Nuclear Magnetic Resonance Studies of the Solution Chemistry of Metal Complexes. XI. The Binding of Methylmercury by Sulfhydryl-Containing Amino Acids and by Glutathione[1]

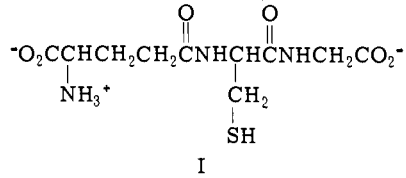
Dallas L. Rabenstein* and Mary T. Fairhurst

Contribution from the Department of Chemistry, University of Alberta, Edmonton, Alberta, Canada. *Received August 6, 1974*

Abstract: The binding of methylmercury, CH_3Hg^{II}, by the amino acids cysteine and penicillamine and by the tripeptide glutathione has been investigated by proton and carbon-13 magnetic resonance spectroscopy. Of the binding sites in these molecules, methylmercury binds most strongly to the ionized sulfhydryl group, with no detectable dissociation of a one-to-one methylmercury–sulfhydryl complex over the pH range 0–14. Evidence is presented for some protonation of the methylmercury-complexed sulfhydryl group of glutathione at pH <2. The nature of the binding of methylmercury in the two-to-one methylmercury–glutathione complex is pH dependent. Starting at low pH, both methylmercury cations are bound to the sulfhydryl group up to pH 4, from pH 4 to 8, one methylmercury shifts from the sulfhydryl group to the amino group, while above pH 10 it dissociates to form CH_3HgOH. The kinetics of the ligand exchange reactions of the glutathione complex were characterized over the pH range 0.5–3 by NMR line-broadening techniques. Rate constants were determined for the proton-assisted dissociation of the complex ($600 \pm 200\ M^{-1}\ sec^{-1}$) and for the displacement of complexed glutathione by sulfhydryl-deprotonated glutathione ($(5.8 \pm 1.9) \times 10^8\ M^{-1}\ sec^{-1}$). The mechanisms of the ligand-exchange reactions are discussed.

It is well known that, of the potential coordination sites in peptides and proteins, the sulfhydryl group binds methylmercury, CH_3Hg^{II}, most strongly.[2-5] The formation constants of the methylmercury complexes of cysteine and glutathione are 5.0×10^{15} and 7.9×10^{15}, respectively, some eight orders of magnitude larger than the formation constants for the binding of methylmercury by the amino group.[1,3] Despite the importance of the binding of methylmercury by the sulfhydryl group, the dynamics of the interaction have not been quantitatively characterized. Simpson, Hopkins, and Hague concluded from a proton magnetic resonance (^1H NMR) study of the binding of methylmercury chloride by the model peptide *N*-acetyl-L-cysteine that exchange of the peptide between the free and complexed forms is dominated by a pathway involving first-order dissociation of the complex.[6] Due to the spin–spin splitting of the broadened resonances, these authors were able only to estimate the rate constant for the first-order dissociation. Their estimated values are suspect, however, since the rate constant predicted for the reaction of methylmercury and *N*-acetyl-L-cysteine from their values and the formation constant of the methylmercury–cysteine complex[3] is six to seven orders of magnitude larger than the rate constants of diffusion controlled bimolecular reactions.[7]

As part of a continuing study of the chemistry of methylmercury,[1,8-10] we have investigated the binding of methylmercury by selected sulfhydryl-containing amino acids and by the tripeptide glutathione (GSH, I). The purpose of the



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present study has been to characterize the kinetics of the binding of methylmercury by sulfhydryl-containing molecules and the nature of the binding for a wide range of solution conditions.

Experimental Section

Chemicals. Methylmercuric hydroxide (Alfa Inorganics) was purified and a stock solution was prepared as described previously.[8] The solution was standardized by titration with sodium chloride in ethanol; the end point was located potentiometrically with a Ag|AgCl indicating electrode. The titration medium was 80% ethanol and the pH was adjusted to 2 to minimize competition of hydroxide ion with chloride for CH_3Hg^{II}.

Cysteine (Nutritional Biochemicals Corp.) was recrystallized from water as the free base. *S*-Methylcysteine (Nutritional Biochemicals Corp.) and penicillamine (Aldrich) were used as recieved. Glutathione (Nutritional Biochemicals Corp.) was washed with a water–ethanol mixture and dried at 110° before use. Tetramethylammonium (TMA) nitrate was prepared by titration of a 25% aqueous solution of TMA hydroxide (Eastman Organic Chemicals) with HNO_3 to a neutral pH.

pH Measurements. All pH measurements were made at 25° as described previously.[8] The pH meter was standardized with Fisher certified buffer solutions of pH 4.00, 7.00, and 10.00 at 25°.

NMR Measurements. Proton magnetic resonance (^1H NMR) spectra were obtained on a Varian A-60-D high resolution spectrometer at a probe temperature of 25 ± 1°. Spectra were recorded at sweep rates of 0.1 Hz/sec for the chemical shift measurements and 0.5 Hz/sec for the spin–spin coupling measurements. Reported data are the average of several scans. Chemical shifts were measured relative to the central resonance of the TMA triplet or the *tert*-butyl resonance of *tert*-butyl alcohol, as described previously,[8] but are reported relative to the methyl resonance of sodium 2,2-dimethyl-2-silapentane-5-sulfonic acid (DSS). Positive shifts correspond to resonances of protons less shielded than those of DSS.

Carbon-13 magnetic resonance (^{13}C NMR) spectra were obtained on a Bruker HFX-90 spectrometer operating at a frequency of 22.63 MHz and equipped with a Nicolet 1085 computer. The Fourier transform mode was used with proton decoupling; 4K pulses were used for chemical shift measurements and 8K for kinetic measurements. 8K data points were taken with an acquisition time of 0.8 sec. The fluorine-19 resonance from C_6F_6 in a coaxial capillary was used for the lock. Chemical shift measurements were made with respect to dioxane, added as an internal reference at a concentration of about 0.068 *M*. Carbon-13 chemical shifts are reported in parts per million relative to dioxane, positive chemical shifts corresponding to carbon nuclei less shielded than those of dioxane. The carbon-13 nuclei of dioxane are 67.4 ppm less shielded than those of TMS.

Sample Preparation. Solutions used in the NMR measurements were prepared in distilled water from the requisite amounts of the crystalline ligand and the methylmercuric hydroxide standard solution under a nitrogen atmosphere to minimize oxidation. TMA nitrate or dioxane was added as a reference compound. Samples

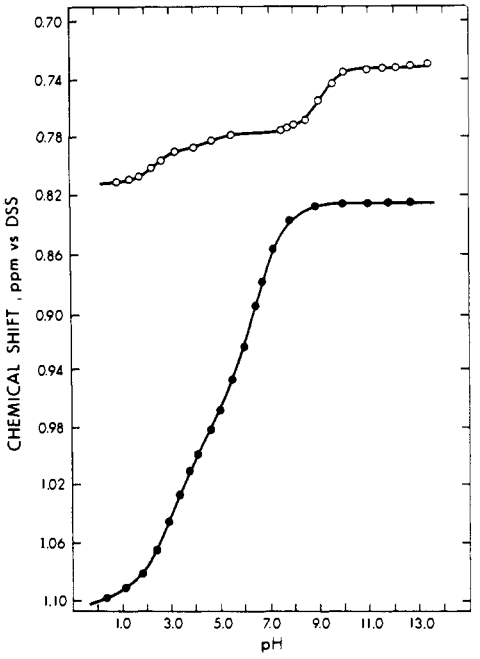


Figure 1. pH dependence of the chemical shift of the methyl protons of methylmercury in an aqueous solution containing 0.190 *M* methylmercury (closed points) and in an aqueous solution containing 0.150 *M* methylmercury and 0.150 *M* cysteine (open points).

were withdrawn from the solution after adjustment of the pH using concentrated HNO_3 or KOH.

Results

The resonance pattern for the methyl protons of methylmercury consists of a singlet flanked symmetrically by two less intense satellite lines.[8] The satellites are due to methyl groups bonded to mercury-199 ($I = \frac{1}{2}$, natural abundance 16.9%) while the central resonance is due to methyl groups bonded to all other isotopes of mercury. The binding of methylmercury by the sulfur-containing amino acids and by GSH was characterized by monitoring the chemical shift of the methyl protons of methylmercury, the mercury-proton coupling constant, and the chemical shifts of ligand protons and selected ligand carbon nuclei as a function of solution conditions.

Characterization of the Complexes. The chemical shift of the methyl protons of methylmercury in a solution containing 0.190 *M* methylmercury and in a solution containing 0.150 *M* methylmercury and 0.150 *M* cysteine is shown as a function of pH in Figure 1. The mercury-proton spin-spin coupling constant for the same solutions is shown in Figure 2. The pH dependence of the chemical shift and the coupling constant of the 0.190 *M* methylmercury solution is due to the following reactions.[4,8,10]

$$CH_3HgOH_2^+ + OH^- \rightleftharpoons CH_3HgOH + H_2O \quad (1)$$

$$CH_3HgOH_2^+ + CH_3HgOH \rightleftharpoons (CH_3Hg)_2OH^+ + H_2O \quad (2)$$

$$CH_3HgOH + (CH_3Hg)_2OH^+ \rightleftharpoons (CH_3Hg)_3O^+ + H_2O \quad (3)$$

The displacement of the chemical shift and coupling constant titration curves when the solution contains cysteine indicates that methylmercury is complexed by cysteine over the pH range 0–14. This is also indicated by changes in the pH-dependent chemical shift behavior of the cysteine carbon resonances (Figure 3) and the multiplet pattern for the cysteine carbon-bonded protons when the solutions contain an equimolar concentration of methylmercury. The mole ratio study in Figure 4 indicates that, of the three potential binding sites of cysteine, the sulfhydryl group is the binding

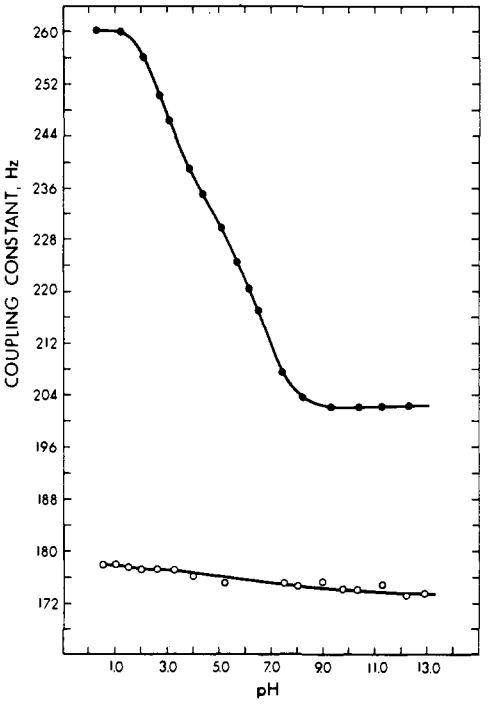


Figure 2. pH dependence of the mercury–proton spin–spin coupling constant of methylmercury in an aqueous solution containing 0.190 *M* methylmercury (closed points) and in an aqueous solution containing 0.150 *M* methylmercury and 0.150 *M* cysteine (open points).

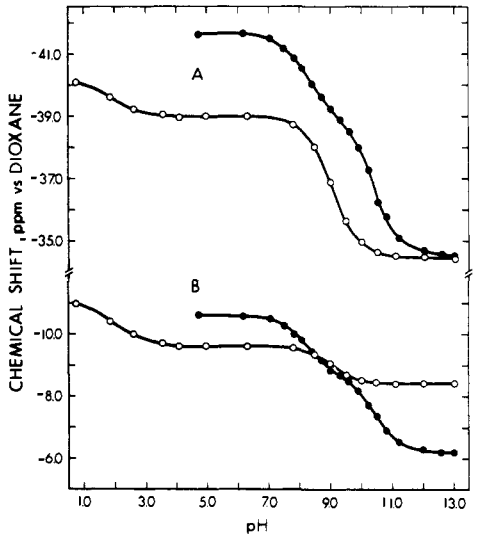


Figure 3. pH dependence of the chemical shifts of the $-CH_2S$ (A) and CHN (B) carbon atoms of cysteine in an aqueous solution containing 0.201 *M* cysteine (closed points) and in an aqueous solution containing 0.200 *M* cysteine and 0.200 *M* methylmercury (open points).

site involved in complexation of the one-coordinate[4] methylmercury in the 1:1 complex. The mole ratio study was performed at pH 13 since the carboxylate and amino groups are known not to be effective binding sites at this pH due to the stronger binding of methylmercury by hydroxide ion.[1,8] The results in Figure 4 for *S*-methylcysteine also indicate that the thioether group is not an effective binding site at pH 13.

The pH dependence of the chemical shift of the methyl protons and of the mercury-proton coupling constant of methylmercury in an equimolar solution of methylmercuric hydroxide and penicillamine is similar to that shown in Figures 1 and 2. These two parameters and the chemical shift behavior of the ligand protons indicate complexation of

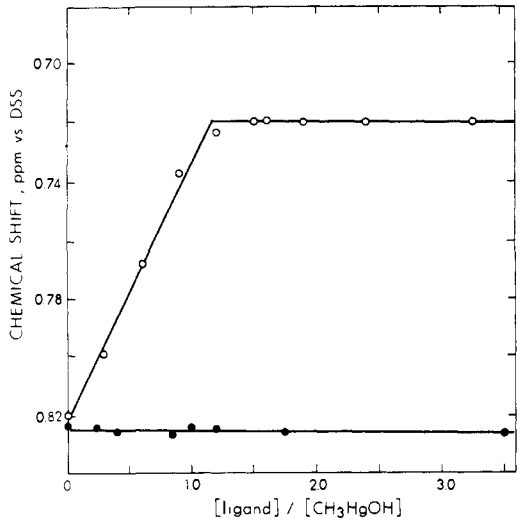


Figure 4. Chemical shift of the methyl protons of methylmercury as a function of the ratio of cysteine to methylmercury (open points) and the ratio of *S*-methylcysteine to methylmercury (closed points), pH 13.0.

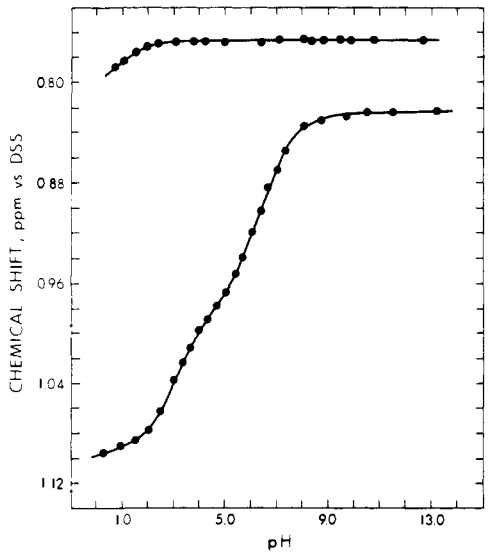


Figure 5. pH dependence of the chemical shift of the methyl protons of methylmercury in an aqueous solution containing 0.190 *M* methylmercury (lower curve) and in an aqueous solution containing 0.150 *M* methylmercury and 0.150 *M* glutathione (upper curve).

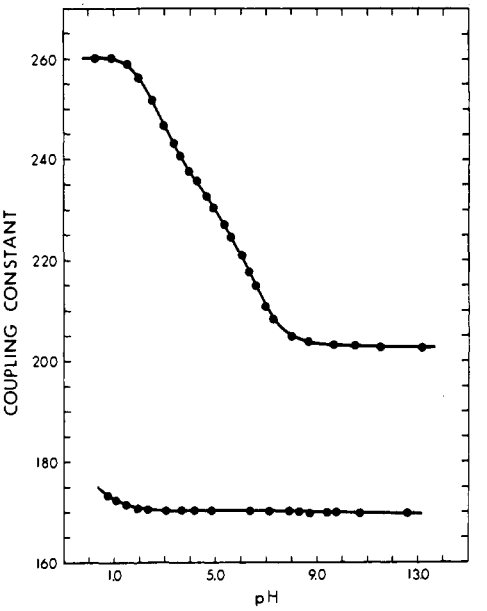


Figure 6. pH dependence of the mercury-proton coupling constant of methylmercury in an aqueous solution containing 0.150 *M* methylmercury (upper curve) and in an aqueous solution containing 0.150 *M* methylmercury and 0.150 *M* glutathione (lower curve).

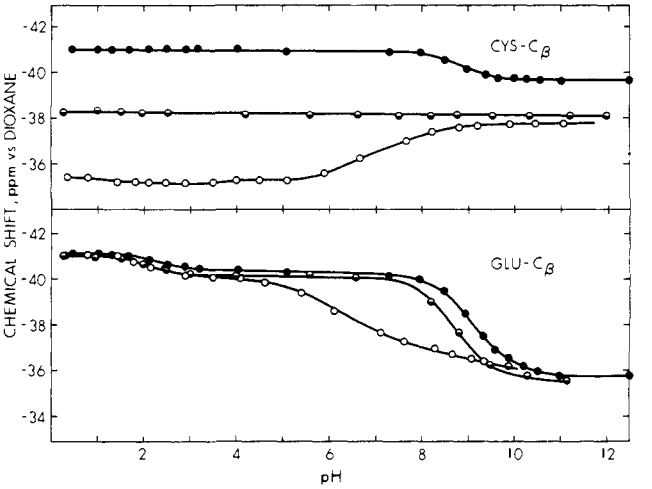


Figure 7. pH dependence of the CYS-C_β and GLU-C_β carbon atoms of glutathione in solutions having methylmercury-to-glutathione ratios of 0 (-●-), 1 (-◒-) and 2 (-○-), 0.22 *M* glutathione.

methylmercury to the sulfhydryl group. Measurements were also made in basic solutions up to 8 *M* KOH. Under these extreme conditions, the resonance for the methyl protons of methylmercury was between that of free methylmercury and the methylmercury in the penicillamine complex, which may indicate partial dissociation of the complex due to competition from hydroxide ion for CH_3Hg^{II} or possibly the formation of a complex of the type $CH_3Hg(OH)(penicillamine)^{2-}$.

The pH dependence of the chemical shift of the methyl protons and of the mercury-proton coupling constant of methylmercury in an equimolar solution of methylmercuric hydroxide and GSH is shown in Figures 5 and 6. These results and chemical shift data for carbon-bonded glutathione protons[9] indicate that methylmercury is coordinated to the sulfhydryl group of glutathione over the pH range 0.5–13.

Methylmercury-complexed glutathione possesses several potential binding sites for additional methylmercury, including the methylmercury-complexed sulfhydryl group.[4] To elucidate the formation of higher complexes from the methylmercury complexed GSH, the chemical shifts of the carbon atoms of GSH in solutions containing no methylmercury and methylmercury at methylmercury-to-glutathione ratios of 1:1 and 2:1 were measured as a function of pH. Chemical shift data for the cysteinyl-C_β (CYS-C_β) and glutamyl-C_β (GLU-C_β) carbon atoms of glutathione are shown in Figure 7. The displacement of the curves for the 2:1 solution from those of the 1:1 solution indicates that over the pH range 0–4 two methylmercury cations are bonded to the sulfhydryl group. The mercury-proton coupling constant of the methylmercury in this species is 212 Hz. As the pH is increased above pH 4, one of the methylmercury cations shifts to the amino group. Mole ratio studies at pH values of 1.0, 4.0, and 8.0 provide additional evidence for the shift of the second methylmercury from the methylmercury-complexed sulfhydryl group to the amino group as the pH is increased. The small differences between the chemical shift curves for the methylmercury:glutathione 1:1 and 2:1 ex-

periments at pH >9 indicates that in this pH region the second methylmercury is partially dissociated from the glutathione, presumably to form CH_3HgOH by analogy with the pH dependence of the binding of methylmercury by amines.[1]

Exchange Kinetics. With each of the ligands studied, ^1H NMR spectra were recorded for the methylmercury in solutions having methylmercury to ligand ratios greater than one over the pH range 0–13. In every case, a single exchange-averaged resonance pattern was observed, indicating exchange of methylmercury between the various methylmercury species to be fast on the NMR time scale.

However, the rate of exchange of ligand between the free and complexed forms in solutions containing an excess of ligand is pH dependent. For the methylmercury–penicillamine system, the line width of the exchange-averaged resonances of a solution having a penicillamine to methylmercury ratio of 2:1 indicated fast exchange except over the pH region 1.7–3.5 where a small amount of broadening of the averaged penicillamine methine proton resonance was observed. For the methylmercury–GSH system, the rate of exchange of GSH between the free and complexed forms in a solution containing an excess of GSH is fast at pH >6 as indicated by sharp, averaged resonances. At pH <6, the rate decreases and is dependent on pH and GSH concentrations. Due to the complexity of the ^1H NMR spectrum of GSH, the exchange was studied by ^{13}C NMR. The CYS-C_α region of the ^{13}C spectra for a solution containing 0.33 *M* glutathione and 0.165 *M* methylmercury is shown as a function of pH in Figure 8. The CYS-C_β resonance is shifted more upon complex formation and thus is more sensitive to kinetic effects; however, overlap of the exchange averaged CYS-C_β resonance with the GLU-C_β resonance precludes analysis of its line shape. At these concentrations, the exchange rate is sufficiently slow at pH 2.12 that separate exchange-broadened resonances are observed. The exchange-broadened resonances of a solution containing 0.56 *M* glutathione and 0.28 *M* methylmercury never separate into the resonances of the free and complexed forms, indicating that the exchange rate is concentration dependent. The concentration dependence is also indicated by line shape changes in mole ratio experiments.

The inverse of the mean lifetime of the methylmercury–glutathione complex is given in Table I for a range of experimental conditions. The mean lifetimes were obtained by matching of experimental and computer-simulated spectra; spectra were simulated as a function of the lifetime of the glutathione in each of the environments using Bloch phenomenological equations modified by the approach of McConnell[11] and Meiboom[12] to account for the transfer of magnetization between the two sites by chemical exchange. The uncertainty of the lifetimes, due mainly to the low signal-to-noise ratio for the exchange-broadened spectra, of which those in Figure 8 are typical, is estimated to be ±15%.

Possible reactions by which glutathione might exchange between the free and complexed forms for the conditions of Table I are

$$CH_3HgSG \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} CH_3Hg^{II} + GS^- \quad (4)$$

$$CH_3HgSG + H^+ \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} CH_3Hg^{II} + GSH \quad (5)$$

$$CH_3HgSG + OH^- \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} CH_3HgOH + GS^- \quad (6)$$

$$CH_3HgSG + \overset{*}{G}S^- \underset{}{\overset{k_4}{\rightleftharpoons}} CH_3HgS\overset{*}{G} + GS^- \quad (7)$$

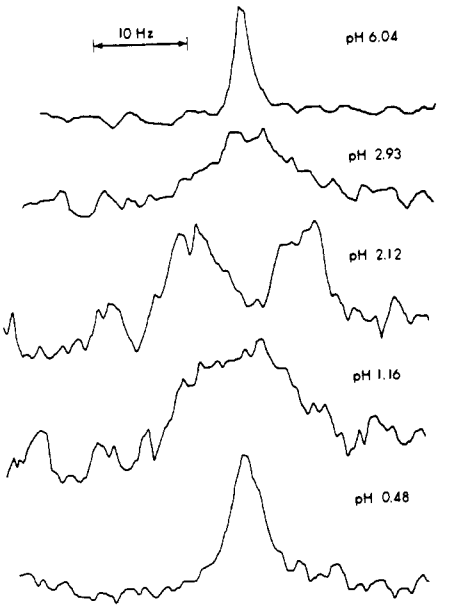


Figure 8. CYS-C_α region of the ^{13}C NMR spectra of a solution containing 0.165 *M* methylmercury–glutathione and 0.165 *M* glutathione as a function of pH, 25°.

Table I. NMR Kinetic Data for the Methylmercury–Glutathione System[a]

pH	[Complexed glutathione], *M*	[Free glutathione], *M*	$1/\tau_{complex}$,[b] sec^{-1}
2.93	0.165	0.165	100
2.52	0.165	0.165	33
2.32	0.165	0.165	25
2.12	0.165	0.165	17
1.98	0.165	0.165	20
1.55	0.165	0.165	33
1.16	0.165	0.165	50
1.03	0.165	0.165	40
0.90	0.165	0.165	50
0.72	0.165	0.165	200
0.65	0.165	0.165	200
0.48	0.165	0.165	240
2.93	0.28	0.28	67
2.54	0.28	0.28	50
2.32	0.28	0.28	50
2.14	0.28	0.28	40
1.93	0.28	0.28	33
1.45	0.28	0.28	40
1.06	0.28	0.28	50
0.95	0.28	0.28	50
0.80	0.28	0.28	67
0.68	0.28	0.28	100
0.47	0.28	0.28	200

[a] At 25°. [b] Obtained by comparison of experimental and computer simulated spectra. Uncertainty in $1/\tau_{complex}$ values is estimated to be ±15%.

$$CH_3HgSG + \overset{*}{G}SH \underset{}{\overset{k_5}{\rightleftharpoons}} CH_3Hg\overset{*}{S}G + GSH \quad (8)$$

where CH_3HgSG and GS^- represent sulfhydryl-complexed and sulfhydryl-deprotonated glutathione. The inverse of the mean lifetime of the complex is related to the rate of decrease in the concentration of the complex by

$$\frac{1}{\tau_{complex}} = -\frac{d[C]}{dt}\frac{1}{[C]} \quad (9)$$

where [C] is the concentration of the complex. The rate of decrease in the concentration of the complex is given by eq 10

$$-\frac{d[C]}{dt} = k_1[C] + k_2[H^+][C] + k_3[OH^-][C] + k_4\alpha_S[F][C] + k_5\alpha_{SH}[F][C] \quad (10)$$

where [F] is the concentration of free glutathione and $\alpha_S = [GS^-]/[F]$ and $\alpha_{SH} = [GSH]/[F]$. Division by [C] leads to the following equation for the inverse of the mean lifetime of the complex.

$$1/\tau_{complex} = k_1 + k_2[H^+] + k_3[OH^-] + k_4\alpha_S[F] + k_5\alpha_{SH}[F] \quad (11)$$

The relative importance of exchange by reaction 4 can be predicted. Assuming the formation reaction in eq 4 to have a diffusion-controlled rate constant of 10^{10} M^{-1} sec^{-1}, k_1 is predicted to be $\sim 10^{-6}$ sec^{-1}, using the formation constant reported by Simpson[3] for the methylmercury-cysteine complex. If the rate of the formation reaction is slower than diffusion controlled, k_1 will be even less. Since reaction 4 is pH independent, it is predicted to contribute a constant, negligible amount, equal to k_1, to $1/\tau_{complex}$. Reaction 6 can also be shown to not contribute significantly to the observed exchange, at least up to pH ~4. If the reaction of OH^- with CH_3HgSG is diffusion controlled and has a rate constant of 10^{10} M^{-1} sec^{-1}, its contribution to $1/\tau_{complex}$ would only be 0.01 sec^{-1} at pH 2 and 1.0 sec^{-1} at pH 4. It is likely, however, that k_3 is somewhat less than diffusion controlled since Simpson reported a value of 1.6×10^4 M^{-1} sec^{-1} for the rate constant for the displacement of CN^- from CH_3HgCN by OH^-,[13] in which case the contribution of reaction 6 to $1/\tau_{complex}$ will be even less.

The relative importance of reactions 5, 7, and 8 can be predicted from their dependence on solution conditions. The spectra shown in Figure 8 and the more extensive data in Table I for the solution containing 0.165 M methylmercury-glutathione and 0.165 M free glutathione indicate that the rate of exchange is at a minimum at pH 2.1. The larger exchange rate at pH <2.1 indicates that exchange occurs by proton assisted dissociation of the complex at these pH values, while the increase in exchange rate at pH >2.1 is due to displacement of complexed glutathione by GS^-, whose concentration increases as the pH increases. If the rate of exchange by pH dependent reactions 5 and 7 is negligible at pH 2.12, the value of $1/\tau_{complex}$ at this pH is equal to the term $k_5\alpha_{SH}[F]$ in eq 11. Since the rate of exchange by reaction 8 will be pH independent for the pH range of the data in Table I,[14] this will also be the contribution of reaction 8 to $1/\tau_{complex}$ at the other pH values. If, however, some exchange is also occurring at pH 2.12 by reactions 5 and 7, the contribution of reaction 8 to the observed $1/\tau_{complex}$ will be even less, indicating that the amount of exchange by reaction 8 for the conditions given in Table I is likely to be small relative to that by reactions 5 and 7. Equation 11 then reduces to

$$1/\tau_{complex} = k_2[H^+] + k_4\alpha_S[F] \quad (12)$$

Rate constants k_2 and k_4 were estimated from the data in Table I by a method of successive approximations. First, a value was calculated for k_2 from each of the lifetimes at pH < 2 by using eq 12 and assuming $k_2[H^+] \gg k_4\alpha_S[F]$. Then a value of k_4 was calculated from each of the lifetimes for pH >2.3 using eq 12 and the mean of the previously determined values for k_2. New values were then calculated for k_2 using the mean of these values for k_4, the mean of which was then used to calculate a new mean value for k_4. The values so obtained are $k_2 = 600 \pm 200$, $k_{-2} = 5.1 \times 10^9$,[15] and $k_4 = (5.8 \pm 1.9) \times 10^8$ M^{-1} sec^{-1}.[16] The value predicted by these rate constants for $1/\tau_{complex}$ for 0.165 M methylmercury-glutathione and 0.165 M free glutathione at pH 2.12 is 20 sec^{-1}, within experimental error of the value given in Table I.

Discussion

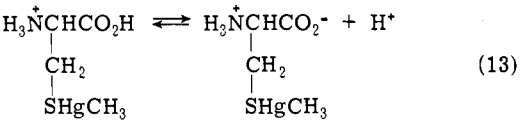
Binding of Methylmercury. Of the potential binding sites in cysteine, penicillamine, and GSH, the sulfhydryl group binds methylmercury most strongly. The results in Figures 1-3 and 5-6 indicate that, in the 1:1 complexes, the other potential coordination sites are not coordinated, consistent with a methylmercury coordination number of one.

Binding of methylmercury by the sulfhydryl-rich protein thionein has been investigated by Chen, Ganther, and Hoekstra,[17] who concluded on the basis of spectral changes that methylmercury has a lower affinity for thionein than does Cd^{2+}. This is opposite to the affinity order obtained from NMR studies of the binding of these metal ions by the sulfhydryl group of GSH.[18] The greater affinity of thionein for Cd^{2+} may result from multiple binding of thionein to Cd^{2+}, which does not occur in the Cd^{2+} complex of GSH below pH 6[18] nor in the methylmercury complex, and presumably not in the methylmercury complex of thionein.

The nature of the methylmercury-glutathione complex formed when the methylmercury concentration is in excess of the concentration of sulfhydryl groups is pH dependent. At pH less than 5, a second methylmercury binds to the sulfhydryl group. As the pH is increased above 5, the excess methylmercury shifts to the amino group indicating that the affinity of the deprotonated amino group for CH_3Hg^{II} is greater than that of the methylmercury-coordinated sulfhydryl group. Simpson, Hopkins, and Hague[6] concluded that two or more methylmercury groups can bond to the sulfhydryl group of *N*-acetyl-L-cysteine, although the pH at which such complexes form was not given, while Carty and coworkers[19] found that in the 2:1 complex of methylmercury with penicillamine isolated from alkaline solution the sulfhydryl and amino groups are methylmercury coordinated.

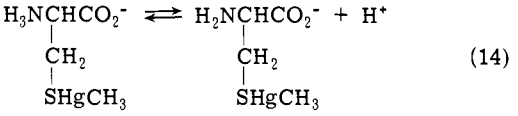
The position of the resonance for the methylene protons of the cysteinyl residue of GSH in a 1:1 methylmercury-GSH complex is essentially independent of pH, indicating that the methylmercury-complexed sulfhydryl group is deprotonated. At pH less than 2, the chemical shift of the methyl protons of methylmercury (Figure 5) and the mercury-proton coupling constant (Figure 6) shift in the direction of the chemical shift and coupling constant of free methylmercury. This could be due to dissociation of the complex through competition of protons with CH_3Hg^{II} for the sulfhydryl group or to protonation of the methylmercury-complexed sulfur. Addition of methionine, which forms a thioether complex with methylmercury at low pH in which the methylmercury chemical shift is 1.12 ppm,[20] caused no detectable change in the methylmercury chemical shift of a pH 0.5 solution of 0.171 M methylmercury and 0.171 M GSH up to a methionine concentration of 0.34 M. These results suggest that the changes in the chemical shift and coupling constant at pH less than 2 are due to protonation of the complexed sulfhydryl group; if dissociation were occurring, the methionine would have caused an increase in the amount of dissociation which would have been indicated by a change in the chemical shift.

Assuming the sulfhydryl group to be deprotonated in the cysteine complex of methylmercury, the pH dependence of the chemical shifts of the cysteine carbons (Figure 3) reflects the state of protonation of the amino and carboxylate groups. Using methods described previously,[21] the data in Figure 3 yield a pK_A of 1.95 ± 0.05 for the carboxyl group (eq 13) and a pK_A of 9.05 ± 0.04 for the amino group (eq



(13)

14) of methylmercury-complexed cysteine. The log K_A values obtained for the analogous reactions of methylmer-

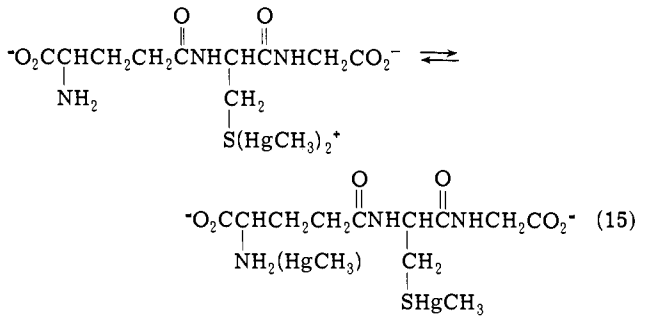


(14)

cury-complexed penicillamine from the chemical shift of the methine proton are 2.0 ± 0.1 and 9.0 ± 0.1. The chemical shift of the methyl protons of the methylmercury yields the same values for the pK_A's of the amino groups, but values calculated for the carboxyl pK_A's from data over the pH range 1–5 are not constant, presumably because the chemical shift also reflects some protonation of the methylmercury-complexed sulfhydryl group.

The results in Figure 3 indicate that, upon protonation of the amino group of $CH_3HgSCH_2CHNH_2CO_2^-$, the resonance for the methylene carbon, which is two bonds removed from the site of protonation, shifts by 4.58 ppm while that for the methine carbon shifts by only 1.18 ppm even though it is one bond closer to the site of protonation. Similarly, complexation of the ionized sulfhydryl group by CH_3Hg^{II} causes the methine carbon, which is separated from the site of complexation by two bonds, to shift by 2.22 ppm whereas the resonance for the methylene carbon shifts by only 0.11 ppm even though it is bonded to the site of complexation. This is analogous to the relative shifts observed in carbon-13 magnetic resonance spectra upon protonation of simple amino acids[22] and upon complexation of Cd^{2+} and Zn^{2+} by the amino group of glutathione.[18]

Exchange Kinetics. For solutions having a methylmercury to ligand ratio greater than 1, a single averaged methylmercury resonance was observed over the range 0–13, indicating fast exchange of methylmercury between the free ($CH_3HgOH_2^+$, $(CH_3Hg)_2OH^+$, and CH_3HgOH) and complexed forms and between the different types of complexed methylmercury, for example, between the sulfhydryl and amino groups of GSH (eq 15). In contrast, exchange of

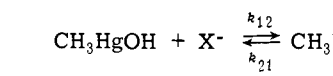


(15)

methylmercury between the somewhat weaker amine (and simple amino acid) complexes and the free forms is slower at pH <5,[23] which suggests that the lability of sulfhydryl-complexed methylmercury is due to the ability of the sulfhydryl group to bind a second methylmercury.

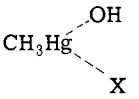
Exchange of GSH between the free and complexed forms in solutions having a GSH to methylmercury ratio greater than one is predominantly by proton-assisted dissociation of the complex at pH <2, presumably via a sulfhydryl-protonated intermediate, and by displacement of complexed GSH by sulfhydryl-deprotonated GSH at pH >2. Simpson, Hopkins and Hague[6] concluded from ^1H NMR measurements that, for the related ligand *N*-acetyl-L-cysteine, exchange between the free and complexed forms is by a pathway involving first-order dissociation of the methylmercury complex. However, as noted in the introductory section, for this reaction to account for the observed exchange would require a formation rate constant six to seven orders of magnitude larger than diffusion-controlled bimolecular rate constants.

Eigen, Geier, and Kruse[24] have shown that, in ligand displacement reactions of the type



(16)

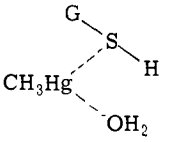
where X^- is a halide ion, k_{12} is strongly dependent on the identity of X while k_{21} is reasonably independent of X and proposed that the reaction proceeds by an associative mechanism. The formation constant of CH_3HgOH is larger than those of the halide complexes, suggesting that once an intermediate of the type



forms, the tendency for X^- to dissociate from the intermediate will be larger than for OH^-. In support of this (a) $k_{12} < k_{21}$ for the halides, (b) both K_f and k_{12} increase in the order $Cl^- < Br^- < I^-$, and (c) when X is CN^-, which forms a methylmercury complex having a log K_f = 14.1 compared to log K_f = 9.37 for CH_3HgOH, k_{21} is less than k_{12} and is approximately five orders of magnitude less than when X is a halide ion.[13]

If displacement of complexed glutathione by GS^- proceeds through an analogous intermediate, there is an equal probability that, once the intermediate forms, it will dissociate to products or reactants. Thus, the rate of ligand exchange is predicted to be one-half the rate of formation of the intermediate, which is expected to be equal to the rate of diffusional encounter or slightly less due to steric effects.

It is not known if the mechanism of complex formation reactions involving $CH_3HgOH_2^+$ is associative or dissociative. However, if the mechanism of its reaction with protonated glutathione, described by k_{-2}, is associative, it will proceed through an intermediate such as



whose rate of formation presumably is diffusion controlled. The experimental results described above as evidence for a sulfhydryl-protonated methylmercury–glutathione complex indicate that the stability of the protonated complex is greater than that of the aquo complex. Thus, by analogy with the dissociation of the intermediate proposed in the reactions involving CH_3HgOH, the tendency will be for the intermediate to dissociate to the glutathione complex and k_{-2} is expected to approach a diffusion controlled value.

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(14) The fractional concentration of GS^- varies from $\sim 10^{-8}$ at pH 1 to 10^{-3} at pH 6.
(15) $k_{-2} = k_2 k_{123} K_f$ where k_{123} is the microscopic acid dissociation constant for the sulfhydryl group[9] and K_f the formation constant.[3]
(16) In the calculation of these rate constants, it was assumed that exchange by reaction 8 is negligible. If, however, the exchange at pH 2.12 is due solely to reaction 8, the rate constants obtained by the above procedure are $k_2 = 400 \pm 250\ sec^{-1}$, $k_{-2} = 3.4 \times 10^9\ M^{-1}\ sec^{-1}$, $k_4 = 2.5 \pm 1.5 \times 10^8\ M^{-1}\ sec^{-1}$, and $k_5 = 110\ M^{-1}\ sec^{-1}$.
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Spectral Studies of Copper(II) Carboxypeptidase A and Related Model Complexes

Robert C. Rosenberg, Charles A. Root, Paula K. Bernstein, and Harry B. Gray*

Contribution No. 4923 from the Arthur Amos Noyes Laboratory of Chemical Physics, California Institute of Technology, Pasadena, California 91125. *Received July 18, 1974*

Abstract: The near-infrared and visible electronic absorption spectrum of $Cu^{II}CPA$ exhibits a maximum at 12,580 cm^{-1} with a molar extinction coefficient of 124. Analysis of the frozen glass EPR spectrum of $Cu^{II}CPA$ in 1:1 ethylene glycol:buffer yields $g_\parallel = 2.327$, $g_\perp = 2.057$, $A_\parallel = 124$ G (13.5), $A_\perp = 15$ G (1.4 mK). Comparison of the electronic spectral and EPR properties of $Cu^{II}CPA$ with those of model Cu(II) complexes indicates that the coordination site is significantly distorted from square planar toward a tetrahedral geometry. Addition of the inhibitor sodium β-phenylpropionate (NaβPP) results in a shift of the principal peak in the electronic absorption spectrum to 11,400 cm^{-1} and an increase in molar extinction coefficient to 180, suggesting that the coordination geometry in $Cu^{II}CPA{\cdot}\beta PP$ is closer to tetrahedral than that in the unsubstituted derivative. A formation constant $K_1 = 1.44 \times 10^2\ M^{-1}$ was measured for the $Cu^{II}CPA{\cdot}\beta PP$ complex.

Carboxypeptidase A (CPA) is a zinc metalloenzyme that exhibits both peptidase and esterase activities.[1-3] An X-ray crystallographic study[4] has indicated that the zinc is probably in a distorted tetrahedral coordination environment at the active site of CPA. The probable donor-atom set in the resting enzyme is N_2O_2, comprised of the N(1)'s of His 69 and His 196, with oxygens furnished by Glu 72 and a water molecule.[4,5]

Numerous other dipositive metal ions have been substituted for the zinc in CPA with varying degrees of retention of peptidase and esterase activities.[6] Our electronic spectroscopic and magnetic susceptibility studies of two active derivatives, $Co^{II}CPA$ and $Ni^{II}CPA$, have demonstrated that the enzyme is flexible enough to accommodate both five- and six-coordinate active-site structures.[7] It appears, then, that as long as the metal center possesses a properly oriented, substitution-labile coordination position, a range of coordination numbers and geometries is possible for a peptidase-active derivative. In order to further examine the question of coordinative flexibility in the CPA system and the relationship of the coordination environment of the metal to enzymatic activity, study of an inactive metalloderivative appeared essential.

The $Cu^{II}CPA$ derivative is known to show neither peptidase nor esterase activity.[6a] Only sketchy information regarding its spectroscopic properties is available in the literature. The wavelength of maximum absorption of $Cu^{II}CPA$ has been mentioned,[8] but no details on the rest of the absorption spectrum appear to be available. A brief report stating that the EPR spectrum of a single crystal of $Cu^{II}CPA$ shows three principal g values and superhyperfine interaction from two equivalent nitrogen ligands has been published,[9] although no g values or hyperfine coupling constants were given. The only other EPR data for $Cu^{II}CPA$ are from a study of freeze-dried samples and pH 5.5 solutions.[10]

In this paper we report the results of our investigation of the electronic absorption and EPR spectra of $Cu^{II}CPA$. The spectroscopic data for $Cu^{II}CPA$ are compared with those obtained for a variety of cupric model complexes, with the aim of elucidating the coordination number and geometry of the metal center. We also report the electronic absorption spectrum and the formation constant of the complex of $Cu^{II}CPA$ with the inhibitor β-phenylpropionate (βPP).

Experimental Section

Materials. Crystalline carboxypeptidase A, isolated by the Cox procedure,[11] was obtained from Sigma Chemical Co. and used without further purification. The Cox method was chosen because it yields a relatively small amount of the undesirable CPA_γ form of the enzyme.[12] Samples were checked for peptidase activity[13] and metal content before and after metal replacement. Hippuryl-L-phenylalanine (Schwarz/Mann) was used as the substrate in all assays. $Cu^{II}CPA$ was prepared by the method of Coleman and Vallee.[14] Peptidase activity was found to be proportional to the zinc content of preparations containing mixtures of $Zn^{II}CPA$ and $Cu^{II}CPA$. The preparations of $Cu^{II}CPA$ used for the spectral studies were found to contain 1-3 mol % residual zinc and had a correspondingly low level activity. Extreme care was taken to prevent contamination of the CPA by adventitious metal ions.[15] Plastic lab ware was used, and all the Tris-HCl buffers were repeatedly extracted with dithizone in CCl_4 prior to use. Cupric ion solutions were made up by dissolving the pure metal in metal-free HCl. Minimum 99.9% pure Cu metal (J. T. Baker Co.) was used. D_2O was obtained from Columbia Organic Chemical Co. The D_2O