Modeling Blue Copper Protein Resonance Raman Spectra with Thiolate–Cu^{II} Complexes of a Sterically Hindered Tris(pyrazolyl)borate

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Abstract: The resonance Raman (RR) spectra of blue copper proteins are unusually complicated, with at least five bands in the 400-cm^{-1} region. To explore the sources of this complexity we have examined RR spectra of LCuSR complexes [L = hydrotris(3,5-diisopropyl-1-pyrazolyl)borate], which are known to be close structural and electronic analogs of the blue Cu site. When the C atom which is attached to the S atom lacks a proton, a single prominent band is seen near 430 cm^{-1}, assignable to the stretching mode of a short (ca. 2.1 Å) Cu–S bond, which is characteristic of the blue Cu site, and of the model complexes. The frequency decreases in the order R = *tert*-butyl > triphenylmethyl > pentafluorophenyl, consistent with the expected effect of increasing electron withdrawal on the Cu–S bond strength. Weaker bands are seen at lower frequencies, which are attributed to thiolate internal bending and to Cu–N[pyrazole] stretching coordinates. These assignments were confirmed with a normal coordinate analysis for the *tert*-butylthiolate complex, which accurately reproduced the frequencies and isotope shifts for perdeuteration of the *tert*-butyl substituent. When R = *sec*-butyl, three prominent bands are seen in the 400-cm^{-1} region instead of one. Normal mode analysis shows extensive mixing of Cu–S stretching with C–C–S and C–C–C bending coordinates in these three modes, occasioned by the inequivalences in the bending coordinates and the involvement of HC–CH torsion. Implications for the blue copper protein RR spectra are discussed.

Introduction

"Blue" copper proteins,[1] which function as electron transport agents in a number of biochemical systems, gain their color from an intense electronic absorption band centered at 600–625 nm. This band arises from a charge transfer transition to the Cu^{2+} ion at the active site from a cysteine thiolate ligand. The unusually low energy of the transition results[2] from the coordination geometry about the Cu^{2+}, which involves a nearly trigonal arrangement of two imidazole N atoms and the thiolate S atom, as shown in Figure 1. A methionine S atom is found along the trigonal axis at a long distance, 2.6–3.1 Å, reflecting a weak bonding interaction; sometimes a peptide carbonyl oxygen atom is located on the other side of the trigonal plane, at an even longer distance. This bonding arrangement, together with a high degree of covalency associated with the short [ca. 2.1 Å] Cu–S bond, leads to reduced values of the $A_{\parallel}$ Cu hyperfine coupling constant. The coordination geometry stabilizes the Cu^{+} oxidation state, and the redox potentials are unusually high in relation to ordinary copper complexes.

Resonance Raman spectroscopy is expected to be a useful probe of the coordination environment in blue Cu proteins, since excitation in resonance with the charge transfer transition should enhance Raman bands associated with Cu–ligand stretching vibrations. Indeed, it has long been known[3] that intense low-frequency bands are detected with RR excitation near 600 nm. But the spectra turned out to be surprisingly complicated, and they are still not understood, despite numerous experimental studies. One might have expected a quite simple RR spectrum, in view of the low effective coordination number of the Cu. The methionine and, where applicable, carbonyl axial ligand atoms are far enough from the Cu that the associated metal–ligand stretching modes should be at quite low frequency, and little enhancement is expected. The two imidazole ligands are expected to produce metal–ligand stretches near 250 cm^{-1}, since the rigid rings move as point masses, and these modes are indeed detected as bands of modest intensity in the RR spectra.[4] Finally, the thiolate ligand should produce a Cu–S stretching vibration near 400 cm^{-1}, in view of the short Cu–S bond. This is the mode that should carry most of the RR intensity, since the Cu–S bond

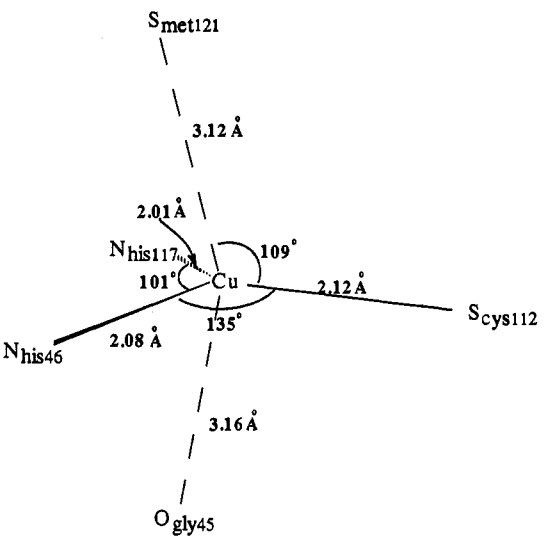

Figure 1. The arrangement of the ligand atoms in the *Alcaligenes denitrificans* azurin[22], with the indicated bond lengths and angles.

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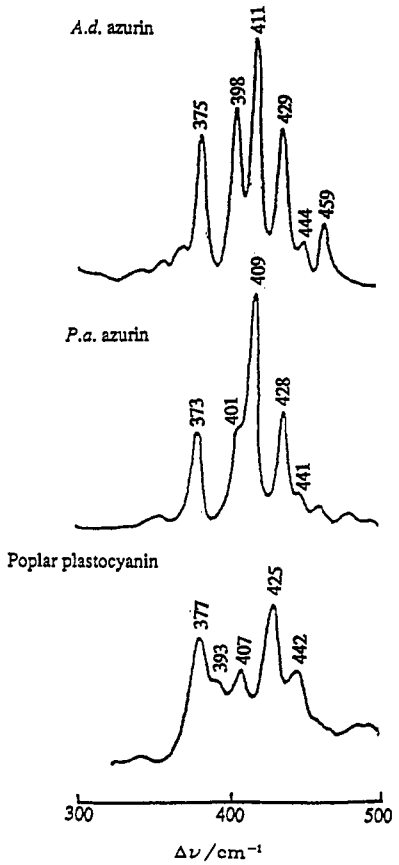

Figure 2. Resonance Raman spectra of the three blue copper proteins: *Alcaligenes denitrificans* azurin at 90 K (568.2 nm excitation);[7] *Pseudomonas aeruginosa* azurin at 12 K (647.1 nm excitation);[6] poplar plastocyanin at 12 K (604 nm excitation).[5] Adapted from the cited references.

extension is expected to be the main distortion coordinate in the charge transfer excited state.[5]

In fact, however, the RR spectra contain *several* strong bands near 400 cm^{-1}. Figure 2 contains three spectra, taken from the literature,[5-7] that illustrate the point. They each contain four to six bands of comparable intensity between 370 and 460 cm^{-1}. Accounting for this embarrassment of riches has been a continuing preoccupation of blue Cu protein RR studies. Since the spectra differ appreciably among different proteins of the class (see Figure 2), they might provide useful information about functional differences.

It is generally accepted that the multiplicity of enhanced bands must reflect vibrational mixing of the Cu–S stretch with other vibrational modes of the proteins. This mechanism was established experimentally in an early study of stellacyanin, using $^{65/63}Cu$ isotope substitution.[4] Stellacyanin has two main bands, at 347 and 385 cm^{-1}, and they are both isotope sensitive, shifting by 1.8 and 1.5 cm^{-1}. The sum of these shifts is that calculated for an isolated Cu–S bond, establishing that Cu–S stretching contributes to both bands. It was proposed[4] that the mixing mode was S-C-C bending, since this coordinate is coupled kinematically to Cu–S stretching, especially if the CuS–CC dihedral angle is near 180°, so that the two coordinates are in line. Protein crystallography has since established that this dihedral is in fact near 180° in the cases so far examined.[1a] Mixing of S–C–C bending with metal–S stretching vibrations has been firmly established in Fe–S proteins and models.[8] Yet, this mixing cannot be the whole story for blue Cu proteins, since most of them have more than two strong bands as Figure 2 illustrates. Other modes must be involved as well.

Urushiyama and Tobari[9] have confronted this problem by calculating all the modes of a 169-atom fragment of protein surrounding the active site. Naturally, many modes were calculated in the 400-cm^{-1} region, and four of them were found to have appreciable (>2%) Cu–S stretching contributions to the potential energy distribution. Surprisingly, the remaining contributors to these ν(Cu–S)-associated modes were heavy atom (C, N, O, and S) deformation coordinates that were widely dispersed throughout the 169-atom fragment. Appreciable contributions were calculated, for example, from a proline ring which is two residues away from one of the histidine ligands, or from a tyrosine ring two residues away from the bound cysteine. Mixing among such distant coordinates is difficult to understand, and raises the question whether so complex a calculation can reliably represent the nature of the normal modes. Experimentally, resolution of this issue will require isotopic data designed to test the role of specific residues.

Because of the complexity of the protein spectra, it is desirable to study spectra of appropriate model compounds, so that the factors responsible for the complexity can be isolated. Blue copper models have, however, been notoriously difficult to construct, partly because of the strong preference of Cu^{2+} for tetragonal coordination and partly because thiolate ligands are readily oxidized by Cu^{2+} to disulfides. These problems have, however, been overcome by using tris(pyrazolyl)borate complexes with isopropyl[10] substituents, which enforce non-tetragonal coordination and inhibit disulfide formation. Thiolate adducts of these complexes have small EPR hyperfine coupling constants (50–70 G), and absorption bands near 600 nm, with extinction coefficients ranging from 3000 to 7000 M^{-1} cm^{-1}. The redox potentials for the complexes are in the –0.1 to 0.3 V region which are comparable to those of the blue copper proteins.[13] Moreover, the crystal structures[11,13] reveal a coordination geometry also very much like that of the proteins (see Figure 3). The Cu atom is near the center of a plane formed by the S atom and two of the pyrazolyl N atoms, while the third pyrazole ligand is located near the trigonal axis, with a Cu–N distance slightly longer than the other two. The Cu–S distance is short, ca. 2.1 Å.

We report RR spectra for a series of these thiolate adducts, which, together with isotopic substitution and normal coordinate calculations, lend new insight into the nature of mode couplings in thiolate ligands, and suggest likely sources of the complexity observed for blue copper proteins.

Experimental Section

The thiolate adducts LCuSR (L = hydrotris(3,5-diisopropyl-1-pyrazolyl)borate; R = *tert*-butyl (**1**), *sec*-butyl (**2**), triphenylmethyl (**3**), and pentafluorophenyl (**4**)) were synthesized as described earlier.[10,11] The four thiolate adducts absorbed strongly in the 600-nm region, with maxima at 608, 630, 630, and 656 nm, for **1**–**4**, respectively, in agreement with published data.[13] The EPR spectrum of compound **1** had identical $A_\parallel$ and g values with those reported previously.[10] The synthesis of the adduct **2** was carried out using the same procedure[10] with particular care in sample handling since **2** is the least stable adduct among the four in this study. The resulting product has the characteristic intense blue color,

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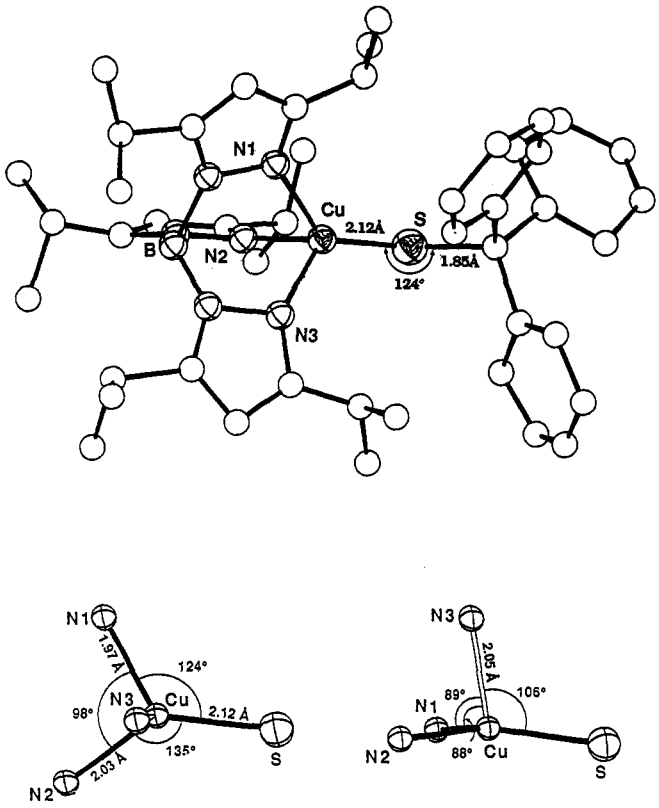

Figure 3. The X-ray structure of $Cu(SCPh_3)(HB(3,5\text{-}ipr_2pz)_3)$; two views of the Cu coordination group are shown (adapted from ref 11).

with $\epsilon_{630} \approx 3000\ M^{-1}\ cm^{-1}$. The EPR hyperfine coupling constant of the complex **2** is ca. 80 G.

The required thiol ligands were purchased from Aldrich as well as the perdeuterated *tert*-butylthiol. The isotopomer for **2**, $HSCD(CH_3)C_2H_5$, was synthesized using the procedures described in ref 12 with slight modifications. Excess thiourea was dissolved in hydrobromic acid and was added to $HOCD(CH_3)C_2H_5$. After the reaction mixture was heated vigorously, sodium hydroxide was added. The product was distilled to obtain the pure thiol. The extent of deuteration was monitored by ^1H NMR spectroscopy.

Resonance Raman spectra were obtained with a Spex 1877 triple spectrograph equipped with a Princeton Instrument diode array detector. Excitation was provided by a Coherent Innova 100-K3 Kr^+ laser (647.1 nm). The laser power was ca. 45 mW at the sample. The slit width was 200 μm, and the acquisition time for each scan was 45 min. The samples were ca. 5 mM solutions in methylene chloride solution. The sample temperature was controlled with liquid nitrogen (77 K) in order to sharpen the spectra and minimize decomposition.

Normal coordinate analyses were carried out by using the updated Fortran IV program written by Schachtschneider.[14] This is an interactive program based on the Wilson F & G matrix method to solve the vibrational secular equations.

Results

1. (Cu–S) Stretching Mode in Models Lacking C^1H. Figure 4 shows RR spectra in the 200–500-cm^{-1} region for LCu^{II} bound to *tert*-butyl-, triphenylmethyl-, and pentafluorophenylthiolate ligands, all of which have a non-hydrogen-bearing carbon atom attached to the S atom. The spectra all show a single dominant band near 400 cm^{-1}, the position expected for the stretching vibration of a short Cu–S bond. The frequencies decrease from 437 to 409 cm^{-1} along the series, consistent with the expected electronic effect of increasing electron withdrawal: *tert*-butyl < triphenylmethyl < pentafluorophenyl. As electron density is withdrawn from the sulfur atom, donation to the Cu atom diminishes, along with the bond strength.

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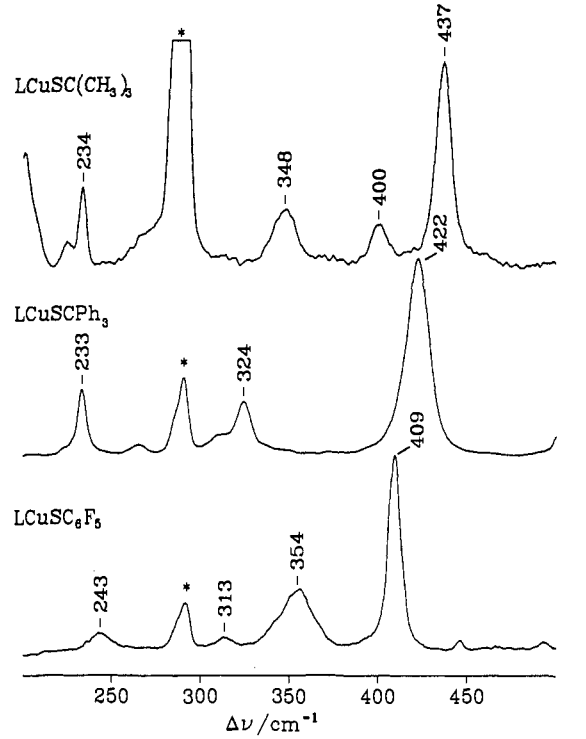

Figure 4. Resonance Raman spectra of LCu^{II} complexes, L = $HB(3,5\text{-}ipr_2pz)_3$, with the indicated thiolate ligands (647.1 nm excitation). Asterisks mark the solvent peak.

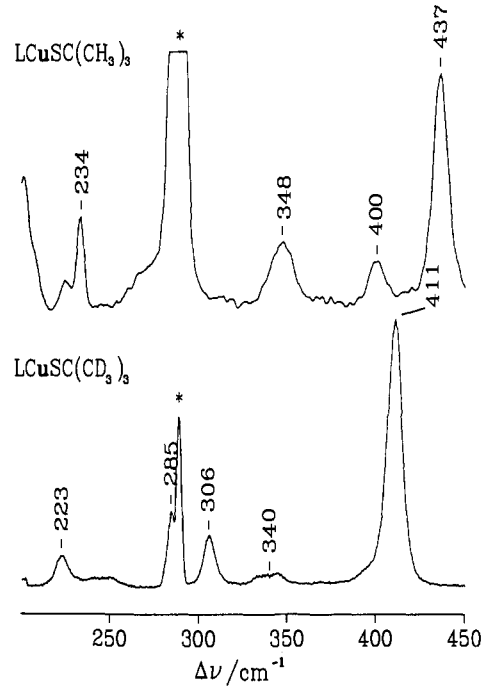

Figure 5. Resonance Raman spectra of natural abundance and perdeuterated *tert*-butylthiolate complex (647.1 nm excitation).

Assignment of the 437-cm^{-1} band of the *tert*-butylthiolate adduct was confirmed by replacing all the *tert*-butyl protons with deuterium. As shown in Figure 5, the 437-cm^{-1} band shifts down to 411 cm^{-1}, due to the increased effective mass of the ligand. Two weaker bands, at 400 and 347 cm^{-1}, shift down even more; these are assigned to C–C–C and C–C–S bending modes. Another weak band, at 234 cm^{-1} is assigned to Cu–pyrazole stretching, by analogy with the ca. 250 cm^{-1} Cu–imidazole stretches[4] in blue copper proteins and in $[Cu(imidazole)_4]^{2+}$. Its deuteration shift, to 223 cm^{-1}, reflects coupling with the Cu–S stretch. A similar

Table 1. Structural Parameters of the Copper Thiolate Complexes (from ref 23)

bond lengths (Å)		bond angles (deg)	
Cu–N1	1.97	N1–Cu–N2	98
Cu–N2	2.03	N1–Cu–N3	89
Cu–N3	2.05	N2–Cu–N3	88
Cu–S	2.12	N1–Cu–S	124
S–C	1.85	N2–Cu–S	135
C–C[a]	1.54	N3–Cu–S	106
C–H[a]	1.09	Cu–S–C	124

[a] Standard values for alkyl group bond distances (C–C = 1.54 Å, C–H = 1.09 Å) and angles (109.5°) were employed. The CuS–CC dihedral angles are given in the text for the *sec*-butylthiolate calculations. For *tert*-butylthiolate, the *tert*-butyl group was oriented so that one of the dihedrals was 180°.

Table 2. Force Constants Used in the Normal Coordinate Calculations[a]

K(S–C)	3.00	K(Cu–S)	1.90
K(Cu–N)	1.76	K(C–C)	4.80
K(C–H)	4.66		
F(S–C–H)	0.620	F(N–Cu–S)	0.064
F(N–Cu–N)	0.164	F(S–C–C)	0.82[b]
F(C–C–C)	1.18	F(H–C–C)	0.620
F(Cu–S–C)	0.300	F(H–C–H)	0.536
t(HC–CH)	0.033[c]		
f(C–S, C–C–S)	0.564	f(H–C–C, S–C–C)	–0.110[c]
f(H–C–S, Cu–S–C)	0.200[c]	f(Cu···C)	0.120

[a] K = stretching force constant (mdyn/Å); F = bending force constant ((mdyn·Å)/rad²); t = torsional force constant; f = interaction force constant ((mdyn·Å)/rad² for bend/bend interaction constants, mdyn/rad for stretch/bend interaction constants); ··· = nonbonded interaction (mdyn/Å). [b] 0.80 for the *tert*-butylthiolate complex. [c] Not applicable for the *tert*-butylthiolate complex.

band is seen in the RR spectra of the other two thiolate adducts (Figure 4). These also contain weaker bands in the 300-cm^{-1} region that are attributed to ligand deformation modes.

The *tert*-butylthiolate assignments are supported by the results of a normal coordinate analysis, using the crystallographic structure parameters which have been determined for the triphenylmethylthiolate complex,[11] and standard bond lengths and angles for the *tert*-butyl group (see Table 1). All *tert*-butylthiolate atoms were included, but the tris(pyrazolyl)borate ligand was approximated as three point-mass pyrazole pseudo-atoms (68.08 amu, the sum of the masses of the ring atoms, plus H in place of the substituents). The force constants used in the calculation are given in Table 2. The ligand force constants were adopted from studies of thiols and thiolate complexes,[15-17] while the Cu–N and Cu–S force constants were taken from our previous calculation on stellacyanin.[4] The high Cu–S force constant (1.90 mdyn/Å) is consistent with the short Cu–S distance, as can be checked by applying Badger's rule.[18] For example, this rule predicts the force constant to decrease to ca. 1.39 mdyn/Å when Cu–S is extended to 2.26 Å, the distance found in bis(thiosemicarbazonato)copper(II),[19] for which a Raman band at 330 cm^{-1} has been assigned to the Cu–S stretch.[20] A frequency of 318 cm^{-1} is calculated for an isolated Cu–S oscillator with this force constant.

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Table 3. Observed and Calculated Frequencies (cm^{-1}), d_9 Isotope Shifts (in parentheses), and Potential Energy Distribution (PED) Contributions (%) for the *tert*-Butylthiolate Complex

		PED			
obs (Δd_9)	calc (Δd_9)	ν(Cu–S)	δ(C–C–C)	δ(C–C–S)	ν(Cu–N)
437(26)	437(21)	45	15	14	8
400(60)	399(60)	0.7	78	2	0
	397(60)	0.2	84	1	0
348(42)	356(37)	7	38	29	11
234(11)	208(13)	30	2	27	35

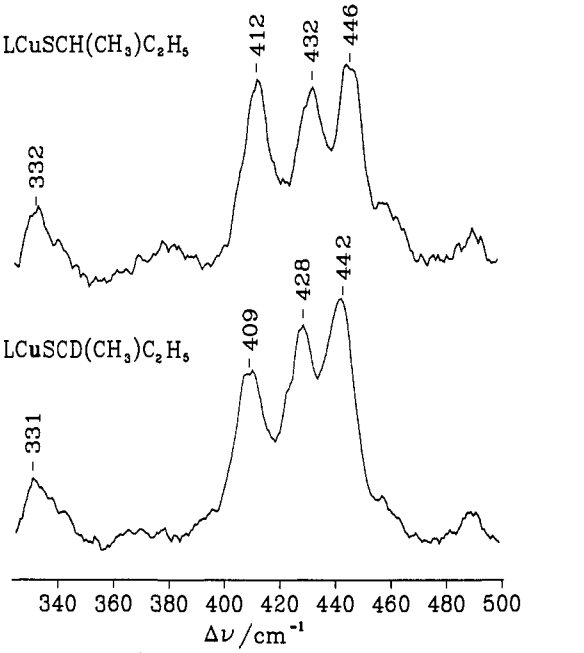

Figure 6. Resonance Raman spectra of the *sec*-butylthiolate complex and its C^1–D isotopomer (647.1 nm excitation).

Table 3 shows good agreement of the calculated and observed frequencies and isotope shifts for the *tert*-butylthiolate complex. The 435-cm^{-1} mode is mainly Cu–S stretching in character, as expected. This coordinate also mixes into the 208-cm^{-1} Cu–pyrazole mode, accounting for its deuteration sensitivity, as well as its moderate resonance enhancement. Two nearly-degenerate C–C–C bending modes are calculated near 400 cm^{-1} and account for the observed band at this frequency. They are exactly degenerate in the 3-fold local symmetry of the *tert*-butyl group, but this symmetry is lowered by the bent Cu–S–C angle. The C–C–C bending coordinate also mixes into the 358-cm^{-1} mode, which is allocated to C–C–S bending. The Cu–S stretch is a minor contributor to the bending modes, but probably accounts, at least in part, for their [weak] intensities. (The intensities are not, of course, proportional to the potential energy contribution, but rather to the square of the bond displacement. A quantitative treatment of the intensities will require a more accurate calculation, and is beyond the scope of this study.)

2. CH Induces Mixing of Angle Bending Modes. A very different RR spectrum is observed for the secondary butylthiolate adduct, as shown in Figure 6. Instead of a single dominant band at 435 cm^{-1}, there are three equally strong bands, at 412, 432, and 446 cm^{-1}. An additional weak band is seen at 332 cm^{-1}. (The spectrum was not extended to the region of the Cu–pyrazole band because of sample instability.) The appearance of three prominent bands above 400 cm^{-1} is a surprising result, since the change from *tert*-butyl- to *sec*-butylthiolate merely involves an interchange of a methyl group with a hydrogen atom at the C^1 and C^2 positions (see Figure 7). This change has two subtle consequences for the normal mode structure, however, which are sufficient to explain the result:

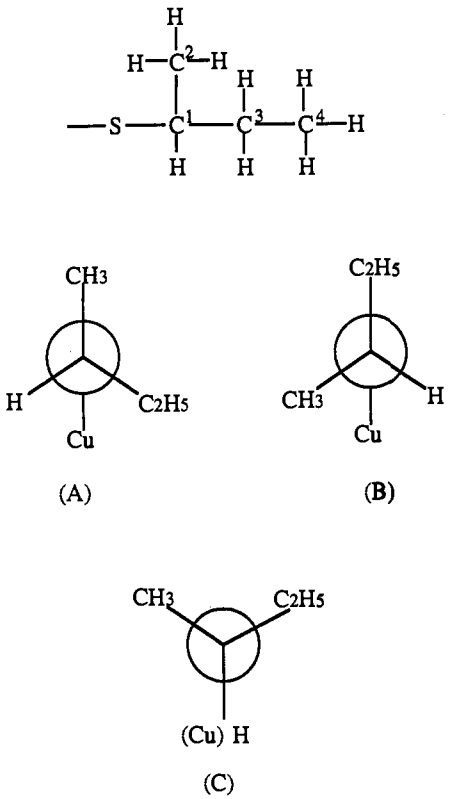

Figure 7. *sec*-Butylthiolate atom numbering and Newman projections down the C–S bond for the three conformations used in the normal coordinate calculations (see text).

(1) The local 3-fold symmetry of the *tert*-butyl group is completely lost, and there are two kinds of C–C–C bending coordinates, one involving the methyl substituent on C^1, the atom attached to S, i.e. C^2–C^1–C^3, and the other involving the ethyl substituent only, i.e. C^1–C^3–C^4. Thus, the formerly nearly degenerate C–C–C bends are now well-separated.

(2) The H atom on C^1 introduces a set of HC–CH torsion coordinates which are not present in the *tert*-butylthiolate. The torsion modes, which are expected in the 200-cm^{-1} region, do not contribute directly to the spectra, and the effect of C^1–H/D substitution is small, as shown in Figure 7. The major bands all shift down by 3–4 cm^{-1} due to the slight increase in effective mass. Nevertheless, the torsion coordinates help to spread the Cu–S coordinate among the three major bands, accounting for their comparable intensities.

These conclusions were reached with the aid of a normal coordinate analysis of the *sec*-butylthiolate adduct, whose results are given in Table 4 (column A). The agreement of observed and calculated frequencies is reasonable, and the deuterium shifts are accurately calculated. The potential energy distributions show the modes to be more highly mixed than in the *tert*-butylthiolate complex. C–C–C and C–C–S bending contribute importantly to all four modes in the 300–450-cm^{-1} region. Significantly, there are major Cu–S stretching contributions to the highest three modes, but not to the lowest one, consistent with the observed intensity pattern.

The force constants were held at the same values as in the *tert*-butylthiolate calculation (Table 2), with the single exception of a slightly elevated S–C–C bending constant, 0.82 instead of 0.80 (mdyn·Å)/rad^2. In addition, force constants associated with the C^1–H bond were added: an HC–CH torsion and two bend–bend interaction constants, H–C–C,S–C–C and H–C–C,Cu–S–C. When the torsion force constant was reduced to zero, the three highest bands were calculated at 441, 424, and 377 cm^{-1}, instead of 446, 432, and 398 cm^{-1}, and the percentage Cu–S contribution changed to 22, 31, and 2 from 30, 13, and 15. Thus, in the absence of the HC–CH torsion, one might have expected two strong bands in the 400-cm^{-1} region instead of three.

Table 4. Calculated Frequencies[a] and C^1–H/D Isotope Shifts (cm^{-1}) and Potential Energy Distributions for the *sec*-Butylthiolate Complex in Three Conformations A, B, and C (shown in Figure 6)

obs[b] (cm^{-1})	PED[c]	A	B	C
ν_1 446(4)		446(5)	450(4)	451(7)
	Cu–S	30	30	36
	C^2–C^1–C^3	3	2	0
	C^1–C^3–C^4	10	11	0
	S–C–C	19	23	22
	Cu–N	4	5	6
ν_2 432(4)		432(4)	434(5)	434(2)
	Cu–S	13	7	0
	C^2–C^1–C^3	7	14	15
	C^1–C^3–C^4	29	21	38
	S–C–C	19	11	11
	Cu–N	3	1	0
ν_3 412(3)		398(2)	379(2)	381(2)
	Cu–S	15	22	17
	C^2–C^1–C^3	22	9	19
	C^1–C^3–C^4	1	12	6
	S–C–C	14	8	11
	Cu–N	6	19	14
ν_4 332(1)		318(1)	331(1)	322(1)
	Cu–S	1	0	1
	C^2–C^1–C^3	23	41	29
	C^1–C^3–C^4	4	2	4
	S–C–C	12	29	17
	Cu–N	47	5	30

[a] Calculated modes are listed which correspond to the four observed RR bands. An additional mode is calculated at 225 cm^{-1}, corresponding to the 208-cm^{-1} Cu–pyrazole mode of the *tert*-butyl adduct (Table 3), but the band was not observed due to the lability of the *sec*-butyl sample. [b] Observed RR frequency and C^1–H/D shift (in parentheses). [c] Percentage contribution to the potential energy distribution for the indicated Cu–X bond stretching or C–C–C or S–C–C angle bending coordinate.

This calculation was carried out with an *sec*-butyl conformation, shown as A in Figure 7, in which the CuS–C^{1}C^2 dihedral angle is 180°, i.e. the methyl group is trans to the Cu atom. Slightly different results, shown in column B of Table 4, were obtained when the conformation was changed so that the ethyl group was in the trans orientation (conformation B in Figure 7). The calculated ν_3 and ν_4 frequencies were respectively lowered and raised, by 21 and 13 cm^{-1}, although the ν_1 and ν_2 frequencies and all the isotope shifts remained essentially the same. The potential energy distribution was altered as well, although substantial Cu–S contributions were again calculated for ν_{1-3} but not for ν_4. Either calculation accounts equally well for the observed RR spectrum. The agreement with experiment worsens significantly, however, when the conformation was changed to C (Figure 7), in which neither the methyl nor the ethyl group is in the Cu–S–C plane (120° CuS–CC dihedral angles). Now the isotope shift is significantly higher than that observed for ν_1, while it is significantly lower than that observed for ν_2. Moreover, only two modes, ν_1 and ν_3, are calculated to have significant Cu–S stretching contributions, so that only two prominent RR bands would be expected.

These results indicate that complexity in the 400-cm^{-1} region of Cu–thiolate RR spectra depends on the availability of multiple bending coordinates of the thiolate group and their mixing with the Cu–S stretch, aided by the torsion coordinates of C–H bonds on the S-bound C atom. Moreover, the mode compositions can be quite sensitive to the conformation of the thiolate ligand.

Discussion

The RR spectra of the thiolate complexes lacking a hydrogen atom on the C^1 atom adjacent to the S atom establish that the natural frequency for stretching a short (ca. 2.1 Å) Cu–S bond is near 430 cm^{-1}, as has generally been assumed for blue Cu

proteins. A single prominent RR band is seen near 430 cm^{-1} for these complexes. This frequency, along with its deuteration shift, is accurately calculated with a normal coordinate analysis of the *tert*-butylthiolate adduct, which also accounts for the frequencies and deuteration shifts of weak bands arising from C–C–C and C–C–S bending and Cu–N[pyrazole] stretching. Cu–S stretching is the main contributor to the 435-cm^{-1} mode, and mixes to a minor extent with the other modes, accounting for their low intensity. Thus, the RR spectrum of the *tert*-butylthiolate adduct is well-behaved, and provides a baseline against which to evaluate the much more complex spectra of the blue copper proteins.

The *sec*-butylthiolate adduct affords insight into the sources of this complexity. The simple switching of the alkyl branching pattern from the *tert*-butyl to the *sec*-butyl side chain dramatically alters the RR spectral pattern. Instead of a single prominent band in the 400-cm^{-1} region, there are now three. Normal mode analysis traces this added complexity to the increase in the number of independent heavy atom [C–C–C and C–C–S] bending coordinates, and to enhanced mixing of these coordinates with Cu–S stretching. This mixing is partially mediated by the HC–CH torsion coordinate, even though the natural torsion frequency is in the 200-cm^{-1} region. In addition, the mixing depends on the *sec*-butyl conformation with respect to the S–C bond. The observed spectrum is best accounted for with a 180° CuS–CC dihedral angle; the C^1–H/D shifts are accurately calculated, and significant Cu–S stretching contributions are found for three bands in the 400-cm^{-1} region. The frequencies and mode compositions are somewhat sensitive to whether the Cu–S-C–C plane contains the methyl group or the ethyl group, although both conformations give results accounting equally well for the observed spectra. When the CuS–CC angle is altered to 120°, however, the mode compositions change significantly. The isotope shifts now show detectable discrepancies from the observed values, and Cu–S stretching contributes appreciably to only two modes in the 400-cm^{-1} region.

What lessons do the model compounds have for the interpretation of blue Cu protein RR spectra? The behavior of the *sec*-butylthiolate adduct implies that multiple heavy atom bending coordinates associated with the cysteine ligand must contribute to the spectral complexity in the 400-cm^{-1} region. The analogs in cysteine of the *sec*-butyl C^1–C^3–C^4 bending coordinate are the C^β–C^α–N and C^β–C^α–C(O) coordinates. Along with the Cu–S stretching and C–C–S bending coordinates, these might account for four bands in the 400-cm^{-1} region. Moreover, the presence of H atoms on C^β and C^α ensures the involvement of the HC–CH torsion coordinate, which is expected to promote Cu–S coordinate mixing. As pointed out by Han et al.,[21] it is significant that the CuS–CC dihedral angle is 180° in all blue Cu proteins for which structures are available. This geometry is expected to maximize kinematic interaction between the Cu–S stretching and C–C–S bending coordinates, and the *sec*-butylthiolate normal coordinate calculations show that mixing with the other heavy atom bending coordinates is enhanced when the CuS–CC dihedral angle is 180°. Involvement of the C^β–C^α–N bending coordinate could account for the sizable D_2O shifts observed in the 400-cm^{-1} region of blue copper protein RR spectra, if the peptide N proton is exchangeable.[21] On the other hand, since five or more prominent bands are generally seen in the 400-cm^{-1} region, the involvement of still more heavy atom bending coordinates is suggested.

To explore these effects, we tried to model the blue Cu protein RR spectra by building the cysteine side chain and its neighboring atoms onto the *sec*-butylthiolate normal mode calculation, using the protein atomic coordinates and additional force constants taken from the peptide literature. We found it possible to calculate five modes in the 400-cm^{-1} region with significant Cu–S contributions provided that the model extended out to or beyond the C^α atoms of the residues on either side of the cysteine. Examination of the potential energy distributions, however, revealed unexpectedly large contributions from bending coordinates involving the terminal heavy atoms (C, O, or N), which truncate the model. This problem was noted also by Urushiyama and Tobari,[9] who carried out trial calculations on small molecular models of the active site. It was for this reason that they increased the size of the model to 169 atoms, at which point the "abnormally strong cooperation with the terminal bending coordinates... almost disappeared ...". Instead, however, their model produces mixing of many spatially distant coordinates, as discussed in the introduction. This kind of mixing may itself be considered to be "abnormal", and remains to be tested experimentally.

The present LCu^{II}–thiolate spectra suggest that the multiple band RR spectra of blue copper proteins arise largely from mixing of Cu–S stretching with bending coordinates involving atoms which are in or adjacent to the cysteine side chain. How this mixing can properly be modeled remains a topic for further research.

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