restricted to iron.[24] These chemical shifts are modulated by bridging protons but only slightly by pseudoprotons such as $[AuPPh_3]^+$. On the other hand, the direct Au-B interaction does not lead to large downfield shifts. Partial encapsulation of the boron by metal can lead to large asymmetries in the electric field gradient at the boron nucleus. The resulting rapid quadrupolar relaxation leads to line broadening to such an extent that boron signals are easily lost in the background noise of a spectrum. Bridging the exposed B-M edges with either protons or pseudoprotons such as $[AuPPh_3]^+$ results in a large reduction in the electric field gradient at the boron nucleus.

Experimental Section

Fenske-Hall calculations[25] were carried out by using geometries derived from solid-state structures or by using reasonable geometries based on model compounds. The calculations employed single-ζ Slater basis functions for the 1s and 2s functions of B, C, and O. The exponents were obtained by curve fitting the double-ζ functions of Clementi[26] while orthogonal functions were maintained; the double-ζ functions were used directly for the 2p orbitals. For hydrogen, an exponent of 1.16 was used. The iron 1s-3d functions were taken from the results of Richardson et al.[27] and were all single-ζ except the 3d function, which is double-ζ and was chosen for the +1 oxidation state. Both the 4s and the 4p exponents were chosen to be 2.0.

The spin-lattice relaxation times (T_1's) for ^{11}B were determined for the series of ferraboranes $[Fe_4(CO)_{12}BH_{3-n}]^{n-}$ by the optimized inversion recovery technique,[28] and the standard software package (T1IRCA) supplied with the Nicolet NT-300 spectrometer was utilized. BH_3·THF was used to obtain proper pulse widths. Parameters used were the following: 180° pulse width, 78 μs; 90° pulse width, 39 μs; delay, 5× the longest τ value. T_1 values were determined from plots of the equation $\ln (A_\infty - A) = \ln 2(A_\infty) - \tau/T_1$, where A_∞ is the limiting value of A at large τ and A is the amplitude following the 90° pulse at time τ.

The ^7Li NMR spectra were acquired on a Nicolet NT-300 spectrometer with a sweep frequency of 116.6 MHz. Solutions were made in THF by freshly prepared lithium salts of the anions. The lithium salts were washed with hexane to remove any excess BuLi. An external reference of 1 M LiCl in D_2O was used, and the same reference was used for each measurement: pulse width, 15 μs; delay, 1 s.

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Infrared Spectroscopy of Half-Methemocyanin Cyanide: Evidence for Two Nonequivalently Bound Cyanides

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Abstract: The cyanide vibrational modes of half-methemocyanin cyanide were studied by infrared spectroscopy to further understand the structure of the binuclear copper hemocyanin active site. Half-methemocyanin (one copper in the +2 oxidation state and the other in the +1 oxidation state) was found to bind one or two cyanide ligands, each having separate infrared vibrational frequencies. The first cyanide had a ν(CN) at 2112 cm^{-1}, consistent with a μ-1,2 bridging geometry. The second cyanide had a ν(CN) at 2092 cm^{-1}, consistent with a carbon end-on-bonded geometry. These assignments were confirmed using $^{13}CN^-$ and $C^{15}N^-$ isotopes. The bridging cyanide binds with $K > 10^3$ M^{-1}, whereas the second, end-on-bonded cyanide binds with $K = 7.1$ M^{-1}. The model that emerged for half-methemocyanin cyanide is completely consistent with the model developed by Solomon and co-workers using EPR and UV-visible spectroscopies. A smaller than expected ^{13}C shift in the end-on-bonded cyanide may be an indication of nonlinearity in the metal cyanide bond, and a 2-cm^{-1} shift upward in the bridging cyanide band in D_2O may indicate that this cyanide forms a weak hydrogen bond with an imidazole.

The binuclear copper active site in hemocyanin, the oxygen-transport protein in molluscs and arthropods, has been the subject of intense investigation over the past 10 years. Many of these investigations have involved spectroscopic studies of oxyhemocyanin and chemically prepared derivatives that contain exogenous ligands bound to either one or both of the copper atoms.[1] The active site in oxyhemocyanin is best described as two antiferromagnetically coupled Cu(II)'s bridged by peroxide dioxygen; each copper has up to three imidazole ligands from histidine amino acid residues, and the coppers may be bridged by an additional endogenous ligand.[2-4] Methemocyanin has two Cu(II)'s with no dioxygen bridge, but the coppers are still antiferromagnetically coupled, implying an endogenous ligand bridge.[5] Methemocyanin binds exogenous ligands such as halogen anions, azide, and thiocyanate.[5] Half-methemocyanin, which has a Cu(II)-Cu(I) site, can bind exogeneous ligands such as halogens, cyanide, azide, and thiocyanate and may have an endogenous ligand bridge.[4]

Solomon and co-workers[4,5] have extensively studied the hemocyanin derivatives using EPR, UV-visible, and CD spectroscopies deriving a "spectroscopically effective" active site structure for oxy-, met-, and half-methemocyanins, which involves an en-

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dogenous ligand bridge such as phenolate (from a tyrosine residue), carboxylate, or hydroxide (termed endogenous because of the ever-present aqueous environment). An endogenous ligand bridge that is responsible for the antiferromagnetic coupling of the copper atoms is deemed necessary in order to account for the diamagnetism in methemocyanin. In methemocyanin both coppers are divalent, and there is no bound dioxygen that could bridge the coppers.[1,4,5] The recent X-ray structure of *Panulirus interruptus* deoxyhemocyanin at 3.2-Å resolution showed no possible phenolate or carboxylate endogenous ligand bridge within 17 Å of the two coppers.[2] The X-ray results do not rule out small endogenous ligand bridges such as oxide, hydroxide, or water. Hydroxide is considered a leading candidate for the bridging ligand in light of the X-ray results and the spectroscopic information. (Reference 6 has a clear, detailed discussion of the endogenous ligand possibilities.)

These results stimulated us to further investigate the properties of the hemocyanin derivatives with bound exogenous ligands. Most of what we know about coupled binuclear copper active sites has been through the direct study of the proteins and protein derivatives using spectroscopic probes. Each time a new spectroscopic tool is applied to the study of the hemocyanin active site, further detail, understanding, or confidence in existing models is gained. We report here the first results from using infrared spectroscopy to study internal vibrational modes of multiatomic exogenous ligands bound to copper in the hemocyanin active site. We present evidence of a bridging cyanide in half-methemocyanin cyanide and of a second nonbridging cyanide in half-methemocyanin in excess cyanide. While these results do not directly address the issue of an endogenous bridging ligand, they are entirely consistent with the model of half-methemocyanin cyanide described by Solomon and co-workers using EPR, UV–vis, and CD spectroscopies.

Experimental Section

Hemocyanin from *Busycon canaliculatum* (marine snail, a mollusc, purchased from Marine Biological Laboratories, Woods Hole, MA) was obtained by heart puncture. The hemolymph was centrifuged at low speed to remove solid debris, and then the supernatant was dialyzed versus 0.05 M sodium phosphate buffer, pH 6.3. Protein concentration was determined by absorption at 280 nm (ϵ = 89 000 M^{-1} cm^{-1} [7,8]), and copper concentration was determined in oxyhemocyanin by absorption at 345 nm (ϵ = 10 000 M^{-1} cm^{-1} [7]).

The half-methemocyanin nitrite (intermediate) and half-methemocyanin cyanide were prepared according to published procedures.[4] Half-methemocyanin nitrite was prepared by reacting oxyhemocyanin (1 mM in copper, 0.5 mM in protein) with a 10-fold molar excess of ascorbic acid and a 10-fold molar excess of sodium nitrite at room temperature for 12–18 h in 0.05 M phosphate buffer, pH 6.3. The reaction was stopped by dialysis versus 0.05 M phosphate buffer, pH 8.0. After exchange with the pH 8.0 phosphate buffer, the half-methemocyanin nitrite was dialyzed versus 0.05 M Tris-HCl buffer, pH 8.2. Half-methemocyanin with a singly bound cyanide was prepared by reacting half-methemocyanin nitrite (1 mM in copper) in 0.05 M Tris-HCl buffer, pH 8.2, with a 25–100-fold molar excess of KCN for 5–10 min at room temperature. The excess cyanide was then removed by exhaustive dialysis in 0.05 M Tris-HCl buffer, pH 8.2. The half-methemocyanin cyanide was then concentrated to 3–4 mM (in copper) by an Amicon (Lexington, MA) ultrafiltration cell with a PM-30 membrane filter. Half-methemocyanin cyanide with two bound cyanides was prepared by simply adding a 25–100-fold molar excess of KCN to half-methemocyanin cyanide or directly to half-methemocyanin nitrite. Spectra of hemocyanin with excess cyanide were taken immediately after addition of the excess cyanide to minimize the effect of copper removal by the cyanide. Half-methemocyanin cyanide in D_2O (99%, Aldrich) was prepared by exchange of D_2O buffer (pD 8.6, estimated as pH meter reading plus 0.4) for H_2O buffer using diafiltration.

Infrared spectra were taken on a Mattson Cygnus 100 Fourier transform infrared (FTIR) spectrometer using a 0.11-mm path length (measured by fringe method) liquid cell with CaF_2 windows and Teflon spacer. Background spectra were of pure buffer. All spectra were recorded at 4-cm^{-1} resolution with 1000 scans. Because of the small absorbances that were measured (0.002–0.10), the spectra were base-line corrected with the standard Mattson software. This causes a distortion in the "wings" of broad, weak peaks (for example, the 1887-cm^{-1} peak in Figure 4). This base-line correction does not quantitatively affect the spectra but improves the presentation.

$K^{13}CN$ and $KC^{15}N$ were obtained from Cambridge Isotope Laboratories, Woburn, MA.

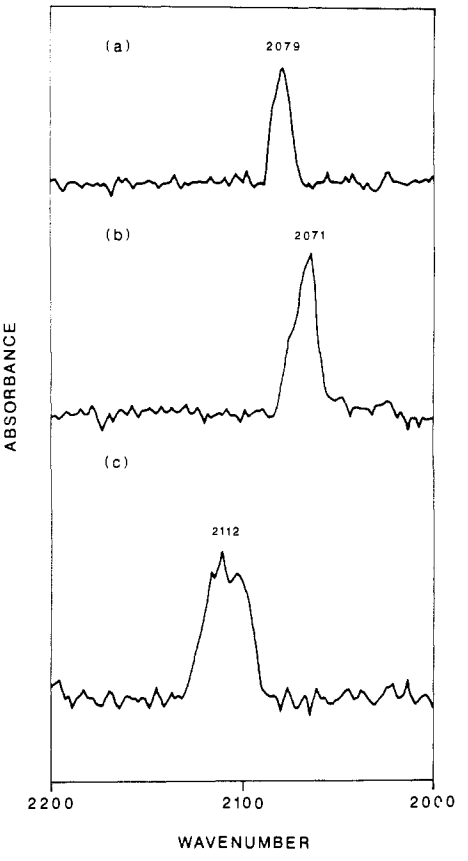

Figure 1. Base-line-corrected infrared spectra of half-methemocyanin cyanide (excess cyanide removed by dialysis), 4 mM (copper) protein concentration, 0.05 Tris-HCl buffer: (a) $C^{15}N^-$, (b) $^{13}CN^-$, (c) CN^-.

Results

Figure 1 shows the infrared spectra in the cyanide stretching region of half-methemocyanin cyanide (excess CN^- removed) prepared with KCN, 99% $K^{13}CN$, and 99% $KC^{15}N$. The weak, broad peak in the natural-abundance spectrum at 2112 cm^{-1} shifts to 2071 cm^{-1} in the $^{13}CN^-$ spectrum and to 2079 cm^{-1} in the $C^{15}N^-$ spectrum. Figure 2 shows the infrared spectrum of half-methemocyanin cyanide in excess CN^- prepared with KCN, 99% $K^{13}CN$, and 99% $KC^{15}N$. Now three bands are apparent: the relatively weak, broad band at 2112 cm^{-1}, which appears as a shoulder; a sharp intense band at 2092 cm^{-1}; and a sharp band at 2080 cm^{-1}, which is due to free CN^- in aqueous solution. The new feature at 2092 cm^{-1} shifts to 2061 cm^{-1} with 99% $^{13}CN^-$ and to 2060 cm^{-1} with 99% $C^{15}N^-$. The peak position and molar absorptivity data are summarized in Table I.

The band at 2092 cm^{-1}, which shifts to 2061 cm^{-1} with $^{13}CN^-$ and to 2060 cm^{-1} with $C^{15}N^-$, could potentially be due to ν(CN) from aqueous HCN.[9] The peak position and isotope shift behavior are nearly identical with those reported for aqueous HCN;[9] however, the conditions in this experiment (up to 100-fold molar excess of KCN, approximately 0.3 M) result in pH 10.71 in the 0.05 M Tris buffer and pH 10.50 in the half-methemocyanin solutions. At pH 10.71, the concentration of HCN in a 0.3 M KCN solution is only 0.015 M (pK_a of HCN is 9.31). Figure 3

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Table I. Values for ν(CN) (Wavenumbers)

cyanide species	medium	$\nu(^{12}C^{14}N)$	$\nu(^{13}C^{14}N)$	^{13}C shift obsd	^{13}C shift calcd[a]	$\nu(^{12}C^{15}N)$	^{15}N shift obsd	^{15}N shift calcd[a]	ϵ,[b] mM^{-1} cm^{-1}
free CN^-	H_2O/D_2O	2080	2036	44	44	2048	32	32	0.03
HCN[c]	H_2O	2093	2060	33	44	2060	33	33	0.03
DCN	D_2O	1887							
$Cu(CN)_3^{2-}$	H_2O	2093	2049	44	44				0.3
half-methemocyanin cyanide									
bridging CN^-	H_2O	2112	2071	41	44	2079	33	33	0.6
bridging CN^-	D_2O	2114							0.6
end-on CN^-	H_2O	2092	2061	31	44	2060	32	32	2.2
end-on CN^-	D_2O	2091							2.2

[a] Calculated using a simple diatomic vibrator model. [b] Per cyanide. [c] Data taken from ref 9.

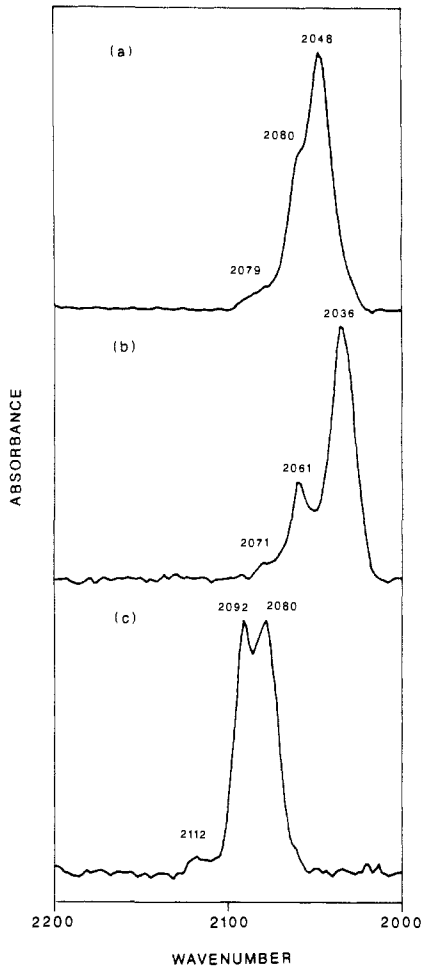

Figure 2. Base-line-corrected infrared spectra of half-methemocyanin cyanide in 25-fold excess cyanide, 4 mM (copper) protein concentration, 0.05 M Tris-HCl buffer: (a) $C^{15}N^-$, (b) $^{13}CN^-$, (c) CN^-.

shows the infrared spectra of 0.3 M KCN in H_2O buffer and 0.3 M KCN in D_2O buffer. In the H_2O buffer, only a peak at 2080 cm^{-1} is observed, due to free CN^-; the peak at 2093 cm^{-1} for HCN is buried under the free CN^- peak. In D_2O buffer, the major peak is still at 2080 cm^{-1} (free CN^-), but now a weak peak at 1887 cm^{-1} due to 0.015 M DCN is seen.[9] The infrared spectra of half-methemocyanin cyanide and of half-methemocyanin cyanide in excess cyanide, both in D_2O buffer, are shown in Figure 4; the peak at 2092 cm^{-1} is still present at nearly the same intensity as in H_2O; it cannot be due to ν(CN) from HCN.

Shown in Figure 5 is the variation of the ν(CN) band intensities with cyanide concentration. The spectrophotometric titration was run in D_2O to avoid the small amount of added intensity at 2093 cm^{-1} due to HCN. The peak at 2114 cm^{-1} (shifted from 2112 cm^{-1} in H_2O) is at its full intensity even with the smallest excess of cyanide; the peak at 2091 cm^{-1} (shifted from 2092 cm^{-1} in H_2O) steadily grows in intensity, reaching a maximum intensity near 50-fold excess cyanide. The equilibrium constant calculated from

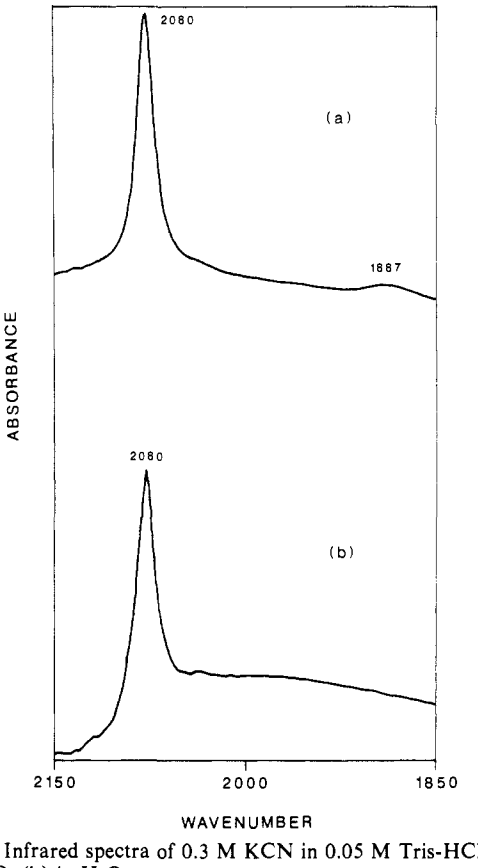

Figure 3. Infrared spectra of 0.3 M KCN in 0.05 M Tris-HCl buffer: (a) in D_2O, (b) in H_2O.

these data for binding of the first cyanide is $K > 10^3$ M^{-1} and for binding of the second cyanide is $K = 7.1$ M^{-1}.

The vibrational bands due to ν(CN) in monovalent copper cyanide complexes have been studied;[10] however, shifts with $^{13}CN^-$ were not reported. The spectrum of the $Cu(CN)_3^{2-}$ complex in the cyanide stretching region is shown in Figure 6, and a single peak at 2093 cm^{-1} is observed. Also shown in Figure 6 is the infrared spectrum of the complex prepared with $K^{13}CN$; a peak due to $\nu(^{13}CN)$ is observed at 2049 cm^{-1}, within 1 cm^{-1} of what is predicted on the basis of a diatomic vibrator model. Because the complex is prepared by mixing KCN with CuCN and we did not use ^{13}C-enriched CuCN, the spectrum contains peaks due to ν(CN) as well as $\nu(^{13}CN)$.

Discussion

The ν(CN) is expected to shift up upon binding to a metal since cyanide is a good σ donor and a poor π acceptor.[12] Electrons donated to the copper are from a σ orbital, which is weakly

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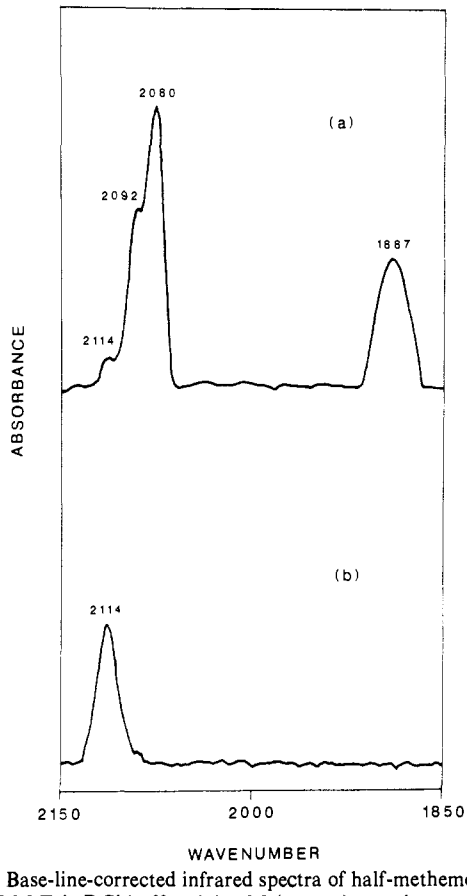

Figure 4. Base-line-corrected infrared spectra of half-methemocyanin in D_2O, 0.05 M Tris-DCl buffer, 3.3 mM (copper) protein concentration: (a) 25-fold excess cyanide, (b) excess cyanide removed by dialysis (note distortion caused by base-line correction in the DCN peak at 1887 cm^{-1}).

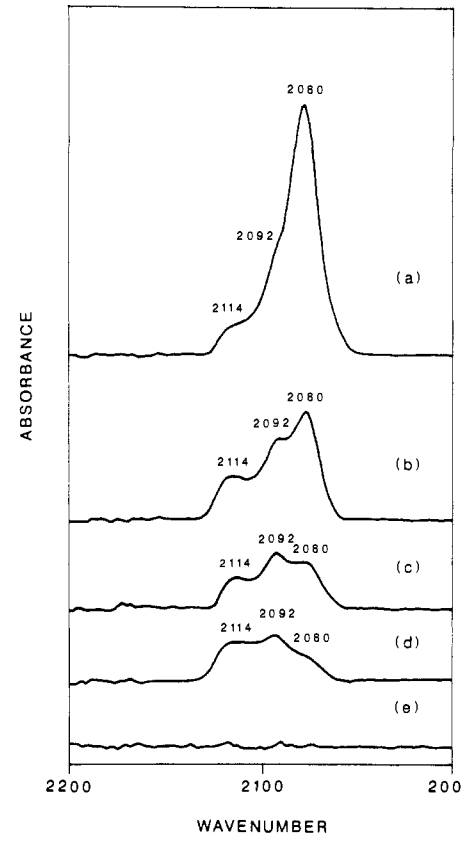

Figure 5. Spectrophotometric titration in half-methemocyanin nitrite with cyanide in D_2O, base-line-corrected spectra, 3.3 mM (copper) protein concentration: (a) 50-fold excess cyanide, (b) 25-fold excess cyanide, (c) 16-fold excess cyanide, (d) 8-fold excess cyanide, (e) no cyanide (half-methemocyanin nitrite).

antibonding; thus, the bond order of cyanide increases upon binding. Extending this logic, it might be expected that ν(CN) for a bridging cyanide would be higher than for a terminally bound cyanide. Although no compounds are reported with cyanide bridging divalent and monovalent coppers, ν(CN) does increase for bridging cyanide versus terminally bound cyanide in complexes of other metals.[12,13] Therefore, we assign the 2112-cm^{-1} band to ν(CN) from a bridging type cyanide and the 2092-cm^{-1} band, which appears in excess cyanide, to a terminally bound cyanide.

The isotope shifts show that the bands are in fact due to ν(CN). Comparison of the actual shifts with those predicted using a free diatomic vibrator model is shown in Table I. We would expect the diatomic prediction to be excellent since the C≡N force constant is so large; cyanide vibrates independently of other atoms. The only significant disagreement between observed and predicted frequencies is the one for $\nu(^{13}CN)$ for nonbridging cyanide. As noted above, this effect has also been observed for the $\nu(^{13}CN)$ in $H^{13}CN$.[9,11] Caughey and co-workers studied the infrared vibrational bands of cyanide bound to Fe(II) and Fe(III) heme proteins.[9] In their work, ν(CN) for $^{13}CN^-$ when attached to the metal was observed within 2 cm^{-1} of the value predicted with a simple diatomic vibrator model. They concluded that this could be used as additional evidence that the cyanide was bound to the metal and that the ν(CN) observed was not due to that from HCN. While this is true for the systems they studied and is also true for the $Cu(CN)_3^{2-}$ complex (Figure 6), it is definitely not true for the cyanide bound end-on to the copper(II) in half-methemocyanin cyanide. In an attempt to account for the observed ^{13}C shift in the vibration of the terminally bound cyanide, we calculated various pairs of ν(CN) and $\nu(^{13}CN)$ using a simple general valence force field for a linear triatomic molecule, Cu—C≡N.[14] Force constants for the Cu–C bond ranging from 1.0 to 2.5 mdyn/Å and force constants for the C≡N bond ranging from 15.8 to 16.5 mdyn/Å were used.[12] No combination could be found that was more satisfactory than the diatomic model. Although we do not have sufficient data to quantitatively test it, we believe that the nonbridging cyanide must be highly coupled to vibrations involving other copper ligands or that the Cu—C≡N is slightly nonlinear in order to account for the $\nu(^{13}CN)$ shift. The possibility of nonlinear metal–carbon–nitrogen has been proposed as a reason for reduced carbon-13 shifts.[9]

The intensities and bandwidths of ν(CN) support our assignments. The molar absorptivity for bound cyanide is usually higher than for free cyanide and can be up to 2 orders of magnitude higher.[9,10,12] The molar absorptivities are listed in Table I. Our results for free cyanide and $Cu(CN)_3^{2-}$ agree with published results,[9,10,12] and the values for ν(CN) bridging and ν(CN) end-on bonded are consistent with the much higher molar absorptivities of bound cyanide versus free cyanide. The bandwidths of ν(CN) of bound cyanide broaden as metal oxidation number decreases and generally broaden in bridging versus nonbridging configurations.[9,13] The 2112-cm^{-1} peak is substantially broader than the 2092-cm^{-1} peak, consistent with both bridging and bonding (carbon end) to Cu(I).

In final defense of our assignments, we must consider the possibility that the two ν(CN) bands observed are due to two different symmetry modes of a dicyano complex. This assignment can be ruled out on the basis of the different rates of intensity increase of the two bands during addition of increasing amounts of cyanide (Figure 5). If the two bands were different symmetry modes of the same complex, their intensities would change in parallel. Aqueous copper cyanide complexes with two, three, or four bound cyanides invariably show a single peak due to ν(CN) in the infrared;[10] therefore, the half-methemocyanin cyanide in

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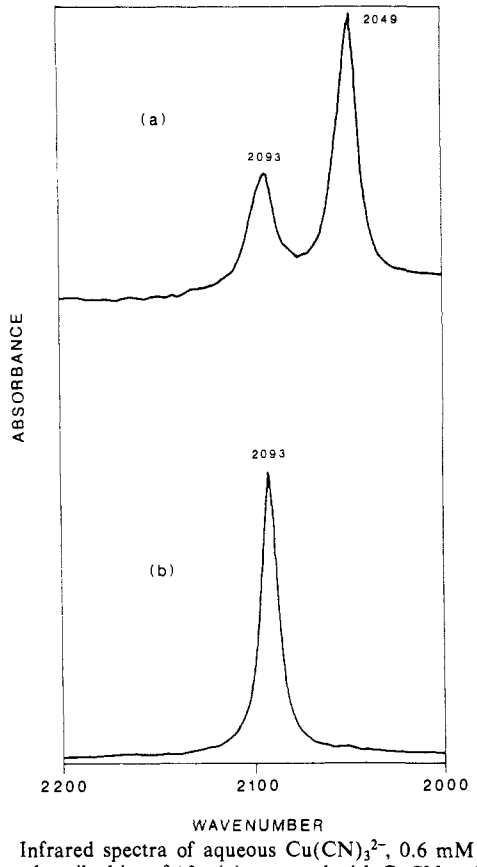
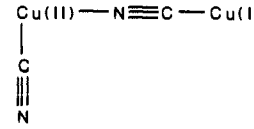


Figure 6. Infrared spectra of aqueous $Cu(CN)_3^{2-}$, 0.6 mM complex prepared as described in ref 10: (a) prepared with CuCN and $K^{13}CN$, (b) prepared with CuCN and KCN.

excess cyanide behaves as two separate (not coupled) copper cyanide species. This brings up a final possibility that the 2112-cm^{-1} band is due to a monocyano complex and the 2092-cm^{-1} band is due to a dicyano complex and that, at a given concentration of cyanide, the mono- and dicyano complexes are in equilibrium. If this were the case, the intensity of the 2112-cm^{-1} band should decrease and the intensity of the 2092-cm^{-1} band should increase with added cyanide. Again, this intensity behavior in the spectrophotometric titration was not observed.

In the Solomon half-methemocyanin cyanide model, the first bound cyanide forms a bridge between the copper atoms.[4] Lack of ^{13}C superhyperfine structure in the EPR spectrum of the Cu(II) leads Solomon and co-workers to conclude that the carbon end of the cyanide was bound to the Cu(I). The fact that the UV–visible spectrum of half-methemocyanin changes when the first cyanide binds leads to the conclusion that the nitrogen end of the cyanide binds to the Cu(II). ^{13}C superhyperfine structure is present in the EPR spectrum of half-methemocyanin in excess $^{13}CN^-$, so the second cyanide binds carbon end-on to Cu(II) and the model looks like I. Our infrared data support this structure.



I

Solomon further proposed that an endogenous bridging ligand was displaced to make room for the bridging cyanide. Our infrared results are direct evidence that the cyanide does in fact form a bridge.

Attributing the reduced ^{13}C shift for the end-on-bonded cyanide to nonlinearity in the metal cyanide bond is plausible in light of the small formation constant. Since most metal cyanides are linear, considerable distortion must occur, destabilizing the bond. The lower equilibrium constant could be the result of the cyanide binding to divalent copper. Monovalent copper has a higher affinity for cyanide than does divalent copper.[15] Nevertheless, the active site in half-methemocyanin cyanide must be under considerable strain. The coppers in deoxyhemocyanin are separated by 3.7 Å[2] and in oxyhemocyanin and methemocyanin by 3.5–3.6 Å.[3] In order for cyanide to bridge the coppers in half-methemocyanin cyanide as shown in I, the coppers must be separated by 5.1–5.2 Å.[5]

The behavior of the bridging cyanide band in D_2O is an upward shift of 2 cm^{-1} from 2112 to 2114 cm^{-1}, a small but real shift. Upward shifts of cyanide bands in D_2O versus H_2O have been attributed to hydrogen-bonding effects[9] as hydrogen bonding is expected to be weakened with deuterium replacing hydrogen.[16] Water does not seem to form strong hydrogen bonds with the nitrogen in terminally bound cyanides, and shifts in D_2O are usually less than 1 cm^{-1}.[9] Imidazole, which can form strong hydrogen bonds, has been suggested as the hydrogen-bonding species responsible for upward shifting ν(CN) in D_2O observed in horseradish peroxidase cyanide derivatives.[9] There are at least six imidazole ligands in the hemocyanin active site,[2] and some could come close enough to the bridging cyanide nitrogen to affect ν(CN). Since the bridging cyanide ν(CN) is affected (weakly) by hydrogen bonding, the Cu(II)–nitrogen bond must be relatively weak. The bridging cyanide band position may turn out to be species sensitive since other spectroscopic data[5] indicate that mollusc, arthropod, and *Limulus polyphemus* (an arthropod subspecies) hemocyanins may differ in the divalent copper axial ligand.

Acknowledgment. This study was supported in part by the funds of the National Science Foundation (R II, 8610679).

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