Comparative Studies of Cu(II) Binding Sites in Collagen, CH_3O-Collagen, and DNP-Collagen

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Abstract

Cu(II) coordination to collagen, CH_3O-collagen and DNP-collagen was analysed by ESR and electronic techniques in the pH interval 3–12. The ESR spectra of the powder samples were shown to have axial symmetry and tetragonal geometry over the whole pH range. A superhyperfine structure is evident from pH 4 in Cu(II)–collagen and Cu(II)–CH_3O-collagen, and from pH 6 in Cu(II)–DNP-collagen.

The absorption band at 16.7×10^3 cm^{-1} for Cu(II)–CH_3O-collagen indicates Cu(II) tetragonal coordination. The protection of carboxylate and free amino groups permits the identification of the order of collagen ligand groups in copper coordination: $-COO^-$, $-NH_2$, imidazole N, peptide nitrogens.

Finally one mol of collagen fixes a number of copper ions equivalent to the histidine residues.

Introduction

In the framework of our studies [1, 2] on the role of metal ions in biological systems, we have examined the characterization of metal collagen interactions depending on the nature of the binding groups and of their interaction strength with metal. Collagen, thought not to be a metallo-protein, interacts with several metal ions [3]. Its interaction with Cr(III) has been investigated and the carboxylate groups are the most important collagen groups reacting with chromium. A clear stoichiometric relationship between chromium fixation and collagen functional groups does not exist. On the other hand, ten thousand grams of collagen fix only 66.5 mg of silver ions, equivalent to the histidine residues.

In this context, we have investigated, by ESR and electronic spectroscopies, copper-collagen interactions with the special purpose of analysing the different, pH dependent, potential binding sites in the protein. Our perspective would be, first, to correlate collagen coordination properties with the analysis of the behaviour of collagen fibrous protein and of some important globular metallo-proteins (insulin, albumin); second, to study the mechanism inducing modifications in the connective tissue (collagen), in arthritic diseases.

Experimental

Materials

Soluble skin rat collagen was obtained by the Gallop method [4]. DNP-collagen and CH_3O-collagen were obtained, respectively, by dinitrophenylation of free NH_2 groups and methyl esterification of carboxylate groups of soluble skin rat collagen according to published procedures [5].

Thus in DNP-collagen and CH_3O-collagen, free NH_2 and carboxylate groups are respectively blocked for Cu(II) coordination. All other reagents were analytical grade from Merk.

Preparation of Cu(II)–collagen, Cu(II)–CH_3O-collagen and Cu(II)–DNP-collagen Samples

Samples made of 3 ml of aqueous solution of collagen, CH_3O-collagen and DNP-collagen (1.6×10^{-5} *M*) were added to 15 μl of $CuCl_2 \cdot 2H_2O$ solution (0.1 *M*).

The sample solutions were adjusted to different pH values in the interval 3–12, by adding microliter quantities of NaOH (1 N) and HCl (1 N). The Cu(II)–collagen and Cu(II)–CH_3O-collagen mixtures were colourless colloidal solutions at pH < 6 and > 11, and were blue-violet gels at intermediate pH. The Cu(II)–DNP-collagen mixtures were yellow solutions over the whole pH range. The samples were shaken and allowed to stand at room temperature for at least 10 minutes. The gels were centrifuged and dessicated under vacuum. The solutions and the colloidal solutions were lyophilized. The copper con-

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**Abbreviations: CH_3O-collagen, collagen with metilated carboxylate groups; DNP-collagen, collagen with nitrated free NH_2 groups.

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centration in the Cu(II)–collagen gels was determined from atomic absorption analysis. ESR spectral patterns of gels indicate that these contain collagen complexed Cu(II), free Cu(II) being present only in the solutions.

ESR Measurements

ESR spectra were recorded at 77 K with a Varian E 109 X band spectrometer. The standard DPPH was introduced in the reference cavity.

Diffuse Reflectance Measurements

Diffuse reflectance spectra were recorded at room temperature with a Varian 2390 spectrophotometer.

Results and Discussion

ESR Spectra of Cu(II)–collagen, Cu(II)–CH_3O-collagen and Cu(II)–DNP-collagen

In the whole pH range ESR spectra of these Cu(II) complexes are characterized by the spectral feature $g_\parallel > g_\perp > 2.0$, with hyperfine structure in the $g_\parallel$ region. The ground state of these complexes is $(d_{x^2-y^2})$, hence the ESR patterns are analogous to those of complexes with tetragonal geometry. ESR parameters are collected in Table I. ESR spectra at pH 4.2, 6.5 and 12.0 are reported in Fig. 1 and Fig. 2. The superhyperfine line in the $g_\perp$ region is evident for Cu(II)–collagen (Fig. 2A) and Cu(II)–CH_3O-collagen starting from pH 4, but only from pH 6 for Cu(II)–DNP-collagen (Fig. 2B″). This is indicative of a covalent bond between the cupric ion and nitrogen atoms. Further, since Cu(II) coordination to DNP-collagen nitrogen binding sites (with blocked N(amino) groups) only occurs at pH ≃ 6, it is likely that Cu(II) in collagen binds first N(amino) and subsequently N(imidazole).

ESR Spectra at Low pH

At pH < 4 one Cu^{2+} signal with $g_\parallel$ 2.36–2.32 and $A_\parallel$ 120–130 Gauss is identical to that obtained from $[Cu(H_2O)_6]^{2+}$. No superhyperfine lines are present in the $g_\perp$ region.

ESR Spectra at Intermediate pH

In Fig. (1A) the ESR spectrum of Cu(II)–collagen at pH 4.2 is shown. Two series of ESR parameters are present ($g_\parallel^1$ 2.36, $A_\parallel^1$ 140 Gauss; $g_\parallel^2$ 2.28, $A_\parallel^2$ 160 Gauss).

Furthermore three superhyperfine lines (A_N 20 Gauss) appear. Just one series of ESR parameters is present in the Cu(II)–DNP-collagen ($g_\parallel$ 2.36, $A_\parallel$ 140 Gauss) and in Cu(II)–CH_3O-collagen ($g_\parallel$ 2.30, $A_\parallel$ 160 Gauss) spectra, which agrees with $g_\parallel^1$ $A_\parallel^1$ and $g_\parallel^2$ $A_\parallel^2$ respectively.

In the last one three superhyperfine lines (A_N 20 Gauss) are evident. Therefore copper binds collagen and in particular binds nitrogen at low pH.

One can notice, at neutral pH, the similarity of the three spectral patterns (Figs. 1B′, B, B″). In particular at pH 7.2, nine lines due to nitrogen superhyperfine splitting have been detected (A_N 15 Gauss) (Fig. 2D″). It should be pointed out here that for the overlapping of the spectral patterns, the assignment of four nitrogen ligands is not possible.

TABLE I. ESR Parameters for Cu(II)–CH_3O-collagen, Cu(II)–collagen and Cu(II)–DNP-collagen samples at 77 K.

pH	Cu(II)–CH_3O-collagen		Cu(II)–collagen		Cu(II)–DNP-collagen	
	$g_\parallel$	$A_\parallel$ (Gauss)	$g_\parallel$	$A_\parallel$ (Gauss)	$g_\parallel$	$A_\parallel$ (Gauss)
3.1	2.36	120	2.34	130	2.32	120
4.2	2.30	160	2.36	140	2.36	140
			2.28	160		
5.1	[a]2.30	160	[a]2.36	140	2.34	140
			2.27	160		
6.5	[a]2.30	160	[a]2.27	160	2.28	160
	2.26	180	2.25	180	2.26	190
7.2	[a]2.30	160	[a]2.27	160	2.28	160
	2.26	180	2.25	180	2.26	190
8.0	[a]2.30	160	[a]2.26	160	[b]	[b]
	2.26	180	2.24	180		
9.5	[b]	[b]	[b]	[b]	2.24	180
					2.20	200
10.5	[a]2.26	180	2.25	180	2.19	200
	2.20	190	2.20	190		
12.0	2.17	200	2.16	200	2.19	200

[a]Gels (the other samples are lyophilized). $g_\perp$: 2.07–2.05; A_N: 15–20 Gauss. [b]The parameters are not determined because of the band widths.

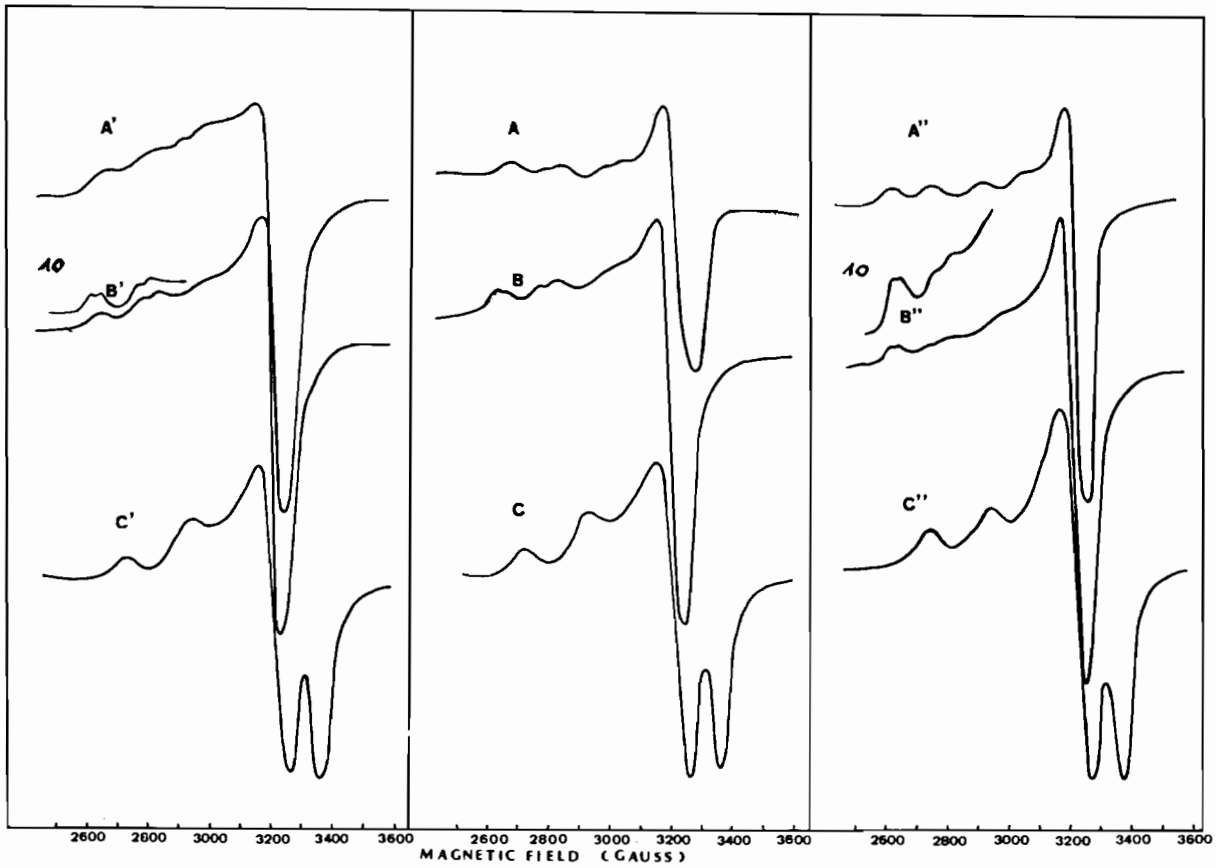

Fig. 1. ESR powder spectra for Cu(II)–CH_3O-collagen, Cu(II)–collagen and Cu(II)–DNP-collagen samples recorded at 77 K A, A′, A″) pH = 4.2; B, B′, B″) pH = 6.5; C, C′, C″) pH = 12.0.

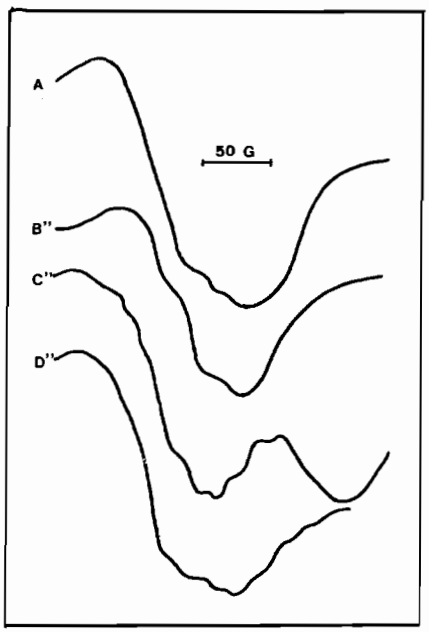

Fig. 2. Nitrogen nuclear superhyperfine structure of A, B″, C″. Nitrogen nuclear superhyperfine structure of Cu(II)–DNP-collagen sample pH 7.2, D″. Expanded magnetic field axis.

ESR Spectra at High pH

At increasing pH, $g_{\parallel}$ decreases and $A_{\parallel}$ increases. Up to pH 11 a mixture of two copper-complexes forms and nine superhyperfine lines are present (Table I). At pH 12 the resonance becomes sharper ($g_{\parallel}$ 2.17, $A_{\parallel}$ 200 Gauss) (Fig. 1C′, C, C″) and the detection of nine nitrogen superhyperfine lines becomes unequivocal (A_N 15 Gauss). This indicates a strong covalent bond between the cupric ion and four nitrogen atoms. The ESR spectral patterns and parameters are similar for the three Cu(II)–complexes series and resemble those given by biuret-type complexes [6]. pH 11 is sufficiently high to ensure the maximum involvement of peptide-bond nitrogens [7] and the coordination of copper to four peptide nitrogens is plausible.

Diffuse Reflectance Spectra of Cu(II)–CH_3O-collagen

The spectral data are collected in Table II. The reported bands are all assignable to transitions derived from the octahedral $^2E_g \rightarrow {}^2T_{2g}$. Figure 3 shows an absorption maximum at 12.5×10^3 cm^{-1} in the pH range 3–5. This signal, attributed to $[Cu(H_2O)_6]^{2+}$, is dominant. Only at nearly neutral pH does a second absorption maximum 16.7×10^3 cm^{-1} appear,

TABLE II. Reflectance Absorption for Cu(II)–CH_3O-collagen lyophilized samples.

pH	4.2	5.1	7.2	9.5	12.0
$\bar{\nu}$ ($cm^{-1} \times 10^3$)	12.5	12.5	12.5	12.5	12.5(sh)
			16.7	17.2	22.2

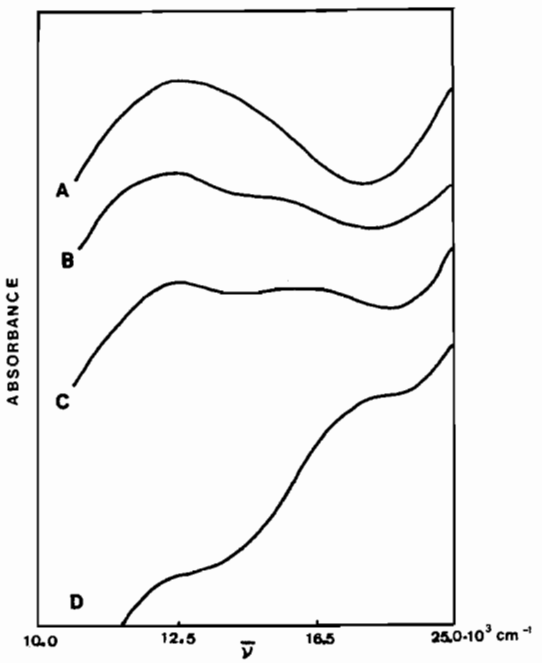

Fig. 3. Reflectance absorption spectra for Cu(II)–CH_3O-collagen lyophilized samples recorded at room temperature. A) pH = 4.2, B) pH = 7.2, C) pH = 9.5, D) pH = 12.0.

characteristic of tetragonal Cu(II) compounds. Since a large copper exaquo absorption masks the spectrum, only at about pH 7 is copper coordination to nitrogen evident. The band shift, at pH 12, to 22.2 $\times 10^3$ cm^{-1} can be attributed to the ligand field variations, as the number of strong field electron donors to Cu(II) increases. This is well known for Cu(II) small peptides [8] and for Cu(II)–protein [9] complexes. In this context, diffuse reflectance data for Cu(II)–CH_3O-collagen samples confirm ESR results and in particular the likely, at high pH, Cu(II) coordination to four peptide nitrogens in collagen.

Conclusions

The analysis of ESR and electronic data concerning collagen, CH_3O-collagen and DNP-collagen copper derivatives indicates that the affinity of Cu(II) for different potential collagen coordination groups is pH dependent. Copper coordinates collagen favoured ligands in the order: $-COO^-$, $-NH_2$, imidazole nitrogen, peptide nitrogens. At physiological pH three or four nitrogen (NH_2, imidazole and peptide nitrogens) are involved in copper coordination. Copper concentration from atomic absorption shows that one copper ion is present in the complexes for each histidine residue. This agrees with the number of silver ions coordinated to collagen [3]. Since skin rat collagen contains fifteen histidine residues, even at physiological pH there is no unique binding site. Such behaviour is different from that of insulin [10] and Human albumin [9] which possess specific Cu(II) binding sites and is similar to that of polypeptide simple models [11]. This can be interpreted in terms of collagen structure, namely triple helix structure, with almost regular aminoacid sequences.

As for arthritic diseases the antiinflammatory action of copper and of some of its compounds has been proved [12]. As copper is thought to be absorbed through the skin, the collagen involvement could be highly suggestive.

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