Supplement

Cisplatin binds human copper chaperone Atox1 and promotes unfolding *in vitro*

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Experimental details (protein preparation, mass spectrometry, Pt NMR)

In vitro thermal unfolding experiments

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Figure legends

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Figures S1-S12

Experimental details

Protein preparation

The procedure for expression and purification of Atox1 is based on M.A. Kihlken et al. (1) with some modifications. The Atox1 gene identity was confirmed by sequencing. Atox1 in a pET21B vector was transformed into BL21(DE3)pLysS cells by electroporation (1700 V, 6 ms). The cells were grown in pre-warmed 2YT at 37 °C in 45 minutes. The culture was plated on LB agar plates (ampicillin, chloramphenicol) and grown over night at 37 °C. One colony was transferred to a test tube with 5 ml LB with 50 mg/L ampicillin and 34 mg/L chloramphenicol. The culture was incubated with shaking at 37 °C for 7 hours. 1 ml culture was transferred to each of two flasks containing 25 ml LB media with 50 mg/L ampicillin and 34 mg/L chloramphenicol. The flasks were incubated with shaking at 37 °C over night. 20 ml culture was transferred to each of 2 L pre-warmed LB media with 50 mg/L ampicillin and 34 mg/L chloramphenicol. The flasks were shake-incubated at 37 °C, 270 rpm. When OD_{600} reached 0.6 the culture was induced with IPTG to a final concentration of 1 mM. The flasks remained shaking at 37 °C for between 4 and 20 hours. The cells were harvested by centrifugation (7000 rpm, 4 °C, 5 minutes).

Pellets were re-suspended in total 80 ml media and transferred to two falcon tubes, which were centrifuged for 5 minutes, 7000 rpm in 4 °C. The supernatant was discarded and the pellet was put in the freezer over night or directly used in next step. The pellet was thawed on ice and thoroughly dissolved. A protease inhibitor cocktail pill was mashed and dissolved in 10 ml lysis buffer (20 mM MES, 1 mM EDTA, 2 mM DTT, pH 5.7), then added to the thawed pellet. The pellet was freeze thawed in three cycles by letting the falcon tubes alter between a EtOH/dry ice bath (-72 °C) and a beaker with room temperatured water (20 °C). DNaseI was added to a final concentration of 0.01 units/μl and $MgCl_2$ was added to a final concentration of 5 mM. The tubes were incubated on ice for 30 minutes. The content in the falcon tubes were combined and lysis buffer was added to a final volume of 100 ml. The suspension was mixed thoroughly and incubated on ice for 15 minutes. Cell debris was pelleted by centrifugation (20000 rpm, 4 °C, 30 minutes). The supernatants were combined and lysis buffer added to a final volume of 500 ml, it was

filtered through a 0.2 μm sterile filter. A SP Sepharose ion exchange column was equilibrated with 5 column volumes (CV) running buffer (20 mM MES, 1 mM EDTA, 2 mM DTT, pH 5.7). The cell extract was loaded and the column was washed with 5 CV running buffer. The protein was eluted with a salt gradient over 10 CV eluting buffer (20 mM MES, 1 mM EDTA, 2 mM DTT, 1 M NaCl, pH 5.7).

Fractions containing protein with the right mass according to SDS-page were combined and concentrated with filter tubes by centrifugation (4 °C, 7000 rpm) to < 5 ml. A S30 Superdex gel-filtration column was equilibrated with running buffer (20 mM MES, 50 mM NaCl, 2 mM DTT, pH 6). The protein concentrate was loaded on the column and Atox1 was eluted as a single peak with 1.2 CV running buffer. Fractions were combined and concentrated (as described above) and purity was confirmed by SDS-page (yield ≈ 10-15 mg/L culture). Absorbance (ε_{280} = 3,105 $M^{-1}cm^{-1}$) was used to determine protein concentration. For ^{15}N labeled Atox1 the above protocol was followed with the following modifications. Transformed cells with Atox1 was taken from a glycerol stock and grown in M9 minimal media containing ^{15}N-labeled NH_4Cl.

Mass spectrometry

Three samples were made for mass spectroscopy analysis: apo-Atox1, Atox1+1 molar eq. cisPt, and Atox1+5 molar eq. cisPt. All samples had a protein concentration of 100 μM. The Atox1 stock (0.8 mM in 20 mM MES, 50 mM NaCl, 2 mM DTT, pH 6) was diluted with a no-salt buffer (20 mM MES, 2 mM DTT, pH 6). Cisplatin (6.7 mM, made as described before) was added to two of the samples to get a final cisPt concentration of 100 μM (1 eq.) and 500 μM (5 eq.) respectively. A Micromass Q-Tof Ultima (Waters Micromass MS Technologies) hybrid tandem mass spectrometer was used for the acquisition of electrospray ionization (ESI) mass spectra. This instrument is equipped with a nanoflow electrospray source. The samples were infused into the mass spectrometer using nanoES capillaries (Proxeon Biosystems, Denmark). The needle voltage was ~2100 V, and the collision energy was 8 eV for the MS analyses. A desalting step, required to exchange the MES-buffer, or for unreacted cisplatin removal, was performed by microsolid phase extraction using ZipTips C18 (Milliore), eluting the samples in a 70 %

ACN, 0.1 % formic acid directly prior the analysis. The Atox1 samples were diluted to approximately 25μM concentration in 50 % ACN, 0.1% formic acid prior to injection. Data analysis was accomplished with a MassLynx data system and Transform deconvolution software MaxEnt 1 (Waters Micromass MS Technologies)

^{195}Pt NMR details

^{195}Pt NMR experiments were conducted at 20°C on a Bruker DRX 500 instrument equipped with a Bruker 10-mm broadband probe, or at 22°C on a Chemagnetics CMX 400 instrument equipped with a 10-mm wideline probe. Spectra were acquired with a spectral sweep width of 100 kHz (DRX) or 500 kHz (CMX), and 4096 complex points. Spectra of cisPt solution were acquired by accumulating 20k scans with a relaxation delay of 20 ms (total acquisition time 24 min). Spectra of mixtures of 1 mM cisPt and Atox1 at a 1:1 molar ratio were acquired by accumulating 300k scans with a relaxation delay of 100 ms (total acquisition time 9h). Use of the longer relaxation delay did not reveal new features in the spectral range -1500 to -4000 ppm, such as resonances due to adduct species with potentially longer T_1 relaxation times, compared to spectra acquired by accumulating $2x10^6$ scans and a relaxation delay of 20 ms. Data processing included an exponential apodization of 100-500 Hz and a polynomial baseline correction. Chemical shifts were referenced using an aqueous solution of $PtCl_6$.

Thermal resistance of metal-Atox1 complexes

Thermal stability was probed by following the CD signal at 220 nm from 20 to 90 °C (scan rate 0.5 °C/min). To check for reversibility, samples were cooled with the same parameters and then reheated. Thermal curves were analyzed using a two-state model (2) to obtain T_m, the thermal midpoint.

Thermal experiments were performed with apo-Atox1, Cu-Atox1, apo-Atox1+cisPt (1:5 molar ratio) and Cu-Atox1+cisPt (1:5 molar ratio). The negative CD spectrum in the far-UV region reports on the

secondary structure content in a protein and is a useful measure of protein unfolding as an unfolded polypeptide has little signal in this wavelength range. Earlier work on apo- and Cu-forms of Atox1 showed that the protein is stable in both forms: thermal unfolding reactions are reversible two-state processes with the holo-form being more stable than the apo-form but with similar cooperativity (3)). We find that the apo-Atox1+cisPt sample unfolds with a lower T_m than that for the apo-form without cisPt added and that the transition is broad (**Fig S10A**). Whereas apo-Atox1 heating is reversible, in the presence of cisPt the process is completely irreversible in terms of return of negative CD signal at 220 nm. Nonetheless, no precipitation is observed after heating and cooling. Heating of the Cu-Atox1+cisPt sample, like for apo-Atox1+cisPt, gives rise to a broad thermal transition that is irreversible and the midpoint is shifted to a lower temperature as compared to for Cu-Atox1 (**Fig S10B**). However, in contrast to the apo-Atox1+cisPt sample, heating of Cu-Atox1+cisPt results in precipitation upon cooling. Taken together, Atox1 (apo- and Cu-form) appears thermally destabilized in presence of excess cisPt; these results give further support that the metal interacts with the Atox1 polypeptide in solution at these concentrations and conditions.

Cell line studies of Atox1 expression

Cell culture. The human melanoma cell line T289 (4) and the human ovarian cancer cell line 2008 (5) were grown in RPMI-1640 supplemented with 5 % fetal bovine serum. One million cells were grown in T75 flasks in monolayer culture at 37˚C in humidified air with 5% CO_2. After four days of growth the cells were ninty percent confluent (9×10^6 cells) and at that time exposed to cisplatin 20 μM for 2h.

The cells were lysed by osmosis for 1h (6) and total protein concentration was determined by colorimetric assay. Aliquots of 35 μg protein/lane were separated on a 15 % gel and transferred to immobilon-P filters prior to Atox1 detection with ECL by a rabbit anti-human Atox1 polyclonal antibody from Novus Biologicals (**Fig S9**). Loading control was performed by stripping of the filter in 1M Glycine-HCl (pH 2.5) for 10 min and 1M Tris-HCl (pH 7.5) for 10 min followed by detection of β-actin

by a mouse anti-actin antibody from SIGMA. Loading density was quantified by the Quantity One® 4.5.6 software.

Figure legends

Figure S1. A. ^{195}Pt NMR spectrum of the cisPt stock solution. The NMR sample contained 6.7 mM cisPt in 50 mM NaCl, 20 mM MES buffer at pH 6.2 and the experiment was acquired at 20 °C on a Bruker DRX 500 instrument operating at 107.3 MHz accumulating 20k scans over 9 h. Resonances at -1840 and -2150 ppm are assigned to cisPt in monoaqua and dichloro forms, respectively (7). **B, and C.** Sequential ^{195}Pt NMR spectra of 1 mM cisPt and Atox1 at a 1:1 molar ratio. The sample buffer contained 20 mM MES, 50 mM NaCl at pH 6.2 and 5 mM DTT. Spectra were acquired at 85.9 MHz on a Chemagnetics CMX 400 instrument accumulating 300k scans over 9 h with a relaxation delay of 0.1 sec (panel **B**, acquisition started 15 min after mixing). The resonance due to the dichloro form of cisPt at -2150 ppm is absent after 9 h (panel **C**).

Figure S2. ESI-MS de-convoluted spectra of a mixture of Atox1 and cisplatin. **A.** Apo-Atox1, **B.** mixture of 1:1 molar ratio of apo-Atox1 to cisPt and, **C.** 1:5 molar ratio of Atox1 to cisPt. Assigned peaks are labelled. Two forms of apo-Atox1 are indicated in the spectra, one without the first residue which is a Met (ApoAtox1) and one with Met still attached (ApoMetAtox1), respectively.

Figure S3. A. Near-UV CD monitored titrations of metals to apo/metallated Atox1 (pH 6, 20 °C) at high protein concentration (0.5 mM). CisPt (up to 5 fold excess as indicated) added to 500 µM Cu-Atox1. **B.** Cu (one eq.; higher amounts resulted in precipitation) added to 500 µM cisPt-Atox1 (1:1).

Figure S4. (Top) Decays of integrated peaks of selected assigned backbone amide resonances in the ^{1}H-^{15}N HSQC spectrum of 50 μM folded apo-Atox1 upon addition of 5 equivalents of cisPt. **(Bottom)** Histogram showing distribution of decay rates of apo-Atox1 HSQC resonances (disappearing due to cisPt-mediated protein unfolding).

Figure S5. Expansions of ^{1}H-^{15}N HSQC spectra of different Atox1 species. The NMR spectra are expanded around glycine 14 which is a marker for Cu binding to Atox1. (A) Apo-Atox1 (red contours, only glycine 14 is apparent in the expansion) superimposed on an apo-sample with 5 equivalents cisPt added (black contours). (B) Holo-Atox1 (red contours, only glycine 14 is apparent in the expansion) superimposed on a holo-sample with 5 equivalents cisPt added (black contours). The arrows indicate a non-assigned peak corresponding to transient cisPt-bound, folded Atox1. The chemical shifts of the transient peak are identical for cisPt loaded apo- and holo-Atox1.

Figure S6. Size-exclusion chromatography inductively coupled plasma mass spectrometry (SEC-ICPMS) chromatogram of Atox1 mixed with 10 molar equivalents of copper and cisplatin after 15 h at 37 °C. Color codes are: black, sulfur (i.e., protein); purple, copper; green, platinum. The intensities do not correlate with concentration and only qualitative conclusions can be made. Since all profiles overlap, it is clear that both copper and platinum interact with the protein at this condition. Free copper will not enter the column, and free cisPt would elute at around 16 min. The SEC-ICPMS system consisted of a 4.6 x 250 mm KW403-4F column (Shodex, Japan) connected to a PerkinElmer/Sciex Elan DRC-e ICPMS instrument. A mix of 30 mM TRIS-HCl

and n-propanol (80:20) was used as mobile phase at a liquid flow rate of 300 µl/min pumped with a PerkinElmer 200series standard HPLC system. (Reference: Y. Nygren PhD thesis Umeå University 2010, "Advances in analytical methodologies for studies of the platinum metallome in malignant cells exposed to cisplatin", ISBN 978-91-7459-085-2.)

Figure S7. Time-dependent cross-peak volumes from ^{1}H-^{15}N HSQC spectra of apo Atox1 (500 µM) mixed with 5 equivalents cisPt. **A**. Decay of selected apo Atox1 resonances fitted to mono-exponential decay functions (Equation 1). The best fitted curves are shown as a solid line for Met10, a dashed line for Cys15 and a dotted line for Cys41. **B**. Decay of cross-peaks assigned to the Atox1:cisPt complex. Best fitted curves to equation 2 are shown as solid lines.

Figure S8. Distribution of transverse relaxation rates in Atox1 metal complexes. Relaxation rates are displayed as histograms with 10 or 15 uniformly spread bins. Histograms are colored as follows: holo-Atox1 (blue), holo-Atox1 with 5 equivalents cisPt (orange), holo-Atox1 in 8 M urea (green).

Figure S9. SDS-Page gel of Atox1 samples (apo and holo forms) incubated at room temperature for various days without (A) and with (B) five equivalents of cisplatin**.** Lane contents from left to right: marker, apoAtox1 4 days, apoAtox1 2 days, apoAtox1 1 day, apoAtox1 4 hours, apoAtox1 fresh, marker, holoAtox1 4 days, holoAtox1 2 days, holoAtox1 1 day, holoAtox1 4 hours, holoAtox1 fresh.

Figure S10. A. Near-UV CD monitored titration of CisPt to Cu-Atox1 (pH 7.3, 37 °C, 160 mM NaCl, 250 µM Glutathione). CisPt (up to 5-fold excess) added to 50 µM Cu-Atox1. **B.** Far-UV CD spectra as a function of time for Cu-Atox1 (50 µM) mixed with 5 eq CisPt and incubated at 37 °C (pH 7.3, 160 mM NaCl, 250 µM Glutathione). **C.** CD changes at 220 nm plotted as a function of time from **Figure S10B** (red), together with selected data from **Fig 2B**.

Figure S11. Increased expression of Atox1 in resistant human tumor cells. The human melanoma cell line T289 is five-fold more resistant to cisplatin then the human ovarian cancer cell line 2008(4, 5). Atox1 expression in unexposed T289 cells (T289 C) and T289 cells exposed for 2 hours with 20 µM cisplatin (T289 DDP) is 3.3 fold higher than in unexposed 2008 cells (2008 C) and 2008 cells exposed for 2 hours with 20 µM cisplatin (2008 DDP).

Figure S12. Thermal stability of various Atox1-metal complexes. **A.** Thermal unfolding curves monitored by CD at 220 nm for apo-Atox1 and (apo-Atox1+5 eq cisPt). Whereas the reaction is reversible for apo-Atox1, it is irreversible for the (apo-Atox1+5 eq cisPt) sample. **B.** Thermal unfolding curves monitored by CD at 220 nm for Cu-Atox1 and (Cu-Atox1+5 eq cisPt). Whereas the reaction is reversible for Cu-Atox1, it is irreversible for the (Cu-Atox1+5 eq cisPt) sample and precipitation is observed.

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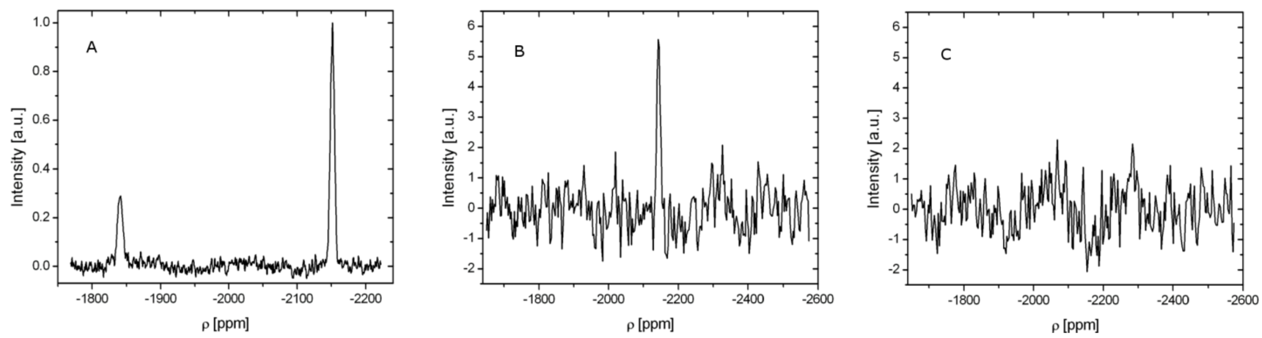
Figure S1



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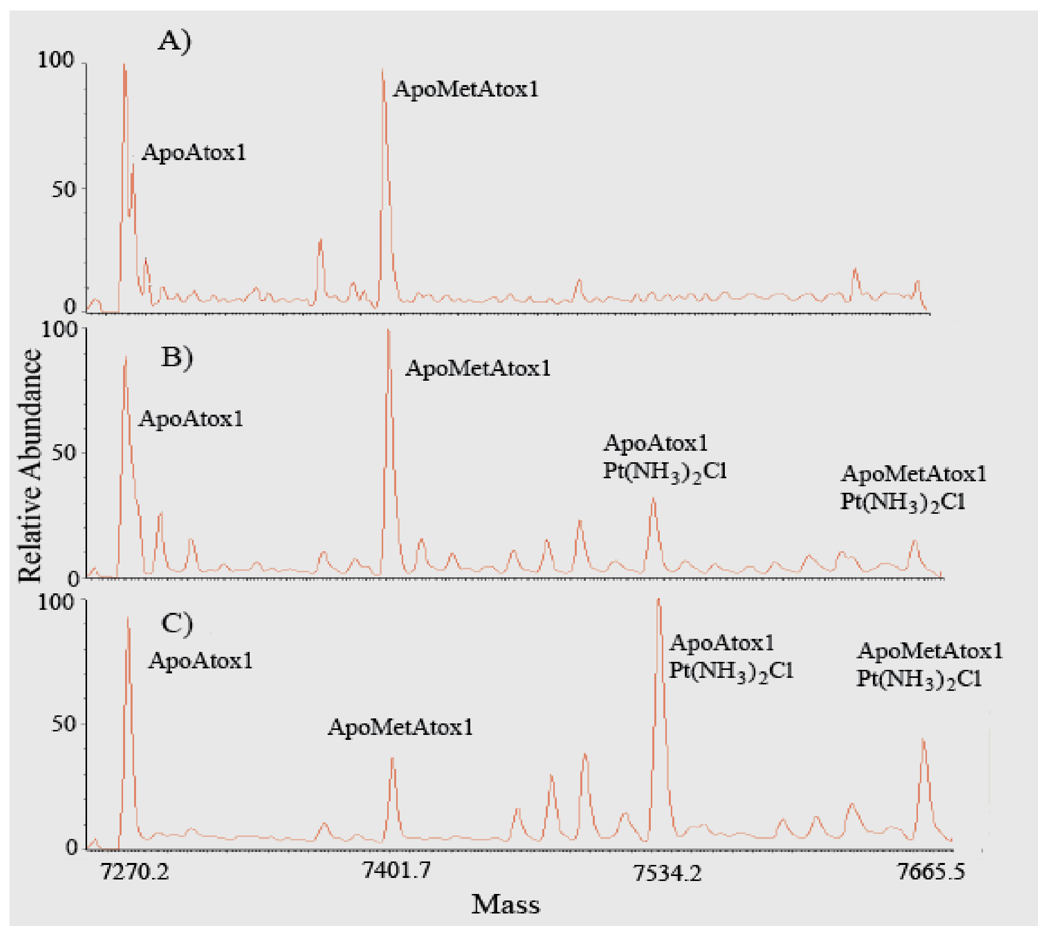

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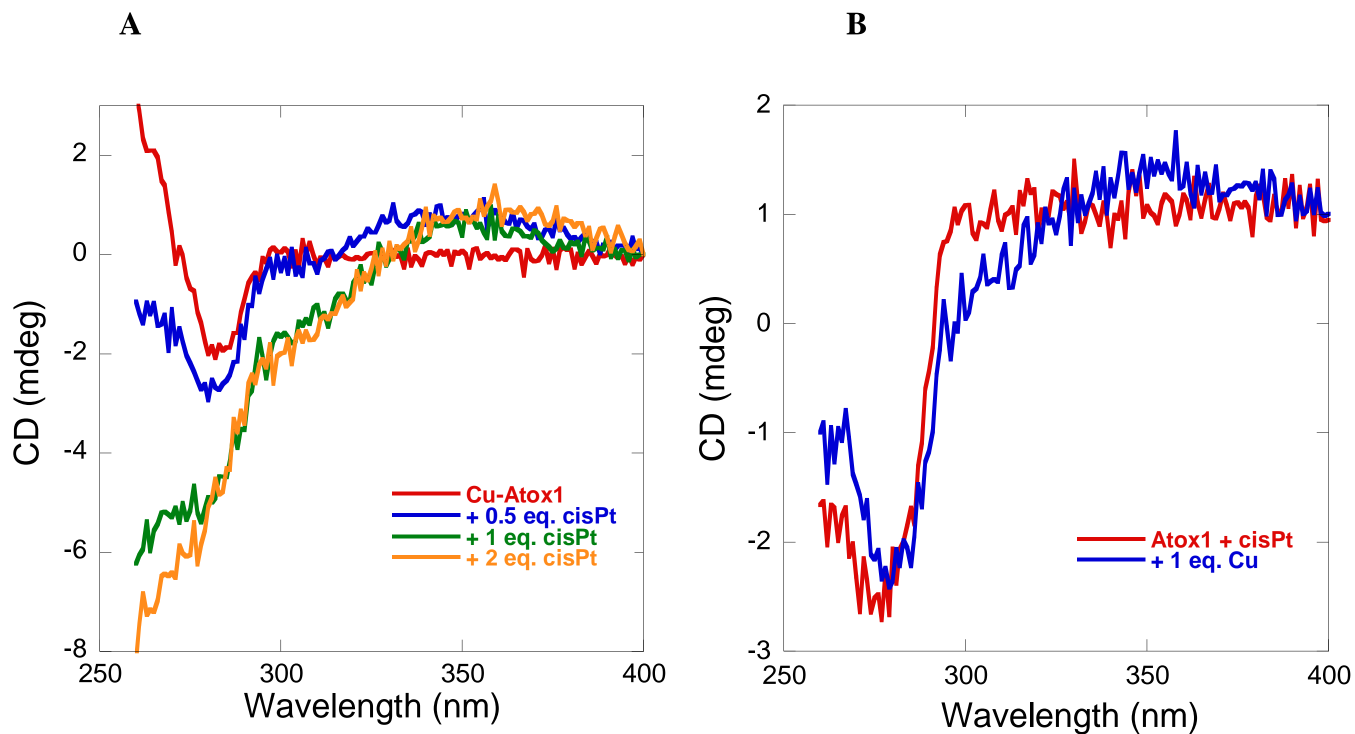

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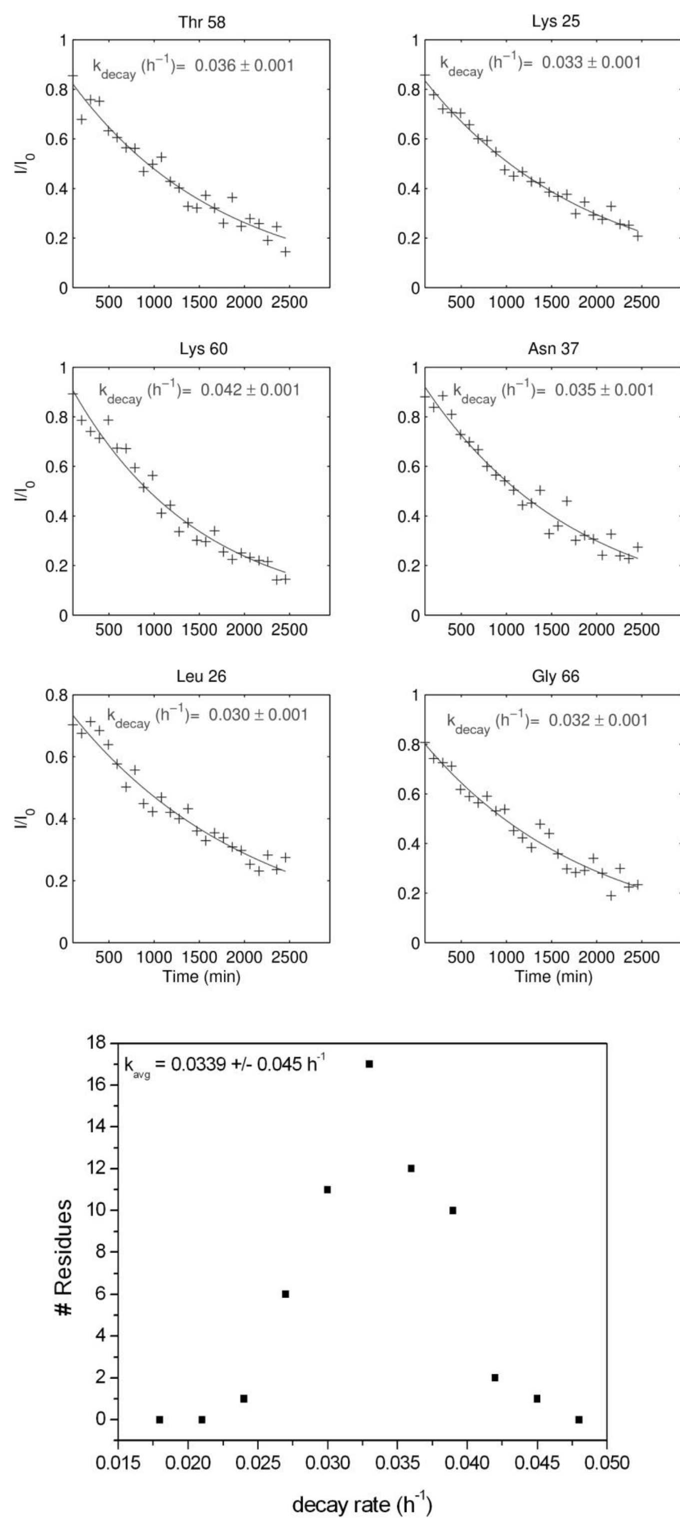

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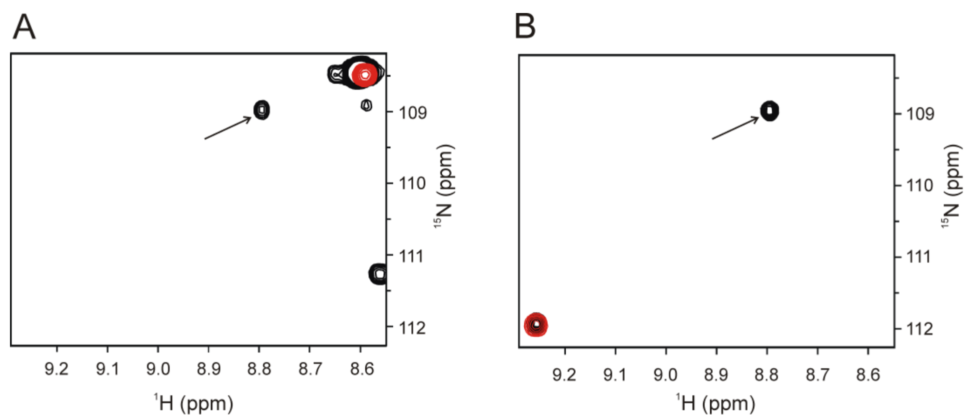

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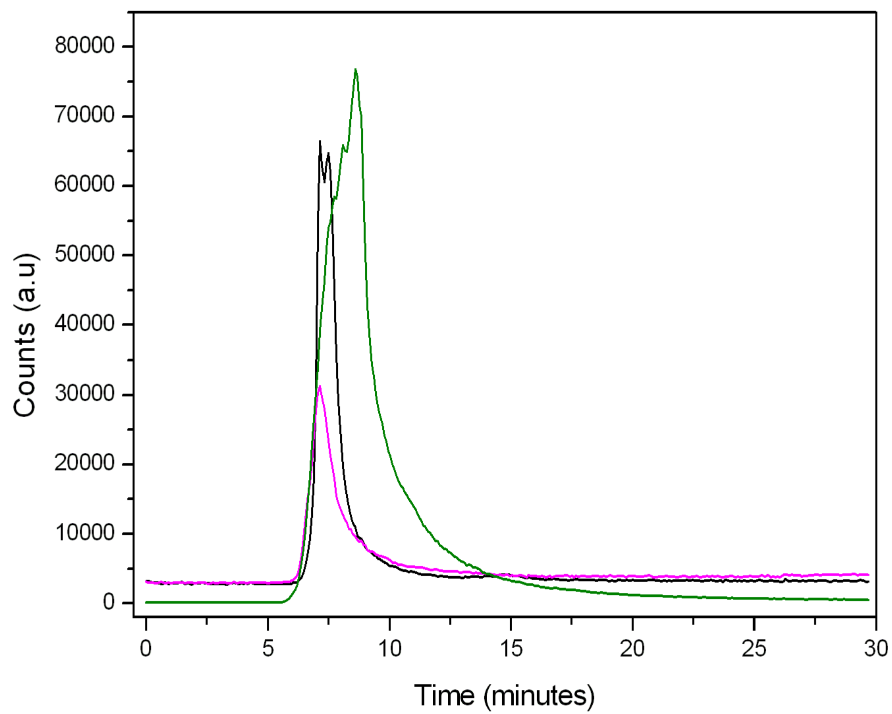

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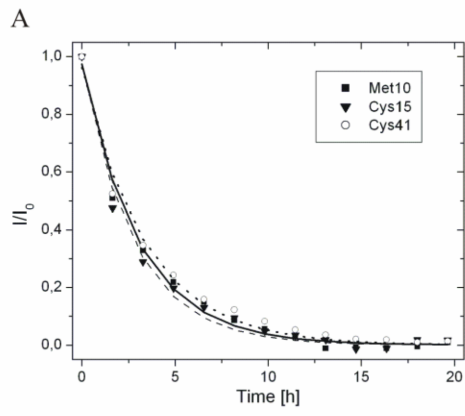

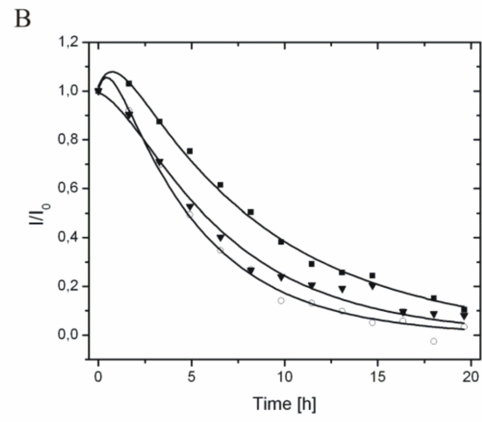

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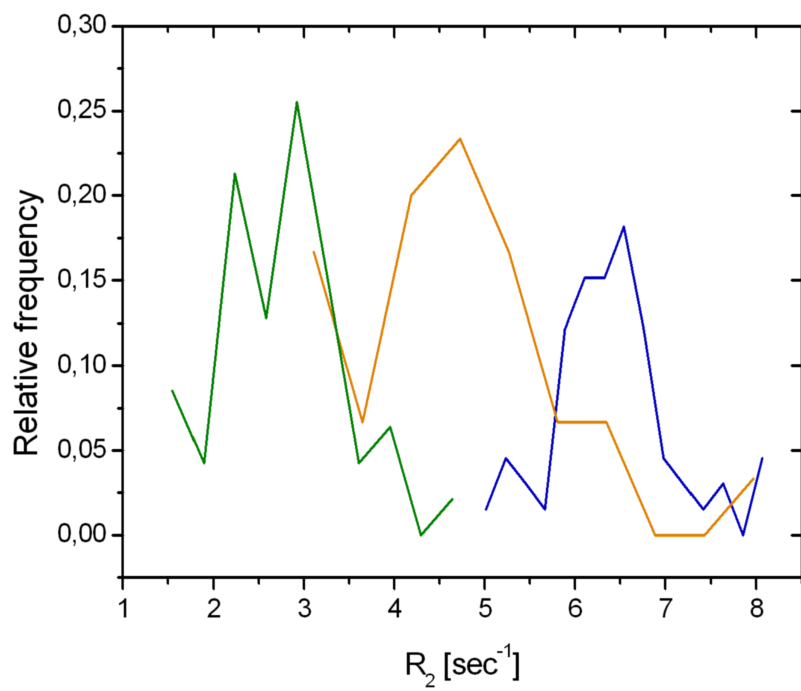
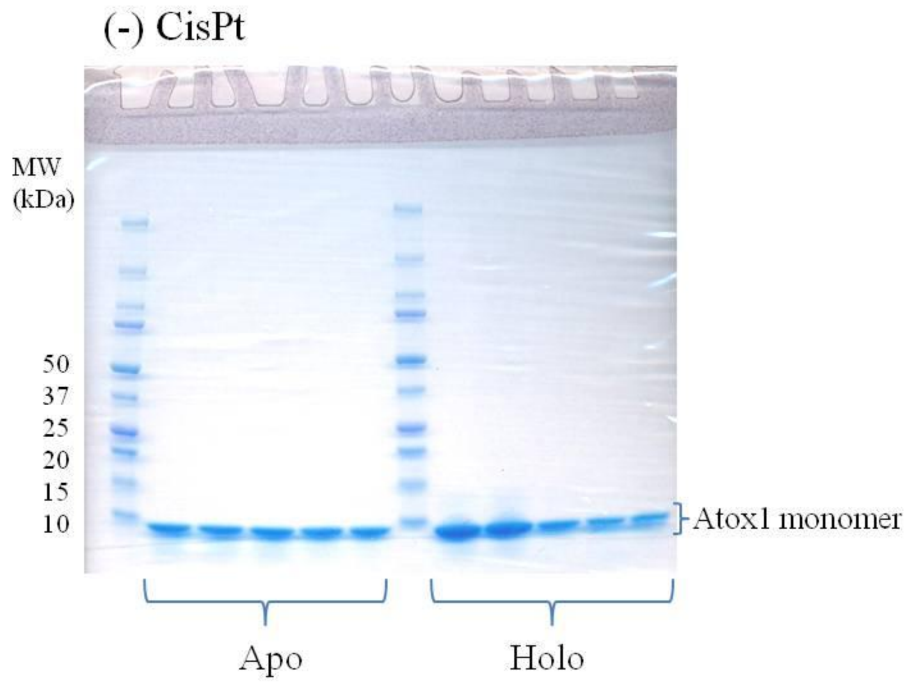


Figure S9

A



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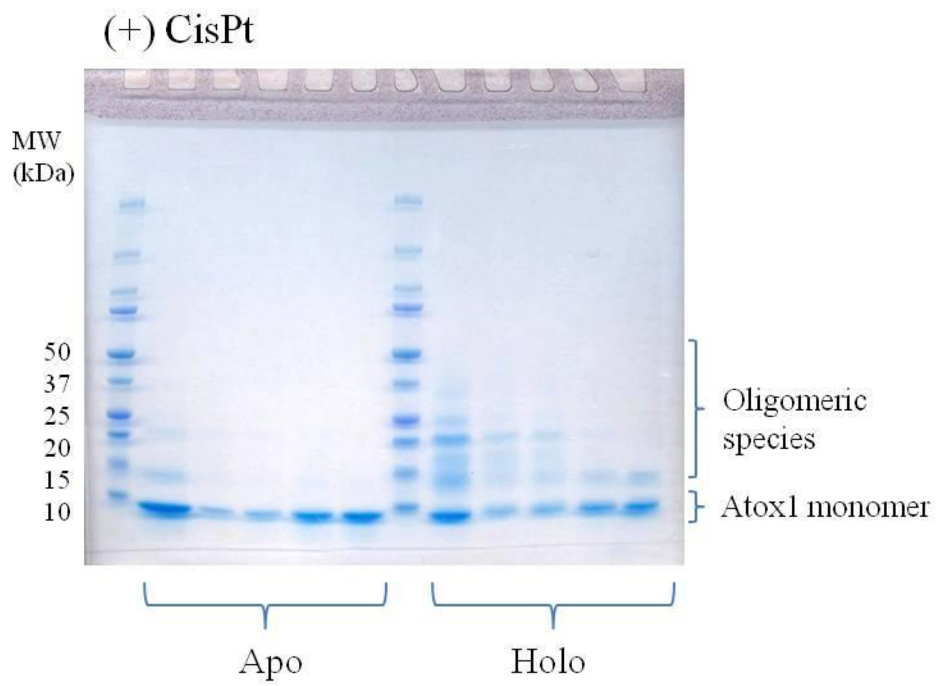

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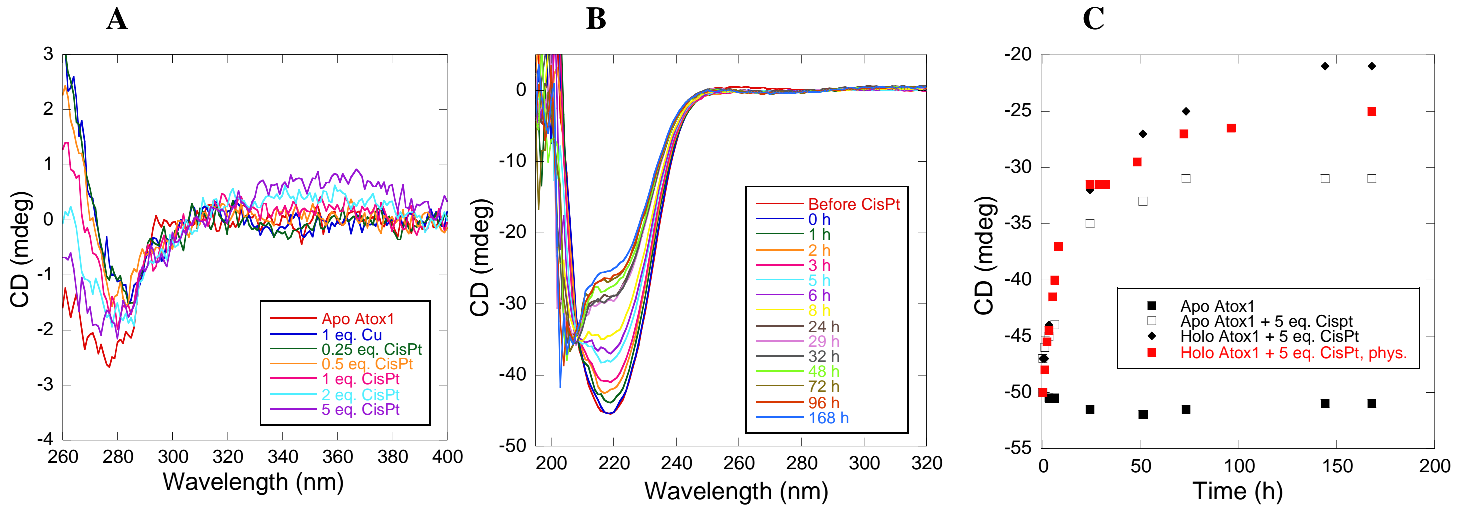

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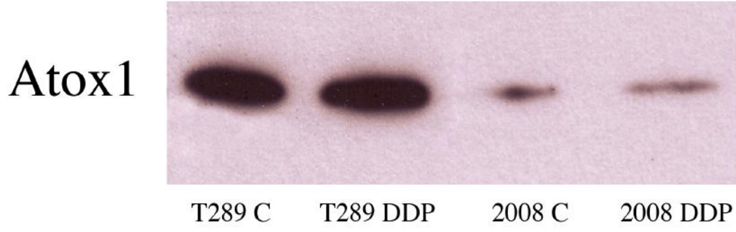

Figure S12

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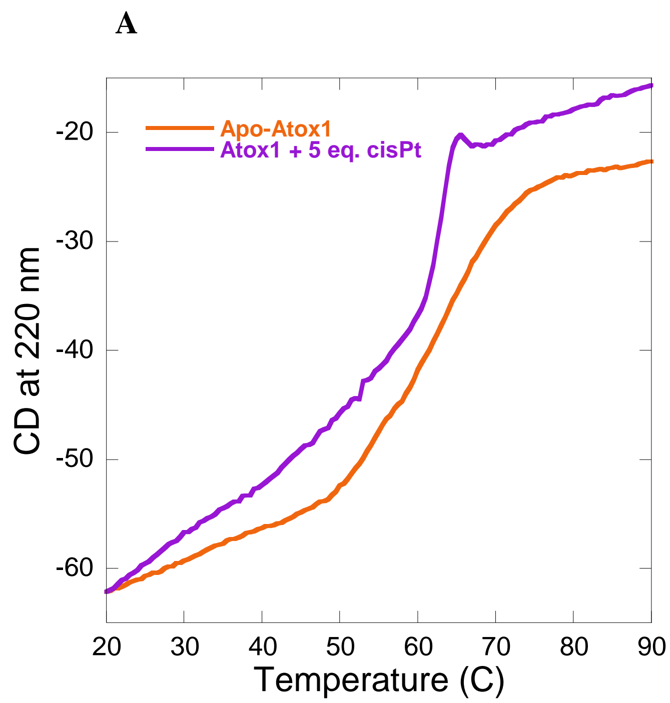

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