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# Metal Complexation by the Peptide-Bound Maillard Reaction Products $N^{\epsilon}$-Fructoselysine and $N^{\epsilon}$-Carboxymethyllysine

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Although the Maillard reaction between proteins and carbohydrates is of central importance for food processing and in vivo processes, only little is known about changes of the metal-binding properties induced by protein glycation. The purpose of this study was to examine the complex formation of the quantitatively important peptide-bound Maillard reaction products (MRPs) $N^{\epsilon}$-fructoselysine and $N^{\epsilon}$-carboxymethyllysine with the biologically relevant metal ions copper(II) and zinc(II). The MRPs were synthesized as the $N^{\alpha}$-hippuryllysine derivatives in order to block the coordination function of the α-amino group. Stability constant measurements were performed in aqueous solution using pH potentiometry. $N^{\alpha}$-Hippuryl-$N^{\epsilon}$-fructoselysine forms moderate Cu(II) complexes ($Log_{10}$ $K_1 = 5.8$; $Log_{10}$ $K_2 = 4.0$) but fails to form any complexes with Zn(II). $N^{\alpha}$-Hippuryl-$N^{\epsilon}$-carboxymethyllysine gives slightly stronger complexes with Cu(II) ($Log_{10}$ $K_1 = 7.3$; $Log_{10}$ $K_2 = 6.3$), but again no complexation with Zn(II) was observed. These results show that post-translational modification of proteins by carbohydrates leads to the formation of new coordination centers for metal ions within a protein chain. Further studies are necessary to clarify the consequences of this phenomenon in terms of protein quality and physiological processes.

**KEYWORDS:** ***N*$^{\alpha}$-Hippuryl-*N*$^{\epsilon}$-fructoselysine; *N*$^{\alpha}$-hippuryl-*N*$^{\epsilon}$-carboxymethyllysine; pH potentiometry; stability constants; Maillard reaction products; zinc(II); copper(II)**

## INTRODUCTION

The Maillard reaction, also referred to as nonenzymatic browning, is a reaction between reducing sugars and amino compounds (*1*). Besides formation of flavor and color, undesirable changes in food may occur during processing and storage (*2*). Furthermore, it is known that Maillard reaction products (MRPs) occur in vivo due to endogenous formation and consumption of heated food (*3*).

Several studies examining the physiological effects of MRPs have been carried out focusing on the observation that MRPs tend to form complexes with selected metal ions (*4*, *5*). For humans, who were given heat-sterilized total parenteral nutrition, an excessive urinary loss of zinc, iron, and copper ions was observed. This phenomenon was ascribed to the potential chelating activity of MRPs formed by glucose and amino acids during the heat sterilization of the total parenteral nutrition (*4*). In an animal experiment it was shown that the feeding of rats with a MRPs-containing diet is associated with an increased urinary loss of zinc (*6*). The fact that in the presence of MRPs some metal-containing enzymes are inhibited (*7*) gives further evidence for the supposition that selected MRPs are able to bind metal ions. To date, however, no detailed information about the exact binding sites and stabilities of the formed complexes with individual MRPs are available. The aim of our study therefore was to determine the stability constants of the complexes formed between the quantitatively important MRPs $N^{\epsilon}$-fructoselysine and $N^{\epsilon}$-carboxymethyllysine with the biologically relevant metal ions copper(II) and zinc(II) using pH potentiometry. Due to the fact that free amino acids form stable chelating complexes with metal ions, the MRPs were synthesized as peptide-bound derivatives, namely, as the $N^{\alpha}$-hippuryllysine derivatives (**Figure 1**).

## MATERIALS AND METHODS

**Chemicals.** All chemicals were of the highest purity available and were used without further purification.

**RP-HPLC.** All semipreparative chromatographic separations were performed with an HPLC system from Knauer (Berlin, Germany), consisting of two pumps K-1001 with 50 mL pump heads, a UV−vis detector K-2501 set at $\lambda = 280$ nm, and a Jetstream 2 plus column oven with temperature set on 20 °C. All samples were separated by a Eurospher-100, C18 column (Knauer; 16 mm × 250 mm, particle size = 15 μm and a precolumn 16 mm × 30 mm filled with the same material).

**Elemental Analysis.** Elemental analysis data were obtained on a Euro EA 3000 elemental analyzer (Eurovector, Milano, Italy).

**Syntheses of the MRPs.** *$N^{\alpha}$-Hippuryl-$N^{\epsilon}$-fructoselysine (HipFruLys).* The synthesis and isolation of HipFruLys were performed according to our procedure (*8*) with the modification of using $N^{\alpha}$-hippuryllysine

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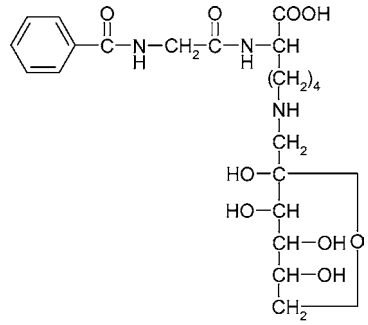
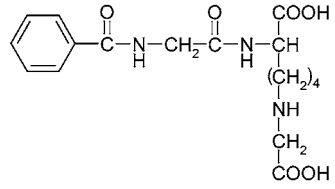
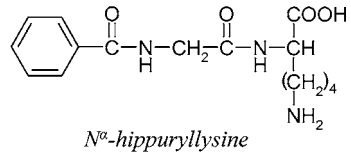


*$N^{\alpha}$-hippuryl-$N^{\varepsilon}$-fructoselysine*
*(HipFruLys)*



*$N^{\alpha}$-hippuryl-$N^{\varepsilon}$-carboxymethyllysine*
*(HipCML)*



*$N^{\alpha}$-hippuryllysine*
*(HipLys)*

**Figure 1.** Structures of the MRPs employed in the present study together with that of HipLys.

instead of $N^{\alpha}$-Boc-lysine. The isolation was carried out using an eluent consisting of 10 mM sodium phosphate (pH 7) and methanol (85:15, v/v) with an isocratic flow of 4 mL/min. Desalting was achieved with an eluent of 50 mM acetic acid and methanol (92/8 v/v) with an isocratic flow rate of 6 mL/min. NMR data corresponded to those obtained in previous studies (*8*). Elemental analysis for $C_{21}H_{31}N_3O_9 \cdot 0.72CH_3COOH \cdot 1.77H_2O$ (544.62) calcd: C, 49.49; H, 6.93; N, 7.72. Found: C, 49.46; H, 6.92; N, 7.71.

*$N^{\alpha}$-Hippuryl-$N^{\varepsilon}$-carboxymethyllysine (HipCML).* Synthesis was performed according to the procedure reported previously (*8*). HipCML was preparatively separated via RP-HPLC at a flow rate of 8 mL/min using isocratic elution with 10 mM sodium phosphate buffer (pH 7) and methanol (97:3, v/v). The fractions containing HipCML were pooled and rechromatographed as above using isocratic elution with a mixture of 50 mM aqueous acetic acid and methanol (90:10, v/v). NMR data were in accordance with those reported previously (*8*). Elemental analysis for $C_{17}H_{23}N_3O_6 \cdot 0.16CH_3COOH \cdot 1.36H_2O$ (399.50) calcd: C, 52.07; H, 6.65; N, 10.52. Found: C, 52.07; H, 6.48; N, 10.51.

**pH Potentiometric Measurements.** The potentiometric titration measurements were carried out at 25 ± 0.1 °C and constant ionic strength ($I$ = 0.15 mol/L; $KNO_3$) under a $CO_2$-free moist nitrogen atmosphere. An automatic titrator 736 GP Titrino with a combined LL-Biotrode 3 mm (Metrohm AG, Herisau, Switzerland) was used for the titration experiments. To calibrate the glass electrode, a titration of a 15 mM $HNO_3$ solution with a $CO_2$-free 0.1 M KOH solution (with 0.13 M $KNO_3$) was carried out. The computer software GLEE (*9*) was used to calculate the calibration constants and additionally the $CO_2$ content. Therefore, all obtained stability constants are concentration constants (*9*, *10*). For the conditions described, a $pK_W$ = 13.77 was determined; this value corresponds with that reported by Pearce et al. (*11*). For the titration, 9 mL of titrand was titrated with a KOH solution to pH 11.5. Thereby the titrand consists of the test ligand (0 or 0.5−3.0 mM), the metal chlorides (0 or 0.5−1.0 mM, concentration ratios of metal ion/ligand of 1:1; 1:2; 1:3, and 1:4), a defined nitric acid concentration (15 mM), and the supporting electrolyte ($KNO_3$). The concentrations of the metal chloride stock solutions and of the acid were checked by EDTA titration, respectively by titration against tris-(hydroxymethyl)aminomethane. The titration data were recorded using the computer software VESUV 3.0 (Metrohm AG), and the stability constants were calculated using HYPERQUAD2000 (Protonic Software, Leeds, U.K.) (*12*). For the determination of protonation or stability constants with metal ions at least three titrations were carried out.

**Table 1.** Stepwise Protonation Constants for Glycine and Lysine and Their Stability Constants with Cu(II) and Zn(II) at 298.1 K and $I$ = 0.15 M ($KNO_3$)[a]

| | glycine | | lysine | |
|---|---|---|---|---|
| | this work | ref *15* | this work | ref *16* |
| protonation constants | | | | |
| $Log_{10}$ $K_1${[LH]/[L]/[H]} | 9.6 (0.05) | 9.60 (0.05) | 10.75 (0.01) | 10.71 (0.08) |
| $Log_{10}$ $K_2${[$LH_2$]/[LH]/[H]} | 2.53 (0.10) | 2.37 (0.07) | 9.10 (0.01) | 9.19 (0.09) |
| $Log_{10}$ $K_3${[$LH_3$]/[$LH_2$]/[H]} | | | 2.15 (0.02) | 2.16 (0.03) |
| stability constants with Cu(II) | | | | |
| $Log_{10}$ $K_1${[CuL]/[Cu]/[L]} | 8.43 (0.05) | 8.20 (0.10) | 7.61 (0.04) | 7.61–7.65[b,c,d] |
| $Log_{10}$ $K_2${[$CuL_2$]/[CuL]/[L]} | 7.04 (0.07) | 6.87 (0.23) | 6.61 (0.08) | 6.32–6.53[b,c,d] |
| stability constants with Zn(II) | | | | |
| $Log_{10}$ $K_1${[ZnL]/[Zn]/[L]} | 5.11 (0.08) | 5.03 (0.10) | 3.98 (0.04) | 4.06[c,d,e,f] |
| $Log_{10}$ $K_2${[$ZnL_2$]/[ZnL]/[L]} | | 4.20 (0.21) | 3.42 (0.06) | 3.47[c,d,e,f] |
| $Log_{10}$ $K_3${[$ZnL_3$]/[$ZnL_2$]/[L]} | | 2.54 (0.31) | | |

[a] Charges are omitted for clarity; standard deviations are given in parentheses. Reference measuring conditions: $T$ = 298 K, $I$ = 0.1–0.2 M (different ionic species), concentration constants, different measuring methods (*b*) $I$ = 0.2 M (KCl), (*c*) unknown whether concentrations or mixed constants, (*d*) standard deviation not specified, (*e*) $I$ = 0.1 M (KCl or $KNO_3$), (*f*) pH potentiometric measurements.

The overall formation constants as well as stepwise protonation and stability constants are reported. Overall formation constants are given as $\beta_{mlh}$ for the reaction

$$m\,\mathrm{M} + l\,\mathrm{L} + h\,\mathrm{H} \rightleftharpoons \mathrm{M}_m\mathrm{L}_l\mathrm{H}_h \qquad \text{with } \beta_{mlh} = \frac{[\mathrm{M}_m\mathrm{L}_l\mathrm{H}_h]}{[\mathrm{M}]^m \cdot [\mathrm{L}]^l \cdot [\mathrm{H}]^h}$$

where M stands for the metal ion, L the deprotonated ligand, and H the proton.

The stepwise protonation constants and stepwise stability constants describe the reaction

$$\mathrm{M}_a\mathrm{L}_b\mathrm{H}_c + \mathrm{H} \rightleftharpoons \mathrm{M}_a\mathrm{L}_b\mathrm{H}_{c+1} \qquad \text{with } k_{c+1} = \frac{[\mathrm{M}_a\mathrm{L}_b\mathrm{H}_{c+1}]}{[\mathrm{M}_a\mathrm{L}_b\mathrm{H}_c] \cdot [\mathrm{H}]}$$

and

$$\mathrm{M}_a\mathrm{L}_b + \mathrm{L} \rightleftharpoons \mathrm{M}_a\mathrm{L}_{b+1} \qquad \text{with } k_{b+1} = \frac{[\mathrm{M}_a\mathrm{L}_{b+1}]}{[\mathrm{M}_a\mathrm{L}_b] \cdot [\mathrm{L}]}$$

Negative constants for H were used to describe complexes with hydroxides. The hydrolysis constant of a metal ion ($\beta_{1x0}$ with $x\mathrm{OH}^-$ as ligands), for example, results from $\beta_{1x0} = \beta_{10-x}xK_W$.

## RESULTS AND DISCUSSION

The hydrolysis constants for Cu(II) were determined as $Log_{10}$ $\beta_{10-1} = -7.79$ and $Log_{10}$ $\beta_{10-2} = -13.72$. For Zn(II) only the hydrolysis corresponding to $Log_{10}$ $\beta_{10-2} = -16.08$ could be determined. These constants agree in order of magnitude with literature data (*13*, *14*). Because of the extensive formation of the metal hydroxides, for Cu(II) and Zn(II) it was not possible to obtain constants for all hydrolysis steps (*13*, *14*); nevertheless, the determined constants appear to be sufficient to represent a reasonable working model of the system.

To compare the stepwise protonation and stability constants of the amino acids glycine and lysine with Cu(II) and Zn(II), the $Log_{10}$ $K$ values were determined under the same conditions as used for the constants obtained for the MRPs (**Table 1**).

**Table 2.** Overall Formation Constants for Protonation and Complexation of HipFruLys, HipCML, and HipLys with Cu(II) at 298.1 K and $I = 0.15$ M ($KNO_3$)[a]

| $Log_{10}\,\beta_{mlh}$ | HipFruLys | HipCML | HipLys |
|---|---|---|---|
| | Protonation Constants | | |
| $Log_{10}\,\beta_{011}$ | 8.81 (0.02) | 9.91 (0.03) | 10.62 (0.01) |
| $Log_{10}\,\beta_{012}$ | 12.00 (0.02) | 13.66 (0.05) | 13.75 (0.01) |
| $Log_{10}\,\beta_{013}$ | | 15.67 (0.05) | |
| | Stability Constants with Cu(II) | | |
| $Log_{10}\,\beta_{110}$ | | 7.34 (0.07) | |
| $Log_{10}\,\beta_{120}$ | | 13.68 (0.04) | |
| $Log_{10}\,\beta_{11-1}$ | −0.89 (0.04) | | |
| $Log_{10}\,\beta_{11-2}$ | −7.90 (0.04) | | |
| $Log_{10}\,\beta_{12-2}$ | −3.90 (0.09) | | |

[a] Charges are omitted for clarity; standard deviations are given in parentheses.

Overall, the data correspond well with the literature values; however, for the Zn(II)−glycine system only the first complexation step was observed. Obviously, in this case there is a pronounced influence due to the presence of the hydroxo complexes (*17*). Due to the synthesis procedure, certain amounts of acetic acid were associated with MRPs, which has to be taken into account. Hence, the acidity and stability constants with Cu(II) and Zn(II) in the present system are conditional. The acidity constant $pK_a = 4.55$ (0.01) corresponds with literature data [$Log_{10}\ K_1 = 4.56 \pm 0.03$, $I = 0.1$ M, $T = 298$ K (*14*)]. Reference literature data (*14*) also show that complexes of acetic acid with Cu(II) and Zn(II) are very weak and, therefore, under our conditions, no significant complex formation could be observed.

On the basis of the experimental data, the overall formation constants for HipFruLys and HipCML with Zn(II) and Cu(II) were quantified (**Table 2**).

The protonation behavior of the free ligands is similar, whereas the individual constants are clearly influenced by the presence of the adjacent functional groups.

HipFruLys forms moderately stable complexes with Cu(II) ($Log_{10}\ K_1\{[CuL]/[Cu]/[L]\} = 5.82$; $Log_{10}\ K_2\{[CuL_2]/[CuL]/[L]\} = 4.00$), whereas HipCML forms slightly more stable complexes with Cu(II) ($Log_{10}\ K_1\{[CuL]/[Cu]/[L]\} = 7.34$; $Log_{10}\ K_2\{[CuL_2]/[CuL]/[L]\} = 6.34$). With Zn(II), no complex formation with either MRP was observed. $N^{\alpha}$-Hippuryllysine (HipLys), which was the initial compound for the MRP syntheses, was also analyzed to serve as a comparison (**Table 2**). Because the $N^{\alpha}$-hippuryl group is connected to lysine by a peptide bond, this compound is a good model for peptide-bound lysine.

Comparison of the stability constants for HipLys and lysine shows that, in contrast to free lysine, peptide-bound lysine forms no complexes with Cu(II) or Zn(II), reflecting the derivatization of the α-amino group. This is accordance with the prior observation that lysine coordinates in a "glycine-like" manner (without substantial influence from the $\epsilon$-amino group) and that in the case of the blocked α-amino group the complexation ability is lost (*16*). The free α-amino group is the preferred binding site in complexes of amino acids (here: together with the carboxylic function) and also for peptides and proteins (here: together with a deprotonated peptide N)—known as the biuret reaction (*18*).

By comparing the stability constants of the glycated products with that of HipLys, it is obvious that the glycation results in the presence of new Cu(II) coordination centers, whereas Zn(II) is still not bound. As HipLys is a model for peptide-bound lysine, HipFruLys and HipCML are the corresponding derivatives of peptide-bound lysine. Thus, we conclude that on the glycation of proteins a new copper-binding center is formed. This new copper-binding center forms in the event of peptide-bound $N^{\epsilon}$-fructoselysine, respectively. $N^{\epsilon}$-Carboxymethyllysine forms 100−1000 times more stable complexes with Cu(II) compared to the well-known imidazole binding site of peptide-bound histidine [references: $N^{\alpha}$-acetylhistidine and $N^{\alpha}$-acetylglycylhistidine (*19*, *20*)].

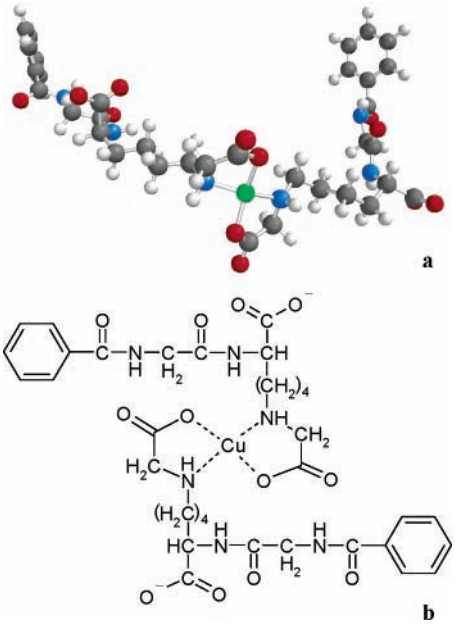



**Figure 2.** Sterical (**a**) and schematic (**b**) model of the $Cu(CML)_2$ complex.

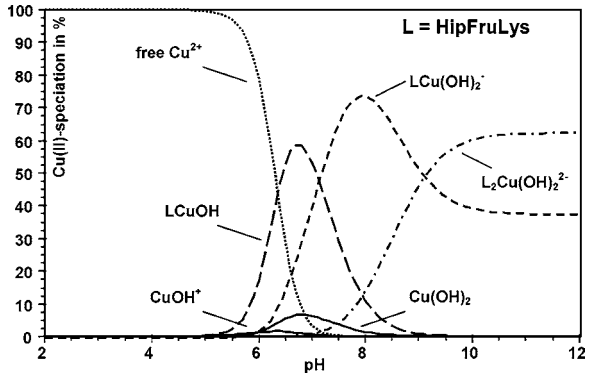



**Figure 3.** Cu(II) speciation for 1.5 mM HipFruLys at a metal ion/ligand concentration ratio of 1:3.

The coordinated Cu(II) is assumed to bind to the N-donor atom of the $\epsilon$-amino group and in the case of HipCML with one O-atom of the neighboring carboxylate group (see **Figure 2**). The nearly similar stability constants for lysine and HipCML, which both have an amino group in an α-position to the carboxylic acid function, are in agreement with this assumption. Because of the amino acid-like structure, the Cu(II) coordination geometry is expected to be distorted planar (*21*).

In the case of HipFruLys, presumably an O-donor of the sugar ring is involved. The second ligand molecule appears to be necessary to achieve a coordination sphere that is typical of Cu(II). Nevertheless, further studies are clearly necessary to define more closely the structure of these complexes.

As shown in **Figures 3** and **4**, Cu(II) is bound by the present glycated products within the physiological pH range and so, from this, physiological consequences of such binding appear likely.

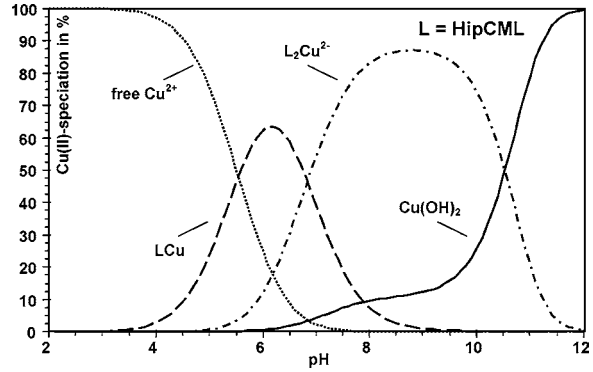



**Figure 4.** Cu(II) speciation for 1.5 mM HipCML at a metal ion/ligand concentration ratio of 1:3.

Feeding studies involving rats (*6*) showed that the uptake of free fructoselysine does not have any influence on urinary excretion of Zn(II) and Cu (II), whereas with heated casein/glucose mixtures an increased excretion was observed. We thus conclude that stability constants of $\mathrm{Log}_{10}\ K = 6$ are not high enough to influence the bioavailability of metals. The stability constants alone, of course, provide no information about the role of MRPs in vivo. Therefore, the influence on bioavailability is not clear without a knowledge of the stability constants involved in competitive physiological processes. For instance, a decrease in resorption of metals by indigestible MRPs, a decrease of plasma metal concentration by urinary-excreted MRPs, and also the inhibition of metalloenzymes are all conceivable. Also, kinetic factors may play a role and need to be considered.

Saxena et al. (*22*) showed that the Cu(II), Ca(II), and Zn(II) binding ability of proteins is related to their carboxymethyllysine content. In this context the binding of the redox-active Cu(II) by proteins can be connected with oxidative stress. For example, metal-catalyzed oxidations were found in some carboxymethyl-rich tissues (*22*).

In summary, these results show that post-translational modification of proteins by carbohydrates leads to the formation of new coordination centers for metal ions within a protein chain. Nevertheless, further studies are necessary to clarify the consequences of such metal ion binding to MRPs for protein quality and physiological processes.

## ABBREVIATIONS USED

HipCML, $N^{\alpha}$-hippuryl-$N^{\epsilon}$-carboxymethyllysine; HipFruLys, $N^{\alpha}$-hippuryl-$N^{\epsilon}$-fructoselysine; HipLys, $N^{\alpha}$-hippuryllysine; MRP(s), Maillard reaction product(s).

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