ESR and Optical Studies on the Interaction between Cu(II) and Pepsin

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The interaction of Cu(II) with the protein pepsin has been investigated by means of electron spin resonance (ESR) and optical spectroscopy. Depending on the molar ratio of Cu(II) and pepsin in aqueous solution two different complexes are formed. A third complex can be detected after a reaction time of several days, attributed to a complex with a conformationally changed pepsin. The presence of inhibitors 1,2-epoxy-3-phenoxypropane (EPP) or diazoacetyl-ethylester (DAE) seems to hinder the conformational change. The structure of the three complexes is discussed.

Introduction

It is well established that the proteolytic enzyme pepsin undergoes total inhibition by epoxy and diazo compounds [1–6]. The epoxy inhibitors modify chemically Asp-32 whereas the diazo inhibitors modify Asp-215. Both of these aspartic acids are in the active center of the enzyme and are important for its enzymatic activity. Measurements from different laboratories have shown that the inhibition of pepsin by diazo substances is increased considerably if Cu(II) ions are present in the reagent mixture [4, 5, 7–10].

Lundblad and Stein [9] and Stein [10] have proposed the formation of an intermediate reactive Cu(II)-carbene complex during the inhibition process. Since the cupric ion is paramagnetic, it is possible to study the interaction between the components of the system pepsin/Cu(II) as well as of the ternary system pepsin/inhibitor/Cu(II) by means of electron spin resonance (ESR) technique.

In this communication we report the findings of these studies which suggest that pepsin coordinates to Cu(II) via two oxygen and two nitrogen atoms. The geometry of the complex is approximately square planar with the ligand atoms located at the corners of the square. A conformational change of the protein determines the complex formation which is influenced, moreover, by temperature and inhibitor.

Material and Methods

Hog pepsin (EC 3.4.4.1), 2-fold crystallized, was a commercial product purchased from Serva (Heidelberg, Germany) and was used without further purification. $Cu(NO_3)_2 \cdot 3\,H_2O$ was obtained from Merck (Darmstadt, Germany) whereas the pepsin inhibitors 1,2-epoxy-3-phenoxypropane (EPP) and diazoacetylethylester (DAE) were purchased from either Riedel-DeHaën (Hannover, Germany), or Roth (Karlsruhe, Germany).

The solutions were prepared in double destilled water. All binary systems were mixed for 2 h at 37 °C before performing the measurements. The ternary systems were prepared by adding a Cu(II) solution to a pepsin-inhibitor solution.

The ESR spectra of the Cu(II)-pepsin complexes were recorded with an X-band Varian E-9 spectrometer using 100 kC field modulation. The microwave power was 10 mW for all samples investigated and the modulation amplitude 2 G if not indicated otherwise. The optical measurements were done with a Zeiss DMR 10 spectrophotometer. The pH value of the solutions was measured with a Knick precision pH-meter with an Ag/AgCl electrode. The time-dependent studies were performed on samples stored at 22 °C (room temperature, RT).

Results

ESR spectra of Cu(II)-pepsin complexes. An aqueous 1 mM Cu(II) solution has been mixed with different pepsin concentrations of up to 800 μM. The ESR spectra obtained at RT (Fig. 1, A–C) and 77 K (Fig. 2, A–C) have been recorded.

As can be seen, the solutions containing 80 μM and 160 μM pepsin, resp., exhibit, at room temperature, ESR spectra with no well resolved Cu(II) hyperfine (hf)-structure (Fig. 1, A, B). At concentrations above 320 μM of pepsin (s. Fig. 1 C) the

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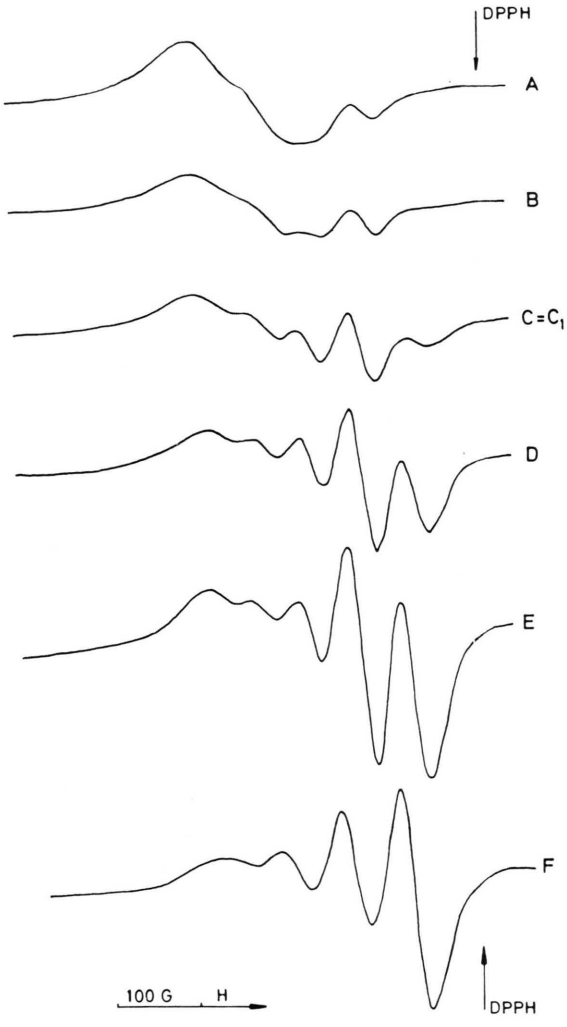

Fig. 1. ESR spectra of aqueous solutions of Cu(II), 1 mM, recorded at RT in the presence of different pepsin concentrations A, B, C, C_1 and various storage times D, E, F. A: 80 μM pepsin; B: 160 μM pepsin; C: 320 μM pepsin, C_1: 800 μM pesin; samples measured after 2 h storage at 37 °C. D, E, and F: 800 μM pepsin solutions measured after 4, 8, and 12 days storage at RT.

hf-structure as well as an additional line in the high field region of the spectrum can be observed.

Some more detailed information can be obtained by the ESR spectra recorded at 77 K. The low field side of the Cu(II) signal of the solution containing 320 μM of the enzyme seems to exist of two quartets belonging to two different Cu(II)-pepsin complexes (Fig. 2 C). The magnetic parameters $|A_{\parallel}|$ and $g_{\parallel}$ of both of the complexes are reported in Table I. An increase in the enzyme concentration results in the domination of one complex (Fig. 2 C_1).

Furthermore, a prolonged storage time will result in the final Cu(II)-pepsin complex with the hf structure shown in Figs 1 F and 2 F.

The optical absorption spectra of the Cu(II)-pepsin solutions are shown in Fig. 3. A shift of the maximum of the Cu(II) d-d absorption band towards shorter wavelengths with increasing enzyme concentrations can be seen in the insert of Fig. 3. Those solutions had been mixed for two hours only. A prolonged storing over 8 days results in a new absorption band at about 520 nm which, again, might resemble the final complex.

ESR spectra of Cu(II)/inhibitor/pepsin complexes. ESR spectra of the ternary system pepsin/inhibitor/Cu(II) with a molar ratio of 1 : 20 : 1, at 77 K, are shown in Fig. 4.

Fig. 4 A shows the ESR spectrum of the ternary system pepsin/EPP/Cu(II). As can be seen, the spectrum is composed of the superposition of two Cu(II) complexes with the magnetic parameters given in Table II. The spectrum does not change either with time or by an increase of Cu(II) up to a 10-fold molar excess. If, however, diazoacetylethylester (DAE) is used as an inhibitor, the ESR spectrum changes with time (Fig. 4 B, C, and D). The ESR spectrum of such a solution which was

Table I. ESR parameters and maxima of the optical d-d transitions of Cu(II)-pepsin complexes as function of the enzyme concentration as well as of the storing time. The samples were mixed for 2 h, at 37 °C, before performing the measurements and stored at room temperature, in a few cases, for 4 to 12 days. Measuring temperature 77 K.

Pepsin + Cu(II) (X μm)	(1 mM)	$\lvert A_{\parallel}^1 \rvert$ ±2 Gauss	$\lvert A_{\parallel}^2 \rvert$ ±2 Gauss	$\lvert A_{\parallel}^3 \rvert$ ±2 Gauss	$g_{\parallel}^1$ ±0.002	$g_{\parallel}^2$ ±0.002	$g_{\parallel}^3$ ±0.002	λ [nm]
X	time							
320	2 h	132	157		2.350	2.298		720±3
320	8 days		152	172		2.295	2.247	654±3 and 524±3
800 *	2 h		157			2.297		700±3
800	8 days		156	172		2.295	2.251	~650 (s) and 520±3
800	12 days			172			2.247	(○)

* Magnetic parameters of the predominant Cu(II)-pepsin complex. (s), Shoulder.
(○), Not recorded because of turbidity of solution.

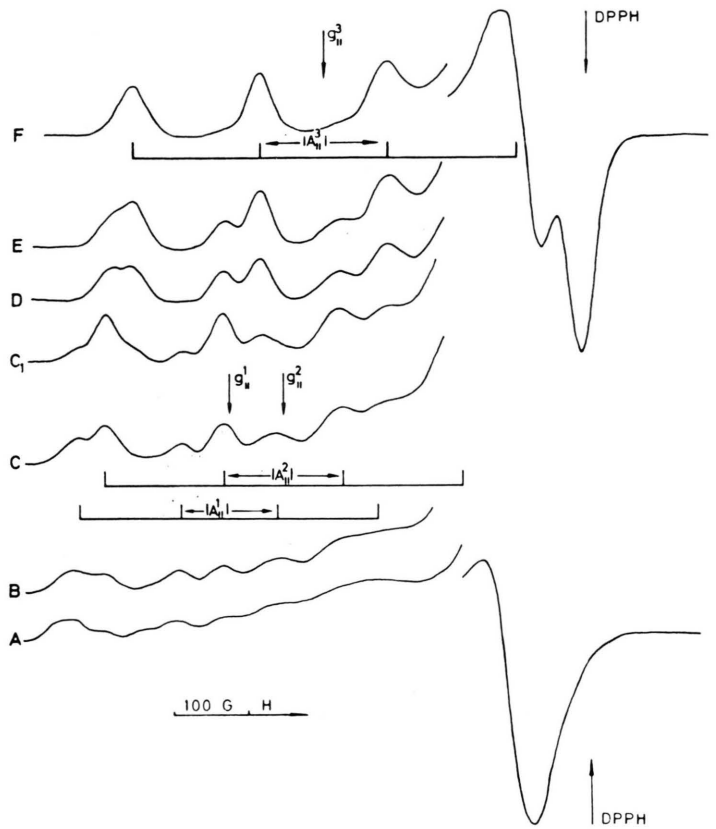

Fig. 2. ESR spectra of aqueous solutions of Cu(II), 1 mM, 77 K, in the presence of different pepsin concentrations A, B, C, C_1 and various storage times D, E, F (for explanation see Fig. 1) (amplification at the low field region x 2.5, modulation amplitude 4 G).

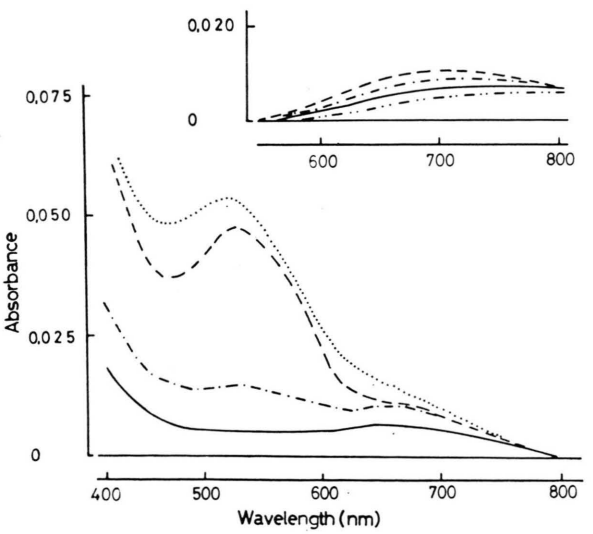

Fig. 3. Optical absorption spectra of aqueous solutions of Cu(II), 1 mM, in the presence of different pepsin concentrations and after 8 days storage at RT: (——) 160 μM; (— · — · —) 320 μM pepsin; (— — —) 640 μM pepsin; (· · · · · ·) 800 μM pepsin. Insert: 2 h after storage at 37 °C. (— · · — · · —) 80 μM pepsin; (——) 160 μM pepsin; (— · — · —) 320 μM pepsin; (— — —) 640 μM and 800 μM pepsin.

stored 12 days at RT and measured at 77 K exhibits the hf structure of one Cu(II)-complex only (s. Fig. 4 D). The magnetic parameters and the absorption maxima are given in Table II.

It should be pointed out that a spectrum with the magnetic parameters $|A_{\parallel}| = 150$ G and $g_{\parallel} = 2.320$ will be obtained when Cu(II) and DAE are mixed first. Addition of pepsin results in a spectrum shown in Fig. 4 B.

Addition of Cu(II) to a pepsin solution containing both inhibitors EPP and DAE, results in an ESR spectrum very similar to that one shown in Fig. 4 A. Again, no change with time can be observed.

Discussion

As can be seen from the ESR spectra in Figs 1 and 2 different complexes are formed in aqueous Cu(II) solutions containing different pepsin concentration. Considering the ESR theory on copper complexes [11 – 13], suggestions on the nature of the ligands and on the symmetry of the complexes can be made.

Two kinds of complexes are present for all pepsin concentrations. The first one (complex I) has a splitting parameter $|A_{||}^1| = 132$ G and a g-value $g_{||}^1 = 2.350$, while the second one (complex II), which is predominant at higher pepsin concentrations, has $|A_{||}^2| = 157$ G and $g_{||}^2 = 2.298$ (s. Table I).

Cu(II)-complexes which have oxygen as well as nitrogen atoms as ligands exhibit, usually, larger splitting parameters $|A_{||}|$ and smaller $g_{||}$-values than has been observed for complex II. Therefore, it is suggested, that in either complex I and II one or two deprotonated carboxyl groups of pepsin contribute to the complex formation. Such a complex should be nearly square planar since $g_{||} > g_{\perp} > 2$ was measured for all spectra.

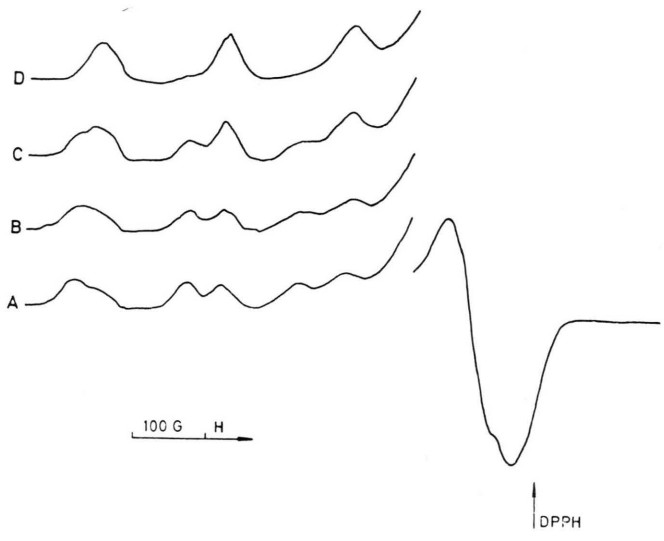

Fig. 4. Time-dependent ESR spectra (77 K) of the ternary system pepsin/inhibitor/Cu(II) with a molar ratio of 1:20:1. Cu(II) concentration was kept constant at 0.5 mM. A: obtained with EPP as an inhibitor; spectrum recorded after 2 h storage at 37 °C; a similar spectrum was also obtained after 12 days storage at RT. B, C, and D: obtained with DAE as an inhibitor; B: spectrum obtained after 2 h storage at 37 °C; C and D: spectra recorded after 6 and 12 days storage at RT. The quaternary system pepsin/EPP/DAE/Cu(II) exhibits a similar spectrum to A when stored either for 2 h at 37 °C or for 12 days at RT after mixing (amplification at the low field region x 2.5, modulation amplitude 4 G).

If Cu(II)-pepsin solutions are stored at room temperature for several days (s. Fig. 1 D, E, F, and Table I), a third type of complex (complex III) between Cu(II) and pepsin is formed with $|A_{||}^3| = 172$ G and $g_{||}^3 = 2.245$. The splitting parameter and the g-values of this complex are about the same as for Cu(II) complexes formed with amino acids [14, 15]. Therefore, two oxygen and nitrogen atoms each might be the ligands of the Cu(II) ion.

The complex III is formed probably by a change of the conformation of pepsin. The d-d absorption maximum of this complex (s. Fig. 3) is relatively strong ($\varepsilon = 210\ \mathrm{M^{-1}\,cm^{-1}}$) and shifted to a shorter wavelength (520 nm). A d-d transition at about this wavelength was already observed for peptide complexes formed with Cu(II) [16, 17]. It has been attributed to a complex in which, at least, one axial position of the metal ion is occupied by a ligand atom which belongs to the peptide.

According to this it is suggested in the case of pepsin, that due to the change in the conformation also axial ligand positions of Cu(II) are occupied by atoms of the pepsin molecule.

It should be mentioned, that the complex described cannot be formed with a denatured pepsin molecule. At pH 9.5, when pepsin is denatured, a completely different complex is formed (not shown here).

The presence of an inhibitor of pepsin causes a certain difference in the complexation behaviour of

Table II. ESR parameters and maxima of the optical d-d transitions of pepsin/Cu(II)/inhibitor(s) systems as function of the storing time. Pepsin, 0.5 mM; Cu(II) 0.5 mM; EPP [a] and DAE [b], 5 mM each. The samples were mixed for 2 h at 37 °C and stored thereafter at RT. Measuring temperature 77 K.

Reactants	Time	$A_{\parallel}^2$ ±2 Gauss	$A_{\parallel}^3$ ±2 Gauss	$g_{\parallel}^2$ ±0.002	$g_{\parallel}^3$ ±0.002	λ [nm]
Pepsin+EPP+Cu(II)	2 h *	157	173	2.291	2.245	625±3
Pepsin+DAE+Cu(II)	2 h	157	172	2.291	2.245	650±3
Pepsin+DAE+Cu(II)	6 days	157	174	2.290	2.245	650±3
Pepsin+DAE+Cu(II)	12 days		174		2.245	640±3
Pepsin+EPP+DAE+Cu^{++}	2 h *	157	172	2.291	2.246	620±3

[a] EPP, 1,2-epoxy-phenoxypropane. [b] DAE, diazoacetyl-ethylester.
* Similar data were obtained for solutions stored 12 days at room temperature.

pepsin with Cu(II). Addition of Cu(II) to a pepsin-DAE solution leads to the formation of two complexes (Fig. 4 B) with nearly identical magnetic parameters as have been obtained for the complexes II and III (Table II). This means that this inhibitor does not influence the complex formed between Cu(II) and pepsin. Again, complex III is the predominant one after several days (Fig. 4 D). A difference, however, is found for the absorption maximum of the d-d transition, which is located at about 640 – 650 nm. The second absorption maximum at about 520 nm, that was found, when complex II and III are in the solution, is no longer detectable.

According to the explanation given above and to the results obtained with peptides [16, 17], it is, therefore, assumed, that also in this ternary system a conformational change of pepsin occurs. The axial position of the Cu(II)-ion, however, is not occupied by a ligand belonging to pepsin.

The same results were obtained when Cu(II) reacted first with either pepsin or DAE and the corresponding third component was added thereafter. The complex between DAE and Cu(II), which can be detected in the ESR-spectrum ($|A_{||}| = 150$ G, $g_{||} = 2.320$, spectrum not shown here) disappears after the addition of pepsin.

The second inhibitor used was EPP, which causes an esterification of Asp-32 of pepsin. Again, addition of Cu(II) to a pepsin-EPP solution results in the formation of complexes II and III (Fig. 4 A, Table II). A change of the ESR spectrum with time however, cannot be observed. The esterification of Asp-32 seems to hinder the conformational change of the enzyme.

The concomitant presence of both inhibitors, DAE and EPP, in a solution containing pepsin and Cu(II), leads to the same results as was obtained with EPP alone. The difference in the absorption maxima of 25 nm between the two complexes pepsin/EPP/Cu(II) and pepsin/DAE/Cu(II) (Table II) may be due to a perturbation of the planar symmetry by different degrees of covalency of the ligand atoms.

In summary the following mechanism for the interaction between Cu(II) and pepsin in the presence of certain inhibitors might be suggested. Cu(II) forms a complex with DAE, which is no longer stable in the presence of pepsin. Thus, the faster inhibition of the enzyme activity caused by the inhibitor DAE does not seem to be due to a very active Cu(II)/DAE complex, as it was suggested by Lundblad and Stein. The faster inhibition of the enzyme activity, if Cu(II) is present together with an inhibitor, may be due to the fact, that the Cu(II)/DAE complex can reach the position of DAE attack in a shorter time. This is followed by an exchange of the DAE ligands of Cu(II) by pepsin ligands and then by the reaction of DAE with pepsin.

Pepsin exhibits a conformational change after forming a complex with Cu(II). Esterification of Asp-215 of pepsin by DAE also allows a conformational change, however, to a lesser extent. Such a conformational change is hindered by the esterification of Asp-32 by EPP. Since the ESR spectra of all the Cu(II)/pepsin complexes studied are almost identical, it might be concluded that Cu(II) does not coordinate with pepsin at the active center of the enzyme.

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