Electronic Supplementary Material (ESI) for Chemical Communications

Electronic Supporting Information

for

Cisplatin Binds to Human Copper Chaperone Cox17: the Mechanistic Implication of Drug Delivery to Mitochondria

Linhong Zhao, Qinqin Cheng, Zhen Wang, Zhaoyong Xi, Dechen Xu, Yangzhong Liu

Experimental Details

Experimental Details

Materials. The ^{15}N-labeled cisplatin (cis-[$PtCl_2(^{15}NH_3)_2$]) was synthesized according to the literature method.[1] The DNA primers using for the construction of recombinant plasmids were synthesized by Sangon Biotech, China. The RNA sequences were purchased from GenePharma, China.

Construction of recombinant plasmids of hCox17. The pST-SG1-hCox17 recombinant plasmid was constructed for prokaryotic expression. The cDNA sequence encoding human Cox17 was amplified from human bone marrow cDNA library by PCR. The sequences of primers were given in Table S1. The PCR products were inserted into pST-SG vector using Ligation Independent Cloning (LIC) method,[2] generating a pST-SG-hCox17 recombinant plasmid. The recombinant plasmid expresses a His6-SG-Cox17 fusion protein with a TEV protease cleavage site between the SG tag and Cox17 protein.

For the eukaryotic expression, the plasmids of pcDNA3.0-Cox17 and pcDNA3.0-Cox17(1-46) were constructed by the insertion genes of hCox17 or Cox17(1-46) into the pcDNA3.0 vector using the Hind III and EcoR I sites.

Regulation of the Cox17 expression levels in HeLa cells. Plasmids or siRNAs were transfected into cells using Lipofectamine™ 2000 (Invitrogen). Generally, DNA, siRNA or transfection reagent was diluted in the median prepared without serum. After a 5 min incubation at room temperature, the diluted DNA or siRNA was added into the transfection reagent. The mixture was incubated at room temperature for 20 minutes before adding to cells. The cells were incubated at 37 °C in a CO_2 incubator. The culture medium was changed after 6 hours and cells were further incubated for 24 h. Then, the relative levels of mRNA were determined by RT-PCR.

Table S1. The sequences of primers and siRNAs.

pST-SG-hCox17	Forward primer	5’-TACTTCCAATCCAATGCGATGCCGGGTCTG GTTGACT-3’
	Reverse primer	5’-TTATCCACTTCCAATGCTATCATATTTTAAAT CCTAGGGCTCTC-3’
pcDNA3.0-hCox17(WT)	Forward primer	5’-CCCAAGCTTATGCCGGGTCTGGTTGAC-3’
	Reverse primer	5’-CCGGAATTCTCATATTTTAAATCCTAG-3’
pcDNA3.0-hCox17(1-46)	Forward primer	5’-CCCAAGCTTATGCCGGGTCTGGTTGAC-3’
	Reverse primer	5’-CCGGAATTCTCATCCACAGTGTTCTTCTCC-3’
SiCox17	sense	5’-GUGAGUCUCAGGAGAAGAATT-3’
	antisense	5’-UUCUUCUCCUGAGACUCACTT-3’
scrambled siRNA sequence	sense	5’-UUCUCCGAACGUGUCACGUTT-3’
	antisense	5’-ACGUGACACGUUCGGAGAATT-3’

Expression and purification of the Cox17 protein. The pST-SG-hCox17 plasmid was transformed into *E. coli* Origami B (DE3) competent cells. The cells were plated on a LB-agar plate supplemented with 100 μg/mL ampicillin and grown overnight at 37 °C. A single colony was picked into 4 ml LB culture containing 100 μg/mL ampicillin and cells were grown with shaking of 250 rpm at 37 °C for 8 h. The 4 mL culture was transferred into 1 L LB medium containing 100 μg/mL ampicillin. The culture was shaken at 37 °C until reaching OD_{600} of 0.8. The protein expression was induced by 0.4 mM IPTG at 25°C for 10 h. Cells were harvested by centrifugation (4000 rpm) for 20 min at 4°C.

The cell pellets were resuspended in 30 mL lysis buffer (50 mM Tris-HCl, 100 mM NaCl, 5 mM imidazole, pH 8.0) and lysed by sonication for 10 min. The lysate was centrifuged at 16000 rpm for 30 min at 4°C. The supernatant was filtered through a 0.45 μm filter membrane and loaded onto the Ni-NTA column pre-equilibrated with the lysis buffer. The column was incubated on a rotatory shaker at 4°C for 20 min for the protein binding. The column was rinsed with 5-column-volume washing buffer (50 mM Tris-HCl, 100 mM NaCl, 10 mM imidazole, pH 8.0), then the protein was eluted with 20 mL elution buffer (50 mM Tris-HCl, 100 mM NaCl, 250 mM imidazole, pH 8.0). The SG tag was cleaved from the fusion protein by 0.1 mg/mL TEV protease at 16°C overnight in the presence of 0.5 mM EDTA. The digestion efficiency was verified using Tricine-SDS–PAGE. The TEV protease digestion resulted in a protein with three additional residues (Ser-Asn-Ala) at the N-terminus of Cox17. The protein contains 66 amino acid residues with the sequence: SNAMPGLVDSNPAPPESQEKKPLKPCCACPETKKARDACIIEKGEEHCGHLIE AHKECMRALGFKI.

The digestion product was concentrated to 5 mL using ultrafiltration and further purified by gel filtration chromatography on an ÄKTA purifier equipped with a Hiload 16/60 Superdex75 Column (GE Healthcare). The protein was loaded and eluted with the buffer (20 mM MES-NaOH, 50 mM NaCl, pH 6.0). The collections were concentrated by ultrafiltration and then frozen at -80 °C. The concentration of Cox17 was determined using Bradford method and further verified with BCA assay.

Control the redox states of Cox17. The redox state of Cox17 was controlled according to the literature method.[3] The Cox17 protein expressed in the *E. coli* Origami B (DE3) strain is always in the oxidation state $Cox17_{3S\text{-}S}$ as this strain lack of TrxB and Gor. The partially reduced $Cox17_{2S\text{-}S}$ was obtained by adding 1 mM DTT into the TEV digestion products. $Cox17_{2S\text{-}S}$ was further purified and DTT was simultaneously removed by gel filtration. ESI-MS data proved the redox states of Cox17 (MW 7180.5 for $Cox17_{3S\text{-}S}$ and 7182.5 for $Cox17_{2S\text{-}S}$, respectively. See Fig. S6 and Table S1). The stability of $Cox17_{2S\text{-}S}$ protein in the absence of DTT was checked by quantification of the thiol groups using Ellman's assay. The results indicated that the protein can be stable at pH 6.0 for at least 12 h (See Fig. S2). The $Cox17_{2S\text{-}S}$ (1 mM protein in 20 mM MES, 50 mM NaCl, pH 6.0) was frozen at -80 °C as the stock solution. The redox state of the protein was checked by Ellman's assay before using.

Characterization of the platination adducts with ESI-MS. The reaction of 100 μM Cox17 with 0.5 mM cisplatin were performed in the presence of 20 mM MES, pH 6.0. Samples were prepared in an anaerobic glove box and incubated at 37 °C for 6 h. The platinated products were separated by HPLC and analyzed by ESI-MS immediately. ESI-MS was carried out on a LTQ Orbitrap XL mass spectrometer (Thermo Scientific) using the positive ion mode.

Quantification of thiols using Ellman's assay. The thiol content in the Cox17 protein was quantified using Ellman's Assay. Protein samples were diluted to a final volume of 1 mL in the presence of 10 μM DTNB and 100 mM Tris-HCl (pH 8.0). After 10 min incubation, the absorbance at 412 nm was measured on an Agilent 8453 UV-visible spectrophotometer.

Copper release assay. The platinum-mediated copper release from Cu^{I}-Cox17 was analyzed using Cu(I) dyer BCA. The reactions of 100 μM Cu^{I}-$Cox17_{2S\text{-}S}$ with 200 μM cisplatin was performed in 20 mM MES buffer (pH 6.0) in the presence of 250 μM BCA in a sealable quartz cuvette. Cu^{I}-Cox17 was prepared by adding $[Cu(CH_3CN)_4]ClO_4$ to equimolar $Cox17_{2S\text{-}S}$. All sample preparations were carried out in an anaerobic glove-box. Then the absorbance at 562 nm was monitored immediately on UV-visible spectrophotometer using a kinetics mode. The cuvette was kept in dark and the temperature was set at 37 °C.

HPLC. HPLC was performed on an Agilent 1200 system equipped with a Jupiter C18 reverse phase column. Samples were directly injected and eluted with a three-step linear gradient with mobile phase A (0.1% TFA/H_2O) and B (100% CH_3OH) in 20 min (0-4 min, 10% B; 4-8 min, 10-50% B; 8-16 min, 50-90% B; and 16-20 min, 90-100% B). HPLC profiles were recorded by UV detector at 220 nm or 280 nm.

The platination of Cox17 were analyzed on the reaction of 100 μM Cox17 with 0.2 mM cisplatin in 20 mM MES (pH 6.0) at 37 °C for 12 h. All samples were prepared and incubated in an anaerobic glove box at 37 °C.

NMR NMR spectroscopy. ^{15}N-labeled cisplatin were dissolved in Milli-Q water to 3 mM. The reactions of 1 mM Cox17 with equimolar ^{15}N-labeled cisplatin was performed in 20 mM MES buffer (pH 6.0) containing 10% (v/v) D_2O. The samples were prepared in an anaerobic glove-box and then incubated at 37 °C in dark for 4 h prior to NMR experiments. ^{1}H-^{15}N HSQC spectra were recorded at 25 °C on a 700 MHz Varian Inova spectrometer.

Quantification of cisplatin accumulation in mitochondria. 2×10^6 HeLa cells were plated in a 10 cm dish and incubated at 37 °C in a CO_2 incubator. After 24 h incubation, 2 μg plasmid or 600 pmol siRNA was transfected into cells using Lipofectamine™ 2000. Cells were cultured for 24 h with the medium change every 6 h. Then the cells were treated with 10 μM cisplatin in the culture medium for 10 h.

Cells were harvested by the trypsin treatment. Mitochondria were isolated from the cells using a Cytoplasmic and Mitochondrial Protein Extraction Kit (Sangon Biotech, China). The mitochondrial protein was quantified with Bradford assay, and mitochondrial platinum content was quantified using ICP-MS.

MTT assay. Cells were seeded in 96-well plates at 3000 cells per well in 100 μL culture medium, and incubated in a 5% CO2 atmosphere at 37 °C for 24 h. The culture medium was then replaced with 100 μL fresh culture medium and the transfection was performed using Lipofectamine™ 2000. The medium was changed after 5 h and cells were further incubated for 24 h. The medium was then replaced with fresh culture medium containing cisplatin at different concentrations, and cells were treated with cisplatin for 48h. MTT solution was added. After incubation for another 4 h, allowing viable cells to reduce the yellow tetrazolium salt (MTT) into dark blue formazan crystals. Finally, 100 μ L of extraction buffer (20% SDS in 50% DMF, pH 4.7) was added to the wells and incubated for another 4 h at 37 °C. The absorbance was measured at 570 nm using a Bio-Rad 680 microplate reader. The IC_{50} values were calculated using GraphPad Prism software (version 5.01), which were based on three separate experiments.

1 S. J. S. Kerrison and P. J. Sadler, *J. Chem. Soc., Chem. Commun*, 1977, 861-863.

2 L. Stols, M. Gu, L. Dieckman, R. Raffen, F. R. Collart and M. I. Donnelly, *Protein Expr. Purif.*, 2002, **25**, 8-15.

3 A. Voronova, J. Kazantseva, M. Tuuling, N. Sokolova, R. Sillard and P. Palumaa, *Protein Expr. Purif.*, 2007, **53**, 138-144.

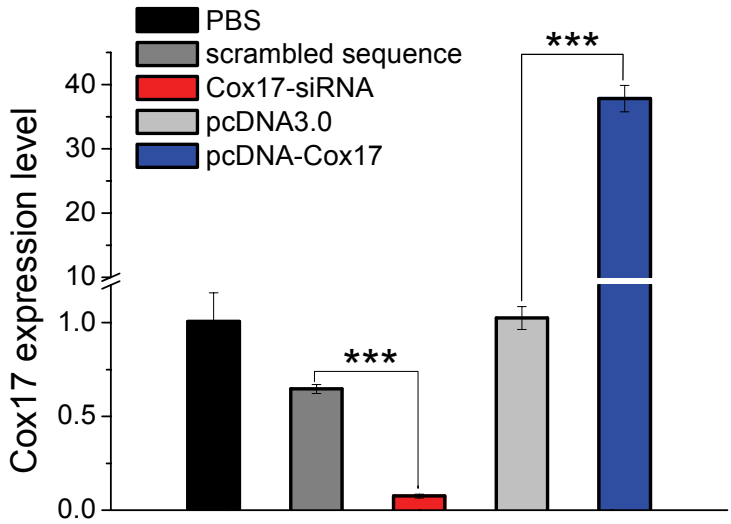

Fig. S1 The mRNA level of Cox17 in HeLa cells with the transfection of PBS buffer, scrambled siRNA sequence, siCox17 dsRNA, pcDNA3.0 vector, and pcDNA3.0-Cox17 recombinant plasmid, respectively. The siRNA sequences were given in Table S1.

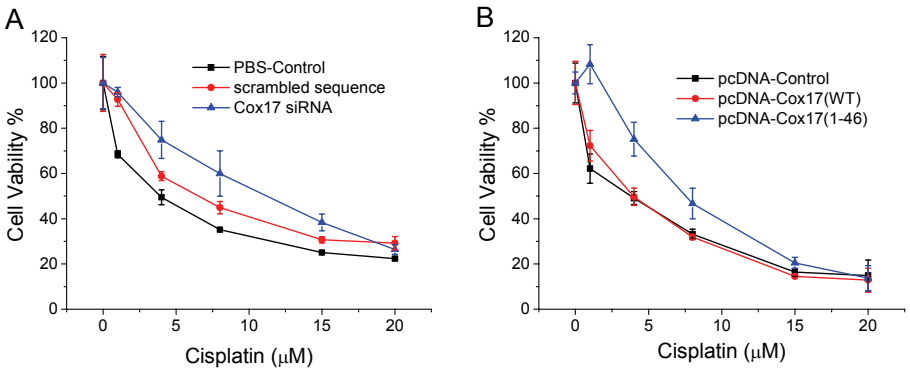

Fig. S2 The effect of the Cox17 level on the inhibitory efficacy of cisplatin to HeLa cells. (A) The effect of down-regulation of Cox17 on the inhibitory efficacy of cisplatin: HeLa cells transfected with PBS (■), scrambled siRNA sequence (●) or Cox17 siRNA(▲); (B) The effect of up-regulation of Cox17 on the inhibitory efficacy of cisplatin: HeLa cells transfected with pcDNA empty vector (■), wild type Cox17 (●) or truncated Cox17 without ITS (▲).

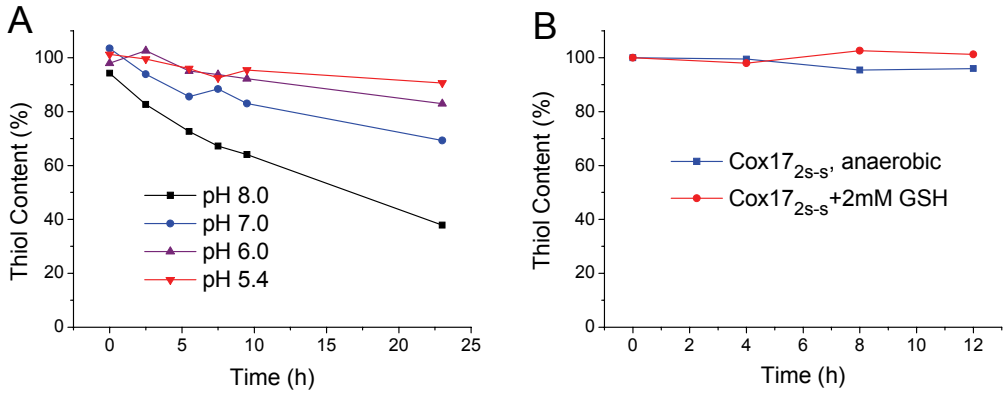

Fig. S3 The redox stability of apo-Cox17$_{2S-S}$ protein. The stability was measured based on the thiol content as a function of time. The thiols were quantified using Ellman's Assay. (A) The oxidation of Cox17 in the air at 25 °C. Samples were prepared with 100 μM Cox17$_{2S-S}$ in the buffer with different pH. (B) The oxidation of Cox17 under anaerobic conditions at 37 °C. 100 μM Cox17$_{2S-S}$ were prepared in 20 mM MES, pH 6.0 in the presence of 2 mM GSH (red circular) or in the absence of GSH (black square). Samples were prepared in 1.5 mL eppendorf tubes in a glove box, then the sealed tubes were incubated out of glove box at 37 °C. The samples containing GSH were desalted to remove GSH before measuring thiols.

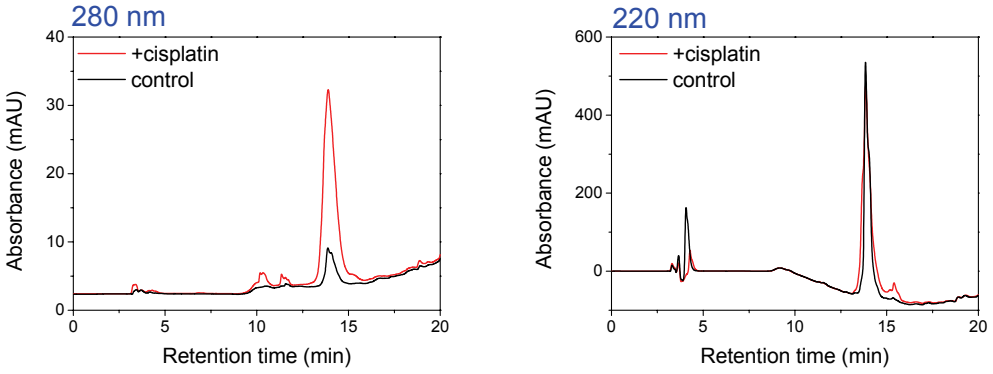

Fig. S4 HPLC monitoring the interaction of Cox17$_{2S-S}$ and cisplatin. 100 μM Cox17$_{2S-S}$ was incubated with 2 molar equivalent of cisplatin at 37 °C for 12 h. The control experiments were performed on the Cox17$_{2S-S}$ in the absence of cisplatin. 50 μL samples were injected and HPLC profiles were recorded with UV detection at 280 nm (left) or 220 nm (right).

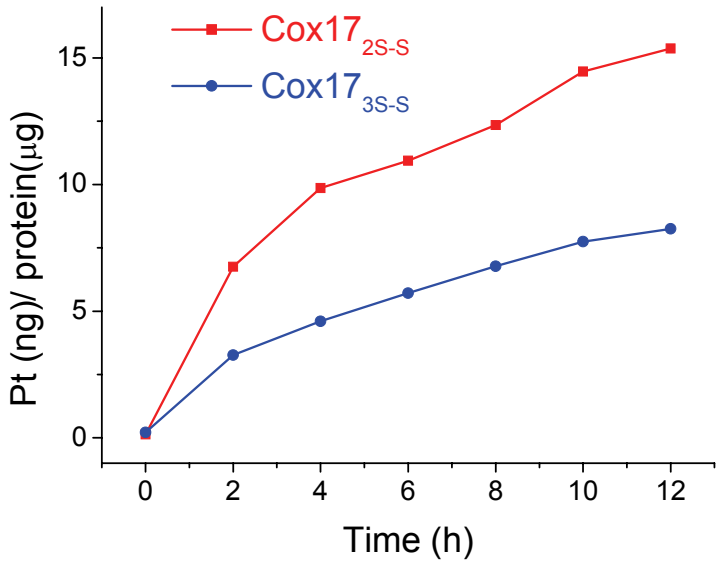

Fig. S5 HPLC-ICP-MS characterize the platination of $Cox17_{2S\text{-}S}$ (■) and $Cox17_{3S\text{-}S}$ (●) versus time. All reactions were performed on 100 μM Cox17 with 200 μM cisplatin in 20 mM MES (pH 6.0) at 37 °C. Samples were prepared in an anaerobic glove box. 100μL samples were injected for HPLC analysis, then the peak corresponding to Cox17 was collected and the platinum content was determined using ICP-MS.

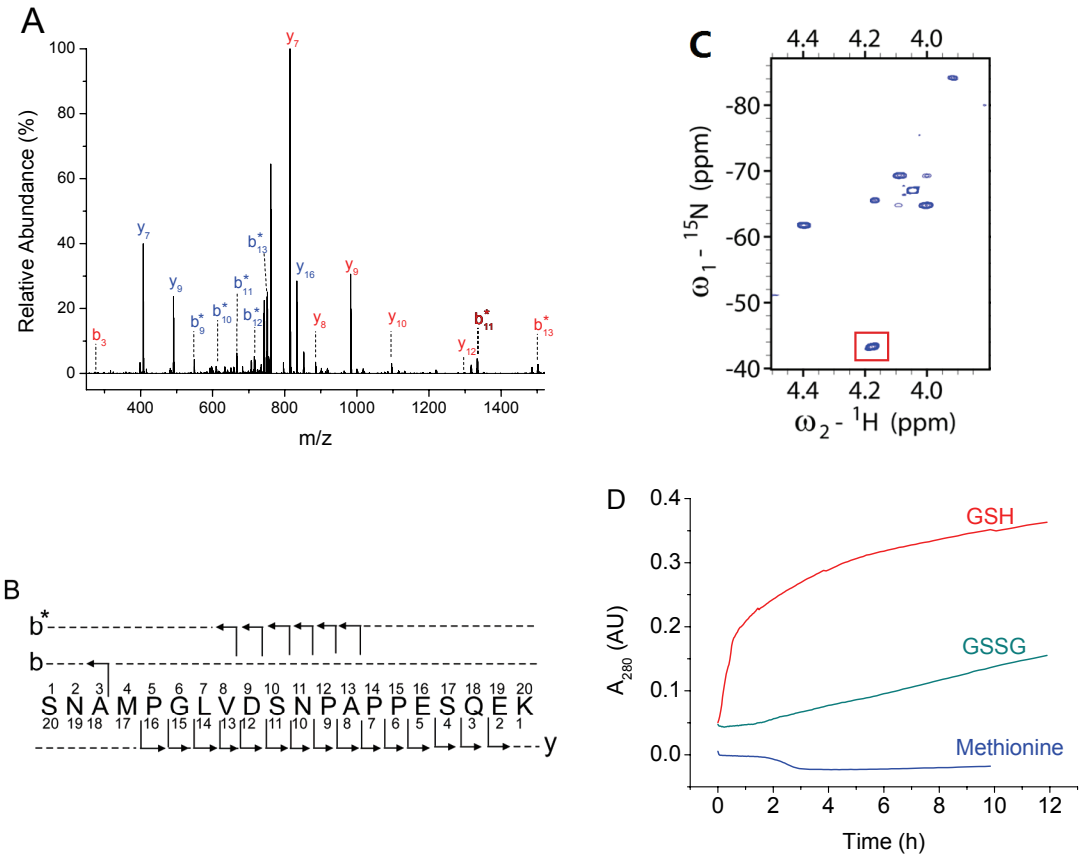

Fig. S6 Identification of platinum binding site in $Cox17_{3S\text{-}S}$. (A) ESI-MS/MS spectra of the platinated peptide (+3 charge, m/z = 777.98) from the trypsin digestion of cisplatin-$Cox17_{3S\text{-}S}$ adducts. Symbols b and y were annotated following the Biemann nomenclature, and the asterisk denotes platinated fragments. Fragments with +1 charge were shown in red and +2 charges were shown in blue. (B) The fragment ions observed in the MS spectrum, indicating the platinum binds to the MPGLV motif. (C) The 2D HSQC NMR spectrum of ^{15}N labeled cisplatin in the reaction of $Cox17_{3S\text{-}S}$ for 4 h. The peak at -43/4.2 ppm in the red box indicates the ammine ligand trans to a sulfur atom. (D) The UV absorbance at 280 nm (A_{280}) versus reaction time. The reactions were performed on 0.2 mM cisplatin with 0.2 mM reduced glutathione (GSH, red), 0.1 mM oxidized glutathione (GSSG, green) and 0.1 mM methionine (blue), respectively, in 20 mM MES (pH 6.0) at 37 °C. Results clearly show that the platination caused the increase of A_{280} in the reactions of GSH and GSSG, but not of methionine.

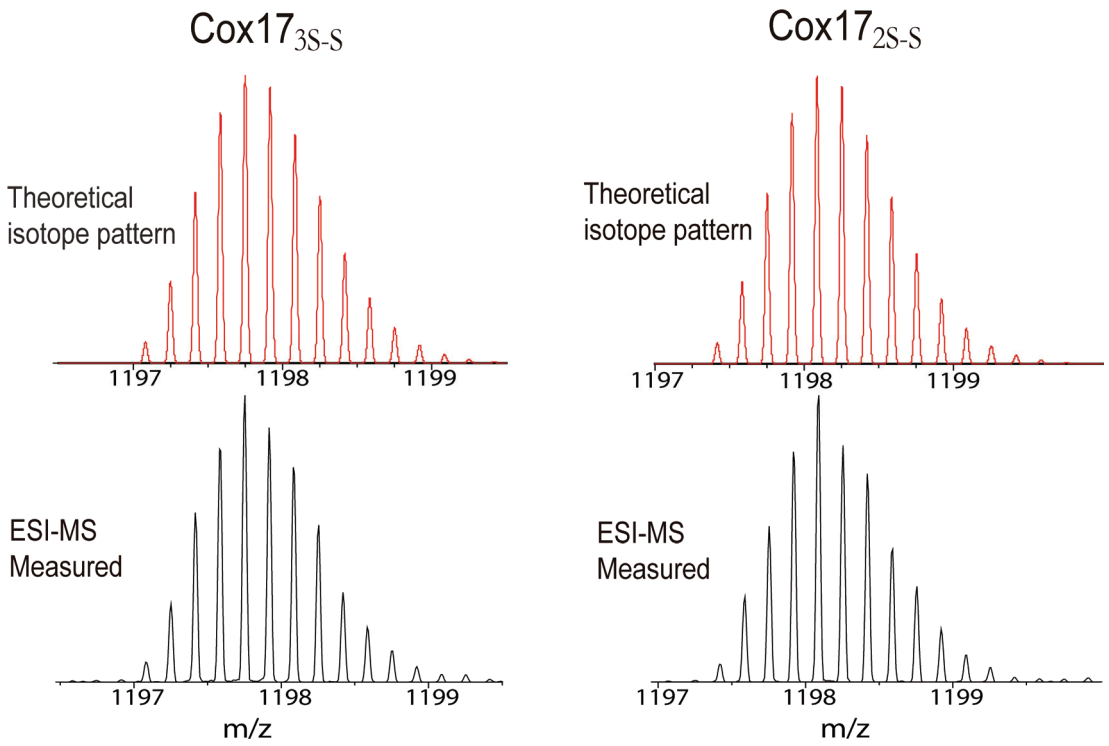

Fig. S7 ESI-MS spectra of apo-hCox17$_{3S-S}$ and apo-hCox17$_{2S-S}$ peaks with 6+ charges. The theoretical isotopic patterns were given for comparison.

Protein	Molecular Formula	m/z (charge)	Molecular Weight	
			observed	calculated
Cox17$_{3S-S}$	$C_{306}H_{495}N_{89}O_{94}S_8$	1197.75 (+6)	7180.50	7180.45
Cox17$_{2S-S}$	$C_{306}H_{497}N_{89}O_{94}S_8$	1198.09 (+6)	7182.54	7182.47

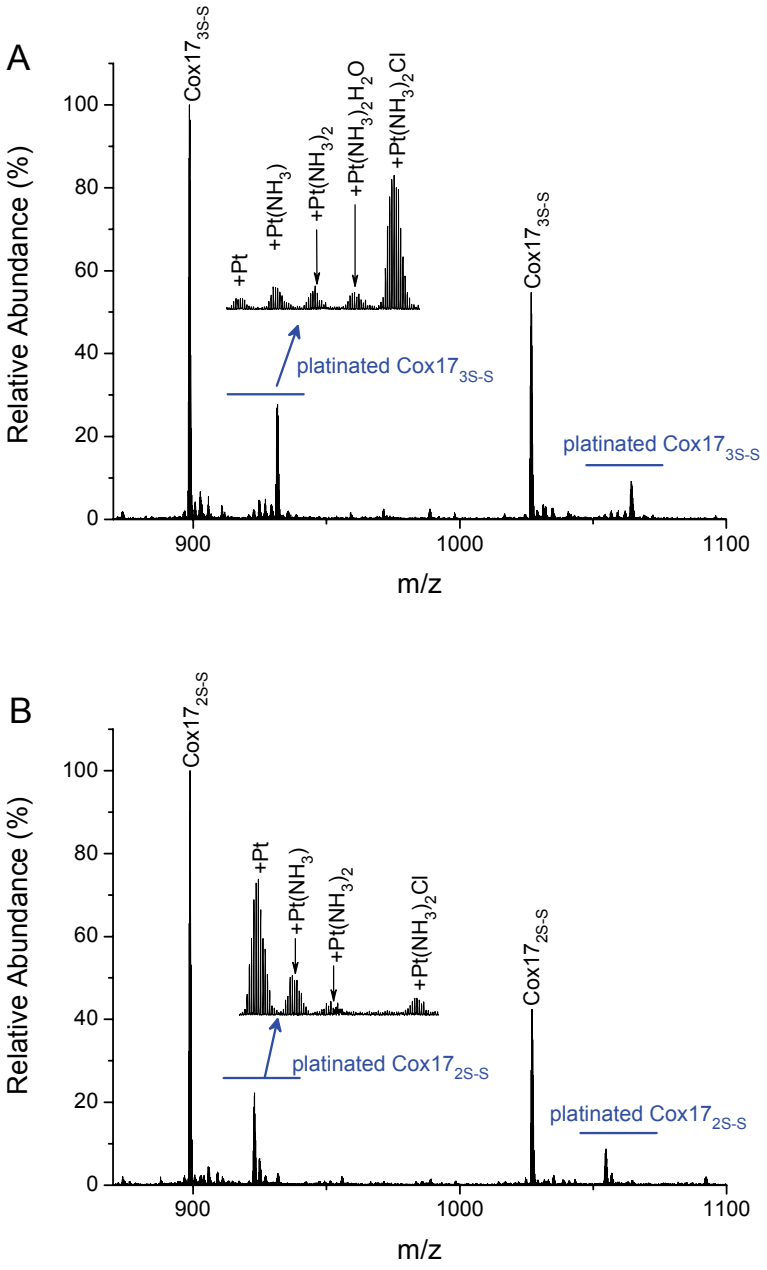

Fig. S8 ESI-MS spectra of 100 μM Cox17 reacted with 5 molar equivalent cisplatin at 37 °C for 6 h. (A) $Cox17_{3S-S}$; (B) $Cox17_{2S-S}$. The peak assignments are listed in Table S2.

Table S2. The analysis of ESI-MS peaks detected in Fig. S8.

Spectra	Composition	Formula	m/z (charge)	Molecular Weight	
				observed	calculated
A	$Cox17_{3S\text{-}S}$	$C_{306}H_{495}N_{89}O_{94}S_8$	898.57 (+8) 1026.79 (+7)	7180.54	7180.45
	+Pt	$C_{306}H_{495}N_{89}O_{94}S_8Pt$	922.68 (+8) 1054.35 (+7)	7375.44	7375.42
	$+Pt(NH_3)$	$C_{306}H_{498}N_{90}O_{94}S_8Pt$	924.69 (+8) 1056.79 (+7)	7391.52	7392.44
	$+Pt(NH_3)_2$	$C_{306}H_{501}N_{91}O_{94}S_8Pt$	927.07 (+8) 1059.07 (+7)	7410.56	7409.47
	$+Pt(NH_3)_2H_2O$	$C_{306}H_{503}N_{91}O_{95}S_8Pt$	929.31 (+8) 1062.07 (+7)	7428.48	7427.48
	$+Pt(NH_3)_2Cl$	$C_{306}H_{501}N_{91}O_{94}S_8ClPt$	931.57 (+8) 1064.50 (+7)	7445.56	7445.45
B	$Cox17_{2S\text{-}S}$	$C_{306}H_{497}N_{89}O_{94}S_8$	898.82 (+8) 1027.08 (+7)	7182.56	7182.47
	+Pt	$C_{306}H_{497}N_{89}O_{94}S_8Pt$	922.94 (+8) 1054.78 (+7)	7377.52	7377.43
	$+Pt(NH_3)$	$C_{306}H_{500}N_{90}O_{94}S_8Pt$	924.94 (+8) 1056.93 (+7)	7393.52	7394.46
	$+Pt(NH_3)_2$	$C_{306}H_{503}N_{91}O_{94}S_8Pt$	927.06 (+8) 1059.37 (+7)	7410.48	7411.49
	$+Pt(NH_3)_2Cl$	$C_{306}H_{503}N_{91}O_{94}S_8ClPt$	931.81 (+8) 1064.79 (+7)	7447.48	7447.46

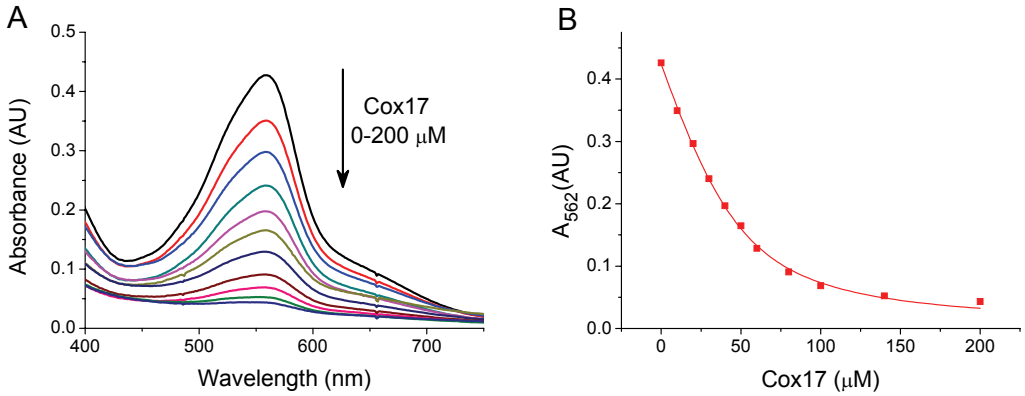

Fig. S9 (A) The UV-visible spectra of $[Cu^{I}(BCA)_2]$ titrated with 0-200 μM Cox17 in 20 mM MES buffer, pH 6.0. The total concentrations of Cu^{I} and BCA were 50 and 300 μM respectively. (B) Variation of the absorbance of $[Cu^{I}(BCA)_2]$ solutions at 562 nm with increasing concentrations of Cox17. Data was fitted using nonlinear least-square method.