Kinetic Study of the Interaction of Methylmercury with the $Fe_2S_2(SR)_4$ Cluster of Adrenodoxin

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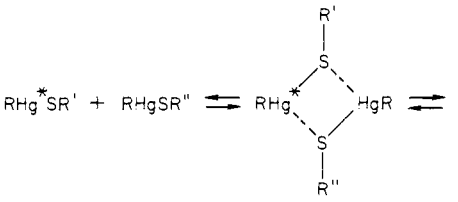
Abstract: A kinetic study of the rate of reaction of the $Fe_2S_2(SR)_4$ cluster of adrenodoxin with CH_3HgOAc and $CH_3HgS-CH_2COOK$ is described. The rate of sulfur extrusion was found to be much faster for methylmercury acetate than for the sulfur-bound CH_3Hg^{II}. A mechanism is suggested that involves bonding of CH_3Hg^{II} at labile sulfur with rate-limiting Fe–S bond rupture.

Since the discovery that many forms of Hg(II) are ultimately converted to CH_3Hg^{II} in the biological cycle,[1] considerable effort has been expended to disentangle the chemical basis for mercury poisoning.[2] The avidity with which alkyl mercurials complex with the sulfhydryl (mercaptan) functional group indicates that essentially all the CH_3Hg^{II} in living systems will be bound to sulfur. For example, the thermodynamic stability of methylmercury mercaptides (CH_3HgSR) is reflected in a formation equilibrium constant of $K_{stab} = 10^{22}$ for mercaptoalbumin[3a] and 10^{16} for cysteine.[3b] Rapid ligand-exchange reactions, which are the key to the bioavailability of alkyl mercurials, are particularly important in the initial stages of toxicity when the RSH groups in blood are present in a large excess relative to the concentration of CH_3Hg^{II}. Anionic exchange of RS– with CH_3HgX is very facile while the comparable ligand exchange with RSH has been shown to be slow on the NMR time scale.[4,5] A second important mechanistic pathway for methylmercury migration involves mercaptide anion exchange in $RHgSR'/RHgSR''$ systems (eq 1).[5] Our NMR



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$$RHg^{*}SR'' + RHgSR' \quad (1)$$

kinetic data provided the first example of bimolecular anion exchange of an alkyl mercurial with the total exclusion of an ionic mechanism. The free energy of activation for ligand exchange was only 5.2 kcal/mol at −138 °C. More recently, we have provided NMR data which established the mechanism of disulfide cleavage by CH_3Hg^{II} to involve a concomitant electrophilic and nucleophilic process.[6]

Ferredoxins (FD) form a class of nonheme iron proteins which are involved in electron-transport reactions that are fundamental to reductive carboxylation, nitrogen fixation, and hydroxylation reactions.[7] Adrenodoxin (AD), which plays a role in the oxidation–reduction process in adrenal mitochondrial steroid hydroxylation, is a single polypeptide chain containing a Fe_2S_2* redox center and 114 amino acid residues,[8] including five cysteine residues per molecule. The iron–sulfur center may be stabilized by chelation and shielding by the protein, which has a marked effect upon the chemical accessibility of the labile Fe_2S_2* core. These highly reactive "labile" sulfurs in iron–sulfur clusters will react with nucleophiles such as triphenylphosphine, extracting labile sulfur quantitatively,[9] and with electrophiles like *p*-chloromercuribenzoate (PCMB).[10] Yoch and Arnon[10d] have reported the increasing relative rates of titration of *Azotobacter vinelandii*, clostridial and spinach FD's, respectively, with PCMB, which presumably reflects the accessibility of the sulfur moiety to electrophilic attack by mercury. Kinetic studies on the dissolution of Fe_4S_4* core ions of several ferrodoxins have also provided a mechanistic rationale for the pH dependence of reaction of these tetrahedral iron complexes with a proton.[11a] Synthetic analogues of the active sites of these iron–sulfur proteins have been prepared, and a detailed study of thiolate substitution reactions, where the core structures remain intact, has been reported.[11b] Despite the extensive use of organomercurials[10] as analytical reagents to measure the oxidation stoichiometry of the iron–sulfur cluster, we find no report of a kinetic investigation of the reaction of these highly reactive sulfur clusters with CH_3Hg^{II}. We now extend our mechanistic investigation of potentially significant biochemical reactions of heavy metals to include the interaction of CH_3Hg^{II} with the iron–sulfur cluster of native adrenodoxin.

Methods and Materials

Adrenodoxin (AD) was isolated from bovine adrenal cortex as previously described.[8a,13] A purity criterion of an absorbance ratio $A_{414}/A_{276} = 0.86$ was utilized, and the protein samples were established to be homogeneous by electrophoresis in a 10% polyacrylamide gel in the presence of dodecyl sulfate. The molar extinction coefficient of 9800 M^{-1} cm^{-1} at 414 nm was used for adrenodoxin. Optical absorbance was measured with a Cary Model 118 spectrophotometer. Studies on temperature dependence were carried out with a thermostated cell holder. Other reagents were obtained from commercial sources.

Results and Discussion

Our mechanistic probe was designed to compare the reactivity of adrenodoxin toward a relatively ionic mercurial like CH_3HgOAc ($\log K_{stab} = 3.6$) to that of the highly covalent sulfur-bound methylmercury derivative CH_3HgSCH_2COOH ($\log K_{stab} = 14$–16).[5] This study is of particular relevance to the toxicity of CH_3Hg^{II} since it is highly probable that most in vivo reactions

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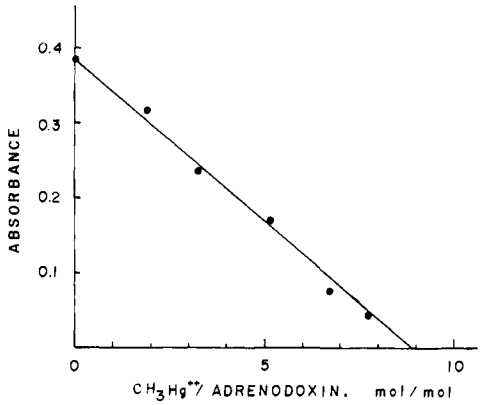


Figure 1. Titration of adrenodoxin with CH_3HgOAc. The reaction mixture contained 3.88×10^{-5} M AD in 10 mM sodium phosphate buffer, pH 7.4. The absorbance at 414 nm was recorded after each addition of CH_3HgOAc at 22 °C.

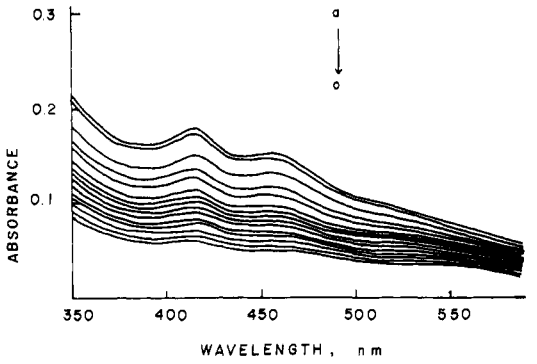


Figure 2. Spectral changes of adrenodoxin upon treatment with CH_3HgSCH_2COOK. The reaction mixture contained 1.84×10^{-5} M AD in 10 mM sodium phosphate buffer, pH 6.4. The reaction was started by the addition of 1 mM CH_3HgSCH_2COOK at 22 °C. Curves a to o represent the reaction times of 0, 1, 12, 25, 43, 57, 75, 91, 110, 138, 159, 207, 241, 282, and 290 min, respectively.

of vital organs with alkyl mercury actually involve an alkyl mercury mercaptide. Secondly, the difference in softness of the two mercurials will provide an indication of the relative selectivity of biological binding of these heavy-metal compounds. Both reagents are soluble at physiological pH and should be sufficiently lipid soluble to readily penetrate the protein and interact with the active site.

Native AD reacted rapidly and quantitatively with CH_3HgOAc at pH 7.4 (10 mM Na, K phosphate buffer) at 22 °C. A plot of [CH_3HgOAc/AD] vs. absorbance at 414 nm (Figure 1) was linear for the first 8 equiv, which were consumed within a 15-s period. A ninth equivalent was consumed more slowly, suggesting that protein penetration may be rate limiting for reaction of the sulfhydryl group of the fifth cysteine residue.

In dramatic contrast to the highly dissociated CH_3HgOAc, methylmercury 2-thioacetic acid (**2**) reacts very slowly with AD and took 5 h for complete extrusion of iron and sulfur from AD at 22 °C (pH 6.4) (Figure 2). Partial unfolding of the polypeptide chain, with the chromophore remaining intact, was achieved by adding the denaturants 4 M urea and 1 M KCl. The rate of reaction with CH_3HgSCH_2COOH increased only slightly, suggesting an equally high collision frequency of the methylmercury mercaptide with the active site that is unimpeded by the tertiary structure of the protein. Under these conditions, we found it possible to follow the progress of this reaction over the pH range 6.3–8.3 without seriously denaturing the protein.

A quantitative measure of the rate of chromophore disappearance was carried out at several temperatures to measure the activation parameters for the reaction of AD with a sulfur-bound mercurial. The rate of electrophilic attack by **2** at labile sulfur was measured under pseudo-first-order conditions with an excess of CH_3HgSCH_2COOK in a 10 mM sodium phosphate buffer

Table I. Effect of [AD] on the Rate Constant for Chromophore Extrusion

10^5[AD], M	$10^3 k_{obsd}$ s^{-1}
1.60	1.53
1.37	1.63
1.14	1.48
0.91	1.82

Table II. Activation Parameters for the Reaction of AD with with CH_3HgSCH_2COOK[a]

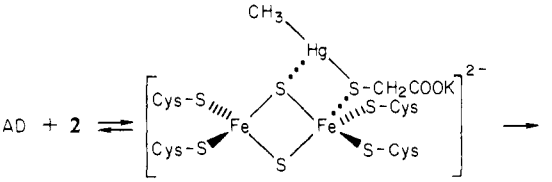
reaction conditions	$\Delta G^{\ddagger}$,[d] kcal/mol	$\Delta H^{\ddagger}$, kcal/mol	$\Delta S^{\ddagger}$,[d] kcal/mol/deg	temp range, K
no urea or 1 M KCl	17.4	10.3	−24	291-314
4 M urea + 1 M KCl[c]	16.9	7.8	−31	283-299

[a] Rates were measured in 10 mM sodium phosphate buffer at pH 7.4. [b] The experiments used 8.78×10^{-6} M AD with $(0.89\text{–}3.57) \times 10^{-3}$ M [CH_3HgSCH_2COOK]. Four different concentrations were used at each of the four temperatures. [c] The concentration of AD = 2.20×10^{-5} M in the presence of 1 M KCl. [d] At 295 K.

solution (pH 7.4) containing 4 M urea–1 M KCl at 23 °C. The rate constant for sulfur replacement was found to be invariant (within experimental error) to the initial adrenodoxin concentration when [CH_3HgSCH_2COOK] = 1.04×10^{-3} M (Table I).

Having established the validity of the rate expression, rate = k_{obsd}[AD] where $k_{obsd} = k_1$[CH_3HgSCH_2COOK], we next examined the temperature dependence on the rate of reaction in the presence and absence of a denaturant at four different temperatures. A linear relationship between ln (k/t) and $1/T$ is obtained for these reaction conditions. The activation parameters given in Table II suggest that the denaturant, which relaxes the tertiary structure of the protein, does not have a marked effect upon the rate at which the mercurial can penetrate the interior of the protein. In contrast, the reactivity of triphenylphosphine with AD was significantly increased by the presence of the denaturants, 1 M KCl and 4 M urea.[9] The high negative entropy is consistent with a bimolecular mechanism. Solvation effects are probably at a minimum since previous ENDOR measurements with solid, aqueous, and deuterated samples of AD indicated that the immediate environment of the Fe_2S_2* core is deficient in water molecules.[14]

In our prior NMR studies,[5] we established that direct exchange of ligands in CH_3HgX/CH_3HgY mixtures proceeded through a four-center transition state as suggested in eq 1. The rate of exchange was shown to increase with *increasing* thermodynamic stability of CH_3HgX. Thus, we observed the fastest rate of anion exchange when X and Y were both sulfhydryl ligands. We attributed this reactivity trend to the more effective bridging of the sulfur ligand between the two metals (eq 1). If such a concerted four-center mechanism is occurring in reactions of AD with CH_3HgX (eq 2), then one would anticipate a rapid reaction with



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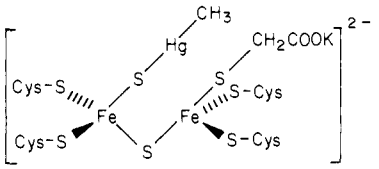

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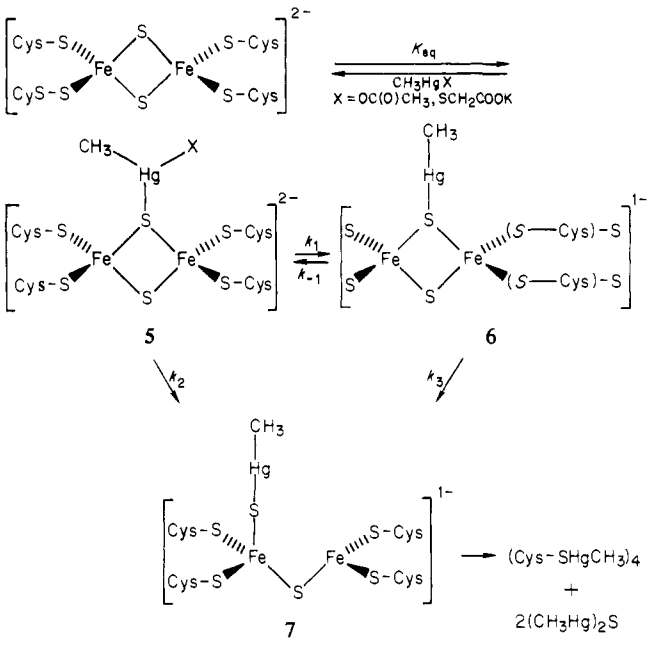
Scheme I



the sulfur-bound mercurial CH_3HgSCH_2COOH. Since we note the *opposite* behavior in the present study, we conclude that the relative electrophilicity of the attacking mercurial is more important than the bridging capacity of the ligands. These observations tend to preclude a concerted four-center transition state such as **3** and suggest an intermediate like **5** where the bioligand is initially bonded to CH_3HgX at the more nucleophilic labile sulfur (Scheme I). The facile extrusion of sulfur from AD with CH_3HgOAc is consistent with a rapid preequilibrium where the effective concentration of complex **5** would be much higher for the more ionic mercurial CH_3HgOAc.[15] In aqueous medium, complex **6** may arise directly either by collision with CH_3Hg^+ or by loss of acetate anion from **5**. Our kinetic data do not allow us to make this distinction. However, with CH_3HgSCH_2COOK, prior ionization to CH_3Hg^+ would be highly improbable, and the rate-limiting step in the reaction should depend upon the ratio k_2/k_1. It seems unlikely that simple ionization with loss of RS^- from **5** (k_1) would be either rapid or reversible in the hydrophobic environment of the iron–sulfur chromophore. We therefore suggest a rate-limiting Fe–S bond rupture in **5** with concerted loss of RS^- (k_2) and subsequent rapid stepwise extrusion of the remaining mercaptides in **7** as a consequence of the disruption of the core ion stability afforded by the intercluster electron delocalization.

In conclusion, we have provided kinetic data which provide a clear distinction between a concerted-type extrusion pathway and one that proceeds by initial attack of CH_3Hg^{II} at nucleophilic sulfur. The observed reaction of an alkyl mercury mercaptide with the iron–sulfur cluster provides yet another demonstration of the tenacity with which mercury bonds to sulfur, providing a target for methylmercury poisoning.

Acknowledgment. We gratefully acknowledge support from the National Institutes of Health (ES 00761 07 and AM 12713-11).

(15) The equilibrium constant for complexation of CH_3SCH_3 with CH_3HgOAc in CH_2Cl_2 is surprisingly small, $K_f = 0.04$. However, complexation of dimethyl sulfide with CH_3HgSCH_3 was too small to measure by our highly sensitive ^{199}Hg NMR method. Since we see no discernable change in the mercury resonance upon addition of excess dimethyl sulfide, we suggest that K_f is at least two orders of magnitude lower than that with CH_3HgOAc (unpublished results).

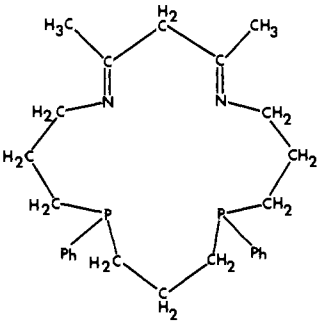
Communications to the Editor

First Synthesis, Characterization, and X-ray Structural Determination of the Macrocyclic Phosphineamine Complex $[Ni(Me_2[16]dieneN_2P_2)](PF_6)_2 \cdot 0.5H_2O$

Sir:

The chemical literature is replete with examples of macrocyclic metal complexes containing tetradentate ligands with N_4, O_4, N_2O_2, and N_2S_2 donor sets.[1,2] Similar complexes with PN_3, P_4, and P_2S_2 type ligands are few in number and have been reported only recently.[3-8] To date, the only method reported for the synthesis of complexes with any mixed macrocyclic P–N ligands has involved refluxing 2,6-diacetylpyridine with the required phosphinodiamine and metal salt.[3,9]

In this communication, we report the synthesis of the first metal complex containing a cyclic N_2P_2 Schiff base ligand, 14,16-dimethyl-5,9-diphenyl-5,9-diphosphino-1,13-diazacyclohexadeca-13,16-diene, hereafter abbreviated $Me_2[16]dieneN_2P_2$.[10] The ligand has two phosphorus and two nitrogen donor atoms equally distributed along the 16-membered ring as shown in I.



I

The macrocyclic structure has been verified by single-crystal X-ray diffraction analysis of the hydrated Ni(II) complex, $[Ni(Me_2[16]dieneN_2P_2)](PF_6)_2 \cdot 0.5H_2O$. This work represents the first X-ray structure determination on any metal complex containing a P–N macrocyclic ligand. Preliminary to the preparation of this 16-membered ring complex, a 14-membered ring complex, $[Ni(Me_2[14]dienatoN_2P_2)] \cdot PF_6$, was prepared as well as two 5-coordinate chlorobis(tertiary phosphino)diamine metal complexes of cobalt(II) and nickel(II). Structures of the latter

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