presence of $C^{16}O$ results in facile displacement of $C^{18}O$, with

$$(\eta^5\text{-}C_5Me_5)_2Ti(C^{18}O)_2 \xrightarrow[h\nu]{C^{16}O} (\eta^5\text{-}C_5Me_5)_2Ti(C^{16}O)_2 \xrightarrow[h\nu]{PF_3} (\eta^5\text{-}C_5Me_5)_2Ti(C^{16}O)(PF_3)$$

intermediate formation of $(\eta^5\text{-}C_5Me_5)_2Ti(C^{16}O)(C^{18}O)$ (ν_{CO} 1926 and 1835 cm^{-1}) and complete formation of $(\eta^5\text{-}C_5Me_5)_2Ti(C^{16}O)_2$ (ν_{CO} 1940 and 1858 cm^{-1}) in a 30-min period. These results imply that the carbonyl ligands in **5**, as in $Cp_2Ti(CO)_2$, are indeed photolabile; however, the reaction intermediate has a surprisingly much greater affinity for CO than for PF_3.

Crystals of **2** suitable for X-ray diffraction studies have been grown by slow sublimation at 50–55 °C (10^{-3} torr). An orange crystal of the compound was sealed in a thin-walled glass capillary. There are two crystallographically independent molecules in the unit cell.[19] One resides on a mirror plane, but the other possesses no site-imposed symmetry. There is, however, no apparent conformational difference between the two; Figure 1 presents a view of both molecules.

In the structure of $Cp_2Ti(CO)_2$,[20] a Ti^{2+} complex, the Ti–C(η^5) distances average 2.347 (13) Å, a value which is *less* than the 2.38 Å standard for complexes of Ti^{4+}.[21,22] This has been taken as evidence that the two d electrons reside in an orbital which is at least slightly bonding with respect to the titanium–cyclopentadienyl interaction. It is interesting to note that in the title compound **2** (also Ti^{2+}), the average of the Ti–C(η^5) lengths, 2.34 (1) Å, is similar. This structural observation is in accord with the fact that CO and PF_3 are similar in ligating ability.[1-3]

The Ti–P bond lengths range from 2.340 (6) to 2.349 (6) Å. Although there are no related compounds available for the purpose of comparison, two indirect estimates of a Ti–P bond of this sort may be obtained. First, the Ti–C(sp^3) length in $(\eta^5\text{-}C_9H_7)_2Ti(CH_3)_2$ is 2.20 Å.[23] Subtracting the C(sp^3) radius of 0.77 Å[24] affords an estimate of the Ti^{4+} radius of 1.43 Å in a Cp_2Ti fragment. The addition of the P(sp^3) covalent radius of 1.10 Å[24] yields an anticipated Ti–P length of ca. 2.53 Å. The second calculation is based on the Ti–Cl distance of 2.368 (4) Å in $(CH_2)_3(\eta^5\text{-}C_5H_4)_2TiCl_2$.[25] If one corrects for the difference between the covalent radii of Cl and P, the projected Ti–P length is 2.48 Å. Thus, by the above estimates, the Ti–P bond distance in **2** is perhaps 0.15 Å less than expected.[26] It should also be pointed out that the Ti–CO length in $Cp_2Ti(CO)_2$ is also approximately 0.1 Å less than that in $(\eta^5\text{-}C_9H_7)_2Ti(CH_3)_2$ after a correction is made for the change in carbon atom hybridization. The similarity between the CO and PF_3 ligands, both capable of substantial back-bonding, is again underscored.

Acknowledgment. We are grateful to the National Science Foundation for support of this research program. We also thank Professor R. Holmes of the University of Massachusetts for a generous gift of PF_3.

Supplementary Material Available: Tables of bond distances, angles, final fractional coordinates, thermal parameters, and observed and calculated structure factor amplitudes (10 pages). Ordering information is given on any current masthead page.

(19) The space group is *C2cm* with cell parameters of a = 7.811 (4), b = 13.724 (5), c = 37.649 (11) Å; D_c = 1.75 g cm^{-3} for Z = 12. Least-squares refinement on the basis of 1037 observed reflections led to a final $R = \sum(|F_o| - |F_c|)/\sum|F_o| = 0.038$. Hydrogen atoms were included with fixed contributions and all nonhydrogen atoms were refined with anisotropic thermal parameters.

(20) Atwood, J. L.; Stone, K. E.; Alt, H. G.; Hrncir, D. C.; Rausch, M. D. *J. Organomet. Chem.* **1977**, *132*, 367-375.

(21) Epstein, E. F.; Bernal, I. *Inorg. Chim. Acta* **1973**, *7*, 211-218.

(22) Mattia, J.; Humphrey, M. B.; Rogers, R. D.; Atwood, J. L.; Rausch, M. D. *Inorg. Chem.* **1978**, *17*, 3257-3264.

(23) Atwood, J. L.; Hunter, W. E.; Hrncir, D. C.; Samuel, E.; Alt, H.; Rausch, M. D. *Inorg. Chem.* **1975**, *14*, 1757-1762.

(24) Pauling, L. "The Nature of the Chemical Bond"; Cornell Press: Ithaca, NY, 1960; p 246.

(25) Davis, B. R.; Bernal, I. *J. Organomet. Chem.* **1971**, *30*, 75-84.

(26) Due to the current lack of data on structurally related Ti^{2+} compounds, no account can be taken for the discrepancy in size between Ti^{2+} and Ti^{4+} in these estimates.

Copper Site of Molluscan Oxyhemocyanins. Structural Evidence from X-ray Absorption Spectroscopy

Man Sung Co and Keith O. Hodgson*

Department of Chemistry, Stanford University Stanford, California 94305

Thomas K. Eccles

EXXON Production Research Company Houston, Texas 77001

René Lontie

Laboratorium voor Biochemie Katholieke Universiteit te Leuven B-3000 Leuven, Belgium

Received August 26, 1980

Knowledge of the molecular architecture around the copper atoms in the "type 3" site of hemocyanins is of importance in understanding how these proteins reversibly bind dioxygen. Analysis of the extended X-ray absorption fine structure (EXAFS) has proven to be useful in the structural elucidation of specific metal sites in metalloproteins under noncrystalline conditions.[1] Indeed, hemocyanin was among the first metalloproteins studied by using EXAFS. This study revealed only low Z atoms (nitrogen or oxygen) in the first coordination sphere and demonstrated that a binuclear copper interaction, if present, had a separation between the two metals of greater than about 2.7 Å.[2] More recently, Spiro and co-workers[3] reported an EXAFS study on *Busycon canaliculatum* hemocyanin. Their work showed four or five low Z atoms coordinated to each copper in the oxygenated form with a Cu–Cu separation of 3.67 Å. Their proposed model consists of two copper atoms each bound to three histidine ligands and bridged by a bound peroxide and an atom from a protein ligand. We report here our EXAFS analysis by using imidazole group fitting[4] on the oxy forms of *Megathura crenulata* and the α and β components of *Helix pomatia* hemocyanin. This EXAFS reveals only two imidazoles and two low Z atoms per copper. Using the EXAFS, as well as the K absorption edge and other spectroscopic data, we propose a model for the binuclear type 3 copper site in which the copper atoms are separated by 3.55 Å. Comparison of the results on oxyhemocyanins from two different species is also discussed.

Hemocyanin is a copper-containing protein which functions as a dioxygen carrier in molluscs and arthropods. The smallest functional subunits, with a molecular weight of ca. 50000 for molluscs and 75000 for arthropods, contain two copper atoms and bind one molecule of dioxygen reversibly. Many studies have been aimed at elucidating the structure of the active site.[5] Results from resonance Raman spectroscopic studies[6] have established that in oxyhemocyanin the bound oxygen is in the form of peroxide (O_2^{2-}). On the basis of near-UV and visible absorption spectroscopy, sulfhydryl groups[7,8] and imidazole groups[8,9] have been suggested to be copper ligands. Acid–base titration studies[10] and photooxidative studies[11] support the binding of imidazole groups

(1) (a) Cramer, S. P.; Hodgson, K. O. *Prog. Inorg. Chem.* **1979**, *25*, 1. (b) Cramer, S. P.; Hodgson, K. O.; Gillum, W. O.; Mortenson, L. E. *J. Am. Chem. Soc.* **1978**, *100*, 3398. (c) Eisenberger, P.; Kincaid, B. M. *Science (Washington, DC)* **1978**, *200*, 1441.

(2) Eccles, T. K. Ph.D. Thesis, Stanford University, Stanford, CA, 1977.

(3) Brown, J. M.; Powers, L.; Kincaid, B.; Larrabee, J. A.; Spiro T. G. *J. Am. Chem. Soc.* **1980**, *102*, 4210.

(4) Co, M. S.; Scott, R. A.; Hodgson, K. O. *J. Am. Chem. Soc.* **1981**, *103*, following paper in this issue.

(5) (a) Lontie, R.; Witters, R. *Inorg. Biochem.* **1973**, *1*, 344. (b) Himmelwright, R. S.; Eickman, N. C.; Lubien, C. D.; Solomon, E. I. *J. Am. Chem. Soc.* **1980**, *102*, 5378.

(6) Thamann, T. J.; Loehr, J. S.; Loehr, T. M. *J. Am. Chem. Soc.* **1977**, *99*, 4187.

(7) Klotz, I. M.; Klotz, T. A. *Science (Washington, DC)* **1955**, *121*, 477.

(8) Amundsen, A. R.; Whelan, J.; Bosnich, B. *J. Am. Chem. Soc.* **1977**, *99*, 6730.

(9) (a) Lontie, R. *Clin. Chim. Acta* **1958**, *3*, 68. (b) Engelborghs, Y.; Lontie, R. *Eur. J. Biochem.* **1973**, *39*, 335.

(10) Salvato, B.; Ghiretti-Magaldi, A.; Ghiretti, F. *Biochemistry* **1974**, *13*, 4778.

0002-7863/81/1503-984$01.25/0

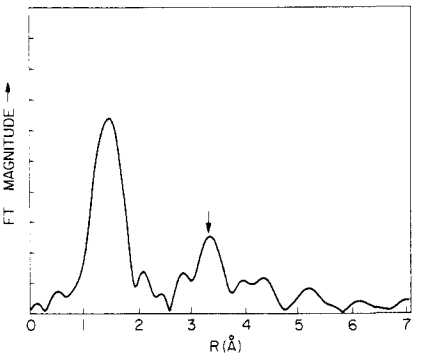

Figure 1. Fourier transform of *Megathura crenulata* oxyhemocyanin over the range 3.5–12.5 Å^{-1}, k^3 weighting. The peak indicated by the arrow suggests the presence of high Z elements in the copper coordination sphere. Our EXAFS curve-fitting analysis shows that this peak results from backscattering by a copper atom at 3.55 Å from the absorbing copper atom.

to copper, predicting around eight imidazoles and six imidazoles bound to each two-copper unit, respectively. More direct evidence concerning imidazole ligands was obtained from resonance Raman studies,[12] but such studies could not predict the number of ligands coordinated. The lack of EPR signals for oxyhemocyanin[13] suggested that the Cu(II) atoms are antiferromagnetically coupled via a bridging ligand.[14] Magnetic susceptibility studies set a lower limit of 550 cm^{-1} on any antiferromagnetic exchange interaction.[15]

Megathura crenulata hemocyanin was prepared from live giant keyhole limpets (obtained from Pacific Biomarine, Venice, CA) as previously described.[2] The hemocyanin was pelleted by centrifuging for 3–4 h at 100000g from a protein solution in 0.1 M phosphate buffer at pH 8.5, with 0.01 M $MgCl_2$. The α and β components of *Helix pomatia* were obtained as a solution (5 mM Cu) in borate buffer at pH 8.2.[16] The X-ray absorption spectra were collected at the Stanford Synchrotron Radiation Laboratory on beam line I-5 and beam line IV-2 by using Si[220] crystal monochromators. Data were collected as fluorescence excitation spectra by using a solid-state NaI scintillation detector system.[17] The sample was inspected at the end of data collection, usually after ca. 10 h of irradiation, and no photobleaching from the beam could be detected. The integrity of the protein was further affirmed by examining the K absorption edge spectra taken at the beginning and at the end of the data collection period. The edge spectra were identical, showing that the copper site remained Cu(II) consistently. Data reduction and analysis were carried out as reported previously.[18] Various studies have indicated that the two copper atoms in each functional subunit are equivalent,[6,21] and the EXAFS analysis described herein makes this assumption.

The X-ray absorption edge spectrum of oxyhemocyanin was examined for evidence concerning the oxidation state of copper in this protein. Comparison of the oxyhemocyanin edge position with a series of Cu(I) and Cu(II) models indicates the presence of Cu(II) in the oxy state. Studies have shown that the structure of X-ray absorption edges can be correlated with the coordination geometry of the absorbing atom.[19] Examination of the edge structures of a series of copper models indicates analogous edge

Table I. Curve Fitting for Oxyhemocyanin

data set[a]	Cu–Im distance, Å	Cu–Im no.	Cu–O distance, Å	Cu–O no.	Cu–Cu distance, Å	Cu–Cu no.
A	2.01	2.0	1.92	2.0	3.55	1.1
B	2.02	2.2	1.92	2.0	3.58	1.2
C	2.02	1.8	1.94	2.4	3.55	1.0

[a] Data from (A) *Megathura crenulata*, (B) β component of *Helix pomatia*, and (C) α component of *Helix pomatia*.

structure among models of similar geometry.[20] The fairly smooth edge structure for oxyhemocyanin is comparable to the edge structure of square-planar $Cu(acac)_2$ and solutions of $Cu(Im)_4^{2+}$ but is different from the edge structure of distorted octahedral, tetrahedral, and square-pyramidal copper complexes. The absorption edge structure for oxyhemocyanin thus suggests the geometry of the copper site to be approximately square planar, although it does not eliminate the possibility of weakly bound axial ligand(s). Such ligands, however, will probably be of less significance in contributing to the ligand field of the copper site.

The background-subtracted, normalized EXAFS data for *Megathura crenulata* oxyhemocyanin was obtained by using published procedures.[18] Fourier transforms of the data over a k range of 3.5–12.5 Å^{-1} (Figure 1) reveal a major peak at 1.5 Å. On the realization that there is always a 0.3–0.6 Å phase shift in R space,[1a] this indicates a first coordination shell at ca. 2 Å from copper. The first major peak in the Fourier transform was filtered and back-transformed for curve-fitting analysis. The curve-fitting method usually gives absorber–scatterer distances to within ±0.02 Å and coordination numbers accurate to within about 20%.[1a,c] Curve-fitting results predict four nitrogen (or oxygen) atoms bound to copper at 1.98 Å. The number of ligands and the copper–ligand distance calculated are within experimental error the same as the results reported for *Busycon canaliculatum*.[3] Because of the similar EXAFS scattering behavior of oxygen and nitrogen atoms, they are not readily distinguishable by such an analysis.

For a more accurate representation of ligands coordinated to metal sites, a new technique of "group" curve fitting has been developed and proven useful.[4] The group fitting approach allows more accurate modeling of the phase and amplitude behavior of second- and third-shell atoms of imidazole groups, allowing other atoms of interest to be more easily identified. When the imidazole group fitting technique is applied to hemocyanin, all the atoms of the imidazole ring can be described as a single group. The distance from the copper atom to the group and the number of such groups constitute the minimal two variables necessary to describe the imidazoles in the copper environment. Fitting the oxyhemocyanin data with only imidazole groups did not reproduce the characteristic beat features of the data and gave a high F value,[22] indicating a poor fit. Fitting with imidazole groups and oxygen atoms at a short distance (ca. 2 Å) improved the quality of the fit, reducing the F value from 0.97 to 0.78. The fit matched the data quite well up to $k = 8$ Å^{-1}, but could not reproduce the characteristic beat features at higher k range. An unusually large peak at 3.3 Å in the Fourier transform (Figure 1) suggests the presence of a heavy atom(s) around 3.6 Å from the copper atom. Sulfur atoms were included but did not reproduce the characteristic beats in the data. These results strongly suggest that there are no sulfur atoms tightly coordinated to copper. A copper atom was then tried, and the quality of the resulting fit was greatly improved, reducing F to 0.61. Careful examination showed that the fit had now reproduced all the characteristic beats in the data, fitting a broad peak at low k and the two characteristic beats at high k. Results from this curve fitting, in which numbers of atoms

(11) Tallandini, L.; Salvato, B.; Jori, G. *FEBS Lett.* **1975**, *54*, 283.
(12) (a) Freedman, T. B.; Loehr, F. S.; Loehr, T. M. *J. Am. Chem. Soc.* **1976**, *98*, 2809. (b) Larrabee, J.; Spiro, T. G. *J. Am. Chem. Soc.* **1980**, *102*, 4217.
(13) Nakamura, T.; Mason, H. S. *Biochem. Biophys. Res. Commun.* **1960**, *3*, 297.
(14) Eickman, N. C.; Himmelwright, R. S.; Solomon, E. I. *Proc. Natl. Acad. Sci. U.S.A.* **1979**, *76*, 2094.
(15) Dooley, D. M.; Scott, R. A.; Ellinghaus, J.; Solomon, E. I.; Gray, H. B. *Proc. Natl. Acad. Sci. U.S.A.* **1978**, *75*, 3019.
(16) *Helix pomatia* hemocyanin samples were prepared in the laboratory of one of the authors (R.L.).
(17) Cramer, S. P.; Scott, R. A., personal communication.
(18) Cramer, S. P.; Hodgson, K. O.; Stiefel, E. I.; Newton, W. E. *J. Am. Chem. Soc.* **1978**, *100*, 2748.
(19) Cramer, S. P. Ph.D. Thesis, Stanford University, Stanford, CA, 1978.
(20) Co, M. S.; Hodgson, K. O., unpublished observations.
(21) Lontie, R.; Vanquickenborne, L. *Met. Ions Biol. Syst.* **1974**, *3*, 183.

(22) Fits are done by minimizing the difference between the observed and calculated EXAFS at each point in k space, squared, and then weighted by k^6. The minimization function value F is given by

$$F^2 = \sum[k^6(\text{data} - \text{fit})^2]/N$$

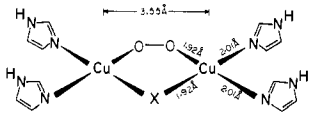

Figure 2. Model proposed for oxyhemocyanin active site. Two copper atoms separated by 3.55 Å are each bound to two imidazoles and two oxygen atoms in an approximately square-planar geometry, bridged by a bound peroxide and a protein ligand X. It should be emphasized that EXAFS analysis alone cannot give orientation information. The model is derived from a synthesis of EXAFS and edge results and other published chemical and spectroscopic data.

were not constrained to have integer values, indicated 2.0 imidazole groups at 2.01 Å, 2.0 oxygen atoms at 1.92 Å, and 1.1 copper atoms at 3.55 Å around each copper atom. This analysis identifies the presence of two imidazole ligands in contrast to the single-filtered-shell method used in the earlier studies[2,3] where only the number of atoms in the first shell was determined and imidazoles were suggested to be present on the basis of absorption edge and other spectroscopic information.

Oxyhemocyanin data from another mollusc, the α and β components of *Helix pomatia*, were collected and gave similar results. All the curve-fitting results are summarized in Table I. These results suggest that the binding sites for the intact *Megathura crenulata* and the α and β components of *Helix pomatia* hemocyanin are identical, within the experimental error of the technique. However, the small difference could also be the result of slight strain on the binuclear copper site due to different protein conformations, which might explain the different oxygenation curves observed for the α and β components of *Helix pomatia*.[23]

From this structural information and earlier spectroscopic results, it is possible to propose a model for the oxyhemocyanin binding site. The model emerging from our EXAFS analysis suggests two copper atoms separated by 3.55 Å. Each copper is in an approximately square-planar geometry and is bound to two histidines with an average Cu–N distance of 2.01 Å and two oxygen atoms at an average distance of 1.92 Å. The Cu–Cu distance of 3.55 Å precludes the possibility of direct Cu–Cu bonding but is reasonable for bridging by an oxygen atom. The bridging concept has also been suggested as a superexchange path for an antiferromagnetic coupling between two Cu(II) ions, explaining the lack of an EPR signal for oxyhemocyanin.[14] The single atom bridging model is consistent with the high coupling constant.[15] Tyrosine has been previously suggested as a possible bridging ligand, on the basis of an observation that tyrosine becomes accessible upon the removal of copper.[10,11] However, the lack of an observed charge transfer between tyrosine and Cu(II) and the high pK_a value for tyrosine make this suggestion questionable. The fourth ligand bound to copper can then naturally be assigned as the bound dioxygen (peroxide). The other oxygen atom from the peroxide cannot be located in the curve-fitting analysis. This can be explained by the minimal contribution to the scattered wave by a single low Z atom at a long distance, in the presence of many other atoms. On the basis of EXAFS and other studies, two models are most feasible, either bridging of the peroxide end to end or bridging through a single oxygen. Resonance Raman studies indicate that the peroxide oxygen atoms are equivalent.[6] These studies and a transition dipole–vector coupling model[14] both favor the end-to-end alternative (Figure 2). Preliminary results from application of the imidazole group fitting technique to EXAFS data on deoxyhemocyanin also indicate the presence of two imidazoles bound to each copper atom,[24] consistent with the proposed oxyhemocyanin model. Studies of deoxy, half-apo, and dimer forms of hemocyanin will be reported in a subsequent communication.[24]

Acknowledgment. We thank Dr. Robert A. Scott and Dr. Patrick Frank for numerous helpful discussions. We also thank Steve Conradson and Jim Hahn for assistance in data collection as well as the Department of Structural Biology at Stanford University for use of biochemical facilities in the Sherman P. Fairchild Center. This work was supported by the National Science Foundation through Grant PCM-79-04915. Synchrotron radiation time was provided by the Stanford Synchrotron Radiation Laboratory, supported by National Science Foundation Grant DMR77-27489, in cooperation with the Stanford Linear Accelerator Center and the U.S. Department of Energy.

(23) Konings, W. N.; van Driel, R.; van Bruggen, E. F. J.; Gruber, M. *Biochim. Biophys. Acta* **1969**, *194*, 55.

(24) Co, M. S.; Hodgson, K. O. *J. Am. Chem. Soc.*, submitted for publication.

Metalloprotein EXAFS. A Group Fitting Procedure for Imidazole Ligands

Man Sung Co, Robert A. Scott, and Keith O. Hodgson*

Department of Chemistry, Stanford University
Stanford, California 94305

Received August 26, 1980

The EXAFS (extended X-ray absorption fine structure) technique has been demonstrated to be useful in extracting detailed structural information about active sites in metalloproteins and metalloenzymes.[1] The curve-fitting techniques now used in our laboratory make use of a parameterized function to model the effect of each single shell of atomic scatterers on the absorption coefficient of the central metal atom.[2] Until recently, for ligands consisting of more than one shell of atoms, it was necessary to include optimizable parameters for each shell. Second-shell atoms required a different set of parameters than first-shell atoms due to differences in scattering characteristics.[3] We have now developed a method for fitting the EXAFS of a *rigid* multiatom ligand as a whole ("group fitting"[4]) and tested its utility for fitting imidazole ligands to copper atoms in several copper proteins. A major advantage of the group fitting technique is that it allows a reduction in the number of variables used in the fit. Another important advantage lies in the technique's ability to account for different phase and amplitude behavior in the second and third shell of scatterers, thereby enhancing the technique's sensitivity toward other outer shell atoms.

The essence of our standard curve-fitting analysis is the adjustment of structure-dependent parameters in the function chosen to model the EXAFS until the fit with the experimentally observed data is optimized by least squares. In general, any atom in the vicinity of the absorber contributes to the EXAFS and the fitting procedure optimizes a distance and number for each atom. The group-fitting technique makes use of the fact that, within a given ligand of n atoms, the distance and number for $n - 1$ of the atoms are highly correlated with these parameters for the other atom. Thus, only one distance and one number need be optimized, with the other parameters being constrained to obey certain relationships with the optimized parameters. These relationships are then defined by fits of model compounds containing only the multiatom ligand.

For example, in order to determine group-fitting parameters for imidazole ligands around a copper atom, $Cu(Im)_4^{2+}$ was used as a model. Three tetrakis(imidazolato)copper(II) compounds (the nitrate, perchlorate, and sulfate salts) were studied. They all give, within experimental error, identical spectra, a typical one being shown in Figure 1. The Fourier transform of the data in

(1) (a) Cramer, S. P.; Hodgson, K. O. *Prog. Inorg. Chem.* **1979**, *25*, 1. (b) Eisenberger, P.; Kincaid, B. M. *Science (Washington, DC)* **1978**, *200*, 1441.

(2) Cramer, S. P.; Hodgson, K. O.; Stiefel, E. I.; Newton, W. E. *J. Am. Chem. Soc.* **1978**, *100*, 2748.

(3) When there are other atoms lying between the absorber and the scatterer of interest, these intervening atoms cause distortions of the primary outgoing wave, resulting in a different absorber–scatterer phase shift and backscattering amplitude function. This distortion cannot be accounted for using only first-shell fitting parameters. (See: Cramer, S. P.; Dawson, J. H.; Hodgson, K. O.; Hager, L. P. *J. Am. Chem. Soc.* **1978**, *100*, 7282.)

(4) A similar technique is also used in refinements of X-ray crystallographic data where a rigid ligand such as a phenyl ring is described as a group having only one x,y,z coordinate and a group temperature factor.

0002-7863/81/1503-986$01.25/0