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AN INFRARED STUDY OF CARBON MONOXIDE COMPLEXES OF HEMOCYANINS. EVIDENCE FOR THE STRUCTURE OF THE CO-BINDING SITE FROM VIBRATIONAL ANALYSIS

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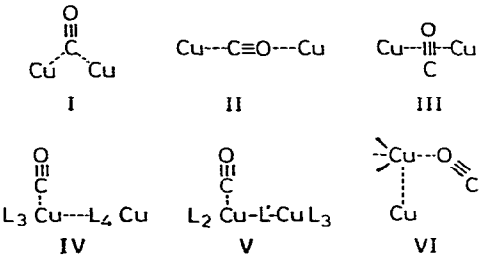
A vibrational analysis was carried out showing that the infrared experimental data of ^{13}C and ^{18}O carbon monoxide complexes of hemocyanin of Fager and Alben (Biochemistry 11 (1972) 4786) are consistent with a coordination of the carbon atom of CO to one of the two copper ions in the active site. This conclusion contradicts the original interpretation of Fager and Alben in which oxygen-coordination to copper was suggested. This vibrational analysis can also be applied to the study of Alben and Caughey (Biochemistry 7 (1968) 175) with ^{13}C and ^{18}O carbonyl hemoglobin, in which oxygen-coordination to iron was suggested. Carbonyl hemocyanins from several sources have also been studied by infrared spectroscopy. The single stretching vibration of CO bound to arthropodal (*Cancer magister*) hemocyanin (ν_{CO}) is at 2042.5 cm^{-1}, while ν_{CO} for gastropod (*Helix pomatia* of the phylum Mollusca) α and β hemocyanin is at 2064.5 cm^{-1} and 2062.5 cm^{-1}, respectively. The intensities of the CO stretching bands were all around 1.5×10^4 M^{-1} cm^{-2}. Calculations show that with the present attainable accuracy it is impossible to detect hydrogen bonding of exchangeable protons to small molecules bound to proteins (for example CO), by comparing its stretching frequencies in H_2O and D_2O buffers.

1. Introduction

Hemocyanin is a multisubunit, copper-containing respiratory protein found in many molluscs and arthropods. Hemocyanin of some gastropods (a class of the phylum Mollusca) contains at least two components, α and β which can be distinguished by their dissociation behavior in 1 M NaCl at pH 5.7 [1].

The ligand binding site of this non-heme protein contains two copper ions and a stoichiometry of one O_2 per two copper atoms has been found [1,2]. Magnetic susceptibility measurements of oxygenated hemocyanin have indicated strong antiferromagnetic exchange coupling between a pair of cupric ions in the active site [3,4]. Resonance Raman spectra have shown that oxygen in oxyhemocyanin is bound as a peroxide ion [5,6] and that the active site has probably a non-planar, μ-dioxygen bridged geometry [7].

Hemocyanin also reversibly binds carbon monoxide with a stoichiometry of one CO per two copper atoms [8,9]. In order to explain this stoichiometry, Williams suggested the bridging structures I-III as unlikely possibilities for the CO binding site [9]. Alben



et al. indicated that structures I and II were inconsistent with a ν_{CO} of 2063 cm^{-1} they found for a molluscan carbonyl hemocyanin [10]. They suggested the non-bridging structures IV and V. A further infrared study of Fager and Alben [11] revealed a ν_{CO} near 2063 cm^{-1} for molluscan species and a ν_{CO} near 2043

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cm^{-1} for crustacean species (phylum Arthropoda). In this study they also measured isotopic frequency ratios ($\nu_{13C16O}/\nu_{12C16O}$ and $\nu_{12C18O}/\nu_{12C16O}$) in carbonyl hemocyanins. The ratio found for ^{13}CO was nearly identical with that for the free gas, whereas the ratio for $C^{18}O$ was larger than that for the free gas. These results ruled out structure III, because the isotopic frequency ratios for ^{13}C and ^{18}O in carbon monoxide would have to change to about the same extent upon binding to hemocyanin in this way.

Model VI in which the oxygen atom of CO is coordinated to one copper atom (Cu—O—C angle near 120°), with the second copper atom of the binding site coordinated only to the protein, was proposed in an attempt to explain, on a rather qualitative level, the observed isotopic frequency ratios [11]. But a conclusion based upon such considerations may be unreliable. Moreover, in all d group transition metal carbonyl complexes known, carbon monoxide coordinates with its carbon to the central metal atom [12—14]. From this point of view the coordination of CO with its oxygen atom as a ligand of copper in hemocyanin seems to be unlikely. Therefore we performed isotopic frequency shift calculations with model structures including oxygen as well as carbon coordination to copper (i.e. Cu—O≡C and Cu—C≡O), in order to see which of these model structures would be consistent with the data of Fager and Alben [11].

We extended our study to infrared measurements of arthropodal (*Cancer magister*) and α and β molluscan (*Helix pomatia*) carbonyl hemocyanins. In addition we attempted to investigate the hydrophobicity of the CO binding site by performing experiments in H_2O as well as in D_2O buffers.

2. Experimental section

2.1. Chemicals

All chemicals were reagent grade and used without further purification.

2.2. Hemocyanins

The preparation of the crustacean (phylum Arthropoda) *Cancer magister* hemocyanin was carried out as described by Thomson et al. [15]. The properties and extinction coefficients of the samples have been described by Moss et al. [3].

α and β gastropod (class of the phylum Mollusca) *Helix pomatia* hemocyanins of which the ratio α : β is 3 : 1 in the hemolymph [16], were isolated and stored according to Heirwegh et al. [17] as modified by Konings et al. [18] and Siezen and Van Driel [19]. The copper concentration was calculated from the determined protein concentration as reported by Konings et al. [18].

The carbonyl hemocyanin solutions were prepared by equilibrating oxyhemocyanin samples with carbon monoxide until the blue color of oxyhemocyanin had disappeared. Although in principle the true pD can be calculated with the glass electrode correction [20,21],

pD = pH meter reading + 0.4,

only pH meter reading values will be given for the D_2O samples [‡1].

2.3. Infrared measurements

Infrared spectra of carbonyl hemocyanins were measured on a Perkin-Elmer 180 infrared spectrophotometer in the absorbance mode at a resolution of about 3.0 cm^{-1}.

The absorption band of carbon monoxide bound to hemocyanin was determined by difference spectroscopy with the carbonyl hemocyanin in a 0.0055 cm CaF_2 cell in the sample beam and H_2O, or D_2O if the hemocyanin solution was in a D_2O buffer, in a variable pathlength CaF_2 cell in the reference beam. This allows the isolation of the absorption band due to bound CO from the broad absorption bands of H_2O or D_2O. Previous studies of protein solutions have shown this spectral region to be devoid of any sharp or intense broad bands except for those of H_2O or D_2O [25].

[‡1] It has been pointed out [22] that the pK values of weak acids in D_2O increase by 0.3 to 0.7 pK units compared to the values in H_2O [23], while Sachs et al. [24] have shown that the histidine imidazole ring pK is 0.4 pK units higher in D_2O than in H_2O. This means that although the pD of a sample in D_2O is 0.4 pH units higher than the pH meter reading [20,21], the state of (de)protonation of the protein is almost identical with the same sample in H_2O with the same pH meter reading.

2.4. Infrared absorption intensities

For intensity determinations, the area under the absorption band of the bound CO was measured over a range of 30 cm^{-1} on each side of the band center by planimetry. The apparent integrated absorption intensity (B) was calculated [26] according to

$$B = \frac{1}{cl} \int A d\nu, \qquad (2)$$

where c is the concentration in M, l is the pathlength in cm and A is the absorbance in log units and ν is the frequency in cm^{-1}.

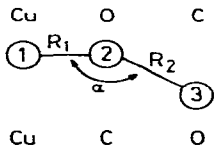
3. Theoretical section

3.1. Triatomic models

As has been pointed out in the introduction the only remaining possibilities for the structure of the CO binding site in hemocyanin are non-bridging models [9–11]. In the calculations we considered only triatomic systems, firstly because it is not known how the copper ion is bound to the protein and to the second copper ion, and secondly because taking more bounds into account would give rise to a larger number of unknown parameters. This would make the calculations cumbersome and the results less reliable. In order to evaluate the effect of this simplification, we performed calculations in which Cu was treated both as an isolated atom (i.e., with a mass equal to the atomic mass of copper) and as an atom rigidly bound to the protein (i.e., with a mass equal to the molecular mass of the whole protein).

3.2. GF matrix method

The calculations are based upon the GF matrix method [27a,28a]. The inverse kinetic energy matrix elements G_{ij} are taken from Decius [29]. The potential energy matrix elements F_{ij} are derived from the Urey-Bradley Force Field (U.B.F.F.) [30]. The force constants describing the U.B.F.F. for a 3-atomic molecule (VII) are: k_1, stretching force constant of ①–②; k_2, stretching force constant of ②–③; H, bending force constant; F, repulsive force constant between non-bonded atoms and F', an additional force constant, for which the usual value of $-0.1\ F$ [28a] is taken.



VII

3.3. Linear model

The most simple model is a linear triatomic molecule. Here only two stretching and no bending vibrations need to be considered, because we are only interested in the C–O stretching frequency, and the bending vibrations do not mix with the stretching vibrations in a linear system. Using this linear model we can take $\alpha = 180°$ and $H = 0$. In order to reduce the number of parameters as much as possible we put $F = 0$ in this model, which means that we neglect an interaction potential between the stretching coordinates, and thus we get two independent coupled harmonic oscillators. Solving $|\mathbf{GF} - \lambda\mathbf{E}| = 0$ then yields two stretching frequencies [31]. Because k_1 is much smaller than k_2 [28c] the highest of these two frequencies belongs to an almost pure CO stretching vibration and is given the symbol ν_{CO}.

3.4. Bent model

The very simplified linear model can be improved by reintroducing a variable Cu–O–C or Cu–C–O angle α, a bending force constant H, and a repulsive force constant between the non-bonded atoms, F (VII). Solving $|\mathbf{GF} - \lambda\mathbf{E}| = 0$ for this bent model yields all three frequencies present [31]: one for a bending vibration δ and two for stretching vibrations ν. The unknown parameters are now: α, F, H, k_1, k_2, R_1 and R_2. But a simple dimensional analysis shows that instead of R_1 and R_2, only the ratio R_1/R_2 can be a parameter in these calculations.

3.5. Computational procedures

The computations were carried out on a CDC Cyber 74-18 computer at the University of Groningen.

All programs were written in FORTRAN IV. The subroutine EIGRF from the International Mathematical and Statistical Library (IMSL) was used for the calculation of the eigenvalues of the GF matrix. The figures were redrawn from computer-plots.

As pointed out before, ν_{CO} can be found at either 2043 or 2063 cm^{-1} [10,11] [‡2]. Neither the frequency of the lower stretching vibration nor that of the bending vibration is known in carbonyl hemocyanin. This means that we have to choose the values for all the unknown parameters independently, except one, which can then be adjusted in such a way that the appropriate value of ν_{CO} is obtained. In the programs used for calculating the isotopic frequency ratios at different combinations of the independent parameters, and in those used for testing the influence of these parameters, it was the k_2 we adjusted before each step in the calculations, because ν_{CO} depends most on k_2. Therefore in the linear model there is only one independent parameter (k_1), while in the bent model there are five (k_1, F, H, α and R_1/R_2).

The computer program of the linear triatomic model was also used in the calculation of hydrogen bonding with the oxygen atom of the CO molecule.

4. Results

4.1. Infrared spectra

Fig. 1A shows the infrared spectrum of carbonyl *Cancer magister* hemocyanin at pH 8.5. Thus this hemocyanin is characterized by a single CO stretching band at 2042.5 cm^{-1} with a width at half-peak height of 11 cm^{-1} (table 1). Upon using D_2O as solvent and lowering the pH [‡1] to 7.1 all infrared spectral data (fig. 1 and table 1) remained constant within experimental errors.

Table 1 shows that the infrared spectral data for α and β carbonyl hemocyanins in H_2O and D_2O were experimentally indistinguishable too. It is important to note that the stretching bands for α and β carbonyl hemocyanins were found at 2064.5 and 2062.5 cm^{-1}, respectively.

[‡2] The value of ν_{CO} = 2054 cm^{-1} for *Limulus polyphemus* carbonyl hemocyanin [11] has not been considered, because *Limulus polyphemus* belongs to a different class in the phylum Arthropoda than *Cancer magister* which belongs to the crustacean class in this phylum.

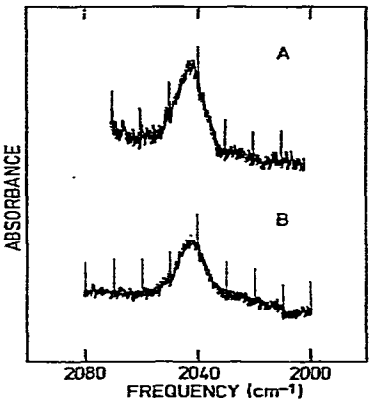


Fig. 1. Infrared spectrum of carbonyl *Cancer magister* hemocyanin. A) Hemocyanin (4.5 mM in copper in 0.1 M Tris-HCl buffer pH 8.5, 10 mM $MgCl_2$) versus H_2O. B) Hemocyanin (2.7 mM in copper in 0.1 M phosphate buffer in D_2O, pH 7.1) versus D_2O.

4.2. Calculations

Fig. 2 shows ($\nu_{^{13}C^{16}O}/\nu_{^{12}C^{16}O}$) and ($\nu_{^{12}C^{18}O}/\nu_{^{12}C^{16}O}$) at ν_{CO} = 2063 cm^{-1} as a function of ν_{Cu-O} assuming a linear Cu—OC model. The frequency ratios are diverging as ν_{Cu-O} becomes larger and even more if the molecular mass of Cu is increased from 63.5 until 9×10^6 the molecular mass of whole *Helix pomatia* hemocyanin [32]. The latter curves were also obtained if instead of the molecular mass of the whole hemocyanin molecule the molecular mass of one functional subunit, 50 000 [33,34], was used. Changing the mass of copper from 63.5 to 9×10^6 at a constant k_{Cu-O} (and ν_{CO}) value causes a decrease of ν_{Cu-O} (horizontal distance between corresponding symbols in fig. 2), and hardly influences the isotopic frequency ratios (vertical distances between corresponding symbols). The two horizontal broken lines in figs. 2, 3 and 4, give the limits between which both isotopic frequency ratios of carbonyl hemocyanins were measured [11] [‡3]. Frequencies of metal-ligand stretch-

[‡3] The area between the two horizontal broken lines was calculated from ref. [11]. Taking the values of ($\nu_{^{13}C^{16}O}/\nu_{^{12}C^{16}O}$) and ($\nu_{^{12}C^{18}O}/\nu_{^{12}C^{16}O}$) for *Limpet hemocyanin* and assuming an error of 0.3 cm^{-1} in each peak position, it can be seen that the frequency ratios should be in between 0.9772 and 0.9778. This area then covers all the isotopic frequency ratios found for several hemocyanins by Fager and Alben [11].

Table 1
Infrared spectral data of carbonyl complexes of hemocyanins [a]

Hemocyanin	Solvent	"C≡O stretching vibration"		
		ν (cm^{-1})	$\Delta\nu_{1/2}$ (cm^{-1}) [b]	$B(10^4\ M^{-1}\ cm^{-2})$ [c]
α Helix pomatia	H_2O [d]	2064.4 ± 0.5	10.5 ± 0.5	1.7 ± 0.2
	D_2O [e]	2064.5 ± 0.5	11.0 ± 0.5	1.7 ± 0.2
β Helix pomatia	H_2O [d]	2062.5 ± 0.5	10.0 ± 0.5	1.5 ± 0.2
	D_2O [e]	2062.3 ± 0.5	10.0 ± 0.5	1.5 ± 0.2
Cancer magister	H_2O [f]	2042.5 ± 0.5	11.0 ± 0.5	1.3 ± 0.1
	D_2O [e]	2042.4 ± 0.5	10.5 ± 0.5	1.4 ± 0.1

a) Spectral data are average-values from 2–3 spectra.
b) Linewidths calculated at half-peak height.
c) Integrated absorption intensities calculated from eq. (2) based upon a stoichiometry of one CO per two copper atoms [8,9].
d) 0.1 M phosphate buffer, pH 7.0.
e) 0.1 M phosphate buffer in D_2O, pH 7.1 (pD = 7.5).
f) 0.1 M Tris-HCl buffer, pH 8.5, 10 mM $MgCl_2$.

ing vibrations are known to be normally in the range from about 200 until 450 cm^{-1}. ‡4 It is obvious from fig. 2 that with this model it is impossible to obtain any reasonable fit between the calculated and experimental [11] ‡3 ratios, and certainly not in the earlier mentioned range. Therefore we also analyzed the linear Cu–CO model, the results of which are shown in fig. 3. Now a reasonable fit is obtained around $\nu_{Cu-C} = 300\ cm^{-1}$.

The bent Cu–CO and Cu–OC models were studied next in order to see whether the simplifications in the

‡4 With metals are meant for example Cu, Co and Fe, and with ligands are meant atoms like C, S, N and O; cf. for example references [6, 12, 28c, 35, 36 and references therein].

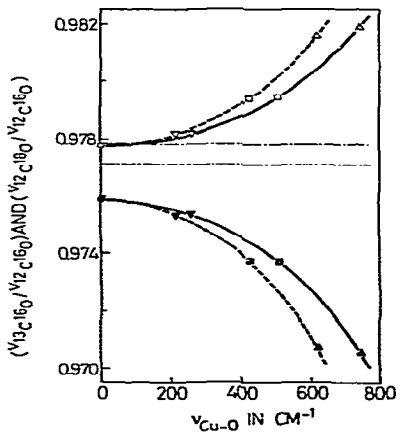


Fig. 2. $\nu^{13}C^{16}O/\nu^{12}C^{16}O$ (open symbols) and $\nu^{12}C^{18}O/\nu^{12}C^{16}O$ (filled symbols) as a function of ν_{Cu-O} at $\nu_{CO} = 2063\ cm^{-1}$ in the linear Cu-OC model. Mass of Cu: 63.5 (——) and 9×10^6 (– – –). k_{Cu-O} (10^5 dyne cm^{-1}): 0 (●); 0.77 (▼); 3.1 (■) and 6.9 (▲). The experimental isotopic frequency ratios are found ‡3 between the two horizontal lines (– . –).

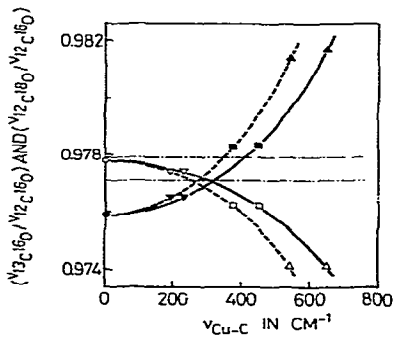


Fig. 3. $\nu^{13}O^{16}O/\nu^{12}C^{16}O$ (open symbols) and $\nu^{12}C^{18}O/\nu^{12}C^{16}O$ (filled symbols) as a function of ν_{Cu-C} at $\nu_{CO} = 2063\ cm^{-1}$ in the linear Cu–CO model. Mass of Cu: 63.5 (——) and 9×10^6 (– – –). k_{Cu-C} (10^5 dyne cm^{-1}): 0 (●); 0.61 (▼); 2.4 (■) and 5.5 (▲). The experimental isotopic frequency ratios are found ‡3 between the two horizontal lines (– . –).

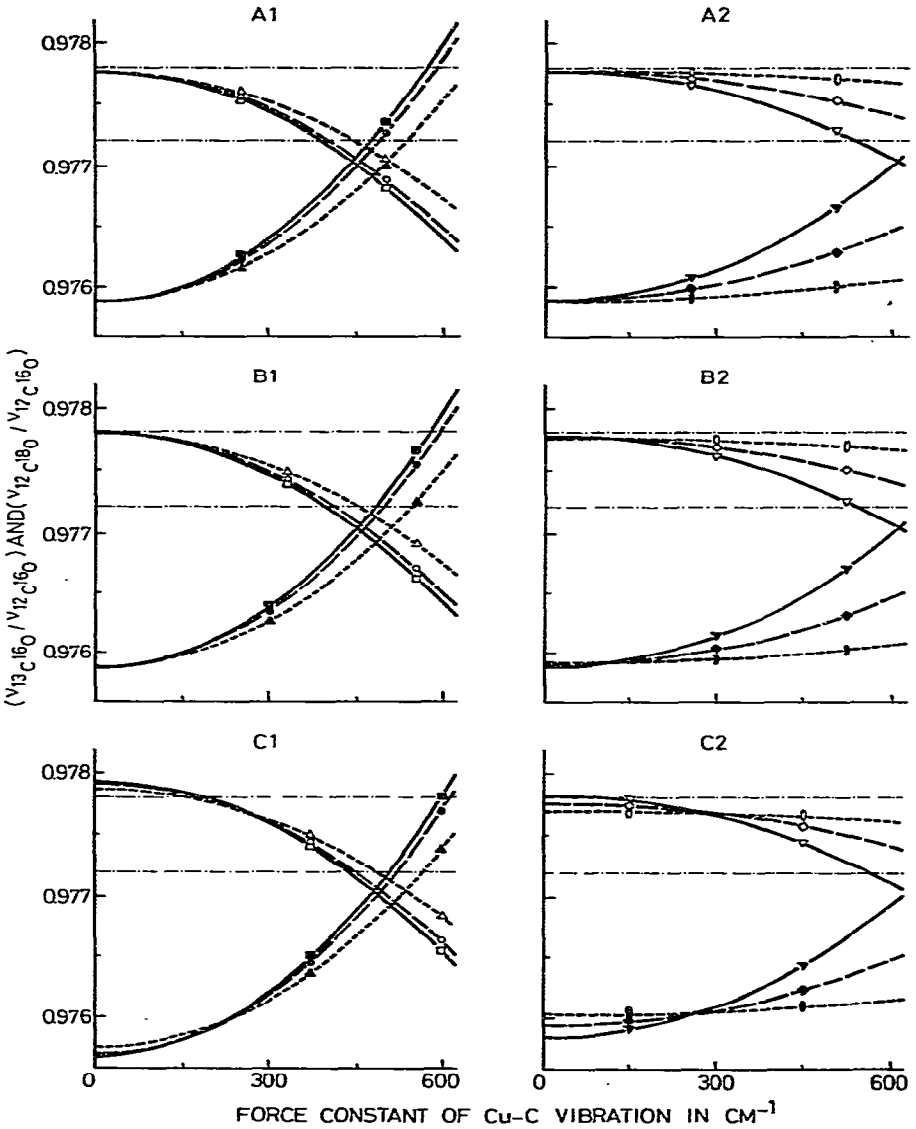


Fig. 4. $\nu_{^{13}C^{16}O}/\nu_{^{12}C^{16}O}$ (open symbols) and $\nu_{^{12}C^{18}O}/\nu_{^{12}C^{16}O}$ (filled symbols) as a function of $k_{Cu\text{-}C} \div 5$, α, F and H at ν_{CO} = 2063 cm^{-1} and with $(R_{Cu\text{-}C}/R_{C\equiv O})$ = 1.6. Mass of Cu: 63.5. Angle α: 180° (▪); 165° (●); 150° (▲); 135° ( ); 120° (◆) and 105° (■). The experimental isotopic frequency ratios are found ± 3 between the two horizontal lines (– . –). A) $F = H = 0$ dyne cm^{-1}, $\delta_{Cu\text{-}CO} = 0$ cm^{-1}; $\nu_{Cu\text{-}C}$ varies from 0–425 cm^{-1} ($\alpha = 180°$) until 0–590 cm^{-1} ($\alpha = 105°$). B) $F = H = 0.53 \times 10^4$ dyne cm^{-1}, $\delta_{Cu\text{-}CO}$ varies from 215 cm^{-1} ($\alpha = 180°$) until 180–150 cm^{-1} ($\alpha = 105°$); $\nu_{Cu\text{-}C}$ varies from 70–430 cm^{-1} ($\alpha = 180°$) until 60–600 cm^{-1} ($\alpha = 105°$) C) $F = H = 3.2 \times 10^4$ dyne cm^{-1}, $\delta_{Cu\text{-}CO}$ varies from 525 cm^{-1} ($\alpha = 180°$) until 440–340 cm^{-1} ($\alpha = 105°$); $\nu_{Cu\text{-}C}$ varies from 170–460 cm^{-1} ($\alpha = 180°$) until 150–670 cm^{-1} ($\alpha = 105°$).

linear model could give rise to a wrong conclusion concerning C or O coordination to Cu. In fig. 4 the isotopic frequency ratios for the bent Cu–CO model at $\nu_{CO} = 2063\ cm^{-1}$ and with $(R_{Cu-C}/R_{C\equiv O}) = 1.6$ (for copper and iron carbonyl complexes the average values of $(R_{M-C}/R_{C\equiv O})$ are about 1.6 [14,37,38, and references therein]), are given as a function of the Cu–C stretching force constant [‡5] k_{Cu-C}, the force constants F and H, and the angle α. The Cu–C force constant was chosen as abscissa, instead of ν_{Cu-C} as in figs. 2 and 3, because at several combinations of F, H, α (<180°) and k_{Cu-C} it was impossible to separate the two lowest frequencies in one with the largest Cu–C stretching vibration character (ν_{Cu-C}) and one with the largest Cu–CO bending vibration character (δ_{Cu-CO}). However, the ranges in which the calculated ν_{Cu-C} and δ_{Cu-CO} can be found, are given in the figure caption. The values of k_{Cu-C}, F and H (F and $H \lesssim 5 \times 10^4$ dyne cm^{-1}) were chosen in such a way that the bending frequency did not exceed a value of about 500 cm^{-1}, which seems to be the upper limit for this frequency as compared with other molecules [28d,39]. The curves in fig. 4 originate from pairs of equivalent values of F and H. Calculations have also been made in which these values were inequivalent, but not much difference was found. Keeping in mind that there are limits on ν_{Cu-C} [6,12,28c,35,36] and δ_{Cu-CO} [28d,39], and that the bending force constant is smaller than the stretching force constant [27b], reasonable fits can only be obtained if the angle α lies between about 135° and 180°. Changing $(R_{Cu-C}/R_{C\equiv O})$ around 1.6 (from 1 to 2) has hardly any influence on the graphs in fig. 4. $R_{C\equiv O}$ in itself is nearly the same in all copper and iron carbonyl complexes, whereas R_{M-C} may slightly vary [14,37,38]. The influence of the equilibrium distance on the force constant of a diatomic molecule is known to be proportional to $1/R^3$ [40]. Therefore the influence of $(R_{Cu-C}/R_{C\equiv O})$ on the isotopic frequency ratios through a varying k_{Cu-C} remains.

No fits could be obtained in the bent Cu–OC model, because all lines were again diverging as in fig. 2. The only difference was that now the lines were less diverging whenever the angle α became less than 180° and ending with about straight lines at an angle of 105°. If a value for F much higher than 5×10^4 dyne cm^{-1} was used, a fit became possible, because at $k_{Cu-O} = 0\ cm^{-1}$ $(\nu_{^{12}C^{18}O}/\nu_{^{12}C^{16}O})$ became larger than $(\nu_{^{13}C^{16}O}/\nu_{^{12}C^{16}O})$, while the shape of the isotopic frequency ratio-graphs (cf. fig. 2) did not change. But in the case of such a fit the limits for ν_{Cu-O} and δ_{Cu-OC} were exceeded. *Thus we can conclude that in carbonyl hemocyanin CO coordinates with its carbon atom to copper.* This conforms to the usual situation [12,14], but is contradictory to Fager and Alben's interpretation of their results [11]. Similar isotopic frequency ratios as in carbonyl hemocyanins [11] have also been found in transition metal carbonyl complexes [41]. No definite statement can be made whether a linear or bent Cu–CO model is favorable. In the latter case, however, the angle α, should lie between about 135° and 180°.

The linear as well as the bent Cu–CO models were used to test the sensitivity of ν_{CO} to all parameters. Fig. 5 shows the influence of a change of ν_{Cu-C} on ν_{CO} assuming the linear model. This means that the degree of mixing of ν_{Cu-C} with ν_{CO} is given. For example, going from $\nu_{Cu-C} = 250\ cm^{-1}$ to about 350 cm^{-1} yields a total $\Delta\nu_{CO}$ of about +20 cm^{-1}, which is the difference in ν_{CO} of the arthropodal and molluscan carbonyl hemocyanins. The sensitivity of ν_{CO} for variation in α, F and H, and $(R_{Cu-C}/R_{C\equiv C})$ was tested in the bent model. In going from $\alpha =$ 100° to $\alpha = 180°$, ν_{CO} increases with a $\Delta\nu_{CO}/\Delta\alpha$ maximum at $\alpha = 135°$. For example, at $k_{Cu-C} = 1.2 \times 10^5$ dyne cm^{-1}, $F = H = 0.53 \times 10^4$ dyne cm^{-1}, and $\nu_{CO} = 2043\ cm^{-1}$, going from $\alpha = 100°$ to 180° yields a total increase of ν_{CO} in the order of 20 cm^{-1}. This effect became slightly smaller whenever F and H were increased.

The influence of F and H in the range from (0–5) $\times 10^4$ dyne cm^{-1} on ν_{CO} is negligible. For example, increasing either F or H or both by a factor of 10 shifts ν_{CO} by 0–5 cm^{-1}, depending upon the initial values of F, H and α. Variation of $(R_{Cu-C}/R_{C\equiv O})$ around 1.6 has a negligible influence on ν_{CO} too [‡6].

[‡5] The force constants are mostly given in dyne cm^{-1}. A stretching force constant in cm^{-1} means the stretching frequency (ν) of the corresponding *free* diatomic molecule in cm^{-1}, based upon the normal masses (i.e. ^1H, ^{12}C etc.) $\nu = (k/\mu)^{1/2}/2\pi c$.

[‡6] Changing $(R_{Cu-C}/R_{C\equiv O})$ from 1 to 2 causes a shift in ν_{CO} of 0–3 cm^{-1}, depending upon the initial values of F, H and α. However, k_{Cu-C} and $k_{C\equiv O}$ were used as independent from R_{Cu-C} and $R_{C\equiv O}$, respectively [40].

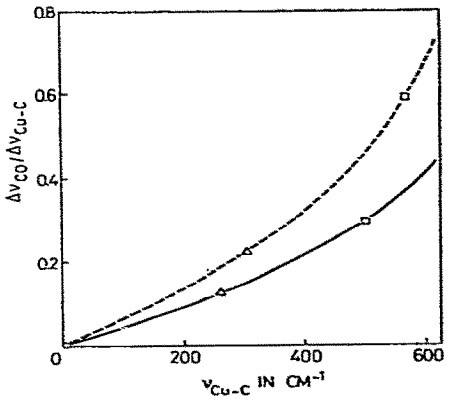


Fig. 5. $(\Delta\nu_{CO}/\Delta\nu_{Cu-C})$ as a function of ν_{Cu-C} at ν_{CO} = 2043 cm^{-1} in the linear Cu–CO model. Mass of Cu: 63.5 (——) and 9×10^6 (– – –). k_{Cu-C} (10^5 dyne cm^{-1}): 0 (○); 0.87 (△) and 3.2 (□).

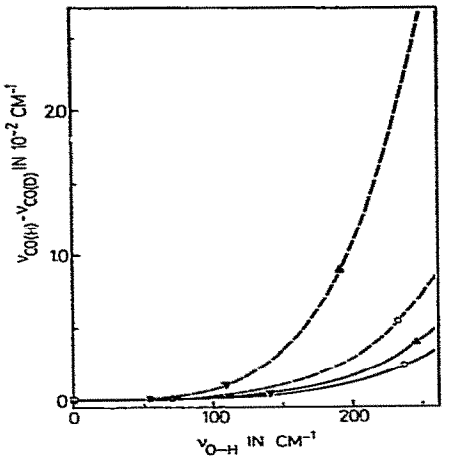


Fig. 6. $(\nu_{CO(H)}-\nu_{CO(D)})$ as a function of $\nu_{O...H}$ at ν_{CO} = 2063 cm^{-1}. Mass of C: 12 (——) and 9×10^6 (– – –). Mass of *H* bound donor: 1 (open symbols) and 18 (filled symbols). $k_{O...H}$ (10^4 dyne cm^{-1}): 0 (■); 0.32 (●); 1.3 (▼) and 3.9 (▲).

Because the carbon atom of carbon monoxide is a ligand of copper, its oxygen atom could be available for hydrogen bonding with protons from amino acid residues or water. In our calculations we simulated H-bonding between the O atom of CO and a proton and computed the ν_{CO} shift in case that H_2O was replaced by D_2O, by using the linear CO...H model. The carbon atom was treated as a 'free' atom with a molecular mass of 12 or as an atom, rigidly bound to the protein, i.e. with the molecular mass of the whole protein, 9×10^6 [32]. Since the hydrogen bonding is much weaker than the bonds within the water molecule, it will be appropriate to consider hydrogen bonding between CO and a whole water molecule instead of a free proton. So in changing from H_2O to D_2O, alterations in the molecular mass of the H-bond donor 1 to 2 as well as from 18 to 20 were used. The alterations in the mass of the hydrogen bond donor cause changes in ν_{CO} which are given as a function of $\nu_{O...H}$ in fig. 6.

Even the strong hydrogen bondings between water molecules do not give rise to stretching frequencies ($\nu_{O...H}$) much higher than 200 cm^{-1} [42]. This means that in the most extreme case the frequency shift will be at most 0.02 cm^{-1} in going from H_2O to D_2O, When anharmonicities and bending vibrations would be taken into account, a value for this frequency-shift much higher than about 0.1 cm^{-1}, is not to be expected. Hence, hydrogen bonding to carbonyl oxygen cannot be detected experimentally.

5. Discussion

On the basis of our calculations it is now clear that the isotopic substitution infrared experiments of Fager and Alben [11], point to a structure in which carbon monoxide is bound through carbon to one of the two copper ions at the active site. This conclusion can be drawn from comparison of figs. 2 and 3, which were obtained using a linear model with two coupled harmonic oscillators without an interaction potential. Fig. 4 shows that using a model, in which the interaction potentials are accounted for by the Urey–Bradley Force constants F and F', does not give rise to much different isotopic frequency ratios, and therefore supports the conclusion mentioned above. Because of this small change of the isotopic frequency ratios it is not to be expected that a more accurate description of the interaction potentials (for example by using a general quadratic valence force field) will cause large alterations in these ratios. This will only increase the number of unknown force constants and

therefore make the calculations and the results unnecessarily complicated.

Results, comparable to those of Fager and Alben [11] were obtained in an infrared study on carbonyl hemoglobin by Alben and Caughey [43] and they too indicated that these results would be more consistent with oxygen coordination to iron in this protein. However, our calculations and conclusions are also valid for carbonyl hemoglobin. It is interesting to note that since the appearance of molecular orbital calculations on carbonyl hemoglobin [44] (quoted by Caughey [45]), which pointed to carbon coordination to Fe, the structure of the CO-binding site was mostly represented in the literature by Fe—C≡O binding. To our knowledge the interpretation of the infrared isotopic data itself [43] has not been questioned earlier in the literature [cf. 46].

Little is known about the structure of the CO binding site of hemocyanin, except that it has to be a non-bridging structure [11,14] and our calculations so far are only able to show carbon to be the ligand of copper in carbonyl hemocyanin. Because no bent carbonyl has been found [47] we slightly prefer a structure in carbonyl hemocyanin, geometrically similar to that in hemoglobin [48,49], i.e. a bond angle at the carbon atom of 180°.

The integrated infrared band intensities for CO bound to the hemocyanins of this study were all around 1.5×10^4 M^{-1} cm^{-2} (table 1) which is half that for carbonyl hemoglobin [50]. In view of the small dipole moment of free CO, this is consistent with less π-donation by copper [51] and thus weaker bonding of carbonmonoxide to hemocyanin than to human hemoglobin. This is also in agreement [11] with a higher ν_{CO} in carbonylhemocyanin than in carbonylhemoglobin [50].

The stretching vibrations, band widths and band intensities, of CO bound to arthropodal and molluscan hemocyanins found in this study confirm the earlier results of Fager and Alben [11], especially the 20 cm^{-1} difference in ν_{CO} between molluscan and arthropodal hemocyanins.

Other spectroscopic measurements indicate differences at the active sites of these hemocyanins too [6,52,53]. The sensitivity analysis of ν_{CO} to all parameters indicates that considerable alterations at the active site seem to be necessary in order to explain the 20 cm^{-1} difference in ν_{CO} (table 1). However, it is important to note that, for example, k_{Cu-C} and k_{CO} were treated in this analysis independent from each other, while it is known that back bonding in metal carbonyls causes a decrease in the CO bound strength when the M—C bond strength increases. This effect is clearly demonstrated by the correlation between ν_{CO} and the ^{13}C chemical shift [54] or the O 1s binding energy [55] and this dominates, with opposite sign, the effect shown in fig. 5 [‡7]. Thus a much smaller change of k_{Cu-C} will be sufficient to cause this difference in ν_{CO}. At the same time this $\Delta\nu_{CO}$ can be caused by a much smaller change in α than mentioned before, because the changing orbital overlap as a function of α will have a much larger effect. Therefore it seems very probable that the CO-binding sites of these two phyla have only slight geometric differences rather than different ligands at their respective active sites, in agreement with earlier suggestions [6]. Nothwithstanding the probably small geometrical differences at the active sites of α and β *Helix pomatia* hemocyanin, they exhibit different oxygen [18,56] and carbon monoxide binding [57] properties.

A small variation in the pH or replacement of H_2O with D_2O did not significantly change the position, width and intensity of the stretching bands of CO bound to hemocyanins (table 1). This means that in the case of *Cancer magister* hemocyanin in this pH range, there is no drastic change in CO-binding. One could be easily tempted to conclude, as has been done before [58,59], from the experiments in which H_2O is replaced by D_2O that there is no interaction of exchangeable protons with carbon monoxide. In that case this would be a confirmation of the earlier suggested hydrophobic environment [60] of the active site in hemocyanins. However, as our calculations show, the effect of replacing H_2O by D_2O will shift ν_{CO} at most by 0.1 cm^{-1}, if hydrogen bonds between CO and exchangeable protons exist. Because the present accuracy of carbonyl frequency measurements is around 0.5 cm^{-1} (cf. table 1) no conclusions with respect to hydrogen bonding can be drawn from such replacing experiments.

[‡7] In the figures, in which ν_{CO} is plotted against the ^{13}C chemical shift [54], and against the oxygen 1s binding energy [55], ν_{CO} should be corrected for the effect described in fig. 5. This correction, however, will be small and probably almost linear in the area where ν_{M-C} can be found.

A recent resonance Raman study of oxyhemocyanin with $^{16}O-^{18}O$ showed one single $^{16}O-^{18}O$ stretching frequency at 728 cm^{-1}, indicating that the bound oxygen atoms are spectroscopically equivalent in oxyhemocyanin. On the basis of these spectral properties Thaman et al. [7] concluded that the structure, which best explains all spectral data of the active site of oxyhemocyanin, has a nonplanar, μ-dioxygen bridged geometry. For further structural and functional work on hemocyanins, it will be of value to know whether other tentative model structures [6] of the active site could also explain the Raman spectral properties. This could be possible, because two peaks with a separation of 3 cm^{-1} or less would have been detected as a single $^{16}O-^{18}O$ peak [7]; and in hemerythrin a separation of 5 cm^{-1} between two $^{16}O-^{18}O$ peaks at 822 cm^{-1} has been interpreted [59] as to be consistent with structures in which the oxygen molecule is bound with one of its atoms to the iron atom(s). Therefore a future isotopic-vibrational analysis, similar to the one we present in this paper, may also prove to be useful in excluding some of the proposed models in the case of oxyhemocyanin.

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