Electronic Supplementary Information

Arsenic binding to human metallothionein-3

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Experimental

MT3 Expression

Recombinant human metallothionein 3 (MT3) was cloned into pET29a plasmids using NcoI and HindIII restriction enzymes (GENEWIZ from Azenta, San Francisco, USA). BL21(DE3) *Escherichia coli* was transformed with the plasmid by heat-shocking for 45 seconds at 42 °C and subsequent growth in SOC media at 37 °C. Transformed cells were grown to an OD_{600} of 0.6 and glycerol stocks containing 9:1 (v/v) cell culture/80% glycerol were prepared and stored at -80 °C. The sequence is as follows (with GS in the N-terminus remaining from the S-tag coded in the plasmid): GSMGKAAA MDPETCPCPSGGSCT CADSCKCEGC KCTSCKKSCC SCCPAECEKC AKDCVCKGGE AAEAEAEKCS CCQKKAAAA. The mass of apo-MT3 is 8211 Da.

A mini-prep was prepared from the glycerol stock in LB broth (BioShop, Toronto, Canada) with 50 mg mL^{-1} kanamycin sulfate (AG Scientific, San Diego, USA) and incubated in a cell shaker at 37 °C overnight. The cell culture was then transferred to 4 x 1 L LB broth cultures containing 1 mL of 50 mg mL^{-1} kanamycin sulfate and 100 µL of 1 M $CdSO_4$ solution. When OD_{600} reaches 0.4, isopropyl-β-D-thiogalactoside (IPTG, BioShop, Toronto, Canada) was added to a final concentration of 0.4 mM. Cells were grown for 30 minutes before 400 µL of 1 M $CdSO_4$ was added to each flask and incubated for 3 hours and 30 minutes. Cells were pelleted through centrifugation (Avanti J-26 XPI fixed rotor centrifuge, Beckman Coulter, Toronto, Canada) and suspended in argon-saturated and pH 7.4 tris-hydroxymethyl-aminomethane hydrochloride (Tris-HCl) buffer (Sigma Aldrich, St. Louis, USA) and stored at -80 °C.

MT3 Purification

Cells were thawed and lysed using a cell homogenizer (Constant Systems, Hull, UK) under pressures of 18-22 kPa. Bovine DNAse (Sigma Aldrich, St. Louis, USA) was used in the cell lysate following the manufacturer's instructions. Cell debris was pelleted by centrifugation and the supernatant was isolated and acidified to precipitate proteolytic proteins. The supernatant was again isolated and saturated with $Cd(OAc)_2$ before pH adjustment to 8.6 to allow excess $Cd(OH)_2$ to precipitate and pelleted. The supernatant containing Cd-bound MT3 was pH adjusted to pH 7 and pushed through a DEAE anion exchange column. The flow-through was collected and concentrated using a stirred ultrafiltration cell (EMD Millipore, Burlington, USA) with a 5 kDa cellulose membrane.

The S-tag was removed with Thrombin CleanCleave™ kit (Sigma Aldrich, St. Louis, USA) for 12 hours at 4 °C and the protein was isolated and pH adjusted to pH 8.6 before loading on five consecutive 5 mL HiTrap™ Q Sepharose™ anion exchange columns (GE Healthcare, Chicago, USA). MT3 was isolated using an elution gradient of 20 mM Tris-HCl with 1 M NaCl via high-performance liquid chromatography (Dionex UltiMate 3000, Germany) and identified by absorption at 250 nm (Cary 50, Agilent Technologies, Santa Clara, USA). MT3 was concentrated and stored at -80 °C.

Apo-MT3 Preparation

MT3 was thawed and demetalated using deaerated and argon-saturated 20 mM ammonium formate adjusted to pH 2 and buffer-exchanged using Amicon Ultra-4 centrifugal tubes (5 kDa MWCO, Millipore, Burlington, USA) according to manufacturer instructions. Apo-MT3 was then desalted and pH adjusted through buffer exchange with 20 mM Tris-HCl at pH 7.4 or pH 3.5 depending on experimental parameters with 1 mM tris(2-carboxyethyl)phosphine (TCEP)-HCl (Soltec Ventures, Beverly, USA). Concentrations of MT3 were determined using $\varepsilon_{250\ nm}$ = 89 000 M^{-1} cm^{-1} in the presence of excess Cd^{2+}.

Solution Preparation

Arsenic trioxide (As_2O_3) (AnalaR, BDH, USA) was weighed and dissolved in dilute NH_4OH (Sigma Aldrich, St. Louis, USA) and pH adjusted to pH 7.4 or pH 3.5 using dilute HCl (Caledon, Ontario, Canada) to 10 mM concentrations.

ESI-MS Procedures

All ESI-mass spectra were collected using a Bruker Micro-TOF II spectrometer (Bruker Daltonics, Toronto, ON) and calibrated using the Bruker calibration mix on positive ion mode. The settings are as follows: scan = 1000-3000 m/z; rolling average = 2; nebulizer = 2 bar; dry gas = 130 °C at 6 L min^{-1}; capillary = 3500 V; end plate offset = -500 V; capillary exit = 175 V; skimmer 1 = 30.0 V; skimmer 2 = 23.5 V; hexapole RF = 800 V. Spectra were collected for 2 minutes and deconvoluted with the Bruker Compass Data Analysis software on maximum entropy mode.

For kinetic experiments, six replicates were done to ensure accuracy. The temperature of the syringe pump was recorded to be 304 K constantly at ambient temperature.

UV-visible Spectroscopy Procedures

All absorption spectra were collected using a Cary 60 UV-visible spectrophotometer (Agilent Technologies, Santa Clara, USA) with a Peltier Cell Holder Accessory (Agilent Technologies, Santa Clara, USA). Air baselines were used to zero the data collection software and spectra were buffer baseline-adjusted subsequent to data collection. The temperature was kept at a constant of 304 K, identical to the temperature recorded for the syringe pump where the metalation kinetics were measured for the mass spectra.

Kinetic Trace Fitting Procedures

The As^{3+} metallation data was transformed into concentration units, as a constant of 20 μM of protein was added for all experiments. Experimentally, we determined ε_{290nm} = 11 000 M^{-1} cm^{-1}. For mass spectra-based speciation, the percentage of each species was converted to concentration units with the total available protein set as 20 μM.

Copasi version 4.30 was used to fit the kinetic traces.[1] The data were fit as a sequence of bimolecular reactions for the mass spectral data and as a single bimolecular reaction for the absorption data. The six replicates of mass spectral kinetics of arsenic binding were individually

fitted and the standard deviations of the rate constants determined. The three replicates of kinetics of arsenic binding as determined by absorption spectroscopy were fitted individually and standard deviations were calculated.

Supplementary Figures

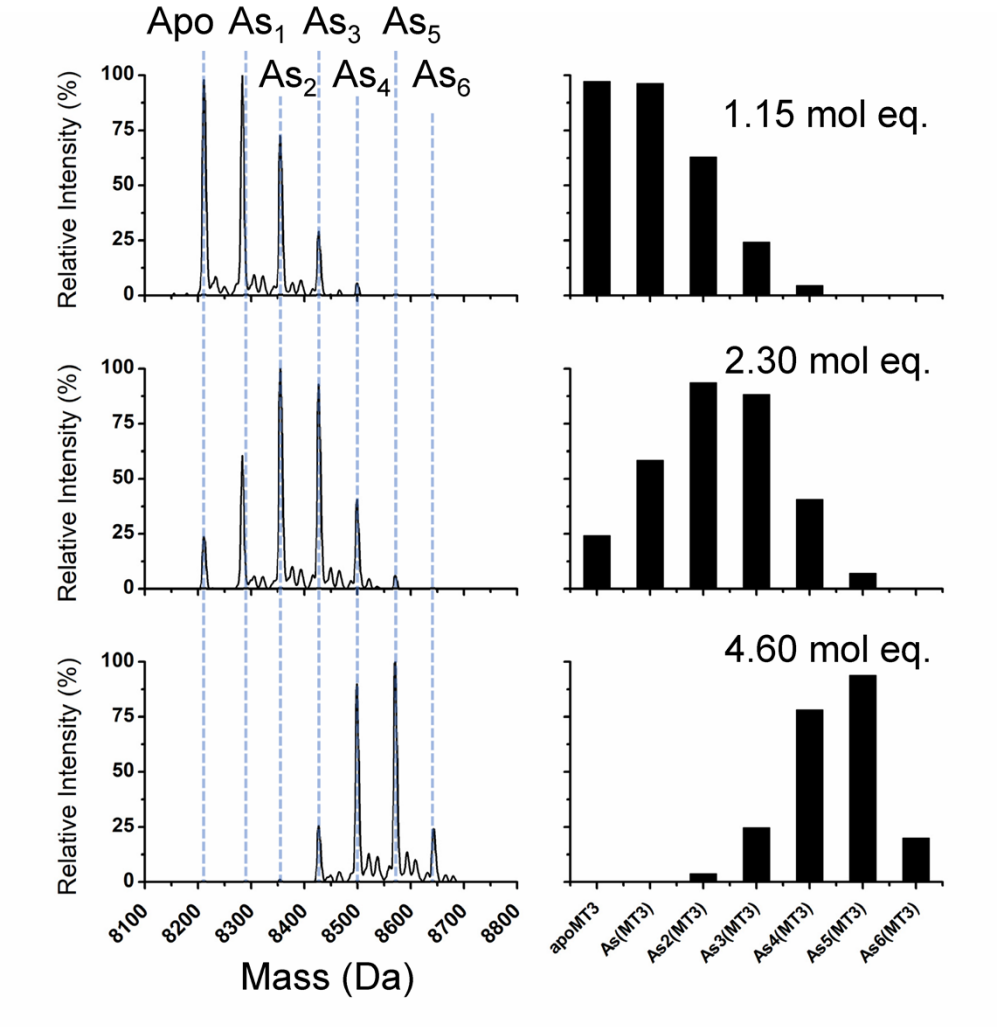

Figure S1 Corresponding mass spectra and simulated mass spectra generated from the log K values used by Hyperquad Simulation and Speciation (HySS) software.

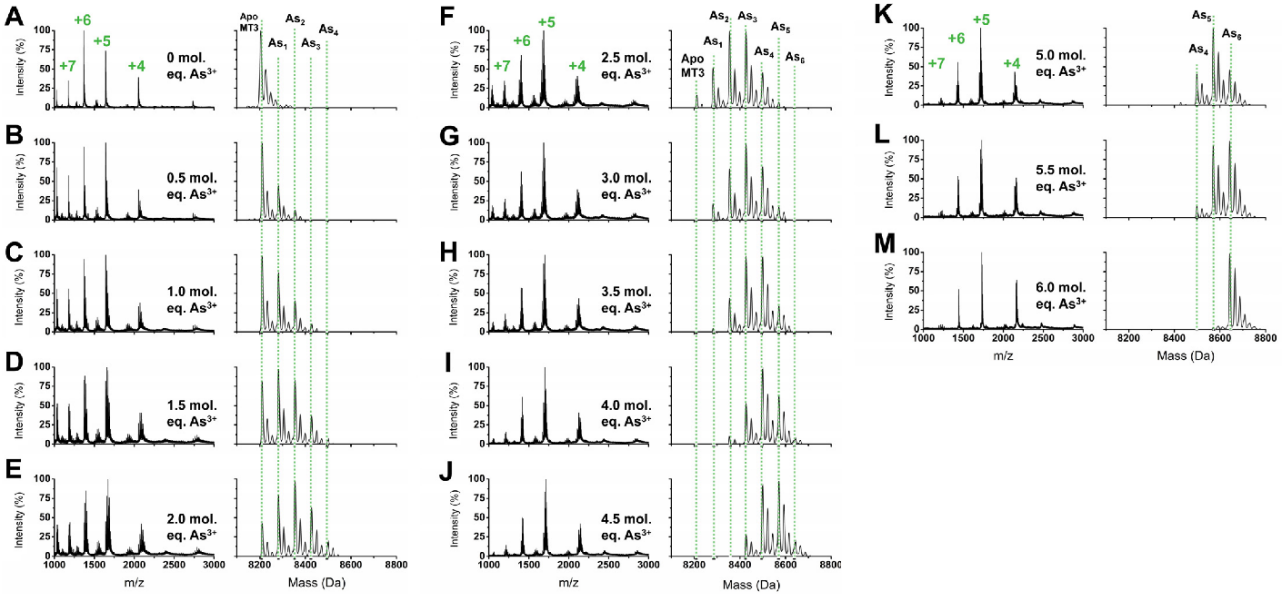

Figure S2 As^{3+} stepwise titration into apo-MT3 at pH 7.4. Charge state spectra (left panels) and deconvoluted spectra (right panels) for As^{3+} at 0 (A), 0.5 (B), 1.0 (C), 1.5 (D), 2.0 (E), 2.5 (F), 3.0 (G), 3.5 (H), 4.0 (I), 4.5 (J), 5.0 (K), 5.5 (L), and 6.0 (M) molar equivalents added. All spectra were recorded for 2 minutes at ambient temperature.

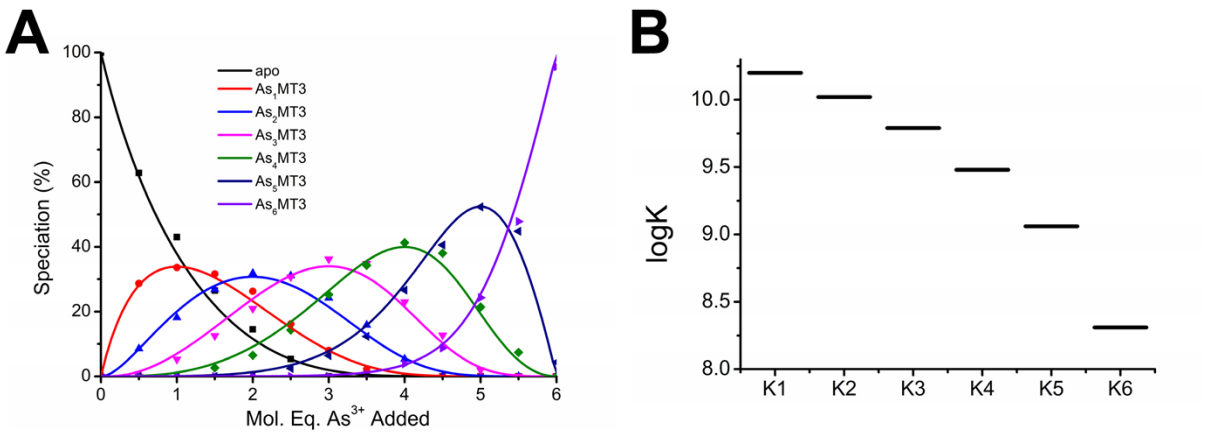

Figure S3 (A) Speciation of As_nMT3 species as a function of mol. eq. As^{3+} added to apo-MT3 at pH 7.4. Dots represent experimental data and solid lines represent model fitted to data. The model was generated by Hyperquad Simulation and Speciation (HySS) software. (B) Corresponding relative binding constants (log K) inputted to HySS.

Supplementary Tables

Supplementary Table S1. A sample of data of species present generated using log K values of As^{3+} binding to MT3 as determined by HySS and the known log β value for $As(GS)_3$. The starting conditions are 1 M of MT3 and 3 M of GSH, the same ratio as experimental conditions. The titration point of 0.7 M As^{3+} was picked as an arbitrary point in the titration to show the difference in free As^{3+} available with both MT and GSH in solution, MT only in solution, and GSH only in solution.

	total As	free As	free MT3	free GSH	$As(GS)_3$	As(MT3)	As_2(MT3)	As_3(MT3)	As_4(MT3)	As_5(MT3)	As_6(MT3)
MT + GSH	0.7	3.91E-09	5.15E-01	2.97E+00	1.02E-02	3.19E-01	1.30E-01	3.14E-02	3.71E-03	1.66E-04	1.33E-06
MT only		3.96E-09	5.10E-01			3.20E-01	1.33E-01	3.25E-02	3.88E-03	1.77E-04	1.43E-06
GSH only		9.60E-06		9.00E-01	7.00E-01						

References

1. S. Hoops, S. Sahle, R. Gauges, C. Lee, J. Pahle, N. Simus, M. Singhal, L. Xu, P. Mendes and U. Kummer, *Bioinformatics*, 2006, **22**, 3067-3074.