The Copper–Poly-L-histidine Complexes. II. Physicochemical Properties[1]

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Abstract: Soluble Cu(II) complexes of poly-L-histidine (PLH) formed at pH 5 and 14 at a molar ratio of His/Cu = 4 were investigated. Titration of PLH showed that in the presence of Cu(II) ions four protons are released per Cu(II) ion in the pH range of 3.0–4.5 and a fifth proton is released between pH 4.5 and 6.5. Absorption spectra in the visible range show a maximum at 543 mμ (ε 83) for the complex formed at pH 5 and at 530 mμ (ε 200) at pH 14. Circular dichroic spectra show that these bands are composite, the main band being positive at pH 5 (λ_{max} 536 mμ (ϵ_{L-R} 0.62)) and negative at pH 14 (λ_{max} 532 mμ (ϵ_{L-R} −0.63)). The two complexes also differ in their epr spectra. It is concluded that in the complex formed at pH 5 the Cu(II) ion is bound to three imidazoles and one peptide nitrogen, whereas at pH 14 four consecutive peptide nitrogens occupy a distorted coordination square and one imidazole group binds at an axial site. These structures were also built from space-filling models.

The understanding of the properties and mode of action of metalloproteins depends to a large extent on our knowledge of the amino acid residues which function as the binding ligands for the metal ions.[2] Whereas in recent years the structures of metalloenzymes like carboxypeptidase[3a] and carbonic anhydrase[3b] have been investigated by X-ray diffraction and the problem solved in a most direct way, our knowledge of the coordination sphere of the metal ion in copper proteins is still very limited and of indicative nature only.[4]

The unique properties of the copper ions bound in proteins[4] and the formal similarity between the reactions catalyzed by simple Cu(II) complexes and these enzymes provoked numerous studies on model systems for these proteins. The two protein residues frequently suggested as ligands for copper ions are the thiol group of cysteine and the imidazol residue of histidine.[5,6] The study of the interaction with the thiol groups suffers from the complication caused by redox reactions between the metal ion and its sulfhydryl ligand.[7] The physical and chemical properties of copper complexes formed with a wide variety of imidazol-containing compounds have been investigated[8,9] in an attempt to reconstruct the unusual visible and epr spectral properties of Cu(II) proteins. Although spectral properties similar to those of the typical copper proteins could be demonstrated in some complexes,[8,10,11] all attempts to prepare such complexes with naturally occurring amino acid residues as ligands found very limited success.

Finally, copper ions catalyze electron transfer reactions whether bound specifically within a copper protein or as the simple aquo complex ions;[12] therefore, numerous investigations were undertaken in an attempt to correlate the nature of ligands added with their effect on the oxidase activity of copper ions. Among the variety of ligands studied for their effect on the catalytic efficiency of copper ions, only in a few cases was enhancement observed.[13,14] It was demonstrated[1,15] that the poly-L-histidine–Cu(II) complex exhibits catalytic efficiency which is of two orders of magnitude higher than that of the aquo copper complex and is comparable to that of ferroxidase (ceruloplasmin). Moreover, the oxidations catalyzed by this complex have a kinetic pattern and specificity which have not been observed hitherto in any model system.[1,15]

In order to gain more understanding of the unique catalytic activity of PLH–Cu(II) and the mode of binding of the Cu(II) to the polypeptide, a detailed study of the physicochemical properties of PLH–Cu(II) has been undertaken.

Experimental Section

Materials. All solutions were prepared from double distilled water. Poly-L-histidine (PLH) was a product of Yeda Research and Development Co., Rehovot, Israel. The polymer was dried *in vacuo* at 100° over P_2O_5 to constant weight. The average molecular weight determined by sedimentation velocity and diffusion was 16,500 (degree of polymerization = 120). *Anal.* Calcd for $(C_6H_7ON_3)_n \cdot 0.5nH_2O$: N, 28.8. Found: N, 28.7.

Poly-L-lysine hydrobromide (n = 90–100) was kindly donated by Dr. A. Yaron, from the Department of Biophysics.

Methods. The concentration of PLH solutions was determined by the micro-Kjeldahl method. The concentration of $CuSO_4 \cdot 5H_2O$ or $CuCl_2 \cdot 2H_2O$ solutions was determined by the biquinoline method.[16] Spectrophotometric measurements were performed on a Cary Model 14 spectrophotometer. The ORD measurements were performed on a Rudolph spectropolarimeter using a mercury arc or a xenon arc. The molecular rotation of the copper chromophore in the PLH–Cu(II) complex was calculated from eq 1, where $[M]_{Cu(II)}$ is the molecular rotation of the copper chromophore, n is

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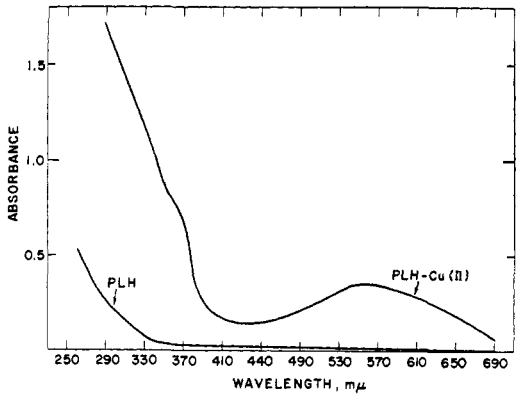

Figure 1. The visible and near-ultraviolet spectra of PLH–Cu(II); the solution contained 5.6 mg/ml of PLH (4.1×10^{-2} M in histidyl residues), 4.1×10^{-3} M Cu^{2+}, and 0.16 M $NaClO_4$ at pH 5.0. For comparison the spectrum of PLH in the absence of Cu^{2+} is given.

$$[M]_{Cu(II)} = \frac{3}{n^2 + 2} \frac{\alpha_{obsd} MR}{dC} \quad (1)$$

the refractive index of the solution, M is the residue molecular weight of histidine, α_{obsd} is the measured rotation, d is the optical path in decimeters, C is the concentration of the polymer in grams per 100 ml, and R is the molar ratio of histidyl residues to Cu(II) in the PLH–Cu(II) complex.

The circular dichroism (CD) spectrum was measured on a prototype dichrograph built by Rehovot Instruments, Ltd., Israel, in the range 300–700 mμ. Results are plotted as ϵ_{L-R}, the difference between the molar extinction coefficients for left and right circularly polarized light, respectively, *vs.* the wavelength.

The epr spectra of PLH–Cu(II) complexes were measured on a Varian-4500 spectrometer.

Sedimentation velocity and diffusion were measured in the Beckman/Spinco model E ultracentrifuge. Before diffusion measurements were carried out, the PLH–Cu(II) solutions were dialyzed against the solvent. The molecular weight of the PLH–Cu(II) complex was calculated from eq 2, where $S°_{20}$ and $D°_{20}$

$$M = \frac{RTS°_{20}}{D°_{20}(1 - \bar{v}\rho)} \quad (2)$$

are the extrapolated values of the sedimentation constant and the diffusion constant, respectively.

All measurements of the physical properties of the PLH–Cu(II) complex were done in unbuffered solutions, containing 0.16–0.26 M NaCl or $NaClO_4$, although it could be shown that except for the sedimentation pattern, none of the physical properties measured was altered in the absence of salt. The desired pH was obtained by adding NaOH to the PLH–Cu(II), in a pH-stat. It should be noted that both PLH and PLH–Cu(II) become insoluble above pH 6.0; hence all measurements were performed below pH 6.0. The PLH–Cu(II) complex redissolves at pH 14.0 to form the classical biuret complex. Potentiometric titrations of PLH and PLH–Cu(II) complex were performed between pH 2.0 and 8.0 using the Radiometer TTT-1 titrator. All measurements were carried out at 25°.

Results

The Molecular Weight of PLH–Cu(II). The diffusion and the sedimentation constants for both PLH and PLH–Cu(II) are summarized in Table I.

Table I. Sedimentation and Diffusion Parameters of PLH and PLH–Cu(II)

Material	$D°_{20} \times 10^7$ cm² sec⁻¹	$S°_{20}$	Mol wt
PLH	12.5	2.1	16,600
PLH–Cu(II)	12.4	2.3	18,700

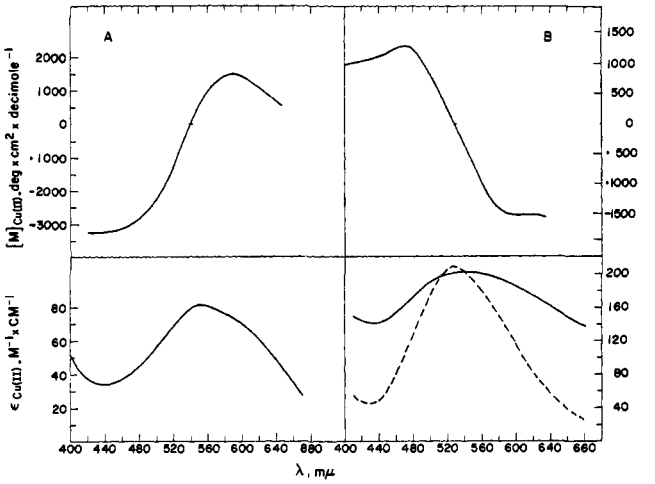

Figure 2. The visible and ORD spectra of PLH–Cu(II): (A) (——) PLH–Cu(II) at pH 5.0; (B) (——) PLH–Cu(II)–Cu(II) at 1.0 N NaOH (pH 14.0); (----) poly-L-lysine–Cu(II) at 1.0 N NaOH. In both cases the molecular rotation of the copper chromophore $[M]_{Cu(II)}$ and the molar absorption coefficient $\epsilon_{Cu(II)}$ are given. The absorption spectrum and the ORD spectrum of the PLH–Cu(II) at pH 14.0 were measured within 1 hr during which racemization is insignificant. The molar concentrations of the polyamino acids and of Cu^{2+} were identical with those in Figure 1.

All measurements were performed at pH 5.0 in the presence of 0.26 M NaCl. In the case of PLH–Cu(II) the molar ratio of histidyl residues to Cu(II) was 10. For the two materials, $1 - \bar{v}\rho$ (eq 2) was taken as 0.25. The concentration range used for the calculation of $S°_{20}$ and $D°_{20}$ was 20–10 mg/ml.

From the sedimentation–diffusion data it is concluded that PLH–Cu(II) does not associate in the concentration range investigated.

Absorption and ORD Spectra. In Figure 1 the visible and the near ultraviolet spectra of the PLH–Cu(II) at pH 5.0 are shown. The complex exhibits a maximum at 543 and a shoulder at 350–380 mμ. This pH value was chosen since it was found by titration (see below) that above pH 5.0 the dissociation of the ionizable hydrogens at the copper binding sites is complete and it can therefore be assumed that the copper atoms are all bound to identical coordination environment. The association constant of this site,[17] calculated on the basis of the titration data (Figure 6) by a curve fitting program, was found to be 10^{19} M^{-1}. The molecular rotation, $[M]_{Cu(II)}$, of the PLH–Cu(II) complex and its molar absorbance in the visible range at pH 5.0 are shown in Figure 2A. For comparison the molar absorbance and the molecular rotation of the classical PLH–Cu(II) biuret complex (in 1 N NaOH) are given in Figure 2B. It was found that the molar absorbance depends neither on the concentration of the PLH–Cu(II) complex, nor on the molar ratio of histidyl residues to Cu(II) in the range 20–4. Also, the molecular rotation of the copper chromophore, $[M]_{Cu(II)}$, within the PLH–Cu(II) complex does not depend on histidyl to Cu(II) molar ratios down to 4 (Figure 3). This confirms the assumption that all Cu^{2+} ions are bound to form a series of identical binding sites on a single PLH–Cu(II) chain. At a lower ratio of histidyl residues to Cu(II), insoluble PLH–Cu(II) complexes are formed.

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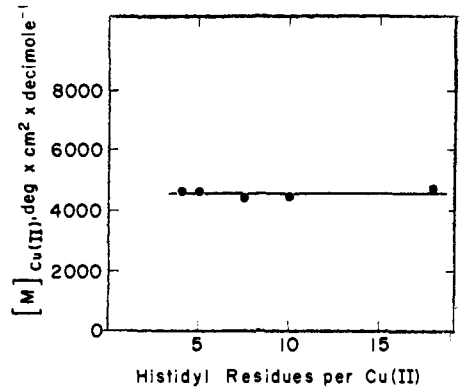

Figure 3. The amplitude of the cotton effect as a function of the His/Cu(II) molar ratio; conditions are identical with those described in Figure 1. Different amounts of $Cu(ClO_4)_2$ were added in order to bring about the His/Cu(II) ratios desired.

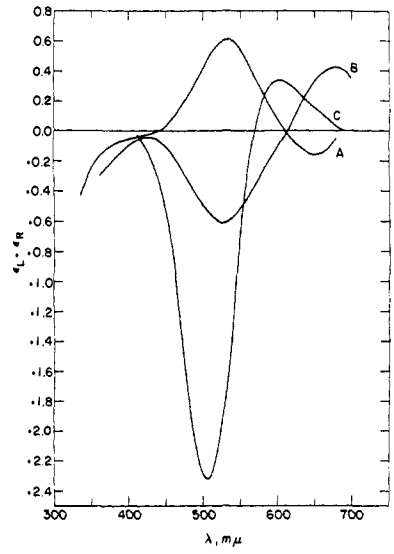

Figure 4. Circular dichroism spectrum of PLH-Cu(II). The solution contains 0.01 M Cu(II) and PLH or poly-L-lysine with His/Cu(II) = 4 or Lys/Cu(II) = 4 and 0.16 $NaClO_4$. The dichroism at pH 14.0 was measured within less than 1 hr during which racemization is insignificant: A, PLH-Cu(II) at pH 5.0; B, PLH-Cu(II) at pH 14; C, poly-L-lysine-Cu(II) at pH 14.

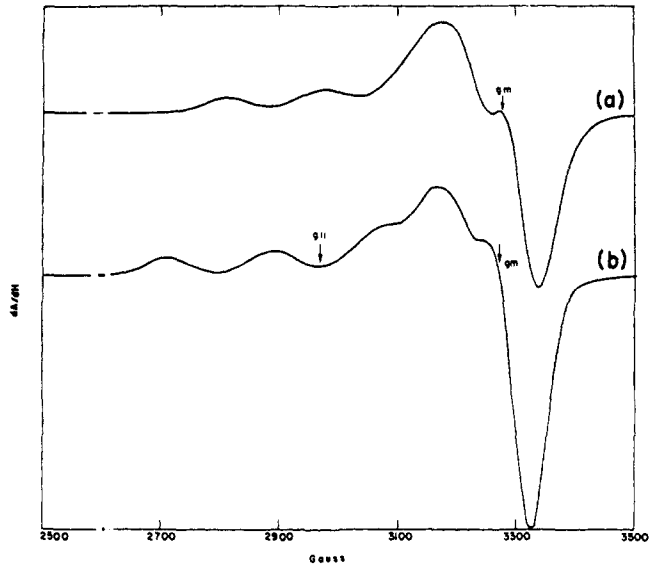

Figure 5. Epr spectra of the PLH-Cu(II) complex. The solution contained 10 mM Cu(II) and 100 mM histidyl residues (8.3×10^{-4} M in PLH) and 0.16 M NaCl: (a) epr of the PLH-Cu(II) biuret complex at pH 14.0; (b) epr of PLH-Cu(II) at pH 5.1.

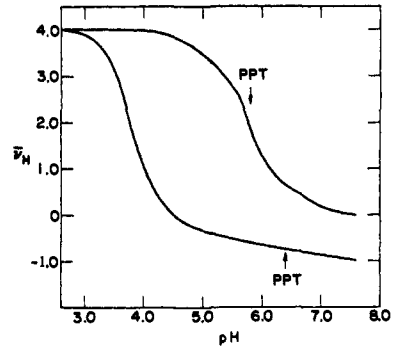

Figure 6. Potentiometric titration of PLH and PLH-Cu(II). The titration was performed on a solution containing 1.04 μmol of PLH (125 μmol in histidyl residues) in the absence and in the presence of 31.2 μmol of $CuCl_2$. The solution was 0.2 M in KCl. The upper curve is the titration of PLH and the lower curve is of the PLH-Cu(II) complex.

From the spectra and ORD of the PLH-Cu(II) complex of pH 5.0 and of the biuret PLH-Cu(II) complex it is evident that the Cu(II) chromophores are different in the two types of the PLH-Cu(II) complexes.

CD Spectra. The asymmetric shape of the absorption bands (Figure 4) of PLH-Cu(II) both at pH 5.0 ("catalytic complex") and at pH 14.0 (classical biuret complex) indicates the composite nature of these bands. In the case of Cu(II) chromophores one should expect two types of electronic transitions, $B_{1g} \leftarrow B_{2g}$ with the energy ΔE_{xy} and $B_{1g} \leftarrow E_g$ with the energy ΔE_{xz}, which occur below 700 mμ.[8] In the present case these bands are optically active and therefore appear in the circular dichroism spectrum (Figure 4). The PLH-Cu(II) complex at pH 5.0 shows two bands, one positive (peak at 536 mμ (ϵ_{L-R} 0.62) and the other negative (trough at 650 mμ (ϵ_{L-R} −0.17)). It may be noted that at wavelenths shorter than 400 mμ another negative dichroic band appears. For comparison, the CD spectrum of the biuret complex of PLH-Cu(II) (pH 14.0) is given. Again, two optically active transitions are observed. one negative at 532 (ϵ_{L-R} −0.63) and one positive at 680 mμ (ϵ_{L-R} 0.43) in contrast to those of the catalytic complex. The biuret complex of poly-L-lysine resembles the pH 14 PLH-Cu(II) complex (Figure 4). The energy values of the two optically active electronic transitions of both PLH-Cu(II) complexes are given in Table II. The values for ΔE_{xy} and ΔE_{xz} were calculated from the CD spectra (Figure 4).

Epr Spectra. The epr spectra of the PLH-Cu(II) complex (pH 5.0) and of the PLH-Cu(II) biuret complex (pH 14.0) are given in Figure 5. With the data from the CD spectra it is possible to estimate the extent of the covalent character[9] in the PLH-Cu(II) complex from the values of the coefficients a and b in eq 3 and 4. In these expressions g_e is a constant (the

$$g_{\parallel} = g_e\left(1 - \frac{a\lambda}{\Delta E_{xy}}\right) \quad (3)$$

$$g_{\perp} = g_e\left(1 - \frac{b\lambda}{\Delta E_{xz}}\right) \quad (4)$$

Table II. Magnetic and Energy Parameters of the PLH–Cu(II) Complexes

Complex	pH	$g_{\|\|}$	g_{max}	ΔE_{xy}, cm^{-1}	ΔE_{zz}, cm^{-1}	a	b	A, cm^{-1}
PLH–Cu(II)	5.0	2.225	2.037	15,385	18,657	2.066	0.389	0.018
PLH–Cu(II)	14.0	2.180	2.032	14,860	19,083	1.592	0.342	0.015

g value of the free electron) and λ is the spin–orbit coupling constant for the free cupric ion (-828 cm^{-1}). $g_{||}$ can be calculated from the epr spectrum and $g_{\perp}$ can be assumed as a first approximation to be close to g_{max}. The approximation $g_{\perp} \sim g_{max}$ does not introduce a large error for the pH 5.0 PLH–Cu(II) complex. For the pH 14.0 PLH–Cu(II) complex the magnetic parameters should be considered as an approximation since the hyperfine structure of the spectrum is not well resolved. The values for the hyperfine constants (A) for both complexes are also given (Table II). For simple ionic binding in planar copper complexes, a and b should have values of 4 and 1, respectively. Departure from these values (see Table II) most probably reflects tetragonal distortion and partial covalent character.[8,9]

Potentiometric Titrations. The potentiometric titration curves of PLH in the absence and presence of Cu(II) ions at a molar ratio of histidyl residues/Cu = 4 are shown in Figure 6. When PLH is titrated, protons start to be released at pH 4.5. In the presence of Cu(II) ions in the above mentioned ratio, protons are released from pH 3, and up to pH 4.5 4 equiv per Cu(II) are titrated. An additional, fifth equiv of protons is almost fully released between pH 4.5 and 6.5 before the complex precipitates. In Table III, the number of protons released per Cu(II) bound is presented for different histidyl residue to Cu(II) molar ratios. From these data it is evident that four histidyl residues are required for the binding, and at this ratio all the binding sites on the macromolecules are fully occupied.

Table III. Proton Equivalents Released on Binding Cu(II) to PLH at Different Molar Ratios of Histidyl Residues to Cu(II)

Molar ratio His residues/Cu(II)	H$^+$/Cu(II) (pH 4.6)
20	3.98
10	4.02
8	4.00
5	4.00
4	3.90
3.5	3.55 (precipitation)

Discussion

In alkaline aqueous solutions all water-soluble polyamino acids (except polyproline) bind Cu(II) ions to form the purple biuret type complex.[18,19] Poly-L-histidine (PLH) binds copper also at low pH (starting at pH 3) with a miximum binding constant of 10^{19} M^{-1} at pH 5.[17] The optical and magnetic properties of these two types of complexes are very different indicating that the copper is bound in different ways. Since the copper binding site in the biuret complex is the peptide group it seems that at low pH the imidazole side chains are the main ligands. At pH 5, where polyhistidine is protonated, addition of one Cu(II) per four histidyl residues causes the dissociation of four protons. However, the spectral properties of this complex strongly indicate that at least one of these protons originates from a peptide group. The Cu(II)–(imidazole)$_4$ complex has its maximum at 596 mμ[19] and in all peptide complexes which absorb light at wavelengths shorter than 600 mμ the peptide nitrogen is known to be deprotonated. For example, Cu(II) histidylhistidine,[20] in which the dissociated peptide nitrogen is complexed, has λ_{max} 560 mμ. It thus seems that it is the fifth proton, titrated above pH 5, which originates from the fourth imidazole group.

Coordinated water cannot be the source of this extra proton since the latter should have a pK of 9 or higher.[20] It may therefore be concluded that three imidazole nitrogens and one peptide nitrogen occupy a distorted coordination square of the copper ion (see Figure 7). The fourth imidazolium either does not participate in complex formation or occupies one of the axial sites, the other axial site, or both, being occupied by a water molecule. The fourth imidazole is titrated with pK_{app} = 6.1 and is probably responsible for the solubility of PLH–Cu(II) at pH lower than 6.5. At higher pH, when most of these imidazolium groups on the polypeptide chains become discharged, the polymer precipitates.

The differences in ORD and CD spectra between the two kinds of PLH–Cu(II) complexes reflect their differences in structure. The biuret type complex, formed at high pH, is similar in its CD spectrum to that of the polylysine biuret complex (Figure 4). It can be assumed that in these complexes, in analogy to the copper complexes of oligopeptides, each copper atom is coordinated with four consecutive peptide nitrogens.[21] The difference in the amplitude of the CD bands between PLH–Cu(II) and polylysine–Cu(II) may be an indication that the side chains occupy the axial coordination sites. Using space-filling models (Corey–Pauling–Koltun atomic models) it could be demonstrated that this is indeed possible (Figure 7).

The low pH complex of PLH shows a CD spectrum in which the two bands are of opposite sign as compared with the high pH complex. This shows that the two complexes are of different geometry. In the most probable conformation of the pH 5 complex, three of the four ligands are imidazole nitrogens and one is a peptide nitrogen, as shown in Figure 7. With the space-filling models this structure can be built without strain so that two axial positions are taken up by water molecules.

Copper complexes with small ligands such as amino acids and peptides differ in their epr spectra from copper containing oxidases[4] such as laccase and ferroxidase[22]

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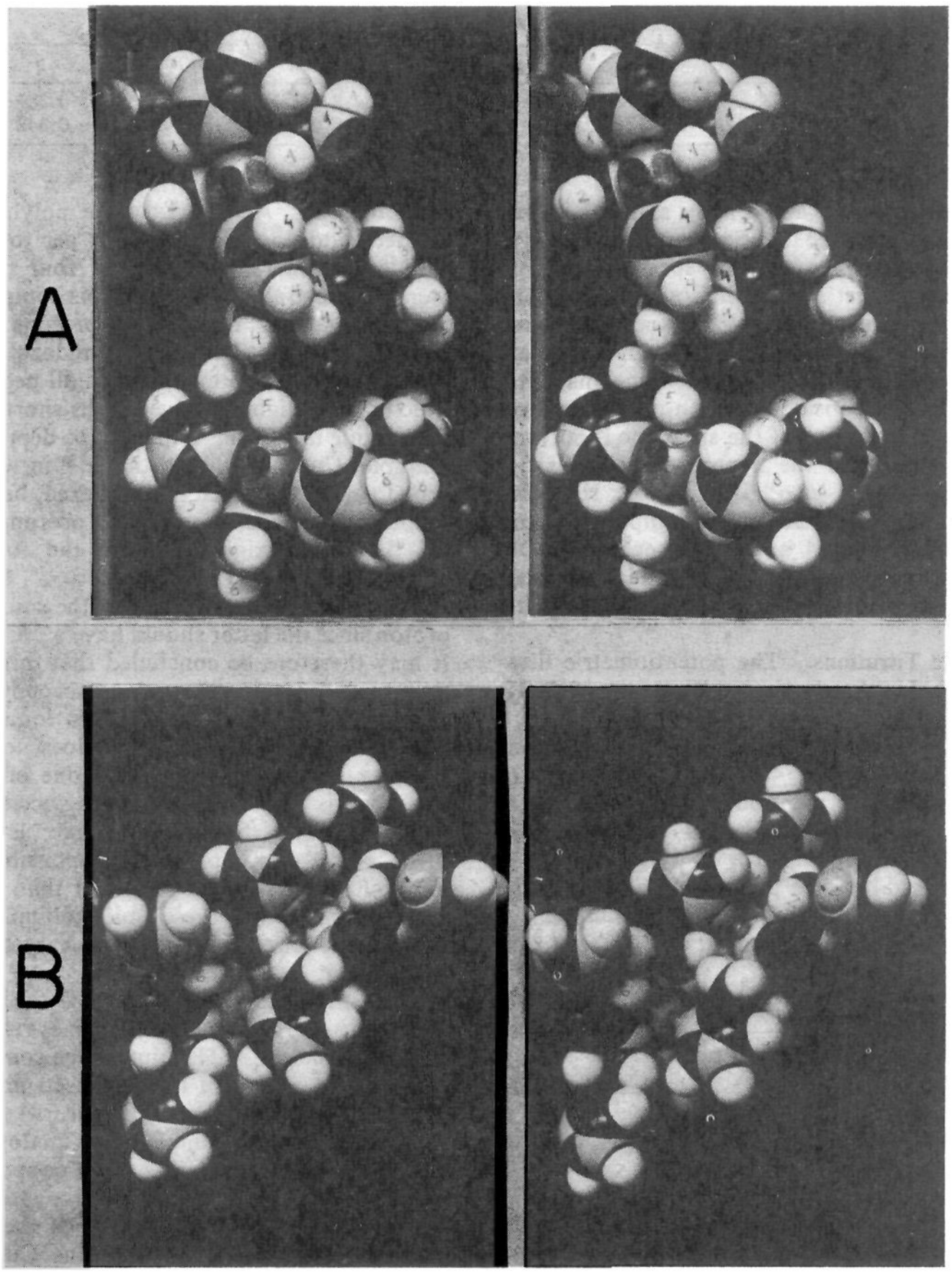

Figure 7. Stereoview of the space-filling models of the PLH–Cu(II) complexes (proposed structure). A. The catalytic PLH–Cu(II) complex formed at pH 5.0; the Cu(II) ion is coordinated to four nitrogen atoms: three of imidazole side chains (residues 1, 2, and 4) and one of a peptide group (residue 3) in a square planar geometry. The two axial sites of the metal ion are exposed and can be occupied by water molecules. The imidazole group of residue 3 can be protonated and is thus responsible for the solubility of the PLH–Cu(II) complex below pH 6. There is free rotation between residues 4 and 5 which makes it possible for a long chain to coil randomly. B. The PLH–Cu(II) biuret complex formed at pH 14. The Cu(II) ion is coordinated to four consecutive peptide nitrogens (residues 1, 2, 3, and 4) occupying the corners of a distorted square.[21] Residues 4 and 8 are on the hidden side of the picture. One axial site may be occupied by the imidazole nitrogen of residue 5, whose peptide nitrogen is coordinated with the next copper atom. The structure shown can be repeated indefinitely, forming a rigid, rather wide helix. The helix axis lies behind the residues shown in the picture, somewhat to the left. It passes through imidazole rings 4, 8, etc. There are 5.5 copper atoms per turn of the helix. The translation per amino acid along the helix axis is less than 1 Å.

(ceruloplasim), in that the hyperfine structure constant of the former ($A = 0.02$) is about twice as big as that of the latter. Some copper proteins such as azurin also have a larger A value.[23,24] Although the PLH–Cu(II), pH 5, is catalytically as active as ceruloplasmin,[1,15] the former has a large A (0.018) and thus it seems that this property cannot always be correlated with the catalytic action of the bound copper.

In conclusion, it can be said that although in some features, such as the high binding constant for Cu(II) at low pH,[17,25] the shoulder in the spectrum at 350–380 mμ,[26] and the pattern of the low pH CD spectrum,[27] the PLH–Cu(II) complex shows a similarity with certain copper

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proteins whereas it does differ in others. The PLH–Cu(II) system thus constitutes at best a partial model for a binding site in copper-containing proteins. It is possible that the similarities observed stem from a similarity in the way Cu(II) is bound, *i.e.*, at least partly at the imidazole groups.

Substrate Specificity of Farnesyl Pyrophosphate Synthetase

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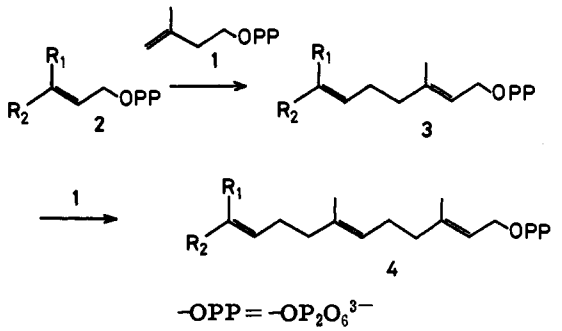
Abstract: The substrate specificity of farnesyl pyrophosphate synthetase of pumpkin fruit was studied with artificial allylic pyrophosphates. In order to determine the upper size limit of the reactive allylic pyrophosphate, *trans*-3-methyl-2-undecenyl (**2a**), *trans*-3-methyl-2-dodecenyl (**2b**), and *trans*-3-methyl-2-tetradecenyl pyrophosphate (**2c**) were assayed in the enzymatic reaction with isopentenyl pyrophosphate (**1**). **2a** and **2b** were reactive, the latter being far less so, whereas **2c** was no longer reactive. Replacement of the methyl group in 3-methyl-2-alkenyl pyrophosphate by ethyl did not cause marked change in the reactivity. *trans*-3-Ethyl-2-heptenyl (**2d**), *trans*-3-ethyl-2-octenyl (**2e**), and *trans*-3-ethyl-2-decenyl pyrophosphate (**2f**) were all reactive. However, branching of the alkyl group in 3-methyl-2-alkenyl pyrophosphate caused a remarkable decrease in the reactivity. Reactivities of *trans*-3,4-dimethyl-2-pentenyl (**5a**), *trans*-3,4-dimethyl-2-hexenyl (**5f**), and 3,6-dimethyl-2-heptenyl (**5c**) pyrophosphate were negligible.

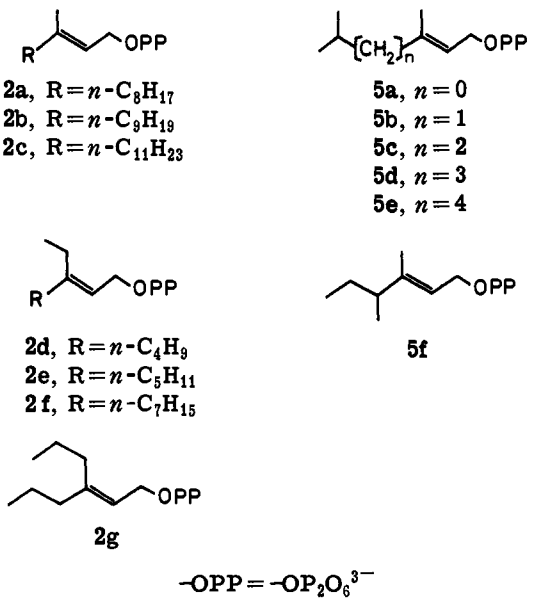
Studies with artificial substrates for farnesyl pyrophosphate synthetase of pig liver and pumpkin fruit[1–5] have shown that the longest carbon chain homolog formed by pumpkin farnesyl pyrophosphate synthetase is a C_{18} compound, trishomofarnesyl pyrophosphate (**4**, $R_1 = CH_3$, $R_2 = n\text{-}C_4H_9$ or $R_1 = n\text{-}C_4H_9$, $R_2 = CH_3$) (see Scheme I) which results from the condensation of two molecules of isopentenyl pyrophosphate (**1**) with *trans*- (**2**, $R_1 = CH_3$; $R_2 = n\text{-}C_4H_9$) or *cis*-3-methyl-2-heptenyl pyrophosphate (**2**, $R_1 = n\text{-}C_4H_9$; $R_2 = CH_3$).[3] It was shown that the longer homolog of the cis series (**2**, $R_1 = n\text{-}C_5H_{11}$; $R_2 = CH_3$) was inactive and that the longer homologs of the trans series (at least up to $R_1 = CH_3$; $R_2 = n\text{-}C_7H_{15}$ in **2**) reacted with one molecule of **1** to give the corresponding geranyl pyrophosphate homologs (**3**),[3] but the longer limit of the trans series remains to be defined. The previous observation that *trans*-3,5-dimethyl-2-hexenyl pyrophosphate (**5b**) cannot be a substrate in contrast to the reactivity of the nonbranching isomer[3] also led us to study the effect of the branching of the alkyl group in 3-methyl-2-alkenyl pyrophosphate on the reactivity as a substrate. The present paper describes these results.

Scheme I



In order to define the longer limit of the reactive homolog, *trans*-3-methyl-2-undecenyl (**2a**), *trans*-3-methyl-2-dodecenyl (**2b**), and *trans*-3-methyl-2-tetradecenyl pyrophosphate (**2c**) were synthesized by a method similar to that reported previously[3] (Scheme II). The Witting reaction of 2-decanone, 2-undecanone, and 2-tridecanone with diethyl methoxycarbonylmethyl phosphonate gave mixtures of methyl *cis*- and *trans*-3-methyl-2-alkenoates. The mixture of the esters was hydrolyzed to the free acids, from which the trans isomers were purified by recrystallization.

Scheme II



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