The ^{199}Hg Chemical Shift as a Probe of Coordination Environments in Blue Copper Proteins

Lisa M. Utschig,[†] Jeffery G. Wright,[†] Gregg Dieckmann,[‡] Vincent Pecoraro,[‡] and Thomas V. O'Halloran*[,†,§]

Department of Chemistry and Department of Biochemistry, Molecular Biology, and Cellular Biology, Northwestern University, Evanston, Illinois 60208, and Department of Chemistry, University of Michigan, Ann Arbor, Michigan 48109

Received November 4, 1994

The sensitivity of ^{199}Hg chemical shifts to the primary coordination sphere of mercury complexes,[1] coupled with the relative ease of Hg(II) substitution into metalloprotein sites,[2,3] makes ^{199}Hg NMR ($I = {}^1/_2$, natural abundance = 16.8%) a potentially powerful tool for elucidating metal-binding sites in proteins. Insight into the coordination environment can be readily obtained by correlating ^{199}Hg chemical shifts with those of structurally characterized compounds.[1] Despite the applicability of this technique, few ^{199}Hg chemical shifts are available for Hg(II)-substituted proteins.[4] Santos et al. have suggested that the large anisotropies expected for Hg(II)−protein environments with low coordination numbers may excessively broaden the ^{199}Hg NMR line widths.[5] We demonstrate here that spectra with reasonable line widths relative to the ^{199}Hg chemical shift range are readily obtained at high-field strength in a few hours for the distorted coordination sites of two mercuric ion substituted blue copper proteins. The relatively fast ^{199}Hg longitudinal relaxation times (T_1) in these sites allow acquisition of good signal-to-noise spectra in a few hours with 30 mg of protein. Furthermore, comparison of spectra for *Pseudomonas aeruginosa* azurin and spinach plastocyanin indicate that ^{199}Hg chemical shifts are sensitive to subtle variations in the coordination chemistry of type 1 copper sites. The copper environment in each family of blue copper proteins affects the active site reactivity.[6] These NMR results clearly reflect the coordination differences previously observed in the crystallographically characterized metalloproteins and suggest that ^{199}Hg chemical shifts obtained from straightforward solution NMR experiments will be useful for defining a variety of metal coordination sites in other biopolymers.

The best understood mercuric ion−protein complex is Hg(II)-substituted plastocyanin. Freeman and co-workers have solved three-dimensional structures of the Hg(II)-substituted,[7] the apo-,[8] and the native copper form of spinach plastocyanin.[9,10] Few changes in protein structure occur when the copper atom of plastocyanin is replaced by Hg(II).[7] The Hg(II) and the Cu(II) ions both bind in a distorted trigonal geometry with primary bonds to a thiolate S atom of cysteine and to two imidazole N atoms of two histidines. The axial mercuric ion−thioether (methionine) bond at 3.02(5) Å is longer than the sum of the covalent radii for Hg−S (2.50Å),[1] indicating a secondary bonding interaction. Considerable variation is observed in the structure and redox properties of other blue copper proteins.[6] The metal centers in all structurally characterized type 1 sites are coordinated to two His and one Cys; however, the slight displacement of the metal from the N_2S plane toward or away from one or more axial ligands depends upon the specific protein and the nature of the substituted metal.

Addition of 1−2 equiv of ^{199}Hg(II) to the plastocyanin and azurin apoproteins[11] leads to quantitative formation of the mercury derivatives.[3,12] The S→Hg LMCT bands were monitored during the addition, and upon reaching a plateau, the Hg(II) binding was deemed complete. Final metal-to-protein mole ratios, determined by ICP−AES analysis after gel filtration, were 0.9 and 0.8, respectively.[13]

The ^{199}Hg NMR spectra of the ^{199}Hg-substituted proteins are shown in Figure 1. The spectra, obtained using a Bruker 600 spectrometer (14.09 T, 107.4 MHz for ^{199}Hg), exhibit chemical shifts of −749 ppm for ^{199}Hg−plastocyanin and −884 ppm for ^{199}Hg−azurin. The ^{199}Hg chemical shift spans a range of over 5000 ppm[14] and is a function of the number and type of ligand atoms, as well as coordination geometry. Thiolate S atoms form strong bonds to Hg(II) and are highly deshielding,[1] whereas amine N atoms are less deshielding. A HgS_4 environment can be over 1000 ppm more deshielded than a HgN_4 center, as can be seen in solid state ^{199}Hg chemical shifts for the distorted tetrahedral compounds $[N(CH_3)_4]_2[Hg(SC_6H_4Cl)_4]$ at −176 ppm[15] and $[Hg(NH_2CH_2CH_2NH_2)_2][(ClO_4)_2]$ at −1272 ppm.[16] While the data correlating shielding with structurally characterized mercuric complexes containing both S and N ligands are limited, the ^{199}Hg chemical shift of −749 ppm for plastocyanin is within the range expected for a N_2SS' coordination environ-

* Corresponding author: Department of Chemistry, Northwestern University, 2145 Sheridan Road, Evanston, IL 60208-3113.

† Department of Chemistry, Northwestern University.

‡ University of Michigan.

§ Department of Biochemistry, Molecular Biology, and Cellular Biology, Northwestern University.

(1) Wright, J. G.; Natan, M. J.; MacDonnell, F. M.; Ralston, D. M.; O'Halloran, T. V. In *Progress in Inorganic Chemistry*; Lippard, S. J., Ed.; Wiley: New York, 1990; Vol. 38.

(2) Morie-Bebel, M. M.; Morris, M. C.; Menzie, J. L.; McMillin, D. R. *J. Am. Chem. Soc.* **1984**, *106*, 3677.

(3) Utschig, L. M.; Wright, J. G.; O'Halloran, T. V. In *Methods in Enzymology*; Vallee, B., Riordan, J., Ed.; Academic Press: San Diego, CA, 1993; Vol. 226; pp 71−97.

(4) Sudmeier, J. L.; Perkins, T. G. *J. Am. Chem. Soc.* **1977**, *99*, 7732.

(5) Santos, R. A.; Gruff, E. S.; Koch, S. A.; Harbison, G. S. *J. Am. Chem. Soc.* **1991**, *113*, 469.

(6) Solomon, E. I.; Baldwin, M. J.; Lowery, M. D. *Chem. Rev.* **1992**, *92*, 521. Solomon, E. I.; Lowery, M. D. *Science* **1993**, *259*, 1575.

(7) Church, W. B.; Guss, J. M.; Potter, J. J.; Freeman, H. C. *J. Biol. Chem.* **1986**, *261*, 234.

(8) Garrett, T. P. J.; Clingeleffer, D. J.; Guss, J. M.; Rogers, S. J.; Freeman, H. C. *J. Biol. Chem.* **1984**, *259*, 2822.

(9) Guss, J. M.; Freeman, H. C. *J. Mol. Biol.* **1983**, *169*, 521.

(10) Coleman, P. M.; Freeman, H. C.; Guss, J. M.; Murata, M.; Norris, V. A.; Ramshaw, J. A. M.; Venkatappa, M. P. *Nature* **1978**, *272*, 319.

(11) Spinach plastocyanin was isolated using the procedure described by Ellefson et al. *P. aeruginosa* azurin was isolated using a modified procedure of Ambler et al. (See: Ellefson, W. L.; Ulrich, E. A.; Krogmann, D. W. In *Methods in Enzymology*; Academic Press: San Diego, CA, 1980; Vol. 69; pp 225−227. Ambler, R. P.; Wynn, M. *Biochem. J.* **1973**, *131*, 485.) Apoplastocyanin was prepared by following the procedure outline by McMillin et al., and the apoazurin was prepared by the method of Blaszak et al. (See: McMillin, D. R.; Rosenberg, R. C.; Gray, H. B. *Proc. Nat. Acad. Sci. U.S.A.* **1974**, *71*, 4760. Blaszak, J. A.; McMillin, D. R.; Thornton, A. T.; Tennent, D. L. *J. Biol. Chem.* **1983**, *258*, 9886.)

(12) The difference spectrum of Hg−plastocyanin is identical to a previous report: Tamilarasan, R.; McMillin, D. R. *Inorg. Chem.* **1986**, *25*, 2037. The difference spectrum of Hg−azurin exhibits λ_{max} at 246 nm and 290 nm with $\Delta\epsilon \sim 9 \times 10^3$ M^{-1} cm^{-1} and 2×10^3 M^{-1} cm^{-1}, respectively.

(13) Isotopically enriched ^{199}HgO (91.1%, Oak Ridge National Laboratories) was dissolved in concentrated acetic acid and diluted with buffer. After addition of ^{199}Hg(II), free metal ions were separated from Hg−protein complexes on Sephadex G25 using a 10 mM sodium phosphate, pH 7.0 buffer for plastocyanin and a 50 mM ammonium acetate, pH 8.5 buffer for azurin. Protein concentrations were determined on the basis of the published extinction coefficients (See ref 12 and: Tennent, D. L.; McMillin, D. R. *J. Am. Chem. Soc.* **1979**, *101*, 2307.) Protein samples were concentrated to a final volume of 0.5−0.6 mL using Centriprep-10 and Centricon-10 filtration devices.

(14) Wrackmeyer, B.; Contreras, R. In *Annual Reports on NMR Spectroscopy*; Academic Press: San Diego, CA, 1992, Vol. 24; pp 267−321.

0020-1669/95/1334-2497$09.00/0

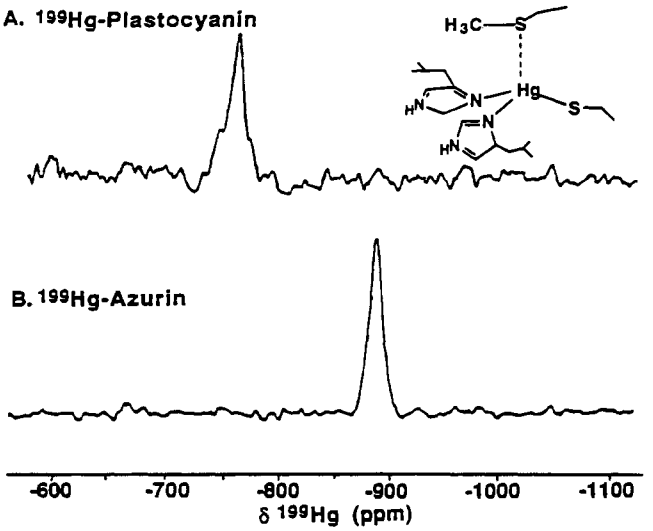

Figure 1. ^{199}Hg NMR spectra at 107.43 MHz of (A) ^{199}Hg−plastocyanin and (B) ^{199}Hg−azurin. Each spectrum was obtained using a broadband tunable 5 mm probe in 3 h with 65 535 scans, a pulse angle of 90°, a sweep width of 125 000 Hz, an acquisition time of 65 ms, and a relaxation delay of 0.1 s. A 500 Hz line broadening function was applied. Spectrum A ($\nu_{1/2}$ = 1600 Hz) was obtained at 10 °C, with Garp ^{1}H decoupling, on a 6.5 mM ^{199}Hg−plastocyanin solution in 10 mM sodium phosphate, pH 7.0. Spectrum B ($\nu_{1/2}$ = 1670 Hz) was obtained at 20 °C, without ^{1}H decoupling, on a 6.2 mM ^{199}Hg−azurin solution in 4 mM ammonium acetate, pH 8.5, 97% D_2O.[29] Chemical shifts are reported relative to $Hg(CH_3)_2$ at 25 °C.

ment. In this regard, the structurally well-defined Hg−plastocyanin complex is an important model compound.

While Hg-L edge EXAFS results for Hg−azurin and Hg−plastocyanin are very similar,[17] comparison of the ^{199}Hg chemical shifts indicates differences between these coordination environments. The upfield 135 ppm shift of ^{199}Hg−azurin relative to −plastocyanin is consistent with a subtly different axial coordination environment that leads to greater shielding. Crystallographic characterization of Cu−azurin indicates a methionine and a carbonyl oxygen from the peptide backbone as distant fourth and fifth ligands to the copper center of the native protein.[18] The crystal structures of Cd- and Zn-substituted azurin reveal a coordination environment similar to the native copper protein; however, the Cd(II) center is displaced away from the methionine and toward the carbonyl oxygen.[19,20] Relative to sulfur and nitrogen, ligation of oxygen atoms generally has a shielding effect on ^{199}Hg chemical shifts.[15] The additional shielding of ^{199}Hg in azurin relative to plastocyanin is consistent with either weak bonding of a carbonyl oxygen to the Hg(II) center in azurin, diminished interaction of the methionine sulfur, or both. In comparison, an upfield shift of only 60 ppm was observed for ^{113}Cd−azurin (*P. aeruginosa*) relative to ^{113}Cd−plastocyanin.[21] The ^{199}Hg chemical shift is clearly more sensitive than ^{113}Cd to subtle differences in metalloprotein coordination environments.

Spin−lattice relaxation times (T_1) are of practical importance in optimizing data acquisition in both 1D and 2D experiments, but no ^{199}Hg T_1 values for a mercury protein have been reported. The T_1 of Hg−azurin was measured using the standard inversion recovery sequence and found to be 13(3) ms at 14.09 T. Similar T_1 values were estimated for Hg−plastocyanin[22] and the binuclear Hg_2S_6 environment of Ga14.[23]

These T_1 values for the ^{199}Hg-substituted blue copper sites are ~10-fold lower than T_1 values for the corresponding ^{113}Cd-substituted proteins; however, the latter were determined at a lower field strength.[21] The relaxation times of the ^{113}Cd−blue copper proteins are much faster than typically found for ^{113}Cd-substituted proteins, where T_1 values range from 0.1 to 8 s, depending on the field strength and temperature.[24,25] Faster acquisition rates in NMR studies of ^{199}Hg proteins can represent a significant advantage over ^{113}Cd in 1D experiments, depending on the protein.[23] These short metal T_1 values do not preclude coherence transfer experiments that establish metal−ligand connectivity such as ^{1}H{^{199}Hg} HMQC[23] and may not be a problem for observing heteroTOCSY spectra.[26]

The rapid relaxation times and broad line widths in these 14.09 T ^{199}Hg-protein spectra are most likely the result of a relaxation mechanism dominated by chemical shift anisotropy (CSA). Solid-state ^{199}Hg NMR experiments of model ^{199}Hg complexes show that these complexes often exhibit large CSA, on the order of 400−7000 ppm.[1,5,15] Nonetheless, the chemical shift anisotropies of the distorted HgN_2SX environments do not excessively broaden ^{199}Hg−protein signals in solution NMR experiments even at high field strength. While narrower lines will be observed at lower field strengths in some ^{199}Hg−protein spectra, experiments at higher field strengths provide additional signal intensity.

As our results indicate, 1D ^{199}Hg NMR spectra of Hg(II)-substituted proteins are observable in a period of a few hours at high field strength and the ^{199}Hg chemical shift obtained accurately reflects subtle differences in metalloprotein environments. This spectroscopic technique could provide useful information about coordination in mercuric ion resistance proteins.[23] Recently, Blake et al. reported relatively large "through-space" ^{1}H−^{199}Hg J couplings[27] and determined the chemical shift of ^{199}Hg-substituted rubredoxin by indirect methods.[28] ^{199}Hg NMR will be useful in exploring the coordination geometry and ligation of a wide variety of copper, zinc, and iron binding sites.

Acknowledgment. This research was supported by a Dreyfus Teacher-Scholar Award and NIH grants (GM38784 and GM45972) to TVO and a NIH Training Grant (GM08061) fellowship for LMU. We thank J. Bryson, S. Watton, D. Kushland, G. Ashley, and D. LeMaster for helpful discussions. The 600 MHz NMR Facility was funded by the W. M. Keck Foundation, the NIH, the NSF, the State of Illinois Technology Challenge Grant Program, and Northwestern University.

IC941278T

(15) Natan, M. J.; Millikan, C. F.; Wright, J. G.; O'Halloran, T. V. *J. Am. Chem. Soc.* **1990**, *112*, 3255.
(16) Bryson, J. W.; O'Halloran, T. V. Unpublished results.
(17) Klemens, A. S.; McMillin, D. R.; Tsang, H.-T.; Penner-Hahn, J. E. *J. Am. Chem. Soc.* **1989**, *111*, 6398.
(18) Baker, E. N. *J. Mol. Biol.* **1988**, *203*, 1071. Adman, E. T.; Jensen, L. H. *Isr. J. Chem.* **1981**, *21*, 8.
(19) Blackwell, K. A.; Anderson, B. F.; Baker, E. N. *Acta Crystallogr.* **1994**, *D50*, 263−270.
(20) Nar, H.; Huber, R.; Messerschmidt, A.; Filippou, A. C.; Barth, M.; Jaquinod, M.; van de Kamp, M.; Canters, G. W. *Eur. J. Biochem.* **1992**, *205*, 1123−1129.
(21) Engeseth, H. R.; McMillin, D. R.; Otvos, J. D. *J. Biol. Chem.* **1984**, *259*, 4822.
(22) At each of the four τ values, 16 384 acquisitions were obtained for Hg−plastocyanin at 10 °C. The T_1 for Hg−azurin was determined for six τ values with 100 000 scans/τ value, with recycle delays of 0.2 s. Data were fit with a nonlinear least-squares method to the two-parameter inversion recovery equation. The T_1 for Hg−plastocyanin was estimated to be ~0.01 s.
(23) Utschig, L. M.; Bryson, J. W.; O'Halloran, T. V. *Science*, in press.
(24) Coleman, J. E. In *Methods in Enzymology*; Vallee, B., Riordan, J., Ed.; Academic Press: San Diego, CA, 1993; Vol. 227; pp 16−43.
(25) Kordel, J.; Johansson, C.; Drakenberg, T. *J. Magn. Reson.* **1992**, *100*, 587.
(26) Gardner, K. H.; Coleman, J. E. *J. Biomol. NMR* **1994**, *4*, 761.
(27) Blake, P. R.; Lee, B.; Summers, M. F.; Adams, M. W. W.; Park, J.-B.; Zhou, Z. H.; Bax, A. *J. Biomolec. NMR* **1992**, *2*, 527.
(28) Blake, P. R.; Lee, B.; Summers, M. F.; Park, J.-B.; Zhou, Z. H.; Adams, M. W. W. *New. J. Chem.* **1994**, *18*, 387.
(29) No difference in line width was observed with or without ^{1}H decoupling.