On the Analogy and Specificity of Copper(II) Binding Sites in Type I and Type IV Collagen

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Abstract**

The complexation of copper(II) with collagen from different tissues (rat skin, calf skin and human placenta), in a molar ratio 1:1 was studied, at nearly physiological pH, by ESR and electronic techniques. The spectral patterns and parameters are similar for Cu(II)–type I collagen and Cu(II)–type IV collagen derivatives, in agreement with their analogous biochemical behaviour. The absorption maxima at 16 900 and 18 500 cm^{-1} are typical of a Cu(II)–$(N)_4$ system in a D_{4h} geometry. The transition energy ΔE_{xy} = 16 900 cm^{-1} can approach a ligand field formed by one N_{imid} and three N_{pept}. The ESR spectral patterns agree with an axial symmetry of the complexes. The ESR parameters $g_{\parallel} = 2.25$, $A_{\parallel} = 180$ G, with not well resolved superhyperfine structure, suggest a distorted D_{4h} geometry for a Cu(II)–$(N)_4$ system in the plane of the nitrogens. Only at pH $\geqslant$ 11 does the complete N_{pept} dissociation, involving also the breaking of hydrogen intramolecular bindings, permit Cu(II) coordination of four nitrogens in an undistorted square-planar geometry.

Introduction

In our previous work [1], we investigated the copper(II)-rat skin soluble collagen (type I) interactions, in order to identify the pH dependence of the metal potential binding sites in the protein. From the ESR and electronic data it appeared that Cu(II) coordination to oxygen and nitrogen ligands is pH dependent, in the order; $-COO^-$, $-NH_2$, N(imidazole), N(peptide), and at physiological pH copper coordinates three or four nitrogens, but there is no unique binding site. In order to better characterize this collagen behaviour we studied the copper(II) complexes with rat skin collagen and their CH_3O- and DNP-derivatives, together with calf skin collagen and human placenta collagen at neutral pH, using two molar ratios to identify a potential copper-specific binding site: (a) 15:1, corresponding to the presence of 15 histidine residues in the tropocollagen molecule; and (b) 1:1.

Our aim was first to individuate and to structurally characterize by ESR and electronic measurements the Cu(II)–collagen complexes; second, to compare the biochemical behaviour of different collagen tissues in the copper-coordination; third, to analyze the copper–collagen affinity related to that of some other metals previously studied and connected to cross-linking formation in mature collagen.

Experimental

Materials

Soluble collagens from calf skin and human placenta, and trypsin were purchased from Sigma. Rat skin, CH_3O-rat skin and DNP-rat skin soluble collagens were obtained in our laboratory according to published procedures [1]. $^{63}CuCl_2$ was obtained by dissolving isotopically pure ^{63}CuO from the Oak Ridge National Laboratory in 0.1 N HCl. All other reagents were analytical grade from Merck. Copper(II) concentrations in all the collagen samples were determined by atomic absorption spectroscopy.

Preparation of Samples of Cu(II)–Calf Skin and Cu(II)–Human Placenta Soluble Collagen

1.6 μl and 24.0 μl of $^{63}CuCl_2$ solution (0.1 M) were added to 10 ml of an aqueous solution of tropocollagen (1.6×10^{-5} M) to have a molar ratio of 1:1 and 15:1, respectively. The samples were adjust-

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**Abbreviations: CH_3O-collagen = collagen with methylated carboxylate groups. DNP-collagen = collagen with dinitrophenylated NH_2 groups, RSKC = rat skin collagen, CSKC = calf skin collagen, HUPC = human placenta collagen.

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ed to the desired pH using NaOH (0.1 N) and were lyophilized.

Preparation of Samples of Cu(II)–Rat Skin, Cu(II)–CH_3O-Rat Skin and Cu(II)–DNP-Rat Skin Soluble Collagen

8.0 μl and 20.0 μl of $^{63}CuCl_2$ solution (0.1 M) were added to 50 ml of an aqueous colloidal solution of tropocollagen (1.6×10^{-5} M). The samples were adjusted to neutral pH using NaOH (0.1 N) and were lyophilized.

Preparation of Samples of Trypsin Collagen and Trypsin Collagen Derivatives

0.5 ml of trypsin solution (0.1%) were added to 20 ml, respectively, of collagen, frozen collagen at 77 K, Cu(II)–collagen, CH_3O-collagen and DNP-collagen (2 mg/ml) aqueous solutions, and the samples were incubated for 3 h at 25 °C.

Viscosity Measurements

We measured the relative viscosity coefficient ν of the collagen and trypsin–collagen samples as a function of incubation time, using an Ostwald capillary viscometer (series 100), with a flow time for water of approximately 40 s at 25 °C.

Diffuse Reflectance Measurements

The spectra were recorded on the lyophilized and dessicated samples at room temperature with a Varian 2390 spectrophotomer.

ESR Measurements

ESR spectra were recorded on the lyophilized and dessicated samples in vacuum vials at 77 K using a Varian E 109 X-band spectrometer equipped with a double cavity.

Results and Discussion

Trypsin Assay and Viscosity Measurements on the Collagen Samples

The ν values of relative viscosity at 25 °C of collagen samples with and without treatment (freezing at 77 K, copper addition, methylation of the carboxylate groups and dinitrophenylation of the NH_2 groups) on addition of trypsin, are constants over the time examined. Hence, treated and untreated collagen samples are not digested by trypsin and the samples are not denatured.

Diffuse Reflectance Spectra of Cu(II)–Rat Skin, Cu(II)–Calf Skin and Cu(II)–Human Placenta Collagen Complexes

The diffuse reflectance spectra of Cu(II)–collagen complexes are similar. Figure 1 shows the spectral pattern of a ^{63}Cu(II)–HUPC sample and displays

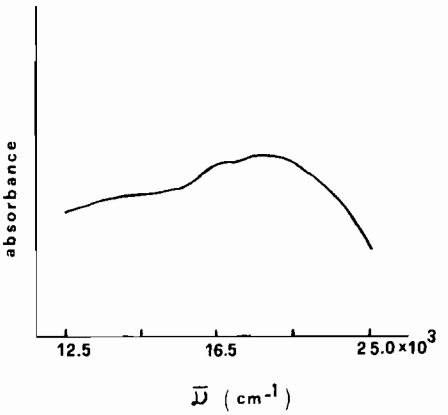

Fig. 1. Reflectance absorption spectra for the ^{63}Cu(II)–HUPC complex, molar ratio 1:1, pH = 6.96.

two sufficiently resolved bands at 16 900 and 18 500 cm^{-1}. The ligand field transition energies ΔE_{xy} = 16 900 cm^{-1} and ΔE_{xz} = 18 500 cm^{-1} can be attributed to the $B_{1g} \leftarrow E_g$ and $B_{1g} \leftarrow B_{2g}$ transitions, in a tetragonal distorsion from octahedral geometry, by the Jahn–Teller elongation along the z axis [2]. This agrees with a Cu(II)–collagen tetragonal complex, where Cu(II) is coordinated to four nitrogen ligands, because amino and imidazole groups, acting without concomitant peptide ligand involvement, give distinctly higher values of λ_{max} [3]. In particular, the absorption maximum at 16 900 cm^{-1} suggests the presence of one imidazole nitrogen of the histidine residue and three peptide nitrogens. Indeed this, compared with the absorption maximum at 19 400 cm^{-1} which is typical of the stronger ligand field containing one NH_2, one N_{imid} and two N_{pept} [4], is in good agreement with the donor power order $N_{imid} < N_{pept} < NH_2$.

Furthermore, we recall that the histidine residues, being in the outer part of the helix [5], possess a good mobility favouring the copper(II) coordination to the adjacent N peptides.

The spectral patterns of the Cu(II)–collagen complexes, molar ratio 15:1, are similar to that of the Cu(II)–collagen complex of Fig. 1, but are broadened due to the presence of more than one complex [1].

ESR Spectra of Cu(II)–Rat Skin, Cu(II)–Calf Skin and Cu(II)–Human Placenta Collagen Complexes

ESR spectra at 77 K of copper(II)–collagen complexes show a spectral feature $g_\parallel > g_\perp > 2$, with hyperfine structure in the $g_\parallel$ region and superhyperfine structure in the $g_\perp$ region (Fig. 2). An axially symmetric Hamiltonian

$$\hat{\mathcal{H}} = \beta g_\parallel H_z S_z + \beta g_\perp (H_x S_x + H_y S_y) + A_\parallel S_z I_z + A_\perp (S_x I_x + S_y I_y)$$

TABLE I. ESR Parameters for ^{63}Cu(II)–Collagen Samples, Molar Ratio 1:1, pH ≃7, at 77 K

	Cu(II)–HUPC	Cu(II)–CSKC	Cu(II)–RSKC	Cu(II)–CH_3O-RSKC	Cu(II)–DNP-RSKC
$g_{\parallel}$[a]	2.24	2.25	2.28	2.25	2.24
$A_{\parallel}$ (G)[b]	180	180	170	180	180

[a] $g_{\perp}$: 2.06–2.07. [b] A_N = 14–15 G.

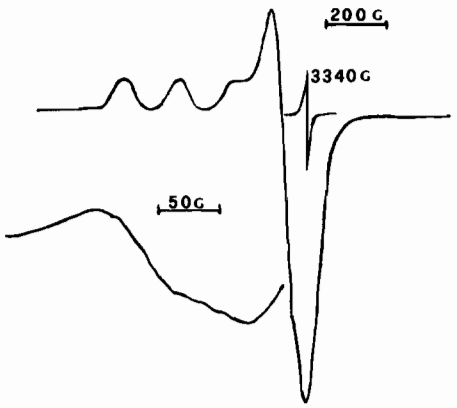

Fig. 2. ESR powder spectra of the ^{63}Cu(II)–HUPC complex, molar ratio 1:1, pH = 6.96, at 77 K. Bottom part: expanded magnetic field axis.

can be used to describe the tetragonal geometry D_{4h} of the Cu(II)–collagen complexes, in which the cupric ion is in the ground state $d_{x^2-y^2}$.

Copper(II)–Collagen Complexes, Molar Ratio 1:1

As one can see from Fig. 2, at pH ≃ 7 the superhyperfine structure in the $g_{\perp}$ region is not resolved and, indeed, it is impossible on this basis to assign the number of copper(II) coordinated nitrogens. At any rate, the decrease in resolution of the superhyperfine structure could be explained as being due to an increased distortion of the D_{4h} geometry. Furthermore, the $g_{\parallel}$ and $A_{\parallel}$ values (Table I) indicate a slow covalence degree.

By analysing also all the Table I parameters, we can see that when –COOH or $-NH_2$ groups are selectively blocked, Cu(II)–CH_3O-collagen and Cu(II)–DNP-collagen parameters are very close to that of Cu(II)–collagen. This signifies that 1 mol of copper binds first and preferentially to one histidine site.

We point out that, like the electronic spectra, the ESR spectral patterns (Fig. 2) and parameters (Table I) of Cu(II) complexes of type I and type IV collagen are similar. On this basis we can conclude that type I and type IV collagens have very nearly the same biochemical reactivity, and their common and preferential Cu(II) binding site is one of the histidine residues. Besides, type IV collagen, which differs from type I collagen only in some amino acid molecular composition, has the same histidine composition (5 histidine residues in the tropocollagen molecule).

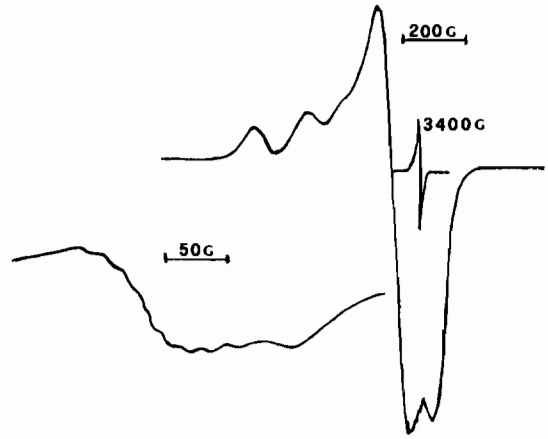

Fig. 3. ESR powder spectra of the ^{63}Cu(II)–CH_3O–RSHC complex, molar ratio 1:1, pH ⩾ 11, at 77 K. Bottom part: expanded magnetic field axis.

On the contrary at pH ⩾ 11, the ESR parameters of the Cu(II)–collagen complexes ($g_{\parallel}$ = 2.19 and $A_{\parallel}$ = 200 G), together with the presence of nine lines (A_N = 14–15 G) on the $g_{\perp}$ region (Fig. 3) suggest the superhyperfine structure indicative of four nitrogens covalently linked to Cu(II).

The approximate values of the g tensor, taking account of the covalent bond, are given by the formulae [2]:

$$g_{\parallel} = 2.0023\left(1 - \frac{a\lambda}{\Delta E_{xy}}\right)$$

$$g_{\perp} = 2.0023\left(1 - \frac{b\lambda}{\Delta E_{xz}}\right)$$

where λ is the spin–orbit coupling constant for the free cupric ion and is equal to –828 cm^{-1}, ΔE_{xy} and ΔE_{xz}, evaluated from electronic spectra, are 16 900 and 18 500 cm^{-1}; a and b, calculated for a simple model Cu(II)–AcGzHG [2] where copper(II) coordinates one N_{imid} and three N_{pept}, at pH 11 are 1.91 and 0.485. The calculated parameters $g_{\parallel}$ = 2.19 and $g_{\perp}$ = 2.04 are in good agreement with the experimental $g_{\parallel}$ = 2.19 and $g_{\perp}$ = 2.05 of our complex.

At pH ⩾ 11 the complete dissocation of the NH peptide groups involves also the breaking of the hydrogen intramolecular linking and makes them available to be covalently bound by Cu(II) in a D_{4h} geometry.

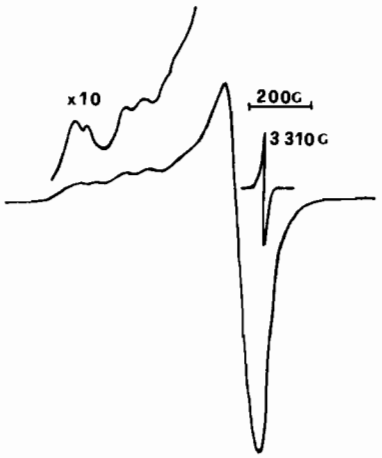

Fig. 4. ESR powder spectra of the ^{63}Cu(II)–HUPC complex, molar ratio 15:1, pH = 6.90, at 77 K.

Copper(II)–Collagen Complexes, Molar Ratio 15:1

The ESR spectra of these complexes with a 15:1 Cu(II)/protein ratio, corresponding to the presence of 15 histidine residues in the tropocollagen molecule, have overlapping spectral patterns. We report the ESR spectra of the ^{63}Cu(II)–HUPC sample (Fig. 4), which has sufficiently good resolution for measuring two series of ESR parameters ($g_{\parallel}^1 = 2.26$, $A_{\parallel}^1 = 160$ G, $g_{\parallel}^2 = 2.21$, $A_{\parallel}^2 = 180$ G). The superhyperfine structure is not reported because the spectral overlapping does not allow us to assign the number of nitrogen ligands [1].

Conclusions

With reference to the ESR and electronic results, we can conclude that at nearly physiological pH: (a) cupric ion can form a chelate Cu(II)–$(N)_4$ type [6], with a D_{4h} distorted geometry, weakly binding one N_{imid} of the one histidine residue and three N_{pept} on the collagen helix; (b) the behaviour of the type **I** and type **IV** collagens, with respect to copper(II) coordination, is the same; (c) when cupric ion binds the imidazole of one histidine residue, it can occupy one of the required sites useful for the cross-linking formation in the mature collagen [7]; (d) even if collagen is not a metallo-protein, several interactions with metal ions have been explored [7, 8] and the nature of the copper–collagen bonds could be correlated to that of silver ions. It is well known that fibrous proteins lacking histidine, e.g., elastin or silk fibroin, do not fix any silver ions [7].

We can still point out that, unlike globular proteins, amino-acid side chains, being on the outside of the collagen molecule, are less involved in molecular stability than in intermolecular interactions. Probably the greatest difference of collagen from other proteins [9, 10] lies in the fibrous, compact conformation and in the nature of the cross-links [5].

Acknowledgements

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