an enhanced interaction with the solution protons. In particular, the hydrogen-bonding network seen in the crystal structure shows that the water molecules in the outer coordination sphere of the manganese complex strongly hydrogen bond to the phosphate and pyridine nitrogen atoms. As noted in Table IV, six water molecules are less than 5 Å from the manganese center.

Studies are in progress to further assess the imaging utility of this novel chelate and to prepare a toxicity profile to support human studies. The results of these studies will be reported elsewhere.

Acknowledgment. We thank Steven Sheffer and Thomas Garrett for their technical assistance and helpful discussions.

Registry No. 1, 118248-92-3; PLP, 54-47-7; DPDP, 118248-91-2; MnH_2DPDP^{2-}, 118248-93-4; $MnDPDP^{6-}$, 118248-94-5; $ZnDPDP^{6-}$, 118248-95-6; $CuDPDP^{6-}$, 118248-96-7; $FeDPDP^{5-}$, 118248-97-8; $H_2NCH_2CH_2NH_2$, 107-15-3; $BrCH_2CO_2H$, 79-08-3; *N,N'*-dipyridoxylethylenediamine 5,5'-bis(phosphate), 118248-90-1.

Supplementary Material Available: Table IS (crystal and data collection details for $CaNa_2MnDPDP{\cdot}21H_2O$), Table IIS (hydrogen atom positional and thermal parameters), Table IIIS (bond distances and angles—a complete list), Table IVS (intramolecular and intermolecular hydrogen-bond parameters), Table VS (least-squares planes and their equations), Table VIS (anisotropic temperatures factors), and Table VIIS (root-mean-square amplitudes of thermal vibration) (11 pages); Table VIIIS (observed and calculated structure factors) (38 pages). Ordering information is given on any current masthead page.

Contribution from the Department of Chemistry,
San Jose State University, San Jose, California 95192

Evidence for the Binding of Bromide to Mercury in Organomercurial–Protein Complexes Obtained by Using Bromine-81 Magnetic Resonance Spectroscopy

Joseph J. Pesek* and Faridah Abhanan

Received April 19, 1988

Bromine-81 magnetic resonance measurements are used to detect the formation of bromide–organomercurial–protein complexes. When an organomercurial compound is placed in a NaBr solution of various proteins, the bromine-81 magnetic resonance line broadens. This phenomenon can only be observed if the mercury coordination number is at least 3 with the sites being occupied by the organic moiety, the protein, and a bromide. Under these conditions quadrupolar relaxation becomes more efficient as bromide exchanges between the bound and the free states. The complexes are characterized by determining their correlation times at the binding site(s) (segmental motion) as well as the bromide ion-exchange rates. A possible fourth coordination site on mercury can be detected by adding DMSO to a NaBr solution containing the bromide–organomercury–protein complex.

Recently[1] we have reported on mercury coordination numbers greater than 2 for methylmercury and *p*-(chloromercurio)benzoic acid in the presence of 0.1 M NaCl and various proteins. This is unusual since the aqueous coordination chemistry of methylmercury and other organomercurials is usually described in terms of a linear geometry.[2] There are a few examples of methylmercury complexes with mercury coordination numbers greater than 2 such as the $CH_3HgX_2^-$ and $CH_3HgX_3^{2-}$ complexes of iodide[3] and thiocyanate.[3-5] In addition, some chelating agents such as 2,2-bipyridine have also resulted in higher coordination numbers for methylmercury.[6-10] Spectroscopic studies have shown that $CH_3HgCl_2^-$ is formed in a number of nonaqueous solvents.[11,12]

The use of halide ion NMR to study metal–protein complexes has been well established.[13] Mercury was first shown to be applicable to such investigations,[14] but zinc[15] and other metals[16] have been reported as well. For chloride, bromide, and iodide, NMR relaxation is dominated by quadrupolar effects such that the spin–lattice decay rate is given by

$$R_1 = KT_c \tag{1}$$

where K is a constant that includes the spin quantum number, the electric field, and the quadrupole moment and T_c is the correlation time for reorientation of the field gradient.[14] For symmetrically solvated halide ions R_1 values are considerably smaller (1200 s^{-1} for Br^-) than those for covalently bound species ($>10^6$ s^{-1} for Br). However if exchange occurs at a moderately fast rate between free and bound halide ions, then a single composite NMR line will be observed. The time-averaged decay rate becomes

$$R_1 = R_2 = P_f R_{1f} + P_b R_{1b} \tag{2}$$

where R_2 is the spin–spin relaxation rate, P_f and P_b are the fraction of halide ions in the free and bound forms respectively, and R_{1f} and R_{1b} are the relaxation rates of the free and bound halide ions respectively. For metal–protein complexes, $R_{1b} \gg R_{1f}$ (by a factor of $\sim 10^5$) so that measurable line broadening is observed at reasonable halide ion concentrations ($\sim$0.1 M) and metal–protein concentrations ($\sim 10^{-5}$ M). This large chemical amplification effect is not present when a smaller ligand is used instead of a protein. The line width increase is defined by

$$R_2 - R_{1f}/\pi = (1/k_2[B] + [X]/R_1[B])^{-1} \tag{3}$$

(1) Pesek, J. J.; Schneider, J. F. *Inorg. Chem.* **1987**, *26*, 3064.
(2) Rabenstein, D. L. *Acc. Chem. Res.* **1978**, *11*, 100.
(3) Barbieri, R.; Bjerrum, J. *Acta Chem. Scand.* **1965**, *19*, 469.
(4) Relf, J.; Cooney, R. P.; Henneike, H. F. *J. Organomet. Chem.* **1972**, *39*, 75.
(5) Petroysan, V. S.; Reutov, O. A. *J. Organomet. Chem.* **1974**, *76*, 123.
(6) Schwarzenbach, G. *Pure Appl. Chem.* **1970**, *24*, 307.
(7) Wong, Y. S.; Taylor, N. J.; Chieh, P. C.; Carty, A. J. *J. Chem. Soc., Chem. Commun.* **1974**, 625.
(8) Anderegg, G. *Helv. Chim. Acta* **1974**, *57*, 1340.
(9) Canty, A. J.; Gatehouse, B. M. *J. Chem. Soc., Dalton Trans.* **1976**, 2018.
(10) Canty, A. J.; Marker, A. *Inorg. Chem.* **1976**, *15*, 425.
(11) Lucchini, V.; Wells, P. R. *J. Organomet. Chem.* **1975**, *92*, 283.
(12) Goggin, P. L.; Goodfellow, R. J.; Hurst, N. W. *J. Chem. Soc., Dalton Trans.* **1975**, 561.
(13) Diehl, P.; Fluck, E.; Kosfeld, R. *Chlorine, Bromine, and Iodine NMR. Physico-chemical and Biological Applications*; Springer-Verlag: New York, 1976; pp 249–75.
(14) Stengle, T. R.; Baldeschweiler, J. D. *J. Am. Chem. Soc.* **1967**, *89*, 3045.
(15) Ward, R. L. *Biochemistry* **1969**, *8*, 1879.
(16) Sudmeier, J. L.; Pesek, J. J. *Anal. Biochem.* **1971**, *41*, 39.

0020-1669/89/1328-0485$01.50/0

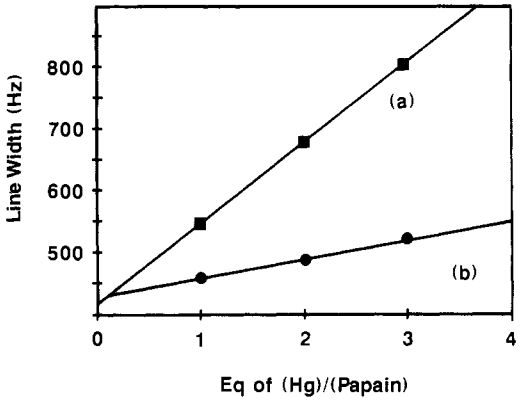

Figure 1. Bromine-81 line width increase vs the mercury:protein ratio for the addition of methylmercury (●) and *p*-(chloromercurio)benzoic acid (■) to 10 μM papain.

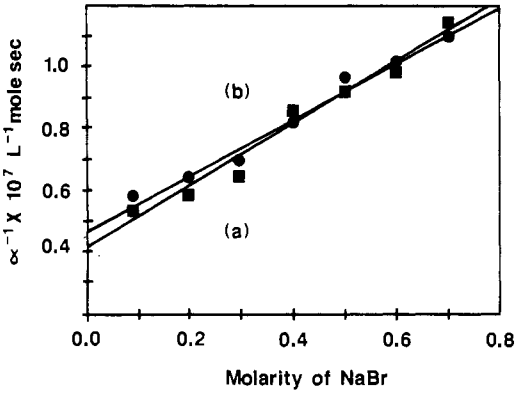

Figure 2. Duplicate determinations of curve α^{-1} vs [Br^-] in the methylmercury–lysozyme complex.

where [B] is the concentration of bound halide, k_2 is the bimolecular halide exchange rate, and [X] is the concentration of free halide. Substituting Equation 1 into eq 3 gives

$$R_2 - R_{1f}/\pi[B] = (1/k_2 + [X]/KT_c)^{-1} = \alpha \qquad (4)$$

Therefore a plot of line broadening ($R_2 - R_{1f}/\pi$) vs [B] yields a straight line with a slope of α. A subsequent plot of α^{-1} vs [X] gives a straight line with a slope of $1/KT_c$ and an intercept of $1/k_2$.

Practically, the above information can be obtained by adding approximately an equimolar amount of metal ion in several increments to a protein solution containing NaBr or NaCl and determining the value of α, assuming that on average a single halide per metal is bound during the time of the NMR measurement. If these determinations are made at several halide ion concentrations, a plot of α^{-1} vs [X] can be generated. This plot will reveal two important pieces of information about the metal–protein complex: the segmental motion at the binding site as determined from T_c and the accessibility of the binding site as determined from k_2. In this study the formation of a number of bromide–organomercurial–protein complexes are detected and these complexes are further characterized by correlation times (T_c) and halide ion exchange rates (k_2).

Experimental Section

Materials. α-Chymotrypsinogen-A, lysozyme, acid phosphatase, urease, and papain were purchased from Sigma while ovalbumin was obtained from Calbiochem (all were the highest purity available). *p*-(Chloromercurio)benzoic acid (Aldrich) and methylmercury(II) chloride (1000 ppm standard, Alfa) were the organomercurials used. All other materials were reagent grade or the highest purity available.

Apparatus. All NMR spectra were obtained on an IBM NR-80 spectrometer using 4K data points. A 90° pulse was employed with a repetition rate of 25.6 ms that was limited by the speed of the A-D converter. T_1 of bromine-81 is less than 1 ms. All spectra were recorded at the probe temperature (23 ± 2 °C). The pH of all solutions was measured with a Corning Model 7 potentiometer equipped with a combination microelectrode.

Procedures. All protein solutions were 10 μM and were verified by measuring the solution absorbance at 280 nm using published extinction coefficients for each protein. Varying amounts of organomercurial (1 mM) or DMSO were added by means of an Eppendorf pipet so that volume changes were less than 5%. The effects of DMSO on the bromine-81 line width were determined by making measurements in the presence and absence of protein. The difference between the two determinations was used for the calculation of α. The bromide ion concentration was varied between 0.1 and 0.9 M. The pH was maintained at 7.0 by means of a 0.1 M phosphate buffer. Line width vs [B] and α^{-1} vs [X] plots were generated on an Apple Macintosh Plus computer employing Cricket Graph for standard linear regression analysis.

Results and Discussion

Figure 1 illustrates the effect of adding methylmercury (MM) and *p*-(chloromercurio)benzoic acid (PMB) to a 0.2 M solution of NaBr containing 10 μM papain. The results are typical of those obtained for each of the proteins studied over the entire range of NaBr concentrations (0.1–0.9 M). These results also are similar to data obtained in the previous study[1] of the same organomercurials added to protein solutions of NaCl. As in the chloride–protein systems, it can be concluded that the coordination number of mercury in both MM and PMB must be at least 3 with the coordination sites occupied by the organic moiety, the protein and one bromide. Only such a three-coordinate structure with moderately fast exchange of the bromide between the organomercury–protein complex and the bulk of solution could account for the line broadening observed. It is only as a result of the chemical amplification effect provided by the halide ion technique that it is possible to detect the coordination of bromide to Hg in the organomercurial–protein complexes.

Table I. Correlation Times and Halide Ion Exchange Rates for Organomercury–Protein Complexes

complex	$10^{11}T_c$, s	$10^{-7}k_2$, L/mol s
PMB–papain	3.0 ± 0.2	7.6 ± 1.5
	2.7 ± 0.1	7.4 ± 0.9
MM–papain	0.7 ± 0.1	2.8 ± 0.8
PMB–acid phosphatase	1.7 ± 0.1	10.0 ± 1.8
	1.5 ± 0.1	12.2 ± 5.2
MM–acid phosphatase	1.2 ± 0.1	5.6 ± 1.2
	1.2 ± 0.1	6.3 ± 1.7
PMB–acid phosphatase–DMSO	3.0 ± 0.3	19.0 ± 10.1
	3.1 ± 0.2	14.3 ± 7.8
PMB–urease	0.6 ± 0.4	1.3 ± 0.9
MM–urease	0.13 ± 0.01	0.3 ± 0.1
PMB–lysozyme	3.1 ± 0.1	5.4 ± 0.5
	3.1 ± 0.1	4.7 ± 0.1
MM–lysozyme	2.7 ± 0.1	2.1 ± 0.1
	2.5 ± 0.2	2.4 ± 0.2
PMB–α-chymotrypsinogen	3.4 ± 0.1	4.2 ± 0.7
	3.3 ± 0.1	5.1 ± 0.3
MM–α-chymotrypsinogen	1.6 ± 0.3	2.3 ± 0.4
	1.2 ± 0.1	3.9 ± 1.5
PMB–ovalbumin	5.2 ± 0.3	2.3 ± 0.1
	4.8 ± 0.3	2.5 ± 0.2
MM–ovalbumin	4.3 ± 0.2	1.7 ± 0.1
	3.7 ± 0.3	1.9 ± 0.2

In addition to the detection of the bromide–organomercurial–protein complexes, the nature of the halide–ion probe experiment allows determination of the segmental motion at the binding site and the bromide exchange rate (eq 4). Bromide ion has the advantage over chloride in that the exchange rate is generally appropriate for the measurement of both T_c and k_2 while for chloride only the determination of T_c is usually possible.[17] Figure 2 is a typical plot of α^{-1} vs [X] for the determination of k_2 and T_c. Two separate experiments are shown for different lysozyme samples as an indication of the reproducibility of the results. Table I gives the T_c and k_2 values calculated for all the MM- and PMB–protein complexes. In several cases a duplicate

(17) Collins, T. R.; Starcuk, Z.; Barr, A. H.; Wells, A. J. *J. Am. Chem. Soc.* **1973**, *95*, 1649.

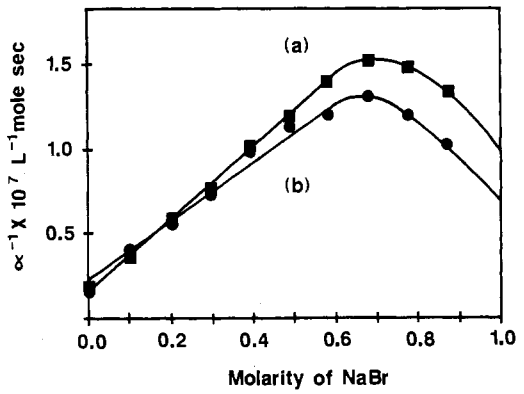

Figure 3. Duplicate determinations of curve for α^{-1} vs [Br^-] in the methylmercury–acid phosphatase complex.

α^{-1} vs [X] curve for the organomercury–protein system was generated in order to verify the results.

A number of interesting observations can be made from the data in the table. In all cases T_c for a particular protein complex with PMB is longer than the value for the corresponding complex with MM. This is quite reasonable since we are measuring segmental motion (the segment includes one or more amino acid residues from the protein, the metal, and the organic moiety of the organomercurial) and the mass of PMB is much greater than the mass of MM. As the mass increases, the motion of the segment should be slower. The results obtained here agree with a previous study[18] on ternary protein–metal–ligand complexes where T_c became longer as the mass of the ligand increased.

Both MM and PMB complexes of some proteins exhibit halide ion-exchange rates that are faster that those for $HgBr_4^{2-}$. This result is consistent with a previous study of ternary protein–metal–ligand complexes,[18] which also measured similar fast exchange rates. This is the expected result when the degree of heterosubstitution increases.[19] Therefore the range of rates observed must be due to steric effects of the protein that control access to the binding site(s). A possible explanation for the consistently faster exchange rate of PMB is the steric effect of the organic moiety. Steric effects were noted for α- and β-alanine complexes[20] of both cobalt(II) and nickel(II). The difference in exchange rates for each of the two ligands on the same metal was almost an order of magnitude. Therefore, it is not surprising that steric factors could lead to differences in the exchange rates for MM and PMB complexes of the same protein.

Table I also presents data for the PMB–acid phosphatase complex in the presence of DMSO. Further line broadening after the effects of DMSO on the protein alone have been subtracted out indicates that this species affects the metal–protein complex. This could be due to an increase in the halide ion-exchange rate without any direct interaction between DMSO and the PMB–acid phosphatase complex. The plot of α^{-1} vs [Br^-] reveals however that the presence of DMSO affects both k_2 and T_c. The most likely explanation for such an observation is the coordination of DMSO to the organomercury–protein complex. This would increase the degree of heterosubstitution accounting for a faster halide ion-exchange rate. The greater mass on the binding site segment(s) due to the presence of DMSO then accounts for the larger value of T_c. Therefore, the four coordination sites on mercury would be occupied by the protein, the organic moiety, DMSO, and a bromide ion. The previously cited methylmercury studies[3-5] have demonstrated that such four-coordinate species can exist. Because the total volume percent of DMSO is less than 1, it is unlikely that unfolding of the protein is responsible for the line-width increase observed.[21] Indeed unfolding of the protein should result in a smaller T_c leading to a decrease in line width—just the opposite of the observed experimental results.

Additional, although not conclusive, evidence for the presence of a fourth coordination site is given in Figure 3. As the concentration of bromide ion increases, the plot of α^{-1} vs [Br^-] begins to decrease. This curvature is observed in all the organomercurial–protein complexes studied. A decrease in α^{-1} could be due to greater line broadening than predicted by the coordination of a single bromide during the lifetime of the NMR measurement. The presence of a fourth coordination site would then accommodate this requirement. An increase in k_2 and/or T_c could be responsible for this additional line broadening. An increase in T_c would certainly be expected for the coordination of another bromide due to a greater mass on the binding site segment. An increase in k_2 might also be expected due to the increase in the degree of substitution. However, in this case there are no previous studies for a direct comparison on mercury when the increase in substitution is the result of coordinating an additional ligand that is identical with one already coordinated. However, an increase in the coordination number for alanine complexes of Ni(II) and Co(II) resulted in faster ligand exchange rates.[20] It should be noted that additional line broadening could also be the result of a conformational change in the protein due to ionic strength effects. Such a conformation change could alter the T_c for the metal binding site segment(s) and/or affect the accessibility of the binding site(s), which would be reflected in k_2. Bromide cannot be disrupting the equilibrium for formation of the organomercury–protein complex since such an effect would result in a decrease in the line width.[16]

In conclusion this study presents spectroscopic evidence for a third and possibly a fourth mercury coordination site in organomercury–protein complexes. These complexes can be characterized by halide ion-exchange rates that measure the accessibility of the binding site(s) and the local correlation time that measures segmental motion at the binding site(s). The halide ion-exchange rate is controlled by steric factors involving both the protein and the size of the moiety on the organomercurial species. The correlation time is mainly dependent on the total mass of the binding site segment(s). However, identification of the specific binding site(s) for the organomercurial on the protein must be done by carbon-13 or other NMR studies.[22]

Registry No. DMSO, 67-68-5; Br^-, 24959-67-9.

(18) Pesek, J. J.; Ronen, S. M.; Alcaraz, A. *Appl. Spectrosc.* **1987**, *41*, 865.
(19) O'Reilly, D. E.; Schacher, G. E.; Schug, K. *J. Chem. Phys.* **1963**, *39*, 1756.
(20) Kustin, K.; Pasternach, R. F.; Weinstock, F. M. *J. Am. Chem. Soc.* **1966**, *88*, 4610.
(21) Fink, A. L. *Cryobiology* **1986**, *23*, 28.
(22) Pesek, J. J.; Schneider, J. F. *J. Inorg. Biochem.* **1988**, *32*, 233.