# Identification of the Cyanide Stretching Frequency in the Cyano Derivative of Cu/Zn-Superoxide Dismutase by IR and Raman Spectroscopy

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Cyanide has been investigated as a potential *ligand-directed* probe of the coordination chemistry of Cu(II) and Cu(I) active sites via vibrational spectroscopic studies of $CN^-$ coordinated to the metal centers in non-blue copper proteins. Native superoxide dismutase (SOD) was found to bind one $CN^-$ that had IR and Raman frequencies at 2137 $cm^{-1}$. The assignment of this band was confirmed by using $^{13}CN^-$, which gave the theoretically expected isotope shift, and by comparison with an inorganic model complex, $[Cu(PMAS)]^{2+}$, which was found to form a monocyano complex with $\nu_{CN}$ = 2125 $cm^{-1}$. Ultrafiltration experiments definitively identified the 2137-$cm^{-1}$ band as $\nu_{CN}$ of a *protein-bound* species. These results are consistent with a linear end-on-bonded $CN^-$ on Cu(II)–SOD as proposed from earlier EPR and EXAFS experiments. Replacing $H_2O$ in the medium by $D_2O$ gave no isotope shift of the 2137-$cm^{-1}$ band, indicating that $CN^-$ is most likely not involved in hydrogen-bonding interactions in the active-site cavity. With increasing concentrations of cyanide, the 2137-$cm^{-1}$ band became weaker and was accompanied by the appearance of strong vibrational modes characteristic of di-, tri-, and tetracyano Cu(I) complexes arising from Cu removal from the protein. These studies demonstrate the potential importance of cyanides (and isocyanides) as *ligand-directed* vibrational spectroscopic probes of non-blue copper proteins.

## Introduction

The coordination chemistry of the Cu(I) centers of copper-containing enzymes is of interest because of the role of Cu(I) in the binding and activation of dioxygen. Although the structural requirements for dioxygen binding have become clearer via spectroscopic and crystallographic studies on hemocyanin[1-3] and inorganic analogues of oxy- and deoxyhemocyanin,[4-6] much less is known about the Cu(I) coordination chemistry of oxidases and monooxygenases. The invisibility of these Cu(I) centers to most *metal-directed* forms of spectroscopy has made *ligand-directed* spectroscopic methodologies especially attractive. Therefore, as part of our continuing studies on copper oxidative enzymes, we have initiated a program of ligand-directed vibrational spectroscopy[7,8] in combination with X-ray absorption of the Cu(I) centers in our efforts to characterize the Cu(I) coordination chemistry[9,10] and compare the properties of the oxidized and reduced forms.

Cyanides and alkyl isocyanides have been invoked as Cu(I)-binding ligands[11] but also often form stable complexes with the Cu(II) forms of copper proteins. Whereas a number of non-blue copper proteins such as amine oxidases,[12] superoxide dismutase,[13] galactose oxidase,[14] and Cu-substituted carbonic anhydrases[15] form stable cyano complexes, others such as dopamine $\beta$-hydroxylase[16] and hemocyanin[17] react with $CN^-$ to form metastable complexes from which the copper atoms are easily lost to form apoproteins. These differences in stability must in some way be related to the coordination environment of the Cu(II) and/or

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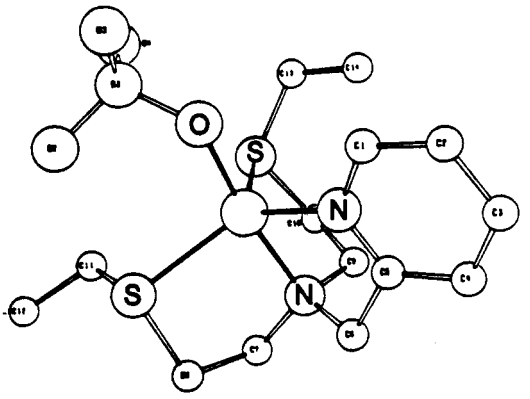
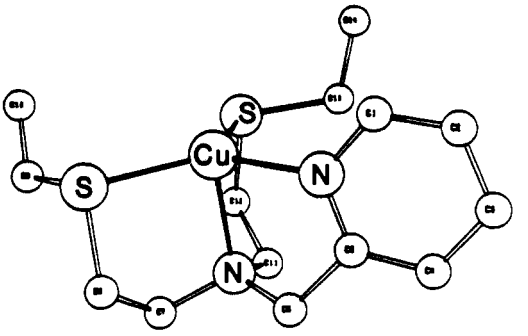
**Chart I**




[Cu(PMAS)][SO$_4$]




[Cu(PMAS)]$^+$

Cu(I) centers in the respective copper proteins. Thus $CN^-$ might be expected to be a good candidate for comparative ligand-directed spectroscopy on the oxidized and reduced forms of the proteins and provide valuable information on redox-induced changes in substitution chemistry.

Although $CN^-$ has been utilized as a vibrational probe in heme systems,[18] few IR or Raman spectroscopic studies of cyanide binding to either Cu(II)- or Cu(I)-protein sites have been reported.[11a] Therefore we have chosen to initiate our studies via the well-characterized Cu(II)-cyano complex of Cu/Zn-SOD as a model copper-protein system.[19-25] As a necessary adjunct to the study of the protein-cyanide interactions, we have also reinvestigated the IR and Raman spectra of simple cyanocuprate(I) complexes in aqueous solution for which the vibrational spectra are quite well understood,[26-28] together with a novel inorganic copper-cyanide complex of the tripodal ligand PMAS[29] (see Chart I).

Here we report the first observation of a cyanide stretching frequency for the cyano complex of Cu(II)-SOD (2137 cm$^{-1}$) together with a full characterization of the interaction of cyanide with the Cu(II)-PMAS model system. Our results unambiguously confirm the formation of an end-on-bonded monocyano-SOD derivative, as previously suggested.[13,19-24] Substitution of $H_2O$ in the medium by $D_2O$ gives no isotopic shift of the 2137-cm$^{-1}$ band, suggesting that the ligated cyanide is most likely not involved in hydrogen-bonding interactions. Excess $CN^-$ leads to reduction of the Cu(II), and under these conditions, we have been unable to obtain evidence for any species other than inorganic Cu(I) cyanocuprates.

## Materials and Methods

**Preparation of Samples for IR and Raman Studies.** All cyanide samples were freshly prepared on the day the spectrum was recorded. Tetrakis(acetonitrile)copper(I) hexafluorophosphate, [Cu(CH$_3$CH)$_4$][PF$_6$], was prepared by literature methods.[30] Aqueous Cu(I)-cyano complexes were prepared by dissolving (with gentle heating) solid [Cu(CH$_3$CN)$_4$][PF$_6$] in a small aliquot of concentrated (1.0 M) aqueous sodium cyanide. When dissolution was complete, deionized water was added to adjust the final concentrations. The pH of these solutions was close to 10. Na$^{13}$CN was purchased from Stohler/KOR Isotopes, Woburn, MA. $D_2O$ was purchased from Sigma (99.8% D). All other materials were of reagent grade unless otherwise stated. Solid CuCN ($^{12}$CN, $^{13}$CN) was prepared on the basis of the low solubility of the compound at a $CN^-$:Cu ratio ($R$) = 1. First, [Cu(CH$_3$CN)$_4$][PF$_6$] solid was dissolved in 5 mL of 10% CH$_3$CN/H$_2$O solvent (with gentle heating) with [Cu] ~ 10 mM. Then, a small aliquot of concentrated aqueous NaCN (1.0 M) was added to the solution to obtain the white precipitate of copper(I) cyanide. Only 80% of the stoichiometric amount of cyanide for CuCN was used to minimize disproportionation of Cu(I). The white precipitate was filtered off and washed with ethanol. The air-dried CuCN solid was stored in a desiccator.

[Cu(PMAS)][SO$_4$] was a gift from Professor K. D. Karlin. This complex was found to form a cyano complex in the Cu(II) oxidation state which was insoluble in water. For this reason, 1:1 DMF/water (v/v) was used as solvent for studies on the PMAS system. The cyano complexes were formed by addition of 20-$\mu$L aliquots of a stock solution of sodium cyanide (1:1 DMF/water, v/v) to 0.4 mL of an 18 mM solution of the Cu(II)-PMAS complex, followed by rapid mixing. The resulting solution was transferred to a 2 mm path length optical cuvette, and the visible absorption spectrum recorded using a Perkin-Elmer $\lambda$9 spectrophotometer. A 100-$\mu$L aliquot of this solution was then transferred into the IR cell and the FTIR spectrum recorded. Since collection of the data took approximately 40 min, the visible absorption spectrum was recorded again immediately after completion of the IR measurement to check for reduction or sample degradation. In all cases, visible absorption spectra were identical before and after the IR experiment. Fresh 200-$\mu$L aliquots of each sample were then transferred to an EPR tube and frozen for subsequent recording of the EPR spectrum.

Bovine erythrocyte SOD was purchased from DDI Pharmaceuticals, Mountain View, CA. Protein samples were dissolved in 10 mM $Na_2B_4O_7$ buffer at pH 8.4. Protein concentration was determined by absorption at 258 nm ($\epsilon$ = 10 300 M$^{-1}$ cm$^{-1}$), and the copper concentration was calculated from the relationship of 2 mol of Cu/mol of protein dimer.[31] A sample of protein stock solution which had been chromatographed in a gel-permeation column prerinsed by Chelex-100-treated buffer gave the same results as that from non-pretreated protein solution. Nonspecifically bound Cu(II) ions were therefore not considered a problem, and the SOD was used without further purification. SOD-CN samples were

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prepared by addition of 5 μL of a concentrated solution of NaCN to 60 μL of native SOD. By varying the concentration of the stock cyanide solution, samples with Cu:CN⁻ ratios ($R$) from 1 to 12 were prepared.

Apo-SOD was prepared by dialysis of the holoprotein against 50 mM sodium acetate, pH 3.8, containing 10 mM EDTA (three buffer changes), followed by exhaustive dialysis against (a) 50 mM sodium acetate, 100 mM sodium chloride, pH 3.8 to remove EDTA (five buffer changes), (b) 100 mM sodium acetate, pH 3.8 to remove sodium chloride (three buffer changes), and (c) 10 mM $Na_2B_4O_7$, pH 8.4 (three buffer changes).

Ultrafiltration was used to separate protein-bound from protein-free cyano species. In these experiments, 100 μL of SOD–CN solution was loaded into an Amicon Centricon ultrafiltration cell fitted with a PM-10 membrane filter and centrifuged until sufficient filtrate was obtained for spectral measurement (20 μL).

The $D_2O$-exchanged sample of cyano–SOD was prepared by first dissolving lyophilized native SOD in 10 mM $Na_2B_4O_7/D_2O$ buffer (pH meter reading 8.4) and then diluting the solution 10-fold in $D_2O$ buffer. The sample was then concentrated by use of a Centricon 10 device. The above dilution and reconcentration steps were repeated two more times. Before the last reconcentration step, the dilute solution was incubated overnight at 5 °C. The same sample but in $H_2O$ was prepared in parallel to serve as a control.

**IR Measurements.** Infrared spectra were recorded on a Perkin-Elmer 1800 series FTIR spectrometer equipped with a room-temperature DTGS detector and interfaced to a Perkin-Elmer 7500 computer. The Cu–PMAS IR titrations utilized a SpectraTech Micro Circle cell with a $^1/_8$ in. diameter ZnSe crystal rod. All other IR measurements utilized a cell constructed of rectangular $CaF_2$ windows separated by a 0.025-mm Teflon spacer (MacArthy Scientific), and the whole assembly was placed in a thermostatable copper block. In all cases, the IR spectrum of a reference (unligated protein, buffer, or solvent) was subtracted from that of the cyanide-ligated system. To keep the path length constant in each pair of data (sample and reference) so as to obtain a flat baseline and the best cancellation of water absorption, a syringe was used to flush the sample into the cell after the spectrum of the blank was recorded, thereby eliminating the introduction of micro air bubbles into the cell. The spectra of inorganic complexes were recorded at room temperature and referenced against solvent. The enzyme samples were studied at 10 °C using a refrigerated constant-temperature circulator (VWR1165) and referenced against the identical concentration of buffered, unligated protein. FTIR spectra of CuCN were obtained by mixing 1 mg of the solid with 2 drops of mineral oil and applying the mull to $CaF_2$ plates. For each sample, 200 scans were collected in the range 800–4000 $cm^{-1}$ with a resolution of 2 $cm^{-1}$. Typical absorbances for the $CN^-$ vibration were 0.0001–0.03 in the range 2000–2300 $cm^{-1}$. Base-line correction using standard Perkin-Elmer software did not affect quantitative aspects of the spectra but did improve the presentation.

**Raman Measurements.** Raman spectra were obtained with an IBM AT/286 interfaced Dilor Z24 spectrophotometer equipped with a cooled Hamamatsu 943-02 photomultiplier tube.[32] Excitation at 514.5 nm was provided by a Coherent Innova 90-6 $Ar^+$ laser. The Raman spectra were collected in a 90°-scattering geometry. Protein samples (contained in standard glass melting-point capillaries) were studied at a temperature of ~5 °C by placement of the capillary in a copper cold finger immersed in an ice/water-filled Dewar flask. The other Raman samples were studied at room temperature. Raman spectra of the solids were obtained on sealed glass capillaries in a backscattering geometry at room temperature. For isotopic comparisons, samples were run consecutively under identical instrumental conditions. Peak positions were determined by abscissa expansion. The Raman instruments were calibrated daily for frequency accuracy with distilled indene, and spectral frequencies are accurate to ±1 $cm^{-1}$.

**EPR Measurements.** EPR spectra were recorded on a Varian E-109 spectrometer equipped with a Macintosh computer. Temperature was controlled to 108 K with a Varian E-257 variable-temperature controller using a cooled nitrogen-gas flow system. The microwave frequency was 9.211 GHz, the power was 1.0 mW, and the modulation amplitude was 5 G.

**X-ray Absorption Measurements.** Samples were studied on beamline 7.1 at SRS, Daresbury Laboratory, with an electron beam energy of 2.0 GeV and a maximum stored current of 300 mA. The data were collected with a Si(111) double crystal monochromator and a focusing mirror which also rejected harmonics. Samples were studied as frozen glasses at 100 K in fluorescence mode using a NaI scintillator–detector array. Energy calibration was achieved by setting the first inflection point of a copper foil spectrum to 8980.3 eV, and the resolution was estimated to be about 3 eV by inspection. EXAFS data reduction and analysis were performed as previously described,[10b] using curved-wave multiple-scattering calculations. The quality of the fits was determined using a least-squares-fitting parameter or fit index, $F$, defined as

$$F^2 = (1/N)\sum k^6(\chi_i^{\text{theor}} - \chi_i^{\text{exp}})^2$$

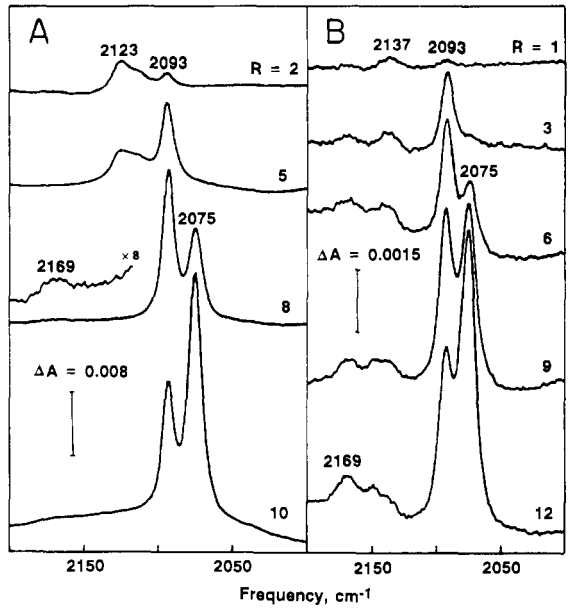


**Figure 1.** FTIR titration of aqueous $[Cu(CH_3CN)_4][PF_6]$ (A) and Cu(II)–SOD (B) with cyanide. $R = [CN^-]/[Cu]$. Copper concentrations were 8 mM, for all spectra, except spectrum A, $R = 2$, where [Cu] = 200 mM.

**Copper Content.** The copper content of the SOD–CN samples was determined using a Varian/Techtron (AA-5) atomic absorption spectrophotometer. Ultrafiltration (1 h, 4000 rpm) was used to separate inorganic copper- from protein-bound copper-containing species. For each analysis, 10 μL of protein–$CN^-$ solution was diluted with 1.5 mL of buffer. The filtrate and retentate solutions were each further diluted by adding 0.6 mL of buffer to give sufficient volume for three absorption measurements (3 × 300 μL). The total volume of filtrate and retentate was estimated by weighing each part of the Centricon before and after addition of sample, assuming the density of the dilute solution was 1. These analyses allowed for the determination of both protein-bound ([Cu in retentate] – [Cu in filtrate]) and inorganic cyanocuprate ([Cu in filtrate]) copper concentrations. The copper concentrations in both fractions of an unligated SOD sample were also measured as a control.

## Results

**Cyanocuprate(I) Complexes.** As a basis for understanding the vibrational spectroscopy of coordinated cyanide, the infrared and Raman spectra of cyanocuprate(I) complexes in dilute aqueous solution were first investigated. IR spectra of solutions of $[Cu(CH_3CN)_4][PF_6]$ with increasing concentrations of sodium cyanide as $R = [CN^-]/[Cu(I)]$ changes from 2 to 10 are shown in Figure 1A. As summarized in Table I, these bands have been assigned to the asymmetric stretching modes of the di-, tri-, and tetracyano Cu(I) complexes, respectively: $[Cu(CN)_2]^-$ (2123 $cm^{-1}$), $[Cu(CN)_3]^{2-}$ (2093 $cm^{-1}$), and $[Cu(CN)_4]^{3-}$ (2075 $cm^{-1}$).[33,34] In addition, we note a very weak and broad band at ~2169 $cm^{-1}$ that is present in all samples. Its intensity does not appear to be significantly affected by changing cyanide concentrations. Using $^{13}CN^-$, the bands at 2123, 2093, and 2075 $cm^{-1}$

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**Table I.** Raman and Infrared Frequencies ($cm^{-1}$) of the $CN^-$ Vibrations in Copper Complexes in Aqueous Solution ($^{13}CN^-$ Isotope Shift)

| species | Raman | IR | vib mode | ref |
|---|---|---|---|---|
| $Cu(CN)_4^{3-}$ | 2075 (–45) | 2075 (–44) | $\nu_{as}$ | 33, 34, 36–38 |
| | 2095 (–46) | | $\nu_s$ | |
| $Cu(CN)_3^{2-}$ | 2095 | 2093 (–45) | $\nu_{as}$ | 33, 34, 36, |
| | 2109 (–46) | | $\nu_s$ | 37, 39 |
| $Cu(CN)_2^-$ | 2138 | 2123 | | 33, 34, 36, 37 |
| CuCN solid | 2170 (–46) | 2169 (–45) | | 38, this work |
| KCN | 2080 (–42) | 2080 | | 36 |
| HCN | | 2093 | | 18c |
| DCN | | 1887 | | 18c |
| $[Cu(PMAS)CN]^+$ | | 2125 (–44) | | this work |
| $[Cu(PMAS)]^{2+}$ + excess $CN^-$ | 2091 | 2093 (–44) | | this work |
| | 2106 | 2165 (–61) | | |
| SOD–CN | 2137 (–46) | 2137 (–46) | | this work |

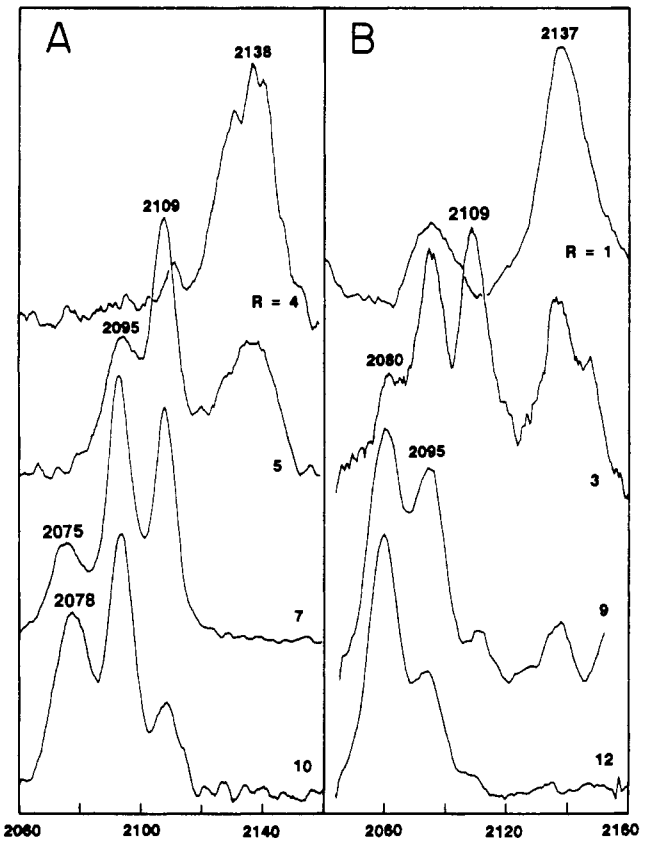


**Figure 2.** Raman spectral titration of aqueous $[Cu(CH_3CN)_4][PF_6]$ (A) and Cu(II)–SOD (B) with cyanide. $R = [CN^-]/[Cu]$. Conditions were the same as for Figure 1.

all shift close to –44 $cm^{-1}$, in agreement with the value calculated for a simple diatomic oscillator.[35] However, the 2169-$cm^{-1}$ band has an abnormally large $^{13}CN^-$ shift of –60 $cm^{-1}$, and its assignment is not clear at this time.

The corresponding Raman spectra of these cyanocuprate complexes are shown in Figure 2A. These spectra agree well with the results previously reported,[34,36–39] and correlate with the stepwise formation of di-, tri- and tetracyanocuprate species (Table I). At high values of $R$, free cyanide with $\nu_{CN}$ = 2080 $cm^{-1}$ contributes to the Raman spectrum, causing the apparent shift of the lowest frequency band to 2078 $cm^{-1}$.

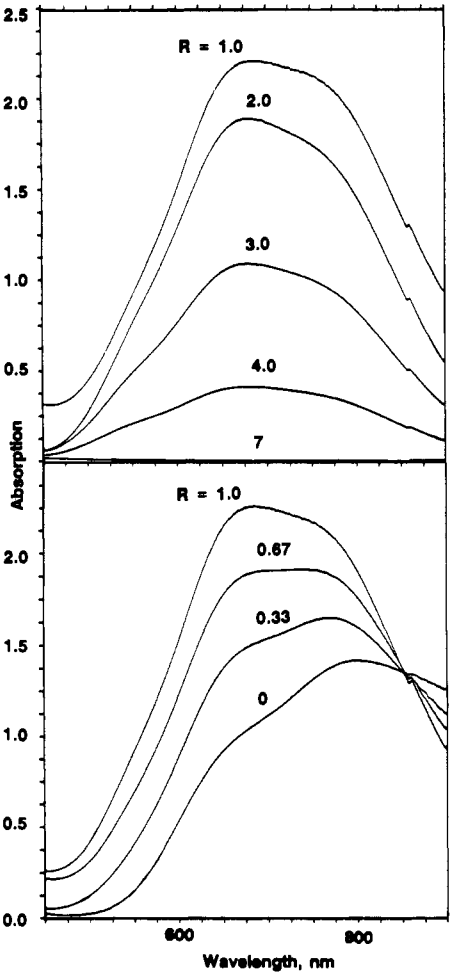


**Figure 3.** Electronic spectral titration of $[Cu(PMAS)][SO_4]$ with cyanide. $[Cu(PMAS)][SO_4]$ concentrations were 18 mM in 1:1 DMF/water. $R = [CN^-]/[Cu(PMAS)]^{2+}$.

**Cyanide Binding to $[Cu(PMAS)]^{2+}$.** To further assess the usefulness of $\nu_{CN}$ as a probe of copper coordination chemistry and biochemistry, we have investigated cyanide binding to a well-characterized inorganic complex which mimics cyanide interaction with the Cu centers in proteins. The tetradentate tripodal ligand PMAS forms complexes with copper in both the Cu(II) and Cu(I) oxidation states, and crystal structures for both complexes have been reported.[29] The Cu(II) complex binds inorganic anions such as $Cl^-$, to form trigonal-bipyramidal pentacoordinate structures in the solid state, and it may be inferred that, in solution, a solvent-accessible vacant coordination site is available similar to the anion-binding sites in non-blue copper proteins.[29] Details of the structures of the Cu(II) and Cu(I) complexes of PMAS are given in Chart I.

Green crystals of $[Cu(PMAS)][SO_4]$ were dissolved in 1:1 DMF/water to give a blue solution with $\lambda_{max}$ = 800 nm. Titration of an 18 mM solution of this solvated species with NaCN resulted in a color change from blue to purple, corresponding to the visible absorption changes shown in Figure 3. Isosbestic behavior was observed up to a $CN^-$:CuPMAS ratio ($R$) of 1, indicating the formation of a monocyano species, consistent with the anion-binding chemistry discussed above. As $R$ was increased above 1, the spectrum remained unchanged but diminished in intensity, and was finally bleached. When the $CN^-$ titration was followed by EPR spectroscopy, similar results were obtained (data not shown). The EPR spectrum of the solvated species converted to a new form with lower $g$ values, the conversion being essentially complete at $R$ = 1. The EPR spectra are nonaxial, consistent with distorted trigonal-bipyramidal coordination.[29] Further addition of $CN^-$ decreased the intensity of the signal. All of these data support the formation of a monocyano complex, [Cu-

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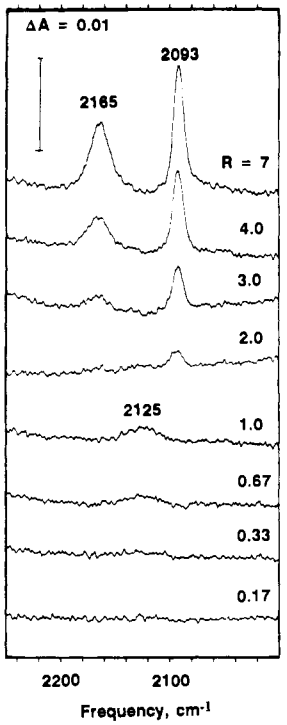



Figure 4. FTIR spectra corresponding to the solutions in Figure 3.

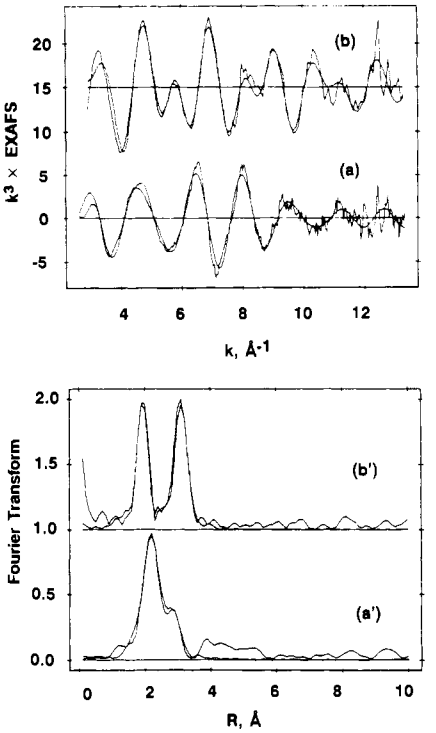



Figure 5. Experimental versus simulated EXAFS and Fourier transforms of $[Cu(PMAS)]^{2+}$ treated with 1 equiv of sodium dithionite (a, a') and with an excess ($R$ = 7) of sodium cyanide (b, b').

$(PMAS)(CN)]^{+}$, which is reduced by excess $CN^-$ to a colorless, diamagnetic Cu(I)-containing species.

**FTIR and Raman Studies of Cyanide Interaction with $[Cu(PMAS)]^{2+}$.** Figure 4 shows IR spectra corresponding to the electronic spectral titrations described in Figure 3. For values of $R$ below 1, a broad band centered at 2125 $cm^{-1}$ grows in parallel with the formation of $[Cu(PMAS)CN]^{+}$. However as $R$ increases above 1, and the Cu(II)–cyano complex is reduced to the Cu(I) complex, new bands appear in the IR spectra at 2093 and 2165 $cm^{-1}$. Confirmation that all these bands are derived from cyano species is provided by the isotope shifts given in Table I. Raman experiments were unable to detect any bands associated with the oxidized [Cu(PMAS)]–cyano species ($R < 1$). For $R > 1$, a doublet of bands was observed at 2106 and 2091 $cm^{-1}$ (data not shown).

The 2125-$cm^{-1}$ band may be assigned with confidence to the $\nu_{CN}$ of a coordinated cyano group in $[Cu(PMAS)CN]^{+}$. The bands which appear in parallel with reduction of the complex by excess cyanide could possibly arise either from $\nu_{CN}$ of a monocyano–Cu(I)–PMAS complex, [Cu(PMAS)CN], or from cyanocuprate(I) species formed by degradation of the $[Cu(PMAS)CN]^{+}$ complex. The weight of evidence supports the latter assignment because the IR (2093 $cm^{-1}$) and Raman (2106, 2091 $cm^{-1}$) frequencies, depolarization ratios, and isotope shifts are identical (within experimental error) to those of $[Cu(CN)_3]^{2-}$ and the noncoincidence of Raman and IR bands implies the presence of more than one cyano group in the complex, giving rise to both symmetric and asymmetric modes. Although the band positions did not convert to those expected for $[Cu(CN)_4]^{3-}$ under conditions of excess $CN^-$ (Table I), we were able to show that in 1:1 DMF/water, the tetracyano species does not form, owing to solvation by DMF (data not shown).

**EXAFS Studies of Cyanide Interaction with $[Cu(PMAS)]^{2+}$.** The identity of the species formed in the PMAS system for $R > 1$ was further investigated by EXAFS spectroscopy, and the results are shown in Figure 5. The experimental and simulated EXAFS data and Fourier transforms for a sample of $[Cu(PMAS)]^{+}$ are shown in Figure 5a and Figure 5a', respectively. The spectrum is dominated by two S ligands in the first coordination sphere, and can be simulated by two N atoms at 2.06 Å, one S at 2.28 Å, and one S at 2.68 Å. The metrical parameters derived from EXAFS analysis are of interest in that one of the thioether S ligands is found at a longer distance than in the crystal structure.[29] We interpret this as the result of solvation by DMF. In marked contrast, it can be seen that the EXAFS spectrum of $[Cu(PMAS)]^{2+}$ plus excess $CN^-$ ($R$ = 7) shown in Figure 5b is dramatically different. The split shell in the FT (Figure 5b') due to S ligation is no longer present, and instead the spectrum is dominated by an intense second shell in the transform, corresponding to higher frequency oscillations in the EXAFS spectrum. We have been able to obtain an excellent fit to the data using single- and multiple-scattering contributions from three cyanide groups with Cu–C = 1.95 Å, C–N = 1.21 Å, and Cu–C–N = 180°. No other ligands were necessary to simulate the data. The distances compare well with the crystal structure of sodium tricyanocuprate which shows average Cu–C and C–N distances of 1.93 and 1.16 Å, respectively.[39] Thus, the EXAFS data confirm that the species produced with excess cyanide has the empirical formula $Cu(CN)_3^{2-}$ and is derived from decomposition of the $[Cu(PMAS)]^{2+}$ complex. The coincidence of the IR and Raman band positions with those of an authentic $[Cu(CN)_3]^{2-}$ species supports its presence as the major species. The identity of the 2165-$cm^{-1}$ band is less clear, although EXAFS suggests it to have a Cu:CN stoichiometry close to 1:3. An assignment as an oligomeric cyanocuprate complex is the most likely, although other assignments including mixed cyano–PMAS–Cu(I) derivatives cannot be ruled out.

**Vibrational Spectroscopy of Cyano–SOD.** The infrared spectra of SOD solutions titrated with cyanide as $R$ changes from 1 to 12 are shown in Figure 1B. At $R$ = 1, a feature centered at 2137 $cm^{-1}$ is obtained together with two very low-intensity bands at 2093 and 2169 $cm^{-1}$. As the $[CN^-]$ concentration increases to $R$ = 6, a fourth band at 2075 $cm^{-1}$ appears. Thereafter, the 2169-, 2093-, and 2075-$cm^{-1}$ bands become more intense, and finally at $R$ = 12, the 2075-$cm^{-1}$ band becomes the most intense. On the other hand, the intensity of the 2137-$cm^{-1}$ band remains almost constant and can still be observed even at $R$ = 12. The behavior of the bands at 2169, 2093, and 2075 $cm^{-1}$ is very similar to that observed for the inorganic cyanocuprate system (Figure

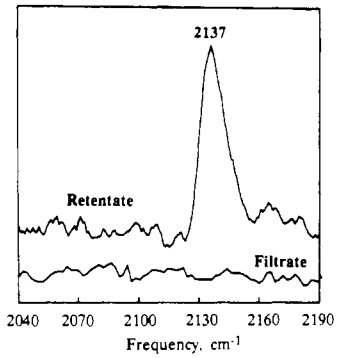



**Figure 6.** Raman spectra of ultrafiltered solutions of SOD–CN at $R$ = 1, [Cu] = 8 mM. The upper trace is that of the retentate and corresponds to protein-bound species (2137 $cm^{-1}$).

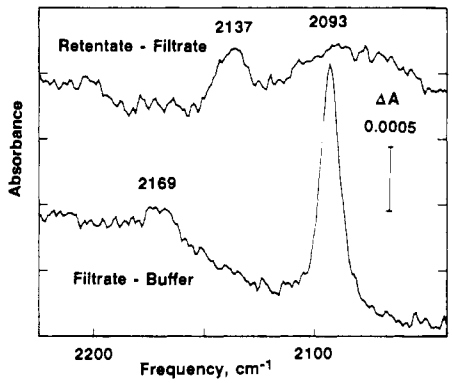



**Figure 7.** FTIR spectra of ultrafiltered solutions of SOD–CN at $R$ = 3, [Cu] = 8 mM. The upper trace is the retentate–filtrate difference spectrum and represents protein-bound species (2137 $cm^{-1}$). The lower trace is the filtrate–buffer difference spectrum and represents low-molecular-weight components (2169, 2093 $cm^{-1}$).

1A). We propose that the 2137-$cm^{-1}$ band is due to $\nu_{CN}$ of a monocyano complex of Cu(II)–SOD.

The corresponding Raman spectra of these SOD–CN solutions are shown in Figure 2B. At the beginning of the titration, a single broad band centered at 2137 $cm^{-1}$ is obtained. It is at the identical frequency as in the IR spectra. With increasing $R$, the 2137-$cm^{-1}$ band diminishes relative to new bands at 2109, 2095, and 2080 $cm^{-1}$, diagnostic of cyanocuprate(I) complexes. At $R$ = 12, the 2137- and 2109-$cm^{-1}$ bands have essentially disappeared relative to the 2080-$cm^{-1}$ band, which is due to both the $[Cu(CN)_4]^{3-}$ complex and free cyanide. In the SOD–CN system over the interval $3 < R < 9$, the 2137-$cm^{-1}$ band coexists with bands due to inorganic cyanocuprate(I) complexes and free cyanide. For the protein-free system, however, the 2138-$cm^{-1}$ Raman band disappears rapidly with increasing $R$, as shown, for example, by the complete absence of this feature in the $R$ = 7 spectrum of Figure 2A. This suggests that the origins of the ~2137-$cm^{-1}$ band are different in the two systems, assignable to $[Cu(CN)_2]^-$ and to copper-bound cyanide in SOD–CN, respectively. The characteristics of this band in many ways resemble those of the 2125-$cm^{-1}$ band of the $[Cu(PMAS)]^{2+}$ system and suggest an assignment as the intraligand stretch of coordinated $CN^-$ in the monocyano–Cu(II)–SOD complex.

Given the ambiguity in the assignment of the ~2137-$cm^{-1}$ band to either a protein-bound or a protein-free species, we sought to obtain more direct evidence by ultrafiltration. Raman and IR spectra of the retentate and filtrate solutions prepared at ratios of $R$ = 1 and 3, respectively, are shown in Figures 6 and 7. Quantitation of the EPR spectra of these solutions shows that the protein-bound copper exists entirely as Cu(II) within experimental error. The Raman spectrum of the protein fraction in the retentate (Figure 6) has a single, broad band at 2137 $cm^{-1}$ ($\Delta[^{13}CN^-]$ = –46 $cm^{-1}$), whereas the filtrate solution shows no peaks in this

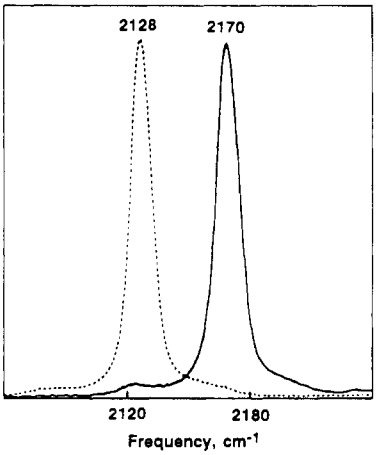



**Figure 8.** Raman spectra and isotope shift of solid CuCN: $^{12}CN$ (solid line); $^{13}CN$ (dotted line).

region (Figure 6). As a control, the Raman spectrum of a sample of apo-SOD was measured under identical conditions of protein and cyanide concentrations, and no 2137-$cm^{-1}$ band was detected. These experiments allow unambiguous assignment of the 2137-$cm^{-1}$ band to the intraligand stretch of cyano–Cu(II)–SOD. The FTIR difference spectrum (Figure 7) for the retentate minus the filtrate spectrum at $R$ = 3 (representing the protein species that cannot pass through the membrane) shows a single broad band at 2137 $cm^{-1}$, giving further confirmation of the assignment. At this ratio ca. 10% of the copper has been removed from SOD by $CN^-$ complexation to form $[Cu(CN)_3]^{2-}$, but this is only observed (2093 $cm^{-1}$) in the IR spectrum of the filtrate. In addition, the weak absorption at ~2169 $cm^{-1}$, already mentioned before (Figures 1 and 4), is also observed only in the filtrate spectrum and proves that this band must be associated with a low-molecular-weight cyano species rather than a protein-bound species.

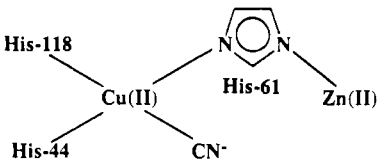
It has been suggested that $CN^-$ binding to Cu/Zn–SOD involves H bonding of the anionic ligand as well as coordination to Cu(II). Vibrational spectroscopy provides a way to detect H-bonding interactions via band shifts induced by proton exchange in $D_2O$. Unexpectedly, replacing $H_2O$ in the medium with $D_2O$ gave no isotope shift of the 2137-$cm^{-1}$ band. The absence of a shift indicates that the ligated cyanide is most likely not involved in strong hydrogen-bonding interactions.

**Copper(I) Cyanide Solid.** To probe the origin of 2169-$cm^{-1}$ band, we studied the IR and Raman spectra of solid CuCN. The CuCN samples give clean IR and Raman spectra, each with a single band at 2169 and 2170 $cm^{-1}$, respectively (Figure 8). In $Cu^{13}CN$, this band shifted approximately –42 $cm^{-1}$, close to the anticipated value of –44 $cm^{-1}$. Therefore, the –60-$cm^{-1}$ shift of the bands in the 2165–2170-$cm^{-1}$ region of the IR spectra, observed both for the inorganic $[Cu(CN)_x]^{n-}$ and [Cu(PMAS)] systems and for the SOD–CN system, rules out the possibility that these bands with the "abnormal" isotope shift arise from microprecipitated CuCN (or related) species.

## Discussion

The results described above have documented the presence of IR bands in the spectra of both $[Cu(PMAS)]^{2+}$ and oxidized SOD which can be assigned with reasonable certainty to the intraligand stretching frequencies of the $CN^-$ ligand in monocyano–Cu(II) complexes. For the model complex, UV/vis and EPR spectra provide good evidence for the formation of a monocyano adduct at or below a $CN^-$:Cu ratio of 1:1. In the case of Cu(II)–SOD, a large number of studies have confirmed binding of $CN^-$ to the Cu(II) centers. Early studies demonstrated that cyanide binds via its carbon end to copper in the ratio of 1 mol/mol of metal.[13] The visible spectrum of SOD–CN is blue-shifted

to 550 nm from 680 nm in the native enzyme, and the EPR spectrum exhibits an axial shape rather than the rhombic spectrum of native SOD.[13] The redox potential of SOD–CN is more negative than that of native SOD, suggesting a preference of $CN^-$ to bind to the Cu(II) rather than the Cu(I) form.[40] Results using electron spin echo spectroscopy indicate that the bridging imidazolate remains intact on cyanide binding and that cyanide binds in an equatorial position, replacing one of histidine ligands on copper.[19] ENDOR spectroscopy suggests a square-planar geometry around copper consisting of three imidazole nitrogen atoms and one $CN^-$ carbon.[20] Of the three imidazole nitrogen atoms, two are magnetically equivalent and strongly coupled; the third nitrogen is inequivalent and weakly coupled and is in a trans position to cyanide. EXAFS studies using a multiple-scattering treatment support these results and provide metrical details of the SOD–CN derivative.[22] These data showed a Cu–CN bond length of 1.97 Å and a Cu–C–N angle of 180°. To address which histidine ligand is displaced by cyanide, Bertini and co-workers have carried out a series of NMR and NOE experiments.[23-25] $CN^-$, $N_3^-$, and $NCO^-$ are all shown to behave similarly in their copper binding and to cause the removal of the same coordinated histidine.[24] Saturation-transfer, steady-state, and transient-NOE experiments have suggested that $CN^-$ removes His-46,[24] rather than His-44,[23] but this is still the subject of debate.[48] The proposed metal site structure of the SOD–CN derivative is shown in the following diagram:




The intraligand stretch associated with cyanide bound at such a site is found at 2137 $cm^{-1}$. This frequency differs from that exhibited by $[Cu(PMAS)CN]^+$ (2125 $cm^{-1}$) but resembles that of $[Cu(phen)_2(CN)]^+$ (2136 $cm^{-1}$).[41] This indicates that $\nu$(CN) is sensitive to the nature of the other ligands in the complex (N vs S) and is a potentially useful probe of Cu(II) coordination. The relationship between $\nu$(CN) and coordination environment is determined by the mode of bonding in the Cu(II)–cyano complexes. The pair of electrons which are donated to Cu(II) are localized on the C atom. These are weakly antibonding in the free ligand but become bonding upon coordination to the metal, because $CN^-$ is a good $\sigma$-donor and a poor $\pi$-acceptor.[27,42,43] As a result, $\nu_{CN}$ is expected to increase upon binding to Cu(II), as is indeed found in the Cu(II)–cyano systems studied in this and related work. One may speculate (on the basis of the limited data available) that the greater the basicity or $\sigma$-donor potential of the other ligands (N vs S), the more the frequency will be increased over that of free cyanide.

Previous Raman studies on metalloproteins from this and other laboratories have established that hydrogen bonding may be detectable via the observation of frequency shifts upon deuteration of the sample. Thus, for example, the symmetric Fe–O–Fe vibrational mode of oxyhemerythrin shifts from 486 to 490 $cm^{-1}$ upon exchange of solvent from $H_2O$ to $D_2O$ due to the presence of a hydrogen bond between the $\mu$-oxo group and the terminally coordinated dioxygen (hydroperoxo) molecule.[44] Similarly, $D_2O$ exchange results in an 8-$cm^{-1}$ increase in $\nu_{CN}$ of HRP(II)CN (HRP = horseradish peroxidase), which has been ascribed to the formation of an H bond between the N of coordinated $CN^-$ and a protonated (deuterated) protein residue.[18c] The literature of anion binding to SOD supports the premise that anions are stabilized in the active-site pocket via H bonding to Arg-141 (bovine) or Arg-143 (human). The evidence for this is derived from the fact that the binding constant for $N_3^-$, $CN^-$, and $SCN^-$ is controlled by the charge at position 143 in the case of the $Cu_2Co_2$ derivative of recombinant human SOD in which Arg-143 has been replaced by Lys, Ile, or Glu.[25] However, in the present study, we have been unable to detect any shift in Raman frequency of cyano–SOD at 2137 $cm^{-1}$ on exchange of solvent from $H_2O$ to $D_2O$. This implies that $\nu_{CN}$ is not coupled to a proton or deuteron vibration and would seem to suggest that H bonding is not important in stabilizing the binding of the $CN^-$ in the active-site cavity. Any stabilization of anion binding to Cu(II) via the positive charge at position 143 would thus appear to be due to nondirectional electrostatic effects.

Excess cyanide leads to progressive reduction of Cu(II) in both the $[Cu(PMAS)]^{2+}$ and SOD systems, associated with new bands in the Raman and IR spectra. These bands exhibit frequencies, isotope shifts, and Raman depolarizations identical to those of aqueous inorganic cyanocuprate species and clearly arise from low-molecular-weight species, since they are not retained by the ultrafiltration membrane. During the reduction phase of the cyanide titrations, no new bands assignable to protein-bound Cu(I)–CN species were observed. Thus it appears that inorganic cyanocuprates act as thermodynamic sinks and monocyano–Cu(I) complexes of either PMAS or SOD are unstable relative to the simple di-, tri-, and tetracyanocuprate complexes. This suggests that, contrary to previous proposals that $CN^-$ is a good ligand for Cu(I),[11] the greater stability of the cyanocuprate species will always lead to breakdown of the complex and removal of copper from the protein. Indeed, the efficiency of $CN^-$ dialysis in producing apoprotein derivatives of metalloproteins is assuredly due to this effect.

The 2169-$cm^{-1}$ band is a new band not previously reported for the cyanocuprate system. An analogous intense band (2165 $cm^{-1}$) is also found in the spectra of the [Cu(PMAS)]–CN system under conditions of excess $CN^-$. It is neither a protein species, because it passes through an ultrafiltration membrane, nor a microcell of CuCN solid, because it has an abnormal $^{13}CN$ isotope shift. It is a low-molecular weight soluble Cu(I)–cyanide species but does not seem to be in equilibrium with $[Cu(CN)_x]^{1-x}$ because the intensity does not correlate with changes in $\nu$(CN) of $[Cu(CN)_x]^{1-x}$. The high energy of this band suggests assignment as a bridging mode.[43,45] The unusually large isotope shift indicates a different mode of cyanide bonding and/or environment. We propose that the 2169-$cm^{-1}$ band may be due to a multinuclear cluster of $[Cu_xCN_y]_n$, with a large contribution of bridged $CN^-$, and similar species have been reported.[46,47] Preliminary studies on the interaction of $CN^-$ with dithionite-reduced SOD have indicated that the 2169-$cm^{-1}$ band is present with much increased intensity, but no data are yet available as to whether in this case the species is protein-bound or protein-free. Further experiments aimed at elucidating the nature of this band in the Cu(I)–protein and model systems are underway.

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