Nuclear Magnetic Resonance Studies of the Solution Chemistry of Metal Complexes. 19. Formation Constants for the Complexation of Methylmercury by Glutathione, Ergothioneine, and Hemoglobin

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Abstract: The three most abundant thiol-containing molecules in the intracellular region of human erythrocytes are glutathione (GSH), ergothioneine, and hemoglobin. The binding of methylmercury (CH_3Hg^{II}) by these molecules in aqueous solution has been quantitatively characterized by ^1H NMR spectroscopy. Formation constants for the GSH complexes, including microscopic formation constants for complexes in which the amino group is protonated and deprotonated, and for the ergothioneine complex have been determined by measuring the extent of competitive complexation of CH_3Hg^{II} by mercaptoacetic acid as a function of pH. The formation constant for the binding of CH_3Hg^{II} by hemoglobin was measured by a similar procedure using GSH as the competing ligand. The entire hemoglobin resonance envelope was selectively suppressed by a technique employing a selective presaturation pulse to observe the GSH resonances in the presence of interfering hemoglobin resonances. The formation constant for the GSH complex at physiological pH is found to be similar to those of other CH_3Hg^{II} complexes with small monothiol ligands, whereas that for the hemoglobin complex is smaller (log K_{FC} = 11.55 and 10.7 for the GSH and hemoglobin complexes). Log K_{FC} for the ergothioneine complex is much smaller (7.9). These results support previous conclusions that, in human erythrocytes, GSH binds more CH_3Hg^{II} than does hemoglobin, while ergothioneine is not an important binding site. The quantitative results obtained for the CH_3Hg^{II}–hemoglobin binding demonstrate the potential of the ^1H NMR method based on competitive complexation for the study of metal binding by proteins.

It has been estimated that in humans exposed to methylmercury (CH_3Hg^{II}), some 5–10% of the total body burden of CH_3Hg^{II} is in the blood.[1] Of that, 90% or more is in the erythrocytes.[1-3] Because of the high affinity of sulfhydryl groups for mercury, all the CH_3Hg^{II} in erythrocytes can be assumed to be complexed by sulfhydryl-containing molecules, the most abundant of which in the intracellular region are glutathione (γ-L-glutamyl-L-cysteinylglycine (GSH)), ergothioneine, and hemoglobin.[4] Less than 5% of the CH_3Hg^{II} in erythrocytes is membrane bound.[5]

In view of the central role of the erythrocyte in binding and transporting CH_3Hg^{II}, it is of interest to characterize quantitatively the binding of CH_3Hg^{II} by the sulfhydryl-containing molecules of the erythrocyte. In a previous in vitro ^1H NMR study of CH_3Hg^{II} in intact human erythrocytes, we found binding by GSH and hemoglobin with no detectable binding by ergothioneine.[6] Exchange-averaged resonances were observed for the intracellular GSH, indicating exchange of CH_3Hg^{II} between sulfhydryl groups to be fast on the NMR time scale. From the exchange-averaged chemical shift of one of the GSH resonances, it was concluded that CH_3Hg^{II} binds more strongly to the sulfhydryl group of GSH than to those of hemoglobin. The importance of the GSH complex in the toxicology of CH_3Hg^{II} is also indicated by the dependence of CH_3Hg^{II} uptake by the erythrocytes and other organs of rats, with the exception of the liver, on cellular GSH levels.[7]

In the present study, we have measured formation constants for the CH_3Hg^{II} complexes of GSH, ergothioneine, and hemoglobin, with the objective of being able to quantitatively describe the distribution of CH_3Hg^{II} among these molecules in human erythrocytes. Formation constants for the GSH and ergothioneine complexes were determined from the equilibrium constants (K_d) for the reaction

$$RS^- + R'SHgCH_3 \rightleftarrows RSHgCH_3 + R'S^- \quad (1)$$

where RS^- is fully deprotonated GSH or ergothioneine and $R'S^-$ is fully deprotonated mercaptoacetic acid. Exchange of CH_3Hg^{II} between the two thiols is fast on the NMR time scale, resulting in a single ^1H resonance for mercaptoacetic acid from which K_d was calculated directly. The formation constants for the GSH and ergothioneine complexes were then calculated from the K_d values and the known formation constant for the mercaptoacetate complex.[8] The same procedure was used to measure the formation constant for the hemoglobin complex, except that GSH was used as the competing thiol. ^1H NMR spectra for GSH in the GSH–hemoglobin–CH_3Hg^{II} solution were measured by selective saturation of the interfering hemoglobin resonances.[9]

Experimental Section

Chemicals. Glutathione and ergothioneine were used as received from Sigma Chemical Co. The determination of purity and the preparation of solutions were similar to procedures described previously for other thiols.[8]

Methylmercury(II) iodide (Alfa Division, Ventron Corp.) was converted to a solution of methylmercury(II) hydroxide and standardized by titration with chloride as described previously.[8,10]

The water used in the preparation of aqueous solutions was doubly distilled and then deionized by passage through a Barnstead D8902 Ultrapure mixed bed ion-exchange resin. The D_2O used in the preparation of D_2O solutions was obtained from Stohler Isotope Chemicals.

Preparation and Assay of Hemoglobin Solutions. Venous blood was collected in Vacutainers (Becton, Dickinson and Co.) containing EDTA solution. The whole blood was centrifuged at 5000 rpm at 4 °C for 15 min, the plasma and buffy coat removed, and 10 mL of packed cells hemolyzed by sonication (Heat Systems–Ultrasonics Model W-225R sonicator) for 30 s. The hemolyzed erythrocytes were centrifuged at 20000 G for 1 h to remove cell debris. the resulting solution was placed in a dialysis bag (Spectrapor dialysis tubing, M_r cutoff 6000–8000) with 0.2 g of CPG-lipoamide beads (Pierce Chemical Co.) and dialyzed against 2 × 200 mL of D_2O phosphate buffer (0.005 M, pD 7.4) for 12-h periods. After dialysis, the solution gave no indication of a significant (>0.2 mM) concentration of any small molecule, as judged by the ab-

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sence of any resonances in the 0–4.5-ppm region of the spin–echo Fourier transform NRM spectrum[11] measured with a between-pulse delay time of 0.060 s. The hemoglobin concentration and the percentage of methemoglobin were determined by a standard spectrophotometric assay.[12] The hemoglobin concentration was found to be 2.06 mM; the percentage of methemoglobin was 2%, as compared to 1–3% in vivo.[12]

The sulfhydryl content of the hemoglobin solution was determined by titration with a standardized CH_3Hg^{II} solution. The end point was determined by NMR, with the change in the chemical shift of the ergothioneine C(4) proton resonance (at ~6.8 ppm) being used to indicate the end point. The results of the sulfhydryl determination combined with the hemoglobin assay indicated 1.96 sulfhydryl groups per hemoglobin molecule, in excellent agreement with the accepted value of 2.[4] The NMR titration method for the determination of sulfhydryl concentration will be described in detail elsewhere.[13]

It is recognized that the solution prepared by the above procedure is not a pure hemoglobin solution. However, it is well established that CH_3Hg^{II} binds much more strongly to sulfhydryl groups than to other potential coordination sites in proteins[14] so that for the purpose of the CH_3Hg^{II} binding study, the only potential interferences will be thiol molecules. The only other thiols present in the intracellular region at significant concentrations relative to that of hemoglobin are GSH and ergothioneine,[4] which are removed by the dialysis step.

pH Measurements. All pH measurements were made at 25 ± 1 °C with an Orion Model 701 digital pH meter equipped with either a standard glass electrode and a porous ceramic junction reference electrode or a microcombination electrode. Fisher Certified buffers of nominal pH values 4.00, 7.00, and 10.00 were used for calibration. The exact pH of each buffer was established, and periodically checked, by comparison with freshly prepared N.B.S. pH standard solutions.[15] For pH measurements made on D_2O solutions, the pH meter was calibrated with aqueous buffers. The pH meter readings were converted to pD values with the relation pD = pH meter reading + 0.40.[15]

NMR Measurements. In the GSH and ergothioneine binding studies, ^1H NMR spectra were obtained on a Varian A60D spectrometer at a probe temperature of 25 ± 1 °C by procedures described previously.[8] In the hemoglobin binding study, ^1H NMR measurements were made at 400 MHZ and 25 °C on a Bruker WH-400/DS spectrometer operating in the pulsed Fourier transform mode. The ^1H NMR spectrum of a solution containing similar concentrations of hemoglobin, GSH, and CH_3Hg^{II}, as measured by the standard single-pulse sequence, is dominated by the multitude of overlapping resonances from hemoglobin, with the resonances from GSH buried under the hemoglobin resonances. To partially eliminate the interfering hemoglobin resonance, we measured ^1H NMR spectra by a technique in which they are selectively suppressed with a presaturation rf pulse.[9] The presaturation pulse was applied to hemoglobin resonances at 8.1 ppm (outside the chemical shift region of interest) for 2 s, during which time the saturation is transferred to other hemoglobin resonances by cross relaxation.

Results

CH_3Hg^{II}–GSH Complex. In solutions containing sulfhydryl ligands and CH_3Hg^{II} with sulfhydryl groups in excess, CH_3Hg^{II} binds exclusively to sulfhydryl groups.[14] Formation constants for the binding of CH_3Hg^{II} by the sulfhydryl group of GSH were determined by the method described previously in which two sulfhydryl molecules compete for the CH_3Hg^{II}.[8] Mercaptoacetic acid (MAA) was used as the competing thiol. Chemical shift data are shown in Figure 1 for MAA in (A) a solution containing only free MAA, (B) a solution containing only the CH_3Hg^{II}–MAA complex, and (C) a solution containing MAA, GSH, and CH_3Hg^{II} in a 1:1:1 mole ratio. A single averaged resonance is observed at all pH values for the three solutions, indicating fast exchange of free MAA (curve A) and sulfhydryl-complexed MAA (curved B) among its various protonated forms as well as between its free and complexed forms (curve C). Curve C lies between curves A and B, indicating displacement of some of the complexed MAA by GSH. The position of curve C relative to curves A and B is pH dependent, indicating the relative affinities of MAA and GSH for CH_3Hg^{II} to be pH dependent.

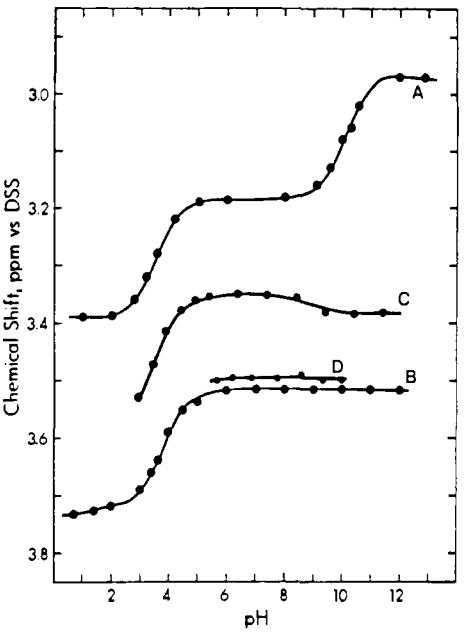

Figure 1. pH dependence of the chemical shift of MAA in solutions containing (A) 0.100 M MAA; (B) 0.100 M MAA and 0.100 M CH_3Hg^{II}; (C) 0.100 M MAA, 0.100 M CH_3Hg^{II}, and 0.100 M GSH; (D) 0.100 M MAA, 0.100 M CH_3Hg^{II}, and 0.232 M ergothioneine. The solid lines are the theoretical curves calculated from equilibrium constants obtained from the data. Approximately one-half the experimental points are shown.

The fraction of MAA in the free form (P_f) was calculated as a function of pH by using the chemical shift data in curve C and eq 2, where δ_{obsd} is the observed chemical shift at a particular pH

$$P_f = (\delta_{obsd} - \delta_c)/(\delta_f - \delta_c) \quad (2)$$

(curve C) and δ_f and δ_c are the chemical shifts of free and complexed MAA at that pH (curves A and B, respectively). The P_f vs. pH data were then computer fitted by using the nonlinear least-squares curve-fitting program KINET[16] to obtain K_d, the displacement equilibrium constant (eq 1) when GSH and MAA are both in their fully deprotonated forms, and the acid dissociation constants of the ammonium and carboxylic acid groups of CH_3Hg^{II}-complexed GSH (Figure 2). K_d was found to be 0.12 ± 0.002, $pK_2' = 3.63 \pm 0.04$, and $pK_3' = 9.25 \pm 0.02$. From this value for K_d, the formation constant for the MAA complex,[8] and k_{1234} and K_3', values of 16.00 and 15.85 were obtained for log K_f and log K_f', respectively. Literature values[17] for the macroscopic and microscopic acid dissociation constants were used in the curve fitting calculations.

CH_3Hg^{II}–Ergothioneine Complex. The structure of ergothioneine is shown in Figure 3, where its tautomerization/acid dissociation/CH_3Hg^{II} complexation equilibria in the pH region studied here are summarized. Due to tautomerization at the sulfur atom, K_2 is not strictly a dissociation constant per se but also includes the thiol/thione equilibrium constant embedded in it. pK_2, corrected to an ionic strength of 0.3, is 10.40 ± 0.04.[18]

The formation constant for the CH_3Hg^{II}–ergothioneine complex was also measured by competing ergothioneine and MAA for CH_3Hg^{II}. Due to the weaker binding by ergothioneine, a MAA/ergothioneine/CH_3Hg^{II} ratio of 1:2.32:1 was used. Curve D in Figure 1 is the chemical shift of the MAA resonance as a function of pH. The very small displacement of curve D from curve B indicates the affinity of ergothioneine for CH_3Hg^{II} to be small relative to that of MAA. Using the curve fitting procedure described above, we calculated K_d (eq 1) to be $(6 \pm 2) \times 10^{-4}$, from which log K_f is calculated to be 13.7 ± 0.2.

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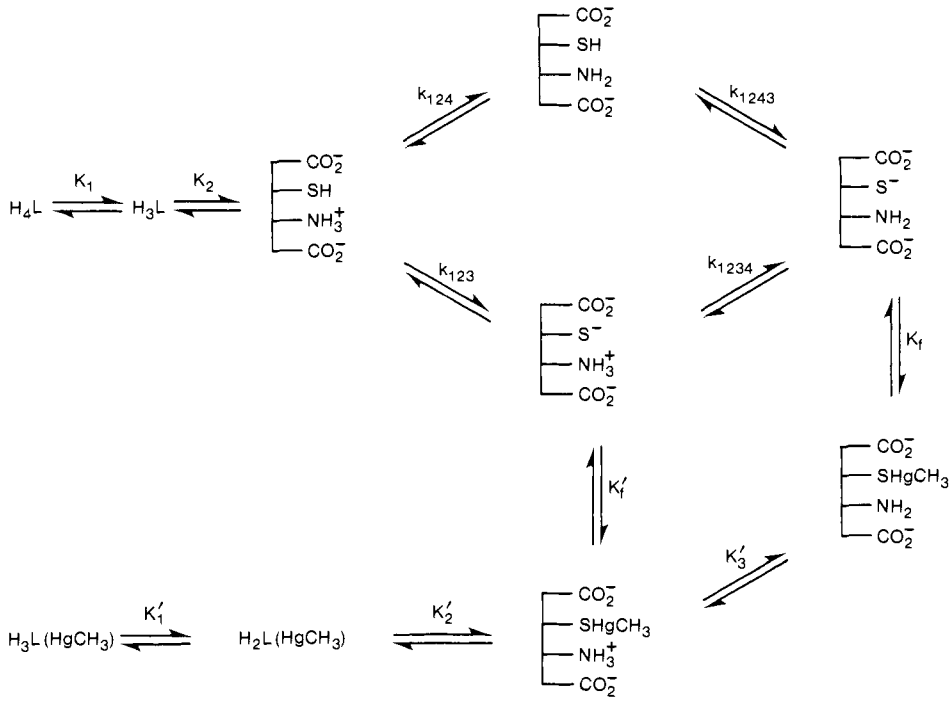

Figure 2. Microscopic acid dissociation and CH_3Hg^{II} complexation scheme for GSH.

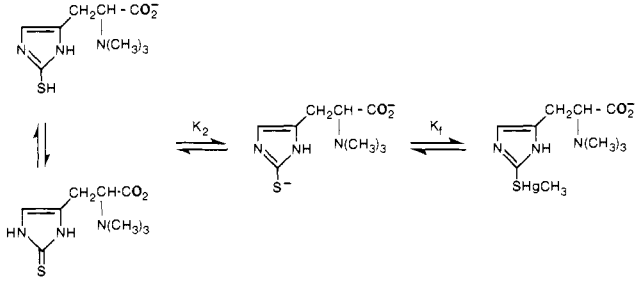

Figure 3. The tautomerization, acid–base, and CH_3Hg^{II} complexation equilibria of importance for ergothioneine in the pH region 5–10.

CH_3Hg^{II}–Hemoglobin Complex. The conditional formation constant for the hemoglobin complex in pD 7.4 phosphate buffer was determined by a procedure similar to that described above, with GSH as the competing ligand. GSH was used since it could be predicted from the results of the previous study of CH_3Hg^{II}-containing erythrocytes[6] that hemoglobin would displace some of the GSH from its CH_3Hg^{II} complex and that the fraction of GSH in the complexed form could be determined from the exchange-averaged chemical shift of the resonance for the methine proton on the α-carbon of the cysteinyl residue. The interfering hemoglobin resonances were selectively suppressed with the saturation method described in the Experimental Section to observe this resonance in the presence of hemoglobin. The spectrum obtained by this method for a solution containing 3.56 mM GSH, 1.82 mM hemoglobin, 2.2 mM ergothioneine, and ~0.5 mM tetramethylammonium (TMA) nitrate is shown in Figure 4. The TMA was added as a chemical shift reference; the ergothioneine was added as an indicator of when the total CH_3Hg^{II} concentration was greater than the total sulfhydryl concentration. Resonance assignments are given in Table I.

Resonance g5 shifts to higher frequency when the sulfhydryl group of GSH is complexed by CH_3Hg^{II}, as shown in Figure 5. The fraction of GSH free from and the fraction complexed by CH_3Hg^{II} were calculated from the chemical shift of resonance g5 by using equations similar to eq 2. The fraction complexed is shown as a function of the total CH_3Hg^{II} concentration in Figure 6. The plot clearly displays a downward curvature, indicating that GSH has a higher affinity for CH_3Hg^{II} than does hemoglobin under these conditions. For comparison, the insert diagram shows the plots expected for GSH binding (relative to hemoglobin sulfhydryl binding) (a) stronger by a factor of 10^3, (b) stronger by a factor of 5, (c) exactly as strong, (d) weaker by a factor of

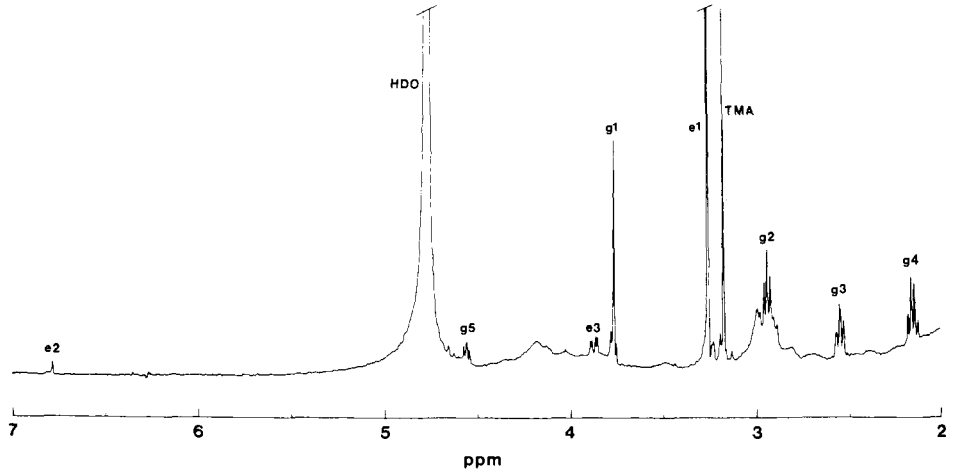

Figure 4. ^1H NMR spectrum (400 MHZ) for a solution containing 1.82 mM hemoglobin, 3.56 mM GSH, 2.2 mM ergothioneine, and ~0.5 mM tetramethylammonium nitrate in pD 7.4 phosphate buffer. Interfering hemoglobin resonances were eliminated by the presaturation method.[9]

Table I. Assignment of Resonances in Figure 3

peak designation	chemical shift (ppm) vs. DDS	assignment[a]
g1	3.76	C(α) of Gly residue of GSH
g2	2.92	C(β) of Cys residue of GSH
g3	2.55	C(γ) of Glu residue of GSH
g4	2.15	C(β) of Glu residue of GSH
g5	4.55	C(α) of Cys residue of GSH
e1	3.25	C of methyls on quaternary nitrogen of ergothioneine
e2	6.77	C(4) of ergothioneine
e3	3.9	C(β) of ergothioneine
TMA	3.176	methyl carbons of tetramethylammonium ion

[a] Position in molecule to which ^1H is attached.

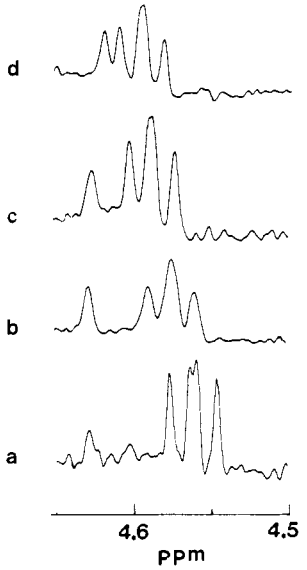

Figure 5. Resonance g5 for GSH in solutions containing CH_3Hg^{II} and equal concentrations of GSH and hemoglobin sulfhydryl at CH_3Hg^{II} to total sulfhydryl ratios of (a) 0.00, (b) 0.231, (c) 0.463, (d) 0.695. Interfering hemoglobin resonances were eliminated by the presaturation method.[9]

5, and (e) weaker by a factor of 10^3 for the same relative GSH and hemoglobin concentrations as in the experimental data. The equilibrium constant for reaction 3[19] was calculated as follows.

$$HbSH + GSHgCH_3 \rightleftarrows HbSHgCH_3 + GSH \quad (3)$$

If C is defined as the total GSH concentration (all forms), which is also the total hemoglobin sulfhydryl concentration for the conditions used, XC as the CH_3Hg^{II} concentration, and P_C as the fraction of GSH complexed, then

$$[GSHgCH_3] = P_C C \quad (4)$$

and

$$[GSH] = (1 - P_C)C \quad (5)$$

From the mass balance for CH_3Hg^{II}

$$[HbSHgCH_3] = (X - P_C)C \quad (6)$$

and from the mass balance for hemoglobin sulfhydryls,

$$[HbSH] = (1 - X + P_C)C \quad (7)$$

By combining eq 4–7, we can express the equilibrium constant for reaction 3 as

$$K = \frac{(X - P_C)(1 - P_C)}{P_C(1 - X + P_C)} \quad (8)$$

The data were fitted to this equation with KINET.[16] A value of 0.15 ± 0.01 was obtained for K_1, indicating that the conditional formation constant for the GSH complex at physiological pH is

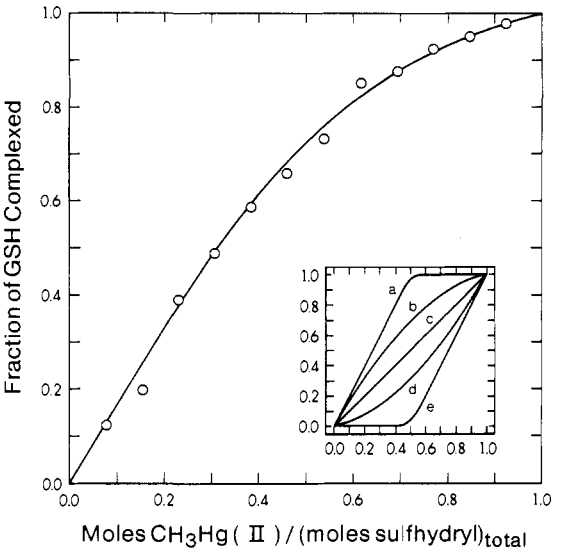

Figure 6. Fraction of the total GSH complexed by CH_3Hg^{II} in a solution containing equal concentrations of GSH and hemoglobin as a function of the CH_3Hg^{II} to total sulfhydryl ratio. The curve drawn through the points is the theoretical curve calculated by using the equilibrium constant obtained for reaction 3 from the data. The inset shows the expected curves for equilibrium constants of (a) 10^{-3}, (b) 0.2, (c) 1.0, (d) 5, (e) 10^3.

6.7 ± 0.4 times higher than that for the complex with a hemoglobin sulfhydryl.

Discussion

The nature of the complexation of CH_3Hg^{II} by GSH is strongly pH dependent. Not only do four complexes form that differ in the protonation state of the carboxylate and amino groups but protonation of the sulfhydryl group and complexation of CH_3Hg^{II} by hydroxide ion also complete[14] with the binding of CH_3Hg^{II}. The formation constants K_f and K_f' provide a measure of the stability of the CH_3Hg^{II} complexes of the fully deprotonated and amino-protonated forms of GSH (Figure 2) and can be used along with similar constants for other complexes[8] to elucidate factors affecting the intrinsic strength of CH_3Hg^{II} binding by sulfhydryl groups. For example, K_f' is less than K_f. Since pk_{123} is less than pk_{1243}, this is consistent with the dependence of the formation constant on the Brønsted basicity of the deprotonated sulfhydryl group.[8] However, because of the competing pH-dependent reactions, it is more convenient to discuss the tendency for complexation to occur under a particular set of conditions in terms of a conditional formation constant K_{fc} for the complexation reaction written as

$$CH_3Hg_f + GSH_f \rightleftarrows CH_3HgSG_{total}$$

where CH_3Hg_f and GSH_f include all free forms of CH_3Hg^{II} and GSH, and CH_3HgSG_{total} all forms of the GSH complex.[14]

Because of the competing protonation of the sulfhydryl group and complexation of CH_3Hg^{II} by hydroxide ion, K_{fc} is orders of magnitude less than K_f over the entire pH range. For example, at pH 7.4, log K_{fc} is calculated to be 11.55, which still is sufficiently large that in solutions containing GSH in excess of CH_3Hg^{II}, all the CH_3Hg^{II} is complexed by GSH. At pH 7.4, log K_{fc} for the ergothioneine complex is 7.9.

The conditional formation constant for the hemoglobin complex at pD 7.4 is found experimentally to be 6.7 times less than that for the GSH complex. With the above value for K_{fc} for the GSH complex, log K_{fc} for the hemoglobin complex is calculated to be 10.7. No literature value could be found for comparison; however, the conditional formation constant for the CH_3Hg^{II} complex of bovine serum albumin (BSA) can be estimated from the data reported by Berg and Miles.[20] In a mixture containing 16.7 mM

(19) HbSH represents hemoglobin sulfhydryl, the concentration of which was determined as described in the Experimental Section.

CH_3Hg^{II}, 5.9 mM BSA, and 18.5 mM cysteine, it was estimated by exclusion chromatography that 46% of the BSA was complexed. From mass-balance relationships and the K_{fc} for the CH_3Hg^{II}–cysteine complex, log K_{fc} for the CH_3Hg^{II}–BSA complex is calculated to be 11.0. This value and the K_{fc} for the hemoglobin complex are both smaller than the conditional formation constants for CH_3Hg^{II} complexes with a variety of monothiols (ranging from 11.1 to 11.7). Because of the dependence of K_{fc} on the acid–base properties of the sulfhydryl groups, which have not been quantitatively characterized for hemoglobin or BSA, it is not possible to draw any conclusions regarding the intrinsic strength of the CH_3Hg^{II}–protein binding.

For purposes of elucidating the chemistry of CH_3Hg^{II} toxicology, however, the conditional formation constants at pH 7.4 are the quantities of interest; the relative magnitudes of the K_{fc} values indicate the relative affinities of the ligands for CH_3Hg^{II}. The conditional formation constants combined with the normal levels of GSH, hemoglobin, and ergothioneine in human erythrocytes[21] lead to the prediction that of the CH_3Hg^{II} in human erythrocytes, 59% will be complexed by GSH, 41% by hemoglobin, and 0.001% by ergothioneine. This prediction is in agreement with results from ^1H NMR measurements[6] on intact human erythrocytes containing CH_3Hg^{II} and gel-filtration studies of CH_3Hg^{II} in hemolyzed erythrocytes.[22]

It is of interest to also compare these conditional formation constants to those of complexes with other biological molecules containing sulfhydryl groups and with sulfhydryl molecules used as antidotes for CH_3Hg^{II} poisoning.[8] Log K_{fc} values at pH 7.4 for CH_3Hg^{II} complexes with other biological molecules are cysteine (11.57) and homocysteine (11.15) and with antidote molecules are mercaptosuccinic acid (11.68), penicillamine (11.33), and *N*-acetylpenicillamine (11.20). K_{fc} for the hemoglobin complex is somewhat less than those of the complexes with the other biological thiols and the antidote molecules. K_{fc} for the GSH complex is similar to that of the cysteine complex; however, GSH is much more abundant than free cysteine in cellular systems.[4] K_{fc} for the mercaptosuccinic acid complex is larger than that for the GSH complex, which correlates with the greater effectiveness of mercaptosuccinic acid as compared to that of penicillamine as an antidote for CH_3Hg^{II} poisoning in animal studies.[23]

The method used to measure K_{fc} for the CH_3Hg^{II} complex of hemoglobin is direct, precise, and should be applicable to the measurement of other metal–protein binding constants. The basis of the method is the measurement of the extent of complexation of a small ligand in the presence of the macromolecule. The key is the selective observation of resonances from the small ligand, which can be done either with the method used here[9] or with the spin–echo Fourier transform method.[11] If exchange of the small ligand between its free and complexed forms is fast on the NMR time scale, the extent of complexation of the small ligand can be obtained directly from the chemical shifts of exchange-averaged resonances. If exchange is slow, the extent of complexation can be obtained from the relative intensities of the free and complexed resonances. We are investigating the application of this method to other metal–protein systems.

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Registry No. GSH, 70-18-8; ergothioneine, 497-30-3.

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Kinetics of the Protonation of Buffer and Hydration of CO_2 Catalyzed by Human Carbonic Anhydrase II

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Abstract: The interaction of external buffer with human carbonic anhydrase II in the catalyzed hydration of CO_2 has been examined for several cationic and zwitterionic buffers over the pH range 6.8–9.0. The rate of change of absorbance of a pH indicator, measured by stopped-flow spectrophotometry, was used to determine initial velocities. We found that the rate-limiting proton transfer between human carbonic anhydrase II and external buffer is dependent on the pK difference between donor and acceptor species in a manner consistent with proton transfer between small molecules. The rate constant for the proton transfer follows a Brønsted curve that reaches a plateau at a value of 1×10^9 M^{-1} s^{-1}, very close to that found for proton transfer between small molecules. The transition region of the Brønsted plot indicates that the donor group on the enzyme has a pK_a = 7.6 ± 0.6, which is consistent with proton transfer directly between the active site and buffer or between a proton shuttle group on the enzyme of pK_a near 7 and buffer.

Carbonic anhydrase is a zinc-containing enzyme that catalyzes the reaction shown in (1), the hydration of CO_2 to produce bicarbonate and a proton.[1] Under physiological conditions the

$$CO_2 + H_2O \rightleftharpoons HCO_3^- + H^+ \quad (1)$$

turnover number for CO_2 hydration catalyzed by human carbonic anhydrase II (the high activity isozyme) is large, near 10^6 s^{-1}. Since the catalyzed reaction produces a proton, it is necessary that a proton be transported out of the active site at a rate at least as rapid as the hydration rate. The rapid rate of proton transfer from the enzyme to solvent cannot be accounted for by proton transfer to water or OH^- at pH near 7, water because it is a poor proton acceptor and OH^- because it is not present at sufficient concentration. It has been pointed out that removal of this proton from the enzyme may be accomplished mainly by buffers in

(1) Pocker, Y.; Sarkanen, S. *Adv. Enzymol. Relat. Areas Mol. Biol.* **1978**, *47*, 149–274.

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