SUPPORTING INFORMATION

Copper trafficking in the CsoR regulon of Streptomyces lividans

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MATERIALS AND METHODS

Preparation of the CopZ-1317 mutants

The CopZ-1317 nucleotide and amino acid sequences. Coloured red are the codons to be changed to create the respective mutations.

ATG	CCC	TCG	AAC	GTC	ACC	GCC	CCC	GTC	ACC	ACC
М	Ρ	S	Ν	V	Т	А	Ρ	V	Т	Т
GCC	TAC	GCC	GTC	GCC	GGC	ATG	AGC	TGC	GGT	CAC
А	Y	А	V	А	G	М	S	С	G	Н
TGC	AGC	GCG	ACC	CTC	ACC	CGG	GTG	ATC	GGC	GAG
С	S	А	Т	L	Т	R	V	I	G	Е
CTG	GAC	GGC	GTG	ACC	GGC	GTC	GAC	GTC	CAA	CAC
L	D	G	V	Т	G	V	D	V	Q	L
GAC	ACC	GGC	CGG	GTC	ACC	GTC	ACC	GCC	GAC	GCG
D	Т	G	R	V	Т	V	Т	A	D	А
GAG	CCG	GAC	GAC	GCC	GCG	ATC	GCC	GAG	GTG	GTC
E	Р	D	D	А	А	I	А	Ε	V	V
GAC	GAA	GCC	GGG	TAC	GAG	CTG	ACC	GGC	CGC	GTC
D	Ε	А	G	Y	Ε	L	Т	G	R	V
TGA										
*										

The forward and reverse mutagenic primers for the H22G and the Y71F mutants were as follows:

H22G mutant

CAC - GGC His to Gly

25-Mers, $T_m = 62 \text{ °C}$, 72 % GC-content

F-5'-CATGAGCTGCGGTGGCTGCAGCGCG-3'

R-5'-CGCGCTGCAGCCACCGCAGCTCATG-3'

 $\frac{Y71F \text{ mutant}}{TAC - TTC}$ Tyr to Phe

25-Mers, $T_m = 62 \text{ °C}$, 72 % GC-content

F-5'-CGAAGCCGGGTTCGAGCTGACCGGC-3'

R-5'-GCCGGTCAGCTCGAACCCGGCTTCG-3'

Concentrations of plasmid DNA (pET28a containing the gene for CopZ-1317) and primers used for PCR were 15 and 75 ng/ μ l respectively. The PCR reaction volume was 30 μ l, consisting of 1 μ l of plasmid DNA, 1 μ l of each forward and reverse primer, 0.6 μ l of 10 mM dNTPs (Fermentas), 0.6 μ l of PFU Turbo polymerase (Stratagene), 3 μ l of 10 X PFU Buffer (Stratagene) and 22.8 μ l of deionized water.

The following program was used to carry out the PCR:

- 1. 95 °C 3 min
- 2. 95 °C − 30 s
- 3. 58 °C 1 min
- 4. 68 °C − 13 min
- 5. Repeat steps 2-4 15 times
- 6. 72 °C 8 min
- 7. 10 °C hold

Determination of Cys pK_a values using Badan

Reduced apo-CopZ proteins (~ 400 μ M) were prepared in an anaerobic chamber in 5 mM MOPS pH 7.5, 25 mM KCl, together with a series of mixed buffer systems containing 10 mM each of potassium acetate, MES, MOPS, Tris and 200 mM KCl with the pH of each solution individually adjusted in increments of 0.5 from pH values of 3 to 10. A 10 mM stock solution of 6-bromoacetyl-2dimethylaminonaphthalene (Molecular Probes) more commonly known as Badan was prepared in DMSO. Under anaerobic conditions reduced apo-proteins were added to a final concentration of $\sim 1 \ \mu M$ to each buffered solution followed by addition of Badan to a final concentration of 13 µM in a reaction volume of 3 ml. The change in fluorescence emission of Badan at 540 nm was monitored every 10 s (excitation wavelength 391 nm) at 20 °C for 1 h on a Perkin-Elmer LS55 fluorescence spectrophotometer. Time courses measured at the different pH values were fitted to either a single or double exponential function to obtain an initial pseudo-first order rate constant (k_0), which was plotted as a function of pH. p K_a values were obtained by fitting the data to equation 1,

$$k_0 = k_{SH} + \frac{k_{S^-} 10^{pH - pK_a}}{1 + 10^{pH - pKa}} \tag{1}$$

describing a single protonation/deprotonation process, where k_o is the initial pseudofirst order rate constant and k_{SH} , k_{S} - are the rate constants for the various protonated and deprotonated forms of the protein. The pK_a values reported are an average of multiple data sets and the error is the standard deviation.

RESULTS

It has been well documented that Cys residues in the ionized thiolate anion form readily react with alkylating reagents, and the measurement of the rate of alkylation at varying pH values can be used to determine the pK_a of Cys thiol groups ¹. The alkylating reagent Badan is a fluorescent probe that has been successfully used in previous studies with proteins containing a CXXC motif to obtain pK_a values for the Cys residues ²⁻⁶. On forming a thioether bond to a solvent exposed Cys residue the fluorescence emission of Badan at ~540 nm significantly increases. The appearance of a second emission peak at a lower wavelength has also been reported and is indicative of Badan binding to a more buried Cys². For both CopZ proteins only a single emission peak at ~540 nm was observed upon reacting with Badan. Representative plots of the fluorescence emission increase at 540 nm as a function of time at varying pH values are illustrated in Figure S1A and C. At certain pH values the fluorescence intensity decays slightly after the initial increase and this has been attributed to local unfolding caused by the addition of two labels to the MXCXXC site². This effect makes it difficult to extract rates of the initial modification phase and was particularly problematic for CopZ-3079 above pH 7.5. For CopZ-1317 initial pseudo-first order rates could be obtained from fits of the data for pH values between 4 and 9, with initial rates above pH 9 again difficult to extract due to the biphasic nature of the time course. The pseudo-first order rate constants for each CopZ protein are plotted as a function of pH in Figures S1B and C with the data fitting well to a single protonation/deprotonation process (equation 3) with average pK_a values of 7.6 (0.2) for CopZ-1317 and 7.4 (0.2) for CopZ-3079 determined.



Figure S1: Determination of the pK_a properties of the Cys thiols by the fluorescent alkylating reagent Badan. Fluorescence intensity traces at 540 nm as a function of time following the addition of apo-CopZ-1317 (A) and apo-CopZ-3079 (C) at different pH values, as indicated. Lines represent a fit to an exponential function to obtain a pseudo first-order rate constant (k_o) at the different pH values, and plotted in (B) for CopZ-1317 (D) for CopZ-3079. The lines show the fits to equation1 and give pK_a values for these data sets of 7.8 for CopZ-1317 and 7.4 for CopZ-3079.



Figure S2: Determining the Cu(I) stoichiometry and affinity using the chromogenic affinity probes BCA and BCS for the H22G mutant of CopZ-1317. (A) The absorbance at 562 nm in the visible spectrum of $[Cu^{I}(BCA)_{2}]^{3-}$ (inset) decreases to zero upon increasing additions of each apo-H22G, with plots of absorbance change at 562 nm as a function of [H22G/Cu(I)] indicating an ~ 1:1 stoichiometry based on the intersection of the lines at the start and end of the titration. (B) Under copper-limiting conditions imposed by $[Cu^{I}(BCS)_{2}]^{3-}$ the absorbance at 483 nm in the visible spectrum of $[Cu^{I}(BCS)_{2}]^{3-}$ (inset) decreases upon addition of each apo-H22G and the $K_{D}(Cu^{I})$ determined using equation 2. The line through the data points represents a best fit to the data using a $K_{D}(Cu^{I})$ of 6.0×10^{-17} M.



Figure S3: Determining the Cu(I) stoichiometry and affinity using the chromogenic affinity probes BCA and BCS for the Y71F mutant of CopZ-1317. (A) The absorbance at 562 nm in the visible spectrum of $[Cu^{I}(BCA)_{2}]^{3-}$ (inset) decreases to zero upon increasing additions of each apo-Y71F, with plots of absorbance change at 562 nm as a function of [Y71F/Cu(I)] indicating an ~ 1:1 stoichiometry based on the intersection of the lines at the start and end of the titration. (B) Under copper-limiting conditions imposed by $[Cu^{I}(BCS)_{2}]^{3-}$ the absorbance at 483 nm in the visible spectrum of $[Cu^{I}(BCS)_{2}]^{3-}$ (inset) decreases upon addition of each apo-Y71F and the $K_{D}(Cu^{I})$ determined using equation 2. The line through the data points represents a best fit to the data using a $K_{D}(Cu^{I})$ of 1.8×10^{-17} M.



Figure S4: Cu(I) transfer between CsoR and CopZ proteins probed by EMSA. The DNA sequences of the individual CsoR operator sites used are indicated above the corresponding EMSA gel, with bold type the consensus operator sequence for the individual targets. In (A) 1 or 10 molar equivalent of Cu(I)-loaded CopZ proteins were incubated with apo-CsoR and in (B) 1 or 10 molar equivalent of apo-CopZ proteins were mixed with Cu(I)-loaded CsoR. The components present in each lane of the gels are indicated with the concentrations as follows: 0.5 μ M [DNA], 4 μ M [apo-CsoR-monomer], 4 μ M [Cu(I)-CsoR-monomer] (equivalent to 0.5 μ M 2 x CsoR tetramer), 4 and 40 μ M [apo-CopZ] or [Cu(I)-CopZ] (equivalent to a 1:1 and a 1:10 CsoR-monomer:CopZ).

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