Copper(II) Complexes of Opiate-like Food Peptides

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The α-casein peptide fragment (90−96) Arg-Tyr-Leu-Gly-Tyr-Leu-Glu, one of the exorphins called "food hormones", is an efficient ligand for Cu^{II} ions. Potentiometric and spectroscopic studies on aqueous solutions indicate that, depending on the pH range, complex species with different coordination modes (involving the amino and the deprotonated amide groups) are formed. The phenolate side chain of the Tyr residue is not involved in the metal coordination.

Keywords: *Arg-Tyr-Leu-Gly-Tyr-Leu-Glu;* α*-casein; exorphins; copper(II) complexes*

INTRODUCTION

Like plasma, milk is necessary for the delivery of essential metals. Milk proteins constitute up to 30% of man's average dietary intake. Among them, casein, lactoferrin, and albumin exhibit the most significant metal-binding properties. Metal ions, by interacting with proteins, can influence their structure and affect the thermodynamics of their reactions. These interactions may have mutual effects on both ligands and metal ions, because (i) the digestibility and the absorbability of proteins are influenced by antagonistic or synergistic interactions with other dietary components and (ii) the binding to proteins can enhance the uptake of metal ions in the intestine.

Peptides with opioid activity have been described in partial enzymatic digests of proteins from foodstuffs and isolated from pepsin hydrolysates of wheat gluten, α-casein, and β-casein (milk proteins). Because of their exogenous origin and morphine-like activity, these peptides were named exorphins. Their discovery introduced a new criterion in evaluating the "nutritive value" of food proteins. Peptides that are "hidden" in an inactive state within the protein sequence may be released by digestive processes in vivo and may act as potential physiological modulators of metabolism during the gastrointestinal passage of the diet. They appear quite resistant to the action of gastrointestinal enzymes. Therefore, once formed in the stomach, they could resist further degradation and elicit a physiological effect. In addition, as shown by the amino acid composition and the chromatographic and solubility properties, they are highly hydrophobic and could easily cross the blood−brain barrier (Brantl et al., 1979, 1981, 1982).

For instance, β-casomorphins are peptide sequences with morphine-like properties corresponding to the 60−70th residues of bovine β-caseins (Brantl et al., 1979). Also, peptides derived from bovine α-casein exhibit opioid activity. The fragments consisting of the 90−96th and 90−95th residues could be isolated from in vitro enzymatic digest with the stomach proteinase pepsin [124 mg of peptides was obtained from 10 g of α-casein, according to Zioudrou et al. (1979)], and they may be expected to survive extensive degradation in the intestine. They correspond to the sequences Arg-Tyr-Leu-Gly-Tyr-Leu-Glu or RYLGYLE (90−96) and Arg-Tyr-Leu-Gly-Tyr-Leu or RYLGYL (90−95) of α-casein. The sequence is different with respect to either endorphins or casomorphins (Brantl et al., 1979). In particular, α-casein (90−96) is the only opioid peptide so far studied that requires an amino acid residue prior to the terminal tyrosine for an optimal opioid activity (Zioudrou et al., 1979; Loukas et al., 1983). Although the real physiological function of biologically active peptides derived from food proteins as exogenous metabolic modulators is not yet well understood (Schlimme et al., 1988), an active role in metal binding has been proposed. Actually, no indication is available to substantiate the assumption that chelation with metal ions is required for the opioid activity. However, it has been reported that some of these peptides (e.g. the nonapeptide corresponding to the 66−74 sequence of α-casein) can enhance the calcium uptake in the intestine (Schlimme et al., 1988).

Among metals, copper has been recognized as an element of basic importance for proteins involved in living processes. Copper(II) ions may act synergistically with peptides by promoting their biological activity (Sharrock et al., 1982; Pettit and Formicka-Kozlowska, 1984). Because of its reactive nature, copper(II) is expected to interact with proteins and amino acid residues of food components. Our previous studies on the Cu^{II}−α-casein (90−95) system have shown that this opiate-like peptide is an efficient chelating agent for metal ions and that the side-chain donor groups such as that of Tyr residue can be involved in metal ion coordination (Chruscinska et al., 1997).

This work was aimed at investigating the complexing ability of α-casein (90−96) toward Cu^{II}. The results indicate that the presence of the Glu residue in casein (90−96) can influence the structure of Cu^{II} complexes.

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Table 1. Stability Constants (log β) of the Proton and Cu(II) Complexes of α-Casein Fragments at T = 25 °C and I = 0.1 M (KNO_3)

species	RYLGYLE α-casein (90−96)	RYLGYL[a] α-casein (90−95)
LH_5	35.03(3)	
LH_4	31.31(2)	30.92
LH_3	26.63(2)	27.08
LH_2	19.71(1)	20.09
LH	10.33(1)	10.60
pK_a(Tyr-OH)	10.33	10.60
pK_a(Tyr-OH)	9.38	9.49
pK_a(NH_3^+) (terminal)	6.92	6.99
pK_a(COOH) (terminal)	3.72	3.84
pK_a(COOH) (Glu)	4.68	
$CuLH_3$	28.71(9)	
$CuLH_2$	24.01(2)	23.73
CuLH	19.13(1)	19.11
CuL	12.11(1)	12.60
$Cu_2L_2H_{-1}$		19.18
$CuLH_{-1}$	4.24(2)	4.56
$CuLH_{-2}$	−5.38(2)	−5.36
$CuLH_{-3}$	−16.07(2)	−16.26
pK_a($CuLH_3$)	4.70	
pK_a($CuLH_2$)	4.88	4.62
pK_a(CuLH)	7.02	6.51
pK_a(CuL)	7.87	8.04
pK_a($CuLH_{-1}$)	9.62	9.92
pK_a($CuLH_{-2}$)	10.69	10.90

[a] Chruscinska et al. (1997).

MATERIALS AND METHODS

Chemicals. RYLGYLE was obtained from Bachem. Copper(II) nitrate, used for the pH metric measurements, was a Merck product. The concentration of the standard metal ion solutions was determined complexometrically.

Potentiometric Measurements. The acidity and stability constants were calculated from pH titration data obtained with a Molspin automatic titrator on aqueous solutions at 298 K using a total volume of 2.0 mL. Alkali was added from a 0.1 mL micrometer syringe calibrated by both weight titration and titration of standard materials. The ionic strength was adjusted to 0.1 mol dm^{-3} with KNO_3. The pH values were recorded using a glass−calomel electrode (Russell TR-CMAW711) calibrated in hydrogen concentration using HNO_3 (Irving et al., 1967). The metal to ligand molar ratio was 1:1.05 and the metal ion concentration 1×10^{-3} mol dm^{-3}. The equilibrium constants were determined from three independent titrations in each system with the SUPERQUAD program (Gans et al., 1985). As usual, the stabilities of the metal complexes are reported as the logarithms of the overall formation constants β_{pqr} = $[M_pL_qH_r]/[M]^p[A]^q[H]^r$, where M stands for the metal ion, H is the proton, and L is the deprotonated form of the ligand (Table 1). The standard deviations reported were calculated by assuming error randomness.

Spectroscopic Measurements. Electronic absorption spectra were recorded with a Perkin-Elmer Lambda 11 spectrophotometer. Electron paramagnetic resonance (EPR) measurements were carried out with a Bruker ESP 300E instrument at the X-band frequency (9.3 GHz) and the temperature of 120 K; 1:2 ethanediol-water was used as a solvent. Circular dichroism (CD) spectra were obtained with a Jobin-Yvon CD-6 spectropolarimeter.

RESULTS AND DISCUSSION

Ligand. The heptapeptide investigated contains five distinct acidic groups, which deprotonate with increasing pH in the 2−11 interval. These are the C-terminal glutamic carboxyl, the glutamic side-chain carboxyl, the N-terminal L-arginine α-ammonium, and the phenolic

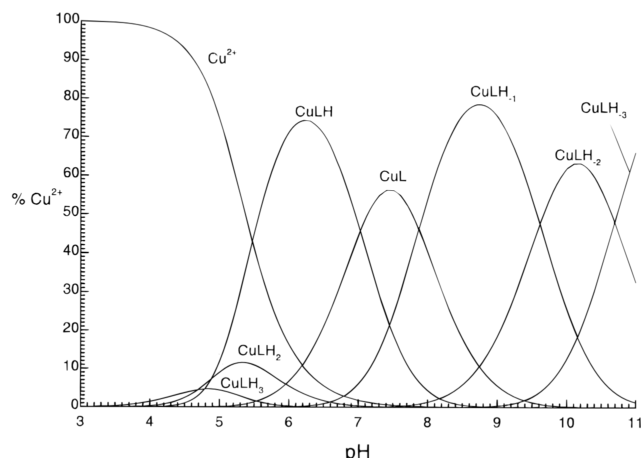

Figure 1. Species distribution curves for the Cu^{2+}−RYLGYLE system at Cu(II) concentration of 1×10^{-3} M and metal to ligand molar ratio 1:1, with varying pH.

groups of two tyrosine residues in increasing order of the pK_a values (Table 1). The values are sufficiently different from each other and can be assigned to individual groups in a fairly straightforward way on the basis of the literature data on amino acids and peptides (Kiss, 1990; Sóvágó, 1990). According to the species distribution diagram, the fully protonated form LH_5 dominates over the low pH range and completely disappears above pH 5. In the physiological pH region the species LH_3 and LH_2 are in equilibrium with each other, while L becomes a major component only above pH 10.

Titrations in the presence of copper(II) ion revealed that the ligand is able to keep 1 mol of metal ion in solution in the whole pH interval. The computed stability constants of the species taking part in the complex system are listed in Table 1.

From the species distribution diagram of the complex system (Figure 1) one can see that the first species is formed above pH 5. Both $CuLH_3$ and $CuLH_2$ are minor species with metal coordination to the terminal amino and nearby carbonyl group. These usually show up in the earlier steps of Cu^{II} complexation by peptides. They are distinguished by a set of weak EPR resonances with parameters similar to those of the analogous species formed by Gly-Gly [$g_{\parallel}$ = 2.332 and $A_{\parallel}$ = 161 × 10^{-4} cm^{-1}; see Sóvágó et al. (1996)]. The species differ from each other because of the number (one or two) of ionized carboxylate groups. However, these do not take part in metal coordination. The equilibrium constant (log K) of the protonation process

$$CuLH_2 + H^+ \rightleftharpoons CuLH_3$$

is 4.70 (vs 4.68 in the free ligand, see Table 1), indicating no significant effect of the metal ion. The major species CuLH exhibits the (NH_2, N^-, CO) donor set because of the deprotonation and coordination of the first amide group (which occurs with pK_a = 4.88) assisted by the next carbonyl oxygen. The d−d transition at 650 nm measured from the electronic absorption spectra (Figure 2) and the EPR parameters $g_{\parallel}$ = 2.249 and $A_{\parallel}$ = 179 × 10^{-4} cm^{-1} are consistent with this donor set (Table 2). In the CD spectra (Figure 3) one observes the charge-transfer band around 320 nm corresponding to amide nitrogen to Cu(II) charge-transfer transition (Pettit et al., 1985). The next species, CuL, is detected above pH

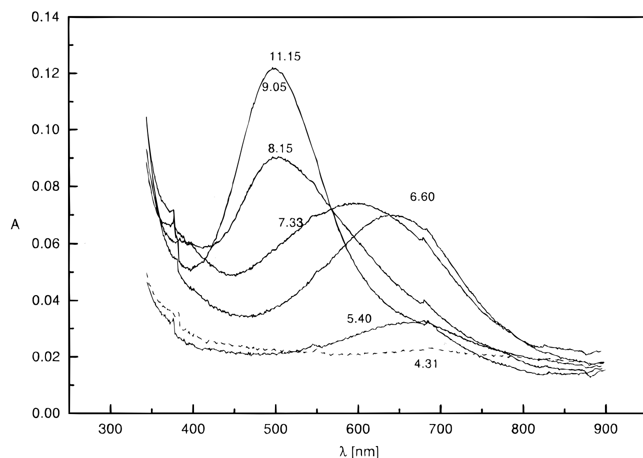

Figure 2. Electronic absorption spectra recorded on the Cu(II)−RYLGYLE system at Cu(II) concentration of 1×10^{-3} M and metal to ligand molar ratio 1:1, with varying pH.

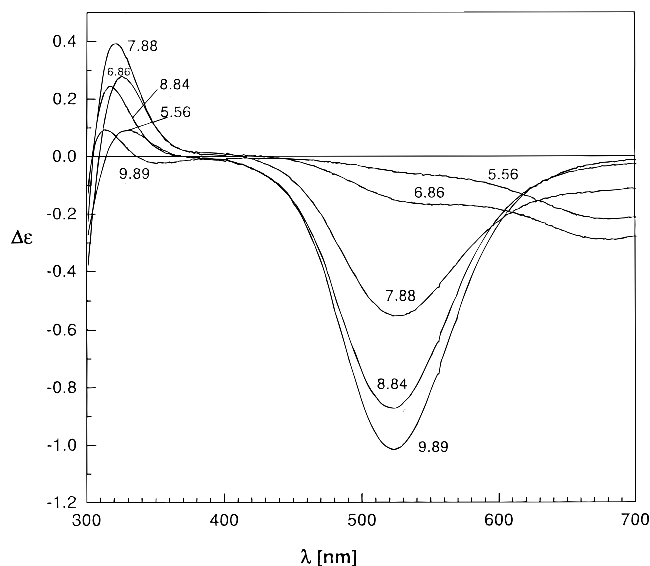

Figure 3. CD spectra recorded on the Cu(II)−RYLGYLE system at Cu(II) concentration of 3×10^{-3} M and metal to ligand molar ratio 1:1, with varying pH.

7 as a major complex. The change of EPR parameters and the ipsochromic shift of the *d*−*d* absorption maximum to ∼590 nm clearly indicate the additional amide binding and the (NH_2, N^-, N^-, CO) coordination (Kozlowski and Micera, 1995; Sóvágó et al., 1996). Above pH 8.5, a $CuLH_{-1}$ complex is observed that exhibits a 4N coordination at the metal ion. The value for the absorption maximum, λ_{max} = 500 nm, EPR, and CD parameters support this mode of binding (Table 2) (Kozlowski and Micera, 1995; Sóvágó et al., 1996). The deprotonation resulting in the species $CuLH_{-2}$ and $CuLH_{-3}$ has no effect on the structure of the complex formed, as seen from the CD and EPR measurements. The spectroscopic parameters remain almost unchanged (Table 2), suggesting the proton release from two noncoordinating Tyr residues.

The comparison of spectroscopic and equilibrium data with those of Cu^{II} complexes formed by analogous peptides reveals a very close similarity. The relevant exception is the absence of the phenolate coordination in the Arg-Tyr-Leu-Gly-Tyr-Leu-Glu copper(II) system. The coordination of the phenolate group of the 94-Tyr residue, to yield monomeric or dimeric species, was earlier observed in the copper(II) complexes of Arg-Tyr-Leu-Gly-Tyr-Leu-Gln and Arg-Tyr-Leu-Gly-Tyr-Leu (Chruscinska et al., 1997). This additional phenolate donor assisted the (NH_2, N^-, CO) chelating set in metal binding and was removed from the metal ion only after deprotonation of a further amide group. The extent of phenolate−Cu(II) binding decreases in the order Arg-Tyr-Leu-Gly-Tyr-Leu-Gln > Arg-Tyr-Leu-Gly-Tyr-Leu > Arg-Tyr-Leu-Gly-Tyr-Leu-Glu. The difference between the complex systems is observed in the absorption spectra. In fact, only the first system exhibits a significant absorption band ∼400 nm (Chruscinska et al., 1997) due to Cu^{II}−phenolate charge-transfer transition. As can be seen in Figure 2, no similar absorption is detected in solution containing Arg-Tyr-Leu-Gly-Tyr-Leu-Glu and Cu^{II}. This may indicate that the replacement of Gln by Glu in the side chain inhibits the dimer formation, probably because of the electrostatic repulsion between the negatively charged monomeric units, which is responsible for the Cu^{II}−phenolate binding.

Conclusions. RYLGYLE, the bovine casein fragment provided with opioid activity, can form stable complexes in the physiological pH region by adopting a "normal" peptide-type coordination, with fused five-membered chelate rings starting from the N-terminal α-amine up to the third deprotonated amide group. The lack of tyrosine side-chain interactions with Cu^{II} ions supports the observation that charge effects can prevent dimer formation.

The difference in opioid activity between the two 90−96 and 90−95 sequences of α-casein was attributed to

Table 2. Spectroscopic Parameters for Cu(II) Complexes of RYLGYLE

species (donor set)	UV−vis		CD		EPR	
	λ_{max}, nm	ϵ, $dm^3\ mol^{-1}\ cm^{-1}$	λ, nm	$\Delta\epsilon$, $dm^3\ mol^{-1}\ cm^{-1}$	$g_\parallel$	$A_\parallel$, $10^{-4}\ cm^{-1}$
CuLH (NH_2, N^-, CO)	650	57	670 325	(−0.30) (+0.28)	2.249	179
CuL (NH_2, $2N^-$, CO)	595	74	526 320	(−0.55) (+0.38)	2.210	201
$CuLH_{-1}$ (NH_2, N^-, N^-, N^-)	500	90	523 317	(−0.87) (+0.24)	2.169	211
$CuLH_{-2}$ (NH_2, N^-, N^-, N^-)	495	113	523 314	(−1.02) (+0.09)	2.168	215
$CuLH_{-3}$ (NH_2, N^-, N^-, N^-)	495	122			2.169	216

[a] The data are measured at the maximum extent of formation of the species; the molar extinction coefficient is referred to the total metal concentration.

the different conformational flexibility of these peptides and, in particular, to the greater conformational freedom in 90−95, as observed by NMR (Brandtl et al., 1979; Loukas et al., 1983). Hence, the complex formation could restrict the conformational freedom of both the peptides and in this way influence (probably increase) their biological activity.

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