at least 95% of the β-diastereomer.[1] The latter observation prompted speculation that in the case of CH_3Cbi the products were under kinetic control, with alkylation of the β-face being sterically favored due to congestion at the α-face from the b, d, and e propionamide side chains and the f secondary amide side chain. We have consequently attempted to determine if for the CF_3CH_2Cbi's the products are under equilibrium control, with initial alkylation occurring at the β-face followed by equilibration of the diastereomers via a pathway that is not available (or more likely of too high energy) in the case of CH_3Cbi. Time-resolved determination of the product ratios for the CF_3CH_2Cbi's under a variety of conditions[13] showed that the final product ratio (i.e. 6.9:1, α:β) is established within 10–20 s of introduction of alkylating agent and remains unchanged throughout the reaction period (up to 30–60 min). Interestingly, both diastereomers of CF_3CH_2Cbi proved to be quite unstable with respect to reductive dealkylation by zinc/acetic acid ($T_{1/2} \sim 1$ min) so that net alkylation in this system represents a steady-state situation. Consequently, at long reaction times (30–60 min) the total yield of alkylcobinamides is reduced, presumably due to depletion of alkylating agent. At 0 °C, the net alkylation yield rose from about 25% to 46% over the first 4 min and then gradually declined to less than 20% at 1 h, while the ratio of diastereomers remained constant throughout. These observations are consistent with kinetic control of products, with the rate of α-face attack exceeding that of β-face attack by about 7-fold, but this conclusion is difficult to accept in light of the contrary results with CH_3Cbi. The results are also consistent with the CF_3CH_2Cbi products being under equilibrium control, provided that the rate of equilibration of the diastereomeric CF_3CH_2Cbi's is quite fast, even exceeding the rate of initial alkylation at 0 °C. The exact mechanism of formation of the diastereomeric CF_3CH_2Cbi's and the extreme difference in behavior of the CF_3CH_2Cbi's and CH_3Cbi are currently under intense investigation.[14]

Acknowledgment. This research was supported by Grant CHE-89-00927 from the National Science Foundation and Grant Y-749 from the Robert A. Welch Foundation, Houston, TX.

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(14) Kraütler and Caderas[15] have reported that treatment of heptamethyl cob(I)yrinate in toluene/tetrahydrofuran with methyl tosylate leads exclusively to the heptamethyl β-methylcobyrinate (75% yield) while treatment with CH_3I yields the heptamethyl α-methylcobyrinate (71% yield) along with 7% of the β-isomer. The reasons for the different outcomes when cobinamide in water and cobester in an organic medium are reductively alkylated with CH_3I are not at all clear. As yet, we have been unable to obtain any products by reductive alkylation of cobinamides with trifluoroethyl tosylate.

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Received March 9, 1990

Sulfur L-Edge XANES Study of Zinc-, Cadmium-, and Mercury-Containing Metallothionein and Model Compounds

We report the first sulfur L-edge (150–250 eV), X-ray absorption near-edge structure (XANES) spectra for the metallothionein (MT) species Zn_7-MT, Cd_7-MT, Hg_7-MT, and Hg_{18}-MT 2. These data are compared with sulfur L-edge spectra obtained for PbS (galena) and ZnS (sphalerite) and the model compounds $[Zn_4(SPh)_{10}](Me_4N)_2$ and $[Cd_4(SPh)_{10}](Me_4N)_2$. The detail in these XANES spectra illustrates the considerable utility of a technique that can provide geometric data for a range of different metal complexes from the same thiolate groups within the metal binding site. Analysis of the data for Zn_7-MT 1, Cd_7-MT 1, and Hg_7-MT 1 shows that Hg_7-MT 1 adopts a similar structure in terms of the sulfur environment, with the same thiolate oxidation state and nearest-neighbor distances as those for the well-known Cd and Zn metallothioneins. The substantially different spectrum for the recently described Hg_{18}-MT 2 species shows that it adopts a significantly different structure in terms of the thiolate environment. Although the XANES spectra of $[Zn_4(SPh)_{10}](Me_4N)_2$ and $[Cd_4(SPh)_{10}](Me_4N)_2$ compounds, which can be considered to be analogues of the α-domain in MT, are similar to each other, these spectra do not closely resemble the data obtained for the protein samples.

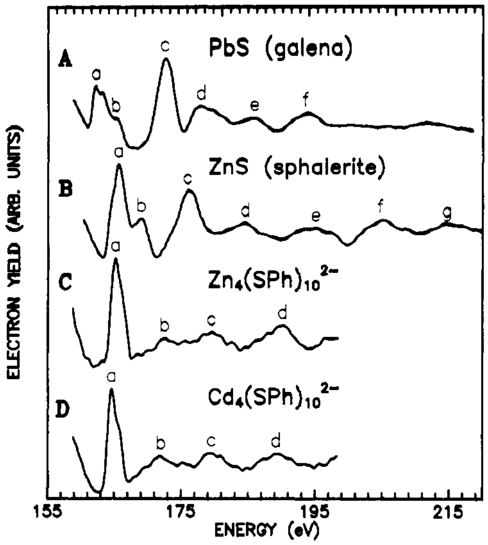

Figure 1. Sulfur L-edge XANES spectra of model compounds: (A) PbS; (B) ZnS; (C) $[Zn_4(SPh)_{10}](Me_4N)_2$; (D) $[Cd_4(SPh)_{10}](Me_4N)_2$.

Metallothioneins are low molecular weight, cysteine-rich proteins containing 20 SH groups per molecule. These proteins bind to a wide range of metal ions both in vivo and in vitro.[1] Extensive studies have been carried out in order to understand the nature of metal binding in metallothionein using ^{113}Cd NMR, ^{1}H NMR, X-ray crystallography, circular dichroism (CD), magnetic circular dichroism (MCD), and Raman spectroscopic techniques.[1,2]

Hasnain et al. have reviewed the available extended X-ray absorption fine structure spectroscopy (EXAFS) data for different metal environments of MT.[1,3,4] Elder et al.[5a] and Laib et al.[5b] have published results from XANES studies, in which the Au L_3 energy region (11–12 keV) was used to determine the oxidation state of Au in Au_{20}-MT. Klemens et al. have recently reported the EXAFS and XANES spectra of the Hg L_3-edge of mercury-substituted copper proteins.[6] Stephan and Hitchcock[7] have reported metal K-edge EXAFS of two model compounds of the metallothionein α-domain. To our knowledge, no sulfur L-edge XANES studies of metallothionein or any other sulfur-containing

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metalloprotein have been published previously. Sulfur L-edge spectra offer the advantage of a considerable increase in resolution over K-edge measurements.

The XANES region in an X-ray absorption spectrum covers the range between the threshold (edge) and approximately 40 eV above the edge. This region contains information about local symmetry and the interatomic distances involved in the bonding of the central absorbing atom and can be used to fingerprint different compounds.[8] Rabbit liver Zn_7-MT 1 used in this study was prepared as previously described.[9] Cd_7-MT 1 and Hg_7-MT 1 were prepared by replacement of zinc in Zn-MT with Cd^{2+} and Hg^{2+} at neutral pH. Hg_{18}-MT 2 was prepared by titrating 18 mol equiv of Hg^{2+} into rabbit liver Zn-MT 2 in 10 mM HCl.[10] All of these MT samples were lyophilized prior to measurement of the XANES spectra. The stoichiometric ratio of metal:protein was determined by analysis of the thiol content using methods described previously[11] and by spectroscopic monitoring of titrations of the metal into the protein solutions. The end points in these titrations are sharp and well defined (refer to Hg CD,[10] Ag CD,[27] and Cd CD[2d]). When previously lyophilized samples were reconstituted in water or HCl, the optical spectral properties were the same as before lyophilization.

Figure 1A–D shows the sulfur $L_{2,3}$-edge XANES spectra[12] for PbS (galena), ZnS (sphalerite), $[Zn_4(SPh)_{10}](Me_4N)_2$, and $[Cd_4(SPh)_{10}](Me_4N)_2$, respectively. We also recorded spectra for $Hg(SR)_2$ (R = *t*-Bu), but this compound was not stable toward the X-ray radiation.

The BAN computer program from McMaster University[13] was used for data analysis. Three spectra for each compound were combined digitally, smoothed to differing extents, and a linear background was subtracted.

These XANES spectra can be conveniently divided into two regions for analysis. The first region, encompassing peaks a–c, is due to electronic transitions from sulfur 2p levels to bound-state, unoccupied, or "trapped" levels. These levels are very sensitive to the electronic state and the local symmetry of the absorbing atom.

The spectra of the model compounds represent different symmetries around the sulfur. The PbS (galena) structure is similar to that of NaCl (cubic), with coordination of sulfur by six nearest neighbors of Pb. On the other hand, although the ZnS (sphalerite) structure is cubic, the sulfur is tetrahedrally bonded to four Zn atoms. Sulfur atoms in the two other model compounds are bonded to the metal atoms in either a terminal or a bridging mode. As with the case for metallothionein, both terminal and bridging sulfurs are bonded to carbon.[14] In these compounds, as in ZnS, the zinc is surrounded by four sulfur atoms.

As can be seen from spectra A and B in Figure 1, the first region of the spectrum (bands a–c) is quite different for PbS compared to ZnS. Not only are the peaks shifted to lower energies, but the relative intensities are also significantly different. The energy shift is more than is normally expected from differences between the binding energies of S 2p levels in the two compounds. (The binding energies of S $2p_{3/2}$ measured by XPS for PbS and ZnS are 160.5 and 161.4 eV, respectively, differing only by 0.9 eV,[15] whereas the shift for peak a in PbS, compared with the corresponding one in ZnS, is 3.4 eV.) At this stage of the investigation we are not attempting to identify the transitions responsible for these peaks but rather to use the spectral envelopes as fingerprints of different sulfur environments, in the manner used by Bianconi et al.[16] and Lytle et al.[17] for systems in which metals are surrounded tetrahedrally with oxygen, in which the XANES features are similar.

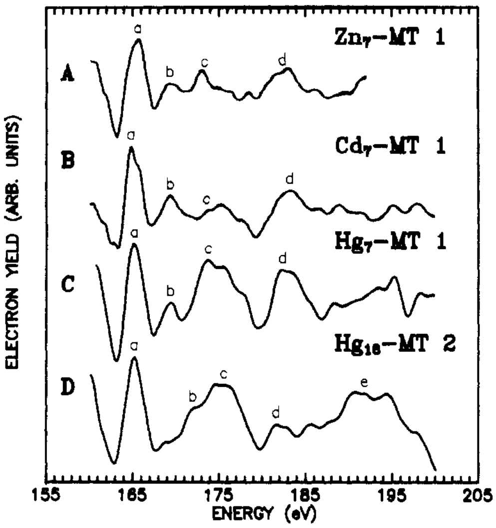

Figure 2. Sulfur L-edge XANES spectra of metallothioneins: (A) Zn_7-MT 1; (B) Cd_7-MT 1; (C) Hg_7-MT 1; (D) Hg_{18}-MT 2.

The features in the first region of the XANES spectra for $Zn_4(SPh)_{10}{}^{2-}$ and $Cd_4(SPh)_{10}{}^{2-}$ (spectra C and D), as anticipated, are identical. The two compounds have a formal thiolate, S^{2-}, oxidation state and a similar local symmetry. However, the spectral features compared to those of ZnS are quite dissimilar. The position of peak a is about the same, but peaks b and c have significantly different energies, which reflects differences in the sulfur environment in these compounds.

The second spectral region, peaks d–f, is due to single- or multiple-scattering events involving electrons ejected to the continuum.[8] It is generally accepted that structure in this region reflects the interatomic distances of several shells surrounding the absorbing atom.[8,17] Indeed, $1/R^2$ correlations have been worked out for several different systems, where R is the scattering path length.[18] As it can be seen from spectrum A in Figure 1, the features in the PbS spectrum peaks c–f are systematically shifted, according to the $1/R^2$ rule, toward lower energies when compared with the corresponding peaks in spectrum B for ZnS. The S–Pb and S–Zn distances for the first shell in PbS and ZnS are 297 and 234 pm, respectively. The features in this region for the other two compounds are essentially identical for both compounds. The S–Cd distance is larger than S–Zn by ~18 pm, which is not large enough to have any significant effects on the position of the peaks. Nevertheless, peaks for the Cd compound are slightly shifted to lower energies. The accuracy of the $1/R^2$ rule at the present time is ±15 pm. The high-energy tails of the spectra for $Zn_4(SPh)_{10}{}^{2-}$, $Cd_4(SPh)_{10}{}^{2-}$, and the MT proteins are truncated at 200 eV due to the interference of the N 1s second-order band that is present in all these substances. As a result, a complete $1/R^2$ correlation cannot be applied to these systems as has been carried out for ZnS and PbS.[18] However, we believe the remaining part of the spectrum can conveniently be used to fingerprint the sulfur environment in protein, on the basis of the information obtained from the model compounds. XANES S K-edge spectra can be used

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to distinguish different forms of organically bound sulfur[19] and inorganically bound sulfur,[20] which suggests that L-edge measurements of MT will be sensitive to differences in coordination environment of the metal-binding, thiolate sulfur groups.

We now turn to the data from the protein samples. Figure 2 shows the S $L_{2,3}$-edge XANES spectra for Zn_7-MT 1 (A), Cd_7-MT 1 (B), Hg_7-MT 1 (C), and Hg_{18}-MT 2 (D). First, we consider Zn_7-MT 1 and Cd_7-MT 1. From previous spectroscopic studies it is known that Zn_7-MT 1 and Cd_7-MT 1 have almost identical structures, with four metals in the α-domain and three metals in the β-domain, forming M_4S_{11} and M_3S_9 clusters, respectively. The tetrahedral metal binding sites are cross-linked by sulfur bridges,[1] with both terminal and bridging thiolate groups involved in each domain.[21] Spectra A and B in Figure 2 show that features in the first region, bands a and b, which are attributed to transitions to bound or "trapped" states, are identical in both spectra. The position of peak a, which is generally associated with the electronic state of the absorbing atom,[22] is very close to that of the two model compounds. The S 2p binding energy from XPS (X-ray photoelectron spectroscopy) measurements, which is 162.5 ± 1 eV, for all MT complexes confirms that structures a and b in the spectra of the protein complexes lie above or at the ionization edge. However, the spectral structure between peaks a and b and the position of peak b are quite different when compared with those of the spectra of the model compounds. This suggests that the local environment of the sulfurs in the MT species may be slightly different. In these spectra we are observing the averaged effects of the two domains and terminal and bridging thiolate groups in the protein. The thiolate groups in the protein not only bind to the metals but are also part of the peptide chain. The configuration of the peptide chain is expected to be similar for Zn_7-MT 1 and Cd_7-MT 1, but in both cases the peptide chain does not resemble the ligands used in the model compounds.

The structures in the second region, apart from some slight changes in the intensity, are again very similar for Zn_7-MT 1 and Cd_7-MT 1. On the basis of these findings, we can conclude that, as far as the sulfur environment is concerned, the two structures are identical and the variation in the bond distances (S–M or S–S) from one protein to another is not large enough to be detected by this technique.

Hg-containing metallothioneins have not been characterized spectroscopically as completely as the Zn- and Cd-containing proteins. Initial attempts at ^{199}Hg NMR measurements of Hg-MT failed to find a signal,[23,24] possibly because of the fast exchange of the mercury atoms between different sites. Analysis of absorption[23,25] CD,[25,26] and MCD[25] spectral data suggest that Hg_7-MT does not involve binding sites that are quite the same as for the Zn and Cd analogues. The XANES spectrum for Hg_7-MT 1 (Figure 2C) is very similar to those of the Zn and Cd analogues, although the intensities of peaks b–d are different, which might arise from differences in the binding site geometry. It has been suggested[23] that this structure might involve both a tetrahedral and linear Hg coordination environment for metal–sulfur clusters.

The XANES spectrum of Hg_{18}-MT 2 is shown in Figure 2D. This is an unusual MT species found at present for both Hg and Ag metallothioneins that we have only recently studied spectroscopically.[10,27] We have previously suggested that it adopts a unique 3-dimensional structure that is very different from the structure adopted by Hg_7-MT 1, Cd_7-MT 1, and Zn_7-MT 1.[10] Except for peak a, the XANES spectrum of Hg_{18}-MT 2 is very different when compared with the spectra from Zn_7-MT 1, Cd_7-MT 1, and Hg_7-MT 1. The consistent energy of peak a among the protein complexes suggests that the formal oxidation state of the sulfur is also 2– in Hg_{18}-MT 2. The shift in the other bands, the significant changes in the overall structure observed throughout the spectrum, and the appearance of the new structure e indicate that the local symmetry of the sulfur is different in this complex. These conclusions support our previous suggestion[10] that Hg_{18}-MT 2 adopts a completely different structure when compared with known M_7-MT species in terms of the sulfur environment. Probable these are no α- or β-domains, just a supercoiled peptide chain.

In summary, this first study of the S L-edge XANES spectra of a metalloprotein suggests that while Zn_7-MT 1, Cd_7-MT 1, and Hg_7-MT 1 have similar structures from the perspective of the coordinating sulfurs, the binding sites in Hg_7-MT are not exactly the same. The data show that Hg_{18}-MT 2 adopts a completely different structure when compared with that of the M_7-MT complexes. We are unsure why the model compounds $[Zn_4(SPh)_{10}](Me_4N)_2$ and $[Cd_4(SPh)_{10}](Me_4N)_2$, which have well-defined tetrahedral metal binding sites, much like the α-domain of M_7-MT, exhibit such different XANES spectra. These results underscore the utility of measuring spectroscopic data from the same chromophore within similar complexes.

Acknowledgment. We gratefully thank Prof. P. A. W. Dean of UWO for providing us the model compounds used in this study and Dr. Margaret Hyland for measuring XPS spectra used in this study. We thank Dr. T. Tyliszczak for the BAN program. We thank the staff at the Synchrotron Radiation Center, University of Wisconsin, for technical support. This study was supported by NSERC Operating and Strategic Grants to M.J.S. and NSERC and NRC Grants to G.M.B.

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Received October 5, 1989

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