Spectroscopic Characterization of Copper(II) Thermolysin

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The compound obtained from zinc-free thermolysin and copper(II) sulphate, which is biologically inactive, has been characterized together with some inhibitor derivatives of the native enzyme through electronic, e.s.r., and n.m.r. spectroscopies. The co-ordination at the metal is suggested to be pseudo-tetrahedral. A comparison with the similar copper(II)-substituted metalloenzyme carboxypeptidase is presented.

THE metalloenzyme thermolysin is a protease produced by *Bacillus thermoproteolyticus* Rokko having a molecular weight of 34 600 with one zinc and four calcium ions.[1] The zinc is essential for the catalysis whereas the calcium stabilizes the protein from autodigestion. *X*-Ray diffraction studies at a nominal resolution of 2.3 Å have been made for the enzyme as well as for several inhibitor derivatives,[2] and on this basis detailed mechanisms for the enzymatic activity have been proposed.[3]

The zinc ion can be replaced by cobalt(II), manganese(II), and iron(II) with restoration at various degrees of enzymatic activity[4] which is also little affected by replacement of the calcium ions with lanthanoids.[5,6] Replacement of zinc by copper(II) or other bivalent ions leads to inactive products which have not been characterized.[4]

Natural copper proteins have been a challenge for co-ordination chemists: a typical example is given by the blue proteins.[7,8] Copper-substituted metalloenzymes also provide interesting examples of copper co-ordination.[9-12] The characterization of this type of derivative should clarify the differences between active and inactive metal-substituted enzymes. With this in mind, the zinc present in native thermolysin has been replaced by Cu^{II} and the resulting product characterized, as well as its derivatives with the inhibitors of the native enzyme.

EXPERIMENTAL

Inhibitors *N*-benzyloxycarbonyl-L-phenylalanine, cbz-L-phe (Sigma Chemical Co.), L-phenylalanine, L-phe (E. Merck), and L-phenylalanyl-L-phenylalaninamide, L-phephe(NH_2) (Vega Fox Co.) were used without further purification. 3-Phenylpropionyl-L-phenylalanine (ppphe) was synthesized by us following described procedures.[13] All the glassware was washed with a 0.1 mol dm^{-3} ethylenediaminetetra-acetate (edta) solution and the buffer solutions extracted with dithizone dissolved in CCl_4.

Thermolysin was obtained from Sigma as a lyophilized, three-times recrystallized, material containing 30% of calcium acetate. The enzyme was recrystallized by dissolving it (50 mg cm^{-3}) in a solution at pH 7.5 containing 0.05 mol dm^{-3} aminotris(hydroxymethyl)methane hydrochloride, 5 mol dm^{-3} NaBr, and 0.01 mol dm^{-3} $CaCl_2$, and then by dialyzing at low ionic strength with the same buffer, but in the absence of NaBr.

Apothermolysin was obtained as follows. The enzyme was dissolved in a solution containing 5 mol dm^{-3} NaBr, 0.05 mol dm^{-3} aminotris(hydroxymethyl)methane hydrochloride, and 0.01 mol dm^{-3} $CaCl_2$ at pH 7.5, and dialyzed against a solution containing 1 mol dm^{-3} NaCl, 0.05 mol dm^{-3} aminotris(hydroxymethyl)methane hydrochloride, 0.01 mol dm^{-3} $CaCl_2$, and 0.002 mol dm^{-3} 1,10-phenanthroline (pH 7.5) at *ca.* 4 °C for 3 d with five changes of the dialyzing solution. The apothermolysin obtained was dialyzed against a solution (pH 7.5) containing 0.05 mol dm^{-3} aminotris(hydroxymethyl)methane hydrochloride and 0.01 mol dm^{-3} $CaCl_2$ until complete precipitation occurred. The precipitate was then dissolved in a solution (pH 7.5) containing 5 mol dm^{-3} NaBr, 0.01 mol dm^{-3} $CaCl_2$, and 0.05 mol dm^{-3} aminotris(hydroxymethyl)methane hydrochloride. The residual activity of apothermolysin was checked with the substrate furylacryloylglycyl-L-leucinamide, by monitoring the decrease in absorption at 345 nm.[14] The activity of apothermolysin was found to be $<10\%$ of that of the reconstituted enzyme. The protein concentration was determined at 280 nm (ε 5.2×10^4 dm^3 mol^{-1} cm^{-1}).[15]

The copper derivative of thermolysin was obtained by adding $^{63}Cu[SO_4]$ solutions in less than the stoicheiometric amount (90%) to apothermolysin solutions.

Electronic spectra were recorded in the range 8 000—20 000 cm^{-1} on a Cary 17D spectrophotometer with 10-mm pathlength cells, using D_2O as solvent and apothermolysin at the same concentration in the reference cell. Proton-relaxation measurements were performed with a Varian CFT 20 spectrometer. T_1 Values were determined with the inversion recovery method. E.s.r. spectra of glassy solutions of copper thermolysin and its inhibitors were recorded on a Varian E9 spectrometer operating at *ca.* 9 GHz, at liquid-nitrogen temperature.

RESULTS

The Copper–Protein System.—To apothermolysin solutions, copper sulphate was added in less than stoicheiometric amounts: up to 1 : 0.9 protein to metal ratios the e.s.r. spectrum simply increased in intensity without any other change. The spectrum can be interpreted as pseudo-axial with $g_{\parallel} = 2.26$, $g_{\perp} = 2.06$, and $A_{\parallel} = 163 \times 10^{-4}$ cm^{-1}. Some superhyperfine structure on the perpendicular part of the spectrum is also evident (Figure 1). When the metal was present in $>90\%$ of the stoicheiometric amount weak signals appeared in the e.s.r. spectrum which indicate that at least one other binding site becomes competitive and that the metal ion is shared between two binding sites. Indeed, it was already known that the zinc(II) ion itself binds to a second specific site and behaves as an inhibitor of the native enzyme.[2] The electronic spectra show a broad absorption at 13.7×10^3 cm^{-1} ($\varepsilon \simeq 90$ dm^3 mol^{-1} cm^{-1}) (Figure 2).

From pH 6 to 9 the e.s.r. spectrum of the copper derivative is not pH-dependent; at pH >9 a new signal appears which shows a pattern of hyperfine and superhyperfine

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splitting characteristic of superoxide dismutase [16] and carbonic anhydrase * at the same pH values. The $A_{\parallel}$ value of 200×10^{-4} cm^{-1} and a new absorption in the electronic spectra at *ca.* 18.5×10^{3} cm^{-1} are typical of tetragonal structures; at these pH values peptidic nitrogens compete for binding to Cu^{II}.[17]

In order to check the presence of a water molecule or in general of a group with exchangeable protons bound to the metal, proton T_1 measurements have been performed on the solvent water.[18,19] The presence of exchangeable protons in the donor group is revealed by an enhancement in T_1^{-1} with respect to the value of an aqueous solution containing the diamagnetic native enzyme. The measured value of T_1^{-1} for a 10^{-3} mol dm^{-3} solution was 6.3 s^{-1} compared to 0.5 s^{-1} for the solution of the native enzyme. Therefore the copper(II) ion is bound to a group with exchangeable protons.

The Inhibitor Derivatives.—It is known that small peptides and L-phenylalanine are inhibitors of the native enzyme. They have also been found to bind to manganese ion in the manganese enzyme.[20] The e.s.r. spectra show that they also bind to the copper enzyme, since the parameters change. For example, all of the inhibitors except

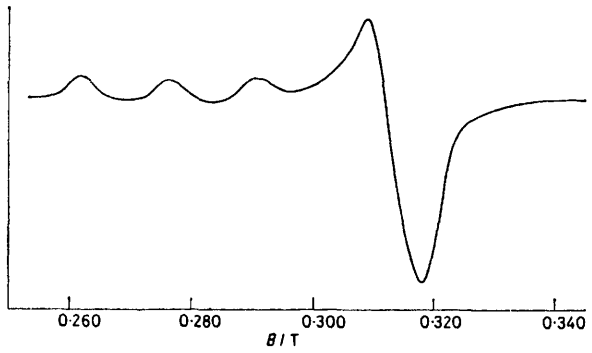

FIGURE 1 E.s.r. spectrum of a frozen solution of apothermolysin (1×10^{-3} mol dm^{-3}) and copper(II) sulphate (9×10^{-4} mol dm^{-3}) at liquid-nitrogen temperature

L-phe show similar g patterns (Table), $A_{\parallel}$ values of about the same magnitude, and the same superhyperfine structure. In contrast, L-phe modifies the superhyperfine structure so that the number of observed lines increases (Figure 3).

contrast to the manganese enzyme, all the inhibitors decrease the T_1^{-1} enhancement, although the resulting values are still far from those of the diamagnetic solution (Table).

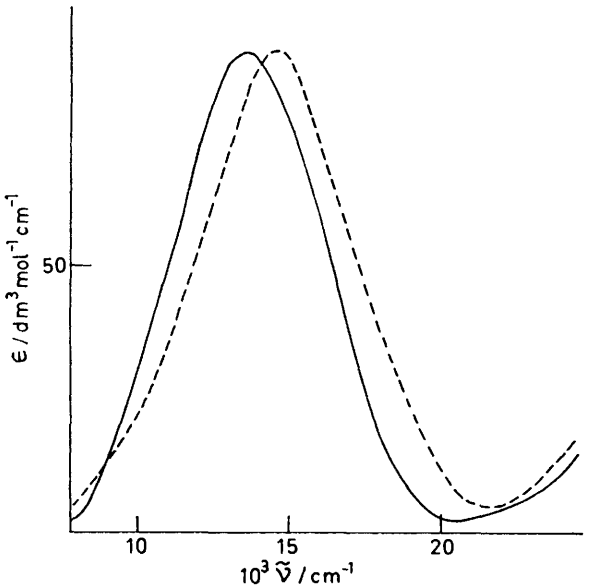

FIGURE 2 Electronic spectra of copper(II) thermolysin in D_2O (——) and of the adduct with L-phenylalanine (— — —)

DISCUSSION

The e.s.r. and electronic spectra as a function of the metal : protein ratio, and compared with those of carboxypeptidase (see below), lead to the conclusion that, at least in the range pH 6—9, a single copper–protein complex is formed. The superhyperfine structure in the perpendicular region of the e.s.r. spectrum is indicative of co-ordinated nitrogen atoms. Unfortunately, the overlapping of the superhyperfine lines and the difficulty in interpretation due to inequivalent nuclear spin-coupling constants prevent a further analysis of the spectrum; however, the five lines observed may be qualitatively

E.s.r. parameters, electronic absorption bands, and proton relaxation times of aqueous solutions of copper(II) thermolysin, copper(II) carboxypeptidase, and the inhibitor derivatives

System	$g_{\parallel}$	$g_{\perp}$	$A_{\parallel}$ [a]	T_1 [b]	$\tilde{\nu}$ [c]/10^3 cm^{-1}
Copper thermolysin	2.26	2.06	163	0.16	13.7 (*ca.* 90)
+L-phe-phe(NH_2)	2.27	2.06	159	0.35	13.7 (*ca.* 90)
+cbz-L-phe	2.30	2.05	150	0.26	13.7 (*ca.* 90)
+ppphe	2.30	2.12—2.07	145	0.26	13.7 (*ca.* 90)
+L-phe	2.25	2.03	174 ($A_{\perp N}$ 12)	0.22	14.9 (*ca.* 90)
Copper carboxypeptidase [d]	2.33	2.05	115	0.38 [e]	12.7 (120)
+3-phenylpropionate [d]	2.35	2.04	110	0.58 [e]	12.8 (125)

[a] 10^{-4} cm^{-1}. [b] For 10^{-3} mol dm^{-3} solutions. [c] Values of ε/dm^3 mol^{-1} cm^{-1} are given in parentheses. [d] Ref. 12. [e] Results from our laboratory.

The electronic spectra do not show any major change upon addition of the inhibitor, again with the exception of the L-phe derivative (Table).

The proton T_1^{-1} values of aqueous solutions containing the enzyme decrease upon addition of inhibitors. In consistent with two equivalent nitrogen atoms bound to the metal. On the other hand, the proton T_1 values indicate that a water molecule is also a reasonable candidate for a donor group. Since in the native enzyme the Zn^{II} is bound to two histidyl nitrogens, a water molecule, and a glutamate residue,[2] it can be

* Unpublished work from our laboratory.

reasonably concluded that the copper ion is bound to the protein at the same site as the zinc ion.

Similarities to Carboxypeptidase.—Carboxypeptidase is a zinc-containing enzyme very similar to thermolysin; although they have evolved independently, the active sites are very similar to each other, the donor-atom set [20] as well as the proposed enzymatic mechanism being the same.[21] In both cases the cobalt-substituted enzyme is active but the copper one is not.[22] The e.s.r. and electronic spectra are also similar for the two copper enzymes [12] (Table).

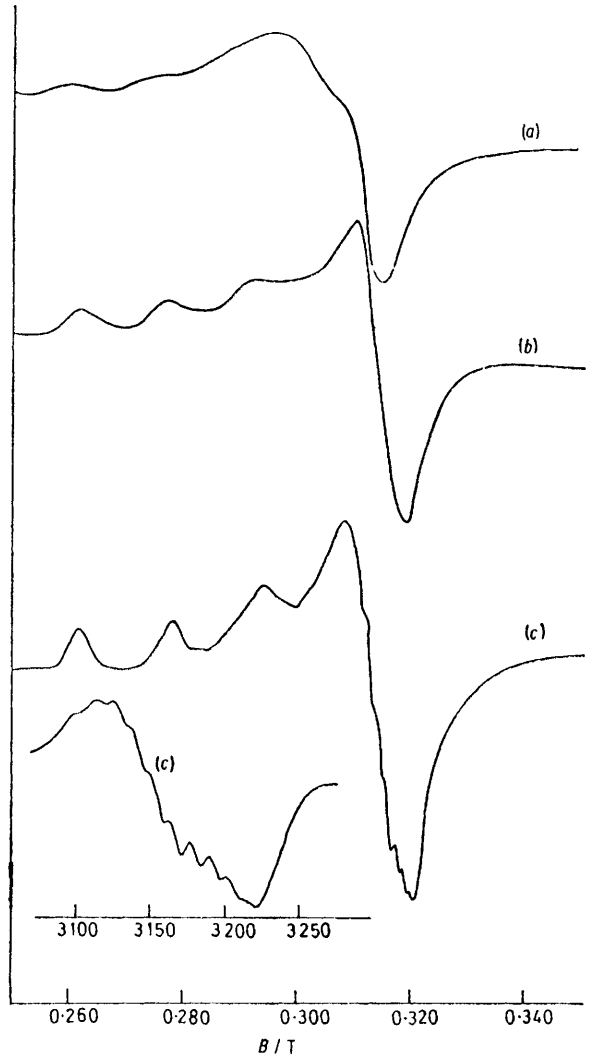

FIGURE 3 E.s.r. spectra of frozen solutions of copper(II) thermolysin at liquid-nitrogen temperature in the presence of the inhibitors (*a*) 3-phenylpropionyl-L-phenylalanine, (*b*) L-phenylalanyl-L-phenylalaninamide, and (*c*) L-phenylalanine. In the inset the normal region of the spectrum of the L-phenylalanine derivative is shown

Geometry of the Co-ordination Polyhedron.—The electronic as well as the e.s.r. spectra of copper thermolysin and its derivatives are similar to those of carboxypeptidase [12] and carbonic anhydrase; [9] however, such spectral properties are not common among inorganic copper compounds. For this reason, attention has been devoted to these spectra. An absorption between 12×10^3 and 14×10^3 cm^{-1} with ε *ca.* 90 dm^3 mol^{-1} cm^{-1} rules out the possibility of a planar or six-co-ordinate geometry, especially if nitrogen atoms are in the donor set, since in such a case absorptions would be expected at $>16 \times 10^3$ cm^{-1}.[23] Also, the e.s.r. data rule out a trigonal-bipyramidal geometry or any geometry with a d_{z^2} ground state since $g_{\parallel}$ would be smaller than $g_{\perp}$.[23] Therefore, the co-ordination polyhedra consistent with these electronic properties are a flattened tetrahedron [24] and distorted five-co-ordination.[25] The co-ordination around Cu^{II} in carboxypeptidase was proposed to be flattened tetrahedral.[12] Monoanionic inhibitors of carbonic anhydrase, such as $[O_2CMe]^-$, I^-, $[N_3]^-$, *etc.*, were found to give rise to five-co-ordinate compounds, whereas copper carbonic anhydrase itself was proposed to be flattened tetrahedral with a possible fifth donor at a distance larger than usual.[9]

Since the inhibitors of thermolysin displace water from co-ordination and give rise to a pattern in which $A_{\parallel}$ increases with the absorption maximum, it is proposed that copper thermolysin and all its derivatives are flattened tetrahedral; indeed, it has been proposed and is consistent with the basic ideas of ligand-field theory that, as the deviation from planarity decreases, $A_{\parallel}$ and the absorption maxima increase. On this basis copper thermolysin would be closer to planarity than copper carboxypeptidase.

The large proton T_1^{-1} values of the solvent water in the presence of inhibitors deserve further comment. If the co-ordinated water is removed the residual relaxation should be significantly lower as found in the case of carbonic anhydrase.[9] The *X*-ray structure of the native enzyme has shown evidence of a water molecule bound to the glu-143 residue in the cavity close to the metal.[3] This molecule is believed to be involved in the hydrolytic cleavage of the peptidic bond of substrates and, as well as the peptidic NH (and the NH_2 in the case of L-phe), can also contribute to the overall proton relaxation rate. Although the equation of Solomon, Blombergen, and Morgan has been recently criticized [26] with respect to the calculation of the number of protons bound to the metal, the difference in T_1^{-1} between the non-inhibited enzyme (6.3 s^{-1}) and the averaged value (3.8 s^{-1}) obtained for the inhibitor derivatives is consistent with two hydrogen atoms for the usual τ_c of 10^{-9} s and a metal–hydrogen distance of 280 pm.[9] The present results differ from those of the manganese thermolysin [20] in the sense that it is not possible to infer a different stereochemical behaviour of the inhibitors from their relaxation values.

Binding Mode of the Inhibitors.—The peptidic inhibitors except L-phe may bind only through the CO or NH groups. The *X*-ray data on the native enzyme have shown that all the inhibitors bind through the CO group.[3] Since the superhyperfine splitting in the e.s.r. spectra does not change upon binding of inhibitors, the CO group is again probably the binding group in the case of copper. When the inhibitor is L-phe, however,

the superhyperfine lines increase from five to seven or even more, indicating that the nitrogen is actually bound instead of CO_2^-, whereas in the case of the native enzyme the latter group is co-ordinated.[3]

Conclusions.—It seems that the major difference between copper carboxypeptidase and thermolysin with respect to the cobalt analogues or the native enzymes is the flattening of the co-ordination polyhedra. It seems also that the co-ordination number by itself is not a factor governing the enzymatic activity of these metal-substituted enzymes since nickel carboxypeptidase is six-co-ordinate and active.[27] Probably, the flattened tetrahedral geometry is most unfavourable with respect to hydrolytic processes. Furthermore, the particularly high reactivity of Cu^{II} towards hard donors is a distinctive property of these copper enzymes; it may be meaningful that L-phe binds the copper ion through the amine nitrogen and the zinc ion through a carboxyl group.

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