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C-Terminal Domain of the Membrane Copper Transporter Ctr1 from *Saccharomyces cerevisiae* Binds Four Cu(I) Ions as a Cuprous-Thiolate Polynuclear Cluster: Sub-femtomolar Cu(I) Affinity of Three Proteins Involved in Copper Trafficking

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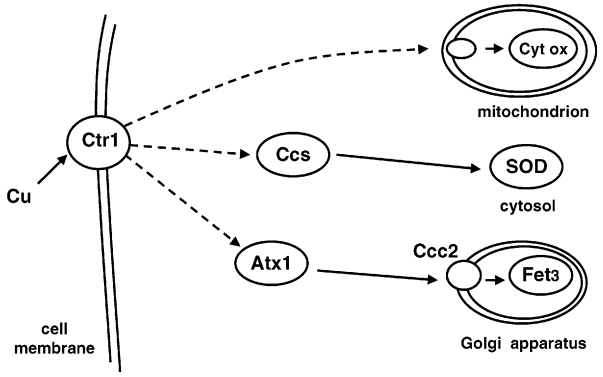
Abstract: The cytosolic C-terminal domain of the membrane copper transporter Ctr1 from the yeast *Saccharomyces cerevisiae*, Ctr1c, was expressed in *E. coli* as an oxygen-sensitive soluble protein with no significant secondary structure. Visible-UV spectroscopy demonstrated that Ctr1c bound four Cu(I) ions, structurally identified as a $Cu^{I}_4(\mu\text{-S}-\text{Cys})_6$ cluster by Xray absorption spectroscopy. This was the only metalated form detected by electrospray ionization mass spectrometry. An average dissociation constant $K_D = (K_1K_2K_3K_4)^{1/4} = 10^{-19}$ for binding of Cu(I) to Ctr1c was estimated via competition with the ligand bathocuproine disulfonate bcs ($\beta_2 = 10^{19.8}$). Equivalent experiments for the yeast chaperone Atx1 and an N-terminal domain of the yeast Golgi pump Ccc2, which both bind a single Cu(I) ion, provided similar K_D values. The estimates of K_D were supported by independent estimates of the equilibrium constants K_{ex} for exchange of Cu(I) between pairs of these three proteins. It is apparent that, in vitro, the three proteins buffer "free" Cu(I) concentrations in a narrow range around 10^{-19} M. The results provide quantitative support for the proposals that, in yeast, (a) "free" copper concentrations are very low in the cytosol and (b) the Cu(I) trafficking gradient is shallow along the putative Ctrlc → Atx1 → Ccc2n metabolic pathway. In addition, both Ctr1c and its copper-responsive transcription factor Mac1 contain similar clusters which may be important in signaling copper status in yeast.

Introduction

Copper is an essential but toxic element for all living cells and its uptake and trafficking must be regulated strictly.[1,2] In the baker's yeast *Saccharomyces cerevisiae*, copper uptake under different conditions is mediated by different copper importers.[3] These include the high affinity plasma membrane transporters Ctr1and Ctr3, and the low affinity transporters Fet4 and Ctr2. The imported Cu(I) ions are delivered to various proteins and compartments by carrier proteins (chaperones; Scheme 1). The mechanism of acquisition of copper by the chaperones is not known. One study suggested that it is unlikely that the plasma membrane transporters and the chaperones interact directly.[3] However, a cyanobacterial copper chaperone was demonstrated to interact directly with its copper importer.[4]

Levels of the high affinity membrane pump Ctr1 in yeast (a dimer or trimer) are regulated at transcription and after translation by the concentration of available copper ions. At limited extracellular levels, transcription is activated by the copper ion sensing factor Mac1.[5−7] When extracellular Cu ion levels are elevated, Ctr1 undergoes endocytosis to cytoplasmic vesicles.[8]

Scheme 1



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At higher Cu concentrations, Ctr1 also degrades directly in the membrane via a process which is independent of endocytosis. Mac1 is required for control of Ctr1 degradation.[9]

The Ctr1 pump has been detected in all eukaryotic organisms screened. The murine form is essential for foetal development.[10] An intriguing recent development has identified Ctr1 as the primary entry point of the cancer drug *cis*-platin into mouse cells.[11] Yeast Ctr1 features three separate structural domains that are proposed to mediate different aspects of Cu transport.[12] In particular, the cysteine-rich C-terminal domain, Ctr1c, is hydrophilic and apparently located in the cytosol. In vitro, it is able to deliver Cu(I) directly to the chaperone Atx1.[13]

Ctr1 proteins are conserved topologically in eukaryotic cells, but the hydrophilic domains vary in primary structure.[14] Although yeast Ctr1c is composed of 125 amino acid residues with six Cys residues, the human and murine forms feature a short sequence of 11 amino acid residues with one Cys residue only and this Cys residue can be mutated without affecting copper transport.[15] In addition, the murine form can complement a yeast strain deficient in high affinity copper uptake.[16] The molecular roles of the individual domains in the different forms of the pumps remain to be defined.

The present work provides initial molecular characterization of yeast Ctr1c. It demonstrates that an expressed soluble form exhibits a high affinity for Cu(I) and can bind up to four copper ions as a $[Cu^{I}_4(\mu\text{-}S{-}Cys)_6]^{2-}$ cluster. It is able to deliver Cu(I) directly to both the chaperone Atx1 and to an N-terminal domain of the Ccc2 protein (Scheme 1).

Materials and Methods

Unless otherwise specified, all characterization and manipulations of Ctr1c and its variants were carried out anaerobically in a glovebox ($[O_2] < 2$ ppm) with de-oxygenated buffers and solvents. The samples were kept in sealed containers, including during transfer from the glovebox for characterization.

Plasmids and Site Directed Mutagenesis. The DNA sequence coding the C-terminal domain (residue 282−406) of Ctr1 protein, Ctr1c, was amplified from *S. cerevisiae* genomic DNA by PCR and cloned into the expression vector pET11a at sites between *Nde*I and *Bam*HI. A less favorable codon (AGA) for arginine at the N-terminus was replaced by a more favorable one (CGC) in the PCR primer. A starting codon (ATG) coding methionine (M) was introduced at the N-terminus to initiate protein expression. The sequence of the inserted Ctr1c fragment was confirmed by DNA sequencing.

Site-directed mutagenesis was carried out by PCR:[17] the template to be mutated was amplified using two sets of gene-specific primers which overlapped the intended mutation site(s). Each overlapped primer contained the desired mutation(s). Two amplified PCR fragments were fused and amplified further to complete the mutation. The PCR products were cloned into the pET11a vector and the sequences and variant sites confirmed by DNA sequencing.

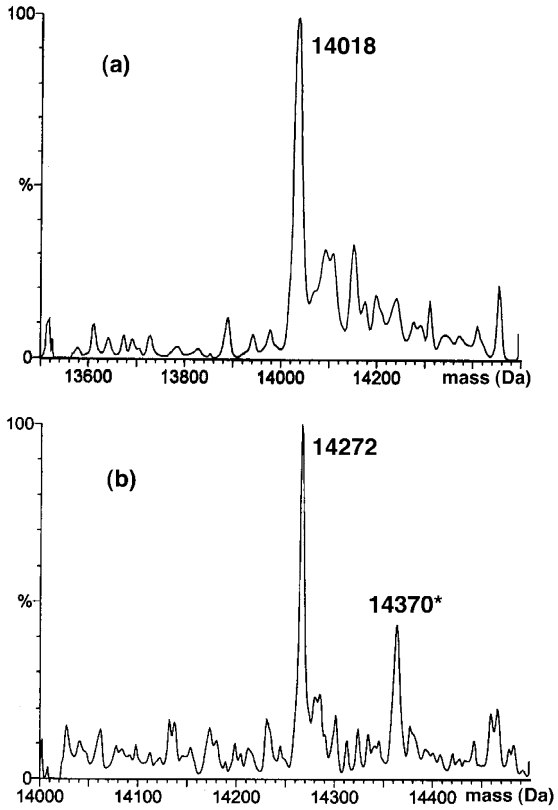

Figure 1. Electrospray ionization mass spectra of (a) apo-Ctr1c and (b) Cu_4Ctr1c in MeOH/H_2O (1:1) with 0.1% acetic acid. * mass consistent with an adduct of Cu_4Ctr1c with PO_4^{3-}.

Protein Expression and Purification. The plasmid pET11a-CTR1c was transformed into *E. coli* BL21(DE3) CodonPlus and protein expression induced with isopropyl-β-*o*-thiogalactopyranoside (IPTG; 0.6 mM) when cell optical density reached about 1. Cells were harvested after a further 3−4 h of growth. A lysis extract was prepared in Tris-HCl buffer (20 mM; pH 8; 10 mM β-mercaptoethanol) and eluted from an anion-exchange DE−52 column (3 × 15 cm) with a NaCl gradient of 0−0.2 M NaCl in the same buffer. Ctr1c-containing fractions were identified by SDS-PAGE. Solid $(NH_4)_2SO_4$ was added to the combined fractions to 40% saturation. Precipitated solids were stirred in a minimum of KPi buffer (50 mM, pH 7; 100 mM NaCl, 100 mM β-mercaptoethanol) and the soluble fraction applied to a Superdex-75 FPLC column. The protein eluted in KPi buffer (50 mM, pH 7) containing 100 mM NaCl and 10 mM dithiothreitol (DTT). About 10 mg of pure protein was obtained per liter of culture. Protein identity was established by N-terminal sequencing (M N R C K I A M L) and by electrospray ionization mass spectroscopy ESI−MS (14 018 Da; Figure 1). The isolated protein (*apo*-Ctr1c) was assayed for metal content by ICP−MS and contained <0.01 atom equiv of Cu, Ni, Co, Cd, Mn, Ag, and <0.05 atom equiv of Zn.

Addition of $CuSO_4$ (0.5 mM) to the growth medium 1 h after IPTG induction provided Ctr1c protein containing 4 molar equiv of Cu as detected by ESI−MS (14 272 Da; Figure 1). This Cu-loaded form can also be obtained by treatment of apo-Ctr1c with Cu(I) reagent. Under anaerobic conditions, concentrated Ctr1c stocks were treated with excess DTT and then transferred into a metal insertion buffer (Tris/Mes; 20 mM; pH 8; 10 mM DTT). Four equivalents of $[Cu^{I}(MeCN)_4]ClO_4$ in MeCN were added and the protein purified on a Superdex-75 FPLC column (see above).

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Isolation of Atx1 and Ccc2n. Atx1 was expressed and purified as described in the literature.[18] DNA coding for Ccc2n, the first 72-residue N-terminal domain of the Ccc2 protein, was amplified from the gene CCC2 of *S. cerevisiae* genomic DNA and cloned into the vector pET11a at sites between *Nde*I and *Bam*HI. Codons at both ends of the gene were optimized for expression in *E. coli* via a change of AGA encoding Arg-2 and Arg-70 to CGC, ATA encoding Ile-5 and Ile-68 to ATT, and CTA encoding the Leu-69 to CTG. A stop codon (TAA) was incorporated after the TCT encoding for Ser-72. After confirmation by DNA sequencing, the plasmid pET11a-CCC2n was amplified and transformed into *E. coli* BL21(DE3) CodonPlus. Protein expression was induced with IPTG (0.5 mM) for cells grown in rich 2YT medium at 37 °C to an OD of 0.7−1.0. Cells were harvested after being induced for 2−3 h at 28 °C. A lysis extract prepared in Tris-HCl buffer (20 mM; pH 8; 10 mM β-mercaptoethanol) was loaded onto an anion-exchange DE−52 column (3 × 15 cm). The bound proteins were washed with 0.1 M NaCl in 300 mL Mes−Na buffer (50 mM, pH 6; 5 mM DTT; 0.1 mM EDTA) and then eluted with a 0.1−0.28 M NaCl gradient in the same buffer (800 mL). Fractions containing Ccc2n were identified by SDS-PAGE. They were pooled and concentrated after addition of extra DTT to ca. 30 mM before being purified further on a Superdex-75 FPLC column with a KPi buffer (50 mM, pH 6.5; 0.1 M NaCl; 5 mM DTT). A yield of about 50 mg of pure protein was obtained per liter of culture. ESI−MS revealed a molar mass of 7943 Da, consistent with the expected full length 72-residue Ccc2n sequence starting with Met-1 and ending with Ser-72. The isolated protein (*apo*-Ccc2n) was metal-free, as confirmed by ICP−MS.

Concentration Assays. Copper concentrations were determined by GC−AAS. Concentrations of *apo*-proteins were estimated directly by absorbance at 280 nm. Quantitative drying of samples in the volatile buffer NH_4HCO_3 provided estimates of $\epsilon_{280} = 7700$ (±500), 4900 (±200), and 1580 (±50) M^{-1} cm^{-1}, respectively, for *apo*-Ctr1c, *apo*-Atx1, and *apo*-Ccc2n, in comparison with respective calculated values of 5700, 3840, and 1280 M^{-1} cm^{-1} of fully reduced forms.

The estimate for *apo*-Atx1 is consistent with that reported value previously,[18] but that for *apo*-Ccc2n is significantly lower (i.e., 1580 vs 2750 M^{-1} cm^{-1}).[19] The Ellman assay[20,21] for free cysteine thiol content of fully reduced *apo*-Ccc2n was consistent with the value reported in this work. Six, two, and six cysteine thiols are present in the reduced *apo* forms of Ctr1c, Atx1, and Ccc2n, respectively. The assay was adapted to use in an anaerobic glovebox. Protein stocks were treated with excess reductant DTT overnight and, prior to assay, were transferred to a Tris/Mes buffer (20 mM; pH 8) via a Bio-Del P-6 DG gel column (Bio-Rad).

Copper(I) Titrations. Purified *apo*-proteins were prepared for Cu(I) titration by anaerobic buffer change into Tris/Mes buffer (20 mM; pH 8) and assayed by Ellman reagent. Stock solutions of $[Cu^I(MeCN)_4]ClO_4$ in MeCN were prepared by dissolving the salt quantitatively in de-oxygenated MeCN. Calculated Cu(I) concentrations were verified independently by GC−AAS. Each sample of reduced *apo*-protein (1 mL; 20−50 μM) was transferred to a quartz cell (1 cm path) sealed with a septum cap and titrated with Cu(I) solution (50× concentration) delivered from an airtight syringe. Absorption was monitored over the wavelength range 200−800 nm. In some cases, the reductant tris(2-carboxyethyl)phosphorane (TCEP) solution was titrated subsequently into the protein solution and the process monitored spectroscopically.

Physical Measurements

ESI mass spectra were recorded on a Micromass Quattro II Triple-Quadrupole mass spectrometer in the positive ion mode with a cone voltage set at 25−35 V and a flow rate at 5 μL min^{-1}. Protein samples were desalted anaerobically with water and diluted to ca. 20 pmol/μL. The diluted samples were mixed with an equal volume of methanol containing 0.1% acetic acid just before delivery to the electrospray probe. The average molar masses were obtained by applying a deconvolution algorithm to the recorded spectra and were calibrated with horse heart myoglobin (16 951.5 Da).

Sedimentation equilibrium experiments were carried out at 20° using a Beckman Optima XL-A analytical ultracentrifuge with an An-Ti60 rotor. Samples for analysis were prepared under anaerobic conditions in potassium phosphate buffer (KPi; 50 mM; pH 6.8), NaCl (100 mM) with or without TCEP (2 mM) by buffer exchange from concentrated protein stocks. The protein concentrations were approximately 50 and 20 μM for apo- and Cu-proteins, respectively. Protein solutions and reference buffers were loaded into the ultracentrifuge cells under anaerobic conditions and sedimentation equilibrium distributions determined from radial scans at 280 nm. The final equilibrium distributions were fitted globally using the program SEDEQ1B (kindly provided by Allen Minton, NIH, Bethesda). The partial specific volume of the protein (0.713 g/mL) was calculated from the amino acid composition.

X-ray Absorption Spectroscopy. Measurements were carried out at the Stanford Synchrotron Radiation Laboratory with the SPEAR storage ring containing 70−100 mA at 3.0 GeV. Spectra were collected on beamline 7−3 using a Si(220) double crystal monochromator, with an upstream vertical aperture of 1 mm and a wiggler field on 1.8 T. Harmonic rejection was accomplished by detuning one monochromator crystal to approximately 50% off-peak and no specular optics were present in the beamline. The incident X-ray intensity was monitored using a nitrogen-filled ionization chamber, and X-ray absorption was measured as the X-ray Cu Kα fluorescence excitation spectrum using an array of 30 germanium intrinsic detectors.[22] During data collection, samples were maintained at a temperature of approximately 10 K, using an Oxford Instruments liquid helium flow cryostat. Spectra were calibrated with reference to the lowest energy inflection point of a copper metal foil (assumed to be 8980.3 eV) which was collected simultaneously with each scan. Data were analyzed using the EXAFSPAK suite of computer programs.[23] The extended X-ray absorption fine structure (EXAFS) oscillations $\chi(k)$ were quantitatively analyzed by curve-fitting.[24] The EXAFS total amplitude and phase-shift functions were calculated using the program FEFF v8.2.[25,26]

Samples for X-ray absorption spectroscopy were prepared in 20 mM KPi (pH 6.8) with or without 2 mM TCEP via buffer change from concentrated protein stocks. Potential interfering ligands, Cl^- and DTT, were removed by extensive dialysis under anaerobic conditions. For the samples containing TCEP, the buffer change was effected on a de-salting column in the glovebox and the protein solution was concentrated with a Centricon filter in air. For other samples, buffer change was

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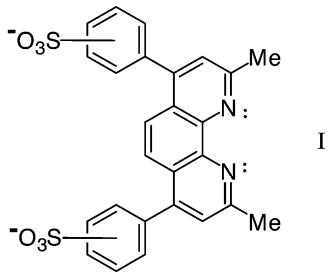
accomplished by extensive dialysis in the glovebox without further concentration after the buffer change. The samples were mixed with glycerol (30 vol %) as glassing agent (to prevent artifacts from crystalline ice) just before loading into sample cells and freezing in liquid nitrogen.

Estimation of Cu(I) Binding Constants. Estimates of "free" Cu(I) concentrations $[Cu]_f$ in the presence of excess proteins P (i.e., at Cu occupancy $\theta < 1$) may be estimated if an effective competition for Cu(I) between *apo*-proteins P and a Cu(I) chelator exists in solution. For the case of bathocuproine disulfonate (Na_2bcs; Structure I; Aldrich)

$$n\mathrm{Cu^I(bcs)_2}^{3-} + \mathrm{P} \leftrightarrow \mathrm{Cu^I}_n\text{-P} + 2n\mathrm{bcs}^{2-} \quad (1)$$

with an assumption that no mixed-ligand complexes form. Cu(I) and bcs form a stable 1:2 complex $Cu(bcs)_2^{3-}$ and the overall association constant $\beta_2 = K_1K_2$ has been determined to be $\beta_2 = 10^{19.8}$.[27] This system exhibits an absorption maximum at 483 nm ($\epsilon = 13\,300\ M^{-1}\ cm^{-1}$)[28] where Cu_n-P *holo*-proteins do not absorb, allowing convenient assessment of the concentrations $[Cu(bcs)_2^{3-}]$ and $[Cu]_f$ at equilibrium for a given total bcs concentration $[bcs]_t$

$$[\mathrm{Cu}]_f = [\mathrm{Cu(bcs)_2}^{3-}]/\{([\mathrm{bcs}]_t - 2\,[\mathrm{Cu(bcs)_2}^{3-}]^2\beta_2\} \quad (2)$$



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With known total concentrations of Cu(I), $[Cu]_t$, and protein, $[P]_t$, the association constants of Cu(I) bound to proteins (K_{CuP}) may be estimated with an additional assumption for Ctr1c that binding of $n = 4$ Cu(I) ions to *apo*-Ctr1c is cooperative under the conditions

$$K_{\mathrm{CuP}} = \{\theta/(1-\theta)\}^{1/n}[\mathrm{Cu}]_f^{-1} \quad (3)$$

$$\theta = [\mathrm{Cu}_n\text{-P}]/[\mathrm{P}]_t \quad (4)$$

With $n = 1$ for CuAtx1 and CuCcc2n, K_{CuP} is the association constant of Cu−P; with $n = 4$ for Cu_4Ctr1c, K_{CuP} as defined in eq 3 refers to the *average* Cu(I) binding constant which equals the 4th root of the overall formation constant of Cu_4Ctr1c. The Cu(I) binding affinity may be also expressed conveniently as the dissociation constant, K_D, which is the inverse of K_{CuP} (eq 3) and represents the "free" Cu(I) concentration at half-loading ($\theta = 0.5$)

$$K_D = \{(1-\theta)/\theta)\}^{1/n}[\mathrm{Cu}]_f \quad (5)$$

The equilibrium constants K_{ex} for exchange of Cu(I) between proteins P1 and P2 are defined by eq 6 and 7. K_{ex} can be predicted if the individual values of K_D are known

$$(1/n)\mathrm{Cu}_n\mathrm{P1} + \mathrm{P2} \leftrightarrow (1/n)\mathrm{P1} + \mathrm{CuP2} \quad (6)$$

$$K_{ex} = K_{D2}/K_{D1} = \{[\mathrm{P1}]^{1/n}[\mathrm{CuP2}]\}/\{[\mathrm{Cu}_n\mathrm{P1}]^{1/n}[\mathrm{P2}]\} \quad (7)$$

Experiments were carried out in both directions of eq 1 with Cu(I) preloaded on either bcs or P. Briefly, for the forward reactions, P was added to a solution obtained by mixing $[Cu^I(MeCN)_4]ClO_4$ and Na_2bcs in the molar ratio, 1:30−70 (to promote effective competition and to ensure formation of the 1:2 complex $Cu(bcs)_2^{3-}$). The mixture was diluted so that the final total concentrations (μM) were 5 for Ctr1c, 15 for Atx1 and Ccc2n, 30 for Cu(I) and 1−2 mM for bcs. Equilibration was attained after incubation for 10 min and transfer of Cu(I) from bcs to P was estimated by the change in absorbance at 483 nm relative to the control solution without protein. For the reverse reactions, similar mixtures were prepared but Cu(I) was pre-loaded onto proteins P. Competition experiments showed that TCEP, bcs and DTT compete for Cu(I) in the order TCEP > bcs > DTT and that both TCEP and DTT may interfere with equilibrium 1. However, glutathione (GSH; up to at least 2 mM) had no effect and was included in the Cu(I) solution to a final concentration of 1 mM after dilution to ensure solubility and to maintain the Cu(I) redox state. Results are presented in Table 1.

Copper Exchange between Ctr1c and Ccc2n. The Ellman assay indicated that the *apo*-Ccc2n protein used in these experiments was fully reduced but *apo*-Ctr1c was ca. 80% reduced only. Consequently, 10 equivalents of fresh DTT were included with *apo*-Ctr1c before the exchange or Cu(I)-loading. Although DTT at high concentration may interfere with equilibrium 1, control experiments showed that the amount of DTT included with Ctr1c had little effect on either copper binding or on copper exchange between Ctr1c and Ccc2n under copper limiting conditions as the Cu(I) affinity of each protein is much higher than that of DTT (see results below). Ctr1c and Ccc2n were loaded with 3.0 and 0.9 equivalents of Cu(I), respectively, to ensure that no excess Cu was present. The exchange experiments were carried out anaerobically in Tris/Mes buffer (20 mM; pH 8) by incubating fixed concentrations of loaded Ctr1c with varying concentrations of *apo*-Ccc2n or the complementary experiments with loaded Ccc2n and *apo*-Ctr1c. The total reaction volume remained constant. After incubation for ca. 30 min, the mixture was loaded onto a 1 mL anion exchange column (DE−52) equilibrated in the same buffer inside the glovebox. The separation was accomplished by stepwise manual addition of buffer with increasing NaCl concentration. The eluted fractions were analyzed for thiol content by Ellman assay, for copper content by GC−AAS and for proteins by SDS-PAGE. DTT was eluted in the column flow-through whereas Ctr1c and Ccc2n eluted at 0.2 and 0.4 M NaCl, respectively. Total Cu, Ctr1c, and Ccc2n were recovered routinely at 100 (±10) %. Results are presented in Table 2.

Results

Protein Expression and Purification. Yeast Ctr1 is predicted to contain three well-defined structural domains.[12] The hydro-

(27) Xiao, Z.; Wedd, A. G. Unpublished data: The stability constants log K_1 and log β_2 for $[Cu^{II}(bcs)_2(OH_2)]^{2-}$ were determined to be 6.1 and 11.9, respectively, based on the Bjerrum titration technique, with an experimental proton dissociation constant of $pK_a = 5.7$ for bcs. The reduction potential for the couple $[Cu^{II}(bcs)_2(OH_2)]^{2-}/[Cu^{I}(bcs)_2]^{3-}$ was reported to be 0.620-(5) V: Lappin, G. A.; Youngblood, M. P.; Margerum, D. W. *Inorg. Chem.* **1980**, *19*, 407−413. The overall association constant log β_2 for $[Cu^{I}(bcs)_2]^{3-}$ was estimated to be 19.8 via the Nernst equation.

(28) The value determined in this work ($13\,300\ M^{-1}\ cm^{-1}$) is somewhat higher than that ($12\,250\ M^{-1}\ cm^{-1}$) reported previously: Blair, D.; Diehl, H. *Talanta* **1961**, *7*, 163−174.

Table 1. Dissociation Constants K_D for Cu(I) Binding to Proteins[a]

protein	Ctr1c		Atx1		Ccc2n	
$[P]_t$, μM	5.0		15		15	
$[bcs]_t$, mM	1.0	2.0	1.0	2.0	1.0	2.0
$[Cu]_t$, μM	30	30	28	29	29	29
$[Cu(bcs)_2^{3-}]$, μM	23	25	21	27	19	23
Cu occupancy θ	0.35	0.25	0.47	0.13	0.67	0.40
$[Cu]_f$, 10^{-19} M	4.0	1.0	3.6	1.1	3.2	1.0
$K_D \times 10^{-19}$ [b]	4.7	1.4	4.1	7.3	1.6	1.4
K_{ex} predicted[c]	0.2−1 for Ctrlc ↔ Atx1		2−5 for Atx1 ↔ Ccc2n		1−3 for Ctr1c ↔ Ccc2n	
K_{ex} expt	0.3−1[d]		1.4[e]		0.7−2.6[f]	

[a] Calculations based on $\beta_2 = K_1K_2 = 10^{19.8}$ for $[Cu^I(bcs)_2]^{3-}$ (see the Experimental Section). [b] From eq 5. [c] From eq 7. [d] See Table S1 and ref 13. [e] Reference 19. [f] See Table 2.

philic C-terminal domain Ctr1c (sequence 282−406; Figure 2) is apparently cytosolic and features six cysteine residues, including two motifs Cys-X-Cys. An expression system, pET11a-CTR1c, was constructed and the protein expressed in *E. coli*. The *apo* form is slightly acidic with a predicted charge of −1 at pH 7. The expressed protein bound weakly to anion-exchange resins allowing purification by conventional chromatography.

Expression and isolation of the soluble *apo*-Ctr1c was possible when thiol reductants such as β-mercaptoethanol or DTT were present throughout the isolation. When they were absent, the protein was oxidized rapidly by O_2 forming aggregated precipitate which could be solubilized upon addition of the reductants.

Some of the cysteine thiol groups in *apo*-Ctr1c appear to be highly reactive and difficult to maintain in fully reduced form. The Ellman assay indicated that samples analyzed immediately after separation from excess DTT were about 90% reduced. However, this thiol content dropped gradually even for samples kept under strict anaerobic conditions.

Addition of $CuSO_4$ to the growth medium after IPTG induction led to isolation of Ctr1c protein containing 4 molar equivalents of Cu. This Cu_4−Ctr1c form bound more strongly to anion-exchange resins than the *apo* form and was somewhat more resistant to oxidative precipitation. Cu_4−Ctr1c can be generated from *apo*-Ctr1c by titration with Cu(I) in a reducing buffer.

Apo-Ccc2n, constructed from the first 72-residues of the N terminal domain of Ccc2, has been expressed previously at about 3 mg per liter culture in the system pET11d/BL21(DE3) with Met at position 1 being replaced by Ala.[19] The present system optimized the expression and allowed Ccc2n isolation in a yield of 50 mg per liter culture. The Ellman assay detected the predicted six cysteine thiols based on protein concentrations estimated via $\epsilon_{280} = 1580\ M^{-1}\ cm^{-1}$. The fully reduced Ccc2n samples were stable indefinitely under anaerobic conditions.

Molecular Characterization of Ctr1c. *Apo*- and Cu_4−Ctr1c proteins migrated anomalously and indistinguishably on reduced SDS-PAGE gels (consistent with dimers) and on G-75 gel filtration columns (consistent with trimers) (Figure 3). However, equilibrium sedimentation analysis indicated that reduced *apo*-Ctr1c existed in solution as a clean monomer while the behavior of Cu_4−Ctr1c could be modeled as >80% monomer with <20% nonequilibrium dimer (Figure 4). The observations are consistent with the presence of intermolecular S−S links (from oxidation of Cys residues by adventitious O_2) or intermolecular S−Cu−S links formed during the titration of Cu(I) into the *apo* protein. The content of dimeric species decreased marginally in the presence of the strong reductant TCEP (2 mM). ESI−MS detected only monomeric molecules of molar mass 14 018 and 14 272 Da, respectively, for *apo*- and Cu_4−Ctr1c (Figure 1).

Absorbance Spectra. Circular dichroism (CD) spectra indicated the absence of significant α-helix or β-strand structure (Figure 5). Binding of Cu(I) did not cause discernible change and the spectra are similar to those of the regulatory domain of the Ace1 transcription factor which also lacks classical secondary structure.[29]

In addition to the absorbance at 280 nm (ϵ, 7700 $M^{-1}\ cm^{-1}$), *apo*-Ctr1c exhibited a weaker broad absorption band between 300 and 350 nm for apo-Ctr1c (Figure 6), as did the Ace1 domain and the CopZ chaperone from *Bacillus subtilis*.[29,30] This feature was also observed for variant *apo*-Ctr1c proteins generated in the present work by substitution of cysteine residues for serine. The exception was the variant C4/24/30/83S (where 4 of 6 Cys residues were replaced by Ser; Figure 2). The possibility that the absorption might originate from adventitious binding of metal ions was precluded by the fact that ICP−MS failed to detect significant quantities of metals in the samples. In another experiment, apo-Ctr1c was precipitated twice with trichloroacetic acid and redissolved in Tris base containing 30 mM DTT and 10 mM EDTA. After changing buffer to 20 mM Tris/Mes (pH 8), an indistinguishable solution spectrum was observed. The broad absorption band between 300 and 350 nm may originate from undefined cysteine-related protein−protein interactions.

This aspect is important as metallothionein proteins that contain $Cu^I_x(\mu\text{-S−Cys})_y$ ($x = 3, 4$) centers absorb characteristically at 310 and 350 (sh) nm.[31] In this context, titration of Cu(I) into *apo*-Atx1 led to an increase of absorbance at 265 nm, assigned to S → Cu charge-transfer transitions (Figure S1). A change in slope at 1 eq Cu(I) (inset, Figure S1) is consistent with the formation of the $Cu(S−Cys)_2$ fragment established for Cu−Atx1 by NMR.[32] Upon titration of additional Cu(I), absorption bands at 310 and 350(sh) nm grow in with changes in slope at 265 and 310 nm at 2 eq Cu(I). The observations are consistent with formation of a $Cu^I_2(\mu\text{-S−Cys})_2$ binuclear unit in Atx1 of the type proposed for the Cop Y protein of *E. hiraie*.[33] However, a single Cu(I) ion only is bound strongly to Atx1: the second one is removed by incubation with bcs or upon gel filtration chromatography.

Cu(I) Titration of Proteins. An absorption at 265 nm appeared upon addition of Cu(I) to *apo*-Ctr1c and is assigned to S → Cu(I) charge-transfer transitions (Figures 6,7). Quantitative titration provided a linear increase in absorbance until a plateau was reached at about 3.5 eq Cu(I) (Figure S2 and 7b). The Ellman assay indicated that the Cys thiols were about 90% reduced in the samples of *apo*-Ctr1c prepared for titration (see Experimental Section). Addition of 10 equiv of reductant TCEP to the titrated solution containing 4.0 equiv Cu(I) induced an additional increase in absorbance at 265 nm, consistent with

(29) Dameron, C. T.; Winge, D. R.; George, G. N.; Sansone, M.; Hu, S.; Hamer, D. *Proc. Natl. Acad. Sci. U.S.A.* **1991**, *88*, 6127−6131.
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(33) Cobine, P.; George, G. N.; Jones, E. C.; Wickramasinghe, W. A.; Solioz, M.; Dameron, C. T. *Biochemistry* **2002**, *41*, 5822−5829.

Table 2. Equilibrium Constant K_{ex} for Exchange of Cu(I) between Ctr1c and Ccc2n

			initial concentrations (μM)[b]				equil. Cu concentrations (μM)[b]		
exp.	θ[a]	ratio Cu:Ctr1c:*Ccc2n*	Ctr1c	Cu in Ctr1c	*Ccc2n*	Cu in *Ccc2n*	in Ctr1c	in *Ccc2n*	K_{ex}[c]
1	0.75	3.0:1.0:0	10	30			29		
2	0.90	0.9:0:1.0			33	30		30	
3	0.37	3.0:1.0:4.0	10	30	40	0	11	18	1.0
4	0.50	3.0:1.0:2.0	10	30	20	0	16	14	2.6
5	0.32	0.90:0.45:1.0	15	0	33	30	18	12	0.7
6	0.48	0.90:0.23:1.0	7.5	0	33	30	10	20	1.8

[a] Overall copper occupancy of total copper sites of two proteins, see eq 4. [b] Concentrations are normalized to starting reaction volume (500 μL). [c] K_{ex} was calculated by eq 7 for exchange reaction 6.

MNR**C**KIAMLK RWDIQREIQK AKS**C**PGFGN**C** Q**C**GRHPEPSP DPIAVADTTS GSDQSTRLEK NNESKVAISE NNQKKTPTQE EG**C**N**C**ATDSG KNQANIERDI LENSKLQEQS GNMDQNLLPA EKFTHN

Figure 2. Protein sequence of the expressed domain Ctr1c.

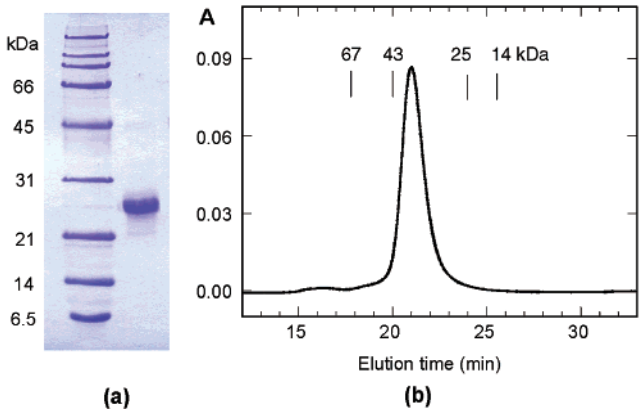


Figure 3. Migration of apo-Ctr1c and Cu_4Ctr1c in buffers containing DTT: (a) SDS-PAGE (16%); (b) elution profile on Superdex-75 gel filtration column (HR10/30; Pharmacia). Conditions: flow rate, 0.6 mL/min; buffer, KPi (25 mM; pH 7); DTT (5mM); NaCl (100 mM). The column was calibrated with Blue Dextran (2000 kDa), albumin (67 kDa), ovalbumin (43 kDa), chymotrypsinogen A (25 kDa) and ribonuclease A (13.7 kDa) as standards (Amersham Pharmacia).

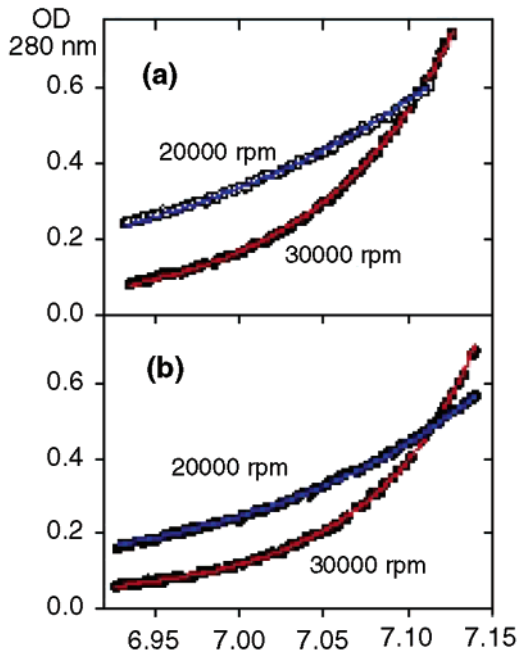


Figure 4. Sedimentation equilibrium profiles for apo-Ctr1 and Cu_4Ctr1c in KPi buffer (50 mM; pH 6.8) containing NaCl (100 mM) and TCEP (2 mM). The equilibrium profiles are presented as the optical density at 280 nm versus the radial distance for (a) apo-Ctr1c, initial concentration 50 μM and (b) Cu_4Ctr1c, initial concentration 20 μM. The lines drawn through the data are best-fit lines assuming the presence of a fixed proportion of monomeric and dimeric species.

binding of 4 equiv Cu(I) (Figure 7a,c). It appears that TCEP scavanged intermolecular S−S and/or S−Cu−S links allowing formation of a full complement of Cu_4 intra-protein clusters. A higher concentration of TCEP led to competition with Ctr1c for Cu(I) (Figure 7c). There were no spectral changes in the range of 600−800 nm that would have suggested Cu(II) ligation (not shown).

A total of five variant forms (C4S, C4/24S, C4/24/30S, C4/24/83S, and C4/24/30/83S) have been generated to investigate the effect of substitution of Cys by Ser upon Cu(I)-binding properties. Residues C30 and C83 form part of the two predicted metal binding motifs C−X−C whereas both C4 and C24 are more isolated in the sequence. Preliminary results indicate that the C4S variant binds 3 eq Cu(I) (Figure S3) and that the other variants bind less copper.

Apo-Ccc2n binds one Cu(I) ion as a $Cu(S-Cys)_2$ fragment.[34] This result was confirmed by following, upon addition of Ccc2n, the decrease in the absorption at 483 nm (characteristic of the $Cu^I(bcs)_2^{3-}$ ion) in the presence of excess bcs ligand (0.2 mM; Structure I). In comparison, Ctr1c bound 3 Cu(I) ions under the same conditions. However, in the absence of bcs, Cu(I) titration of Ccc2n resulted in a linear increase in absorption around 265 nm, characteristic of S → Cu(I) charge-transfer transitions, until a plateau was reached at about 4 eq Cu(I) (Figure S4).[35] CD spectroscopy revealed that the overall secondary structure of Ccc2n remained unchanged upon addition of 1 eq Cu(I), but was disrupted after titration with more than 1 eq Cu(I) (Figure S5).

X-ray Absorption Spectroscopy. The Cu K near-edge spectra of Cu_4−Ctr1c are shown in Figure 8, compared with the spectra of the Cu(I)Ace1 truncate[36] and two different model compounds containing two- and three-coordinate thiolatocopper-(I) centers. As discussed previously,[37] the intensity of the feature

(34) Banci, L.; Bertini, I.; Ciofi-Baffoni, S.; Huffman, D. L.; O'Halloran, T. V. *J. Biol. Chem.* **2001**, *276*, 8415−8426.
(35) Upon blocking of all Cys residues with iodoacetamide, equivalent titrations with Cu(I) lead to negligible increase in the absorbance tail between 240 and 300 nm.
(36) Brown, K. R.; Keller, G. L.; Pickering, I. J.; Harris, H. H.; George, G. N.; Winge. D. R. *Biochemistry* **2002**, *41*, 6469−6476.

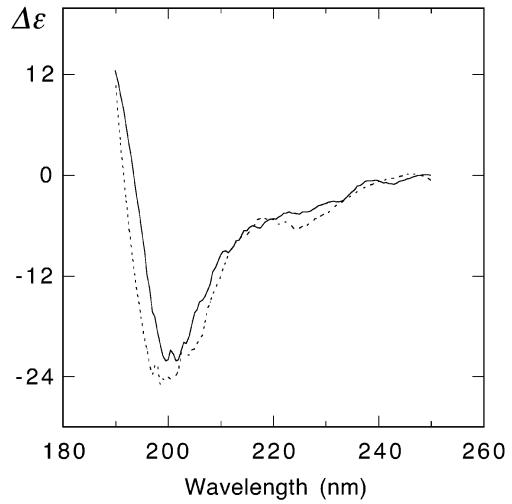


Figure 5. CD spectra of apo-Ctr1c (- - -) and Cu_4Ctr1c (—). Concentration: 13 μM in KPi buffer (20 mM; pH 6.5).

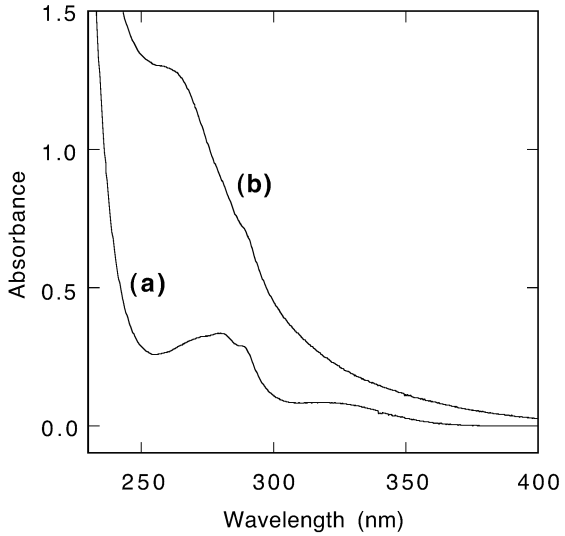
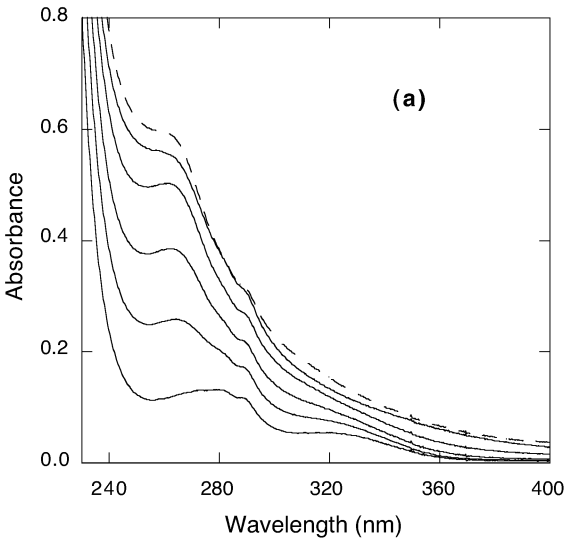


Figure 6. Absorption spectra of (a) apo-Ctr1c and (b) Cu_4Ctr1c. Concentration: 43 μM in Tris/Mes buffer (20 mM; pH 8).

at ~8983 eV is an indicator of coordination geometry and the spectrum of Cu_4—Ctr1c is typical of a trigonally coordinated thiolatocopper(I) center.[38] The XAS spectra (both near-edge and EXAFS) showed no change with addition of TCEP. The spectra from the two proteins show a striking resemblance, indicating a very similar copper coordination, and this is indicated by the essentially featureless difference spectrum shown in Figure 8d.

The Cu—K-edge EXAFS spectra and Fourier transforms of Cu_4Ctr1c are shown in Figure 9. The Fourier transforms show two major peaks, attributable to Cu—S backscattering at about 2.25 Å for the more intense peak and to Cu···Cu backscattering at about 2.7 Å for the less intense one. This observation of Cu···Cu interactions confirms the presence of a polycopper cluster in Cu_4Ctr1c. Consistent with the near-edge spectra, the EXAFS spectrum of Cu_4Ctr1c strongly resembles that of Ace1,[36] and the structural parameters derived from curve-fitting analysis (Table 3) are very similar to those of Ace1. The analysis



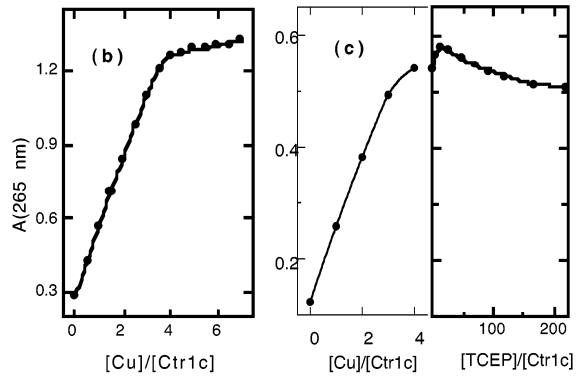


Figure 7. (a) Titration of apo-Ctr1c (17 μM) in Tris/Mes buffer (20 mM; pH 8) with $[Cu^I(CH_3CN)_4]^+$ (850 μM) in MeCN. (b) Absorbance at 265 nm versus Cu:Ctr1c ratio. (c) As for (b) but TCEP (40 mM) was added after the addition of 4 equiv Cu(I). Absorbances corrected for dilution. Dashed line in (a) was the spectrum after 10 equiv of TCEP was added.

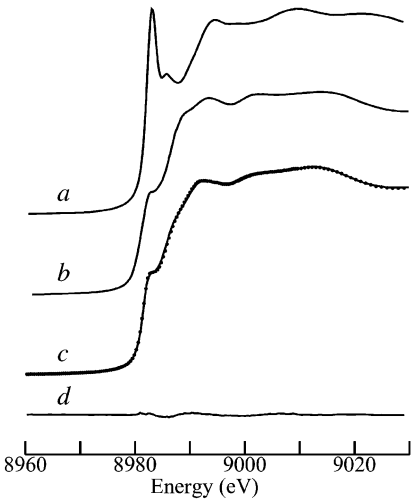


Figure 8. Comparison of the copper K-edge X-ray absorption near-edge spectra of Cu_4—Ctr1c, Ace1, and model compounds. (a) digonally coordinated $[Cu(SC_{10}H_{12})_2]^{2-}$; (b) trigonally coordinated $[Cu_4(SPh)_6]^{2-}$; (c) Cu_4—Ctr1c (points), Ace1 (line); (d) difference spectrum Cu_4—Ctr1c—Ace1.

indicates the presence of three Cu—S interactions at 2.25 Å and two different Cu···Cu interactions at 2.72 and 2.90 Å with coordination numbers of 2 and 1, respectively (Table 3). The Cu···Cu EXAFS from these two different interactions are nearly

(37) Pickering, I. J.; George, G. N.; Dameron, C. T.; Kurtz, B.; Winge, D. R.; Dance, I. G. *J. Am. Chem. Soc.* **1993**, *115*, 9498—9505.
(38) Kau, L.-S.; Spira-Solomon, D. J.; Penner-Hahn, J. E.; Hodgson, K. O.; Solomon, E. I. *J. Am. Chem. Soc.* **1987**, *109*, 6433—6442.

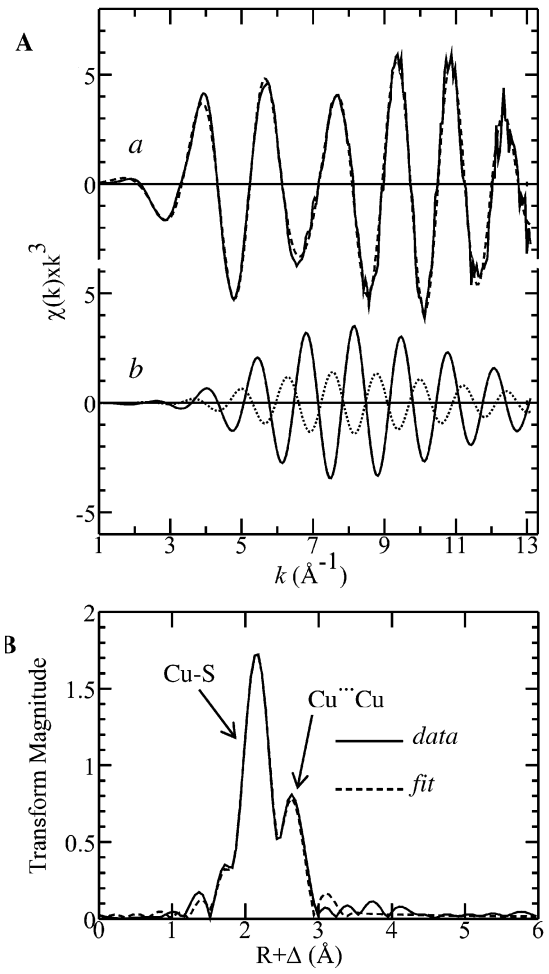


Figure 9. EXAFS oscillations (A) and EXAFS Fourier transforms (B) of Cu_4−Ctr1c. In A, plot *a* shows the best fit (broken line) to the experimental data (solid line), and *b* shows the two different Cu···Cu interactions that contribute to the best fit at 2.72 (solid line) and 2.90 Å (dotted line). The parameters obtained from curve-fitting analysis are given in Table 3.

Table 3. Results of EXAFS Curve Fitting for Cu_4−Ctr1c[a]

model[b]	interaction	N	*R* (Å)	σ^2 (Å^2)	ΔE_0 (eV)	error
A	Cu−S	3	2.248(1)	0.0043(1)	−18.3(3)	0.1736
	Cu···Cu	1	2.706(2)	0.0045(2)		
B	Cu−S	3	2.254(1)	0.0043(1)	−16.7(3)	0.1460
	Cu···Cu	2	2.715(2)	0.0066(2)		
	Cu···Cu	1	2.898(4)	0.0071(2)		

[a] *N* is the coordination number, *R* is the interatomic distance, σ^2 is the Debye−Waller factor (the mean square deviation in interatomic distance) and ΔE_0 is the threshold energy shift. Values in parentheses are the estimated standard deviations from the diagonal elements of the covariance matrix. These values are precisions, and the accuracies (generally larger) are difficult to estimate. For bond lengths, the commonly accepted upper limit is between ±0.001 and ±0.002 Å. [b] Fits are shown for the best-integer fits (the best fits for different integer values of *N*) for the two cases of one (A) and two different (B) Cu···Cu interactions.

out of phase (Figure 9A(b)) and so the resultant intensity of the 2.7 Å absorption is reduced substantially, as observed also for the regulatory domains of the Cu(I)-loaded Ace1 and Mac1 proteins.[36] The analysis is consistent with the copper stoichiometry of four per Ctr1c molecule. Due to the excellent signal-to-noise ratios of the data, weaker transform features in the vicinity of 3.7 Å were detected (Figure 9B). Similar features were also observed for Ace1 and, in that case, a detailed multiple scattering analysis suggested that they were due to multiple

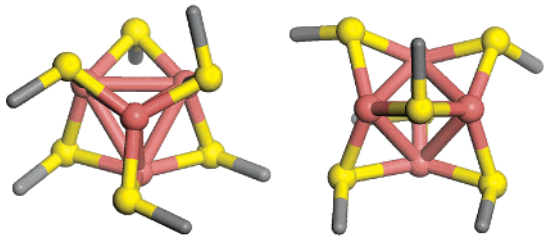


Figure 10. Model of the $Cu_4(\mu$-S−Cys$)_6^{2-}$ cluster, based upon structure of $Cu_4(\mu$-SPh$)_6^{2-}$.[39] Cu and S atoms are represented by darker and lighter spheres, respectively.

interactions involving more than two coppers in the cluster, providing independent evidence for the presence of a tetracopper cluster. While similar in-depth analysis was not performed for Cu_4Ctr1c, the presence of similar features in Figure 9B supports the presence of a tetracopper cluster in Cu_4Ctr1c. Taken together, the data are consistent with a $Cu^I_4(\mu$-S−Cys$)_6$ cluster closely related to that established for the synthetic anion $[Cu^I_4(\mu$-SR$)_6]^{2-}$ (R = Me, SPh; T_d core symmetry)[39] (Figure 10).

Estimation of Exchange Constants. Rapid exchange of Cu(I) has been observed in vitro between Atx1 and Ccc2n ($K_{ex} \approx 1.4$; eq 6, 7; Scheme 1).[19] We have demonstrated previously that Ctr1c also exchanges rapidly with Atx1.[13] It is now apparent that Ctr1c can bind 4 eq Cu(I) ($n = 4$) rather than the 2 eq estimated originally, leading us to reevaluate our original experimental data. The assumption that binding of Cu(I) to Ctr1c is cooperative allows application of eqs 3−7. For 24 separate experiments with overall Cu occupancy $\theta < 0.35$, K_{ex} was estimated to be 0.7(4) (Table S1). Experiments with $\theta > 0.35$ were insensitive as Atx1 ($n = 1$) becomes saturated with Cu(I). The original conclusions that, in vitro, Atx1 equilibrates Cu(I) rapidly with both Ctr1c and Ccc2n are confirmed (Scheme 1).

It has been suggested that, in the absence of Atx1, Ctr1 may traffic copper to Ccc2 directly.[40] Consequently, equilibration experiments were also carried out between Ctr1c and Ccc2n with Cu(I) ions pre-loaded onto either Ctr1c or Ccc2n. As a control, Cu(I) was demonstrated to bind to *either* protein tightly enough to resist competition with DTT (present to promote full reduction of Ctr1c). Cu(I) ions were recovered quantitatively in the protein fractions and Cu(I) was not detected in the DTT-containing fractions (experiments 1 and 2 in Table 2). Experiments with $0.3 < \theta < 0.5$ provided $K_{ex} = 1.5(1.1)$ (Table 2). On the other hand, experiments outside this range were insensitive as Ccc2n ($n = 1$) was saturated with Cu(I) for $\theta > 0.5$ while Cu(I) was removed quantitatively by Ctr1c ($n = 4$) for $\theta < 0.3$. These experiments assume that estimation of K_{ex} is not compromised by the chromatographic separation of the components (which is also assumed in refs 13, 19).

Estimation of Dissociation Constants. Competition experiments for Cu(I) between the Ctr1c, Atx1 and Ccc2n proteins and the Cu(I) chelator bcs were carried out. Concentrations of bcs^{2-} in excess over the proteins were necessary to induce competition and to ensure that the only bcs complex present in solution was $Cu^I(bcs)_2^{3-}$ (eq 1; Figure 11). The association

(39) Dance, I. G.; Bowmaker, G. A.; Clark, G. R.; Seadon, J. K. *Polyhedron* **1983**, *2*, 1031−1043.
(40) Lin, S.-J.; Pufahl, R. A.; Dancis, A.; O'Halloran, T. V.; Culotta, V. C. *J. Biol. Chem.* **1997**, *272*, 9215−9220.

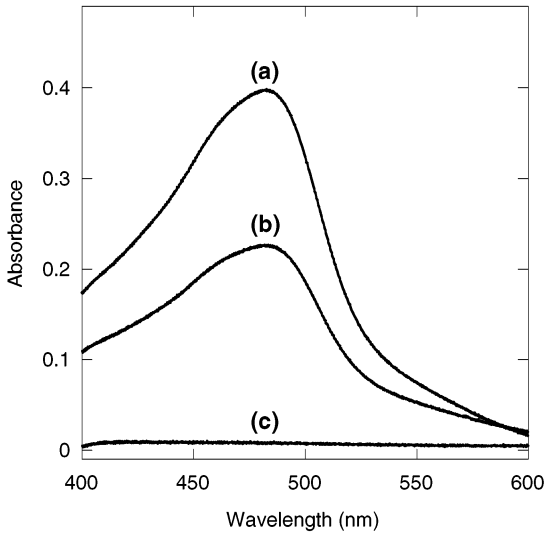


Figure 11. Absorption spectra in Tris/Mes buffer (50 mM; pH 8) of (a) Cu(I) (30 μM) and bcs (200 μM); (b) as in (a) after addition of apo-Ctr1c (5 μM); (c) as in (a) after addition of apo-Ctr1c (60 μM).

constant of $Cu^{I}(bcs)_2^{3-}$ has been determined to be $\beta_2 = K_1K_2 = 10^{19.8}$,[27] allowing the average Cu(I) dissociation constant K_D (eq 5) to be estimated for each protein (Table 1).

Both Atx1 and Ccc2n bind one Cu(I) ion tightly and both K_D values are estimated to be $\sim 10^{-19}$. The results predict that K_{ex} (eq 7) for exchange of Cu(I) between these proteins should fall in the range 2−5, consistent with the independent estimate of 1.4 (Table 1). The average $K_D = (K_1K_2K_3K_4)^{1/4}$ for Ctr1c is also about 10^{-19}, again consistent with the independent estimates for exchange of Cu(I) between this protein and Atx1 and Ccc2n (Tables 1, 2). The assumptions inherent in this analyis should be noted. In the present work, GSH was included in the reactions to ensure solubility and to maintain copper in oxidation state I. It may bind as an extra copper ligand. In addition, excess protein at low values of overall Cu occupancy θ may also function as an interprotein copper ligand.[41] These complications may affect the estimation of K_D. Nevertheless, the close agreement of the two independent estimates of K_{ex} (Table 1) provide some confidence in the validity of the present approach. The three proteins buffer "free" Cu(I) concentrations in a narrow range around 10^{-19} M (Table 1).

Discussion

The isolated cytosolic domain *apo*-Ctr1c was monomeric in solution with little regular secondary structure. It was highly susceptible to oxidation of Cys residues. It exhibited high Cu(I) affinity in vitro and could bind four Cu(I) ions per protein molecule with an average $K_D \approx 10^{-19}$. Titration of Cu(I) into *apo*-Ctr1c provided a linear increase in the absorbance at 265 nm with increasing concentration of Cu(I), necessary but not sufficient evidence of cooperative binding of 4 Cu(I) ions within a single Ctr1c molecule (Figures 7, S2). ESI−MS detected Cu_4−Ctr1c as the only Cu(I)-loaded form (Figure 1). *Apo*-Ctr1c was sometimes detected as a minor component of aged solutions, but Cu, Cu_2, and Cu_3 forms of Ctr1c were never detected. *Apo*- and Cu_4−Ctr1c only were observed in solutions containing 2 eq Cu(I). Addition of 2 extra eq Cu(I) converted all *apo*-Ctr1c to Cu_4Ctr1c (and possibly other Ctr1c species with higher copper contents). These observations suggest that Cu_4Ctr1c is the most stable form of Cu(I)-loaded Ctr1c.

For fully Cu-loaded Ctr1c, EXAFS analysis indicated the presence of a $Cu_4(\mu\text{-S}-\text{Cys})_6^{2-}$ cluster, in which a distorted tetrahedral core of Cu atoms is bound by a distorted octahedron of bridging thiolates, one above each of the six edges of the tetrahedron (Figure 10). The three-dimensional structure of the protein is very likely to be strongly influenced by the metal binding site. Equilibrium sedimentation analysis suggested that Cu_4−Ctr1c samples may contain as much as 20% of Cu(I)-bridged dimers. Considering the structural flexibility of the molecule, the Cys-4 side chain, well separated from other Cys residues (Figure 2), may act as an interchain ligand forming a dimer bridged by two metal clusters in a head-to-tail manner. X-ray absorption spectroscopy would be unlikely to distinguish the metal sites in monomeric and dimeric molecules.

The XAS spectrum of Cu_4Ctr1c is almost identical with that observed for the regulatory domain of Ace1 which also features four Cu(I) ions per protein molecule (Figure 8).[36] The regulatory domain of Mac1 possesses a structurally similar cluster to that in Ace1, as indicated by their similar EXAFS spectra. Remarkably similar Cu(I) clusters have been detected in isolated domains of each of these three proteins.

Acel and Mac1 are copper-sensing transcription factors in yeast that regulate copper levels under different conditions of copper availability.[5−8,29] In response to elevated intracellular Cu levels, Acel triggers the expression of the metallothionein Cup1 which sequesters Cu(I) as a stable and nonexchangeable Cu_7 cluster. Mac1 activates the expression of Ctr1 genes at copper starvation conditions and also (posttranslationally) controls the degradation of Ctr1 protein at elevated copper levels. Intriguingly, a Ctr1 variant with some cysteine residues replaced by serine in the C-terminal domain showed resistance to such degradation.[9] Thus if the same Cu(I) cluster is assembled in vivo in the C-terminal domain, then its formation may be important for communication with Mac1. Notably, both Ace1 and Mac1 copper sensors appear to be unique to yeast cells and so is the C-terminal domain of yeast Ctr1. Therefore, another important role of Ctr1c may be to function as an indicator of copper levels in the cytosol and to direct the action of Mac1. At lower copper levels, assembly of the Cu(I) cluster in Ctr1c may be limited, signaling Mac1 to activate the expression of other copper uptake systems such as Ctr1 and Ctr3. At higher copper levels, the ready assembly of the Ctr1c cluster may instruct Mac1 to act in the opposite direction.

Both Mac1 and Ace1 and their respective transcriptionally controlled gene products Ctr1 and Cup1 have no appreciable regular secondary structures, and show little similarity in metal binding sequence. However, each can bind Cu(I) to form structurally similar metal clusters. The structural flexibility of the *apo*-proteins would appear to contribute importantly to the stability of the metal cluster. The structures of the Cu(I) forms seem to be dictated by the metal cluster binding, as revealed in the case of Cup1.[42] On the other hand, although Ccc2n also carries six cysteine residues, it exhibits a well-defined ferredoxin-type structure and binds a single Cu(I) ion.[34] We have demonstrated that this molecule can also bind up to 4 Cu(I)

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ions per protein molecule (Figures S4,S5) but at the cost of destruction of its secondary structure. This may represent another aspect of the toxicity of uncontrolled "free" copper in living systems.

Whether copper chaperones can interact with and acquire Cu(I) ions *directly* from Ctr1c in vivo, as suggested by the model of Scheme 1, has yet to be demonstrated. However, Ctr1c exchanges Cu(I) with both Atx1 and Ccc2n rapidly and cleanly in vitro. These aspects provide some support for an interesting in vivo observation: while Cu(I) is loaded onto Ccc2 primarily via the copper chaperone Atx1, a second independent pathway may involve endocytic vesicles of Ctr1 which may bypass Atx1 and deliver Cu(I) to Ccc2 directly.[40]

Sophisticated homeostastic mechanisms regulate uptake and distribution of copper within cells.[1,2] Although the intracellular copper concentration is estimated to be about 70 μM, the free copper concentration has been proposed to be very low ($<10^{-10}$ M, i.e., less than 1 free copper ion per yeast cell).[43] This provocative proposal attributes an extraordinary copper binding capacity to the various copper proteins in the cytosol, enabling them to act effectively as copper buffers. A similar situation of controlled ultralow metal ion concentration may exist for Zn(II). There is experimental evidence indicating that zinc export pumps are switched on in *E. coli* when "free" zinc levels in the cytosol exceed 10^{-15} M.[44]

The present work demonstrates that the chaperone protein Atx1 and isolated cytosolic domains of the membrane pumps Ctr1 and Ccc2 in yeast can buffer at concentrations 10^{-19} M (Table 1). The buffering ranges are similar at equivalent copper loadings, consistent with the Cu(I) trafficking gradient being shallow along the putative Ctrlc → Atx1 → Ccc2n route in yeast (Scheme 1).[2] The question then arises as to why these proteins can buffer at about 10^{-19} M Cu(I) when a capacity of about 10^{-10} M would ensure less than one "free" copper atom per cell. This may be a consequence of the tight thermodynamic control needed to maintain discrete metabolic pathways (Scheme 1) in the presence of competition from other proteins and from GSH, a competing cysteinyl ligand, whose cytosolic concentration is about 5 mM.

Abbreviations. bcs: bathocuproine disulfonate; CD, circular dichroism; Ctr1c: protein expressed as residues 282−406 of the Ctr1 protein of *Saccharomyces cerevisiae*; *Ccc*2n: protein expressed as residues 1−72 of the *Ccc*2 protein of *Saccharomyces cerevisiae*; DTT: DL-dithiothreitol; ESI−MS: electrospray ionization mass spectrometry; GC−AAS: graphite cuvette atomic absorption spectroscopy; GSH: glutathione; ICP−MS: inductively coupled plasma mass spectrometry; IPTG: isopropyl-β-*o*-thiogalactopyranoside; KPi, potassium phosphate buffer; TCEP: tris(2-carboxyethyl)phosphorane.

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Supporting Information Available: Titrations of *apo*-Atx1, *apo*-Ctr1c, *apo*-Ctr1c-C4S, and *apo*-Ccc2n in Tris/Mes with $[Cu^{I}(CH_3CN)_4]^+$ in MeCN (Figures S1−S4); CD spectras of Ccc2n (Figure S5); and the direct exchange of Cu(I) between Ctr1c and Atx1 (Table S1). This material is available free of charge via the Internet at http://pubs.acs.org.

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