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A Quantum-Mechanical Study of Lead Coordination in Sulfur-Rich Proteins: Mode and Structure Recognition in UV Resonance Raman Spectra

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Abstract

Resonance Raman spectra are computed applying the weighted gradient methodology with CIS and DFT gradients to determine the characteristic spectral patterns for Hg(II) and Pb(II) loaded sulfur-rich proteins while excited to a characteristic LMCT electronic transition band. A framework of structure-spectrum relationships is established to assess lead coordination modes via vibrational spectroscopy. Illustrative calculations on Hg(II) complexes agree with experimental data demonstrating reliability and accuracy of the applied methodology. In contrast to Hg(II) complexes, a unique 3-center-4-electron hypervalent C_6H_5-S interaction present in lead-sulfur complexes was established and suggested to play a key role in the strong preference for lead versus other metal ions in lead specific proteins such as PbrR691. The characteristic Pb-S symmetric stretching bands, predicted without additional refinements such as scaling of a force field or frequencies, are found around 238 cm^{-1} for 3-coordinated lead-sulfur domains, and around 228 cm^{-1} for 4-coordinated lead-sulfur domains. These results present an experimental challenge for clear detection of lead coordination via solely UVRR spectroscopy. In addition to predicted UVRR spectra, UVRR excitation profiles for relevant vibrational bands of lead-sulfur domains are presented.

Introduction

The commonly accepted view on lead poisoning attributes toxic effects to an unanticipated change in coordination preferences forced by the entry of a poisonous ion. Consequently, this new environment frequently imposes structures that do not stabilize the proper protein forms and effectively disrupt their functions in living organisms¹. Indeed, divalent lead exhibits a rich and interesting coordination chemistry which has been studied both experimentally and theoretically and is frequently discussed in light of the structural effects of the chemically inert but stereochemically active $6s^2$ lone pair orbital². Complexes with high coordination numbers (more than 8) usually adopt a homodirected (symmetrical) geometry, whereas complexes with low coordination numbers (less than 6) are often a hemidirected (asymmetrical) geometry where ligand displacement is likely due to increased effects of the inner lone pair orbital. Recent quantitative analysis on lead(II) coordination in proteins listed in the Protein Data Bank suggests that oxygen, sulfur and nitrogen are the most common donor atoms³. Domains with highest coordination numbers, six to eight have

Associated Content

Supporting Information. Tables of calculated electronic excitations, intensities and individual contributions to weighted gradients at CIS and TD-BP86 levels of theory. Comparison plots of computed UVRR spectra and excitation profiles of selected bands. This material is available free of charge via the Internet at <http://pubs.acs.org>.

been reported mainly for oxygen atoms. However, when lead binds to sulfur-rich proteins, it typically coordinates only three sulfurs to form the binding site⁴. Originally it was suggested that lead in poisoned proteins may coordinate four sulfur atoms since it was observed that Pb(II) commonly targets the tetrahedral domains of Zn(II) found in zinc finger proteins⁴. Although, both 3- and 4-coordinations of lead are potentially equally disrupting and poisonous, a specific identification of coordination and possible structural variations of protein lead domains remain unknown. Hence, structural and spectroscopic detection followed by full characterization of lead poisoned sites should play a key role in understanding the molecular mechanisms of lead poisoning.

Presently, the only known lead(II)-specific binding protein found in nature is PbrR691 from *Ralstonia* (or *Cupriavidus*) *metallidurans* CH34 or its homologues in the same bacterium⁵. The protein belongs to the MerR family transcriptional factors that regulate the concentrations of a range of toxic or essential metal ions in bacteria. The prototype is the Hg(II)-binding MerR that uses three highly conserved Cys residues to selectively bind Hg(II) in proposed trigonal geometry. The sequence alignment of the PbrR691 indicates that the three Cys residues of MerR are conserved as Cys78, Cys113 and Cys122 in PbrR691 which have been suggested to form the metal binding pocket⁶. Recent investigations of the metal-binding site of lead(II)-loaded PbrR691 by extended X-ray absorption fine structure (EXAFS) spectroscopy showed the Pb(II) likely adopts a three-coordinate Pb-(S-Cys)₃-binding mode, with an average Pb-S distance of 2.67 Å⁶. However, the best spectral fits could not exclude a possible binding of the fourth ligand. Both models with a first-shell coordination of three or four sulfur atoms successfully fit the data with comparable error values and therefore give inconclusive results for lead coordination.

An alternative analytical probe for successful detection of specific coordination of lead in lead(II)-binding sites may be provided by vibrational spectroscopy. Especially ultraviolet resonance Raman (UVR) spectroscopy, with its known sensitivity of vibrational frequencies to molecular and electronic structure conferred by resonance enhancement, could be a sensible choice to extract structural details. Apart from immense UVR studies of the heme proteins and related metalloporphyrins, rubredoxin was one of the first biological molecules to which resonance Raman spectroscopy was applied to identify the metal-ligand stretching and bending modes and determine the tetrahedral coordination of the Fe(Cys)₄ domain⁷. Shortly after, the spectroscopic and structural characterization of [2Fe-2S], [4Fe-4S] and [3Fe-4S] cluster proteins successfully followed⁸. More recently, UVR experiments were successfully applied to monitor the histidine and cysteine ligand environment in copper-, cobalt- and cadmium-substituted zinc-binding peptides by resonant excitations to the ligand-to-metal charge transfer transitions⁹. Also, the mercury ligand environment was probed in the Hg(II)-loaded MerR protein¹⁰ and its structural models; an aliphatic 3-coordinated compound, [Hg(SBut)₃]⁻¹¹, and a Hg-dicysteamine complex, Hg(S-CH₂-CH₂-NH₂)₂¹¹.

In general, UVR spectroscopy is capable of monitoring the lead protein environment by utilizing the characteristic sulfur-to-lead charge transfer bands, showing up as a strong signal at approximately 260 nm or a moderated intensity signal at approximately 330 nm. However, no successful experiments have yet been reported for lead proteins since the experiment presents a few technical challenges¹². We wondered if such an experiment when effectively performed may be helpful in structural determination and clear identification of 3-coordinated vs. 4-coordinated Pb(II)-Cys structural sites. The present studies investigate whether, in principle, vibrational spectroscopy can distinctly and conclusively reveal the coordination number of lead(II)-binding sites in poisoned proteins. We are hopeful that the calculations presented here could be helpful in clarifying or solving some of the challenges,

which exist in performing a successful measurement and identification of UVRR lead signals in poisoned proteins.

Methods

All calculations reported here were carried out by the Gaussian 09 program package¹³. Molecular structures are found by full geometry optimization at the B3LYP/6-31G* level of density functional theory (DFT) in combination with cc-pVDZ-PP relativistic effective-core potential for lead to describe its core electrons along with the [4s, 3p, 2d] contracted Gaussians, correlation consistent compositions of Pb valence orbitals (5s²5p⁶5d¹⁰6s², i.e., 20 electrons for Pb²⁺ ion)¹⁴, and with SBKJC VDZ relativistic effective-core potential for mercury along with comparable [4s, 4p, 3d] contracted Gaussians, correlation consistent compositions of Hg valence orbitals (5s²5p⁶5d¹⁰, i.e. 18 electrons for Hg²⁺)¹⁵ that were treated explicitly in electronic structure calculations. Both basis sets were obtained from the Basis Set Exchange Library¹⁶. Computed frequencies of all structures are positive, indicating that the structures are at real minima of their ground-state potential-energy surfaces. Further refinement of DFT force constants and frequencies for Hg model compounds were achieved by employing scaled quantum-mechanical (SQM) formula¹⁷ that scaled solely Hg-S stretch force constants by a factor of 1.224. The value of 1.224 was determined by fitting DFT modeling to experimental data on Hg(S-iBut)₃[−] and Hg(SCH₂CH₂NH₂)₂ compounds. An analogous refinement of DFT force constants and frequencies for Pb models were not implemented. Hence, all computed spectra for lead models were reported with un-scaled DFT frequencies. Based on preliminary tests and previous SQM studies¹⁸ with heavy and transition metals, it may be expected that Pb-S stretching force constants calculated at applied level of theory are underestimated and scaling should increase the reported frequencies by at least 10–15% (a scaling factor estimated at least 1.10–1.15) to achieve the best correlation with plausible experimental data.

Besides Hg(S-iBut)₃[−] and Hg(SCH₂CH₂NH₂)₂ compounds used in modeling, two model ligands were chosen to imitate various Metal-(Cys)_n coordinations in proteins; a smaller, ethanethiolate (S-Et) ligand, and a larger, closer representing cysteine residue, S-Cys* ligand. The S-Cys* ligand, which is a modified cysteine (S-Cys) was used to avoid problems associated with amino acid zwitterions. In the S-Cys* ligand the hydroxy group and one of the hydrogen atoms on amino group of cysteine (two linkage groups in the formation of the protein's backbone) have been replaced by methyl groups. For illustration, 3-coordinated lead structures with S-Cys* ligands are shown in Scheme 2a.

Resonance Raman spectra of Hg compounds were computed based on the weighted-gradient approach which combines computed ground state geometry and force field with a manifold of excited state gradients¹⁹. The CIS calculations in combination with the same basis sets used for the ground state were employed for the 60 lowest electronic excitations and their gradients. In addition CIS computations were embedded in an aqueous environment mimicked by a polarizable cavity, i.e., the Polarizable Continuum Model (PCM)²⁰ as implemented in Gaussian 09. The weighting factors were found by applying the Lorentzian profile parameterized by a single half-bandwidth $\Gamma = 0.350$ eV, for all computed excitations. As expected due to known systematic errors associated with computation of UV spectra at the CIS level of theory, the CIS excitation energies are overestimated leading to lower computed wavelengths than experimentally observed. These errors do not contribute to the computed RR intensities since they depend only on the accuracy of the computed gradients. An analogous computational strategy to simulate the CIS-based UVRR intensities has been successfully applied in studies on aromatic amino acids¹⁹. It was demonstrated that an experimental wavelength of the excitation photon could be easily recalibrated by the 65-nm

down-shift to find the corresponding wavelength of the excitation on the CIS energy scale. Thus, the experimental excitations at 238 nm and 229 nm applied UVRR spectra for Hg compounds correspond to excitations at 173 nm and 164 nm in a CIS computed wavelength scale. Analogously, the predicted UVRR spectra for Pb corresponding to 260 nm are evaluated at a 195 nm excitation line on a CIS computed wavelength scale.

In addition to CIS-based calculations, time-dependent DFT (TDDFT) calculations using BP86 functional and PCM model were employed to predict UV spectra, UVRR excitations profiles for Pb-S stretching bands and UVRR spectra within 100 lowest electronic excitations for $\text{Pb}(\text{S-Cys}^*)_3^-$ and $\text{Pb}(\text{S-Cys}^*)_4^{2-}$ complexes. No scaling or excitation adjustments were utilized for TDDFT-based electronic spectra. Spectral plots and weighted factors for TDDFT excitations were determined by applying the Lorentzian profile parameterized by a single half-bandwidth $\Gamma = 0.200$ eV for all computed excitations. Comparison of UVRR spectra computed based on TD-DFT vs. CIS gradients is included in supplementary information (Figure S1).

Results and Discussion

Structure and coordination of Hg(II) model complexes

All structures of Hg(II) compounds used in the present study are shown in Scheme 1a–1b. The Hg-S and Hg-N bond lengths are selectively listed in Table 1. Optimized Hg-di-thiolate complexes are constrained to C_2 point group symmetry and Hg-tri-thiolate structures are constrained to C_{3h} point group symmetry, besides a $\text{Hg}(\text{S-Cys}^*)_3^-$ model that is constrained to C_3 symmetry with a nearly planar geometry around the metal. Also, other isomers of $\text{Hg}(\text{S-Cys}^*)_3^-$ with various special orientation of ligand chains were computed but the reported C_3 symmetry structure has been found as the most stable and might as well represent key structural features of the compound. The Hg-S bond lengths are invariable with size of sulfur-donor ligands, however the bond expands when the number of coordinated ligands is increased. On average, the computed Hg-S distance for two-coordinated Hg(II) compounds is around 2.41 Å and for three-coordinated Hg(II), it is approximately 2.54 Å. These values when compared to experimental data^{21–24}, are consistently overestimated by about 0.07 Å for two-coordinated Hg(II) and by 0.1 Å for three-coordinated Hg(II) (See Table 1). Similarly, the Hg-N distance in Hg-dicysteamine complex is overestimated and computed around 2.74 Å while experimentally averaging at approximately 2.60 Å. The degree of inaccuracy for computed Hg-S and Hg-N distances is in agreement with other computational studies on Hg(II) complexes¹¹ and is likely due to expected systematic errors associated with basis set truncation and incomplete treatment of electron correlation within applied level of theory. However, after a 0.1-Å refinement of computed Hg-S bond distances (~2.54 Å), the experimentally measured Hg-S distance of 2.43 Å in Hg-MerR could be easily assigned to three-coordinated Hg(II) in a protein. Also the refined Hg-S bond lengths for the two-coordinated Hg(II) model compounds give much shorter distances that consequently also supports the three-coordinated Hg(II) in Hg-MerR.

Structure and coordination of Pb(II) model complexes

Similarly to Hg(II) models we have considered simple thiolates; $\text{Pb}(\text{S-Eth})_3^-$ and $\text{Pb}(\text{S-Eth})_4^{2-}$, as well as larger models; $\text{Pb}(\text{S-Cys}^*)_3^-$ and $\text{Pb}(\text{S-Cys}^*)_4^{2-}$ to investigate structural and UVRR spectral features of three- vs. four-coordinated lead in sulfur-rich proteins.

Unlike Hg(II), Pb(II) complexes exhibit the hemidirected coordination attributed to the effects of the stereochemically active lone pair orbital. Generally, the preference for an asymmetrical coordination induces closer arrangement of the ligands which consequently might impose unique ligand-ligand interactions. Indeed, all optimized structures of lead

models show a specific ligand arrangement that aligns one of two hydrogens of the C_β atoms with a sulfur atom of the neighboring thiolate. This distinctively built hydrogen bond network is represented by attenuated lines shown in molecular displays in Scheme 2a and 2b. More accurately this intra molecular interaction between coordinated thiolates is referred to as generic 3-center-4-electron hypervalent interaction²⁶. A comparable hydrogen bond network has not been found in any corresponding Hg(II)-bounded models. Therefore, we naturally wonder whether the CH---S network could be enforced or pre-organized in a Pb(II)-specific binding protein such as PbrR691 where it is designed to purposely recognize a Pb(II) ion and effectively discriminate against the binding of other metal ions. The present study does not attempt to answer this notable question, however, more elaborate study on the significance of the CH---S network is under investigation.

When the H-bond network between coordinated thiolates is formed it greatly increases plausible conformations of Pb(II) domains and complicates their analysis. The network hinders the rotational freedom along S- C_β bond and consequently the relative orientation of the C_α atom, resulting in two distinct rotational minima for each coordinated ligand, depending on which of the two hydrogen atoms on C_β is involved in the H-bond interaction. This produces 4 distinctive rotational isomers for three-coordinated lead and 9 isomers for four-coordinated lead models. In principle, the hydrogen atoms of C_β might be considered chiral – at least due to dissimilar environments – and their chirality (H_R or H_L) is used in tracking and labeling these lead isomers.

For three-coordinated $Pb(S-Eth)_3^-$ isomers, two C_3 -symmetry structures could be recognized; the *RRR*-conformer where three H_R hydrogen atoms built in the CH---S network place three C_α methyl groups in a very crowded compact conformation, and the *LLL*-conformer where these methyl groups are stretched out, in a more extended conformation (Scheme 2a). Energetically, the compact *RRR*-isomer has been found as the least stable form of $Pb(S-Eth)_3^-$ while the extended, *LLL*-isomer was computed as most stable, both forms separated by only 0.17 kcal/mol. Other two isomers, *LLR*- and *LRR*- that break the C_3 symmetry are only slightly higher than the most stable *LLL*-structure, i.e., by about 0.09 kcal/mol and 0.13 kcal/mol, respectively.

Although these energy differences are quite negligible and unlikely to discriminate one isomer over the other at room temperature, the differences might become more substantial when a protein environment is considered. We expect that *LLL*-conformation of Cys residues that gives less crowded and sterically more favorable arrangement is more likely to form in proteins. Hence, a complete spectral analysis is limited to the *LLL*-conformer of $Pb(S-Cys^*)_3^-$ but in case of $Pb(S-Eth)_3^-$, spectra of all isomers are reported.

In the four-coordinated lead complexes, the four ligands are not equivalent. There are two strongly bound equatorial ligands and two weakly bound axial ligands. Similarly, they build hydrogen bond bridges that anchor the S- C_β rotations to produce numerous stable rotomers. Calculations of $Pb(S-Eth)_4^{2-}$ structures identified that the rotation along the S- C_β bond of the weakly bound axial ligand is virtually free with a negligible, 0.02 kcal/mol, energetic preference for the *R*-conformation. However, the rotation barrier of the strongly bound equatorial ligands is significantly higher, estimated at 0.61 kcal/mol and it also favors the *R*-conformation of the ligands. Therefore, the most stable structure of four-coordinated lead is the *R*-(*RR*)-*R*-conformation and the least stable is the *L*-(*LL*)-*L*-conformation, where two inner letters designate the orientation of the strongly bound equatorial ligands, and two outer letters designate the orientation of the weakly bound axial ligands. Again, even though these energetic differences are quite negligible in the model compounds they might become more substantial in protein environments. This could be demonstrated already in case of the larger $Pb(S-Cys^*)_4^{2-}$ models where the *R*-(*RR*)-*R* isomer is favored over the *R*-(*LL*)-*R* isomer by

3.12 kcal/mol, where the extra stabilization of the *R*-(*RR*)-*R* isomer is due to two new much stronger NH---S bonds, shown in Scheme 2c, that certainly cannot be formed in smaller $\text{Pb}(\text{S-Eth})_3^-$ models.

Computed Pb-S bond lengths of two $\text{Pb}(\text{S-Eth})_3^-$ models; *LLL*- and *RRR*-conformers, and the two most representative $\text{Pb}(\text{S-Eth})_4^{2-}$ models; *R*-(*RR*)-*R*- and *R*-(*LL*)-*R*-conformers, are listed in Table 1. These bond lengths are expected to be overestimated by up to 0.1 Å due to limitations of the theory, as it was previously discussed in case of Hg(II)-thiolates. Similarly to Hg-S, computed Pb-S bond lengths are invariable with the size of sulfur-donor ligands. However, when the number of coordinated ligands is increased, the bond distance of weakly bound axial ligands dramatically expands, changing from around 2.72 Å for 3-coordinated lead to around 3.09 Å for axial ligands of 4-coordinated lead. The Pb-S bond length of strongly bound equatorial ligands also expands but much less radically to around 2.78 Å. The shorter and stronger equatorial Pb-S bond of four-coordinated lead is quite comparable to the length found for three-coordinated lead, and after a simple 0.1 Å refinement, both are in quite good agreement with experimental values of 2.63 Å found in lead-coordinated peptides and 2.67 Å found in PbrR691 protein. The resemblance of Pb-S bond lengths found for 3-coordinated and for the equatorial ligand of 4-coordinated complexes might be one of the plausible reasons why both coordination models successfully fit the EXEFS data giving inconclusive results on lead coordination in proteins.

Computed CH---S distances ranging from around 2.8 Å to 3.2 Å for both coordination models of Pb(II) complexes (Table 1) well complement other experimental data²⁷. The hydrogen bond network is a little more bound for three-coordinated (averaging around 2.85 Å) than for four-coordinated (around 3.00 Å) complexes. However, the presence of two additional NH---S bridges (around 2.79 Å) in the *R*-(*RR*)-*R* form of $\text{Pb}(\text{S-Cys}^*)_4^{2-}$ clearly tightened the CH---S network. Again, this may suggest that in principle, a pre-organized or uniquely formed hydrogen bond network could form and be designed to purposely favor 4-coordinated lead domains in lead-specific proteins.

Interestingly, a very similar formation of the CH---S network was recently reported and detected by hyperfine-shifts in ¹³C NMR spectra verified by DFT calculations in rubredoxin, iron-sulfur protein²⁸. Reported typical CH---S distances and angles for the iron-sulfur protein closely resemble values found in lead-sulfur structures, listed in Table 1.

Resonance Raman Spectra of Hg(II) model complexes

Computed UVRR spectra for Hg(II) complexes in the low frequency region (from 175 cm⁻¹ to 875 cm⁻¹) are directly compared to Hg-MerR spectrum and other available experimental spectra of two- and three-coordinated Hg(II) thiolates^{10,11}. Linear, 2-coordinated Hg(II) complexes are shown in Figure 1 and planar trigonal, 3-coordinated complexes are shown in Figure 2. Computed and available experimental frequencies of the Hg-S stretching mode are listed in Table 1.

The Hg-S stretching mode for linear models; $\text{Hg}(\text{SCH}_2\text{CH}_2\text{NH}_2)_2$, $\text{Hg}(\text{S-Eth})_2$ and $\text{Hg}(\text{S-Cys}^*)_2$ are computed at 336 cm⁻¹, 318 cm⁻¹ and 333 cm⁻¹, respectively. The frequency computed for Hg-dicysteamine complex agrees very well with experimental value found at 339 cm⁻¹. However, the Hg-S stretching predicted for other models that intend to represent two-coordinated Hg(II) domain in the Hg-MerR protein clearly disagrees with the experimental spectrum. The band is observed for the Hg-MerR protein about 45 cm⁻¹ lower at 288 cm⁻¹ than computed for $\text{Hg}(\text{S-Cys}^*)_2$. This spectral mismatch, however, is expected since it is in agreement with previous independent conclusions that the metal in Hg-MerR is bonded with three cysteine residues, not two. Therefore, the spectral disagreement is due to

clear structural differences between modeled and tangible coordination of Hg(II) in the protein.

The Hg-S stretching for three-coordinated models; $\text{Hg}(\text{S-iBut})_3^-$, $\text{Hg}(\text{S-Eth})_3^-$ and $\text{Hg}(\text{S-Cys}^*)_3^-$ are computed at 201 cm^{-1} , 217 cm^{-1} and 283 cm^{-1} , respectively. These frequencies are clearly lower than those for two-coordinated models. The computed band for $\text{Hg}(\text{S-Cys}^*)_3^-$ agrees well with 288 cm^{-1} observed in Hg-MerR and the band for $\text{Hg}(\text{S-iBut})_3^-$ agrees well with observed value at 207 cm^{-1} . Also, the large up-shift of Hg-S stretching mode observed for Hg-MerR vs. $\text{Hg}(\text{S-iBut})_3^-$ complex is well reproduced in calculations. Similar trends of elevated metal-ligand frequencies in the protein environment vs. its modeled compounds are observed for other metals. For instance, the 314 cm^{-1} signal of Fe-S stretching mode observed in rubredoxin⁷ was found at 298 cm^{-1} in the model $\text{Fe}(\text{S}_2\text{-}o\text{-xyl})_2]^-$ (ligand = *o*-xylene- α,α' -dithiolate) complex^{8e}.

Demonstrated accuracy achieved in prediction of the Hg-S stretching mode allows us to computationally confirm that the structure of Hg(II) in Hg-MerR protein is indeed a 3-coordinated domain where three cysteine residues are bonded to the metal within the planar trigonal geometry. Even more importantly for this study, it allows us to gain the confidence in the accuracy of our computational procedure to predict experimentally undetermined position of the Pb-S stretching mode and UVRR spectral patterns in PbrR691 and other sulfur-rich proteins upon Pb(II) bounding.

Besides Hg-S stretching modes, a few other bands in that spectral region may be observed in UVRR. Commonly, these are C-C torsions, S-C-C bending and C-S stretching modes. The C-C torsions are computed at 264 cm^{-1} and 265 cm^{-1} for $\text{Hg}(\text{S-Eth})_2$ and $\text{Hg}(\text{S-Eth})_3^-$ models, respectively. However, when Hg coordinates with S-Cys* ligands the mode shifts dramatically down to around 30 cm^{-1} . For Hg-dicysteamine complex, the C-C torsion frequency is predicted at 203 cm^{-1} and agrees with observed at 217 cm^{-1} . For $\text{Hg}(\text{S-iBut})_3^-$ the mode is much higher, computed at 302 cm^{-1} and also agrees with a weak signal observed at 315 cm^{-1} . Generally, for 2-coordinated models the C-C torsional mode gives an appreciable signal while for 3-coordinated models the signal is quite weak.

The S-C-C bending modes, are computed at 357 cm^{-1} and 364 cm^{-1} for 2- and 3-coordinated S-Eth ligands, respectively. However, when the metal is coordinated to S-Cys* ligands, the modes shift down to 289 cm^{-1} for 2-coordinated and 258 cm^{-1} for 3-coordinated complexes. For Hg-dicysteamine complex, the mode is computed at 273 cm^{-1} that perfectly agrees with observed band at 273 cm^{-1} . For $\text{Hg}(\text{S-iBut})_3^-$ the band shifts to a very low range of frequencies and is not observable.

The C-S stretching modes, are evaluated at 640 cm^{-1} and 644 cm^{-1} for S-Eth and at 631 cm^{-1} and 637 cm^{-1} for S-Cys* ligands within 2- and 3-coordinated complexes, respectively. The C-S stretching is found in the same region for Hg-dicysteamine complex at 657 cm^{-1} , but it is shifted down to 582 cm^{-1} for $\text{Hg}(\text{S-iBut})_3^-$ model which agrees with observed band at 588 cm^{-1} .

Remaining UVRR bands that still might show up are characteristic for certain ligand structures. The CH_2 rocking modes for $\text{Hg}(\text{S-Eth})_2$ are predicted as weak signal at 779 cm^{-1} and for $\text{Hg}(\text{S-Eth})_3^-$ at 782 cm^{-1} . The N-C torsional mode is computed at 367 cm^{-1} for Hg-dicysteamine complex, which likely is hidden in the experimental spectrum under the solvent peak at 385 cm^{-1} . For $\text{Hg}(\text{S-iBut})_3^-$ complex, a symmetric and two asymmetric – $\text{C}(\text{CH}_3)_3$ deformations are found at 377 cm^{-1} , and 333 cm^{-1} and 421 cm^{-1} , respectively. Both asymmetric bands are observed in experiment at 341 cm^{-1} and 430 cm^{-1} that agree with calculations quite well. The band of a symmetric (umbrella) deformation perhaps is

masked by the solvent peak at 382 cm^{-1} . Also the C-C stretching band for the S-iBut ligand is predicted at 826 cm^{-1} and observed at 820 cm^{-1} .

Also, a few deformation modes characteristic for S-Cys* ligands are predicted in UVRR spectra. For 2-coordinated complex, the bending modes of N-C-C, C-C-C and C-C-O are predicted at 347 cm^{-1} , 380 cm^{-1} and 477 cm^{-1} , respectively. Out of these three modes only N-C-C at 347 cm^{-1} gives a relatively strong UVRR signal, which is likely due to its proximity to Hg-S stretching band at 333 cm^{-1} . Also a band assigned to the N-H out of plane deformation, computed at 813 cm^{-1} , gives appreciable signal for the 2-coordinated structure. For 3-coordinated complex with S-Cys*, the bending modes of N-C-C, C-C-C and C-C-O are found at 316 cm^{-1} , 342 cm^{-1} and 506 cm^{-1} , respectively. All of them predicted as relatively weak UVRR signals. Also the weak band observed in Hg-MerR spectrum at 852 cm^{-1} can be easily assigned to CH_2 rocking computed at 860 cm^{-1} for $\text{Hg}(\text{S-Cys}^*)_3^-$. A moderately intense signal in calculated spectrum at 730 cm^{-1} for the 3-coordinated model is not observed in the experiment. The computed band is assigned to deformations of the terminal methyl groups of S-Cys* ligand. Therefore, it is simply an artifact of computational model and it is not anticipated to show up as intense or even present in the HgMerR protein.

UV spectra and Excitation Profiles for Pb(II)-S stretching modes

CIS and TDDFT computed UV spectra along with predicted excitation profiles for Pb-S stretching modes for three representative complexes of lead domains in proteins (i.e., the *LLL* conformer of $\text{Pb}(\text{S-Cys}^*)_3^-$, and *R-(LL)-R* and *R-(RR)-R* conformers of $\text{Pb}(\text{S-Cys}^*)_4^{2-}$) are shown in Figure 3 and their maximal peaks are listed in Table 1S of supplementary information. A common UV spectrum of lead gives two characteristic lead-sulfur LMCT bands, which experimentally are observed as a moderate signal around 330 nm and a strong signal 260 nm (a red trace²⁸ in Figure 3). At the CIS level of theory, this intensity pattern, all though downshifted by about 65 nm, is reproduced reasonably well. For tri-coordinated lead, the computed spectrum shows two LMCT bands; a moderate intensity band at 241 nm and a stronger band around 194 nm, which after a simple 65-nm adjustment results in 306 nm and 259 nm, respectively (a black trace in Figure 3). For four-coordinated lead, the spectrum shows a weaker band around 220 nm for the *R-(LL)-R* conformer (a green trace in Figure 3) and around 238 nm for the *R-(RR)-R* conformer (a blue trace in Figure 3), while a stronger band is found around 189 nm for both conformers. These values again, after a simple 65-nm refinement, agree well with experimental values. Interestingly, CIS-computed UVRR excitation profiles of PbS stretching modes produce a significantly stronger enhancement for a three-coordinated lead than for a four-coordinated lead. A maximum enhancement for a three-coordinated domain is clearly shown around 195 nm (260 nm after 65-nm refinement), while a corresponding enhancement for a four-coordinated lead is predicted almost 4 times weaker. This difference in enhancement strength could easily explain and certainly contribute to spectroscopic challenges associated with recognition of four-coordinated lead domains, especially when they coexist with three-coordinated domains.

At the TD-DFT level of theory, as it is generally expected, the electronic excitation bands are greatly improved over those from CIS calculations (see lower panel of Figure 3). For a three-coordinated model BP86 density functional theory predicts a strong band around 245 nm and a weaker band around 290 nm (a black trace in Figure 3), which slightly underestimate experimental bands (a red trace). Similarly, for the *R-(LL)-R* conformer (a green trace in Figure 3) of a four-coordinated model, the characteristic bands are found around 242 nm and 310 nm. In the case of the *R-(RR)-R* conformer (blue trace in Figure 3), each of the computed characteristic transitions give a clear split to two close lying bands with comparable intensity; one pair at 242 nm and at 252 nm, and another pair at higher wavelengths around 301 nm and at 333 nm.

Similarly to the CIS results, the TD-DFT predicts much stronger enhancement of Pb-S stretching modes in three-coordinated domains. The excitation profile for the band shows a strong and quite steady enhancement in the entire region of Pb-S electronic transitions, with clear equally intense maxima around 267 nm and 328 nm. Corresponding enhancements for four-coordinated domains are again about 4 times weaker, raising concerns whether these bands are detectable in UVRR even when these domains are formed.

Intriguingly, although the excitation profiles for the symmetric Pb-S stretching modes computed for the *R*-(*LL*)-*R* and the *R*-(*RR*)-*R* conformers (solid green and blue trace, respectively) give comparable UVRR enhancements, the 229 cm⁻¹ UVRR signal for the *R*-(*LL*)-*R* form has been predicted at least twice as intense as for the *R*-(*RR*)-*R* form (Figure 1S of supplementary information). Closer analysis of computed Pb-S signal at 229 cm⁻¹ revealed that the *R*-(*LL*)-*R* configuration has two close lying frequencies, one computed at 229 cm⁻¹ with major contribution to Pb-S stretching and the other at 230 cm⁻¹ with comparable contribution and UVRR enhancement (green solid and green dashed trace, respectively shown in Figure 3). Both modes effectively elevate the 229 cm⁻¹ signal of the *R*-(*LL*)-*R* form.

In case of the *R*-(*RR*)-*R* form, however, the Pb-S signal consists of only one band with major contribution to Pb-S stretching computed at 228 cm⁻¹ (blue solid trace in Figure 3). The other band is shifted up by 17 cm⁻¹ and found at 245 cm⁻¹ as a high-frequency shoulder of the main signal. This mode has much lower UVRR enhancement and is mainly composed of a torsional deformation along a C_β-C_α bond.

UV Intensities

TD-DFT computed UV bands satisfactorily agree with experimental data giving generally, greater reliability than those computed by CIS level of theory. Comparison of TD-DFT spectra of three- and four-coordinated lead models in Figure 3 clearly show elevated intensities around the 300-nm range for four-coordinated structures. Giving that computed TD-DFT intensities are reliable enough, this might suggest a plausible method to determine a relative abundance of four- *versus* three-coordinated lead domains simply by measuring the intensity ratios of two characteristic bands.

Based on TD-DFT bands (Table 1S of supplementary information) the intensity ratio for Pb(S-Cys*)₃⁻, I₂₄₅/I₂₉₀ is around 2.8, while for the *R*-(*LL*)-*R* form of Pb(S-Cys*)₄²⁻, I₂₄₂/I₃₁₀ is around 1.0 and for the *R*-(*RR*)-*R* form, I₂₅₂/I₃₃₃ is around 1.3. An experimentally intensity ratio I₂₅₉/I₃₂₆ for a Cys₃ peptide²⁹, which spectrum is shown in Figure 3, is about 3.3. Indeed, this value when compared to TD-DFT theoretical ratios indeed agrees with the three-coordinated lead domain. Other intensity ratios determined for experimental bands also give noteworthy correlations. For instance, intensity ratios, from previously published UV spectra of sulfur-rich peptides and proteins^{4a-b}, are estimated around 4.6 for Cys₃His and 4.1 for Cys₄ peptides. Lower intensity ratio observed for a Cys₄ peptide than that for a Cys₃His peptide supports a larger abundance of four-coordinated lead in a Cys₄ peptide, as it is naturally expected. Furthermore, giving that the intensity ratio is indeed driven solely by a coordination mode and TD-DFT computed intensity ratios are reliable, a simple ratio of these values 4.1/4.6 = 0.89 could suggest that only about 11% of a four-coordinated lead is present at room temperature in a Cys₄ peptide and the rest is three-coordinated lead.

An independent evaluation of the coordination abundance can be attained from computed energies of lead compounds. We have estimated that Pb(S-Cys*)₃⁻ *LLL* form is more stable than Pb(S-Cys*)₄²⁻ *R*-(*RR*)-*R* form by about 20.3 kcal/mol which includes vibrational and rotational energy corrections. Thermodynamically, the energy gap of that magnitude implies that about 3.5% of four-coordinated lead could be present at room temperature, which might

be considered a reasonable agreement with the roughly determined value of 11% in a Cys₄ peptide. However, we are very aware that this kind of interpretation of spectral intensities is very exploratory at the moment and needs more careful analytical consideration before its application.

Resonance Raman Spectra for Pb(II) model complexes

The UVRR low frequency spectra of lead-sulfur complexes are expected to be dominated by the symmetric Pb-S stretching mode due to the resonant sulfur-to-lead charge transfer transition observed around 260 nm. Spectra computed employing CIS gradients and enhanced at 195 nm wavelength (CIS wavelength scale) are shown in Figure 4 (3-coordinated lead models) and Figure 5 (4-coordinated lead models). Analogous calculations of UVRR intensities employing TD-DFT gradients and 260-nm excitation photon show no major difference between the two levels of theory. A direct comparison of UVRR spectra, computed based on both CIS and TD-DFT gradients for 3- and 4-coordinated Pb(S-Cys*) domains, is shown in Figure 1S of supplementary information. Additionally, Table 2S of supplementary information lists gradient contributions of individual electronic transitions to the weighted gradient required to model these spectra.

Figure 4 shows UVRR spectra for all four conformers of Pb(S-Eth)₃[−] (*LLL*-, *LLR*-, *LRR*- and *RRR*-) and only one *LLL*-conformer of Pb(S-Cys*)₃[−]. As it is expected, the signal of the symmetric Pb-S stretching mode dominates in all computed UVRR spectra. With no additional refinement of DFT frequencies, the stretching band is found at 204 cm^{−1} and 238 cm^{−1} for *LLL*-forms of Pb((S-Eth)₃)[−] and Pb(S-Cys*)₃[−], respectively. Interestingly, when at least one of the S-Eth ligands is locked in the *R* conformation, the Pb-S stretching frequency increases by approximately 12 cm^{−1}, and the band is found at 216 cm^{−1} for all *LLR*-, *LRR*- and *RRR*- forms of 3-coordinated Pb(S-Eth)₃[−].

Figure 5 shows UVRR spectra of 4-coordinated complexes that are dominated by the symmetric Pb-S stretching mode of the equatorial bonds. Similar to 3-coordinated models, the band slightly varies with conformation (*R*- vs. *L*-) of the ligand. However, the spectral divergence is negligible for the weakly bonded axial ligands and more pronounced for the equatorial ligands. For the two forms of Pb(S-Eth)₄^{2−} complex, *x*-(*LL*)-*x* and *x*-(*RR*)-*x* which have different equatorial ligand configurations, the Pb-S stretching modes are found at 188 cm^{−1} and 209 cm^{−1}, respectively. While for Pb(S-Cys*)₄^{2−} complex the band is at 229 cm^{−1} for *R*-(*LL*)-*R* and 228 cm^{−1} for *R*-(*RR*)-*R* structures. Generally, all Pb-S stretching bands for 4-coordinated models are found at slightly lower frequency than the bands for 3-coordinated.

It is also worth noting that the computed spectra are not refined and frequencies are not scaled, therefore, reported Pb-S spectral bands very likely are underestimated by at least 10% to 15%. This suggests that experimentally the symmetric Pb-S stretching modes might fold into a 261–274 cm^{−1} region for 3-coordinated lead domains and around a 252–263 cm^{−1} region for 4-coordinated structures.

The symmetric Pb-S stretching mode of axial bonds is much weaker and expected at much lower frequency. The mode is computed at 125 cm^{−1} for both *x*-(*LL*)-*x* and *x*-(*RR*)-*x* forms of Pb(S-Et)₄^{2−}, while for Pb(S-Cys*)₄^{2−}, it is found at 89 cm^{−1} and 177 cm^{−1} for *R*-(*LL*)-*R* and *R*-(*RR*)-*R* conformers, respectively. Considering comparable distances and diagonal force constants (Table 1) for axial Pb-S bonds in these models, it is surprising to observe such a large difference in frequencies for the Pb(S-Cys*)₄^{2−} model.

What is the source of this difference in frequencies observed for axial symmetric mode? We suggest the difference is attributable to the effects of environmental changes on the mode

motions. The fact that the diagonal force constants of these modes are quite comparable (Table 1) strongly suggests that a presence of extra NH---S interactions, which significantly strengthen the CH_{ax}---S_{eq} interactions in the *R*-(*RR*)-*R* structure, anchors the axial ligands and elevates the Pb-S frequency by decreasing the effective mass. Since the axial ligands are bonded much weaker than equatorial ligands, they are more sensitive to these environmental changes. Similar phenomena of elevated frequencies for particular vibrations were observed in molecular crystals when vibrational modes were anchored by a crystal lattice³⁰.

Still other vibrational modes are expected to show up in UVRR below 400 cm⁻¹. For the most part, these are C_β-C_α torsional modes and S-C_β-C_α bending modes. The torsional modes are calculated at 268 cm⁻¹ for *LLL* and at 280 cm⁻¹ for *RLL*, *RRL*, and *RRR* forms of Pb(S-Eth)₃⁻, but relatively lower at 215 cm⁻¹ for *LLL* structure of Pb(S-Cys*)₃⁻. For 4-coordinated lead, these modes are found at 269 cm⁻¹ for *x*-(*LL*)-*x* and at 290 cm⁻¹ for *x*-(*RR*)-*x* of Pb(S-Eth)₄²⁻. While for Pb(S-Cys*)₄²⁻ forms the mode is found in a lower range at 187 cm⁻¹ and around 168 cm⁻¹ for *R*-(*LL*)-*R* and *R*-(*RR*)-*R* forms, respectively. Also for these last two forms, an additional signal of C_β-C_α torsions of the axial ligands is found at 286 cm⁻¹ and 245 cm⁻¹, respectively.

The S-C_β-C_α bending modes are generally found at slightly higher frequency than the dominant Pb-S stretching mode. For 3-coordinated lead, the bending is calculated at 348 cm⁻¹ for *LLL* and at 332 cm⁻¹ for *RLL*, *RRL*, and *RRR* forms of Pb(S-Eth)₃⁻. However, for the *LLL* structure of Pb(S-Cys*)₃⁻ the mode is lower at 275 cm⁻¹. The signal for 4-coordinated lead is computed at 346 cm⁻¹ and 328 cm⁻¹ for *x*-(*LL*)-*x* and *x*-(*RR*)-*x* of Pb(S-Eth)₄²⁻, respectively, and at 312 cm⁻¹ and 341 cm⁻¹ for *R*-(*LL*)-*R* and *R*-(*RR*)-*R* of Pb(S-Cys*)₄²⁻.

Above 400 cm⁻¹, a relatively weak signal of C-S stretching mode is calculated at 663 cm⁻¹ for *LLL* and at 659 cm⁻¹ for *RLL*, *RRL*, and *RRR* forms of Pb(S-Eth)₃⁻. For Pb(S-Cys*)₃⁻ model the band is predicted relatively high at 732 cm⁻¹. Similarly, for 4-coordinated structures, *x*-(*LL*)-*x* and *x*-(*RR*)-*x* of Pb(S-Eth)₄²⁻ the band is found at 663 and 658 cm⁻¹. However, for *R*-(*LL*)-*R* and *R*-(*RR*)-*R* of Pb(S-Cys*)₄²⁻ the band is lower at 607 cm⁻¹ and 593 cm⁻¹, respectively.

Also, it is worth noting that a very visible and unique band calculated at 268 cm⁻¹ solely for the *R*-(*LL*)-*R* structure of Pb(S-Cys*)₄²⁻ (Figure 5) is assigned to torsions along the terminal N-CH₃ bond in the model. Therefore, as such the band is a computational artifact and should not be expected in experimental UVRR spectra of lead poisoned peptide or protein spectra.

Conclusion

We report quantum mechanical studies on structure and vibrational UVRR spectra for two heavy metal ions; Hg(II) and Pb(II). Our primary interest is to establish a reliable structure-spectra relationship that could be experimentally detectable in Hg(II) and Pb(II)-loaded proteins. The Hg(II) predicted spectra are in accord with experimental data supporting the three-coordinated Hg(Cys)₃ domain in Hg-MerR protein. The computational results for Hg(II) serve as an illustration of accuracy and reliability of an applied computational procedure to simulate UVRR spectra for Pb(II) in sulfur-rich proteins.

As far as we know, successful experimental UVRR measurements of Pb(II) loaded sulfur-rich proteins or peptides provide numerous challenges and have not yet been reported. Presented here, state-of-the-art quantum-mechanical studies explore a possibility of structural determination and clear identification or classification of plausible Pb-Cys domains by UVRR spectroscopy.

Unfortunately, based on our modeling, a successful detection of 3- vs. 4-coordination of lead by UVRR experiment might be problematic since the main band, symmetric Pb-S stretching mode, for 4-coordinated models (predicted at 229 cm^{-1}) is found near the corresponding signal for 3-coordinated models (predicted at 238 cm^{-1}). It is also expected that the resonant enhancement is stronger for 3-coordinated lead than the signal for 4-coordinated lead. Coexistence of 3- and 4-coordinated lead domains is quite plausible in the poisoned environment. In principle, the fourth, weakly bonded ligand, could be exchangeable and may exist in a dynamic equilibrium with 3-coordinated lead. Hence, the UVRR signal of 4-coordinated lead might be effectively screened by stronger signals of 3-coordinated lead domains and never detected experimentally. In light of the present study, we conclude that identification of 3- vs. 4-coordinated lead in sulfur-rich proteins is a difficult and challenging problem. The most successful approach to address this problem should combine various spectroscopic techniques such as EXAFS, NMR, UVRR and possibly deeper analysis of UV spectra, supported by computational modeling.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Comparison of experimental and calculated UVRR spectra of 2-coordinated Hg(II) sulfur-complexes. Frequencies of PbS stretching bands are shown by bold font. Experimental spectra adopted from references 10 and 11. Solvent and quartz experimental bands are labeled as (s) and (q) respectively.

Figure 2. Comparison of experimental and calculated UVRR spectra of 3-coordinated Hg(II) sulfur-complexes. Frequencies of PbS stretching bands are shown by bold font. Experimental spectra adopted from references 10 and 11. Solvent and quartz experimental bands are labeled as (s) and (q) respectively. Terminal methyl deformations of S-Cys* (computational artifact) band is labeled as (*).

Figure 3.

Experimental and modeled UV CIS and TD BP86 spectra along with corresponding modeled UVRR excitation profiles of characteristic Pb-S stretching modes. *Color Legend:* BLACK: LLL form of Pb(S-Cys*)₃⁻ and 238 cm⁻¹ band; GREEN: solid, R-(LL)-R form of Pb(S-Cys*)₄²⁻ and 229 cm⁻¹ band, (dot-dashed, 230 cm⁻¹ band – see text); BLUE: R-(RR)-R form of Pb(S-Cys*)₄²⁻ and 228 cm⁻¹ band; RED: experimental data²⁹ and partially labeled on the plot.

Figure 4.

Calculated based on CIS gradients UVRR spectra of 3-coordinated Pb(II) sulfur-complexes.

Color Legend: BLACK: solid, *LLL*; dashed, *LLR*; RED: solid, *RRR*, dashed *RRL* forms of $\text{Pb}(\text{S-Et})_3^-$; BLUE: *LLL* form of $\text{Pb}(\text{S-Cys}^*)_3^-$ and partially labeled on a plot.

Figure 5. Calculated based on CIS gradients UVRR spectra of 4-coordinated Pb(II) sulfur-complexes. Each of spectral panel for $x\text{-(LL)}\text{-}x$ (black) and $x\text{-(RR)}\text{-}x$ (red) show two overlapped spectra of $\text{Pb}(\text{S-Eth})_4^{2-}$ where x represents R or L conformations of weakly bonded axial ligands. *Color Legend:* Labeled on the plot.

1a

1b

Scheme 1.

Structures of 2-coordinated (a) and 3-coordinated (b) Hg(II) sulfur complexes shown in their energy-minimum geometry.

2a

L-(LL)-L *L-(RR)-L* *R-(LL)-R* *R-(RR)-R*

2b

2c**Scheme 2.**

Structures of 3-coordinated Pb(II) complexes (a) 4-coordinated Pb(S-Eth)₄²⁻ complexes (b) and Pb(SCys*)₄²⁻ complexes (c) shown in their energy-minimum geometry (Out-of-plane displacement of lead and its stereochemically active lone-pair is pointing towards the reader)

Table 1

Selected Bond Distances (Å), Angles (deg), Frequencies (cm^{-1}) and Diagonal Force Constants (mdyn/Å) for 2-, 3- and 4-sulfur coordinated Hg(II) and Pb(II) complexes. Experimental values and those for computed for S-Cys* complexes are shown in bold face.

	Bond Distances (Å)				Frequency Me-S symm stretch		Diagonal Force Constant Me-S
	Hg-S		Hg-N		calc	expt	
2-coordinated Hg(II)	calc	expt	calc	expt	calc	expt	calc
Hg(SC ₂ H ₄ NH ₂) ₂	2.439	2.36,2.37 ^a	2.742	2.54,2.66 ^a	336	339 ^b	1.912
Hg(S-Eth) ₂	2.396	2.32–2.36 ^c			318		2.118
Hg(S-Cys*) ₂	2.400				333		2.095
<hr/>							
	Me-S		CH---S				
3-coordinated Hg(II) and Pb(II)	calc	expt	calc				
Hg(S-Eth) ₃ ⁻	2.535		3.595		217		1.274
Hg(S-But) ₃ ⁻	2.539	2.436,2.438,2.451 ^d	n/a		201	207 ^e	2.720
Hg(S-Cys*) ₃ ⁻	2.547		3.599		283		1.313
Hg-MerR		2.43^f				288^e	
<hr/>							
Pb(S-Eth) ₃ ⁻ LLL	2.716		2.841 (145.24)		204		0.880
Pb(S-Eth) ₃ ⁻ RRR	2.724		2.883 (148.33)		216		0.865
Pb(S-Cys*) ₃ ⁻ LLL	2.728		2.830 (143.41)		238		0.889
PbrR691		2.67^g					
Pb CadC		2.66^h					
Pb (TRL16C) ₃ ⁻		2.63^h					
Pb(CP-CCCC)		2.64ⁱ					
4-coordinated Pb(II)	Pb-S(eq) calc	Pb-S(ax) calc	CH(ax)-S(eq) calc	CH(eq)-S(ax) calc	equatorial	axial	axial
Pb(S-Eth) ₄ ²⁻ R-(LL)-R	2.783	3.110	3.018 (142.83)	3.165 (141.04)	188	125	0.657
Pb(S-Eth) ₄ ²⁻ R-(RR)-R	2.796	3.102	2.965 (144.75)	2.992 (152.83)	209	125	0.644
Pb(S-Cys*) ₄ ²⁻ R-(LL)-R	2.764	3.060	3.142 (134.71)	2.932 (151.39)	229	89	0.732
							0.278

	Bond Distances (Angles)		Hg-N	Frequency Me-S symm stretch	Diagonal Force Constant Me-S	
	Hg-S					
Pb(S-Cys*) ₄ ²⁻ R-(RR)-R	2.788	3.086	2.935 (135.90) 2.792 (156.37) ^j	228	0.752	0.286
^a Reference [21]						
^b Reference [11]						
^c Reference [22]						
^d Reference [23]						
^e Reference [10]						
^f Reference [24]						
^g Reference [6]						
^h Reference[25]						
ⁱ Reference [4c]						
^j NH-(eq)---S(ax) calculated distance and angle						