Presence of a Copper(I)−Thiolate Regulatory Domain in the Copper-Activated Transcription Factor Amt1[†]

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ABSTRACT: The Amt1 transcription factor from *Candida glabrata* is activated by the formation of a tetracopper−thiolate cluster. Recombinant Amt1 (residues 1−110) is isolated as a Cu,ZnAmt1 complex. Previous mapping studies [Farrell et al. (1996) *Biochemistry 35*, 1571−1580] revealed that the Zn(II) site is enfolded by an independent, N-terminal domain consisting of residues 1−40. One prediction from the mapping study is that the tetracopper cluster is enfolded by residues 41−110. A truncated Amt1 peptide consisting of residues 37−110 was expressed and isolated as a CuAmt1 complex with 4 mol equiv of Cu(I) bound. The bound Cu(I) ions in the truncated Amt1 complex were spectroscopically similar to Cu(I) ions bound in the 110-mer Amt1 molecule in the energies and intensities of the ultraviolet S → Cu charge transfer transitions and luminescence. Copper K-edge extended X-ray absorption fine structure spectroscopy (EXAFS) of the truncated CuAmt1 complex revealed the same 2.26 Å mean Cu−S bond distance as in the Cu,ZnAmt1 complex. A diagnostic feature of the polycopper-thiolate cluster in Cu,ZnAmt1 is the short 2.7 Å Cu−Cu distance determined by Cu K-edge EXAFS. The truncated CuAmt1 complex had the same short 2.7 Å Cu−Cu distance. The truncated CuAmt1 complex bound DNA specifically and with high affinity consistent with residues 41−110 being an independent domain stabilized by the tetracopper cluster. Thus, Amt1 consists of three independent and contiguous domains, an N-terminal Zn module (residues 1−40), an adjacent Cu regulatory domain (residues 41−110), and a C-terminal transcriptional activation domain. Cu(I) activation of Amt1 appears to consist of conversion of the 70-residue Cu regulatory domain from an inactive conformer to a structure containing the tetracopper cluster.

The ability of yeast to propagate in medium containing copper salts is largely dependent on the expression level of metallothionein (MT) (Fogel et al., 1983; Karin et al., 1984). The synthesis of MT in yeast is coupled to the intracellular copper concentration through Cu(I)-induced transcriptional activation of MT gene expression (Thiele & Hamer, 1986; Furst et al., 1988). The effect of Cu(I) ions on MT gene transcription in *Saccharomyces cerevisiae* and *Candida glabrata* is mediated by the homologous transcription factors Ace1 and Amt1, respectively, in the two yeast (Thiele, 1988; Welch et al., 1989; Zhou & Thiele, 1991; Zhou et al., 1992).

The mechanism of Cu-induced expression of yeast MT genes lies in the Cu(I) activation of Ace1 and Amt1. The activation process involves formation of a polycopper cluster with cysteinyl thiolates as ligands (Dameron et al., 1991; Nakagawa et al., 1991; Thorvaldsen et al., 1994). Both polypeptides consist of at least two segments with distinct functions (Furst et al., 1988). The binding of Ace1 and Amt1 to DNA promoter sequences maps to the N-terminal 100−120 residues of each protein (Furst et al., 1988; Thorvaldsen et al., 1994). The N-terminal segments contain multiple cysteinyl residues in Cys-X-Cys or Cys-X-X-Cys sequence motifs (Furst et al., 1988; Buchman et al., 1989; Zhou et al., 1992). Eleven cysteinyl residues are critical for Ace1 function in *S. cerevisiae* (Hu et al., 1990), and these cysteinyl residues are conserved in Amt1 (Zhou et al., 1992). The C-terminal segment of Ace1 is functionally the transactivation domain (Furst et al., 1988; Chaudhuri et al., 1995).

We have previously demonstrated that expression of the N-terminal halves of Amt1 and Ace1 in bacteria cultured in the presence of copper salts results in the isolation of proteins containing 4 and 1 mol equiv of copper and zinc, respectively (Thorvaldsen et al., 1994; Farrell et al., 1996). Two lines of evidence suggest that the four Cu(I) ions are bound within a single polycopper cluster. First, titration studies followed by mass spectrometry revealed that four Cu(I) ions bind to Amt1 in an all-or-nothing manner (Thorvaldsen et al., 1994). Second, extended X-ray absorption fine structure spectroscopy (EXAFS) of the CuAmt1 and CuAce1 complexes showed the presence of a short 2.7 Å Cu−Cu distance that was identical with the Cu−Cu separation in a synthetic $[Cu_4(SPh)_6]^{2-}$ cage cluster (Dance, 1986; Dameron et al., 1991; Nakagawa et al., 1991; Pickering et al., 1993; Thorvaldsen et al., 1994). EXAFS of a series of tetracopper-thiolate

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clusters revealed a correlation between the mean Cu−S bond distance and the fraction of trigonal Cu(I) ions (Pickering et al., 1993). The mean Cu−S bond distances in Ace1 and Amt1 predict trigonal Cu(I) coordination (Thorvaldsen et al., 1994; Pickering et al., 1993).

Mutagenesis was used to locate the tetrahedral Zn(II) ligands in Amt1 and Ace1 (Farrell et al., 1996). The four ligands consist of three cysteinyl residues and a histidyl imidazole that are located within the N-terminal 25 residues in each protein. The Zn module appears to be an independent domain of 40 residues (Farrell et al., 1996). The setting of the boundary at residue 40 is based on sequence homology of three known yeast proteins and three candidate yeast gene products.

One prediction from the mapping studies on Amt1 is that the tetracopper module is enfolded by residues 41−110. The candidate tetracopper module would consist of four Cu(I) ions and eight cysteinyl thiolates with multiple μ-bridging thiolates holding the cluster together (Farrell et al., 1996). To test the prediction that the tetracopper cluster is enfolded by a submodule consisting of residues 41−110, we constructed a truncated Amt1 molecule consisting of residues 37−110. We report presently that expression of this fragment in *Escherichia coli* results in the isolation of a protein with an intact tetracopper center. The truncated CuAmt1 complex resembles the intact Cu,ZnAmt1 complex in spectroscopic properties and DNA binding. The implication is that residues 41−110 form a copper regulatory domain. Cu(I) triggering appears to involve the formation of the tetracopper−thiolate cluster within the regulatory domain.

EXPERIMENTAL PROCEDURES

Construction of Mutant Amt1 Genes. The DNA fragment of *AMT1* coding for residues 37−110 was amplified from pET9d-ABC containing the *AMT1* synthetic gene (Thorvaldsen et al., 1994). The oligonucleotides used for the polymerase chain reaction (PCR) were 5′-CGC GGG AAT TCC ATG GGT CGT CCG CCG ACC-3′ (*AMT1*) and 5′-CTA GTT ATT GCT CAG CGG TGG-3′ (T7 terminator sequence). A 0.22 kb *Eco*RI/*Bam*HI fragment was subcloned into the *Eco*RI/*Bam*HI multiple-cloning site of pBluescript II SK (Stratagene). An expression vector containing the truncated Amt1 gene product was constructed by subcloning the *Nco*I/*Bam*HI fragment into pET-3d (Novagen). The sequence was confirmed by DNA sequencing with Sequenase (U.S. Biochemicals).

The truncated chromosomal *AMT1* gene was constructed in which codons 8−26 were deleted. Primers for PCR amplification were synthesized as follows: 5′-GGG GAA GCT TGA TAG ACC CT T AAA GAT ACT AAA GCC-3′ (*AMT1*) and 5′-GGC TTT AGT ATC TTT AAG GGT CTA TCA AGC TTC CCC-3′ (vector sequence). The PCR was performed using Vent DNA polymerase (New England Biolabs) and YEp-*AMT1* (Thorvaldsen et al., 1993) as the DNA template. The resulting PCR product containing *AMT1* codons 27−265 was cloned into pUC19 to produce pJS99 and its sequence confirmed. The *Hin*dIII/*Sac*I fragment of pJS99 containing the truncated *AMT1* gene was subcloned into pAKS109 which contains the first seven codons of the *AMT1* coding sequence and 510 bp 5′ to the *AMT1* coding sequences to produce pJS104. The Sac91 ARS fragment of *C. glabrata* previously described (Thorvaldsen et al., 1995) was inserted at the *Sac*I site resulting in pJS105. Plasmid pJS105 was transformed into *C. glabrata* strain 85/038 *amt1-1* (Ura$^-$) generously provided by D. J. Thiele (Zhou et al., 1992).

Standard yeast culturing and transformation conditions were used (Kaiser et al., 1994). Copper tolerance was assessed by plating out serial dilutions of transformed cells on selective medium containing various concentrations of $CuSO_4$.

Protein Purification. Cu,ZnAmt1 was purified as described previously (Farrell et al., 1996; Thorvaldsen et al., 1994). The same protocol was used in the purification of the tetracopper module of CuAmt1, designated $CuAmt1_t$. Purity was assessed by the presence of a single Coomassie-stained band after SDS−polyacrylamide gel electrophoresis. The metal binding stoichiometry was assessed by metal analysis performed on a Perkin-Elmer 305A spectrometer. Protein was quantified by amino acid analysis after hydrolysis in 5.7 N HCl containing 0.1% phenol *in vacuo* at 110 °C with analysis performed on a Beckman 6300 analyzer. Edman sequencing was carried out on an Applied Biosystems model 475 sequencer with on-line high-performance liquid chromatographic analysis of phenylthiohydantoin derivatives. Luminescence was measured on a Perkin-Elmer 650-10s fluorimeter with excitation at 300 nm. Ultraviolet absorption spectroscopy was carried out on a Beckman DU-65 spectrometer.

XAS Data Collection. Copper K-edge data 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 of 1.8 T. Harmonic rejection was accomplished by detuning one monochromator crystal to approximately 50% off peak. 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 with an array of 13 germanium intrinsic detectors (Cramer et al., 1988). Samples were maintained at a temperature of approximately 10 K during data collection using an Oxford Instrument liquid helium flow cryostat. Eight 35 min scans were accumulated for each sample, and the absorption of a copper metal foil was measured simultaneously by transmittance. The X-ray energy was calibrated with reference to the lowest energy inflection point of the copper foil spectrum which was assumed to be 8980.3 eV.

The extended X-ray absorption fine structure (EXAFS) oscillations $\chi(k)$ were quantitatively analyzed by curve-fitting using the EXAFSPAK suite of computer programs (available upon request to G.N.G.) using *ab initio* theoretical phase and amplitude functions generated with the program *feff* version 6.01 (Rehr et al., 1991; Mustre de Leon et al., 1991).

DNA Binding Assay. Gel shift assays were performed as described previously using a 25 bp oligonucleotide duplex (Thorvaldsen et al., 1994). The oligonucleotide duplex spanned the Amt1 binding site of the *AMT1* gene. Two 18 bp oligonucleotide duplexes representing two different segments of the AMT1 5′ sequence were used for competition binding studies. Only one competing duplex (D1) contained the complete upstream activation sequence.

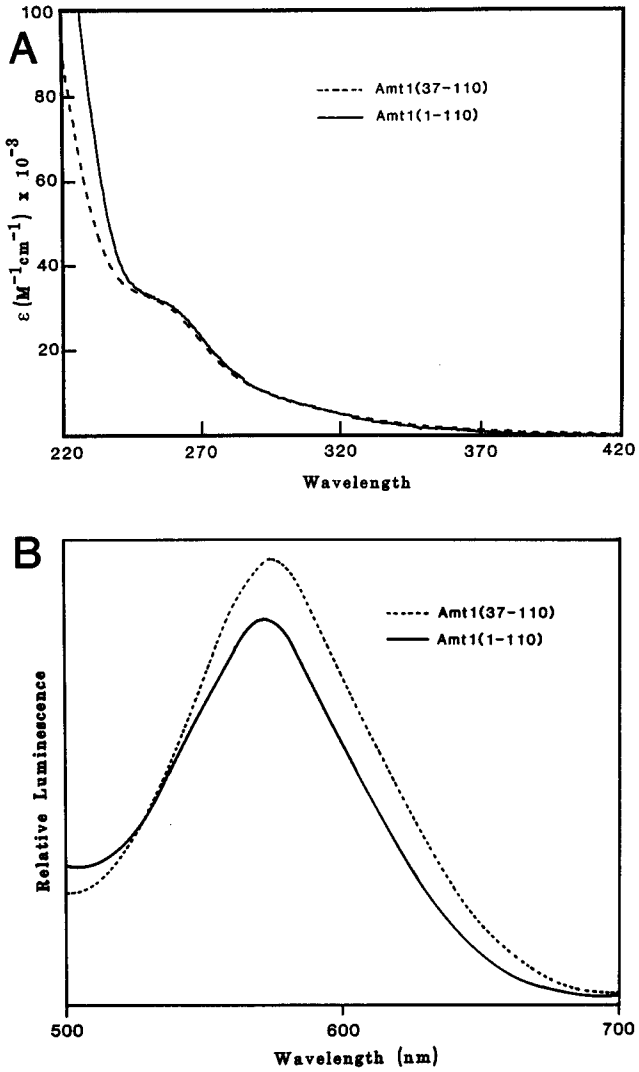

FIGURE 1: Ultraviolet absorption and emission spectra of Cu,ZnAmt1 and CuAmt1$_t$. Panel A contains the absorption spectra of Cu,ZnAmt1 (solid line) and CuAmt1$_t$ (dashed line) at concentrations of 7.5 and 10.5 μM, respectively. Samples were buffered in 20 mM Tris at pH 7.4 containing 50 mM NaCl. Emission spectra shown in panel B were recorded using 5.9 μM samples of Cu,ZnAmt1 (solid line) and CuAmt1$_t$ (dashed line). Excitation was at 300 nm, and the samples were buffered as noted for the absorption experiments above.

RESULTS

Isolation of the Tetracopper Module of Amt1. Our mapping of metal modules in Amt1 and Ace1 indicated that the N-terminal 40 residues of each molecule enfolded the single Zn(II) site (Farrell et al., 1996). It follows that the candidate tetracopper module may be enfolded by residues 41−110. We constructed an expression vector in which a DNA fragment corresponding to codons 37−110 of Amt1 was subcloned in a pET vector. Expression of the Amt1 peptide (designated Amt1$_t$) in cells induced with IPTG in the presence of 1.4 mM $CuSO_4$ resulted in the accumulation of the CuAmt1$_t$ peptide. The CuAmt1$_t$ peptide complex was purified to homogeneity as judged by a single Coomassie-stained band on polyacrylamide gel electrophoresis. Edman sequencing revealed the purified peptide had the expected N-terminal sequence but lacked the initiator methionine. The amino acid composition of the peptide was consistent with the expected composition of residues 37−110.

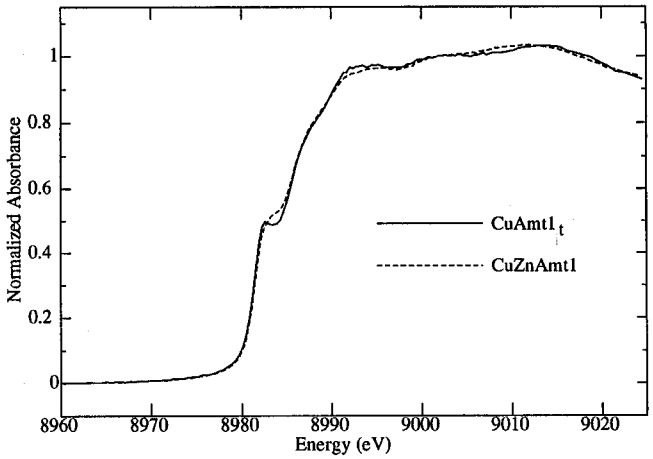

FIGURE 2: Comparison of the copper K-edge near edge spectra of Cu,ZnAmt1 and the truncated CuAmt1$_t$ complex.

The peptide was isolated as a copper-binding component. Quantitation of the bound Cu(I) revealed a stoichiometry of 4.2 ± 0.3 ($n = 3$) Cu(I) ions bound per Amt1$_t$ peptide. The molecule was devoid of Zn(II). The Cu complex exhibited S → Cu charge transfer transitions in the ultraviolet similar in energy and intensity to those of the intact Cu,ZnAmt1 complex (Figure 1A). The differences in absorbance in the samples below 240 nm arise from the higher polypeptide backbone absorbance in Cu,ZnAmt1 due to the greater number of peptide bonds in this protein. The similar absorbance intensity and energies imply that the four Cu(I) ions in Amt1$_t$ are ligated similarly to the four Cu(I) ions in the 110-mer Cu,ZnAmt1 complex.

The copper ions were deduced to be in the cuprous oxidation state from the luminescence of the complex. The intensity and energy of the emission of the CuAmt1$_t$ peptide complex and the Cu,ZnAmt1 complex were similar (Figure 1B). Emission of Cu(I) complexes is typically quenched if Cu(I) ions are accessible to solvent (Lytle, 1970). The similar relative quantum yield of emission of the CuAmt1$_t$ sample and intact Cu,ZnAmt1 sample implies that the Cu(I) ions in both aqueous samples are solvent shielded.

Copper K-Edge EXAFS of Cu,ZnAmt1 and CuAmt1$_t$. Figure 2 shows the copper K-edge X-ray absorption edge spectra of the Cu,ZnAmt1 and CuAmt1$_t$ complexes. The spectra are broadly similar to each other, with subtle differences, and are indicative of copper in the cuprous oxidation state (Kau et al., 1987) with thiolate ligation to the metal (Pickering et al., 1993).

The Cu K-edge EXAFS and Fourier transforms of the Cu,ZnAmt1 and the truncated CuAmt1$_t$ complexes are presented in Figures 3 and 4 with results of curve fitting presented in Table 1. The prominent first shell Cu−S interaction at 2.26 Å in Cu,ZnAmt1 (Figure 3) is in excellent agreement with the Cu−S distance of 2.26 Å determined for a previous sample of Cu,ZnAmt1 (Thorvaldsen et al., 1994). The mean bond length of 2.26 Å was consistent with predominantly trigonal Cu(I) coordination (Pickering et al., 1993). A second smaller peak in the Fourier transform of Cu,ZnAmt1 was observed at 2.71 Å (Figure 3). This peak was best fit as a Cu−Cu interaction and is indicative of a polycopper−thiolate cluster. The main difference between the EXAFS of the present versus the original Cu,ZnAmt1 samples is that the Cu−Cu coordination number (N) was 2, unlike the N of 1 in the earlier study.

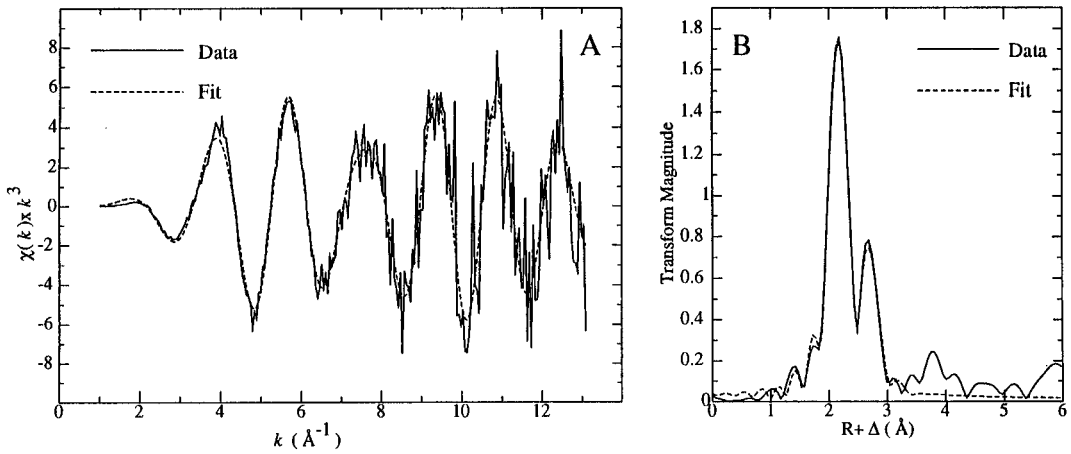

FIGURE 3: Copper K-edge EXAFS (A) and EXAFS Fourier transform (B) of Cu,ZnAmt1. The solid lines show the experimental data and the broken lines the best fit using the parameters given in Table 1. The EXAFS Fourier transforms (B) have been phase-corrected for Cu−S backscattering.

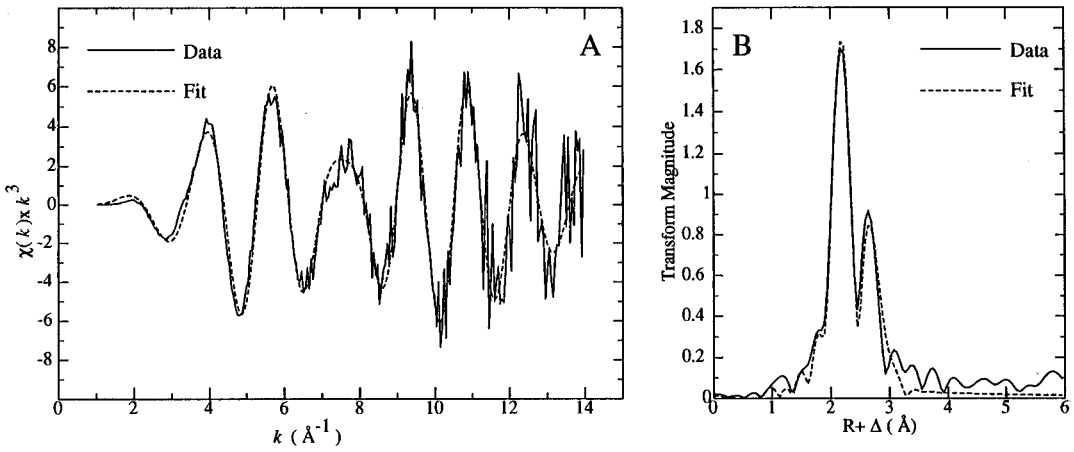

FIGURE 4: Copper K-edge EXAFS (A) and EXAFS Fourier transform (B) of CuAmt1$_t$. The solid lines show the experimental data and the broken lines the best fit. The EXAFS Fourier transforms (B) have been phase-corrected for Cu−S backscattering.

Table 1: Cu K-Edge EXAFS Curve-Fitting Results[a]

parameter	Cu,ZnAmt1	CuAmt1$_t$
	Cu−S	
N	2.8	2.8
R (Å)	2.265 (6)	2.266 (5)
σ^2 (Å^2)	0.0029 (6)	0.0029 (5)
	Cu−Cu	
N	2.0	2.0
R (Å)	2.709 (16)	2.703 (11)
σ^2 (Å^2)	0.0079 (18)	0.0065 (11)
F_{fit}	2.218	1.577
fit range (Å^{-1})	1−13	1−14

[a] The phase and amplitude functions used in the fit were as described previously (Pickering et al., 1993). Coordination numbers were chosen to give the best fit to the nearest integer, ±0.2 for Cu−S and ±0.5 for Cu−Cu. The precision, in the form of estimated 95% confidence limits, is given in parentheses for each of the parameters floated in the fit. The goodness of fit parameter is defined as $F_{fit} = [\Sigma(\chi_0 - \chi_c)^2\kappa^6]/(\eta_{obs} - \eta_{var})$, where χ_0 and χ_c are the observed and calculated EXAFS, respectively (κ^3-weighted), and η_{obs} and η_{var} are the number of observations and variables floating in the refinement, respectively.

The Fourier transform of the EXAFS of the CuAmt1$_t$ peptide complex is dominated by two scatter peaks (Figure 4). The first peak arises from the first shell Cu−S bonding at 2.26 Å (Table 1). The prominent outer shell peak was best fit as a Cu−Cu interaction with a mean Cu−Cu distance of 2.70 Å. These values are very similar to the corresponding values for the Cu,ZnAmt1 complex (Table 1) and suggest that a similar polycopper cluster exists in the intact Cu,ZnAmt1 molecule and the CuAmt1$_t$ complex. Thus, N-terminal truncation of the Amt1 peptide did not perturb the environment of the tetracopper cluster.

DNA Binding of the Truncated CuAmt1t Complex. We demonstrated previously that *in vitro* DNA binding by Cu,ZnAmt1 was not significantly attenuated by removal of Zn(II) from the complex (Thorvaldsen et al., 1994). In contrast, DNA binding was abolished by removal of the bound Cu(I) ions. An interpretation of these studies is that much of the stabilization energy for the Amt1:DNA complex arises from DNA contacts by the tetracopper portion of Amt1. One prediction is that the truncated CuAmt1$_t$ complex would retain high-affinity DNA binding activity. The purified CuAmt1$_t$ complex was compared to the intact Cu,ZnAmt1 complex in quantitative gel shift assays. The CuAmt1$_t$ complex bound DNA with an affinity of 20 nM (Figure 5). The binding affinity was attenuated only 10-fold in comparison with Cu,ZnAmt1. Competition binding studies were carried out using two subfragments of the Amt1 binding site to show that CuAmt1$_t$ formed a specific protein:DNA complex (Figure 6). The addition of unlabeled DNA consisting of the A/T-rich sequence and the core GCTG sequence resulted in a concentration-dependent competition in binding (duplex D1 in Figure 6). In contrast, the addition

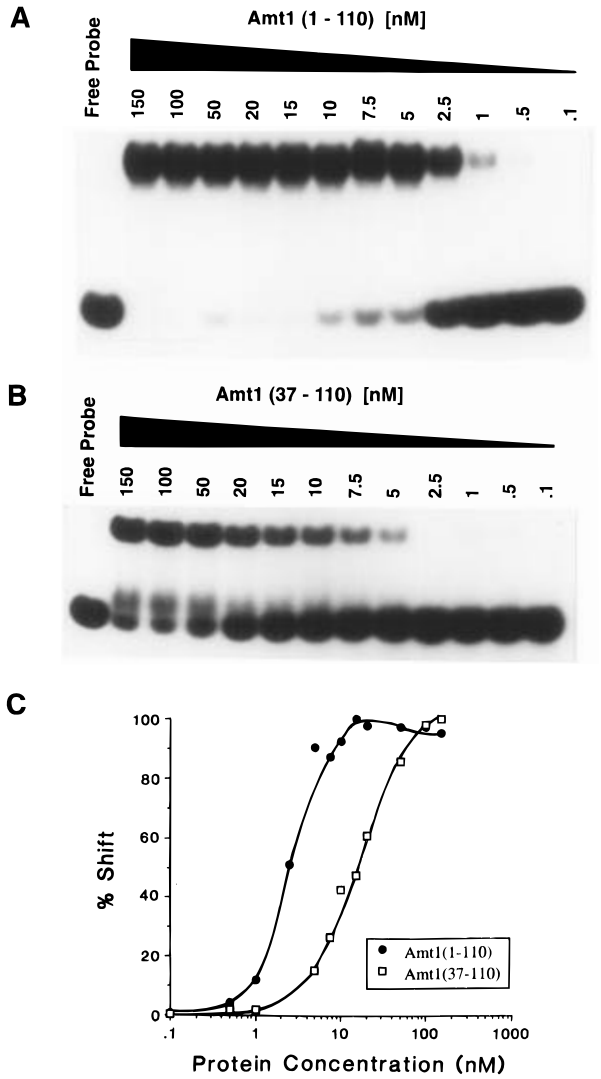

FIGURE 5: Gel retardation assay of Cu,ZnAmt1 (panel A) and $CuAmt1_t$ (panel B). Protein was mixed with a labeled 25 bp DNA duplex containing the Amt1 binding site within the *AMT1* 5′ sequence. The numbers refer to the concentration of each protein within the incubation mixture. The quantitation of the shifts in panels A and B is shown in panel C. The apparent DNA binding affinities for Cu,ZnAmt1 and $CuAmt1_t$ were similar from two different gel exposures.

of a unlabeled DNA duplex consisting of the A/T-rich sequence but lacking the core GCTG sequence failed to compete (duplex D2 in Figure 6).

In Vivo Activity of Amt1 Lacking the Zn Module. The observed high-affinity *in vitro* protein:DNA complex with the truncated $CuAmt1_t$ suggested that an *AMT1* gene lacking only codons for the N-terminal Zn module may retain limited *in vivo* function. A deletion was made in the *AMT1* gene resulting in the deletion of codons 8−27. The deletion results in a gene product lacking all four Zn(II) ligand residues. This truncated gene was placed under its own *AMT1* promoter in a vector containing a *C. glabrata* autonomous replication sequence and used to transform a strain of *C. glabrata* lacking a functional *AMT1* gene. Transformants containing the wild-type *AMT1* gene restored copper resistance to 1 mM $CuSO_4$ (Figure 7). Transformants containing the truncated *AMT1* gene exhibited resistance up to 0.1 mM $CuSO_4$ (Figure 7). In contrast, transformants harboring an empty vector failed to propagate in medium containing 0.1 mM $CuSO_4$. Deletion of the bulk of the Zn module in Amt1 attenuated, but did not abolish, the ability of Amt1 to mediate Cu-induced expression of MT genes. Thus, the copper regulatory domain alone shows partial functionality.

DISCUSSION

Activation of Ace1 and Amt1 requires the formation of a tetracopper cluster in each transcription factor. The existence of a polycopper cluster comes primarily from the detection of the Cu−Cu scatter interaction by EXAFS (Pickering et al., 1993; Thorvaldsen et al., 1994). The nuclearity of the cluster cannot be assessed by EXAFS. The all-or-nothing formation of a 4 Cu(I) center in the 110-residue Amt1 molecule (Thorvaldsen et al., 1994) is suggestive that the actual polycopper cluster is tetranuclear.

Evidence is provided that a fragment of the 265-residue Amt1 polypeptide consisting of residues 37−110 enfolds a similar CuS cluster as the intact 110-residue Amt1 molecule. First, the two Cu(I) complexes exhibit the same extinction coefficients for the ultraviolet S → Cu charge transfer transitions. Second, the two Cu(I) complexes luminesce with a similar relative quantum yield. Third, $CuAmt1_t$ and Cu,ZnAmt1 have the same mean Cu−S bond distance of 2.26 Å and a similar short 2.7 Å Cu−Cu distance determined by copper K-edge EXAFS. Fourth, the truncated $CuAmt1_t$ complex retains significant DNA binding affinity and specificity. The integrity of the tetracopper−thiolate cluster in the truncated Amt1 complex suggests that this segment is an independent domain.

Previous mapping studies showed that the N-terminal 40 residues form a stably folded independent domain (Farrell et al., 1996). The C-terminal boundary of the Zn domain at residue 40 is based on loss of homology between Ace1 and Mac1 after residue 40 (Jungmann et al., 1993). Thus, Amt1 and Ace1 appear to consist of three separate domains: residues 1−40, the Zn(II) domain; residues 41−110 (100 in Ace1), the tetracopper domain; and residues 110−C-terminal end, the transactivation domain. A model for the organization of Ace1 and Amt1 is shown in Figure 8.

Since Amt1 and Ace1 are activated to become DNA-binding proteins by formation of the tetracopper cluster, the tetracopper domain is functionally a Cu regulatory domain. This Cu regulatory domain is also found in the *S. cerevisiae* Lpz8p and *Yarrowia lipolyitica* Crf1 sequences. From these sequences the following consensus sequence can be derived as: $C\text{-}X_2\text{-}C\text{-}(X)_{12,14}\text{-}C\text{-}X\text{-}C\text{-}(X)_{10-27}\text{-}C\text{-}X\text{-}C\text{-}X_5\text{-}C\text{-}X\text{-}C$.

There is only one spacing that shows length variability, and that is the linker separating the two halves containing four cysteines each. Cu(I) activation of Amt1 or Ace1 appears to consist of conversion of the 70-residue regulatory domain from an apoconformer or inactive Zn(II) conformer to a structure containing the tetracopper cluster. It is likely that the inactive state is a Zn(II) conformer, as Amt1 is isolated from cultures grown in the absence of added $CuSO_4$ as a Zn protein.

The presence of a tetracopper cluster within residues 41−110 in Amt1 and Ace1 implies that eight thiolates are available for Cu(I) coordination. If all thiolates exist as μ-bridging thiolate ligands, only six cysteinyl residues are necessary to form the cluster based on known structures of synthetic $[Cu_4(SPh)_6]^{2-}$ cage clusters. The presence of eight

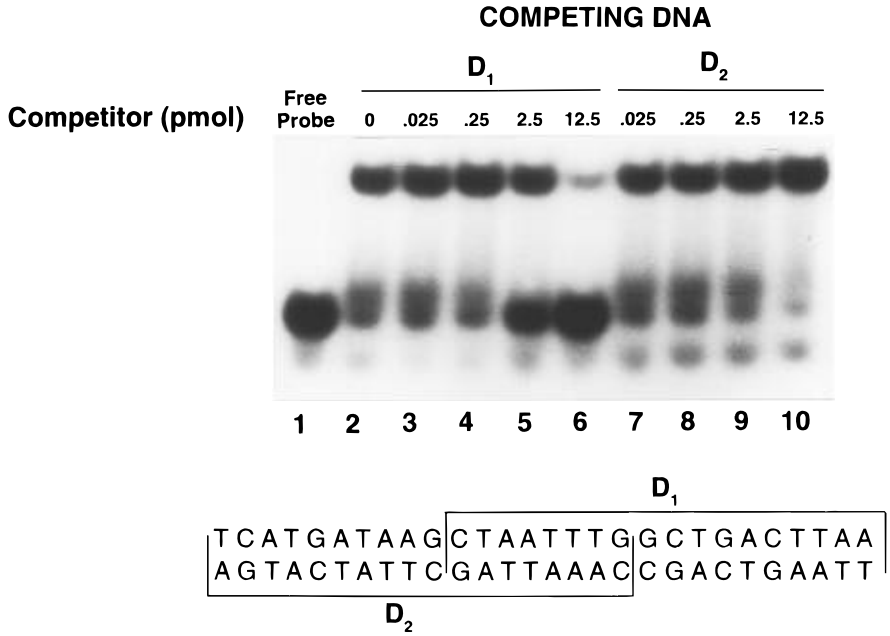

FIGURE 6: Competition DNA binding of CuAmt1$_t$. The radiolabeled DNA duplex (Figure 4) was mixed with 369 nM CuAmt1$_t$ and increasing concentrations of unlabeled oligonucleotides. The two oligonucleotides used in the competition studies were 18 bp duplexes of sequences shown in the figure. The numbers at the top refer to the concentration of added unlabeled oligonucleotide.

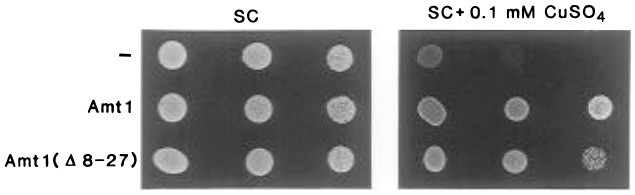

FIGURE 7: Copper tolerance of *C. glabrata* transformed with wild-type or mutant *AMT1*. Cells transformed with wild-type *AMT1*, mutant *AMT1* with a deletion of codons 8−27, or an empty vector were serially diluted and plated on synthetic, selective medium in the presence or absence of 0.1 mM $CuSO_4$. The number of cells plated was 6×10^6, 6×10^5, and 6×10^4 for the serial dilutions.

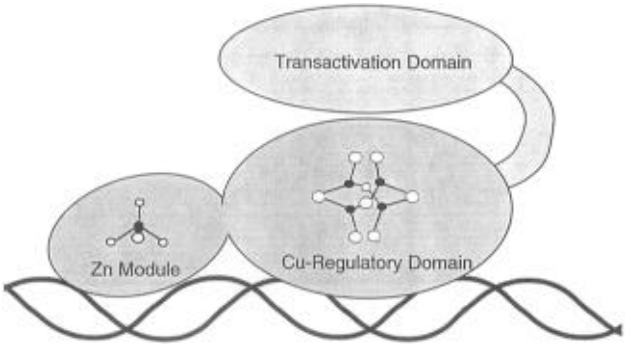

FIGURE 8: Model of the domains of Cu,ZnAmt1 and Cu,ZnAce1. Each protein is depicted having a N-terminal Zn domain and a Cu-(I) regulatory domain followed by the transactivation domain.

cysteinyl thiolates in this domain suggests that either the cluster contains two free thiolates or alternatively the cluster contains a mixture of μ-bridging and terminal thiolates. Cluster integrity is expected to be maintained by μ-bridging thiolates with minimal Cu−Cu bonding interactions as is true with the synthetic cage clusters (Dance et al., 1983; Nicholson et al., 1985; Dance, 1986).

The polycopper cluster may be considered a distorted tetrahedron of Cu(I) ions. By EXAFS, a tetrahedrally symmetric Cu_4 cluster would have three equivalent Cu(I) scatterers per absorbing Cu atom (Pickering et al., 1993). In the two isolates of Cu,ZnAmt1 evaluated, a difference existed in the mean number (N) of Cu(I) scatterers per Cu atom (N values of 1 vs 2). The observation that the Cu−Cu N value in Cu,ZnAmt1 is less than 3 implies that the Amt1 Cu(I) cluster deviates from the symmetrical ideal. Deviation of symmetry in Cu,ZnAmt1 may arise from polypeptide constraints. The difference in the two Cu,ZnAmt1 samples may arise from some plasticity in the cage structure. Similar deviation from symmetry is observed in the heptacopper−thiolate cluster in CuCup1. EXAFS analyses of CuCup1 revealed a Cu−Cu N value near 1 (George et al., 1988; Pickering et al., 1993).

There are two significant advantages of having a polycopper cluster as the structural unit of the activated forms of Ace1 and Amt1. First, a polycopper cluster provides metal ion specificity. The function of Amt1 and Ace1 in mediating metal-induced transcription of a subset of genes in *S. cerevisiae* and *C. glabrata*, respectively, is specific for Cu(I) and Ag(I) ions. The formation of a tetrametallic cluster is one way the process can be restricted in specificity to Cu-(I) and Ag(I) ions which are known to form similar polymetallic thiolate clusters (Dance, 1978). Second, formation of a tetracopper center in an all-or-nothing manner provides a concentration-dependent responsiveness of Ace1 and Amt1 in the activation of MT gene transcription.

The function of the Cu regulatory domain is in DNA binding. Detailed studies have been carried out mapping the protein:DNA contacts for Ace1 and Amt1 (Buchman et al., 1990; Dobi et al., 1995; Koch & Thiele, 1996). Both proteins make major groove contacts within a conserved GCTG core sequence and minor groove contacts in a conserved A/T-rich region. Recently, the protein region responsible for the minor groove A/T contacts was identified as the GRP sequence (residues 37−39) within the conserved Zn module (Koch & Thiele, 1996). An Arg38Lys substitution in this sequence reduced the *in vitro* DNA binding affinity at least 5-fold yet dramatically attenuated the *in vivo* activity of Amt1 (Koch & Thiele, 1996). Likewise, a

Gly37Glu substitution in Ace1 diminishes *in vitro* DNA binding affinity 7-fold (Buchman et al., 1990). Cells harboring this mutant *ACE1* were hypersensitive to copper salts in the growth medium (Welch et al., 1989). The observed 10-fold diminution of the truncated $CuAmt1_t$ complex in DNA binding affinity *in vitro* is consistent with loss of the minor groove contacts. Although the truncated $CuAmt1_t$ complex has the GRP sequence, its position at the N-terminus of the truncated molecule may compromise its ability to make the appropriate DNA contacts. Thus, it is expected and observed that cells harboring *AMT1* lacking codons 8−27 are hypersensitive to copper salts in the growth medium. The observed 20 nM DNA binding affinity of the $CuAmt1_t$ complex is suggestive that the Cu regulatory domain of Ace1 and Amt1 makes major groove contacts within the conserved GCTG sequence in the Cu responsive promoters in *S. cerevisiae* and *C. glabrata*. Footprinting studies are underway to test this prediction.

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