Distinct Metal Binding Configurations in ACE1†

C. T. Dameron,‡ G. N. George,§ P. Arnold,‡ V. Santhanagopalan,‡ and D. R. Winge*,‡

University of Utah Medical Center, Salt Lake City, Utah 84132, and Stanford Synchrotron Radiation Laboratory, Stanford University, P.O. Box 4349 Bin 69, Stanford, California 94309

Received January 26, 1993; Revised Manuscript Received April 28, 1993

ABSTRACT: The ACE1 protein of *Saccharomyces cerevisiae* mediates the metal-induced expression of the *CUP1* metallothionein (MT) genes. Curiously, ACE1 resembles the MT protein in the types of metal complexes that form. ACE1 binds Cd(II) and Cu(I) ions in distinct configurations, but only the Cu(I) conformer of ACE1 forms a high-affinity and specific complex with DNA. Cu(I) ions associated with ACE1 are known to assemble in a polymetallic Cu^I–thiolate cluster that resembles Cu–metallothionein in metal coordination properties [Dameron, C. T., Winge, D. R., George, G. N., Sansone, M., Hu, S., & Hamer, D. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 6127–6131]. In contrast to the Cu(I) nuclearity of 6-7 mol equiv in ACE1 and 7 mol equiv in yeast MT, divalent ions, including Cd(II), Zn(II), and Co(II), bind with a maximal stoichiometry of near 4 mol equiv in ACE1 and 4 mol equiv in yeast MT. Charge-transfer bands consistent with metal:thiolate coordination were observed in CdACE1 and CoACE1. Spectroscopic studies of Co^{II}ACE1 and EXAFS analysis of Cd^{II}ACE1 revealed tetrahedral coordination geometry in these complexes. Similar tetrahedral coordination complexes were observed with Co(II) and Cd(II) complexes of MT from *S. cerevisiae*. Metal binding in ACE1 is clearly similar to that in MT, and therefore the MT–metal clusters appear to be a good structural model of the metal center of ACE1.

Metallothionein (MT)[1] functions in zinc and copper homeostasis an in the detoxification of nonessential metal ions (Bremner, 1987). Regulation of MT expression permits control of the intracellular concentration of certain metal ions by formation of metal:MT complexes (Hamer, 1986; Winge et al., 1992). The expression of MT genes in eukaryotic organisms is metalloregulated, permitting the concentration of MT to vary in response to the intracellular concentration of particular metal ions (Hamer, 1986; Palmiter, 1987; Imbert et al., 1990; Thiele, 1992). Metalloregulation of gene expression for gene products involved in metal homeostasis and/or detoxification is common in all species (Ralston & O'Halloran, 1990; Mehra & Winge, 1991).

Expression of MT genes in animal cells is regulated by a variety of metal ions including Zn(II), Cd(II), and Cu(I), whereas expression of MT genes in the yeasts *Saccharomyces cerevisiae* and *Candida glabrata* is typically restricted to Cu(I) and Ag(I) ions (Palmiter, 1987; Imbert et al., 1990; Thiele, 1992). MT expression in yeast is regulated by metal-responsive transcriptional activator proteins designated ACE1 (for activator of the MT gene, *CUP1*, expression) or CUP2 in *S. cerevisiae* and AMT1 (for activator of *MT1* expression) in *C. glabrata* (Furst et al., 1988; Welch et al., 1989; Zhou & Thiele, 1991; Thiele, 1992). CuACE1 also functions in the copper-regulated expression of the gene for Cu,Zn-superoxide dismutase in *S. cerevisiae* (Gralla et al., 1991).

CuACE1 stimulated transcription of the genes by binding to specific promoter sequences in the 5′ flanking region of the MT gene (Furst et al., 1988; Culotta et al., 1989; Buchman et al., 1990). Binding of ACE1 to DNA is Cu-dependent (Furst et al., 1988). A major *cis*-acting element in the *CUP1* locus is the upstream activation sequence, designated UAS_c (Thiele & Hamer, 1986; Furst et al., 1988; Huibregtse et al., 1989; Evans et al., 1990; Thiele, 1992). The importance of this element in the CuACE1-induced expression of the *CUP1* MT gene was demonstrated by the UAS_c dependency of Cu-activated transcription of reporter genes in fusion constructs (Furst et al., 1988; Culotta et al., 1989; Buchman et al., 1990; Hu et al., 1990).

The molecular basis for metal ion specificity in MT induction in yeast is unknown. The goal of this study was to compare the coordination chemistry of the active CuACE1 and inactive CdACE1 molecules in an attempt to understand the nature of the metal specificity of MT gene expression. Metal binding properties of the Cu(I)-activated form of ACE1 have been reported (Dameron et al., 1991; Nakagawa et al., 1991). CdACE1 is nonfunctional in transcriptional activation of MT gene expression in *S. cerevisiae* (Ecker et al., 1986; Furst et al., 1988). We previously demonstrated that the Cu(I)-activated form of ACE1 contains a Cu^I–thiolate polynuclear cluster with each of the 6-7 Cu(I) ions being ligated in predominantly trigonal geometry (Dameron et al., 1991). The clusters mimic CuS cages observed in CuMT and certain synthetic model compounds (Dance, 1986; Winge et al., 1993a).

The lack of hydrophobic core and periodic secondary structural elements in CuMT and CuACE1 suggest that CuS cluster formation in both proteins may substantially influence the tertiary fold. Cluster formation appears to be driven by the strength of the Cu–S bond and not dictated predominantly by the polypeptide fold. The tertiary fold of mammalian MT is clearly influenced by metal ion binding. The apo-MT protein is devoid of structure (Vasak et al., 1980). Mammalian MT binds Cu(I) and Zn(II) or Cd(II) in distinct clusters which alter the tertiary fold (Nielson et al., 1985; Robbins et al., 1991). If metal clusters in ACE1 resemble metal centers in MTs, distinct cluster structures in CuACE1 and CdACE1

† D.R.W. acknowledges support by a grant (ES 03817) from the National Institutes of Health. C.T.D. was supported by a postdoctoral research training grant fellowship (5T32 DK 07115). The Stanford Synchrotron Radiation Laboratory is funded by the Department of Energy (Office of Basic Energy Science, Office of Health and Environmental Research) and the National Institutes of Health (Biotechnology Resource Program, Division of Research Resources).

* Author to whom correspondence should be addressed.

‡ University of Utah Medical Center.

§ Stanford University.

[1] Abbreviations: ACE1, activator of *CUP1* metallothionein expression; MT, metallothionein; EXAFS, extended X-ray absorption fine structure.

0006-2960/93/0432-7294$04.00/0

may explain the metal ion specificity observed in Cu(I)-mediated MT gene expression in *S. cerevisiae*. The prediction is that Cd(II) induces a metal:thiolate center in ACE1 distinct from the Cu(I) cluster, as is the case with MT (Nielson et al., 1985). Cu(I) specificity may reside in the cluster coordination chemistry. In this report, we have evaluated the binding of different divalent metal ions, Zn(II), Cd(II), and Co(II), to ACE1. Cd(II) and Co(II) are widely used as probes of Zn(II) sites in metalloproteins due to their electronic absorption properties and the nuclear spin of certain Cd isotopes (Vallee & Wacker, 1970; Gettins & Coleman, 1982; Bertini & Luchinat, 1984). These three divalent metal ions exhibit similar coordination structures in many proteins (Vallee & Wacker, 1970; Gettins & Coleman, 1982; Bertini & Luchinat, 1984).

MATERIALS AND METHODS

Purification of ACE1 and MT. The N-terminal segment of ACE1 (122 residues and 21 residues from the vector) was expressed in an *Escherichia coli* T7 expression system and purified as described previously (Dameron et al., 1991). Purity was verified by amino acid analysis and SDS–PAGE. apo-ACE1 was prepared by incubation with 20 mM KCN at pH 6.8 followed by sample concentration by ultrafiltration and subsequent acidification and chromatography on Sephadex G-25 equilibrated with 0.025 N HCl. The apoprotein was reduced with 50 mM DTT at neutral pH and rechromatographed on Sephadex G-25 equilibrated with 0.025 N HCl. The concentration of apo-ACE1 was determined by quantitative amino acid analysis after 24 h of hydrolysis in 5.7 N HCl *in vacuo* at 110 °C. The extent of cysteine reduction was monitored by titration with dithiodipyridine (Grassetti & Murray, 1967).

S. cerevisiae MT was purified from strain 301N as CdMT as described previously (Narula et al., 1991). apo-MT was prepared by acidification of the sample to pH 1 followed by gel filtration on Sephadex G-25 equilibrated in 0.02 N HCl. The protein was quantified by amino acid analysis, and the extent of reduction was determined as described above for ACE1.

Reconstitution of ACE1 and MT. Reconstitutions with metal ions were performed anaerobically at 23 °C in one of two protocols. The first protocol involved mixing apoproteins at pH 2 with a predetermined mole equivalency of Cd(II), Co(II), Cu(I), or Zn(II) from metal stock solutions in 25 mM HCl. The Cu(I) stock solution was 0.1 N HCl containing 0.4 M NaCl or a Cu:glutathione (GSH) complex containing a 10-fold molar excess of GSH. The mixtures were neutralized with 0.2 M potassium phosphate buffer to achieve the desired pH within a range of 6.5–8.5. A 1 M solution of Tris Cl, pH 8.5, was used to neutralize Co^{II}–protein samples. Metal concentrations were verified by analysis on a Perkin-Elmer 305A atomic absorption spectrometer. In a second protocol the apoproteins were premixed with 4 mol equiv of Zn(II) as described in the first protocol and subsequently incubated at pH 7 with increasing quantities of Cu(I) presented as Cu:GSH (1:10 molar concentrations). Additional quantities of glutathione were added to certain samples as described.

Spectroscopy of Metallo Forms of ACE1 and MT. Ultraviolet/visible absorption spectroscopy was carried out on a Beckman DU-65 spectrometer. Proteins reconstituted with Co(II) were scanned spectrophotometrically in anaerobic cuvettes shortly after preparation of the samples. Deleterious oxidative changes in the samples did not occur until after several hours. Luminescence measurements were conducted at 23 °C on a thermostated Perkin-Elmer 650-10S fluorimeter. X-ray absorption spectroscopy was performed at the Stanford Synchrotron Radiation Laboratory on beam line SB07-3, using a wiggler field of 1.8 T and a Si(220) double-crystal monochromator. The storage ring SPEAR contained 50-100 mA at 3 GeV during data collection. The cadmium K-edge X-ray absorption was monitored as the Cd Kα excitation spectrum by using a 13-element Ge detector. Samples were prepared in 67 mM potassium phosphate, pH 7, containing 10% glycerol and were frozen in lucite sample cells by immersion in liquid nitrogen. The samples were maintained at a temperature close to 5 K during data collection in an Oxford Instruments CF1204 liquid helium flow cryostat. The EXAFS oscillations were analyzed as previously described (George et al., 1989), using curved-wave theoretical phase and amplitudes generated by using the program of Rehr and co-workers (Rehr et al., 1991; Mustre de Leon et al., 1991).

Gel Mobility Analysis. DNA binding assays contained a ^{32}P-labeled 22-bp oligonucleotide containing the left half of the UAS_c promoter element of the *S. cerevisiae* MT gene (Hu et al., 1990). The labeled oligonucleotide (100 000 cpm) was mixed with 4-pmol ACE1 samples in potassium phosphate buffer, pH 7.8, in the presence of 3% glycerol, 0.03% NP40, and tRNA and poly(dI–dC) at final concentrations of 30 μg/mL. The samples were incubated for 15 min at room temperature before electrophoresis on 4% polyacrylamide gels (pre-electrophoresed) for 1 h at 30 mA followed by autoradiography (Carey, 1991).

RESULTS

Formation of Cd^{II}ACE1 Complex. An *E. coli* phage T7 expression system was used to produce the N-terminal segment of ACE1 containing the first 122 amino acids (Dameron et al., 1991). The apoprotein prepared by acidification of the purified CuACE1 sample was used for reconstitution studies. Binding studies with Cu(I) reported previously suggested that maximal Cu(I) binding occurred between 6 and 7 mol equiv. Similar reconstitution studies were carried out with divalent metal ions. apo-ACE1 renatured with Cd(II) was monitored by ultraviolet absorption spectroscopy (Figure 1). Increasing the mole equivalency of Cd(II) resulted in the appearance of transitions in the ultraviolet. The difference spectrum of CdACE1 and apo-ACE1 was consistent with thiolate→Cd^{II} ligand-to-metal charge-transfer transitions (Figure 2, inset) (Kagi & Vallee, 1961). The intensity of the charge-transfer transitions was maximal between 3 and 4 mol equiv of Cd(II) and did not change with additional quantities of the metal ion (Figure 1, inset). In contrast, the charge-transfer transitions in CuACE1 were maximal near 6 mol equiv of Cu(I) (20). The charge-transfer bands of CuACE1 and CdACE1 differed in the ultraviolet, as expected when cysteinyl thiolates are ligands for each metal (Figure 2). We previously demonstrated that maximal Cd(II) binding to yeast MT occurred at 4 mol equiv (Winge et al., 1985), unlike the maximal Cu(I) stoichiometry of 7 (Narula et al., 1991).

The CdACE1 complex chromatographed as a single monomeric component. Chromatography of ACE1 reconstituted with 3 mol equiv of Cd(II) resulted in the recovery of CdACE1 in the eluate containing close to 4 mol equiv of bound Cd(II) (data not shown). The CdACE1 complex was partially dissociated in the presence of 50 μM EDTA and totally dissociated above 100 μM EDTA. The yeast CdMT complex was likewise largely dissociated by chromatography in buffers containing 100 μM EDTA.

Spectroscopy of Co^{II}ACE1 Complexes. Reconstitutions with Co(II) were performed to examine the coordination

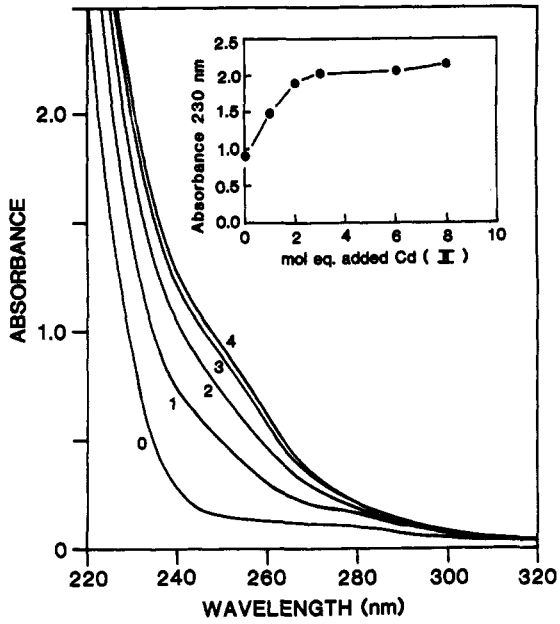


FIGURE 1: Cd(II) reconstitution of ACE1. Samples (15 nmol/mL) of apo-ACE1 were reconstituted with increasing mole equivalency of Cd(II), and after neutralization to pH 7 the samples were scanned spectrophotometrically. The numbers refer to the mol equiv of Cd(II) added to the samples. The inset shows the rise in absorbance at 230 nm as a function of added Cd(II).

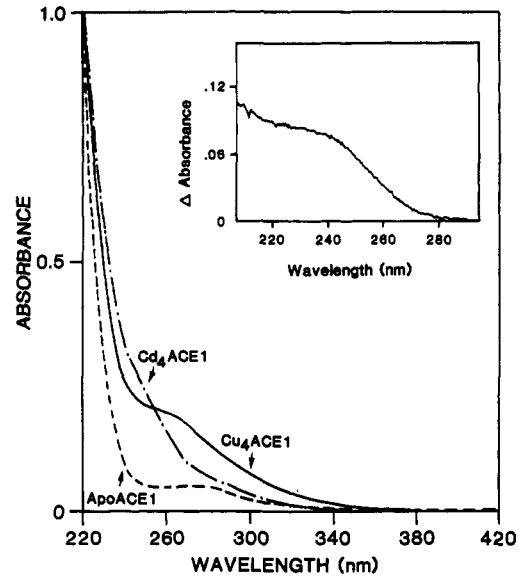


FIGURE 2: Ultraviolet absorption spectra of ACE1 reconstituted with Cu(I) and Cd(II). Samples (4.5 nmol) of apo-ACE1 were reconstituted with 4 mol equiv of either Cd(II) or Cu(I). A difference spectrum of CdACE1 and apo-ACE1 is shown in the inset.

geometry of the metal sites in ACE1. The electronic spectrum of apo-ACE1 reconstituted with increasing quantities of Co(II) showed maximal Co(II) binding at 4 mol equiv of Co(II) (Figure 3A). The resulting CoACE1 samples were blue-green in color when preserved anaerobically. The spectrum was dominated by d–d transitions with maxima at 605 [ϵ = 345 per Co(II)], 681 (ϵ = 441), and a shoulder near 740 nm (ϵ = 256). These transitions are typical of the spin-allowed ligand field $\nu_3[^4A_2 \rightarrow {}^4T_1(P)]$ transitions of T_d Co(II) complexes with tetrathiolate coordination (Lane et al., 1977; May & Kuo, 1978; Maret et al., 1979; Johnson & Schachman, 1983; Bertini & Luchinat, 1984; Giedroc & Coleman, 1986). Tetrathiolate Co(II) complexes exhibit d–d bands between 700 and 800 nm. The shape of the d–d envelope did not

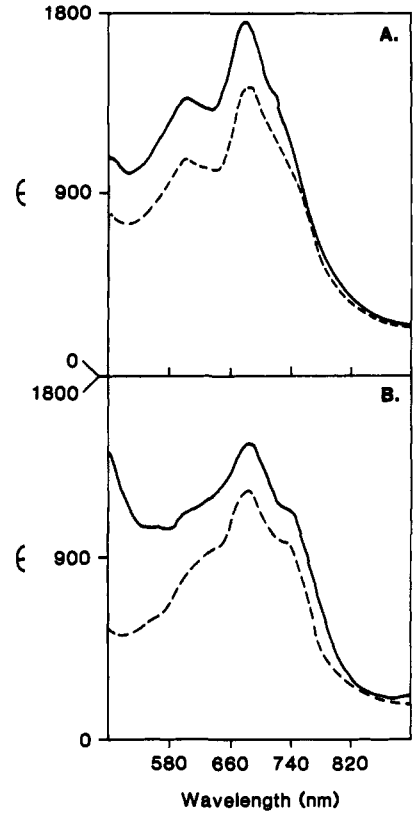


FIGURE 3: Visible absorption spectra of Co^{II}ACE1 (A) and Co^{II}MT (B). Samples (19.2 nmol) reconstituted with either 2 (- - -) or 4 mol equiv of (—) Co(II) were scanned. The extinction coefficient is based on the protein concentration.

change with increasing mole equivalency of Co(II) at either pH 7 or 8.5. This is in contrast to the d–d band envelope of mammalian Co^{II}MT with submaximal and maximal Co(II) equivalents at pH 8.5 (Vasak & Kagi, 1981; Schaffer, 1991). Charge-transfer transitions in Co^{II}–thiolate complexes usually occur in the near-ultraviolet with extinction coefficients of 800-1300 per Co–S–Cys bond (Lane et al., 1977; May & Kuo, 1978; Maret et al., 1979; Johnson & Schachman, 1983; Giedroc & Coleman, 1986). Absorption shoulders are evident in CoACE1, although there is less resolution of individual transitions than in other proteins containing Co^{II}–thiolate coordination (May & Kuo, 1978; Maret et al., 1979; Giedroc & Coleman, 1986) (Figure 4A). The molar absorptivity of the shoulder feature at 305 nm in CoACE1 is 770 per Co–S bond.

CoACE1 resembles CoMT from *S. cerevisiae* in binding stoichiometry and geometry. Reconstitution of apo-MT with Co(II) showed d–d transitions at similar wavelengths with molar absorptivities similar to those of CdACE1 (Figure 3B). CoMT showed a more prominent shoulder near 740 nm characteristic of tetrathiolate complexes. A broad Co–S charge-transfer band was observed near 310 nm (ϵ = 680 per Co–S bond (Figure 4B). The d–d electronic transitions in CoACE1 and CoMT from yeast were highly oxygen-sensitive; the CoMT complex was significantly more sensitive than CoACE1.

EXAFS of Cd^{II}ACE1 Complex. Additional structural information on the Cd(II) binding sites in ACE1 and MT was obtained from EXAFS spectroscopy. The cadmium K-edge EXAFS spectra of Cd_4ACE1 and Cd_4MT are shown in Figure 5A, together with the EXAFS Fourier transforms in Figure 5B. The Cd EXAFS spectra of the two proteins are strikingly similar. The data are dominated by scattering from a single

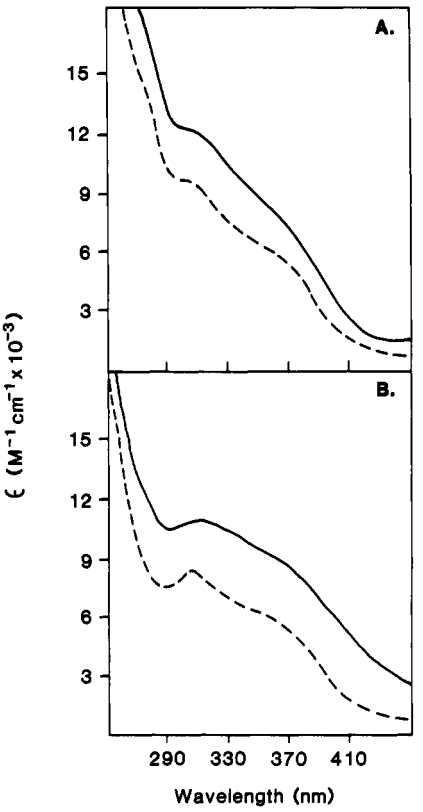


FIGURE 4: Absorption spectra of Co^{II}ACE1 (A) and Co^{II}MT (B) in the charge-transfer spectral region. Details are the same as those listed in the legend for Figure 3.

Table I: Cd–S EXAFS Curve-Fitting Results of CdACE1 and CdMT[a]

sample	N	R (Å)	σ^2 (Å^2)	ΔE_0 (eV)	fit error
Cd_4MT	4.0 (7)	2.529 (9)	0.0037 (9)	−14 (2)	0.392
Cd_4ACE1	3.9 (9)	2.517 (13)	0.0031 (14)	−10 (3)	0.528

[a] The coordination numbers, N, bond lengths, R, Debye–Waller factors, σ^2, and threshold energy displacements, E_0, were freely floated in the curve-fitting. The values in parentheses indicate the 95% confidence limits estimated by the curve-fitting algorithm. It should be noted that the accuracy may in fact be slightly lower than indicated by the curve-fitting due to lack of transferrability of the EXAFS phase and amplitude functions. Nevertheless, the comparative values between the two proteins should be exact. The fit error is the root mean square k^3 weighted residuals, divided by the root mean square of the weighted data.

shell of ligands, with no other obvious contributions. Quantitative curve-fitting analysis indicates that both the CdMT and CdACE1 samples possess Cd–S coordination with Cd–S bond lengths of 2.53 and 2.52 Å, respectively. The results of curve-fitting analyses are given in Table I. The inclusion of lighter scatterers such as Cd–N or Cd–O did not improve the fit.

There are no indications of any outer shell Cd–Cd interactions in the data. These results agree with previous reports of Cd EXAFS from rat liver CdMT (Abrahams et al., 1985). The lack of any observed Cd–Cd interactions might, at first sight, seem at odds with the published crystal structure of rat MT (Robbins et al., 1991). The crystal structure of Cd,ZnMT indicates the presence of two discrete cysteine–thiolate-bridged metal–sulfur clusters: a Cd_4 cluster and a Zn_2Cd_1 cluster. Both clusters have very low symmetry, with the four-metal cluster containing Cd–Cd distances ranging between 3.7 and 4.2 Å and with a single longer Cd–Cd vector at 6.1 Å (Robbins et al., 1991). Such a distribution of interatomic distances would produce a large static EXAFS Debye–Waller factor of about 0.032 Å^2, which will attenuate the outer shell EXAFS. Assuming that our sample of yeast Cd_4MT contains a four-metal cluster similar to the four-metal cluster of the rat CdMT, we have simulated the expected EXAFS by using the crystallographic blood lengths, together with chemically reasonable Debye–Waller factors (0.0055 Å^2). This value may be too small for some of the longest Cd–Cd interactions. Nevertheless, bearing in mind that smaller Debye–Waller values correspond to larger EXAFS amplitudes, we prefer smaller values in order to present as demanding a test as possible. The results, which are shown in Figure 5C, show no Cd–Cd interaction. The distribution in interatomic distances effectively smears out the Cd–Cd interaction. Thus, the lack of any resolved Cd–Cd interaction in the EXAFS is entirely consistent with the crystal structure of rat Cd,ZnMT.

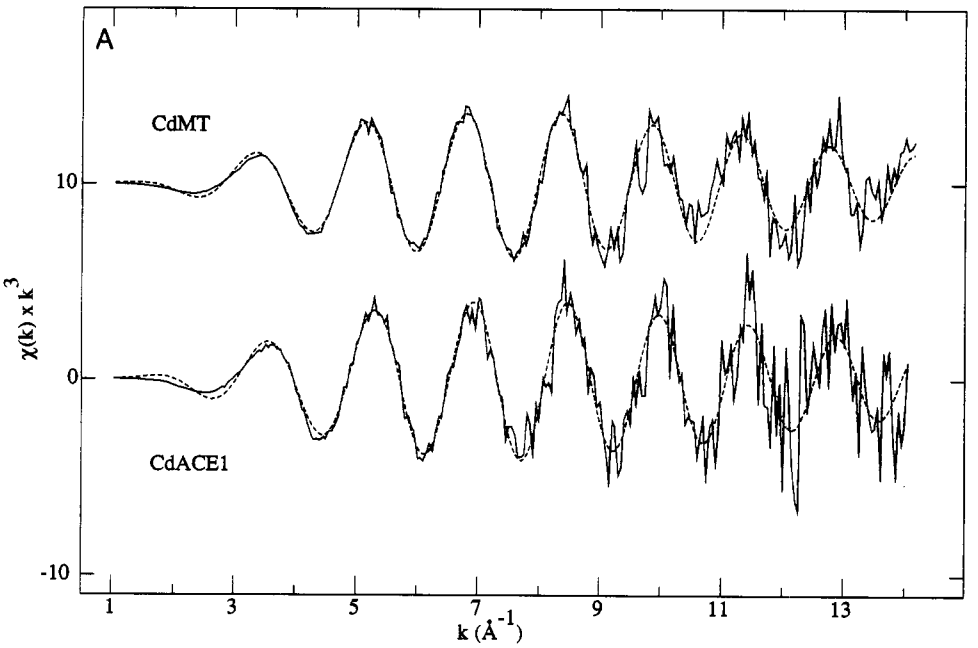
Lack of DNA Binding of Cd^{II}ACE1. Cd(II) salts are unable to induce expression of yeast MT genes (Furst et al., 1988). In addition, CdACE1 fails to form stable complexes with *CUP1* promoter sequences. ACE1 reconstituted with 4 mol equiv of Zn(II) or Cd(II) does not bind to promoter sequences in the presence of competing DNA (Figure 6). In contrast, CuACE1 and AgACE1 form high-affinity complexes with *CUP1* DNA promoter sequences (Figure 6). These complexes are dissociated by incubation with 1 mM KCN.

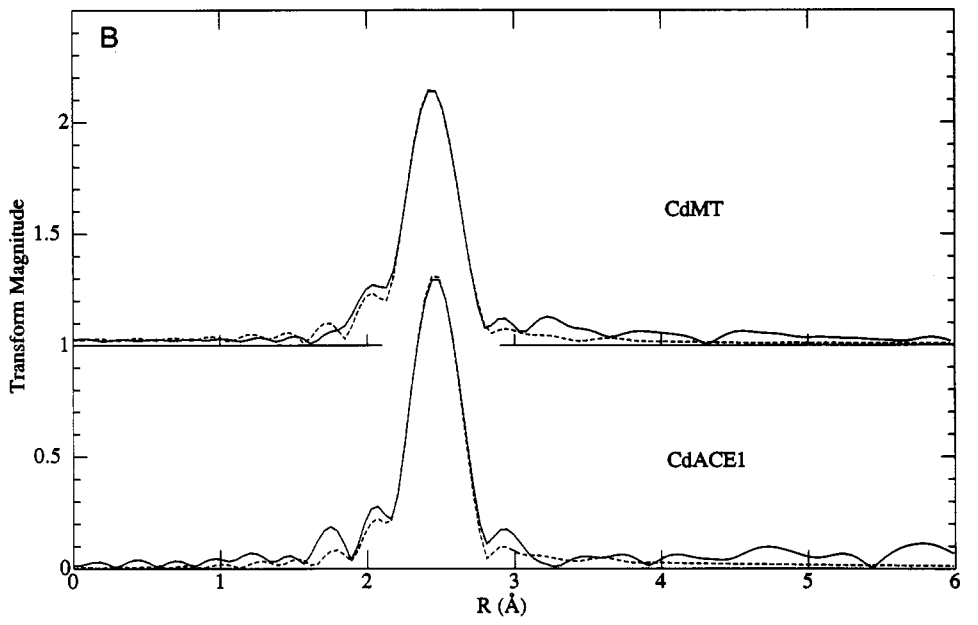
Metal Displacement in ACE1. Cu(I) can activate either apo-ACE1, ZnACE1, or CdACE1 for specific DNA binding. The Cu(I)-mediated activation of ZnACE1 and CdACE1 occurs via a metal displacement reaction. To characterize this reaction, the luminescence of the CuACE1 product was monitored. ZnACE1 was incubated at neutral pH with an increasing mole equivalency of Cu(I) stabilized as Cu^I:glutathione (GSH). Cu(I)-specific emission was observed in each case (Figure 7). Emission was also observed in a ZnACE1 sample renatured with Cu(I) in the presence of 1 mM GSH, but the high concentration of GSH reduced the quantum yield of Cu_6ACE1 samples. The displacement reaction was rapid; complete exchange occurred within 2 min. Rapid metal exchange was also observed with CdACE1 and Cu(I) donors (data not shown).

DISCUSSION

ACE1 resembles yeast MT in binding Cd(II) and Cu(I) in distinct configurations. It is likely that distinct configurations discriminate active and inactive functional conformers of ACE1. The only metal ions known to induce MT gene expression in yeast are Cu(I) and Ag(I) (Furst et al., 1988), and these ions are known to form similar metal:thiolate clusters (Dance, 1978; Narula et al., 1991).

CuACE1 was shown previously to contain a multinuclear Cu^I–cysteinyl sulfur cluster that resembled CuMT in coordination properties (Dameron et al., 1991; Nakagawa et al., 1991). The metal nuclearity of the clusters is 6-7 in ACE1 and 7 in yeast MT (Dameron et al., 1991; Narula et al., 1991). Trigonal geometry of Cu(I) coordination is observed in both MT and ACE1 proteins (Dameron et al., 1991; Nakagawa et al., 1991), although two Cu(I) ions in yeast MT may be digonally coordinated (Narula et al., 1991). The observation of two-coordinate Cu(I) ions in yeast MT is suggested from heteronuclear multiple quantum coherence NMR studies of AgMT (Narula et al., 1991). The similiarity in long-range NOEs observed between AgMT and CuMT is suggestive that CuMT likewise may contain two digonal Cu(I) ions (Narula et al., 1991). In an EXAFS study of synthetic Cu–thiolate compounds, a correlation was observed between the mean


A
CdMT
CdACE1
χ(k) x k^3
k (Å^-1)


B
CdMT
CdACE1
Transform Magnitude
R (Å)

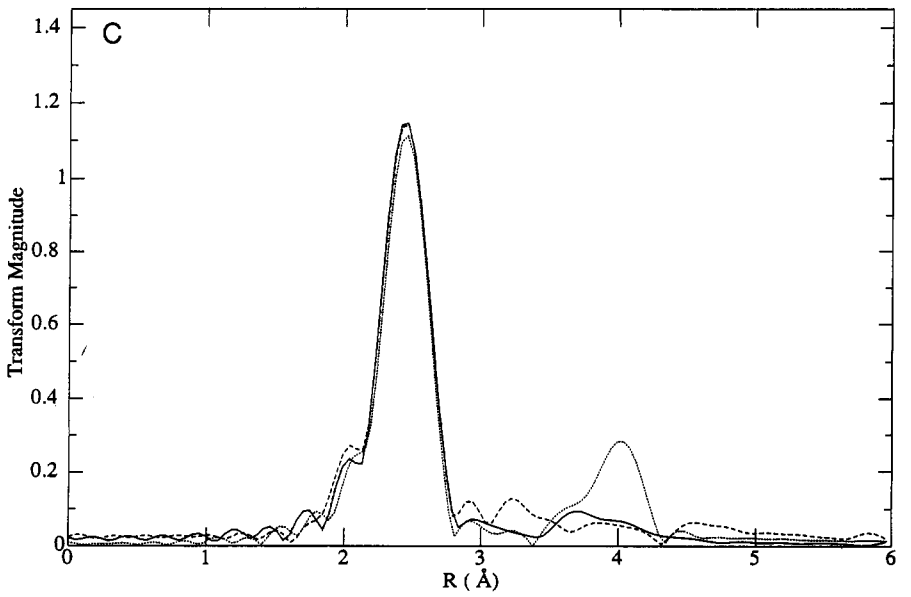

C
Transform Magnitude
R (Å)

FIGURE 5: Cadmium EXAFS of Cd^{II}ACE1 and Cd^{II}MT. (A) The Cd K-edge EXAFS data of Cd_4MT and Cd_4ACE1 (—), together with the best fits (- - -). (B) The Cd K-edge EXAFS Fourier transforms of Cd_4MT and Cd_4ACE1 (—), together with the Fourier transforms of the best fits (- - -). The Fourier transforms have been phase-corrected for Cd–S interactions, and the k range was 1-14 Å^{-1}. (C) The EXAFS Fourier transform of Cd_4MT (- - -), together with the transform of a simulation (—) using the Cd–S distance measured with EXAFS and the Cd–Cd distances obtained from crystallography (Robbins et al., 1991). The transform of a simulation using a single Cd–Cd distance at the mean distance indicated by crystallography is also shown (···) for comparison. The Fourier transforms have all been phase-corrected for Cd–S interactions, and the k range was 1-14 Å^{-1}.

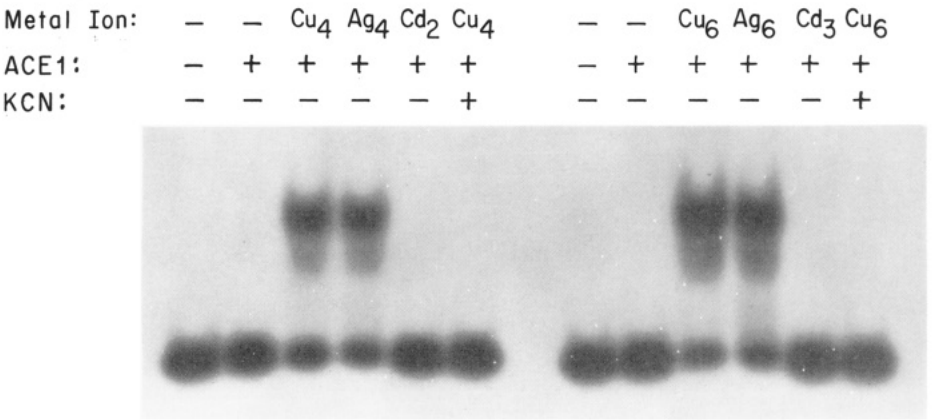


FIGURE 6: Gel retardation assay of Cu^{I}ACE1, Ag^{I}ACE1, and Cd^{II}ACE1. Samples of ACE1 (1.5 nmol) were reconstituted with either Cu(I), Ag(I), or Cd(II) at the equivalency listed in the figure, and 5-pmol samples were used in each reaction. KCN was added to a final concentration of 1 mM.

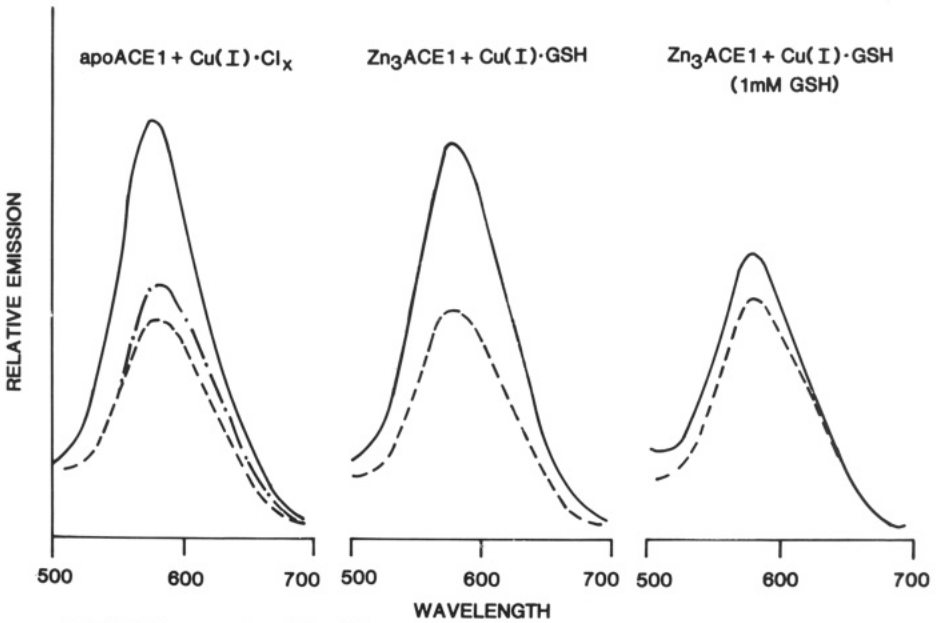


FIGURE 7: Luminescence of Cu^{I}ACE1 reconstituted by different protocols. ACE1 was reconstituted with Cu(I) in three protocols to a final stoichiometry of either 4 (- - -) of 6 (—) mol equiv of Cu(I). In the first procedure (shown on the left side spectra), apo-ACE1 was renatured with Cu(I) stabilized as $(CuCl_x)^-$. In the experiment shown by the central spectra, ACE1 was initially reconstituted with 3 mol equiv of Zn(II) and subsequently incubated with Cu(I) stabilized with glutathione (GSH) in a 10:1 molar excess of GSH. The third protocol (spectra at right) involved preforming Zn_3ACE1 as in the second procedure, but Cu^{I}:GSH was added to a ZnACE1 solution at a final GSH concentration of 1 mM. The addition of GSH to a final concentration of 1 mM to the Cu_6ACE1 sample prepared by the first protocol resulted in the spectrum shown in the left group (– • –).

Cu–S bond distance and the fraction of digonal Cu(I) ions (Winge et al., 1993). Comparison of the mean Cu–S bond distance in CuACE1 with the correlation suggests that CuACE1 contains predominantly trigonal Cu(I) ions with at most 1-2 digonally coordinated Cu(I) ions (Winge et al., 1993b).

The metal center in ACE1 formed with a group of divalent ions including Zn(II), Cd(II), and Co(II) is distinct from the Cu(I) polymetallic cluster. The maximal binding stoichiometry of these divalent ions in ACE1 was found to be near 4 mol equiv. Charge-transfer bands consistent with metal:thiolate coordination were observed in CdACE1 and CoACE1. Metal:thiolate coordination was also implicated in spectroscopic studies with Co(II) and EXAFS analysis of Cd(II)-substituted ACE1. Tetrahedral coordination geometry is predicted on the basis of the energy of the d–d band envelope in the visible spectral region and EXAFS analysis. The d–d transitions presumably arise from the ν_3 $^4A_2 \rightarrow {}^4T_1(P)$ spin-allowed transitions of tetrahedral complexes (May & Kuo, 1978; Maret et al., 1979; Johnson & Schachman, 1983; Bertini & Luchinat, 1984; Giedroc & Coleman, 1986). The centroid of the d–d band envelope near 680 nm in CoACE1 and CoMT with an extinction coefficient of less than 500 per Co(II) and the low-energy shoulder transition above 700 nm are consistent

with Co–S ligation in both proteins. The combination of absorption spectroscopy of Co^{II}–proteins and EXAFS clearly points to tetrahedral coordination geometry in ACE1 and MT containing bound divalent ions. The observed Cd–S bond distance of 2.52 Å in both CdACE1 and CdMT is also consistent with Cd–S distances in other proteins and compounds with tetrahedral coordination geometry (Hagen & Holm, 1983; Lacelle et al., 1984; Abrahams et al., 1985; Robbins et al., 1991).

The energy separation of the ligand field transitions within the d–d band envelope is more pronounced in MT than in ACE1, suggestive of greater distortion from tetrahedral geometry in MT. Titrations of apo-ACE1 and apo-MT from yeast with Co(II) did not show the spectral changes in the ν_3 d–d band observed with mammalian MT at alkaline pH (Vasak & Kagi, 1981; Schaffer, 1991). These spectral changes were shown to arise from the conversion of purely terminal thiolate ligation of Co(II) ions at low equivalency to clustered Co(II) ions with some bridging thiolates at maximal stoichiometries (Vasak & Kagi, 1981). At alkaline pH, metal binding to mammalian MT occurs in a noncooperative manner, unlike neutral pH conditions (Good et al., 1988). The lack of spectral changes in yeast CoMT and CoACE1 does not necessarily imply the absence of cluster formation. Rather, the environment of the Co(II) ions in the two yeast proteins may not change during the binding process or, alternatively, binding to yeast proteins at both neutral and alkaline pH conditions may be cooperative.

The $^4A_2 \rightarrow {}^4T_1(P)$ transition of Co^{II}ACE1 shows a prominent absorption near 630 nm. This component of the d–d envelope is not as major in CoMT from yeast or mammals (Vasak & Kagi, 1981; Hartmann & Weser, 1985). In a series of small Co^{II}–thiolate complexes this high-energy component of the $^4A_2 \rightarrow {}^4T_1(P)$ envelope was most prominent in complexes containing bridging Co–S–Co units (Dance, 1979). This conclusion cannot be universally true as titrations of mammalian MT with Co(II) under conditions of noncooperative binding do not show an enhancement of this high-energy d–d component in going from only terminal thiolate binding to bridging Co(II) coordination (Vasak & Kagi, 1981; Schaffer, 1991). Small synthetic complexes containing bridging thiolates also gave only broad absorption in the near-UV metal–ligand charge-transfer (MLCT) spectral region (Dance, 1979). Co(II) complexes with only terminal thiolates give prominent absorption bands in the MLCT region. This may imply that Co^{II}ACE1 contains bridging thiolates and, therefore, a CoS polynuclear cluster.

Metal binding in ACE1 is clearly similar to that in MT. MT appears to be a good structural model of metaled ACE1 with respect to the metal centers. The paucity of hydrophobic residues in MT and ACE1 suggests that metal ion binding induces structure in both proteins. The distinct metal centers formed in MT and ACE1 by Cu(I) and Cd(II) ions is consistent with these metal ions inducing different structural conformations in both MT and ACE1. The magnitude of the structural alterations induced by the two metal ions will be addressed in future multidimensional NMR studies.

There are two implications of distinct metal ion coordination in ACE1. First, distinct protein conformations induced by Cu(I) and Cd(II) binding to ACE1 may explain the observed Cu(I) ion specificity in MT gene expression. Cd(II) binds to ACE1, but the CdACE1 complex cannot form high-affinity complexes with DNA containing *CUP1* promoter sequences. Secondly, Cu(I) activation of ACE1 may occur by interconversion of conformers. The MT molecule is proteolytically unstable in the absence of metal ions. If apo-ACE1 is similarly unstable, ZnACE1 may be the basal unactivated form of ACE1. It should be noted, however, that no direct evidence of ZnACE1 in *S. cerevisiae* exists to date. Cu(I) activation of ACE1 may involve the displacement of Zn(II) ions in ZnACE1 by Cu(I) and subsequent structural reorganization. The Cu(I) displacement of Zn(II) in ACE1 is rapid *in vitro* and may therefore be a significant factor in activation. Metal exchange in mammalian MT is facile (Otvos et al., 1989) and may occur via a number of solvent-exposed cysteinyl sulfurs (Robbins et al., 1991).

REFERENCES

Abrahams, I. L., Garner, C. D., Bremner, I., Diakun, G. P., & Hasnain, S. S. (1985) *J. Am. Chem. Soc. 107*, 4596–4597.

Bertini, I., & Luchinat, C. (1984) *Adv. Inorg. Biochem. 6*, 72–111.

Bremner, I. (1987) *Experientia Suppl. 52*, 81–107.

Buchman, C., Skroch, P., Dixon, W., Tullius, T. D., & Karin, M. (1990) *Mol. Cell. Biol. 10*, 4778–4787.

Carey, J. (1991) *Methods Enzymol. 208*, 103–117.

Culotta, V. C., Hsu, T., Hu, S., Furst, P., & Hamer, D. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 8377–8381.

Dameron, C. T., Winge, D. R., George, G. N., Sansone, M., Hu, S., & Hamer, D. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 6127–6131.

Dance, I. G. (1976) *Aust. J. Chem. 31*, 2195–2206.

Dance, I. G. (1979) *J. Am. Chem. Soc. 101*, 6264–6273.

Dance, I. G. (1986) *Polyhedron 5*, 1037–1104.

Ecker, D. J., Butt, T. R., Sternberg, E. J., Neeper, M. P., Debouck, C., Gorman, J. A., & Crooke, S. T. (1986) *J. Biol. Chem. 261*, 16895–16900.

Evans, C. F., Engelke, D. R., & Thiele, D. J. (1990) *Mol. Cell. Biol. 10*, 426–429.

Furst, P., Hu, S., Hackett, R., & Hamer, D. (1988) *Cell 55*, 705–717.

George, G. N., Kipke, C. A., Prince, R. C., Sunde, R. A., Enemark, J. H., & Cramer, S. P. (1989) *Biochemistry 28*, 5075–5080.

Gettins, P., & Coleman, J. E. (1982) *Fed. Proc. 41*, 2966–2973.

Giedroc, D. P., & Coleman, J. E. (1986) *Biochemistry 25*, 4969–4978.

Good, M., Hollenstein, R., Sadler, P. J., & Vasak, M. (1988) *Biochemistry 27*, 7163–7166.

Gralla, E. B., Thiele, D. J., Silar, P., & Valentine, J. S. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 8558–8562.

Grassetti, D. R., & Murray, J. F., Jr. (1967) *Arch. Biochem. Biophys. 119*, 41–49.

Hagen, K. S., & Holm, R. H. (1983) *Inorg. Chem. 22*, 3171–3174.

Hamer, D. H. (1986) *Annu. Rev. Biochem. 55*, 913–951.

Hartmann, H.-J., & Weser, U. (1985) *Biochem. Biophys. Res. Commun. 132*, 277–283.

Hu, S., Furst, P., & Hamer, D. (1990) *New Biol. 2*, 544–555.

Huibregtse, J. M., Engelke, D. R., & Thiele, D. J. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 65–69.

Imbert, J., Culotta, V., Furst, P., Gedamu, L., & Hamer, D. (1990) *Adv. Inorg. Biochem. 8*, 139–164.

Johnson, R. S., & Schachman, H. K. (1983) *J. Biol. Chem. 258*, 3528–3538.

Kagi, J. H. R., & Vallee, B. L. (1961) *J. Biol. Chem. 236*, 2435–2442.

Lacelle, G., Stevens, W. C., Kurtz, D. M., Richardson, J. W., & Jacobson, R. A. (1984) *Inorg. Chem. 23*, 930–935.

Lane, R. W., Ibers, J. A. Frankel, R. B., Papaefthymiou, G. C., & Holm, R. H. (1977) *J. Am. Chem. Soc. 99*, 95–98.

Maret, W., Andersson, I., Dietrich, H., Schneider-Bernlohr, H., Einarsson, R., & Zeppezauer, M. (1979) *Eur. J. Biochem. 98*, 501–512.

May, S. W., & Kuo, J.-Y. (1978) *Biochemistry 17*, 3333–3338.

Mehra, R. K., & Winge, D. R. (1991) *J. Cell. Biochem. 45*, 30–40.
Mustre de Leon, J., Rehr, J. J., & Zabinsky, S. I. (1991) *Phys. Rev. B. 44*, 4146–4156.
Nakagawa, K. H., Inouye, C., Hedman, B., Karin, M., Tullius, T. D., & Hodgson, K. O. (1991) *J. Am. Chem. Soc. 113*, 3621–3623.
Narula, S., Mehra, R. K., Winge, D. R., & Armitage, I. M. (1991) *J. Am. Chem. Soc. 113*, 9354–9358.
Nielson, K. B., Atkin, C. L., & Winge, D. R. (1985) *J. Biol. Chem. 260*, 5342–5350.
Otvos, J. D., Petering, D. H., & Shaw, C. F. (1989) *Comments Inorg. Chem. 9*, 1–35.
Palmiter, R. D. (1987) *Experientia Suppl. 52*, 63–80.
Ralston, D. M., & O'Halloran, T. V. (1990) *Adv. Inorg. Biochem. 8*, 1–31.
Rehr, J. J., Mustre de Leon, J., Zabinsky, S. I., & Albers, R. C. (1991) *J. Am. Chem. Soc. 113*, 5135–5140.
Robbins, A. H., McRee, D. E., Williamson, M., Collett, S. A., Xuong, N. H., Furey, W. F., Wang, B. C., & Stout, C. D. (1991) *J. Mol. Biol. 221*, 1269–1293.
Schaffer, A. (1991) *Methods Enzymol. 205*, 529–540.
Thiele, D. J. (1988) *Mol. Cell. Biol. 8*, 2745–2752.
Thiele, D. J. (1992) *Nucleic Acids Res. 20*, 1183–1191.
Thiele, D. J., & Hamer, D. H. (1986) *Mol. Cell. Biol. 6*, 1158–1163.
Vallee, B. L., & Wacker, W. E. C. (1970) *Proteins 5*, 94–128.
Vasak, M., & Kagi, J. H. R. (1981) *Proc. Natl. Acad. Sci. U.S.A. 75*, 6709–6713.
Vasak, M., Galdes, A., Hill, H. A. O., Kagi, J. H. R., Bremner, I., & Young, B. W. (1980) *Biochemistry 19*, 416–425.
Welch, J., Fogel, S., Buchman, C., & Karin, M. (1989) *EMBO J. 8*, 255–260.
Winge, D. R., Nielson, K. B., Gray, W. B., & Hamer, D. H. (1985) *J. Biol. Chem. 260*, 14464–14470.
Winge, D. R., Dameron, C. T., & George, G. N. (1993a) *Adv. Inorg. Biochem.* (in press).
Winge, D. R., Dameron, C. T., George, G. H., Pickering, I. J., & Dance, I. G. (1993b) in *Bioinorganic Chemistry of Copper* (Karlin, K. D., & Tyeklar, Z., Eds.) pp 110–123, Chapman and Hall, New York.
Zhou, P., & Thiele, D. J. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 6112–6116.