laxation delays, and filtered identically, depends on the ratio of the two bandwidths.[20] If the audio filter cutoffs are set at the Nyquist frequency, that ratio becomes the ratio of the acquisition times (t_2^{max}). The relative sensitivity is approximately proportional to the ratio of the acquisition times divided by the ratio of the delays between pulses.[20] When the sweep width is reduced while acquiring the same number of sample points, the acquisition time increases from 102.4 to 256 ms; however, the delay between pulses, ~2.5 s, does not change significantly. Consequently, the sensitivity is improved by the square root of the ratio of acquisition times, $(256/102.4)^{1/2} \sim 60\%$. Intuitively, the signal/noise ratio improves because the noise bandwidth is reduced; the sensitivity increases because a larger portion of the spectrometer time is spent acquiring data rather than waiting for the spins to relax.

Acknowledgment. It is a pleasure to thank Mr. David Koh for synthesizing DNA templates and Ms. Barbara Dengler for managing the laboratory. This research was supported in part by the National Institutes of Health Grant GM 10840, the Department of Energy, Office of Energy Research, Office of Health and Environmental Research Grant DE-FG03-86ER60406, and through instrumentation grants from the U. S. Department of Energy, DE-FG05-86ER75281, and the National Science Foundation, DMB 86-09305 and BBS 86-20134.

Establishment of the Metal-to-Cysteine Connectivities in Silver-Substituted Yeast Metallothionein

Surinder S. Narula,[†] Rajesh K. Mehra,[§] Dennis R. Winge,[§] and Ian M. Armitage*[,†,‖]

Contribution from the Department of Molecular Biophysics and Biochemistry and Department of Diagnostic Radiology, Yale University School of Medicine, 333 Cedar Street, New Haven, Connecticut 06510, and Department of Biochemistry and Medicine, University of Utah Medical Center, Salt Lake City, Utah 84132. *Received April 26, 1991*

Abstract: To elucidate the three-dimensional solution structure of yeast copper-metallothionein (MT) from *Saccharomyces cerevisiae*, silver-substituted yeast metallothionein has been prepared as an isomorphic, NMR active, metal ion derivative. Using Ag-MT, $^{1}H-^{109}Ag$ heteronuclear multiple quantum coherence transfer (HMQC) experiments have been performed to identify the individual ^{109}Ag resonances and their associated cysteine ligands, respectively. Specific factors associated with the optimized execution of the $^{1}H-^{109}Ag$ HMQC experiments are identified and discussed. Analysis of the HMQC data has established the specific connectivities between 10 of the 12 cysteine residues and seven bound $^{109}Ag(I)$ metal ions. The data confirm the exclusive involvement of cysteine thiolates in metal coordination and indicate that a minimum of eight cysteines are involved as bridging, shared ligands. Additionally, the present data suggest the existence of a mixed coordination number (2 and 3) for the seven bound Ag(I) ions.

Introduction

The metallothioneins (MTs) are small, cysteine-rich polypeptides 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 the several different isoform genes that are frequently present in a single organism.

The yeast *Saccharomyces cerevisiae* contains a metallothionein encoded by the *CUP1* locus.[1-3] In most strains of this yeast, the *CUP1* gene is transcriptionally regulated by only Cu(I) and Ag(I) ions.[4] One strain of *S. cerevisiae* has been shown to regulate the *CUP1* gene expression by Cd(II) as well as Cu(I).[5] The *CUP1* locus functions in copper detoxification by regulating the free Cu(I) ion concentration within the cells. Disruption of the *CUP1* MT gene results in hypersensitivity to copper-mediated cytotoxicity.[6] Detoxification is achieved by sequestration of copper ions in a stable complex with MT.

The 12 cysteine residues in yeast MT are all believed to serve as ligands for the eight bound copper ions.[7,8] Luminescence studies revealed that the copper ions are in the Cu(I) valence state and are bound in a solvent-inaccessible environment.[8] The Cu(I) state was confirmed by X-ray absorption edge spectroscopy (EXAFS).[9] The X-ray absorption edge features were similar to those observed for three coordinate Cu(I) model complexes.[9,10] These observations led to a proposed structure of a distorted cubic Cu_8S_{12} polynuclear cluster in which each Cu(I) ion is trigonally coordinated and each cysteinyl thiolate bridges two Cu(I) ions.[9] No folding intermediates were observed in the Cu(I) reconstitution studies analogous to the domains of mammalian MT. Reconstitution studies confirmed that Cu(I) ions bind to MT in a cooperative, all-or-none manner but were not able to confirm the existence of a single polynuclear cluster.[8]

Although the primary sequence of yeast MT is only distantly related to mammalian MTs, its metal coordination properties are similar. Both classes of MTs bind copper as Cu(I) in clusters with

* Address correspondence to this author at the Department of Molecular Biophysics and Biochemistry, Yale University School of Medicine, 333 Cedar Street, P.O. Box 3333, New Haven, CT 06510-8024.
[†] Department of Molecular Biophysics and Biochemistry, Yale University.
[§] Department of Biochemistry and Medicine, University of Utah.
[‖] Department of Diagnostic Radiology, Yale University.

(1) Fogel, S.; Welch, J. W. *Proc. Natl. Acad. Sci. U.S.A.* **1982**, *79*, 5342-5346.
(2) Karin, M.; Najarian, R.; Haslinger, A.; Valenzuela, P.; Welch, J.; Fogel, S. *Proc. Natl. Acad. Sci. U.S.A.* **1984**, *81*, 337-341.
(3) Butt, T. R.; Sternberg, E. J.; Gorman, J. A.; Clark, P.; Hamer, D.; Rosenberg, M.; Crooke, S. T. *Proc. Natl. Acad. Sci. U.S.A.* **1984**, *81*, 3332-3336.
(4) Wright, C. F.; Hamer, D. H.; McKenney, K. *J. Biol. Chem.* **1988**, *263*, 1570-1574.
(5) Inouhe, M.; Hiyama, M.; Tohoyama, H.; Joho, M.; Murayama, T. *Biochem. Biophys. Acta* **1989**, *993*, 51-55.
(6) Hamer, D. H.; Thiele, D. J.; Lemontt, J. E. *Science* **1985**, *228*, 685-690.
(7) Winge, D. R.; Nielsen, K. B.; Gray, W. R.; Hamer, D. H. *J. Biol. Chem.* **1985**, *260*, 14464-14470.
(8) Byrd, J.; Berger, R. M.; McMillin, D. R.; Wright, C. F.; Hamer, D. H.; Winge, D. R. *J. Biol. Chem.* **1988**, *263*, 6688-6694.
(9) George, G. N.; Byrd, J.; Winge, D. R. *J. Biol. Chem.* **1988**, *263*, 8199-8203.
(10) Kau, L-S.; Spira-Solomon, D. J.; Penner-Hahn, J. E.; Hodgson, K. O.; Solomon, E. I. *J. Am. Chem. Soc.* **1987**, *109*, 6433-6442.

0002-7863/91/1513-9354$02.50/0 © 1991 American Chemical Society

a proposed trigonal metal ion coordination geometry.[11] The solution structure of mammalian Cd_7–MT has been elucidated from ^{113}Cd and ^1H NMR data;[12-14] however, similar structural information on yeast Cu–MT is not yet available. The determination of the 3-D solution structure of Cu–MT from *Neurospora crassa* by 2D NMR methods has been hampered by the scarcity of long range ^1H–^1H NOEs.[15] The deficiency of these structural constraints can be partially circumvented by establishing specific metal-to-cysteine connectivities through scalar coupling. However, Cu–MT is unsuitable for such a study due to the quadrupole moment of Cu ($I = ^5/_2$) and its insensitivity to NMR detection. This limitation can be overcome by the use of the Ag(I)-substituted form of MT as both of the abundant Ag isotopes (107 and 109) are NMR active and have a nuclear spin $I = ^1/_2$. More importantly, Ag(I) and Cu(I) exhibit similar coordination chemistry,[16,17] and Ag(I) appears to be an isomorphic replacement for Cu(I) in MTs as evidenced by their identical binding stoichiometries.[11]

Experimental Procedures

Materials. *S. cerevisiae* strain 2186 was grown in synthetic complete medium in the presence of 1 mM $CuSO_4$. The cells were collected by centrifugation from the stationary cultures, and the cell extract was prepared with an X-Press. The soluble protein fraction was initially chromatographed on Sephadex G-75 (5 × 100 cm), equilibrated with 10 mM Tris-HCl (pH 7.4) containing 0.1% β-mercaptoethanol. MT-containing fractions were identified by copper analysis with atomic absorption spectroscopy. These fractions were pooled and applied directly to a column of Whatman DE-52 (2.5 × 5 cm) equilibrated with the same buffer. The resin was washed with two column volumes of the starting buffer, and elution was accomplished with a linear gradient of 0 to 0.2 M KCl in the starting buffer. MT eluted in fractions with a conductivity near 3 $m\Omega^{-1}$ under these elution conditions. Fractions from ion exchange chromatography were pooled and lyophilized. The concentrated sample was chromatographed on Sephadex G-50 (2.5 × 100 cm), equilibrated with 10 mM potassium phosphate, pH 6. The K_{av} of MT elution on Sephadex G-50 is 0.47. The MT fractions were concentrated by lyophilization and incubated with 0.2 M KCN in 10 mM Tris-HCl (pH 7.4) for 1–2 h. Copper cyanide was separated from apoprotein on Sephadex G-25 (1.5 × 25 cm) equilibrated in the abovementioned Tris buffer. The apo-MT was identified by monitoring fractions for absorbance at 214 nm. These fractions were lyophilized and subsequently incubated in the Tris buffer containing 6 M guanidinium chloride and 0.1 M DTT. After an overnight incubation, the reduced sample was desalted on Sephadex G-25 equilibrated with 0.1% trifluoroacetic acid. The concentration of apo-MT was verified by quantitative amino acid analysis following overnight hydrolysis in 5.7 N HCl at 110 °C. The extent of cysteine reduction was determined by thiol titrations with dithiodipyridine.[18]

Apometallothionein was reconstituted with Ag(I) by the addition of 7.5 mol equiv of silver acetate in 0.1% trifluoroacetic acid. The solution pH was neutralized with potassium phosphate to a final pH of 6.5. The protein solution was exchanged with 99.8% ^2H_2O using YM2 ultrafiltration under argon pressure. The final protein NMR concentration was 6 mM in 18 mM phosphate buffer (p^2H 6.5). The solution was changed to 90% H_2O and 10% ^2H_2O for the experiments in which H_α-to-NH connectivities were sought.

NMR Methods. The pulse sequence and experimental setup for the heteronuclear correlation through the indirect detection of X-nuclei has been previously described.[19-22] These experiments have been successfully applied to the indirect detection of nuclei such as ^{31}P, ^{15}N, and ^{113}Cd.[23-25] The successful application of these heteronuclear multiple quantum coherence methods (HMQC) to Ag–MT critically depended on the following factors:[26] (a) A knowledge of the magnitude of the ^1H–^{109}Ag coupling constant. Cysteine $H_\beta H_{\beta'}$ cross-peaks in the 2D ^1H–^1H double quantum filtered correlation spectrum (DQF-COSY) of yeast Ag–MT do not show the characteristic splitting arising from X-nuclei coupling, but they do exhibit additional broadening. A peak-to-peak separation of 17–32 Hz is measured, which is the sum of all of the active and passive couplings. The observed ranges of proton coupling constants for $^3J_{\alpha-\beta}$ and $^2J_{\beta-\beta'}$ are 3–9 and 12–15 Hz, respectively, which suggest that the additional separation arises from couplings to one or more Ag(I) ions. The small value of this coupling (<10 Hz), therefore, requires a longer time (>50 ms) for the preparation and refocussing intervals for maximum sensitivity in the HMQC experiments, and, unfortunately, the inherently short T_2 values result in significant signal decay during these long intervals with the consequent reduction in the sensitivity of the experiment.

(b) Owing to the large Ag chemical shift range, >1300 ppm,[27] the effective bandwidth of the transmitter pulse may present a limitation in the absence of a priori knowledge of the Ag resonance frequency. Unfortunately, due to the very low sensitivity for direct ^{109}Ag (or ^{107}Ag) detection,[28] this information could not be obtained from a normal 1D ^{109}Ag NMR spectrum on a 6 mM Ag–MT sample.

Even though 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. HMQC experiments were carried out on a Bruker AM500 NMR spectrometer where the resonance frequency for ^{109}Ag is 23.276 MHz. A reverse probe was used for these experiments which had a 90° pulse length for ^{109}Ag of 30 μs. HMQC experiments were acquired in the following three different forms: (i) half-HMQC, acquiring the ^1H free induction decay (FID) without any refocussing delay where the signals are *antiphase*; (ii) full-HMQC, acquiring the ^1H FID after a refocussing interval equal to the preparation interval which results in *in-phase* signals; and (iii) relay-HMQC, acquiring the ^1H FID after applying a 90° ^1H pulse at the end of the refocussing delay. This experiment enables the correlation of cysteine H_α's to specific Ag chemical shifts.

Experiments with different preparation intervals were acquired to optimize for the range of couplings present in Ag–MT. Quadrature detection was used in both dimensions, and between 44 and 128 t_1 values were acquired. Depending on the type of HMQC experiment, 256 to 2000 scans were acquired for each t_1 value. Each FID consisted of 1024 complex data points with an acquisition time of 0.2 s. The time domain signals were multiplied by a shifted sine bell of $\pi/4$ in both the ω_1 and ω_2 domains before Fourier transformation. For the half-HMQC data sets, the time domain signals along ω_1 and ω_2 were multiplied by a shifted sine bell of $\pi/4$ and zero degrees, respectively. The 2D ^1H–^1H DQF-COSY experiment was recorded with 800 t_1 values from 1 μs to 44 ms with 96 scans for each t_1 value. Each FID consisted of 2048 complex data points, with an acquisition time of 0.45 s. Water saturation was used only during the relaxation delay. Shifted sine bells of $\pi/12$ and 0 degrees were used along the ω_1 and ω_2 domains, respectively. Clean TOCSY (HOHAHA) (total correlation spectroscopy) was recorded with 512 t_1 values with 96 scans each, and a spin lock interval of 62 ms using the MLEV-17 sequence and Z-filters.[29] Shifted sine bells of $\pi/8$ and $\pi/32$

(11) Winge, D. R. In *Proceedings of the 2nd International Meeting on Metallothioneins and other Low Molecular Weight Metal-Binding Proteins*, Zürich, August 21–24, 1985; Kägi, J. H. R., Kojima, Y., Eds.; Birkhäuser Verlag: Basel, 1987; Vol. 52, pp 213–218.
(12) Otvos, J. D.; Armitage, I. M. *Proc. Natl. Acad. Sci. U.S.A.* **1980**, *77*, 7094–7098.
(13) Arseniev, A.; Schultze, P.; Wörgötter, E.; Braun, W.; Wagner, G.; Vašák, M.; Kägi, J. H. R.; Wüthrich, K. *J. Mol. Biol.* **1988**, *201*, 637–657.
(14) Schultze, P.; Wörgötter, E.; Braun, W.; Wagner, G.; Vašák, M.; Kägi, J. H. R.; Wüthrich, K. *J. Mol. Biol.* **1988**, *203*, 251–268.
(15) Malikayil, J. A.; Lerch, K.; Armitage, I. M. *Biochemistry* **1989**, *28*, 2991–2995.
(16) Hathaway, B. J. In *Comprehensive Coordination Chemistry (Vol. 5): (Late Transition Elements) The Synthesis, Reactions, Properties and Applications of Coordination Compounds*; Wilkinson, G., Gillard, R. D., McCleverty, J. A., Eds.; Pergammon Press: Oxford, 1987; Vol. 5, Chapter 53.
(17) Lancashire, R. J. In *Comprehensive Coordination Chemistry (Vol. 5): (Late Transition Elements) The Synthesis, Reactions, Properties and Applications of Coordination Compounds*; Wilkinson, G., Gillard, R. D., McCleverty, J. A., Eds.; Pergammon Press: Oxford, 1987; Vol. 5, Chapter 54.
(18) Grassetti, D. R.; Murray, J. F., Jr. *Arch. Biochem. Biophys.* **1967**, *119*, 41–49.
(19) Müller, L. *J. Am. Chem. Soc.* **1979**, *101*, 4481–4484.
(20) Bax, A.; Griffey, R. H.; Hawkins, B. L. *J. Am. Chem. Soc.* **1983**, *105*, 7188–7190.
(21) Bax, A.; Griffey, R. H.; Hawkins, B. L. *J. Magn. Reson.* **1983**, *55*, 301–315.
(22) Bendall, M. R.; Pegg, D. T.; Doddrell, D. M. *J. Magn. Reson.* **1983**, *52*, 81–117.
(23) Live, D. H.; Davis, D. G.; Agosta, W. C.; Cowburn, D. *J. Am. Chem. Soc.* **1984**, *106*, 6104–6105.
(24) Frey, M. H.; Wagner, G.; Vašák, M.; Sørensen, O. W.; Neuhaus, D.; Wörgötter, E.; Kägi, J. H. R.; Ernst, R. R.; Wüthrich, K. *J. Am. Chem. Soc.* **1985**, *107*, 6847–6851.
(25) Sklenar, V.; Mujashiro, H.; Zon, G.; Miles, H. T.; Bax, A. *FEBS Lett.* **1986**, *208*, 94–98.
(26) Ernst, R. R.; Bodenhausen, G.; Wokaun, A. In *Principles of NMR in One and Two Dimensions*; Breslow, R., Goodenough, J. B., Halpern, J., Rowlinson, J. S., Eds.; Clarendon Press: Oxford, 1987; pp 467–485.
(27) Endo, K.; Yamamoto, K.; Matsushita, K.; Deguchi, K.; Kanda, K.; Nakatsuji, H. *J. Magn. Reson.* **1985**, *65*, 268–281.
(28) Pople, J. A.; Schneider, W. G.; Bernstein, H. J. In *High-Resolution NMR*; Hume, D. N., King, E. L., Stork, G., Herschbach, D. R., Pople, J. A., Eds.; McGraw Hill Book Co., Inc.: New York, 1959; Appendix A, pp 481–485.

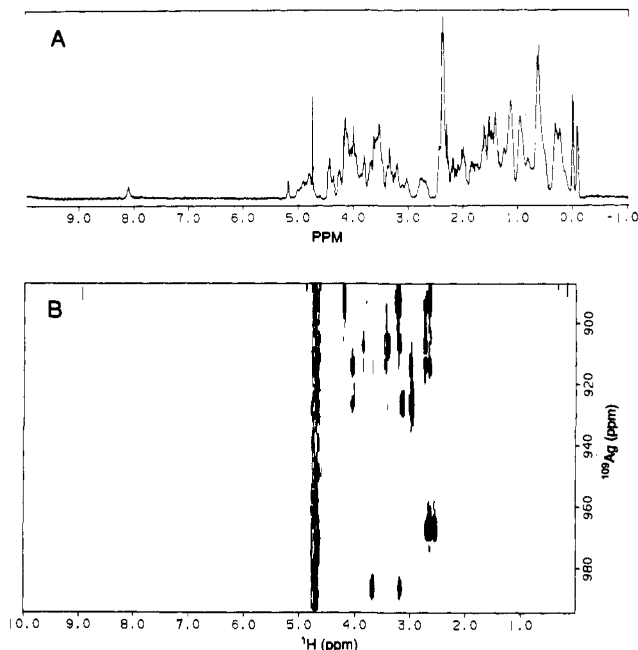

Figure 1. Heteronuclear ^1H–^{109}Ag multiple quantum coherence spectrum of yeast Ag-MT acquired *antiphase* on a Bruker AM500 NMR instrument. The preparation period ($1/2J$) of 50 ms (J = 10 Hz) was used, and 44 t_1 data points were acquired. Both positive and negative levels are plotted without distinction. Many ^{109}Ag resonances are folded along ω_1 in this particular data set. The concentration of the protein was 6 mM in 18 mM phosphate buffer in 99.8% ^2H_2O (p^{2}H 6.5), and the data were acquired at 298 K. At the top is shown a one-dimensional ^1H NMR spectrum. Cross-peaks are seen only in the cysteine H_β chemical shift region.

degrees were used along ω_1 and ω_2, respectively, before Fourier transformation.

For ^{109}Ag, the resonance from a 1.0 M $AgNO_3$ solution was used as an external reference at 0.0 ppm. For ^1H, the residual HDO peak was used as an internal reference at 4.75 ppm at 303 K and 5.02 ppm at 283 K. The most upfield resonance, which is assigned to the γ-CH_3 of T28, is calibrated with respect to the HDO resonance at 303 K and thereafter used as the reference for other temperatures.

Results and Discussion

The success in the application of the HMQC experiment on yeast Ag–MT in spite of the small value of the heteronuclear couplings and a limited knowledge of the ^{109}Ag chemical shift parameters can be judged from Figure 1. This figure shows the results of a ^1H-^{109}Ag HMQC experiment, acquired *antiphase* with a preparation period of 50 ms (J = 10 Hz) on yeast Ag–MT at 298 K. The spectrum shows cross-peaks only for those protons that are coupled to ^{109}Ag. All other proton peaks show very good cancellation except the inevitable HDO line. This spectrum also indicates that the histidine side chain is not involved in binding to the Ag(I) ions, as there are no HMQC cross-peaks at the resonance positions of the histidine side chain protons which have assigned chemical shifts of 8.79 and 7.36 ppm, respectively. This first HMQC spectrum acquired on Ag–MT had many resonances folded along the ω_1 axis due to the nonoptimized value of the spectral offset for ^{109}Ag. All of the cross-peaks in Figure 1 are shown on an expanded scale in Figure 2, which shows six distinct, albeit with several foldovers, ^{109}Ag resonances along ω_1. Due to the *antiphase* nature of these cross-peaks (along ω_2), the intensity of some of these peaks is significantly reduced, and in certain situations there is almost complete cancellation. To alleviate this problem, full-HMQC data sets were acquired where all of the cross-peaks are *in-phase*, even though the sensitivity is reduced in this experiment due to the introduction of a refocussing delay (data not shown).

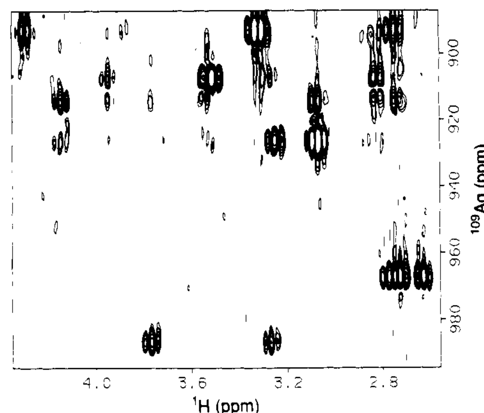

Figure 2. Expanded portion of the ^1H–^{109}Ag HMQC spectrum shown in Figure 1 permitting the identification of six ^{109}Ag resonances along the ω_1 axis.

A ^1H–^{109}Ag relay-HMQC experiment acquired with a preparation period of 30 ms (J = 16.6 Hz) is shown in Figure 3. This data set was acquired at a different spectral offset for ^{109}Ag to eliminate the foldovers observed in Figure 1. Seven well-resolved ^{109}Ag resonances can now be detected, and these are labelled **I** through VII according to their increasing chemical shifts. The foldover of resonance VI was obscured in the half-HMQC spectrum shown in Figure 1 because of its coincidental overlap with the foldover for resonance I. Also detected in this experiment are some of the cysteine H_α protons. With an even longer preparation period (45 ms), this experiment resulted in a significant loss in sensitivity (data not shown); however, a few additional peaks, which are marked by blank circles in Figure 3, were observed, indicative of the smaller values of the ^1H-to-^{109}Ag coupling constants.

At present, the coordination number for silver in this protein is uncertain; however, Cu EXAFS studies on Cu(I)–MT suggest this number to be three.[9] Ideally, the number of cysteines coordinating each Ag(I) ion can be obtained from the full analysis of the HMQC cross-peak pattern. However, the almost certain difference in the magnitude of the ^{109}Ag coupling to the two H_β protons suggests caution at this stage of our investigation. It is essential, therefore, that all of the cysteine spin systems be identified, and for this purpose, 2D ^1H DQF-COSY (Figure 4) and TOCSY NMR experiments were performed. The analysis of this data has been done at two different temperatures to remove the coincidental degeneracy in the chemical shifts of some H_β protons and shift the water resonance. A portion of the DQF-COSY spectrum is shown in Figure 4. The objective was to identify a cross-peak in the DQF-COSY spectrum which corresponded to the observed cross-peaks in the HMQC experiments. The sequential assignment of these cysteine residues has subsequently been accomplished through the use of 2D ^1H nuclear Overhauser spectroscopy using established protocols.[30] Of particular note in these extended 2D ^1H NMR studies was the observation of a doubling of the resonances for most of the amino acids following N40 in the C-terminus. A descriptive model for these conformational substates has not yet been deduced. For the study reported herein, it suffices to indicate that this exchange phenomenon has precluded the sequential assignment of two residues in the protein, C49 and C50 (Table I). Surprisingly, no cross-peaks were observed in the HMQC data sets to either of the C-terminal cysteine residues (49 and 50), providing the first suggestion that they may not participate in metal ligation. Interestingly, such a situation would lend support to the observation of no differences in the properties/stoichiometry of the metal clusters between the wild type and a truncated mutant form of the protein lacking the two C-terminal cysteine residues.[31] The

(29) Griesinger, C.; Otting, G.; Wüthrich, K.; Ernst, R. R. *J. Am. Chem. Soc.* **1988**, *110*, 7870–72.

(30) Wüthrich, K. In *NMR of Proteins and Nucleic Acids*; John Wiley & Sons, Inc.: New York, 1986; pp 130–161.

(31) Thrower, A. R.; Byrd, J.; Tarbet, E. B.; Mehra, R. K.; Hamer, D. H.; Winge, D. R. *J. Biol. Chem.* **1988**, *263*, 7037–7042.

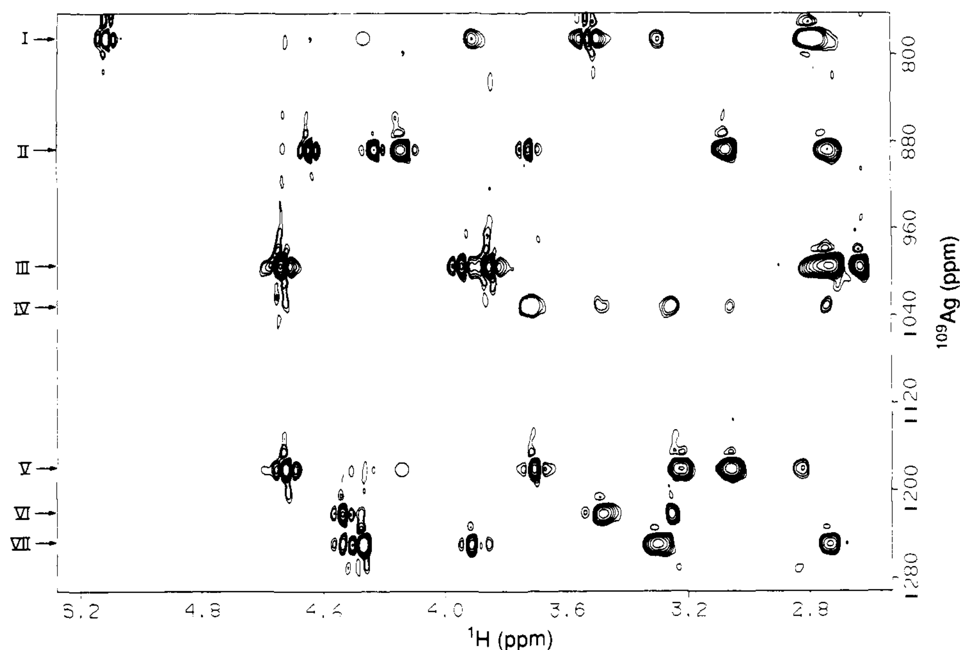

Figure 3. $^1H-^{109}Ag$ HMQC spectrum of yeast Ag-MT acquired with an additional 90° ^1H pulse applied just after the refocussing interval to transfer magnetization to the H_α protons. For this data set, 94 t_1 values were acquired at $T = 308$ K using a preparation period of 30 ms ($J = 16.6$ Hz). The additional cross-peaks in this spectrum result from both the extra pulse on the proton channel and from the use of a different preparation period. The seven ^{109}Ag resonances are marked by Roman numerals according to their increasing chemical shifts. The blank circles indicate the position of observed cross peaks in a similar experiment acquired with a preparation period of 45 ms ($J = 11.1$ Hz).

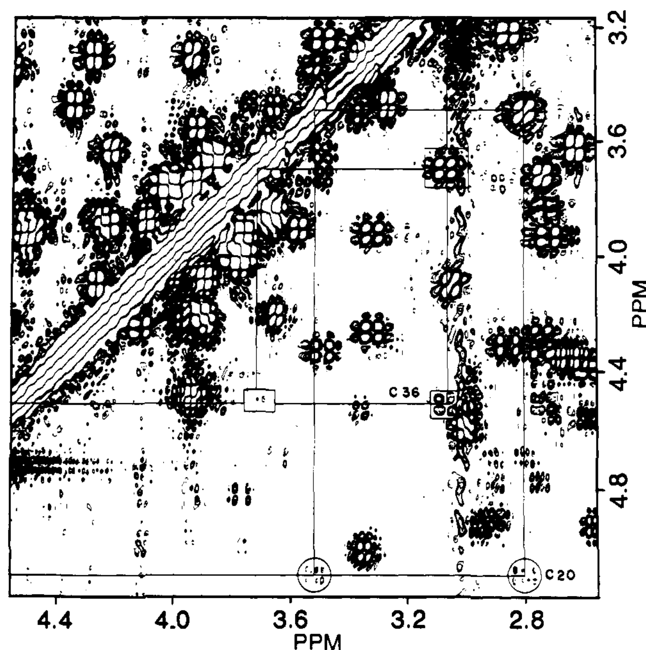

Figure 4. 2D $^1H-^1H$ DQF-COSY spectrum of yeast Ag-MT at 303 K. As a representative example, constructs are drawn for side chain spin systems of C20 and C36. The protein concentration for this experiment was 6 mM in 90% H_2O in 18 mM phosphate buffer (pH 6.5).

^1H and ^{109}Ag chemical shift data are tabulated in Tables I and II, respectively, and a schematic representation of the Ag(I)-Cys coordination deduced from the HMQC experiments is shown in Figure 5.

The unusually large range of the ^{109}Ag chemical shifts, 460 ppm (Table II), for a protein where Ag(I) ligation is exclusively through cysteine thiolates is suggestive of the presence of mixed Ag(I) coordination numbers.[27,32-34] Unfortunately, no ^{109}Ag NMR chemical shift data are available for 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 versus terminal thiolates. Mixed coordination number (2 and 3) complexes, however, have been reported for both Cu(I) and Ag(I).[10,35,36] The

Table I. ^1H Chemical Shift Data (ppm) of Cysteine Residues in Yeast Ag-MT at pH 6.5 and 303 K

no.	NH	H_α	H_β, $H_{\beta'}$
C7	8.55	4.28	3.31, 3.92
C9	8.24	4.35	2.73, 4.31
C11	8.11	4.44	2.73, 3.73
C14	7.14	4.36	3.26, 3.51
C20	7.50	5.12	2.80, 3.52
C24	8.55	4.53	2.73, 3.86
C26	9.29	4.32	2.85, 3.24
C30	7.62	4.27	3.07, 4.12
C36	7.23	4.49	3.08, 3.72
C38	8.20	3.95	2.64, 2.71
C49/C50[a]	7.84	4.50	3.10, 3.26
C49/C50[a]	7.97	4.38	2.95, 3.06

[a] Two additional cysteine spin systems have been found in the 2D ^1H NMR data sets which have not yet been identified sequentially. No cross-peaks are observed to these resonances in the HMQC experiments and they have been tentatively assigned to the remaining two cysteines (49 and 50) in the C-terminus.

Table II. ^{109}Ag Chemical Shift Data (ppm) for Yeast Ag-MT at 303 K[a]

metal no.	chemical shift (ppm)	metal no.	chemical shift (ppm)
I	790	V	1183
II	890	VI	1220
III	1000	VII	1250
IV	1037		

[a] The data are referenced to an external silver nitrate sample (1.0 M).

(32) Haberkorn, R. A.; Que, L., Jr.; Gillum, W. O.; Holm, R. H.; Liu, C. S.; Lord, R. C. *Inorg. Chem.* **1976**, *15*, 2408-2414.
(33) Henrichs, P. M. In *NMR of Newly Accessible Nuclei*; Laszlo, P., Ed.; Academic Press: New York, 1983; Vol. II, pp 319-336.
(34) Lacelle, S.; Stevens, W. C.; Kurtz, D. M., Jr.; Richardson, J. W., Jr.; Jacobson, R. A. *Inorg. Chem.* **1984**, *23*, 930-935.
(35) Dance, I. G. *J. Chem. Soc., Chem. Commun.* **1976**, 103-104.

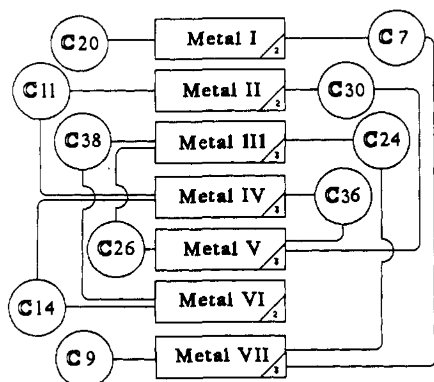

Figure 5. A schematic representation of the metal-to-cysteine connectivities. Metals (Ag(I)) are numbered by their increasing ^{109}Ag chemical shift and are denoted by Roman numerals (Figure 3). Cysteines are labeled by their primary sequence numbers, and in the right lower corner of each rectangle, the coordination number of each Ag(I) ion is indicated.

dependence of the magnitude of the multiple quantum coherence in the HMQC experiment on the preparation period, $(1/2J)$, nevertheless, cautions against excluding the possibility that the two coordinate Ag(I) sites identified in Figure 5 may be missing their connectivities to a third cysteine due to a very small (<5 Hz) heteronuclear coupling constant. This uncertainty is unfortunately not resolved by the near-equivalence in the H_β proton chemical shifts for four cysteines (C9, C11, C24, C38), Table I, which allowed for the unequivocal identification of only three (C11, C24, C38) as bridging cysteines on the basis of observed connectivities to both of their H_β protons. Thus, to account for the large Ag(I) chemical shift dispersion (460 ppm) in Ag-MT, our present model invokes a mixed Ag(I) coordination number (2 and 3) involving 10 of the 12 cysteine thiols in this protein, with eight participating as bridging ligands and two as terminal ligands.

The observation of only seven Ag(I) resonances for the reconstituted Ag-MT protein is at odds with the reported metal-binding stoichiometry of eight for the Ag(I)- or native Cu(I)-MT.[7,8] It is unlikely that this results from the fact that slightly less (7.5 mol equiv) than the reported 8 mol equiv of required metal ion was intentionally added to the apoprotein to purposely avoid the possible aggregation which excess metal ions have been shown to initiate in the mammalian forms of this protein.[37] With the reported cooperative nature of metal ion binding,[8] 93% of the sample would still contain its full complement of eight metal ions. Other possible explanations such as degeneracy of two Ag(I) resonances and limitations in the size of the ^{109}Ag(I) spectral window, which covered the range of 545 to 1551 ppm, seem equally unlikely. The possibility remains, nevertheless, that the eighth resonance is exchange-broadened, and one is reminded of the fact that no cross-peaks were observed in the HMQC data sets to the H_β protons from the two C-terminal cysteine residues (C49 and C50).

Our current model, therefore, suggests that the factors affecting the chemical shifts of the seven Ag(I) thiolate resonances in Ag-MT includes both (a) coordination number of the metal ions[27,32,33] and (b) the bond distances, bond angle, and number of bridging versus terminal thiolates.[34] These metal-to-cysteine connectivities (Figure 5) will provide crucial constraints for the determination of the three-dimensional solution structure of Ag-MT by NMR methods that is currently in progress. Also planned are HMQC experiments and the full 3D solution structural NMR studies on the mutant MT which lacks the five C-terminal residues, which include two cysteines. Together, these studies should provide unequivocal determination of the Ag(I) and, therefore, presumably the Cu(I) metal ion stoichiometry and coordination number in MT from *Saccharomyces cerevisiae*.

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

Registry No. cysteine, 52-90-4; copper, 7440-50-8.

(36) Dance, I. G. *Aust. J. Chem.* **1978**, *31*, 2195-2206.

(37) Otvos, J. D.; Engeseth, H. R.; Wehrli, S. *Biochemistry* **1985**, *24*, 6735-40.

Communications to the Editor

Direct Enantioseparation of β-Adrenergic Blockers Using a Chiral Stationary Phase Prepared by Molecular Imprinting

Lutz Fischer,† Ralf Müller,† Björn Ekberg,‡ and Klaus Mosbach*,†

Department of Pure and Applied Biochemistry University of Lund, P.O. Box 124, 22100 Lund, Sweden BIO-SWEDE AB, IDEON Sölvegatan 41, 22100 Lund, Sweden

Received May 13, 1991

β-Adrenergic blocking agents, or β-blockers, are important drugs used for the treatment of hypertension, arrhythmia, and angina pectoris. There is a strong need to apply optically pure enantiomers since the stereoisomers express varying pharmacological activity and, in some cases, can even be used against different symptoms.[1] Thus, there is intense ongoing research in the preparation of optically pure β-blockers such as the use of asymmetrical synthesis[2] (including biocatalysts[3]), fractionated crystallization,[4] and indirect[5] or direct[6] chromatographic separation of the enantiomeric forms. In this communication we describe,

† University of Lund.
‡ BIO-SWEDE, IDEON.

(1) (a) Richards, R.; Tattersfield, A. E. *Br. J. Clin. Pharmacol.* **1987**, *24*, 485-491. (b) Kloosterman, M.; Elferink, V. H. M.; van Iersel, J.; Meijer, E. M.; Hulshof, L. A.; Sheldon, R. A. *Trends Biotechnol.* **1988**, *6*, 251-256. (c) Walle, T.; Webb, J.; Bagwell, E.; Walle, K.; Daniell, H.; Gaffney, T. *Biochem. Pharmacol.* **1988**, *37*, 115-124. (d) Lindner, W.; Rath, M.; Stoschitzky, K.; Semmelrock, H. J. *Chirality* **1989**, *1*, 10-13.

(2) (a) Katsuki, T. *Tetrahedron Lett.* **1984**, *25*(26), 2821-2822. (b) Klunder, J. M.; Ko, S. Y.; Sharpless, K. B. *J. Org. Chem.* **1986**, *51*, 3710-3712.

(3) (a) Matsuo, N.; Ohno, N. *Tetrahedron Lett.* **1985**, *26*(45), 5533-5534. (b) Phillips, G. T.; Bertola, M. A.; Marx, A. F.; Koger, H. S. *Eur. Pat. Appl. EP 256586*, **1988**, 9 pp.

(4) Oy Star AB. *Neth. Appl. NL 8500939*, 1988, 27 pp.

(5) (a) König, W. A.; Ernst, K. *J. Chromatogr.* **1983**, *280*, 135-141. (b) Isaksson, R.; Lamm, B. J. *J. Chromatogr.* **1986**, *362*, 436-438.

(6) (a) Armstrong, D. W.; Ward, T. J.; Armstrong, R. D.; Beesley, T. E. *Science* **1986**, *232*, 1132-1135. (b) Okamoto, Y.; Aburatami, R.; Hatano, K.; Hatada, K. *J. Liq. Chromatogr.* **1988**, *11*(9-10), 2147-2163. (c) Aboul-Enein; Hassan, Y.; Islam, M. R. *J. Chromatogr.* **1990**, *511*, 109-114. (d) Erlandsson, P.; Marle, I.; Hansson, L.; Isaksson, R.; Pettersson, G.; Pettersson, C. *J. Am. Chem. Soc.* **1990**, *112*, 4573-4574.

0002-7863/91/1513-9358$02.50/0 © 1991 American Chemical Society