Copper- and Silver-Substituted Yeast Metallothioneins: Sequential ^{1}H NMR Assignments Reflecting Conformational Heterogeneity at the C Terminus†

Surinder S. Narula,‡ Dennis R. Winge,§ and Ian M. Armitage*,‡,∥

Departments of Pharmacology and Diagnostic Radiology, Yale University School of Medicine, 333 Cedar Street, P.O. Box 3333, New Haven, Connecticut 06510, and Department of Biochemistry and Medicine, University of Utah Medical Center, Salt Lake City, Utah 84132

Received December 29, 1992; Revised Manuscript Received April 15, 1993

ABSTRACT: Complete ^{1}H NMR sequential assignments have been made for copper(I)- and silver(I)-substituted metallothionein (MT) from *Saccharomyces cerevisiae* using standard 2D ^{1}H NMR methods. The fingerprint region of the COSY spectrum of both metalloproteins shows a doubling of a few backbone proton resonances from residue K41 onward in the C terminus. This doubling of resonances is absent in the spectrum of the truncated mutant protein that lacks the five C-terminal residues which includes two cysteines. Concurrently, it has been established from a comparison of the heteronuclear ^{1}H–^{109}Ag multiple-quantum coherence transfer (HMQC) spectrum on the silver-substituted mutant and the wild-type protein that metal ligation is similar in both molecules. Thus, the 2 C-terminal Cys are not essential for metal cluster formation in the wild-type yeast MT and only 10 of the 12 Cys present in this protein appear to be involved in ligating the 7 mol of bound metal ions. A qualitative analysis of the coupling constant, hydrogen exchange, and NOE data indicates the presence of many type I β-turns and the lack of any other regular secondary structural elements. A comparison of chemical shifts and NOE data for native copper- and silver-substituted yeast MT indicates a high degree of conservation of structural elements in both proteins. Therefore, it seems reasonable to conclude that the metal to Cys connectivities which are obtained directly from the HMQC data on silver-substituted metallothionein are conserved in the native copper protein. Interestingly, a mixture of both 2 and 3 coordination was found for the bound Ag(I) ions in a single Ag_7Cys_{10} cluster. This mixed coordination number and a single cluster arrangement is most probably also shared with the Cu(I) ion coordination in the native protein.

The metallothioneins (MTs)[1] are small, cysteine-rich proteins that bind both essential heavy metals (e.g., Cu and Zn) and nonessential metals (e.g., Cd and Hg). MT gene transcription is inducible by the same heavy metals that are subsequently found bound to the MT protein, thereby providing a mechanism for cells to protect themselves against metal stress/overload. This metal response occurs in all eukaryotic organisms and tissues that synthesize MT and is operative for several different isoform genes that are frequently present in a single organism.

The yeast *Saccharomyces cerevisiae* contains a metallothionein encoded by the *CUP1* locus (Fogel & Welch, 1982; Karin et al., 1984; Butt et al., 1984). In most strains of this yeast, the *CUP1* gene is transcriptionally regulated by Cu(I) and Ag(I) ions (Wright et al., 1988). One strain of *S. cerevisiae* (strain 301N) has been shown to regulate the *CUP1* gene expression by Cd(II) as well as by Cu(I) (Inouhe et al., 1989). Disruption of the *CUP1* MT gene results in hypersensitivity to copper-mediated cytotoxicity as detoxification is achieved by sequestration of copper ions in a stable complex with MT (Hamer et al., 1985).

Both yeast and mammalian MTs bind copper as Cu(I) in clusters with a proposed trigonal metal ion coordination geometry (George et al., 1986, 1988). Spectroscopic data suggested that all 12 cysteine residues in yeast MT served as ligands for the 8 bound copper ions (Winge et al., 1985; Byrd et al., 1988). Luminescence studies revealed that the copper ions are in the Cu(I) valence state and that they are bound in a solvent-inaccessible environment (Byrd et al., 1988). The Cu(I) state was confirmed by X-ray absorption near edge structure (XANES) analysis (George et al., 1988). Additionally, since the XANES features were similar to those observed for the three coordinate Cu(I) model complexes (Kau et al., 1987), a distorted cubic Cu_8S_{12} polynuclear cluster was proposed in which each Cu(I) ion is trigonally coordinated and each cysteinyl thiolate bridges two Cu(I) ions (George et al., 1988). Reconstitution studies confirmed the cooperative nature of Cu(I) binding but were unable to confirm the existence of a single polynuclear cluster (Byrd et al., 1988).

To probe the nature of the Cu–thiolate cluster, a study of mutant forms of yeast MT was carried out (Thrower et al., 1988). Mutant MT molecules were produced with pairwise Cys → Ser substitutions and C-terminal truncations (Wright et al., 1986). Each mutant protein contained 10 of the 12

† This work was supported by grants from the National Institutes of Health (DK18778 to I.M.A. and ES03817 to D.R.W.). NMR instrumentation and computational facilities were provided from NIH (RR03475), NSF (DMB8610557), and ACS (RD259).

* Author to whom correspondence should be addressed at the Department of Pharmacology.

‡ Department of Pharmacology.

§ Department of Biochemistry and Medicine.

∥ Department of Diagnostic Radiology.

[1] Abbreviations: Ag-MT, silver(I)-substituted yeast metallothionein; Ag-T48, silver(I)-substituted yeast mutant metallothionein; COSY, homonuclear two-dimensional correlated spectroscopy; Cu-MT, copper(I) yeast metallothionein; Cu-T48, copper(I) yeast mutant metallothionein; DQF-COSY, double-quantum filtered COSY; DQ, double-quantum (two-quantum; 2-Q) spectroscopy; EXAFS, extended X-ray absorption fine structure; HMQC, heteronuclear multiple-quantum coherence spectroscopy; MT, metallothionein; NMR, nuclear magnetic resonance; NOE, nuclear Overhauser effect; NOESY, two-dimensional NOE spectroscopy; ppm, parts per million; Relay-COSY, two-dimensional relayed *J*-correlated spectroscopy; ROESY, rotating frame Overhauser effect spectroscopy; TOCSY (HOHAHA), two-dimensional total correlation spectroscopy; TPPI, time-proportional phase incrementation; WT, wild type; Xanes, X-ray absorption near edge structure.

0006-2960/93/0432-6773$04.00/0

native Cys thiolates, and they were designated 9/11, 24/26, 36/38, and 49/50, where the numbers corresponded to the sequence positions of Cys → Ser conversion. Analysis of the Cu content of these mutant proteins showed that all of the mutant molecules except 24/26 bind the same number of metal ions as the WT-MT. Surprisingly, two truncated mutants, one with 5 C-terminal residues deleted, which included 2 Cys, and the other with 18 C-terminal residues deleted, which included 4 Cys, were still found to bind Cu(I) ions in a stoichiometry comparable to that of the WT-MT (Byrd et al., 1988). One implication of these results is that the yeast MT polypeptide can adopt multiple conformations in forming its metal complexes.

For comparison, in mammalian MTs, which have 20 Cys thiolates available for metal binding, formation of Ag_6-MT, Ag_{12}-MT, and Ag_{18}-MT has been reported from the analysis of the CD and luminescence spectra of rabbit MT2 (Stillman et al., 1988; Zelazowski et al., 1989). The existence of this wide range in the metal ion coordination stoichiometry in this related metal thiolate protein is again suggestive of considerable flexibility in the structural requirements for MT to function in Cu detoxification.

Expression of MT genes is metalloregulated (Kägi & Kojima, 1987). The protein that mediates the metal responsiveness of the *S. cerevisiae* MT gene is the ACE1 transcription factor (also called CUP2). The N-terminal 122 amino acids of the ACE1 protein, which contains 12 cysteinyl residues, are important for specific DNA binding that is dependent on the presence of bound Cu(I) ions (Furst et al., 1988; Buchman et al., 1989). Spectroscopic studies of Cu-ACE1 have shown that this protein contains a polynuclear Cu(I)–Cys thiolate cluster with six or seven Cu(I) ions coordinated to Cys thiolates with distorted trigonal geometry (Dameron et al., 1991; Casas-Finet et al., 1992). Recently, a gene encoding for a metal-activated, sequence-specific, DNA binding protein called AMT1 has been isolated in yeast *Candida glabrata* (Zhou & Thiele, 1991). This protein contains 11 Cys in its amino terminus and bears a strong resemblance to the sequence of the ACE1 protein.

The Cu(I) polymetallic clusters in ACE1 and yeast MT appear similar in stoichiometry and coordination, so structural studies on yeast Cu-MT may provide insight into the Cu(I) center in ACE1. Elucidation of the solution structure of yeast Cu-MT through NMR methods can be expected to depend critically on the establishment of specific metal-to-Cys ligand connectivities as was required in similar studies on the mammalian Cd-MT (Messerle et al., 1990). However, Cu-MT is unsuitable for such studies due to its quadrupole moment and its insensitivity to NMR detection, a limitation that could fortunately be overcome by the application of ^1H–^{109}Ag HMQC transfer experiments on the silver-substituted derivative of this MT (Narula et al., 1991). Most importantly, Ag(I) and Cu(I) exhibit similar coordination chemistry (Hathaway, 1987; Lancashire, 1987), and Ag(I) appears to be an isomorphic replacement for the Cu(I) in MTs as evidenced by their identical binding stoichiometries (Winge, 1987).

EXPERIMENTAL PROCEDURES

Preparation of Ag-MT and Ag-T48. Metallothionein was purified from cultures of *S. cerevisiae* (strain 301N) grown in synthetic complete medium in the presence of 0.5 mM $CdSO_4$. The cell extract, prepared from the harvested cells with a French press, was clarified by centrifugation at 30000*g* and chromatographed as described previously (Narula et al., 1991). Cadmium analysis was used to assay for the presence of Cd-MT. Cd(II) was displaced from the purified MT protein by acidification to pH 2 followed by gel filtration on Sephadex G-25 equilibrated with 0.01 N HCl. The MT concentration was verified by amino acid analysis following overnight hydrolysis in 5.7N HCl at 100 °C *in vacuo*. The extent of cysteine reduction was determined by thiol titration with dithiodipyridine (Grassetti & Murray, 1967). The addition of Ag(I) to apoMT was carried out as described previously (Narula et al., 1991).

To monitor the protein homogeneity, Ag-MT and Ag-T48 samples were chromatographed on reversed-phase HPLC using a C_{18} column equilibrated with 0.1% TFA, and elution was achieved with a linear gradient of 0–60% acetonitrile. Chromatography was carried out at both 23 and 65 °C. In the elution profile, both metalloproteins showed one predominant component (~85%), but additional peaks were observed even though all peaks exhibited the expected amino acid composition. ApoMT showed the same family of peaks, and again, each component when purified by successive HPLC showed the expected native molecular mass as determined by electrospray mass spectrometry. The heterogeneity is, therefore, unrelated to the degree of metalation or to sequence heterogeneity. If multiple conformers exist in yeast apoMT and metalated MT, chromatography at 65 °C does not reverse the process.

Preparation of Cu-MT and Cu-T48. Wild-type Cu-MT was purified from *S. cerevisiae* strain 2180 as described previously (Byrd et al., 1988). The purified Cu–protein was directly used for NMR without *in vitro* metal ion reconstitution. Truncated Cu-MT (T48) was purified as described (Byrd et al., 1988). For metal replacement with Ag(I) in T-48 MT, the protocol used for the wild-type Cu-MT was followed (Narula et al., 1991).

NMR Methods. NMR spectra were recorded on a Bruker AM-500 spectrometer. To resolve spectral overlap, data were acquired at temperatures of 10, 15, and 30 °C in H_2O (i.e., 90% H_2O and 10% ^2H_2O) and 99.9% ^2H_2O. All of the 2D spectra were acquired in the phase sensitive mode using TPPI (Marion & Wüthrich, 1983). The water resonance was suppressed in all of the experiments by its irradiation during a relaxation delay of 0.9–1.5 s. In ^1H–^1H 2D NMR experiments, a spectral width of 6024 Hz was covered in both dimensions with the carrier set on the water resonance. The decoupler was also set on the water resonance, and both channels were phase locked for better suppression of the water resonance (Zuiderweg et al., 1986). For NOESY spectra (Jeener et al., 1979; Kumar et al., 1980), mixing times between 60 and 175 ms were used, and for TOCSY spectra (Bax & Davis, 1985), MLEV-17 spin lock times between 35 and 151 ms were used with 440–600 increments in t_1 (64–192 scans per increment) and 2048 complex data points in t_2. For COSY (Aue et al., 1976; Bax & Freeman, 1981), Relay-COSY (Eich et al., 1982; Bax & Drobny, 1985), and Ag-filtered COSY experiments (Otting & Wüthrich, 1990), 400–512 increments in t_1 (32–96 scans per increment) and 2048 complex data points in t_2 were acquired. To eliminate baseline distortions in all NOESY and TOCSY spectra, the relative receiver phase and the delay between the last pulse and the start of acquisition were adjusted such that the zero- and first-order phase corrections along ω_2 were 90° and 180°, respectively (Marion & Bax, 1988). The data were processed with the FTNMR program (Hare Research Inc.) running on a Sun 4/280S computer. The time domain signal along ω_2 was multiplied by a sine bell or shifted sine bell of 22° before Fourier

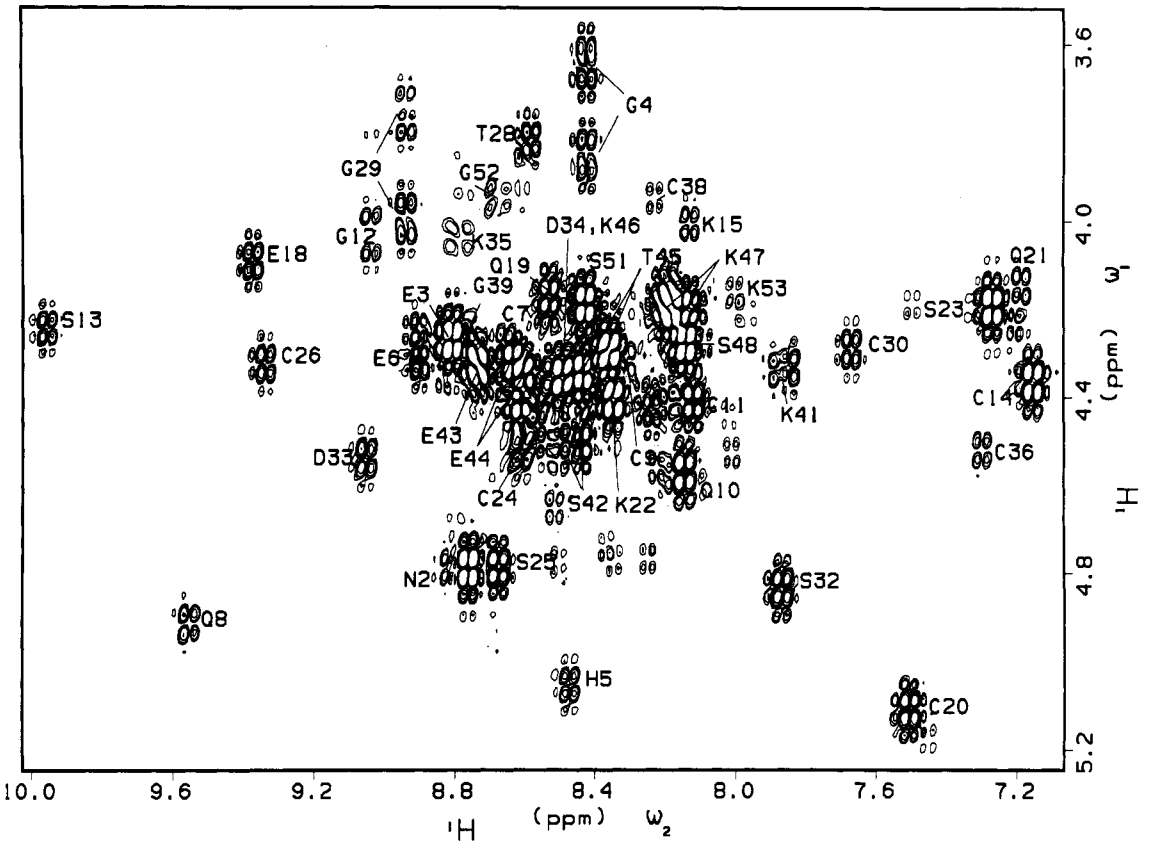

FIGURE 1: Fingerprint region of a phase sensitive COSY spectrum of a 5 mM sample of silver-substituted yeast metallothionein at 10 °C in 90% H_2O/10% D_2O. Both positive and negative levels are drawn without distinction. The resonance assignment of cross-peaks is indicated. The pH of the sample is 6.5 in 18 mM phosphate buffer.

transformation for resolution enhancement. The signal along ω_1 was similarly multiplied by a sine bell or shifted sine bell of 22° and then zero filled to 2048 complex points, resulting in a 2K × 2K real matrix.

HMQC experiments were carried out on the same Bruker instrument, where the resonance frequency for ^{109}Ag was 23.276 MHz. Although both isotopes of silver, ^{107}Ag and ^{109}Ag, have a spin quantum number of $^1/_2$ and almost the same natural abundance, ^{109}Ag was used for detection in our studies. The details of this experiment have been previously described (Narula et al., 1991). The ^{109}Ag chemical shifts are referenced to an external 8 M solution of silver nitrate.

For ^{1}H spectra, the residual water resonance was assigned as reference at 4.75 ppm in a 30 °C spectrum. The most upfield resonance at 1.17 ppm, which is assigned to the γ-CH_3 of T28, is calibrated with respect to the HDO resonance at 30 °C and thereafter used as the reference at other temperatures. All of the spectra are shown as contour plots with positive and negative contours shown for COSY and HMQC experiments and only positive levels shown for NOESY and TOCSY spectra. The horizontal and vertical axis represent ω_2 and ω_1, respectively.

Measurements of Coupling Constants. For measurements of NH/C$^\alpha$H coupling constants, COSY data sets at 30 °C were analyzed. Each row corresponding to a cross-peak in the 2D matrix was transformed back to the time domain signal where it was zero filled to 8 K points and then Fourier tranformed again to provide a resolution of 0.73 Hz/point.

Measurements of Amide Proton Exchange Rates. Due to the denaturation sensitivity of this protein to lyophilization, qualitative measurements of hydrogen exchange of the backbone amide protons were made by exchanging the protein solvent with 99.9% ^2H_2O in an Amicon apparatus (4–5 h) and then recording a 2D TOCSY experiment at 15 °C, which took approximately 16 h. All of the NH/C$^\alpha$H cross-peaks observed in this experiment were interpreted to arise from the slow-exchanging amide protons.

RESULTS

The fingerprint region of the 500-MHz COSY ^{1}H NMR spectrum of silver-substituted yeast metallothionein at 10 °C in 90% H_2O and 10% ^2H_2O is shown in Figure 1. Similar data sets were also acquired at 15 and 30 °C, the procedure used to shift the water resonance and observe the cross-peaks which were obscured by the water resonance at 10 °C. The NH/C$^\alpha$H cross-peaks were identified using the two-stage procedure described by Wüthrich (1986), which consists of the analysis of the spin systems followed by their sequential assignments.

Analysis of Spin Systems. The assignment of the spin systems was performed using COSY, Relay-COSY, and TOCSY experiments in H_2O and ^2H_2O at three different temperatures. Yeast metallothionein is composed of 53 amino acid residues which includes 12 cysteines (Winge et al., 1985). In the absence of any overlap, 55 NH/C$^\alpha$H cross-peaks are expected; however, if the low-intensity peaks in Figure 1 are counted, approximately 69 cross-peaks are observed. The presence of a contaminant in the sample contributing to the excess number of cross-peaks can be confidently excluded from the electrospray mass spectrometry and amino acid composition data. Therefore, these extra cross-peaks must arise from some form of MT self-association (e.g., dimer or higher order aggregates) or from the presence of slow exchange between distinct MT conformers. As will be revealed in subsequent studies (*vide infra*), the extra cross-peaks arise

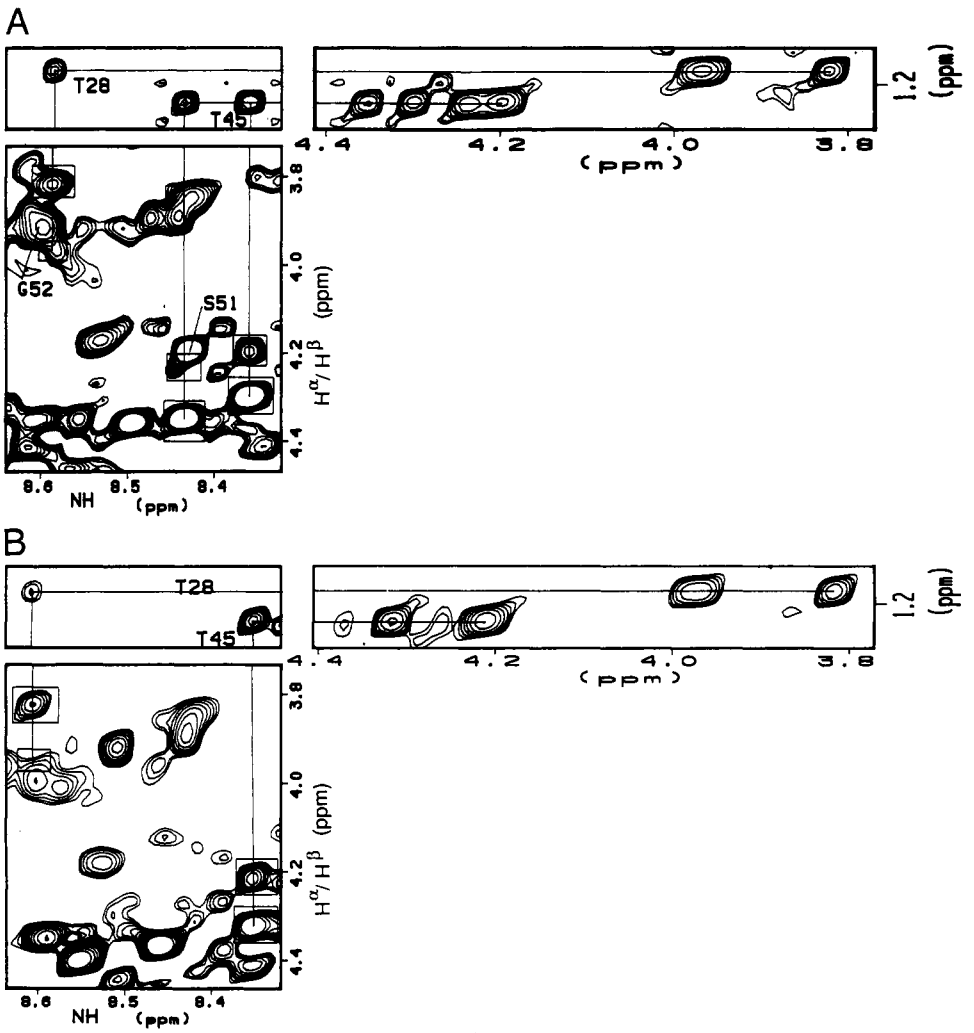

FIGURE 2: Selected regions of the TOCSY spectrum of yeast Ag-MT acquired at 10 °C and mixing time of 60 ms (A) and their comparison with the TOCSY spectrum acquired under similar conditions for Ag-T48 (B). Constructs and boxes are drawn highlighting the Thr spin systems.

from slow conformational exchange occurring in the C terminus of the protein.

The only aromatic residue present in this protein is a histidine at the fifth position, and its ring protons ($C^{\delta}H$ and $C^{\epsilon}H$) could easily be distinguished and assigned due to their characteristic chemical shift and relatively sharp line shapes. Of the five glycine residues, NH/$C^{\alpha}H$ cross-peaks of four could be unambiguously identified in COSY spectra by their characteristic fine structure. Two Gly spin systems were found to have degenerate chemical shifts of their two H^{α}, which was confirmed from the presence of a remote cross-peak at ω_2 of NH and at ω_1 of twice the H^{α} frequency in a 2-Q ^1H spectrum (Braunschweiler et al., 1983). The only methyl-group-containing amino acids in the primary structure (Winge et al., 1985) were two Thr which could be easily distinguished and assigned on the basis of the observed relay cross-peak from amide proton to one $C^{\beta}H$ and $C^{\gamma}H_3$ group protons in TOCSY experiments. However, in the present case, three amide protons showed relay cross-peaks to $C^{\gamma}H_3$ group protons (Figure 2A), indicating the presence of three Thr spin systems of which two had degenerate chemical shifts of their $C^{\gamma}H_3$ group protons. As will be described later, various data sets from native Cu-MT also showed the presence of three Thr spin systems.

Random coil ^1H chemical shifts were used as a guide to assign side-chain protons beyond the $C^{\beta}H$ position of various long-chain amino acids (Wüthrich, 1986). The side-chain spin systems of all but one of the seven Lys were confirmed from the presence of H^{α} to H^{ϵ} in a long mixing time TOCSY dataset. There are 10 Glu/Gln and 7 Asp/Asn spin systems, and the distinction between these two spin systems was made on the basis of the observation of cross-peaks between amide proton and H^{γ} for Glu/Gln and between amide proton and H^{β} for Asp/Asn in a TOCSY experiment. Side-chain NH_2 protons of Asn and Gln were identified in all but one case by NOE connectivities between the NH_2 protons and H^{β} or H^{γ}, respectively. The $C^{\beta}H$ protons of 10 of a total of 12 Cys spin systems were distinguished from all other protons in this protein by the presence of $H^{\beta}H^{\beta'}$ cross-peaks in an Ag-filtered COSY experiment (Otting & Wüthrich, 1990). The assignment of the Cys spin systems was completed by combining this information with the data from the Relay-COSY and TOCSY experiments. The two Pro spin systems could be easily assigned from the comparison of downfield (NH) and upfield regions of a long mixing time (151 ms) TOCSY spectrum. Of the seven Ser spin systems, five showed chemical shift degeneracy of their $C^{\beta}H_2$ protons.

The same procedure was repeated to assign the various spin systems in Cu-MT. This time the task was less laborious as the chemical shifts for most of the non-Cys residues were very close to the assigned Ag-MT data. Again, 65 cross-peaks were observed in the fingerprint region when only 55 peaks were theoretically expected. The best resolved and most obvious illustration was again the observation of three Thr

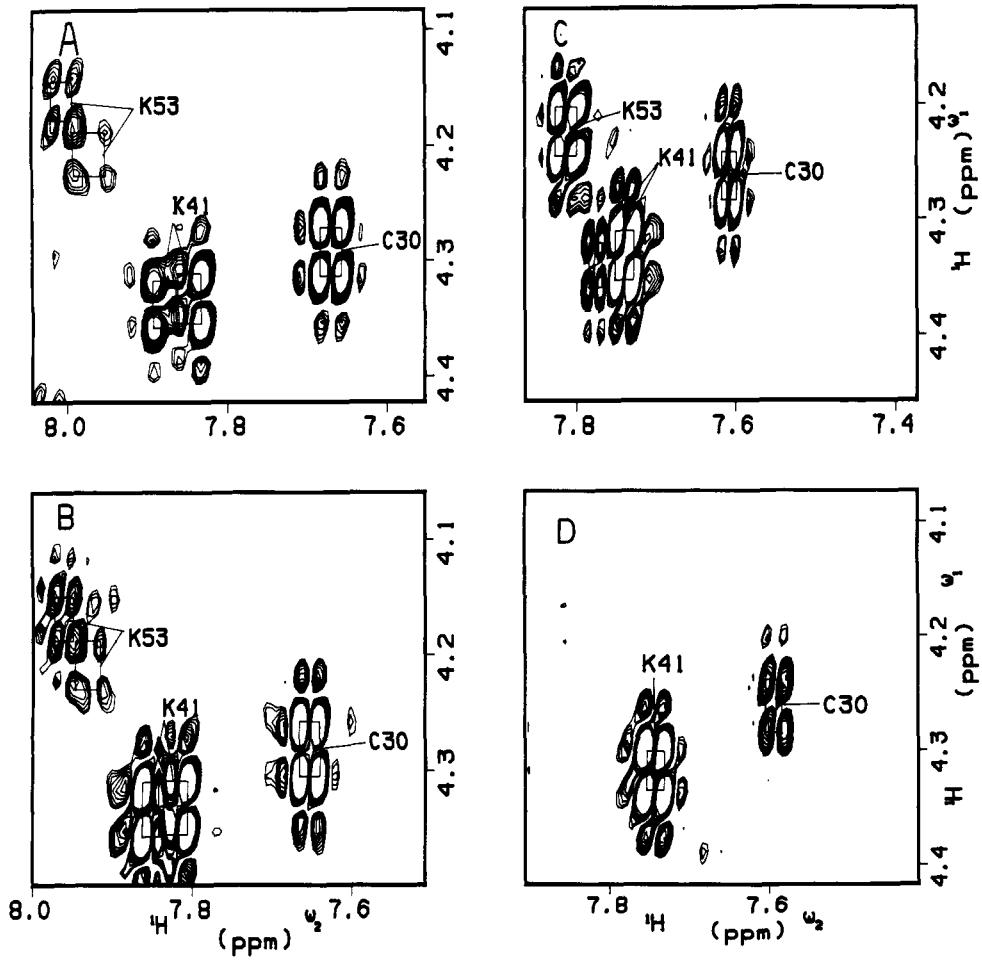

FIGURE 3: Comparison of a selected portion of the fingerprint region of a phase sensitive COSY spectrum of yeast Ag-MT at 10 (A), 15 (B), and 30 °C (C) and of Ag-T48 at 30 °C (D). Other experimental conditions are as described for Figure 1.

spin systems, when only two Thr are known to be present from the amino acid sequence.

The presence of these extra cross-peaks in these protein data sets prompted us to examine the NMR spectrum of an available mutant of this protein (T48) which lacks the C-terminal five residues (C49-C50-S51-G52-K53). Although this mutant protein lacks two Cys (C49 and C50), it has been reported to bind the same number of Cu(I) ions as the wild-type protein (Byrd et al., 1988). The fingerprint region of the 2D COSY spectrum of Cu-T48 showed 50 cross-peaks when 49 are expected. Now for the first time, only two cross-peaks were observed from amide protons to one $C^{\beta}H$ and $C^{\gamma}H_3$ group for the two Thr residues in the protein primary structure (Figure 2B). The temperature dependence of a selected cross-peak region from the COSY spectrum of Ag–MT is shown in Figure 3A–C along with the same region from Ag-T48 at 30 °C for comparison (Figure 3D). Naturally missing from the latter is a cross-peak for K53, but more important to note is that the persistent doubling of the K41 cross-peak over this temperature range (Figure 3A–C) is removed in the Ag-T48 spectrum (Figure 3D).

Sequential Assignments. The second step of the assignment procedure involved correlating cross-peaks in the fingerprint region of the COSY spectrum with the specific amino acids in the protein sequence using d_{NN}, $d_{\alpha N}$, and $d_{\beta N}$ connectivities derived from the NOESY spectrum (Wüthrich, 1986).

A representative example of this assignment procedure is shown in Figure 4A,B for Ag-MT. Sequential connectivities from D2 to N16 were straightforward. Due to the near degeneracy in the chemical shift values of the backbone protons of N16 and N17 and the relatively broad amide proton resonance for these residues, the sequential connectivity between these two residues could not be established. However, the presence of two Asn spin systems with H^{α} chemical shifts close to 4.70 ppm could be confirmed from TOCSY data in a ^2H_2O sample. A weak sequential $d_{\alpha N}$ NOE between K15 and N16 (or N17), as well as a strong $d_{\alpha N}$ between one (or both) of these residues and E18, supports the correctness of this assignment (Figure 4). Once again, the sequential assignments from E18 to C26 were straightforward and terminated upon encountering P27. However, NOEs are observed from the amide proton of C26 to $C^{\delta}H_2$ protons of P27 and also from H^{α} of P27 to amide, H^{α}, and $C^{\gamma}H_3$ group protons of T28. Sequential NOEs are observed from T28 to C36, but sequential connections between C36 and P37 were not observed. The sequential $d_{\alpha N}$ NOE between N31 and S32 was not observed at 10 °C due to overlap of the H^{α} resonance with the water resonance, but this could be seen in NOESY data acquired at 30 °C (data not shown). NOEs were observed from H^{α}, $C^{\beta}H_2$, and $C^{\gamma}H_2$ protons of P37 to the amide proton of C38. Sequential NOEs were observed from C38 to E43 with the exception of $d_{\alpha N}$ between N40 and K41 due to overlap of the H^{α} resonance of N40 with the water resonance at 10 °C. However, this NOE could be seen in the NOESY data acquired at 30 °C. The intraresidue NOE $d_{N\alpha}$ (COSY type) cross-peak for K41 in the NOESY spectrum appears as one broad peak even though in the COSY spectrum the corresponding peak appeared as two partially overlapped cross-peaks (Figure 3A–C). The assignment of E44 was done by exclusion because this was the only unassigned Glu spin

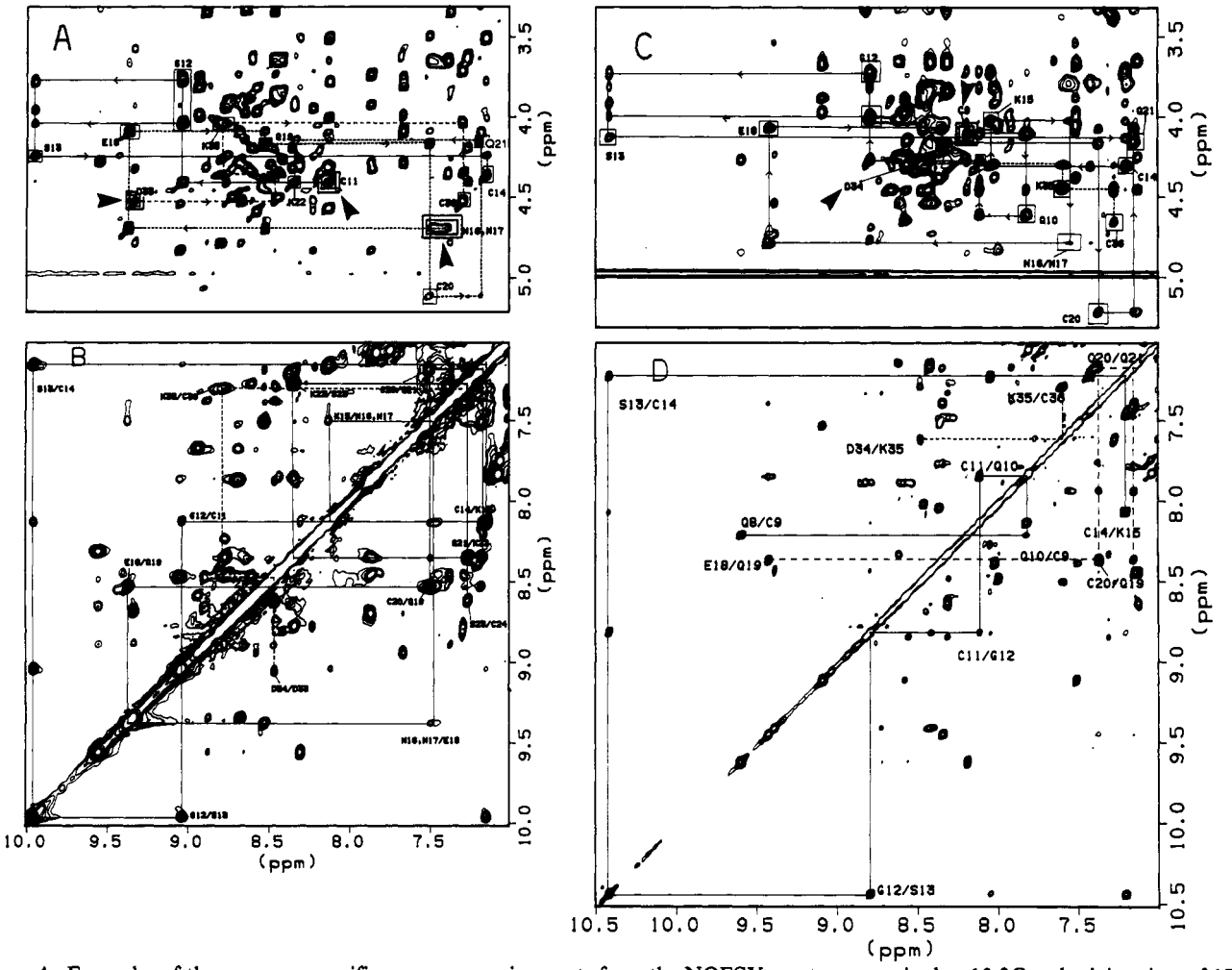

FIGURE 4: Examples of the sequence-specific resonance assignments from the NOESY spectrum acquired at 10 °C and mixing time of 175 ms for Ag-MT (A and B) and Ag-T48 (C and D). The positions of intraresidue NH/C$^{\alpha}$H cross-peaks were determined from the corresponding COSY spectrum and are identified by boxes. Constructs are drawn for selected sequential assignments with double boxes indicating the starting point. A few representative NH/NH cross-peaks are labeled (in B and D) by ω_2 of the first residue and ω_1 of the second residue. Other experimental conditions are the same as those given in Figure 1.

system. The assignment of the remaining two Thr spin systems with the degenerate chemical shifts of their C$^{\gamma}$H$_3$ group protons to T45 (Figure 2A) was supported by the sequential NOEs from one of these Thr spin systems to a Lys spin system which helped to assign it to the T45-K46-K47 segment. The presence of extra cross-peaks in the fingerprint region of the COSY spectrum and the presence of two unassigned Ser (S48 and S51) and Cys (C49 and C50) spin systems among the remaining six unassigned residues prompted us at this point to direct our attention to the sequential assignment of the mutant protein (T48).

The sequential assignment of Ag-T48 proceeded very similarly to that of the wild-type protein. Again, the backbone proton resonances of N16 and N17 were broad and degenerate in Ag-T48. The assignment of the unassigned Ser spin system in Ag-T48 to S48 was done by exclusion, since no sequential NOE was observed. A comparison of the ^{1}H NMR data from Ag-MT and Ag-T48 enabled the assignment of S51, G52, and K53 in the former. However, the sequential assignment of the two Cys spin systems at C49 and C50 remained incomplete due to the lack of any sequential NOEs in this portion of this protein. The patterns of the observed sequential NOEs were essentially the same for Ag-MT and Ag-T48, and this is illustrated in Figure 4. The chemical shift data for the two proteins are given in Table I. Analogous sequential assignment procedures were used for Cu-MT and Cu-T48, and these data are given in Table II.

Stereospecific Assignments of Cys H$^{\beta}$. The motion around the torsion angle χ_1 in the Cys residues is expected to be restricted due to the involvement of the S$^{\gamma}$ group in metal coordination. The characterization of this angle and stereospecific assignment of the two prochiral H$^{\beta}$ has been determined by the use of the two $^3J_{\alpha\beta}$ couplings and the intensity of the NOE cross-peaks between the amide and the two H$^{\beta}$ protons and also between the H$^{\alpha}$ and the H$^{\beta}$ protons (Wagner et al., 1987). The stereospecific assignments for the 10 assigned Cys spin systems are listed in Tables I and II for Ag-MT and Cu-MT, respectively. The two H$^{\beta}$ are distinguished and labeled *R* and *S* according to the IUPAC–IUB (1970).

HMQC Data. Heteronuclear multiple-quantum coherence transfer experiments involving ^{1}H–^{109}Ag couplings were carried out in Ag-MT and Ag-T48. The data for Ag-MT showed seven distinct ^{109}Ag resonances spanning a chemical shift range of 460 ppm (Narula et al., 1991). Moreover, these data also showed that S$^{\gamma}$ of only 10 of the 12 Cys were involved in Ag ligation and that the side chain of His was not involved in metal ligation. The HMQC spectrum for Ag-T48 shown in Figure 5 was acquired *antiphase* with a preparation period of 40 ms (J = 12.5 Hz) at 30 °C. This spectrum shows cross-peaks for only those protons which have couplings to ^{109}Ag.

Table I: ^{1}H Chemical Shifts (in Parts per Million) for Silver-Substituted Metallothionein and the T48 Mutant at pH 6.5, 10 °C, in 18 mM Potassium Phosphate

	NH		H^α			
residue	Ag-MT	Ag-T48	Ag-MT	Ag-T48	H^β [b]	others[a]
Q1			4.43	4.43	2.45, 2.58	H^γ 1.93, 2.09
N2	8.76	8.76	4.79	4.80	2.75, 2.89	$N^\delta H$ 7.07, 7.75
E3	8.81	8.80	4.27	4.28	1.97, 2.08	H^γ 2.30
G4	8.42	8.42	3.65, 3.85	3.65, 3.85		
H5	8.48	8.48	5.06	5.07	3.35[c]	H^δ 7.38, H^ϵ 8.87[c]
E6	8.90	8.90	4.31	4.33	1.95, 2.10	H^γ 2.36, 2.43
C7	8.66	8.66	4.25	4.27	3.32(*R*), 3.93(*S*) [3.34(*R*), 3.98(*S*)]	
Q8	9.56	9.55	4.92	4.91	1.95	H^γ 2.39, 2.52; $N^\epsilon H$ 6.91, 7.61
C9	8.31	8.31	4.34	4.35	2.73(*S*), 4.30(*R*) [2.75(*S*), 4.30(*R*)]	
Q10	8.15	8.13	4.58	4.58	1.66	H^γ 2.37, 2.46; $N^\epsilon H$ 7.00, 7.33
C11	8.13	8.14	4.41	4.42	2.74(*S*), 3.74(*R*) [2.74(*S*), 3.79(*R*)]	
G12	9.04	9.04	3.78, 4.04	3.78, 4.04		
S13	9.96	9.95	4.24	4.25	3.97	
C14	7.16	7.15	4.37	4.37	3.27(*R*), 3.49(*S*) [3.27(*R*), 3.52(*S*)]	
K15	8.13	8.13	4.01	4.02	1.62, 1.75	H^γ 1.36; H^ϵ 2.95
N16	7.45[d]	7.40[d]	4.70	4.70	2.63, 3.65	$N^\delta H$ 7.28, 7.39
N17	7.49[d]	7.50[d]	4.70	4.70	2.75, 3.04	$N^\delta H$ 7.08, 7.84
E18	9.38	9.37	4.10	4.10	2.13	H^γ 2.35, 2.40
Q19	8.53	8.53	4.17	4.18	2.18	H^γ 2.49; $N^\epsilon H$ 6.96, 7.65
C20	7.51	7.51	5.11	5.11	2.80(*R*), 3.52(*S*) [2.82(*R*), 3.50(*S*)]	
Q21	7.19	7.19	4.15	4.16	2.15, 2.24	H^γ 2.46, 2.78; $N^\epsilon H$ 6.70, 8.14
K22	8.35	8.35	4.41	4.42	1.68, 1.89	H^γ 1.44, 1.48; H^δ 1.81; H^ϵ 3.02
S23	7.27	7.27	4.19	4.19	3.65	
C24	8.62	8.61	4.53	4.54	2.73(*R*), 3.85(*S*) [2.72(*R*), 3.88(*S*)]	
S25	8.68	8.67	4.79	4.79	3.91, 4.11	
C26	9.34	9.34	4.33	4.33	2.85(*S*), 3.24(*R*) [2.86(*S*), 3.25(*R*)]	
P27			4.59	4.59	1.97, 2.37	H^γ 2.07, 2.17; H^δ 3.57, 3.93
T28	8.58	8.61	3.81	3.82	3.96	$C^\gamma H_3$ 1.17
G29	8.94	8.93	3.76, 3.99	3.76, 3.99		
C30	7.67	7.67	4.29	4.30	3.07(*S*), 4.12(*R*) [3.02(*S*), 4.10(*R*)]	
N31	8.70	8.69	4.92	4.91	2.86, 2.90	$N^\delta H$ 6.85, 7.53
S32	7.87	7.87	4.84	4.85	3.78, 3.94	
D33	9.05	9.05	4.54	4.55	2.59, 2.83	
D34	8.47	8.47	4.35	4.36	2.64	
K35	8.80[e]	8.77[e]	4.04	4.05	1.82, 1.98	H^γ 1.34, 1.38; H^δ 1.65, 1.72
C36	7.30	7.30	4.52	4.53	3.08(*R*), 3.72(*S*) [3.07(*R*), 3.72(*S*)]	
P37			4.84	4.84	2.07, 2.34	H^γ 1.96, 2.18; H^δ 4.23, 4.55
C38	8.23	8.23	3.96	3.96	2.64(*S*), 2.72(*R*) [2.61(*S*), 2.72(*R*)]	
G39	8.77	8.77	3.91, 4.21	3.90, 4.20		
N40	8.35	8.34	4.95	4.92	2.56, 2.95	$N^\delta H$ 6.94, 7.68
K41[f]	7.85, 7.88	7.86	4.33	4.32	1.83, 1.93	H^γ 1.45; H^δ 1.77; H^ϵ 3.07
S42[f]	8.44, 8.47	8.44	4.50	4.51	3.90	
E43	8.73	8.73	4.33	4.32	1.97, 2.11	H^γ 2.29
E44[f]	8.61,[g] 8.63	8.64	4.33, 4.35[g]	4.33	1.99, 2.08	H^γ 2.29
T45[f]	8.36,[h] 8.43	8.35	4.30,[h] 4.35	4.32	4.23	$C^\gamma H_3$ 1.24
K46	8.49	8.48	4.36	4.37	1.80, 1.89	H^γ 1.46; H^δ 1.70; H^ϵ 3.02
K47[f]	8.13, 8.18	8.17	4.20	4.20	1.73, 1.85	H^γ 1.42; H^δ 1.72; H^ϵ 3.01
S48	8.14	8.13	4.28	4.28	3.86	
C49/50[i]	8.01		4.53		3.10, 3.26	
	8.19		4.41		2.95, 3.06	
	8.24		4.43		2.57, 2.98	
	8.25		4.77		3.20, 3.30	
S51	8.42		4.18		3.98	
G52[d,f]	8.60,[j] 8.68		3.91,[j] 3.95			
K53[f]	7.97,[k] 8.00		4.16, 4.21[k]		1.82, 1.97	H^γ 1.33, 1.39; H^δ 1.64, 1.71, H^ϵ 3.00

[a] The data for side-chain protons (except for Cys) of Ag-T48 are not listed. For a few long-chain amino acids, all the side-chain proton resonances could not be located. [b] The chemical shifts for H^β of the Cys in Ag-T48 are listed in parentheses. [c] The chemical shifts for H^β, H^δ, and H^ϵ of H5 in Ag-T48 are 3.36, 7.39, and 8.90 ppm, respectively. [d] The resonance is broad. [e] A broad and very temperature sensitive resonance (a variation of 0.44 ppm is observed for the NH resonance when the temperature is varied from 10° to 30 °C). [f] The residue shows two sets of resonances for its backbone protons in Ag-MT. [g] The NH resonance at 8.61 ppm is connected to H^α at 4.35 ppm. [h] The NH resonance at 8.36 is connected to H^α, H^β, and H^γ at 4.30, 4.19, and 1.24 ppm, respectively. [i] Four Cys spin systems are listed. However, their sequential assignments could not be established. [j] The NH resonance at 8.60 is connected to H^α at 3.91 ppm. [k] The NH resonance at 7.97 ppm is connected to H^α at 4.21 ppm.

Table II: ^{1}H Chemical Shifts (in Parts per Million) for Copper Metallothionein and the T48 Mutant at pH 6.5, 10 °C, in 18 mM Potassium Phosphate

	NH		H^α			
residue	Cu-MT	Cu-T48	Cu-MT	Cu-T48	$H^{\beta b}$	others[a]
Q1			4.39	4.43	2.43, 2.56	H^γ 1.89, 2.07
N2	8.72	8.74	4.77	4.75	2.72, 2.88	$N^\delta H$ 7.04, 7.72
E3	8.80	8.78	4.27	4.27	1.95, 2.05	H^γ 2.30
G4	8.41	8.41	3.67, 3.83	3.66, 3.81		
H5	8.31	8.32	4.98	5.04	3.31	H^δ 7.31, H^ϵ 8.72
E6	8.82	8.78	4.30	4.29	2.07	H^γ 2.34, 2.42
C7	8.56	8.51	4.28	4.27	3.15(*R*), 3.94(*S*) [3.15(*R*), 3.93(*S*)]	
Q8	9.59	9.57	4.85	4.85	1.90	H^γ 2.38, 2.48; $N^\epsilon H$ 6.90, 7.55
C9	8.19	8.16	4.10	4.10	2.73(*S*), 4.43(*R*) [2.71(*S*), 4.42(*R*)]	
Q10	7.83	7.83	4.61	4.59	1.62	H^γ 2.33, 2.41; $N^\epsilon H$ 6.94, 7.34
C11	8.11	8.10	4.28	4.26	2.87(*S*), 3.81(*R*) [2.85(*S*), 3.80(*R*)]	
G12	8.79	8.81	3.72, 4.00	3.71, 3.99		
S13	10.42	10.41	4.12	4.12	3.92	
C14	7.20	7.18	4.30	4.28	3.21(*R*), 3.71(*S*) [3.19(*R*), 3.68(*S*)]	
K15	8.05	8.03	4.02	4.01	1.63, 1.69	H^γ 1.39; H^ϵ 2.94
N16	7.52[c]	7.53[c]	4.78	4.78	2.71, 3.79	$N^\delta H$ 7.19, 7.42
N17			4.78	4.78	2.78, 2.90	
E18	9.43	9.43	4.07	4.06	2.11	H^γ 2.32, 2.38
Q19	8.34	8.32	4.17	4.16	2.19	H^γ 2.47; $N^\epsilon H$ 6.94, 7.68
C20	7.37	7.37	5.20	5.20	2.67(*R*), 3.32(*S*) [2.65(*R*), 3.31(*S*)]	
Q21	7.15	7.14	4.14	4.13	2.14, 2.19	H^γ 2.49, 2.68; $N^\epsilon H$ 6.69, 7.91
K22	8.42	8.40	4.44	4.44	1.62, 1.90	H^γ 1.40, 1.44; H^ϵ 3.00
S23	7.13	7.11	4.24	4.25	3.65	
C24	8.61	8.62	4.54	4.55	2.73(*R*), 4.01(*S*) [2.72(*R*), 3.98(*S*)]	
S25	8.41	8.45	4.72	4.72	3.79, 4.17	
C26	9.39	9.35	4.24	4.22	2.99(*S*), 3.07(*R*) [2.96(*S*), 3.05(*R*)]	
P27			4.65	4.65	1.94, 2.34	H^γ 2.05, 2.17; H^δ 3.53, 3.85
T28	8.58	8.59	3.88	3.87	3.98	$C^\gamma H_3$ 1.20
G29	9.09	9.07	3.67, 3.97	3.66, 3.97		
C30	7.51	7.50	4.37	4.35	2.91(*S*), 4.05(*R*) [2.90(*S*), 4.03(*R*)]	
N31	8.36	8.37	4.92	4.92	2.81, 2.97	$N^\delta H$ 6.88 7.49
S32	8.02	8.02	4.87	4.87	3.82, 3.91	
D33	9.08	9.08	4.47	4.47	2.59, 2.76	
D34	8.47	8.46	4.30	4.27	2.65, 2.74	
K35	7.60[d]	7.57[d]	4.44	4.44	1.69	H^γ 1.31, 1.39; H^δ 2.08; H^ϵ 3.00
C36	7.27	7.25	4.65	4.63	3.17(*R*), 3.36(*S*) [3.15(*R*), 3.36(*S*)]	
P37			4.98	4.98	1.93, 2.38	H^γ 1.79, 2.21; H^δ 4.36, 4.55
C38	8.31	8.31	3.82	3.82	2.44(*S*), 2.67(*R*) [2.44(*S*), 2.67(*R*)]	
G39	8.46	8.46	3.88, 4.07	3.82, 4.04		
N40	8.00	7.98	4.85	4.82	2.61, 2.95	$N^\delta H$ 6.97, 7.65
K41[e]	8.04, 8.08	8.05	4.28	4.28	1.77, 1.89	H^γ 1.44; H^δ 1.69; H^ϵ 2.90
S42[e]	8.48, 8.50[f]	8.44	4.47, 4.49[f]	4.44	3.88	
E43[e]	8.66, 8.67[g]	8.64	4.27,[g] 4.30	4.29	1.93, 2.08	H^γ 2.28
E44	8.58	8.55	4.29	4.29	1.95, 2.04	H^γ 2.29
T45[e]	8.31, 8.39[h]	8.33	4.29, 4.32[h]	4.30	4.19	$C^\gamma H_3$ 1.22
K46	8.46	8.45	4.33	4.32	1.77, 1.85	H^γ 1.45; H^δ 1.69
K47	8.15	8.13	4.18	4.16	1.65, 1.72	H^γ 1.39; H^δ 1.82
S48	8.11	8.11	4.28	4.26	3.85	
C49/50[i]	8.01		5.03		3.29, 3.47	
	8.23		5.14		2.82, 3.54	
			4.37		3.47, 3.71	
			4.69		2.79, 2.91	
S51[e]	8.22,[j] 8.23		4.22,[j] 4.26			
G52[e]	8.69, 8.76[k]		3.95, 3.96[k]			
K53[e]	7.90, 7.94		4.19		1.68, 1.83	H^γ 1.35

[a] The data for side-chain protons (except for Cys) of Cu-T48 are not listed. For a few long-chain amino acids, all the side-chain proton resonances could not be identified. [b] The chemical shifts for H^β of the Cys in Cu-T48 are listed in parentheses. [c] The resonance is broad. [d] A chemical shift variation of 0.21 ppm is observed for the NH resonance when the temperature is changed from 10 to 25 °C. [e] The residue shows two set of resonances for its backbone protons in Cu-MT. [f] The NH resonance at 8.50 ppm is connected to H^α and H^β at 4.49 and 3.92 ppm, respectively. [g] The NH resonance at 8.67 ppm is connected to H^α and H^β at 4.27 and 1.96 ppm, respectively. [h] The NH resonance at 8.39 is connected to H^α, H^β, and H^γ at 4.32, 4.21, and 1.22 ppm, respectively. [i] Four Cys spin systems are listed. However, their sequence-specific assignment could not be made. [j] The NH resonance at 8.22 ppm is connected to H^α at 4.22 ppm. [k] The NH resonance at 8.76 ppm is connected to H^α at 3.96 ppm.

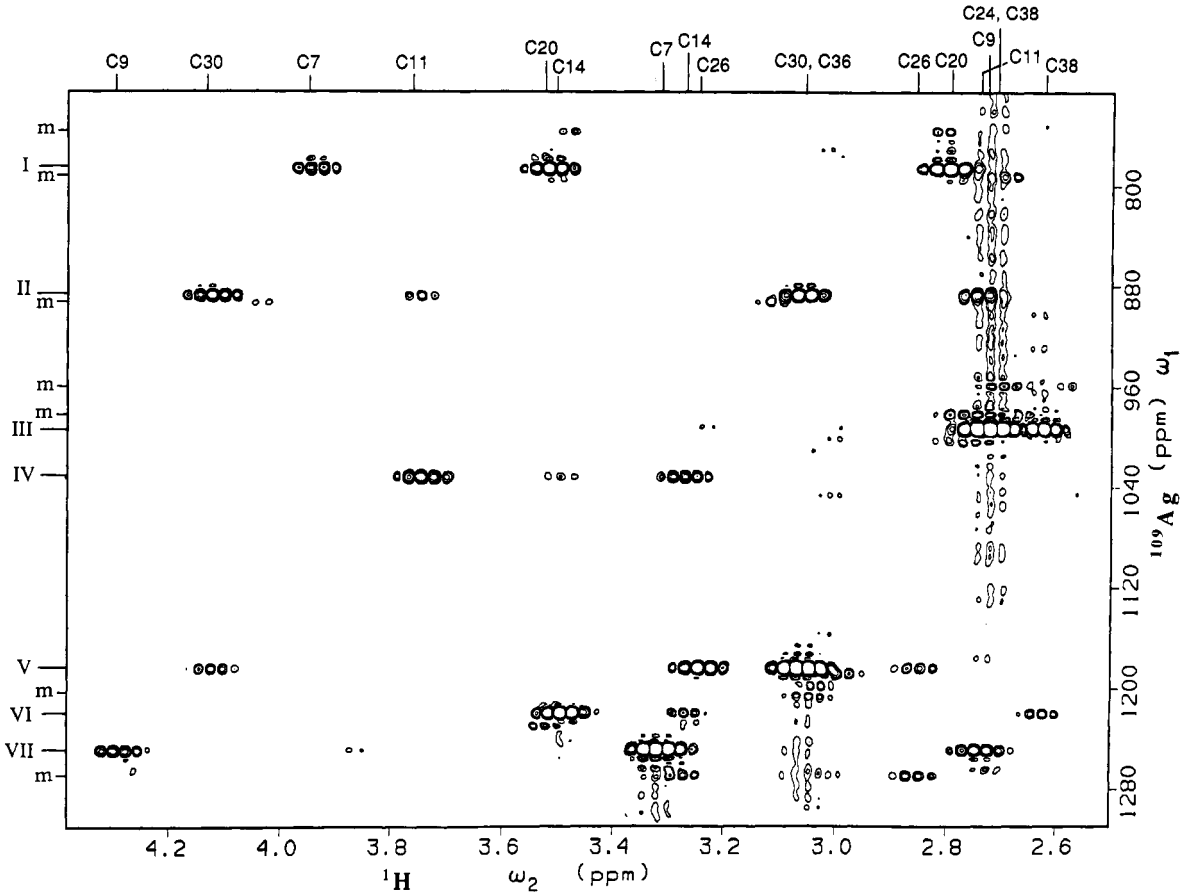

FIGURE 5: Heteronuclear ^{1}H–^{109}Ag multiple-quantum coherence transfer spectrum of yeast Ag-T48 acquired *antiphase* on a Bruker AM500 NMR instrument. The preparation period ($1/2J$) of 40 ms (J = 12.5 Hz) was used, and 224 t_1 data points were acquired at 30 °C. Both positive and negative levels are plotted without distinction. The seven ^{109}Ag resonances are labeled by Roman numerals according to their increasing chemical shifts. The positions of seven low-intensity ^{109}Ag resonances are indicated by the letter **m**. The assignments of various cross-peaks along ω_2 to specific H$^\beta$ of Cys obtained from sequential assignment methods are indicated on the top of the diagram.

The metal stoichiometry of both Ag-MT and the Ag-T48 protein is reported to be eight (Byrd et al., 1988), but only seven major Ag resonances are observed with a spectral range of 1006 ppm covered to minimize the possibility of an eighth Ag resonance falling outside the spectral window. The seven observed resonances are labeled I–VII by their increasing ^{109}Ag chemical shifts. All of the cross-peaks correspond to the chemical shifts of the assigned Cys H$^\beta$ protons (Table I; the data in this table are at 10 °C). Seven low-intensity, ≤10%, ^{109}Ag resonances denoted "m" are also observed in this spectrum. These peaks presumably arise from the presence of some heterogeneity or isoform in the solution.

Table III: ^{109}Ag Chemical Shift Data[a] (in Parts per Million) for Silver-Substituted Wild-Type Metallothionein and Its T48 Mutant at 30 °C

metal resonance	Ag-MT	Ag-T48
I	790	788
II	890	888
III	1000	994
IV	1037	1033
V	1183	1184
VI	1220	1220
VII	1250	1249

[a] The data are referenced to an external 8.0 M silver nitrate solution.

DISCUSSION

Metal Ligation and Cluster. In the present NMR studies, the identity of the coordinating ligands in Ag-substituted MTs has been obtained from ^{1}H–^{109}Ag HMQC data sets. For both Ag-MT and Ag-T48, the HMQC spectra are identical with the observed cross-peaks corresponding to Cys H$^\beta$ chemical shifts (Figure 5 and Table I). The side-chain protons of another potential metal ligand, H5, do not show any cross-peaks in the HMQC spectra consistent with either their noninvolvement in Ag ligation in both proteins or a very small heteronuclear coupling constant. Interactive analysis of the HMQC and sequentially assigned data sets indicated that only 10 Cys residues participate in metal coordination through their S$^\gamma$ group. No evidence was found for the involvement of the two C-terminal Cys (C49 and C50) in metal coordination and, moreover, only seven major Ag resonances were observed when the reported metal stoichiometry is eight (Byrd et al., 1988). Seven minor ^{109}Ag resonances denoted "m" are also observed for both WT and mutant MT samples which must arise from the presence of some heterogeneity in the protein samples such as molecules having different numbers of bound

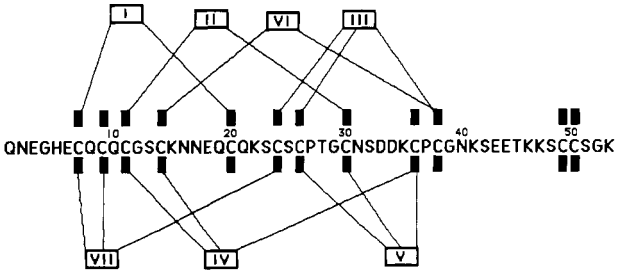

FIGURE 6: Schematic representation of the metal-to-cysteine connectivities obtained from the heteronuclear ^{1}H–^{109}Ag multiple-quantum coherence transfer data on Ag-T48. Ag(I) metal ions are identified by Roman numerals as in Figure 5.

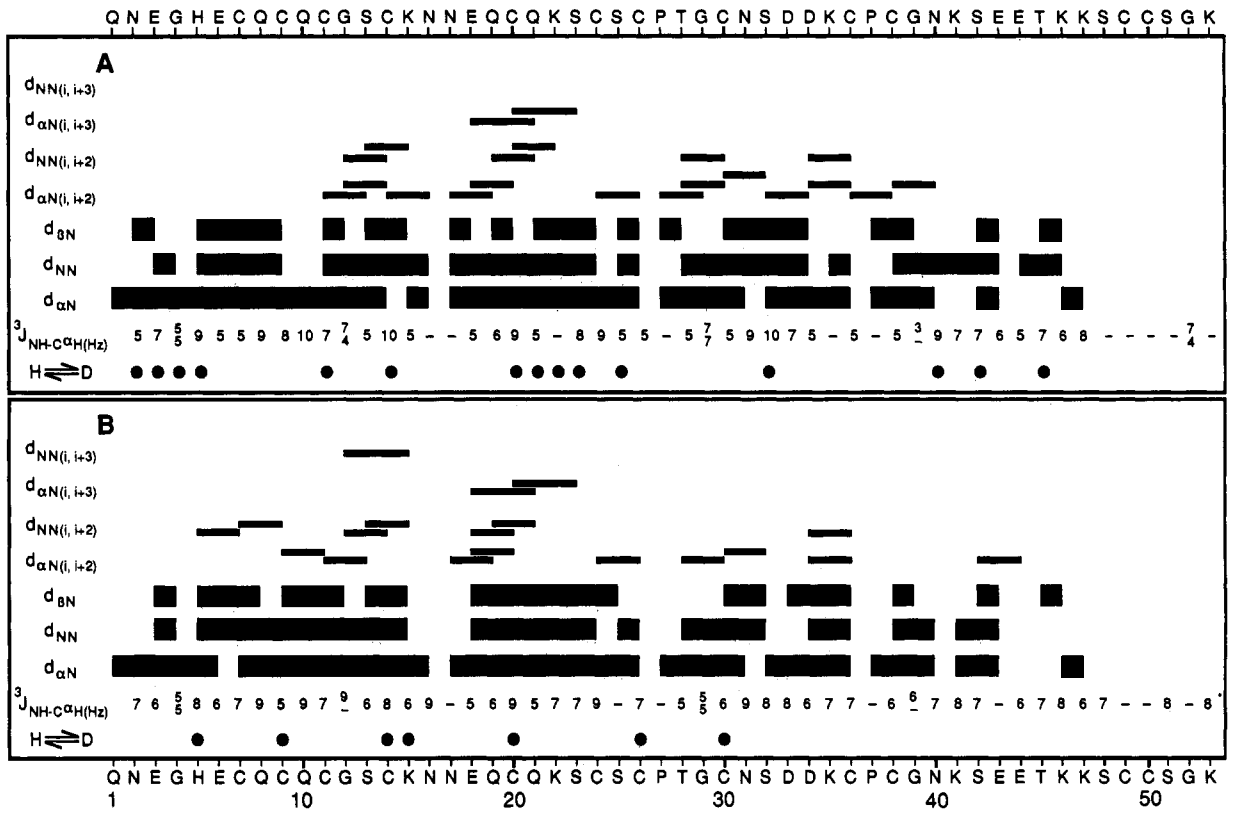

FIGURE 7: Amino acid sequence and summary of the sequential and medium-range NOE connectivities, $^3J_{NH-C\alpha H}$ coupling constants, and amide proton exchange rates observed for Ag-MT (A) and Cu-MT (B). The slow-exchanging amide protons are indicated by solid circles.

Ag(I) ions. During the preparation of the Ag-substituted proteins, slightly less than 8 mol equiv (7.5 mol equiv) of the required metal ion was intentionally added to the apoprotein to purposely avoid possible aggregation which excess metal ions have been shown to initiate in the mammalian forms of this protein (Otvos et al., 1985). With the reported cooperative nature of metal ion binding (Byrd et al., 1988), 93% of the protein would still contain its full complement of eight metal ions. However, if the metal stoichiometry is seven, which appears to be the case from the HMQC data, then even the 7.5 mol equiv of added metal ions during the reconstitution represents a slight excess of metal ions. Possible reasons for the absence of an eighth metal ion resonance have been mentioned previously (Narula et al., 1991).

The unusually large range of ^{109}Ag chemical shifts observed for the seven Ag resonances (Table III) in a protein where Ag(I) ligation is exclusively through Cys S$^\gamma$ groups is suggestive of the presence of mixed Ag(I) coordination number (Haberkorn et al., 1976; Henrichs, 1983; Lacelle et al., 1984; Endo et al., 1985). Unfortunately, no ^{109}Ag NMR chemical shift data are available for stable model thiol complexes of known geometries with different coordination numbers to correlate the dependence of the Ag chemical shift on coordination number, bond distance/angle, and number of bridging vs terminal thiolates. Mixed coordination numbers (2 and 3), however, have been reported for both Cu(I) and Ag(I) model complexes, e.g., $[Cu_5(SPh)_7]^{2-}$, $[Ag_6(SPh)_8]$, and $[Ag_9(SCH_2CH_2S)_6]^{3-}$ (Dance, 1976, 1978, 1981; Kau et al., 1987; Henkel et al., 1988). A schematic representation of the metal-to-Cys connectivities obtained from the analysis of the HMQC data on Ag-T48 is shown in Figure 6, which is exactly the same as was found for Ag-MT (Narula et al., 1991). The most upfield shifted metal resonances, I and II, are found coordinated to two S$^\gamma$ groups, while the downfield shifted metal resonances III–VII (with the exception of VI) are each coordinated by three S$^\gamma$ groups. Most probably, resonance VI, which falls in the chemical shift range of trigonally coordinated Ag(I) ions, is missing its connection to the third Cys ligand in the HMQC spectrum due to the very small value of its ^{1}H–^{109}Ag coupling. There are only two terminal Cys (C9 and C20) which show a linkage to only one metal ion, and we would suggest that one of these two Cys may be ligated to metal VI with the remaining Cys available for further metal ligation. In this proposed model, which is based upon the observed chemical shift dispersion of the seven Ag(I) resonances, there will be 2 metal ions having a coordination number of 2 (diagonal) and 5 metal ions with a coordination number of 3 (trigonal) in a single cluster involving 7 metal ions and 10 Cys. Such a model is consistent with a biochemical study in which it was reported that 25% of the metal ions in yeast-MT are very labile and can react with chelators such as bathocuproine disulfonate and cuproine within seconds (Weser & Hartmann, 1988). In our proposed model, the diagonally coordinated metal ions I and II should be more labile than the other five trigonally coordinated metal ions. It is unlikely, but nevertheless still possible, for the remaining terminal Cys (C9 or C20) to interact with the eighth metal ion by itself for which no resonance is observed in the HMQC experiments. Yet another possibility for our failure to detect the eighth metal resonance is mentioned under Observed Structural Heterogeneity.

Secondary Structural Elements in Ag-MT. The sequential and medium-range NOEs of Ag-MT along with the $^3J_{NH-C\alpha H}$ couplings and amide proton exchange rates are given in Figure 7A. The pattern of the observed $d_{\alpha N}$ and d_{NN} along with the coupling constant values indicates the absence of any regular secondary structural elements, such as α-helix and β-sheet, in this protein. The pattern of observed d_{NN}, $d_{\alpha N}(i,i+2)$, and $d_{NN}(i,i+2)$ in the C11-G12-S13-C14 segment and the slow exchange of the C14 amide proton along with the values of $^3J_{NH-C\alpha H}$ for G12 and S13 indicate the presence of a type I β-turn. Similar analysis showed the presence of additional

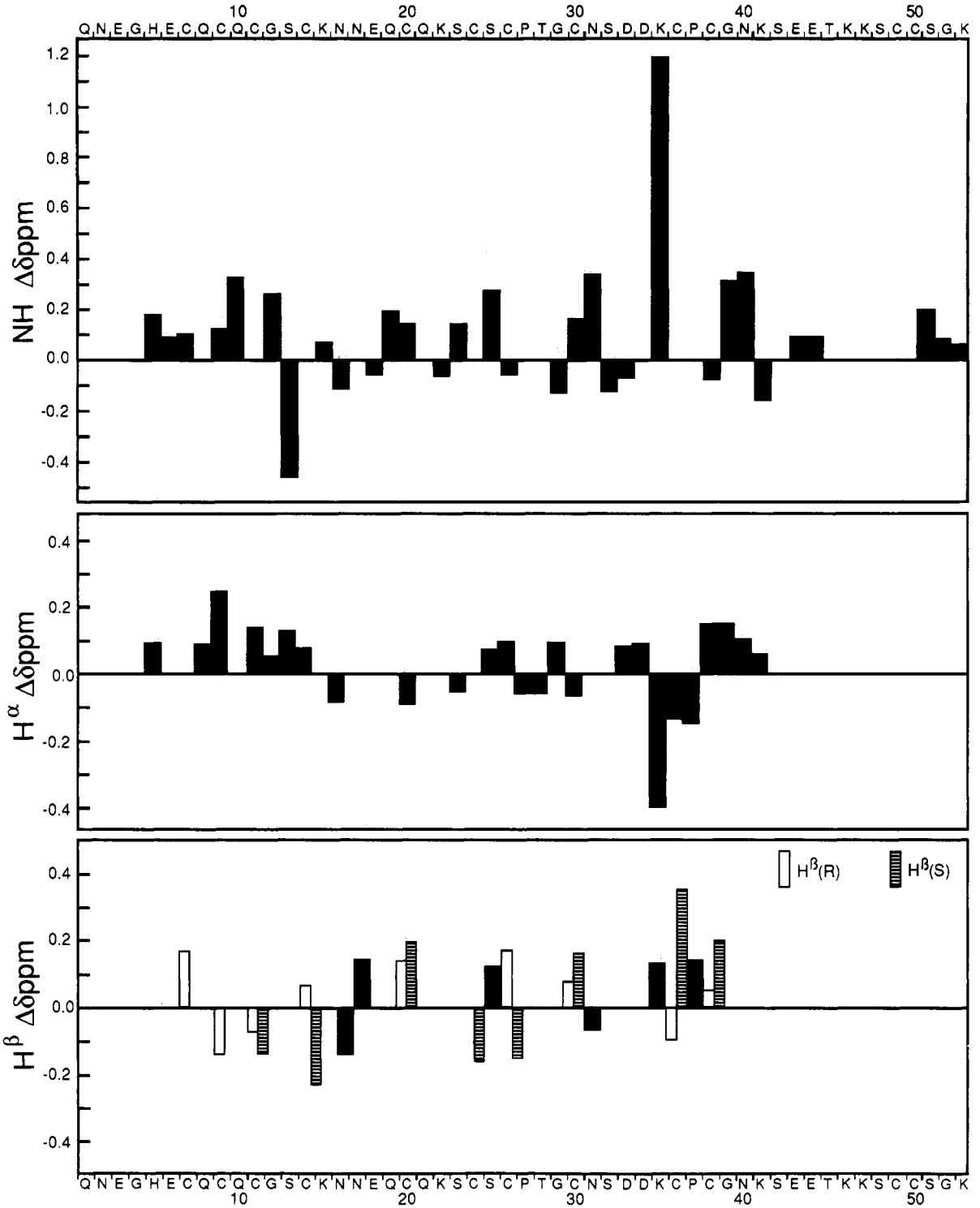

FIGURE 8: Chemical shift changes, $\Delta\delta$, of NH, H^{α}, and H^{β} accompanying the change of Ag(I) to Cu(I) in yeast MTs. $\Delta\delta$ is obtained from $\delta_{Ag} - \delta_{Cu}$ at 10 °C.

type I β-turns at E18-Q19-C20-Q21, P27-T28-G29-C30, and D33-D34-K35-C36. Other turns may be revealed following the completion of ongoing structural refinement calculations.

In the primary sequence of this protein, a CXC repeat is found four times and a CXXC is found only once. In the former category, C7,C9 and C24,C26 are ligated to the same metal (Figure 6, metal VII for the case of C7 and C9 and metal III for the case of C24 and C26). However, the other two members of this class, C9,C11 and C36,C38, do not ligate a common metal ion. In the CXXC category, C11 and C14 share a common metal ion (IV). A similar disposition of the Cys in these repeat units was observed for mammalian Cd_5-Zn_2-MT (Robbins et al., 1991). The eventual calculation of the three-dimensional solution structure from the experimentally determined distance and dihedral angle constraints and the metal–Cys connectivities will undoubtedly reveal additional structural features. However, at this stage it can be said that the polypeptide chain wraps around the seven metal ions to form one cluster and in this folding process various type I β-turns are utilized.

Comparison of Cu-MT and Ag-MT. The ^{1}H chemical shift data for these two proteins are given in Tables I and II. The chemical shift difference, $\Delta\delta$, for NH, H^{α}, and H^{β} on going from Ag-MT to Cu-MT is represented graphically in Figure

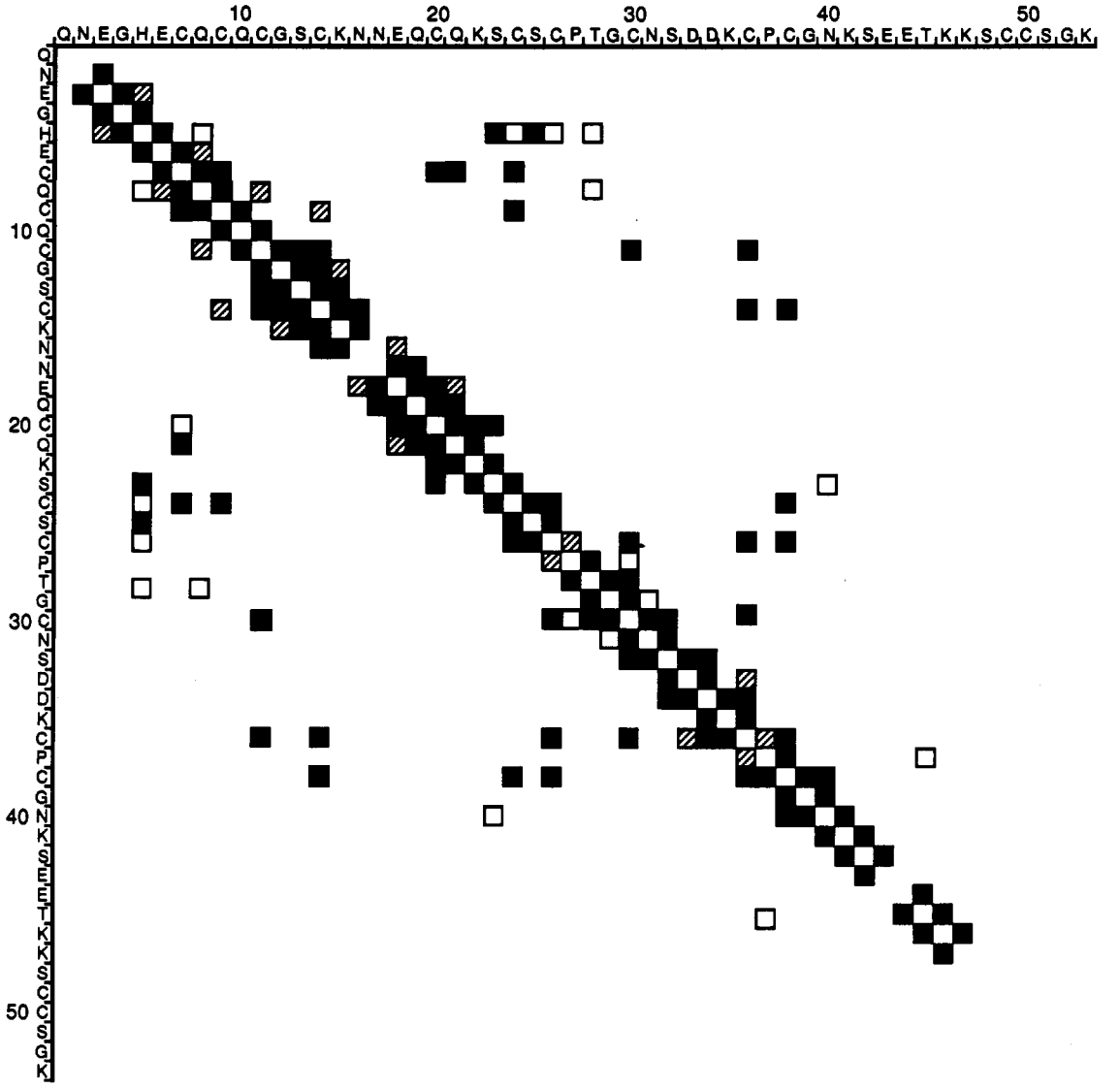

FIGURE 9: Diagonal plot of NOEs observed in Ag-MT with the amino acid sequence represented on both axes. A solid square at the position (x,y) indicates a NOE between backbone protons (NH or H^{α}) of the two residues in the sequence location x and y. A shaded square indicates a NOE between NH or H^{α} of one residue and a side-chain proton in another residue. A blank square indicates a NOE between side-chain protons of two residues. A square with double shading indicates the involvement of two residues (Cys) in the ligation of the same Ag(I) ion. Where two residues are connected by more than one NOE, only the one that involves the largest number of backbone protons is shown.

8 for all changes ≥0.05 ppm. Since stereospecific assignments for the H^{β} of Cys were determined, $\Delta\delta$ for both H^{β} is represented. Not unexpectedly, the most significant values for the $\Delta\delta$ are found for the backbone protons, with the largest being observed for the backbone protons of the K35 residue. Factors which are known to affect the chemical shifts, such as pH, temperature, and buffer conditions, were kept constant in the two samples, suggesting that the observed differences result from minor changes in the secondary structure perhaps reflective of variations in the hydrogen bonding involved in the many β-turns (Wüthrich, 1986).

Among the different $\Delta\delta$ observed for H^{β} resonances, the majority correspond to Cys residues. The chemical shifts for Cys H^{β} protons in a random coil secondary structure fall in the range 2.96–3.28 ppm (Wüthrich, 1986). The observed chemical shift ranges for Cys H^{β} protons are 2.61–4.30 and 2.44–4.43 ppm for Ag-MT and Cu-MT, respectively. This large chemical shift dispersion for the Cys H^{β} in Cu-MT and Ag-MT must arise from the coordination of the Cys S^{γ} to the metal ions and the resulting structure generated by the folded polypeptide chain through these linkages. Additionally, the different magnetic susceptibilities of these metal ions will affect the chemical shifts of coupled protons (Pople et al., 1959). As a result of the exquisite sensitivity of the $\Delta\delta$ values, therefore, the relatively large range of $\Delta\delta$ values observed for Cys H^{β} represents only a small perturbation in the protein structures between the two metaled forms. Diagonal plots of sequential and medium- and long-range NOEs observed for Ag-MT and Cu-MT are shown in Figures 9 and 10, respectively. In the former, Ag-to-Cys connectivities obtained from the analysis of ^{1}H–^{109}Ag HMQC data (Narula et al., 1991) are also indicated. In Ag-MT, the HMQC and NOESY data support each other. For example, HMQC data indicate that C7 and C24 share a common Ag ion (Figure 6), and a NOESY spectrum shows NOE cross-peaks between the H^{β} of the two residues. Examples of regions where similar NOEs are observed for both Ag-MT and Cu-MT include the two regions of long-range NOEs between the protons of H5 and the C24-S25-C26 segment and those between C7 and Q21,C24. A number of interresidue NOEs involving H^{β} of various Cys residues are observed for both Ag- and Cu-MT. Even though the patterns of NOEs are similar for both proteins (Figures 9 and 10), the number of observed interresidue NOEs is greater for Cu-MT. As an example, NOEs are observed between H^{β}

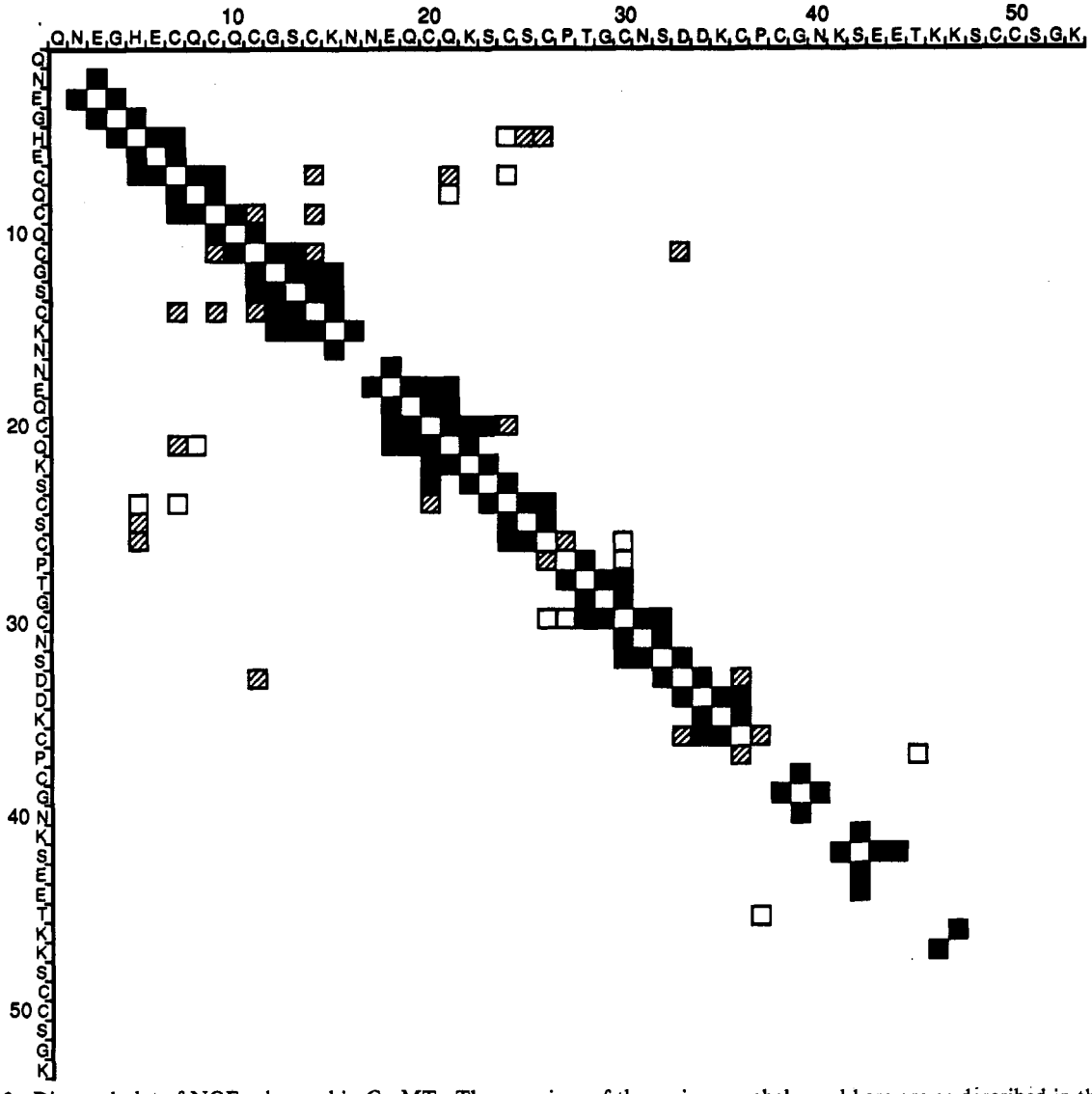

FIGURE 10: Diagonal plot of NOEs observed in Cu-MT. The meanings of the various symbols used here are as described in the legend for Figure 9.

of C26 and C30 for both molecules, but additional NOEs between H^{α} of C26 and H^{β} of C30 are observed in Cu-MT which are not present in the NOESY spectrum of Ag-MT. These experimental observations support the similarity of the metal clusters in both proteins with the presence of additional cross-peaks in Cu-MT being consistent with a more compact structure because of the smaller ionic radius of Cu(I) (0.95 Å) compared with that of Ag(I) (1.15 Å)(Cotton & Wilkinson, 1980). Direct HMQC experiments are not feasible for Cu-MT and, therefore, metal-to-Cys connectivities cannot be independently established as was done for Ag-MT.

Comparison of Wild-Type and Mutant Proteins. The ^{1}H NMR data for Ag-MT and Ag-T48 presented in Table I are very similar. These include the ^{1}H chemical shifts of the Cys H^{β} which are involved in the coordination of the seven Ag(I) ions. It is, therefore, reassuring that the ^{109}Ag chemical shifts obtained from the ^{1}H–^{109}Ag HMQC spectra are very similar for both proteins (Table III). Additionally, the two regions of long-range NOEs are observed in both molecules. These experimental observations along with all of the other physical/chemical measurements which include metal stoichiometry, fluorescence rate constant, luminescent characteristics, and affinity for bathocuproine disulfonate, are consistent with similar tertiary structures stabilized by Cys S^{γ} to metal coordination in both proteins (Thrower et al., 1988; Byrd et al., 1988).

The present NMR have provided the first experimental data showing that the two C-terminal Cys (C49 and C50) are not involved in the Ag cluster formation. This is reflected in two ways: (1) identical Ag chemical shifts from HMQC data of Ag-MT and Ag-T48 and (2) observation of no cross-peaks for the Cys H^{β} protons of C49 and C50 in the HMQC spectrum of Ag-MT. The biochemical studies that showed these two Cys did not titrate at neutral pH under nondenaturing conditions was perhaps an early indicator of their anomalous behavior (D. R. Winge, unpublished results). A close correspondence in the ^{1}H chemical shift data for Cu-MT and Cu-T48 presented in Table II and the same pattern of long- and medium-range NOEs observed in these two proteins suggest very similar structures for the Cu proteins as well.

Observed Structural Heterogeneity. Several amino acid residues from K41 to K53 show a doubling of cross-peaks for their backbone protons in COSY spectra of both Ag-MT (Figures 1 and 2) and Cu-MT. The absence of this cross-peak doubling in the COSY spectrum of Ag-T48 (Figures 2B and 3D) as well as in Cu-T48 indicates that the structural nonrigidity present in the C-terminal domain of the wild-type protein is removed when the five C-terminal residues are

truncated. The doubling of the C-terminal resonances in both the native Cu-MT and the reconstituted Ag-MT excludes its origin being from the metal reconstitution procedure (see Experimental Procedures). It is possible that the two Cys, C49 and C50, which were surprisingly found not to be involved in metal ligation, are interacting transiently either with one another or in some other way, yet unknown, with the residues in the K41–K53 segment, resulting into two exchanging conformations in this segment. It is also possible that in the WT proteins C49 and C50 are involved in loosely ligating an eighth metal ion which is not part of the metal cluster formed by the other seven metal ions and which is more easily lost during protein purification. The ratio of cross-peak intensities for these doubled C-terminal residues' resonances is also temperature dependent, as illustrated for the two peaks corresponding to K41 in the temperature range 10–30 °C (Figure 3A–C). This is illustrated again in the different ratio of cross-peak intensities corresponding to $NH/C^{\gamma}H_3$ of the two T45 spin systems in the TOCSY experiments at two different temperatures, a phenomenon characteristic of conformational exchange. However, no exchange cross-peaks could be observed in a ROESY spectrum acquired at 10 °C with a mixing time of 100 ms. The expected exchange cross-peaks would have been very close to the diagonal, however, because of the limited dispersion of their chemical shifts in the two conformations which may have precluded their observation (Table I and II). No transfer of magnetization was observed in 1D saturation transfer experiments involving the two amide protons of T45, indicating that the exchange rate between the two conformations is less than 10/s. It is interesting to speculate that the presence of this structural heterogeneity in the native protein might have prevented the growth of stable crystals for X-ray investigation (C. D. Stout, personal communication). Since NMR shows a close correspondence between the structures of the wild-type and mutant proteins, one should perhaps consider trying to crystallize the mutant protein.

Conclusion. Similar global fold of the yeast metallothionein is predicted for both the native Cu(I) and reconstituted Ag(I) forms. The single metal cluster is, however, somewhat more compact in the Cu(I) form. The doubling of resonances from the 13 C-terminal residues is removed upon truncation of the 5 C-terminal residues which includes 2 Cys residues. Both the silver-substituted wild-type protein and mutant protein which lacks the five C-terminal residues show seven ^{109}Ag NMR resonances, indicating that there are only seven distinct sites in the metal cluster of these proteins and that the two C-terminal Cys are not directly involved in the formation of the single metal cluster in the WT protein.

ACKNOWLEDGMENT

We thank Bruce Johnson for initiating these studies on the yeast copper metallothionein while in this laboratory and prior to joining Merck & Co., New Jersey.

REFERENCES

Aue, W. P., Bartholdi, E., & Ernst, R. R. (1976) *J. Chem. Phys. 64*, 2229–46.

Bax, A., & Davis, D. G. (1985) *J. Magn. Reson. 65*, 355–60.

Bax, A., & Drobny, G. P. (1985) *J. Magn. Reson. 61*, 306–20.

Bax, A., & Freeman, R. (1981) *J. Magn. Reson. 44*, 542–61.

Brauschweiler, L., Bodenhausen, G., & Ernst, R. R. (1983) *Mol. Phys. 48*, 535–60.

Buchman, C., Skroch, P., Welch, J., Fogel, S., & Karin, M. (1989) *Mol. Cell. Biol. 9*, 4091–95.

Butt, T. R., Sternberg, E. J., Gorman, J. A., Clark, P., Hamer, D., Rosenberg, M., & Crooke, S. T. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 3332–36.

Byrd, J., Berger, R. M., McMillin, D. R., Wright, C. F., Hamer, D., & Winge, D. R. (1988) *J. Biol. Chem. 263*, 6688–94.

Casas-Finet, J. R., Hu, S., Hamer, D., & Karpel, R. L. (1992) *Biochemistry 31*, 6617–26.

Cotton, F. A., & Wilkinson, G. (1980) in *Advanced Inorganic Chemistry*, p 14, Wiley, New York.

Dameron, C. T., Winge, D. R., George, G. N., Sansone, M., Hu, S., & Hamer, D. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 6127–31.

Dance, I. G. (1976) *J. Chem. Soc., Chem. Commun.* 103–04.

Dance, I. G. (1978) *Aust. J. Chem. 31*, 2195–206.

Dance, I. G. (1981) *Inorg. Chem. 20*, 1487–92.

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*, 16895–900.

Eich, G., Bodenhausen, G., & Ernst, R. R. (1982) *J. Am. Chem. Soc. 104*, 3731–32.

Endo, K., Yamamoto, K., Matsushita, K., Deguchi, K., Kanda, K., & Nakatsuji, H. (1985) *J. Magn. Reson. 65*, 268–81.

Fogel, S., & Welch, J. W.. (1982) *Proc, Natl. Acad. Sci. U.S.A. 79*, 5342–46.

Furst, P., Hu, S., Hacket, R., & Hamer, D. (1988) *Cell, 55*, 705–17.

George, G. N., Winge, D. R., Stout, C. D., & Cramer, S. P. (1986) *J. Inorg. Biochem. 27*, 213–20.

George, G. N., Byrd, J., & Winge, D. R. (1988) *J. Biol. Chem. 263*, 8199–203.

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

Haberkorn, R. A., Que, L., Jr., Gillum, W. O., Holm, R. H., Liu, C. S., & Lord, R. C. (1976) *Inorg. Chem. 15*, 2408–14.

Hamer, D. H., Thiele, D. J., & Lemontt, J. E. (1985) *Science 228*, 685–90.

Hathaway, B. J. (1987) in *Comprehensive Coordination Chemistry (Vol. 5): (Late Transition Elements) The Synthesis, Reactions, Properties and Application of Coordination Compounds* (Wilkinson, G., Gillard, R. D., & McCleverty, J. A., Eds.) Chapter 53, pp 533–774, Pergamon Press, Oxford, U.K.

Henkel, G., Krebs, B., Betz, P., Fietz, H., & Saatkamp, K. (1988) *Angew. Chem., Int. Ed. Engl. 27*, 1326–29.

Henrichs, P. M. (1983) in *NMR of Newley Accessible Nuclei* (Laszlo, P., Ed.) Vol. II, pp 319–34, Academic Press, New York.

Inouhe, M., Hiyama, M., Tohoyama, H., Joho, M., & Murayama, T. (1989) *Biochim. Biophys. Acta 993*, 51–55.

IUPAC–IUB Commission on Biochemical Nomenclature (1970) *J. Mol. Biol. 52*, 1–17.

Jeener, J., Meier, B. H., Bachmann, P., & Ernst, R. R. (1979) *J. Chem. Phys. 71*, 4546–53.

Kägi, J. H. R., & Kojima, Y. (1987) in *Proceedings of the 2nd International Meeting on Metallothioneins and other Low Molecular Weight Metal-Binding Proteins*, Zürich, Aug 21–25, 1985 (Kägi, J. H. R., & Kojima, Y., Eds.) Vol. 52, pp 25–61, Birkhaüser Verlag, Basel.

Karin, M., Najarian, R., Haslinger, A., Valenzuela, P., Welch, J., & Fogel, S. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 337–41.

Kau, L.-S., Spira-Solomon, D. J., Penner-Hahn, J. E., Hodgson, K. O., & Solomon, E. I. (1987) *J. Am. Chem. Soc. 109*, 6433–42.

Kumar, A., Ernst, R. R., & Wüthrick, K. (1980) *Biochem. Biophys. Res. Commun. 95*, 1–6.

Lacelle, S., Stevens, W. C., Kurtz, D. M., Jr., Richardson, J. W., Jr., & Jacobson, R. A. (1984) *Inorg. Chem. 23*, 930–35.

Lancashire, R. J. (1987) in *Comprehensive Coordination Chemistry (Vol. 5): (Late Transition Elements) The Synthesis, Reactions, Properties and Application of Coordination Compounds* (Wilkinson, G., Gillard, R. D., & McCleverty, J. A., Eds.) Chapter 54, pp 775–859, Pergamon Press, Oxford, U.K.

Marion, D., & Bax, A. (1988) *J. Magn. Reson. 80*, 528–33.
Marion, D., & Wüthrich, K. (1983) *Biochem. Biophys. Res. Commun. 113*, 967–74.
Messerle, B. A., Schäffer, A., Vašák, M., Kägi, J. H. R., & Wüthrich, K. (1990) *J. Mol. Biol. 214*, 765–79.
Narula, S. S., Mehra, R. K., Winge, D. R., & Armitage, I. M. (1991) *J. Am. Chem. Soc. 113*, 9354–58.
Otting, G., & Wüthrich, K. (1990) *Q. Rev. Biophys. 23*, 39–96.
Otvos, J. D., Engeseth, H. R., & Wehrli, S. (1985) *Biochemistry 24*, 6735–40.
Pople, J. A., Schneider, W. G., & Bernstein, H. J. (1959) in *High Resolution NMR* (Hume, D. N., King, E. L., Strok, G., Herschback, D. R., & Pople, J. A., Eds.) Appendix A, pp 480–85, McGraw-Hill Book, New York.
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*, 1269–93.
Stillman, M. J., Zelazowski, A. J., & Gasyna, Z. (1988) *FEBS Lett. 240*, 159–62.
Thrower, A. R., Byrd, J., Tarbet, E. B., Mehra, R. K., Hamer, D. H., & Winge, D. R. (1988) *J. Biol. Chem. 263*, 7037–42.
Wagner, G., Braun, W., Havel, T. F., Schaumann, T., Gō, N., & Wüthrich, K. (1987) *J. Mol. Biol. 196*, 611–39.
Weser, H., & Hartmann, H.-J. (1988) *Biochim. Biophys. Acta 953*, 1–5.
Winge, D. R. (1987) in *Proceedings of the 2nd International Meeting on Metallothioneins and other Low Molecular Weight Metal-Binding Proteins*, Zürich, *Aug 21–25, 1985* (Kägi, J. H. R., & Kojima, Y., Eds.) Vol. 52, pp 213–18, Birkhaüser Verlag, Basel.
Winge, D. R., Nielsen, K. B., Gray, W. R., & Hamer, D. H. (1985) *J. Biol. Chem. 260*, 14464–70.
Wright, C. F., Hamer, D. H., & McKenney, K. (1986) *Nucleic Acids Res. 14*, 8489–99.
Wright, C. F., Hamer, D. H., & McKenney, K. (1988) *J. Biol. Chem. 263*, 1570–74.
Wüthrich, K. (1986) *NMR of Proteins and Nucleic Acids*, Wiley, New York.
Zelazowski, A. J., Gasyna, Z., & Stillman, M. J. (1989) *J. Biol. Chem. 264*, 17091–99.
Zhou, P., & Thiele, D. J. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 6112–16.
Zuiderweg, E. R. P., Hallenga, K., & Olejniczak, E. T. (1986) *J. Magn. Reson. 70*, 336–43.