Resonance Raman Active Vibrations of Blue Copper Proteins. Normal Coordinate Analysis on 169-Atom Model

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Normal coordinate analyses were performed for three crystallographically defined blue copper proteins: *Alcaligenes denitrificans* azurin, *Pseudomonas aeruginosa* azurin, and poplar leaf plastocyanin. A maximum of 485 internal coordinates were specified from a model constructed by the analyzed coordinates of the 169 atoms around the type-1 blue copper site. Mass-group approximation was employed for CH, CH_2, CH_3, NH, and NH_2 groups throughout. With the use of reasonable force constant values, the standard GF calculation method always gives rise to extensive couplings of Cu-S(cysteine) stretching coordinate to the bending coordinates widely distributed in the molecule. Several normal vibrational modes having appreciable PED values of the Cu-S(cysteine) stretching coordinate are correlated with the characteristic RR lines of the blue copper proteins in the 370—450 cm^{-1} region. Our data indicate that the protein-sensitive variation of the RR pattern might be ascribed to a difference in the peptide structure around the blue copper site, rather than to the structure of the Cu coordination sphere, itself.

Blue copper proteins containing a type-1 site (azurin, pseudoazurin, plastocyanin, amicyanin, stellacyanin, laccase, ascorbate oxidase, ceruloprasmin, etc.) all show in their oxidized form prominent resonance Raman (RR) lines in 370—450 cm^{-1} on excitations within the characteristic visible absorption bands.[1-10] The RR spectra also display vague lines in the 250—280 cm^{-1} region and a group of minor lines in the 750—900 cm^{-1} region. X-Ray crystal structure analyses of two azurins (*Pseudomonas aeruginosa* (P. a.)[11] and *Alcaligenes denitrificans* (A. d.)[12] and one plastocyanin (pc) from poplar leaf (*Populus nigra var italica*)[13] showed similar ligation structures around the copper site. Two histidine-imidazole N's (~2.0 Å) and one cysteine S (~2.0 Å) are trigonally coordinated; an additional linkage by a methionine S (abbreviated by S*) (~2.6 Å) completes a distorted trigonal pyramidal coordination environment. However, the RR spectral pattern of these blue copper proteins varies considerably, having different numbers of lines, frequencies, and relative intensities.

Extensive studies have been carried out in order to ascribe this RR spectral variation to the structural characteristics of the blue copper site. These include: detailed RR measurements in the cryogenic state,[4,6,7,11] a ^{63}Cu—^{65}Cu isotope shift,[5,7] a deuterization,[5,7,9,14] excitation profile,[1-3,8,9] and normal coordinate analysis.[6,15] The early work[15] ascribed most of the prominent lines in the 370—450 cm^{-1} region to Cu-N (histidine imidazole) stretchings. However, normal coordinate analysis on a five-atom model suggested that the 370—450 cm^{-1} bands are Cu-S (cysteine) stretching vibrations, and several authors[5-7] have repeatedly emphasized the importance of the mixing of Cu-S (cysteine) stretching, the S-C_β-C_α (cysteine) bending and C_α (cysteine) deformation. The near planar Cu-S-C_β-C_α sequence in these proteins has been pointed out to be advantageous for these couplings. The possibility of coupling to the histidine imidazole deformations has also been invoked.

However, large chain molecules, such as *n*-paraffins[16] and polypeptides,[17] have several normal modes in the 300—600 cm^{-1} region that arise from skeletal vibrations of the C-C-C, C-C-N and C-N-C bendings involved in the molecules. Thus, it is highly possible that the polypeptide skeleton of the blue copper proteins also has normal vibrations in this frequency range. These skeletal vibrations are expected, in the blue copper proteins, to cooperate with the Cu-S (cysteine) stretching vibration, which has been proved[3,6] to have a natural frequency of about 400 cm^{-1}. Therefore, for a better understanding of the vibrational characteristics of the RR lines of the blue copper proteins it is required that the consideration not be restricted to within the CuNNSS* coordination sphere or the nearest neighbours of the blue copper site.

In the present study, normal coordinate analyses were carried out for molecular models which are constructed by a maximum of 169 atoms around the copper site. Serious coupling of the Cu-S (cysteine) stretching and the bond-angle bending displacements in the polypeptide skeleton and side chains widely distributed into the molecules will be described. The importance of this coupling is emphasized in considering the RR spectra of type-1 blue copper proteins.

Method and Computations

A principal problem concerning normal coordinate analysis of protein molecules would be focused on the presence of subsidiary forces characteristic of protein molecules, however, the problem is divided in that the enormous dimension of internal coordinates should be treated in association with practical difficulties. The particular tertiary structure of the protein molecules is held by hydrogen bondings, hydrophobic interactions and charge interactions in the peptide structure. Therefore, the force potentials working between the atoms in the protein molecule must be highly

complex and, at the same time, are expected to involve serious anharmonicity. For a complete analysis of the normal coordinates of the protein molecules, these subsidiary forces must be taken into account quantitatively by an established formalism. An attempt of this highly difficult (at the present time) procedure is not our purpose; in the present study analyses were limited to within the frequency region of the RR active modes regarding the experimental aspect (RR spectra) of the following two forms of evidence.

It should be noted that a group of minor lines appearing in the 700—900 cm^{-1} region have succesfully been assigned to the overtones and combinations based on the fundamental frequencies of vibration (the RR lines in 370—450 cm^{-1} region) without any particular consideration of anharmonic correction.[7,9,11] This fact would be one of a corroboration that the above-mentioned subsidiary forces do not play a primary role as far as the normal vibrations in the RR active region of 350—450 cm^{-1} are concerned.

These subsidiary forces are expected to have their primary significance in the considerably low-frequency (say>100 cm^{-1}) motions which have been investigated as "concerted motions" of protein molecules. The possible presence of these low-frequency chain motions presents another problem regarding the present study. That is, the corresponding potentials would possibly have many potential minima along with these modes. This means a possible presence of many different tertiary structures in solution.

Regarding this possibility, one position is possible from the experimental evidence that the RR signals of the blue copper proteins are always observed as sufficiently sharp lines, the typical half-width of which can be estimated as ~20 cm^{-1}; the temperature effect exhibits only a slight resolutional improvement (at low temperatures) without any dramatic change in the spectra. These experimental observations suggest that the couplings between these low-frequency motions and the RR active vibrations having a frequency of about 400 cm^{-1} would be small and that the molecules populated in one of the potential local minima (if present) would have RR active vibrations that are not much different in frequency. In other words, the tertiary structures in the potential local minima (if present) would not be so much different from each other than they would give rise different RR active vibrations (vide infra).

At present, we do not have any complete evidences that no significant change take place in the tertiary structure of the studied proteins from the crystal state (X-ray structures) into an aqueous solution in which RR measurements were performed. The most changeable parts in the tertiary structure included in our molecular models would be CYS112-MET121 in azurin[11,12] and CYS84-MET92 in plastocyanin,[13] which are located at a turning part of the 7th and 8th strands of the β sheets in the blue copper protein barrel and are exposed boundary to a solvent in solution.

In order to confirm the preferable tertiary structure in solution, we performed molecular mechanics calculations within the above-mentioned parts of our models, including the Cu center. Two ring structures through the Cu coordinations included in these parts provided a relatively easy procedure for an energy-minimum calculation,[18] with the use of appropriate additional potentials for the Cu-coordination and peptide bondings. Although details are not given here, after each several-hundred iterations of the energy-minimum calculation, a variety of starting structures including the X-ray structure yielded the last structures, which are essentially identical to the X-ray structure. This calculation suggests the highly conservative nature of the X-ray crystallographic structure, even in an isolated (solution) state, at least within the molecular region of our models. Therefore, in the present work the X-ray analyzed atomic coordinates, which were read from Protein Data Bank (PDB) at the Computer Center of the University of Tokyo, were directly used for the construction of the G-matrices.

On the basis of the above consideration, the present normal cordinate analyses were performed by an ordinary Wilson's CF method. Normal coordinate analyses were carried out using a HITAC 680H computer at the Computer Center of the University of Tokyo, using library programs BGLZ and LSMB.[19] The former were utlized for computations of the G- and F-matrix elements (Urey-Bradley type force field), and the latter for computations of the eigenvalues and other applications of molecular vibronics. These programs were revised for use concerning very large molecules, mainly regarding size of the array dimension, as well as input and output control procedures. Molecular mechanics calculations were performed by a NEC PC-9801VM2 personal computer using a MM2PP program (Torey System Center Co., LTD.).

Internal Coordinate. In the present study, molecular models were constructed by a maximum of 169 atoms surrounding the Cu center (vide infra and Figs. 4—8). To avoid an enormous increase of internal coordinates in the calculation, a mass-group approximation was employed in which the mass of hydrogen atoms was included in those of the attached atoms. By using this approximation, the skeletal vibrations around 400 cm^{-1} involved in the peptide linkage of the proteins had to be sufficiently deduced, as was the analyzed case of hydrocarbon molecules by Mizushima et al.[20] and Hayashi.[21]

All possible stretching and bending coordinates in the skeleton, as well as out-of-plane bendings for CH_2-(benzene ring), OH-(benzene ring), CH_2-(imidazole ring), CH_2-(indole ring) and for carbonyl groups, were specified in the molecular models (vide infra). Torsoinal coordinates were introduced only around the peptide C(=O)-N(H) bonding (hereafter abbreviated by C+N). As already mentioned, the coordinates concerned with the hydrogen bonding, hydrophobic interactions and charge interactions expected in the molecules were not introduced.

Force Constant. Firstly, we carried out a separate analysis of a toluene molecule in a mass-group approximation in order to determine reasonable values of the in-plane (Urey-Bradley type) and out-of-plane bending forces (diagonal value) for C-(benzene ring). The values of $H(CH_3$-benzene ring)=0.220, $F(CH_3$-benzene ring)=0.300, and $\pi(CH_3$-benzene ring)=0.300 mdyn $Å^{-1}$ in our mass-group approximation yielded normal modes comparable with the results by Lau et al.,[22] in which a full introduction of hydrogen atoms was performed. The corresponding values for the CH_n-CH_n-OH bending and the CH_2-(imidazole ring) in-plane and out-of-plane bendings were comparably set in our calculations.

As already described, Mizushima et al.[20] and Hayashi[23] have successfully analyzed the skeletal vibrations of some hydrocarbon molecules in a mass-group approximation using the Urey-Bradley force constant values, $H(CH_3$-CH_2-

CH_3)=0.200 mdyn Å^{-1}, $F(CH_3\text{-}CH_2\text{-}CH_3)$=0.330 mdyn Å^{-1}. This set of values is a good guide for the present analyses in the same type of approximation for C-C-C, C-C-N (peptide bonding), and C-N-C (peptide bonding) bendings. Nestor et al.[6] have completed precise extraporations of some force constant values (K(Cu-S(cysteine)), K(Cu-N(histidine)), K(Cu-S*(methionine)), etc.) and performed analyses on the 4- and 5-atom models. These force constants were also appropriately used as one of the starting values of the present study. Other force constant values were chosen within a reasonable range according to values from the literatures (vide infra),[5,15,17,23-27]

Results

The theoretical aspect of the resonance Raman scat-

Table 1. Calculated Normal Frequencies and Observed Resonance Raman Lines of Blue Copper Proteins, Frequency/cm^{-1}

Azurin (*Alcaligenes denitrificans*)					
Calc.		428.8	417.0	398.7	377.4
PED	(Cu-S)[a]	11.3	32.7	9.3	9.2
	(Cu-N)[b]	1.8	2.3	1.2	1.7
	(Cu-N')[c]	0.0	1.9	0.9	0.7
	(Cu-S*)[d]	0.1	0.4	0.3	0.2
Obsd[a]		429.4	411.2	397.9	375.0
RR Intensity		s	vs	s	s
Isotope shift					
^{63}Cu-^{65}Cu	calc.	−0.5	−1.0	−0.5	−0.4
	obsd.[9]	−0.4	−1.0	0.0	−0.4
H-D	calc.	−0.9	−0.1	−0.1	0.0
Azurin (*Pseudomonas aeruginosa*)					
Calcd		442.4	419.1	392.9	380.0
PED	(Cu-S)[a]	2.6	33.8	4.2	14.1
	(Cu-N)[b]	0.1	0.9	0.7	0.4
	(Cu-N')[c]	0.0	1.0	0.0	1.7
	(Cu-S*)[d]	0.0	0.1	0.0	0.1
Obsd[7]		427.9	408.6	400.5	372.6
RR Intensity		m	s	m,sh	m
Isotope shift					
^{63}Cu-^{65}Cu	calc.	0.0	−0.9	−0.2	−0.6
	obsd[7]	−0.2	−0.6	−0.6	−0.6
H-D	obsd	−1.2	−1.0	—	−0.9

Plastocyanin (poplar leaf)							
Calcd	424.8	419.3	414.6	414.0	407.5	389.0	364.5
PED (Cu-S)[a]	14.8	2.0	2.1	2.2	28.1	3.6	8.1
(Cu-N)[b]	2.0	0.2	0.7	0.5	1.7	0.4	0.8
(Cu-N')[c]	0.6	0.2	0.0	0.1	3.8	0.7	0.1
(Cu-S*)[d]	0.1	0.0	0.0	0.1	0.3	0.1	0.0
Obsd (spinach)[5]	441.5	406.5	393	425.3	387	377.3	
RR Intensity	m	m	m	s	m	m	

[a]Cu-S: Cu-S (cysteine), [b]Cu-N: Cu-N ((HIS87) for azurins: Cu-N (HIS56) for plastocyanin, [c]Cu-N': Cu-N (HIS117) for the azurins: Cu-N (HIS96) for plastocyanin, [d]Cu-S*: Cu-S (methionine)
*another normal modes having relatively large PED value of the Cu-S (cysteine) stretching coordinate are 420.9 cm^{-1} (PED: 1.6), 411.4(1.6), 394.3(1.2), 387.8(3.9) for A.d. and 453.3(1.2), 393.3(1.0), 391.3(1.0), 385.9(1.1), 381.0(1.1) for P.a. and 434.4(1.3), 400.5(1.0), 385.8(1.3), 383.8(1.6), 378.5(1.5), 347.5(1.4), 343.2(1.4), 335.5(1.1) for pc.

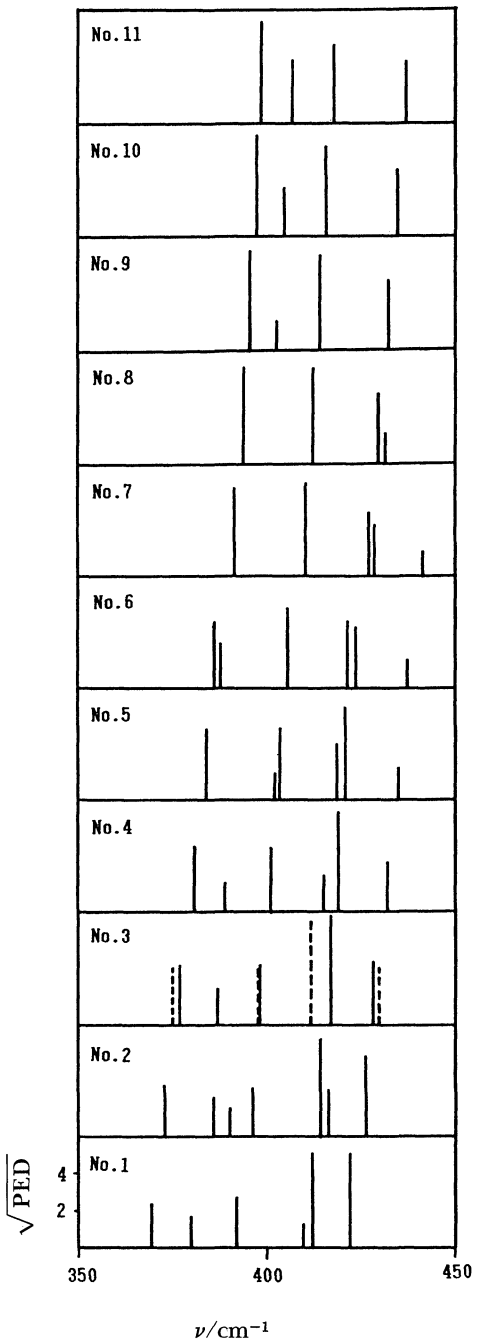

Fig. 1. Normal vibrations, which have Cu-S (cysteine stretching contribution in PED value larger than 2.0, obtained by use of various sets of force constants; a set No. 1 involves Urey-Bladley forces of $H(CH_n\text{-}CH_n\text{-}CH_n)$=$H(CH_n\text{-}CH_n\text{-}NH)$=0.180, $F(CH_n\text{-}CH_n\text{-}CH_n)$=$F(CH_n\text{-}CH_n\text{-}NH)$=0.270 mdyn Å^{-1}. Each of these values are increased progressively by 0.010 mdyn Å^{-1} to the last values in the set No. 11 of 0.280 and 0.370 mdyn Å^{-1}, respectively. The other values of force constants are given in Table 2 except for the out-of-plane bending forces of CH_2-(benzene ring) and CH_2-(imidazole ring) which are increased to be 0.200 and 0.220 mdyn Å, respectively, in the sets No. 4—8 and 0.220 and 0.250 mdyn Å in the sets No. 9—11 for better matching of the increased bending forces. Broken lines included in No. 3 are the observed RR signals (A. d.) with their relative intensities described in hights.

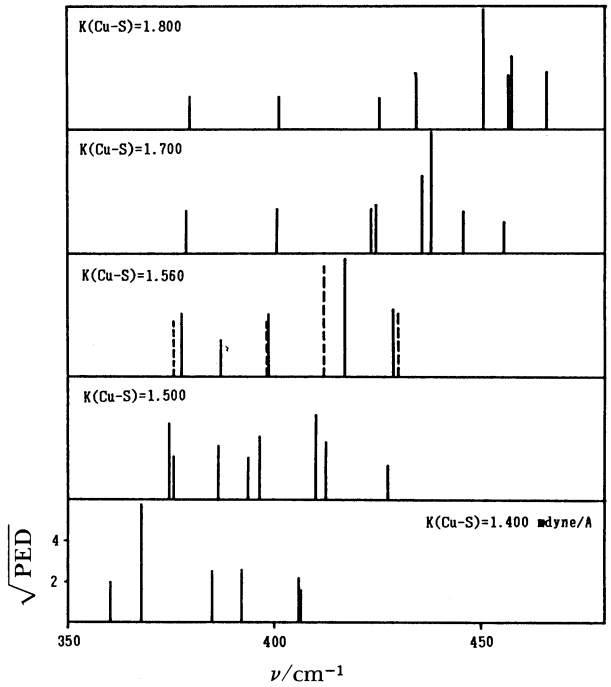

Fig. 2. Calculated normal vibrations of the azurin (A. d.), which have Cu-S (cysteine stretching contribution in PED value larger than 2.0, resulted by sweeping of the values of K(Cu-S(cysteine)). The other force constants are of the values given in Table 2 (set 3).

tering mechanism is given in the literature.[28-34] The Raman scattering tensor, $(\alpha_{\sigma\rho})$, is sensitive to the amount of δ which is a shift of the potential minimum of the concerned electronic excited state to the ground state, along with a considered normal coordinate, Q, if the vibration is totally symmetric (Albrecht's A term). In the present case of the protein models, all normal modes are totally symmetric because of the C_1 point group of the models. The electronic structure of Cu(II) in the type-1 blue copper site was extensively studied by Solomon et al.[35] and Penfield et al.[36] The characteristic intense absorption bands (ε=1000—5000 $mol^{-1}dm^3cm^{-1}$) of the blue copper chromophore in the visible region were assigned to the charge-transfer transitions of S(cysteine)p→Cu d(x^2-y^2), as well as the bands appearing in the near-ultraviolet region to a histidine N→Cu charge transfer, and the weak bands in the near-infrared to d-d transitions of the Cu(II). An excitation profile study[9] indicated that the RR lines in the 370—430 cm^{-1} region are in resonance enhanced (enhancement ratio~3000) with the visible S(cysteine)p→Cu charge-transfer transitions. Therefore, it is expected that the positions of the potential minima of these S→Cu charge-transfer excited states are primarily displaced along the Cu-S(cysteine) stretching coordinate, compared with that of the electronic ground state (elongation of the Cu-S bond in the electronic excited state). In the present study, a quantitative estimation of the (relative) intensity of

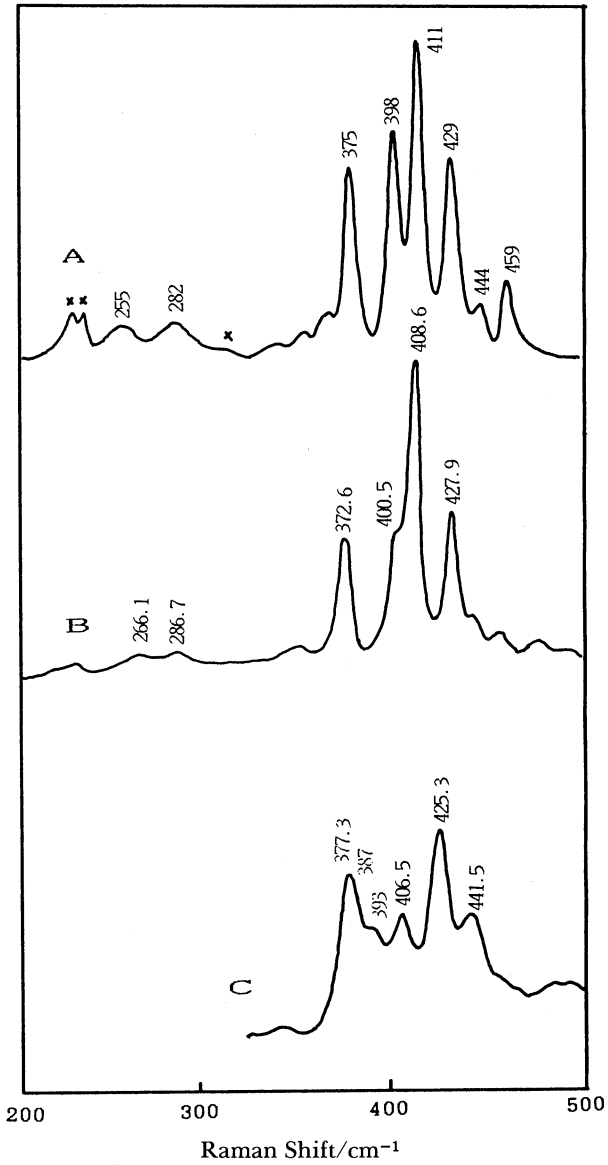

Fig. 3. Resonance Raman spectra of the three blue copper proteins. A: azurin (A. d.) at 90 K (568.2 nm excitation);[9] B: azurin (P. a.) at 12 K (647.1 nm excitation);[7] C: plastocyanin (poplar leaves) at 12 K (604.0 nm excitation).[5]

the RR signals in connection to the actual modes of vibration was not developped. However, it is acceptable that the fractional Cu-S (cysteine) stretching character involved in the normal coordinates is the most important source of RR enhancement on the excitation within the visible S(cysteine)-Cu charge-transfer bands. Thus, we expediently compare the PED values of the Cu-S(cysteine) stretching coordinate in the normal modes to the observed RR line intensities in Table 1 and Figs. 1 and 2.

Trial calculations in which the force constant values are appropriately varied yielded the following general features (1—4).

1. Normal vibration which consists of almost a pure displacement of the Cu-S (cysteine) stretching

Table 2. Urey-Bradley Force Constants; **K, H, and F/mdyn Å^{-1}, π and τ/mdyn Å

K(NH_n-CH_n)=4.200	K(CH_n-CH_n)=3.700	K(C=O)=9.000
K(C+N (peptide bond))=5.000	K(CH_n-S)=2.000	K(CH_n-OH)=4.600
K(CH-CH in imidazole and benzene ring)=6.000		K(Cu-N)=1.200
K(Cu-S (cysteine))=1.560	K(Cu-S*(methionine))=0.364	

H(CH_n-CH_n-NH_n)=0.200	H(NH+C=O)=0.400
F(CH_n-CH_n-NH_n)=0.290	F(NH+C=O)=0.600
H(CH_n-CH_n-CH_n)=0.200	H(CH_n+NH_n-CH_n)=0.220
F(CH_n-CH_n-CH_n)=0.290	F(CH_n+NH-CH_n)=0.300
H(CH_n-CH_n+NH)=0.220	H(CH_n-CH_n-S, S*)=0.150
F(CH_n-CH_n+NH)=0.300	F(CH_n-CH_n-S, S*)=0.160
H(C-C=O)=0.400	H(CH_2-S*-CH_3)=0.200
F(C-C=O)=0.600	F(CH_2-S*-CH_3)=0.260
H(CH_2-imidazole and indole ring, in-plane)=0.210	
F(CH_2-imidazole and indole ring, in-plane)=0.300	
H(CH_2-benzen ring, in-plane)=0.220	
F(CH_2-benzen ring, in-plane)=0.300	
H(CH-CH (or NH)-CH (or NH) in imidazole, indole, benzene ring)=0.480	
H(CH-CH (or NH)-CH (or NH) in imidazole, indole, benzene ring)=0.850	
H(NH_n-CH_2-OH)=0.220	
F(NH_n-CH_2-OH)=0.600	
H(Cu-imidazole and indole ring, in-plane)=0.140	
F(Cu-imidazole and indole ring, in-plane)=0.120	
H(CH_n-N-CH_n)=0.200	
F(CH_n-N-CH_n)=0.300	
H(Cu-S-CH_2 cysteine)=0.150	H(N-Cu-N)=0.085
F(Cu-S-CH_2 cysteine)=0.040	F(N-Cu-N)=0.085
H(Cu-S*-CH_3 methionine)=0.100	H(S, N-Cu-S*)=H(N-Cu-S)=0.085
F(Cu-S*-CH_3 methionine)=0.100	F(S, N-Cu-S*)=H(N-Cu-S)=0.085

π((C_α, NH) C=O)=0.850	π(CH_2-imidazole ring, out-of-plane)=0.190
π(Cu-imidazole ring, out-of-plane)=0.100	
π(CH_2-benzene ring, out-of-plane)=0.300	
π(CH_2-indole ring, out-of-plane)=0.900	
τ(CH_n+NH, peptide bonding)=0.150	

** Force constant set No. 3: see text.

coordinate is not obtained in any frequency region.

2. Instead, in the interesting region of 350—450 cm^{-1}, about 30 vibrational modes appear, which are the cooperative motions of a large number of bond angle bending displacements in the peptide skeleton, including side chains.

3. A limited number (5—8) of the above-mentioned vibrations actually couple to the Cu-S (cysteine) stretching displacement.

4. These important normal vibrations, therefore, are sensitive to the values of the bending force constant, as well as that for the CH_2-(imidazole ring) out-of-plane bending forces, in the peptide skeleton including side chains. However, the variations in the bond stretching and torsional force values do not have much effect for these modes.

Figure 1 shows the effect of a systematic increase in the values of the skeletal bending forces. In each set of force constants, differnces in the skeletal bending values were held constant. The relative values of these forces were appropriately set up refering to the work of Scheraga and co-workers[18] in a molecular mechanics investigation of various peptide molecules.

With the use of the appropriate force constant sets, among the many resulting normal vibrations in the 370—450 cm^{-1} region, the normal modes having an appreciable amount of the Cu-S (cysteine) stretching character are appeared in good accordance with the distribution of the observed RR lines. Table 1 summarizes the important normal vibrations resulting from the force constant set No. 3 (see the caption of Fig. 3) with the observed RR lines. In set No. 3 (Table 2), the important bending force constants are H(CH_n-CH_n-CH_n)=0.200, F(CH_n-CH_n-CH_n)=0.290, H(CH_n-CH_n-NH)=0.200, F(CH_n-CH_n-NH)=0.290, H(CH_n-CH_n+NH)=H(CH_n-NH_n+CH_n)=0.220, and F(CH_n-CH_n+NH)=F(CH_n-NH_n+CH_n)=0.300 mdyn Å^{-1}. They are quite similar to the values of Mizushima et al.[28] and Hayashi[21] (H(CH_3-CH_2-CH_3)=0.200, F(CH_2-CH_2-CH_3)=0.300 mdyn Å^{-1}). Similar trial calculations suggested the optimal value of the stretching force constant K(Cu-S(cysteine))= 1.560 mdyn Å^{-1} (Fig. 2).

Figure 3 shows the RR spectra of the blue copper proteins studied in the present work. Figures. 4—7 represent the PED values of the important normal modes distributed in the azurin model. For the plastocyanin model, the total PED values of the important six modes are represented in Fig. 8.

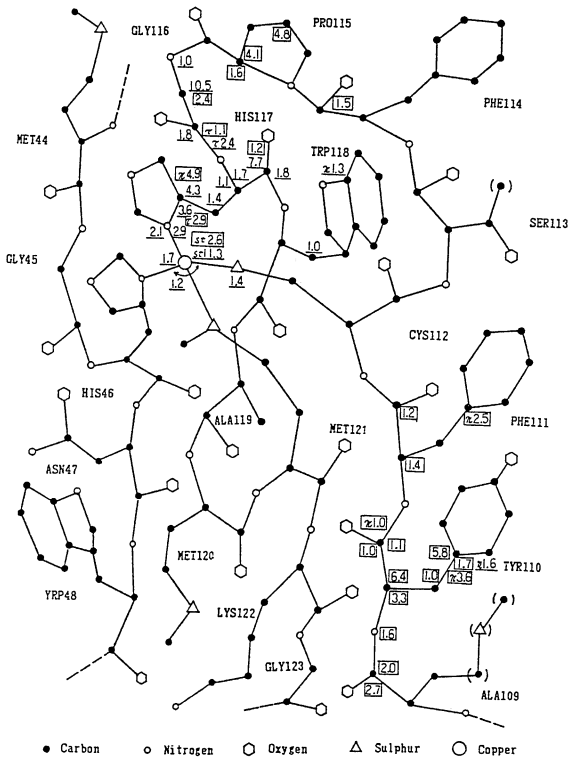

Fig. 4. PED values (larger than 1.0) of the normal vibrations of 428.8 cm^{-1} (A. d.: underlined) and 442.4 cm^{-1} (P. a.: bracketed), the values are depicted for the bond angle bending coordinates (including out-of-plane bending (π) and torsional (τ) coordinates) except for the Cu-S (cysteine) stretching (st) coordinate. Atoms of P.a. are in parentheses.

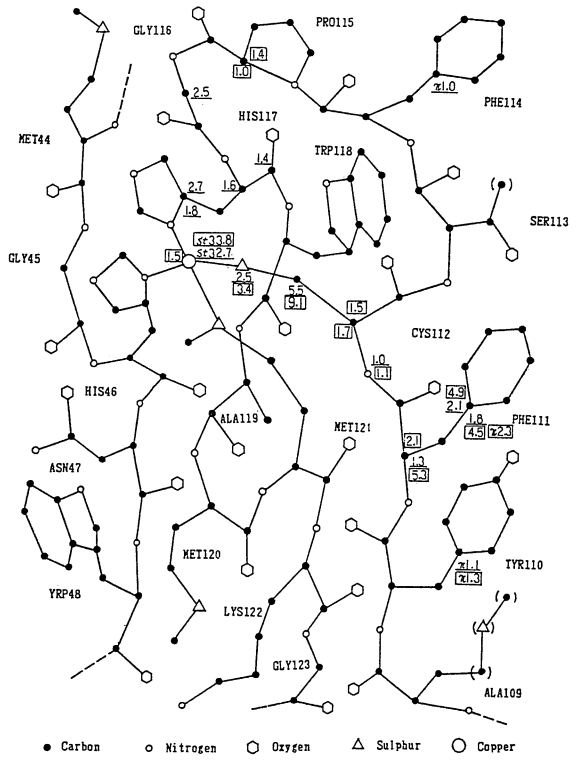

Fig. 5. PED values of the normal vibrations of 417.0 (A. d.: underlined) and 419.1 (P. a.: bracketed) cm^{-1}. The description is the same as Fig. 4.

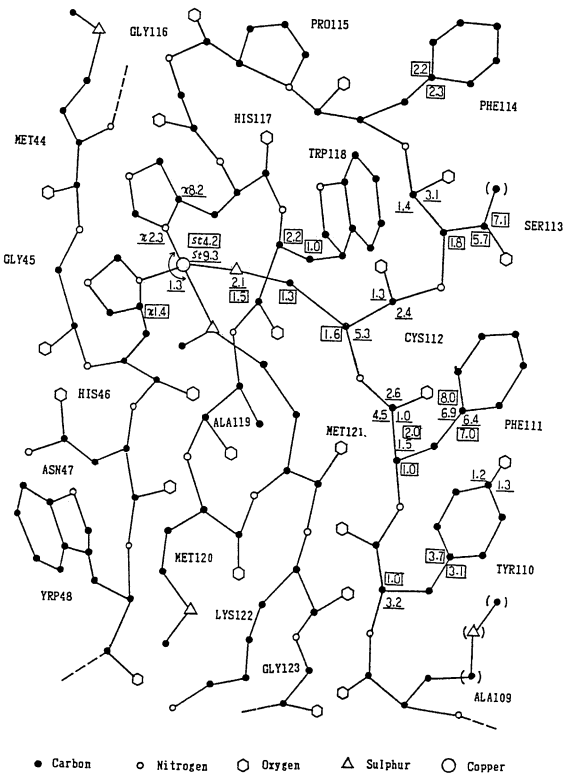

Fig. 6. PED values of the normal vibrations of 398.7 (A. d.: underlined) and 392.9 (P. a.: bracketed) cm^{-1}. The description is the same as Fig. 4.

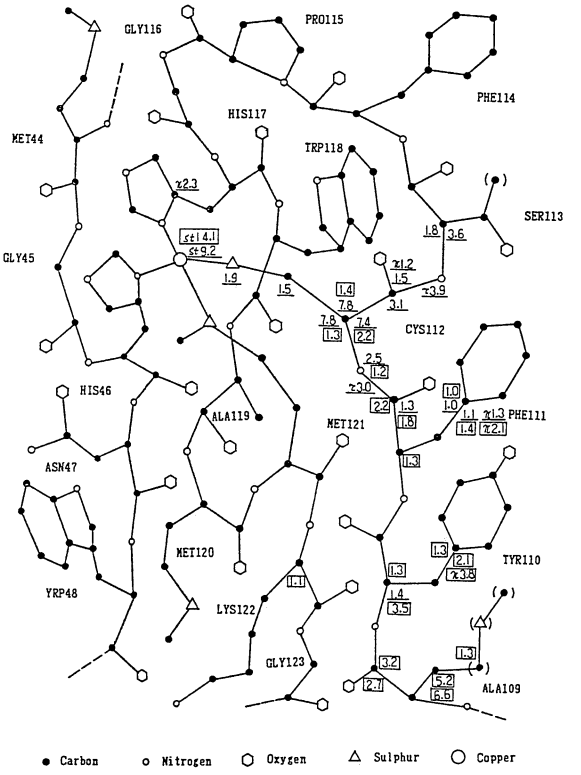

Fig. 7. PED values of the normal vibrations of 377.4 (A. d.: underlined) and 380.0 (P. a.: bracketed) cm^{-1}. The description is the same as Fig. 4.

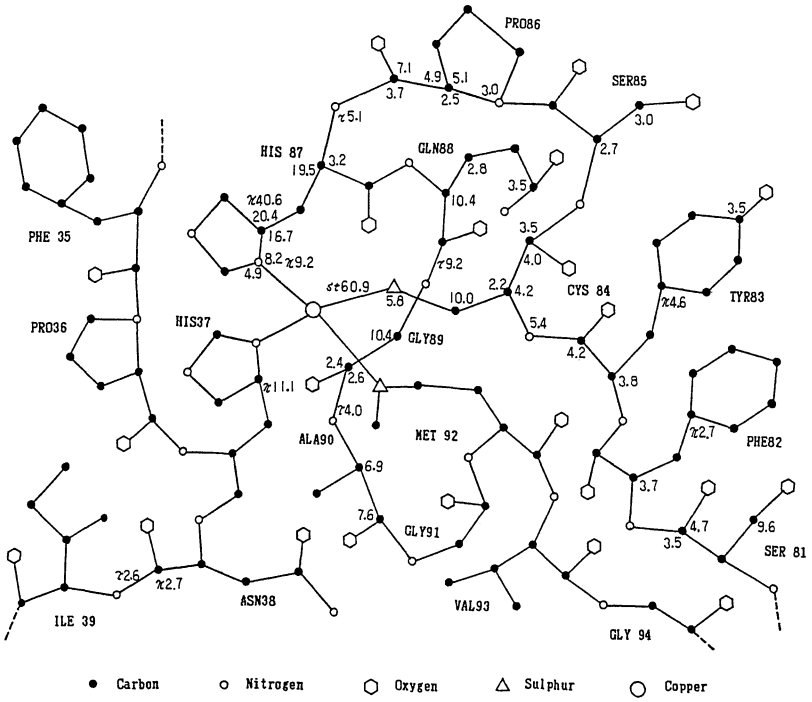

Fig. 8. PED values of the normal vibrations of the plastocyanin, the total values of PED (larger than 2.0) of normal vibrations of 424.8, 419.3, 414.6, 414.0, 407.5, 389.0 and 364.5 cm^{-1} are depicted for each of the coordinates.

Discussion

Figures 4—8 show that the Cu-S (cysteine) stretching displacement couples with a large number of the skeletal bending deformational displacements around the Cu center. Many trial calculations show that these couplings always occur, unless abnormally small values of the skeletal bending forces are assumed (not an accident regarding the particular set of values of the force constants).

We have analysed many molecular models in which succesively increased numbers of atoms centered around the Cu site are introduced. The resulting coupling schema of the Cu-S (cysteine) stretching coordinates in the normal modes were very different from model to model. That is, the abnormally strong cooperation between the Cu-S(cysteine) stretching and the bending coordinates specified by the terminal atoms of the peptides in the models always appeared, particularily in the small molecular models. Thus, the normal coordinates based upon the small molecular models, as well as those of the 4- and 5-atom models,[6,15] are not accepted as good approximations for the RR active modes. Finally, analyses of the 169-atom models reported here were carried out, in which abnormally strong cooperation with the terminal bending coordinates (e.g., concerning with Ala 109 of A.d. azurin) almost disappeared (but not negrigible) and the internal coordinates coupled to the Cu-S (cysteine) stretching coordinates are stably distributed into the models.

A further introduction of atoms into the molecular models was not attempted because of a similar widely distributed coupling between the Cu-S (cysteine) stretching and the skeletal bending coordinates would result.

The normal coordinate analyses performed by 4- and 5-atom models have indicated a relatively large mixing between the Cu-S (cysteine) and Cu-N (histidine) stretcing vibrations.[6,15] However, the present analyses indicate that the mixing of the Cu-S (cysteine) and Cu-N (histidine) stretching is very small if reasonable values of the forces (K(Cu-N(histidine)=1.200 mdyn $Å^{-1}$) is employed, which is a direct quotation from Nestor et al.[6] As a matter of course, analyses of the 4- and 5-atom models could not explain the presence of the plural RR lines commonly observed for the blue copper proteins, and the discussion has been limited to making suggestions concerning the couplings of the Cu-S(cysteine) stretching displacement and the intra-ligand deformational modes of the coordinating histidines, Cu-S-C_β (cysteine), S-C_β-C_α (cysteine), and C_α (cysteine) deformational displacements. The mixing of the Cu-S (cysteine) and the Cu-N (histidine) stretching was discussed by Sakurai et al.[8] from their RR measurements of ultra-violet excitation. However, the present analyses indicate that even though the Cu-S-C_β (cysteine) and S-C_β-C_α (cysteine) bending and the deformational modes around the C_α (cysteine) indeed

contribute to these important normal modes, they do not have any particular significance in their PED values, as shown in Figs. 4—8. The present results also indicate that coupling between the Cu-S (cysteine) stretching and the intra-ring deformational modes of coordinating histidines is small, but that the out-of-plane bending displacement of the CH_2-(histidine imidazole ring (HIS 117 of the azurins: HIS 87 of the plastocyanin) has obvious significance.

Seven normal modes of vibration were estimated in the 254—271 cm^{-1} region, all of which involve in-phase stretching displacements of the two Cu-N (histidine) bonds. The rather small PED values of the Cu-N (histidine) stretching coordinates were estimated for these normal modes (for example for A.d.: 254 cm^{-1}, (PED 1.4 (Cu-N HIS 47), 1.5 (Cu-N HIS 114), 257 (0.2, 0.4), 259 (2.2, 0.9), 266 (2.3, 0.1), 268 (0.7, 0.4), 270 (1.3, 1.8), and 271 (3.8, 1.1)). However, the crowded frequency location of these modes has a likely relevance to observations of the RR signals, provided that the in-phase displacement of the two Cu-N stretching coordinates has a RR activity due to Albrecht's *A*-term, even upon visible excitations far from the higher-energy Cu←N charge-transfer absorption bands. The vague RR bands of the azurins observed near 250—280 cm^{-1} were, thus, explainable. The normal modes involving out-of-phase Cu-N stretchings appeared in a slightly higher frequency region, e.g., for A.d.: 283.0 cm^{-1} (10.0, 11.9), 296.4 (0.1, 6.8), 309.0 (0.3, 5.1) and 312.7 (6.3, 10.8), than those of the in-phase vibrations.

The large extent of mixing of the Cu-S (cysteine) stretching and the bond-angle bending coordinates analysed here are further supported by the calculated ^{63}Cu-^{65}Cu isotope shifts which agree well with the observed values (Table 1). In this respect, the 5-atom models[6] resulted in about 3-times larger values of the ^{63}Cu-^{65}Cu shifts, compared to the observed values.

Since the coupling of the Cu-S (cysteine) stretching coordinate to the skeletal bending coordinates is quite serious, the Cu-S (cysteine) stretching coordinate has even small contributions to the important normal modes in the 370—450 cm^{-1} region. For example, in the case of azurin (A.d.), the PED values of the Cu-S (cysteine) stretching coordinate were estimated to be only 11.3 (428.8 cm^{-1}), 32.7 (417.0 cm^{-1}), 9.3 (398.7 cm^{-1}), and 9.2, (377.4 cm^{-1}). This theoretical result indicates that the RR signals from blue copper proteins are not the "Cu-S (cysteine) stretching vibration" but "skeletal deformational vibrations around the Cu active center that particularily could couple to the Cu-S (cysteine) stretching displacement." Accordingly, the arguments in the RR spectra of the type-1 blue copper proteins should be developped while taking into consideration the larger parts of the protein structure around the blue copper site than has been done in the literature.[6,7]

Our standpoint is confirmed by our model calculations. The tertiary structures of the two azurins, A.d. and P.a., are quite similar (in the modeled region). Accordingly, the normal coordinates were found to be comparable (Table 1, and Figs. 4—7). On the other hand, the distinct structural differences between the azurins and the plastocyanin must be mentioned in Cu-CYS112-SER113-PHE114-PRO115-GLY116-HIS117-Cu (A.d.) and Cu-CYS84-SER85-PRO86-HIS87-Cu (pc) in the modeled region. The former forms a 22-membered ring structure and the latter a 16-membered ring. Accordingly, the RR active modes of plastocyanin were found to be very different in pattern from those of the azurins, in spite of the retention of all the force constant values (Table 1). The present analyses indicate that the sensitive nature of the RR spectra of the blue copper proteins must come from a difference in the peptide structure surrounding the central copper. The observed wide variations in the RR spectra of blue copper proteins may be explained in this way. Of course, a prescise determination of the normal coordinates of such the large molecules as blue copper proteins is not possible unless an enormous number of vibrational assignments (in Infrared and Raman spectroscopy) is confirmed and a quantitative formalism of the subsidiary forces in the protein molecules is established. However, emphasis was primary focused in the present work on the importance of the extensive coupling between the Cu-S (cysteine) stretching coordinate and the skeletal deformational modes.

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