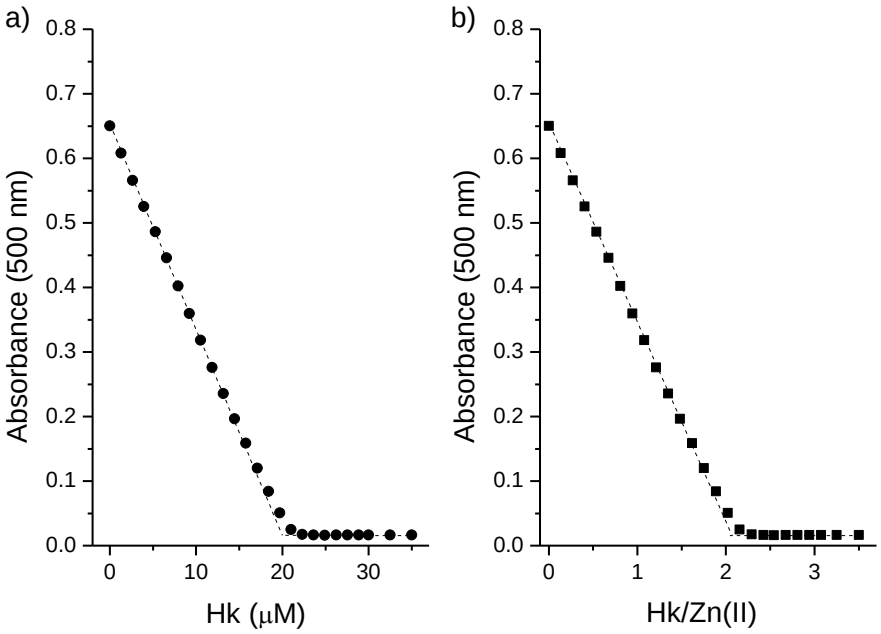


Figure S3. The competition titration of 10 μM $Zn(PAR)_2$ complex with HkL in 50 mM Tris·HCl, pH 7.4 (I = 0.1 M from NaCl), 200 μM TCEP. The dashed line indicates absorbance decrease in the case of $Zn(PAR)_2$ titration with strong chelator EDTA. a) Function of total Hk concentration and absorbance decrease at 500 nm. b) Function of molar ratio of Hk/Zn(II) and absorbance decrease at 500 nm.

6. Determination of K_d values of zinc hook peptides complexes

The dissociation constants of fluorescent zinc hook peptides complexes (Table S1) were determined using a series of metal buffers to maintain constant free Zn(II) concentrations ($[Zn(II)]_{free}$). Briefly, 1 mM concentration of appropriate Zn(II) chelator (EDTA, HEDTA or TPEN) was used with various concentrations of $ZnSO_4$ (0.05-0.95 mM) in 50 mM HEPES, 100 mM NaCl, 50 μM TCEP at pH 7.4 to maintain the free Zn(II) concentration at a constant subnano- and pico- (HEDTA), low pico- and femto- (EDTA) or low femtomolar- (TPEN) level.[6-8] TCEP compared to DTT reducing agent has a minor affinity for Zn(II) and may be used in equilibria studies without any corrections.[2, 9] The concentration of zinc hook peptides in all studies was 5 μM. The sets of peptides in metal buffers (1.4 ml) were equilibrated over 24-48 h depending on the peptide affinity. Complex formation was measured using FRET between Trp and Dns residues located at both ends of the zinc hook peptides. For that purpose samples were excited at 280 nm and spectra were collected in the range of 290-600 nm with maximum emission of 543 nm using an FP-750 spectrofluorimeter (Jasco). Accurate pZn values ($-\log[Zn(II)]_{free}$) were calculated based on pH-independent protonation constants of EDTA (β_{HL} = 10.17, β_{H_2L} = 16.28, β_{H_3L} = 18.96, β_{H_4L} = 20.96, β_{H_5L} = 22.47), HEDTA (β_{HL} = 9.81, β_{H_2L} = 15.18, β_{H_3L} = 17.78), TPEN (β_{HL} = 7.19, β_{H_2L} = 12.04, β_{H_3L} = 15.36, β_{H_4L} = 18.31) and their stability constants with Zn(II) (β_{ZnHL}^{EDTA} = 19.44, β_{ZnL}^{EDTA} = 16.44, β_{ZnL}^{HEDTA} = 14.6, β_{ZnL}^{TPEN} = 15.4) using MINEQL 4.6 software (Table S2).[8]

Table S2. The chemical components of Zn(II) buffers used in this study and related free Zn(II) concentration values.

Competitor	total Zn(II) (mM)	$[Zn(II)]_{free}$ (M)	pZn
1.0 mM TPEN	0.1	7.08×10^{-17}	16.15
1.0 mM EDTA	0.05	1.17×10^{-15}	14.93
1.0 mM EDTA	0.1	2.51×10^{-15}	14.60
1.0 mM EDTA	0.15	3.98×10^{-15}	14.40
1.0 mM EDTA	0.2	5.62×10^{-15}	14.25
1.0 mM EDTA	0.3	9.55×10^{-15}	14.02
1.0 mM EDTA	0.4	1.51×10^{-14}	13.82
1.0 mM EDTA	0.5	2.24×10^{-14}	16.65
1.0 mM EDTA	0.6	3.39×10^{-14}	13.47
1.0 mM EDTA	0.7	5.25×10^{-14}	13.28
1.0 mM EDTA	0.8	9.12×10^{-14}	13.04
1.0 mM EDTA	0.85	1.29×10^{-13}	12.89
1.0 mM HEDTA	0.2	2.04×10^{-13}	12.69
1.0 mM HEDTA	0.4	5.50×10^{-13}	12.26
1.0 mM HEDTA	0.6	1.23×10^{-12}	11.91
1.0 mM HEDTA	0.8	3.31×10^{-12}	11.48
1.0 mM HEDTA	0.9	7.41×10^{-12}	11.13
1.0 mM HEDTA	0.95	1.62×10^{-11}	10.79
1.0 mM HEDTA	1.0	1.12×10^{-9}	8.95

The change of pZn values of the metal buffers due to Zn(II) binding to peptides during equilibration was neglected. As a consequence of the stoichiometry of the complex formed, at 5 μM zinc hook peptide concentration a maximum 2.5 μM of Zn(II) can be bound. This amount of transferred Zn(II) changes the initial pZn value of metal buffers from 0 to 0.01 of the logarithmic scale as based on our previous calculations (data not shown).

Changes of donor (*Trp*) and acceptor (*Dns*) fluorescence intensities were normalized to the ZnL_2 complex fraction at a certain pZn value according to the procedure presented below.

R is ratio of fluorescence intensities of *Dns* and *Trp*

f represents the fraction of ZnL_2 complex, which varies from 0 to 1. These values of the fraction corresponds to 0 - 2.5 µM ZnL_2 concentration in the experiment.

For any given *f* values, the fraction of free (unbound) L ($1 - f$) can also be calculated; in this case it corresponds to an absolute L concentration of 0 - 5 µM.

Dns_{min}, Dns_{max} are minimal and maximal intensities of *Dns*. *f* is then 0 or 1, respectively.

Trp_{min}, Trp_{max} are minimal and maximal intensities of *Trp*. *f* is then 1 or 0, respectively.

$$R = \frac{f \times Dns_{\max} + (1 - f) \times Dns_{\min}}{f \times Trp_{\min} + (1 - f) \times Trp_{\max}}$$

Rearranging the equation to solve for *f* gives:

$$f = \frac{Dns_{\min} - Trp_{\max} \times R}{Dns_{\min} - Dns_{\max} - (R \times Trp_{\max}) + (R \times Trp_{\min})}$$

To calculate pZn values at f = 0.5 ($pZn_{0.5}$) and the cooperativity factor (n) a particular isotherm was fitted using Hill's equation.

$$f = f_{min}\left(\frac{[Zn^{2+}_{free}]^n}{[Zn^{2+}_{free}]^n + Zn_{0.5}}\right) + f_{max}\left(\frac{Zn_{0.5}}{[Zn^{2+}_{free}]^n + Zn_{0.5}}\right)$$

It should be noted that due to ZnL_2 complex stoichiometry, f = 0.5 correspond to half of the maximal concentration of ZnL_2 complex (0.5 × 2.5 µM = 1.25 µM). For the same reason the value of $Zn_{0.5}$ is not equal to the dissociation constant value as in case of metal ion to peptide complex stoichiometry 1 : 1. The K_d values were calculated as follows:

$$K_d = \frac{[Zn^{2+}] \times [L]^2}{[ZnL_2]} = \frac{[Zn_{0.5}] \times (5\ \mu M - 2 \times 1.25\ \mu M)^2}{1.25\ \mu M}$$

7. Construction of plasmids

7.1. pET-15b-CFP and pET-15b-YFP

Plasmids encoding fluorescent proteins (cyan – CFP and yellow – YFP) with a C-terminal optimized zinc hook motif (HkR^{449}A) were generated using the expression vector pET-15b. The full-length CFP and YFP encoding sequences were amplified by PCR reaction from pcDNA3-CFP and pcDNA3-YFP (gift from Dr. Doug Golenbock, Addgene plasmids: #13030 and #13033) using primers CFP_F and CFP_R (Table S3). The PCR reaction removed the STOP codon from the original sequence and introduced *NdeI* and *XhoI* restriction sites on 5' and 3' ends of the sequence, respectively. PCR products, as well as pET-15b vector, were digested with *NdeI* and *XhoI* and separated by size via electrophoresis in agarose gels. The resulting purified PCR product and linearized vector were then ligated using T4 DNA ligase. The ligation products were used to transform chemically competent *Escherichia coli* DH-5α. Resulting pET-15b-CFP and pET-15b-YFP plasmids were isolated from an ampicillin-resistant transformants and verified by DNA sequencing (Genomed, Poland). The resulting plasmids were then used to construct pET15b-CFP-Hk and pET-15b-YFP-Hk. Lyophilized oligonucleotides Hk_1 and Hk_2 encoding the optimized zinc hook motif HkR^{449}A (Table S4) were resuspended in 10 mM Tris·HCl, 1 mM EDTA, 50 mM NaCl (pH 8.0) buffer to a final concentration of 25 μM and mixed in equimolar amounts. Annealing was performed by heating the mixture to 95°C for 3 min and then cooling to 10°C at the rate of 1°C/min. The resulting dsDNA as well as pET-15b-CFP and pET-15b-YFP were digested in two steps using *XhoI* and *BamHI*. Following DNA digestion, the DNA fragments and linearized plasmids were separated by electrophoresis in agarose gels. The purified DNA fragment was then ligated into pET-15b-CFP or pET-15b-YFP vector using T4 DNA ligase to produce pET-15b-CFP-Hk and pET-15b-YFP-Hk. Ligation products were transformed into chemically competent *E. Coli* DH-5α cells. Plasmids pET-15b-CFP-Hk and pET-15b-YFP-Hk were isolated from ampicillin-resistant transformants and verified by DNA sequencing (Genomed, Poland).

7.2. pET31b-MT2A

Plasmid encoding metallothionein isoform 2A (MT2A sequence: MDPNCSCAAGDSCTCAGSCKCKECKCTSCKKSCCSCCPVGCAKCAQGCICKGASDK CSCCA) was constructed using the expression vector pET-31b+. DNA encoding MT2A was ampfilifed from pET-45b-MT2A encoding human MT2A optimized for codon usage in

E.Coli (obtained from GeneArt*)* using MT2A_F and MT2A_R primers (Table S3). The resulting PCR product contained MT2A coding sequence with stop codon substituted for the codon encoding methionine and flanked by *AlwNI* and *XhoI* restriction sites. PCR products, as well as pET-31b+ vector, were digested with *AlwNI* and *XhoI* and separated by size via electrophoresis in agarose gels. The resulting purified PCR product and linearized vector were then ligated using T4 DNA ligase. The ligation products were used to transform chemically competent *E. Coli* DH-5α. Plasmids were isolated from ampicillin-resistant transformants and verified by DNA sequencing (Genomed, Poland).

Table S3. Sequences of oligonucleotides used in this study.

Name	**Sequence**
CFP_F	GGGGCATATGAGCAAGGGCGAGGAGCTGTTC[a]
CFP_R	GGGGCTCGAGCTTGTACAGCTCGTCCATGCCGAG[a]
Hk_1	GGGGCTCGAGGCGAAAGGCAAATGCCCGGTGTGCGGCGCGGAATTGACCGATTAAGGATCCGGGG
Hk_2	CCCCGGATCCTTAATCGGTCAATTCCGCGCCGCACACCGGGCATTTGCCTTTCGCCTCGAGCCCC
MT2A_F	GACACAGATGCTGATGGATCCGAACTGCAGCTGTGCGG[a]
MT2A_R	TGTCCTCGAGCATCGCGCAGCAGCTGCATTTATCGC[a]

[a] The underline sequences anneal to the template plasmid.

8. Protein expression and purification

8.1 Expression and purification of CFP-Hk and YFP-Hk proteins

pET-15b-CFP-Hk and pET-15b-YFP-Hk expression plasmids were transformed into BL21(DE3) *Escherichia coli* strain. For large-scale production of CFP-Hk and YFP-Hk proteins, cells transformed with appropriate plasmids were grown at 37°C in LB medium containing 100 μg/ml ampicillin until the OD600 reached 0.5. At this point, IPTG was added to a final concentration of 0.8 mM and incubation was continued at 30°C for 18 h. The cultures were then harvested by centrifugation, and the cell pellet was resuspended in lysis buffer (50 mM Tris-HCl, 300 mM NaCl, 10 mM imidazole, 0.5 mM TCEP pH 8.0). Cells were disrupted by sonication (100 W, 10 min total time using 10 s pulses followed by 10 s rest) on ice and samples were centrifuged at 12 000 × *g* for 20 min at 4°C. The supernatants were incubated with slurry Ni(II)-NTA agarose beads (previously equilibrated in lysis buffer)

with rocking. After 1 h incubation at 4°C, the bead mixtures were transferred to empty columns and washed with buffer (50 mM Tris-HCl, 300 mM NaCl, 25 mM imidazole, 0.5 mM TCEP pH 8.0). Proteins bound to the resin were eluted with 50 mM Tris HCl, 300 mM NaCl, 250 mM imidazole, 0.5 mM TCEP pH 8.0 buffer. The eluted proteins were dialyzed into 50 mM Tris-HCl, pH 7.4, 100 mM NaCl, 0.5 mM TCEP.

The identity of purified proteins was confirmed using MALDI-TOF measurements (Figure S4 and Table S4). Mass spectra were performed in positive ion mode. Purified samples (1µl) were spotted onto the metal plate of an MS 4800 Plus MALDI TOF/TOF Analyzer (AB Sciex). After drying 1 µl of sinapic acid (10 mg/ml) in 50% ACN in 0.1% TFA was added. The spectra were recorded within the 20 000 – 150 000 Da range. For each spot, spectra were obtained from 1000 laser shots with 200 s^{-1} laser shot frequency and laser power of 3800-5100 AU.

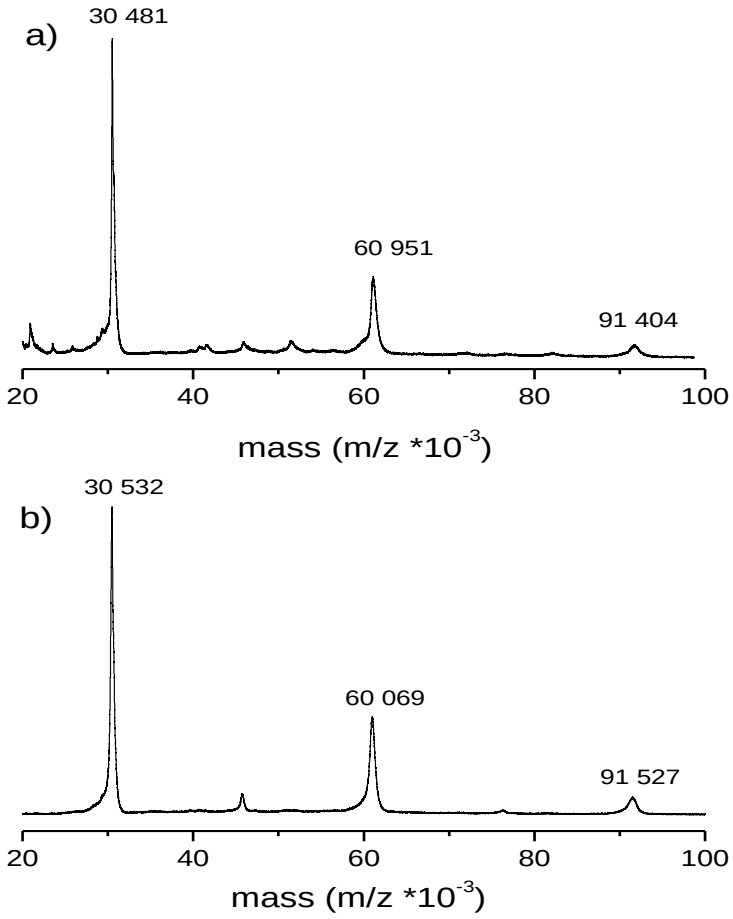


Figure S4. MALDI-TOF mass spectra of CFP-Hk - 30 481 *m/z* (a) and YFP-Hk 30 532 *m/z* (b). Peaks at higher *m/z* values represent non-covalent oligomers and their relative intensities vary at different laser power. Sinapic acid was used as the matrix.

Table S4. The amino acid sequences and molecular weights (experimental and calculated) of CFP-Hk and YFP-Hk. Experimental MW were determined by MALDI-TOF. Bolded and underlined sequence represent zinc hook peptide motif incorporated into fluorescent protein sequence.

Protein	**Amino acid sequence**	**$MW_{exp.}$**	**$MW_{cal.}$**
CFP-Hk	GSSHHHHHHSSGLVPRGSHMSKGEELFTGVVPILVELDGDVNGHRFSV SGEGEGDATYGKLTLKFICTTGKLPVPWPTLVTTLTWGVQCFSRYPDH MKQHDFFKSAMPEGYVQERTIFFKDDGNYKTRAEVKFEGDTLVNRIEL KGIDFKEDGNILGHKLEYNYISHNVYITADKQKNGIKAHFKIRHNIED GSVQLADHYQQNTPIGDGPVLLPDNHYLSTQSALSKDPNEKRDHMVLL EFVTAAGITLGMDELYKLE**AKGKCPVCGAELTD**	30481	30485
YFP-Hk	GSSHHHHHHSSGLVPRGSHMSKGEELFTGVVPILVELDGDVNGHKFSV SGEGEGDATYGKLTLKFICTTGKLPVPWPTLVTTFGYGLKCFARYPDH MKQHDFFKSAMPEGYVQERTIFFKDDGNYKTRAEVKFEGDTLVNRIEL KGIDFKEDGNILGHKLEYNYNSHNVYIMADKQKNGIKVNFKIRHNIED GSVQLADHYQQNTPIGDGPVLLPDNHYLSYQSALSKDPNEKRDHMVLL EFVTAAGITLGMDELYKLE**AKGKCPVCGAELTD**	30532	30520

8.2. Expression and purification of apo and holo forms of metallothionein MT2A.

Human MT2A was expressed in as CNBr-cleavable N-terminal ketosteroid isomerase (KSI) / C-terminal His-Tag fusion protein. pET-31b-MT2A expression plasmid was transformed into BL21(DE3)pLysS *E. Coli* strain. A single colony was used to inoculate 3 l of LB media supplemented with 100 μg/ml of ampicillin and 34 μg/ml of chloramphenicol and incubated at 37 °C until the OD600 reached 0.5. At this point, IPTG was added to a final concentration of 1 mM and incubation was continued for 4 h. Bacterial culture was then harvested by centrifugation, and the cell pellet was resuspended in lysis buffer (40 mM Tris-HCl, 500 mM NaCl, 5 mM imidazole, 0.5 mM TCEP, pH 7.9). Cells were disrupted by sonication (100 W, 10 min total time using 10 s pulses followed by 10 s rest) on ice and samples were centrifuged at 12 000 × *g* for 20 min at 4°C. The supernatant was discarded and the pellet was resuspended in denaturating buffer (40 mM Tris-HCl, 500 mM NaCl, 5 mM imidazole, 6 M GdmCl, 0.5 mM TCEP, pH 7.9) followed by centrifugation at 12 000 × *g* for 20 min at 4°C. The supernatant was incubated with slurry Ni(II)-NTA agarose beads with rocking. After 1 h incubation at 4°C, the bead mixture was transferred to empty column and washed with buffer (40 mM Tris-HCl, 500 mM NaCl, 16 mM imidazole, 6 M GdmCl, 0.5 mM TCEP pH 7.9). Protein bound to the resin was eluted with 40 mM Tris-HCl, 500 mM NaCl, 300 mM imidazole, 0.5 mM TCEP, 6 M GdmCl, pH 7.9 buffer. The eluted protein was dialyzed into

10 mM Tris-HCl, 0.5 mM TCEP, pH 7.4. The protein precipitate formed during dialysis was pelleted by centrifugation at 2000 × *g* for 10 min at 4°C. The recovered protein precipitate was then dissolved in 75% formic acid and 3-fold molar excess of $Na_2S_2O_3$ over cysteine residues and 20-fold molar excess of CNBr over methionine residues were added. The solution was bubbled with argon and reaction was conducted in dark at room temperature with moderate stirring. After 18 h, DTT to a final concentration 40 mM and EDTA to 5 mM were added and pH was slowly adjusted to 8 using concentrated NaOH under argon atmosphere. The reaction mixture was then centrifuged at 5000 × g for 15 min at 4°C, and MT2A was purified from supernatant by HPLC (Dionex Ultimate 3000) on Vydac C18 column. Briefly, the supernatant was filtered through a 0.22 um filter and 5ml of the filtrate was injected on column, and a gradient of 0 - 35% ACN / 0,1% TFA (0% ACN / 0.1% TFA. over 10 min, then 0-35% ACN / 0.1% TFA for 30 min) was ran. The identity of purified MT2A was confirmed using mass spectrometry, using an API 2000 instrument (Applied Biosystems) with electrospray ionization using a mixture of 50% MeOH, 49% H_2O and 1% HCOOH. The experimental mass was 6012.82 Da, the calculated mass was 6012.09 Da. Purified MT2A was subsequently used to obtain an apo (T-thionein) and reconstituted holo (Zn_7T – metallothionein) forms using previously described method.[10]

9. Protein dimerization studies

Freshly purified fluorescent proteins (CFP-Hk and YFP-Hk) were analyzed in terms of Zn(II) and thiol concentrations. For that purpose Ellman and PAR assays were applied independently. To eliminate any reductant (TCEP) or metal ions contamination in the formulation buffer, proteins were washed five times with chelexed buffer using Centriprep YM-10 with 10 kDa cutoff membrane (Merck Millipore). Thiol concentration was determined in 50 mM HEPES buffer, pH 7.4, 100 mM NaCl, 5 mM EDTA using 1 mM DTNB. The absorbance values measured after 1 h at 412 nm were corrected in terms of fluorescent protein-self absorbance and subsequently converted to thiol concentration with molar extinction coefficient of 14 150 M^{-1} cm^{-1}.[11] Similarly the concentration of Zn(II) bound to the zinc hook motif in recombinant proteins was determined in 50 mM HEPES buffer, pH 7.4, 100 mM NaCl using 100 μM PAR that contained 1 mM of DTNB as an oxidant.[10] A stock solution of EDTA was added to the cuvette after 1 h, which resulted in rapid absorbance decrease. The difference between maximum absorbance value and that recorded after EDTA equilibration was used to calculate Zn(II) concentration using $Zn(PAR)_2$ molar coefficient, 65

000 M^{-1} cm^{-1}.[4, 10]. All above analyses were performed in triplicate. Protein concentrations were determined at 433 and 514 nm in the case of CFP-Hk (ε_{433} = 26 000 M^{-1} cm^{-1}) and YFP-Hk (ε_{514} = 84 000 M^{-1} cm^{-1}), respectively.[12] Table S5 presents molar ratios of particular protein, Zn(II) and thiol concentrations. These results demonstrate that both CFP-Hk and YFP-Hk proteins are present as dimers after purification, where one metal ion is present per 2 monomer molecules.

Table S5. Molar ratios of Zn(II) and thiols (reduced Cys residues) present in the purified fluorescent proteins CFP-Hk and YFP-Hk, respectively.

Protein	FP protein/Zn(II)	Cys/FP protein	Cys/Zn(II)
CFP-Hk	2.02 ± 0.05	1.95 ± 0.07	3.93 ± 0.04
YFP-Hk	1.87 ± 0.05	2.09 ± 0.03	3.92 ± 0.08

In order to study CFP-Hk and YFP-Hk protein dimerization freshly purified proteins were first treated overnight with 10 mM EDTA in the presence of 500 µM TCEP in 50 mM Tris-HCl, pH 7.4, 100 mM NaCl buffer in order to remove metal ion and to form monomer species of the proteins. EDTA was subsequently eliminated from protein solution by washing samples with chelexed 50 mM HEPES, 100 mM NaCl, 50 µM TCEP at pH 7.4 buffer at least five times using Centriprep YM-10 with 10 kDa cutoff membrane (Merck Millipore). Such prepared *apo*-CFP-Hk and *apo*-YFP-Hk were then used in homo and hetero-FRET based experiments.

Dynamic light scattering (DLS) was used to measure the hydrodynamic size of the protein particles. DLS measurements were performed with a DynaPro NanoStar instrument (Wyatt Technology Corporation) using a thermostatized 1 µl quartz cuvette (Wyatt Technology Corporation). Purified protein samples in formulation buffer (50 mM Tris-HCl buffer, pH 7.4, 100 mM NaCl, 100 µM TCEP) were freshly filtered through 0.22 µm filter and 1.5 µl of filtered sample was disposed into the cuvette. The DLS signal was monitored at 25°C and Dynals algorithm (Wyatt Technology Corporation) was used with set cut-off correlation function low 1 µs and high cut-off 6 s.

Figure S6. DLS size distribution of protein particles and related molecular weights of freshly purified CFP-Hk and YFP-Hk proteins (20 μM). *Pd* demonstrates percentage of particle polydispersity.

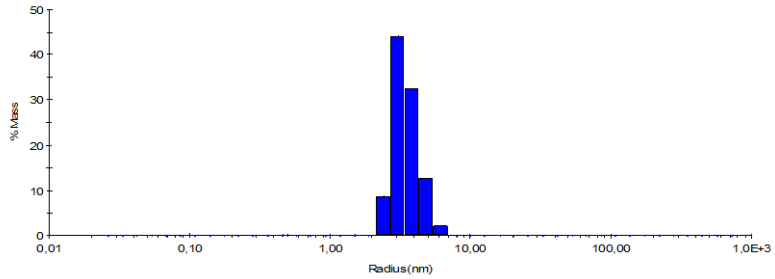
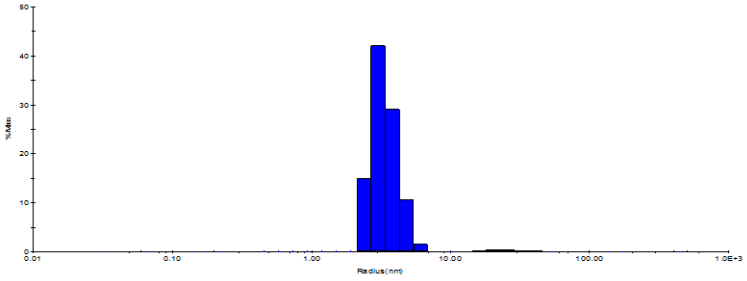
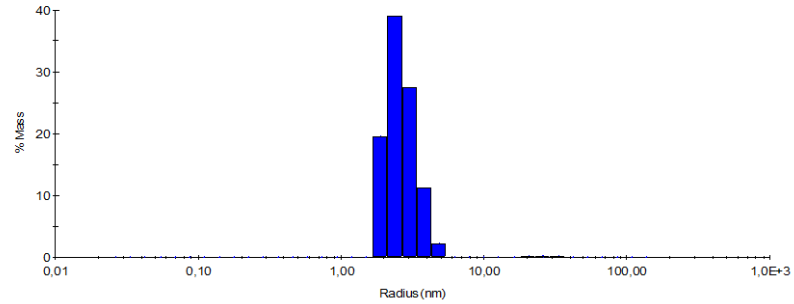
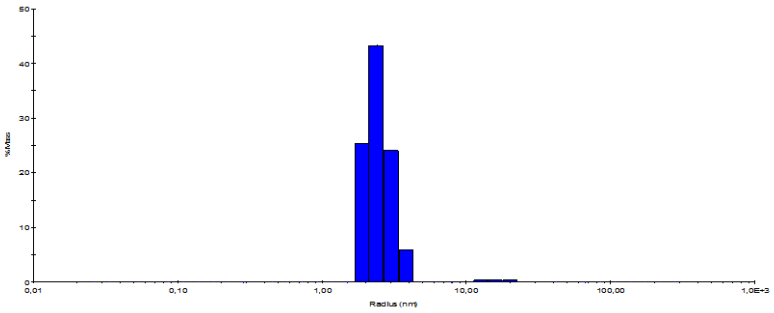
	Radius (nm)	MW (Da)
	3.548 (*Pd*: 22.5 %)	65 000
	3.431 (*Pd*: 23.1 %)	60 000

Figure S7. DLS size distributions of protein particles and related molecular weights of CFP-Hk and YFP-Hk incubated overnight with 10 mM EDTA. *Pd* demonstrates percentage of particle polydispersity.

	Radius (nm)	MW (Da)
	2.695 (*Pd*: 25.1 %)	34 000
	2.510 (Pd: 21.1 %)	29 000

Steady-state fluorescence anisotropy measurements of CFP-Hk and YFP-Hk were obtained using a Jobin Yvon Fluoromax-3 spectrofluorimeter (Horiba) at 25°C. Freshly prepared *apo*-CFP-Hk and *apo*-YFP-Hk were mixed with metal buffers (details above) to a final concentration of 2 μM and incubated for 24 h in the presence of 50 μM TCEP. The excitation and emission wavelengths were set to λ_{ex} = 434 nm and λ_{em} = 477 nm for CFP-Hk and λ_{ex} = 515 nm and λ_{em} = 528 nm for YFP-Hk. The integration time and the bandpass were adjusted for each protein in order to ensure ≈ 1.0 x 10^6 - 1.5 x 10^6 counts per second for all samples in the VV polarization mode. Measurements at each metal buffer were repeated 10 times and averaged to increase the signal to noise ratio. Fluorescence anisotropy changes were fitted using Hill's equation to calculate dissociation constants.

Similarly, heterodimerization studies were performed in the same metal buffers. FRET changes between CFP and YFP domains that accompany Zn(II) complexation were measured at 25°C using an FP-750 spectrofluorimeter (Jasco). *Apo*-protein samples (both 2.5 μM) mixed together were incubated for 24 h in the presence of 50 μM TCEP. Spectra were recorded in the wavelength range from 440 to 650 nm with the excitation wavelength set at 434 nm. Spectrum measured at half-protein concentration was subtracted from obtained spectra to eliminate contributions of proteins forming homodimers. FRET changes were normalized similarly to fluorescent zinc hook peptides (details above) and finally converted to ZnL_1L_2 (dimer) complex fraction that was used subsequently for determination of the dissociation constant.

10. Zn(II) complexation of zinc hook peptides in T/MT buffering system

Purified thionein (T) and metallothionein (Zn_7T) were mixed in 50 mM HEPES, 100 mM NaCl, 0.5 mM TCEP at pH 7.4, to prepare a set of buffers with various T/T+MT ratios. T concentration varied within a range of 0 – 30 μM while Zn_7T remained constant at 10 μM. Zinc hook peptides were added at a concentration of 1 μM and equilibrated over 4h. FRET changes were measured and normalized as described above. Equlibira calculations were performed using published log β values ($4Zn^{2+} + T \rightleftarrows Zn_4T$ log β = 47,2; $Zn^{2+} + Zn_4T \rightleftarrows Zn_5T$ log β = 57,6; $Zn^{2+} + Zn_5T \rightleftarrows Zn_6T$ log β = 67,6; $Zn^{2+} + Zn_6T \rightleftarrows Zn_7T$ log β = 75,6).[10]

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