Metal-Binding Ability of Leu-Enkephalin, Related Glycoconjugates and Peptidomimetics

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Abstract: Both the chemistry and consequences of the nonenzymatic reaction between reducing sugars and reactive amino groups of amino acids, peptides and proteins (known as the Maillard reaction), have received considerable attention in food and health science fields. This initial reaction results in Amadori and similar products formation, followed by degradation to advanced glycation end products (AGEs). It is well established that AGEs are associated with color and odor of thermally processed or stored food, as well as with pathogen products in a number of diseases. The model systems of early stage Maillard reaction products (MRP) were prepared between endogenous opioid peptide leucine enkephalin (**1**) and D-glucose / D-glucuronic acid. The complexation ability of prepared MRP with metal ions (Ca^{2+}, Zn^{2+}, Al^{3+}, Pb^{2+} and Cu^{2+}) was investigated and compared to the complexation ability of parent peptide using ECD and FTIR spectroscopic measurements.

Keywords: Amadori compound, hydroxypyridinium, imidazolidinone, Maillard reaction, metal complex.

INTRODUCTION

TRANSITION metal ions are found in living organisms, coordinated to different biomolecules and participating in many biochemical reactions where they play a crucial role. Carbohydrates, although involved in many biochemical processes, immunological events, and pathological conditions, exhibit relatively poor coordinating properties and form weak complexes with metal ions, however, an increased metal-binding ability has been linked to the glycated proteins formed in Maillard reaction.[1–4] In such reversible reaction, reducing sugars react with amino acids, peptides or proteins, producing Amadori, Heyns, and other similar compounds, which can further form irreversible AGE (Advanced Glycation End) products. There is accumulating evidence for the ability of Maillard reaction products (MRP) to complex metal ions.[5] It is well known that metal ions can form complexes of various properties with MRPs, oxidize Amadori compounds as well as their derivatives, and catalyze further interactions of these compounds.[5–7] In addition, transition elements accelerate the formation of Amadori compounds, as well as their polymerization products during the final stage of the Maillard reaction.[6–14]

Nevertheless, the information about MRP complexes and whether complexations of individual MRPs are likely to influence internal metabolism *in vivo* is not currently available.

We know that the type of cation is important for the chelating ability of MRPs, as well as its role in the stability of the complexes formed, and the accumulation of chromophores in reaction systems.[5,15,16] Moreover, the browning reaction rate studied in heated solutions of Amadori compounds depends not only on the amounts of metal salts added, but also on the valence of the cations,[6,17] and pH of the solution.[18]

Since formations of metal complexes are associated with different but unknown MRP ligands, we have explored the ability of several MRPs with known structures to complex with several biologically relevant metal ions.[19] For instance, the importance of copper in human metabolism is its involvement in oxidative processes.[20–25] Aluminum was chosen due to its high presence in the environment and extensive use in modern daily life (diets and medication). However, there is no known physiological role for aluminum within the body and hence this metal may strongly alter normal cellular metabolic pathways and produce adverse physiological effects.[26] Calcium is important for

healthy bones, but the question is if it is responsible for kidney stones formation, and calcification of blood vessels. The strong and selective binding of calcium(II) ions to proteins is related to the regulation of many physiological functions.[27] Zinc is known to be essential for all highly proliferating cells in the human body, especially the immune system, but high dosage of zinc evokes the same negative effects as those observed with zinc deficiency.[28] The toxicological effects of lead include neurotoxicity, carcinogenicity, reproductive toxicity, and neurobehavioral/developmental effects.[29] Thus, investigation concerning difference in complexation ability for these metal ions of natural peptides and their MRPs is of essential importance. The ability of complexation of Zn^{2+}, Al^{3+}, Ca^{2+}, Pb^{2+} and Cu^{2+} multivalent cations with endogenous opioid peptide Leu-enkephalin (**1**)[30,31] and four different Leu-enkephalin MRPs **2–5** was investigated. Electronic circular dichroism (ECD) and Fourier transform infrared (FTIR) spectroscopy, as complex spectroscopic methods, were used to characterize the conformation and the cation-induced conformational changes of peptides **1–5**.

EXPERIMENTAL

Leu-enkephalin (**1**) was purchased from Bachem. Amadori compounds, *N*-(1-deoxy-D-fructos-1-yl)-L-tyrosylglycylglycyl-L-phenylalanyl-L-leucine (**2**) and *N*-(1-deoxy-D-fructofuranos-1-yl-uronic acid)-L-tyrosyl-glycylglycyl-L-phenylalanyl-L-leucine (**4**) were synthesized under the conditions described by Horvat *et al.* (1998)[32] and Horvat *et al.* (2007).[33] Imidazolidinone derivative, *N*-{[2-(D-*gluco*-pentitol-1-yl)-4-(4-hydroxybenzyl)-5-oxoimidazolidin-1-yl]acetyl}glycyl-L-phenylalanyl-L-leucine (**3**) was obtained as described by Roščić and Horvat (2006).[34] *N*-[2-(4-hydroxybenzyl)-2-(3-hydroxypyridin-1-ium)ethanoyl]glycylglycyl-L-phenylalanyl-L-leucine (**5**) was obtained as described by Horvat *et al.* (2007).[33] All compounds were desalted using an octadecylsilica solid-phase extraction (SPE) cartridge (500 mg, 2.8 mL). The following compounds all purchased from Sigma-Aldrich were used as metal ions source: $Al(ClO_4)_3 \times 10H_2O$, $Ca(ClO_4)_2 \times 4H_2O$, $Cu(ClO_4)_2 \times 6H_2O$, $Zn(ClO_4)_2 \times 6H_2O$ and $Pb(ClO_4)_2 \times 3H_2O$. ECD titrations were performed at peptide : metal ratios ranging from 1 : 0.25 to 1 : 5, by adequately mixing stock solutions of peptides (0.4 mM) and different metal ions (25 mM). The concentrations of peptides were between 0.2–0.4 mg mL^{-1} and concentrations of metal salts between 1–6 mg mL^{-1}. The solvents for the spectroscopic studies were 2,2,2-trifluoroethanol (TFE; Aldrich, NMR grade), and double distilled water.

ECD Measurements

Electronic circular dichroism (ECD) spectra were recorded on a Jasco J-810 spectropolarimeter at room temperature

in a quartz cell with 0.2 mm path length in the 185–280 nm (far UV) and with 10.0 mm path length in the 250–320 nm (near-UV) region. The peptide:metal ratio is expressed with r_{cat} = cation / peptide and titration was running at r_{cat} = 0.25–5. ECD band intensities are expressed in mean residue ellipcity ($[\Theta]_{MR}$; deg cm^2 dmol^{-1}).

FTIR Measurements

FTIR spectra of 2 cm^{-1} resolution were recorded on a Bruker Equinox 55 spectrometer equipped with liquid N$_2$-cooled MCT detector. The spectra were obtained at room temperature in a CaF$_2$ cell of 0.2 mm path length. The sample concentration in TFE (or a TFE solution containing 2, 8 or 12 mM Ca perchlorate) was 4 mM in each measurement. The spectra were corrected with the background.

RESULTS AND DISCUSSION

ECD Spectroscopic Characterization of Compounds and Their Metal Complexes

Investigation of the endogenous opioid peptide Leu-enkephalin under Maillard reaction conditions (nonenzymatic glycation) lead to the chemical transformations of Leu-enkephalin (**1**) (Figure 1). Two glycation products resulted from the reaction of D-glucose (Glc) and Leu-enkephalin (**1**) as early glycation products. The first product is Amadori compound **2** in which the linear peptide is alkylated at the N-terminal position by a 1-deoxy-D-fructose unit. The second product in the reaction is compound **3** which has characteristic rigid 5-membered imidazolidinone ring. In this adduct an imidazolidinone moiety connects the acyclic sugar residue with the linear peptide chain. Early glycation products in the reaction of D-glucuronic acid (GlcA) and Leu-enkephalin are Amadori compound **4** and aromatic 3-hydroxypyridine derivative **5**.

In aim to determine the complexation potential of compounds **1–5** first of all ECD spectra of these synthetic peptides were recorded (Figure 2). According to the spectrum and previous investigation it was concluded that Leu-enkephalin **1** is present in TFE as a mixture of conformers with no dominant recognizable conformation.[35,36] The ECD spectra of Amadori compounds **2** and **4** do not differ significantly from that of **1** which suggests no strong difference in distribution of backbone conformers from peptide **1**. The ECD spectrum of the imidazolidinone-derivative **3** shows a broad positive band centered at $\lambda \sim 206$ nm suggesting the influence of imidazolidinone ring on the conformation of Leu-enkephalin. The 3-hydroxypyridinium derivative **5** shows a markedly different ECD spectrum featuring with a weak positive band centered at ~ 220 nm, a negative band at 202 nm and a positive one at 191 nm due to structure of 3-hydroxypyridinium ring. It is
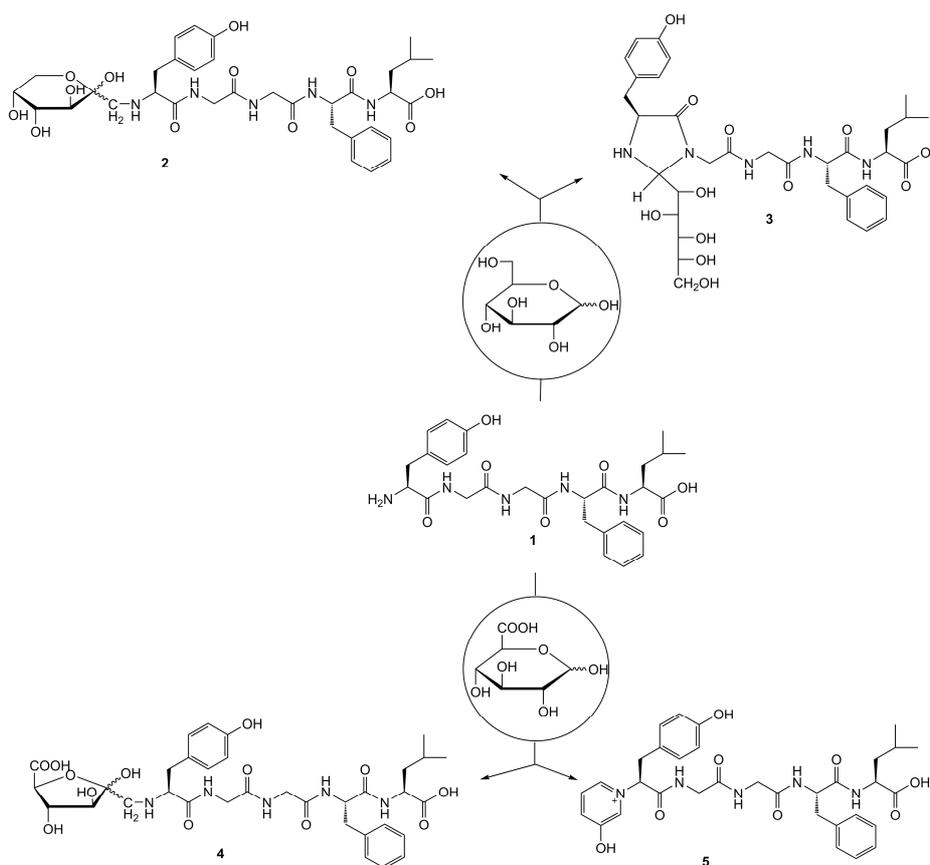
Figure 1. Formation of early glycation products of Leu-enkephalin (**1**) with glucose (Glc) and glucuronic acid (GlcA).

obvious that different MRPs of Leu-enkephalin have different effect on the structure of peptide **1**, which can produce difference in peptide stability and activity.[7] ECD spectra in TFE of MRP peptides **2**–**5** were measured in the presence of different types of cations and at their different ratio to study the conformational effect / binding type of multivalent cations Zn^{2+}, Al^{3+}, Ca^{2+}, Pb^{2+}, and Cu^{2+}. There have been many attempts to categorize various metal ions and anions to predict reactivity, solubility, etc. According to Pearson (1963)[37] Al^{3+} and Ca^{2+} are hard Lewis acids, Zn^{2+} is strong Lewis acid, Cu^{2+} and Pb^{2+} soft Lewis acids are in borderline; while carboxyl, amide and hydroxyl groups belong to the hard Lewis bases. Hard acids and bases are small, compact, and non-polarizable. Hard acids tend to bind to hard bases to form adducts with ionic character while soft acids tend to bind to soft bases and theirs adducts are more covalent in nature. In oligopeptides both terminal groups, the amide and carboxyl group and the functional side chain groups (carboxyl-, amino-, thioether-, thiyl-, imidazolyl group) are potential binding sites for cations. If the amino terminal group is free, the side chain primarily serves as anchor of

metal ion. Since Al^{3+} and Ca^{2+} are *strong Lewis acids* and hard metals, they are expected to react with the *O*-donors, such as *e.g.* carboxylate ion (C-terminal or side chain COO^- of peptides), phenolate (Tyr(O$^-$)) and also deprotonated hydroxyl group from carbohydrates to form glycopeptide-metal complexes. Zn^{2+} and Cu^{2+} ions can bind to the terminal amino and/or carboxyl groups (mostly they are labile). More stable complex (chelate-type) is expected to form involving the terminal amino and either the neighboring amide carbonyl groups (in the case of Zn^{2+}) or deprotonated amide nitrogen atom. The formation of complexes through peptide backbone will affect the character of the ECD spectra.

The aim of the present study was twofold: i) studying the ability of complexation of each compound **1**–**5** with various cations; ii) comparison of the complexation ability of MRPs **2**–**5** with the ability of complexation of peptide **1**. The cation-induced ECD spectral changes in spectra of peptides **1**–**5** were measured from "stable complexes" at r_{cat} where increasing cation concentration caused no more ECD-changes. Compounds **2**–**5** as Amadori, imidazolidinone and 3-hydroxypyridinium derivatives of Leu-enkephalin (**1**)
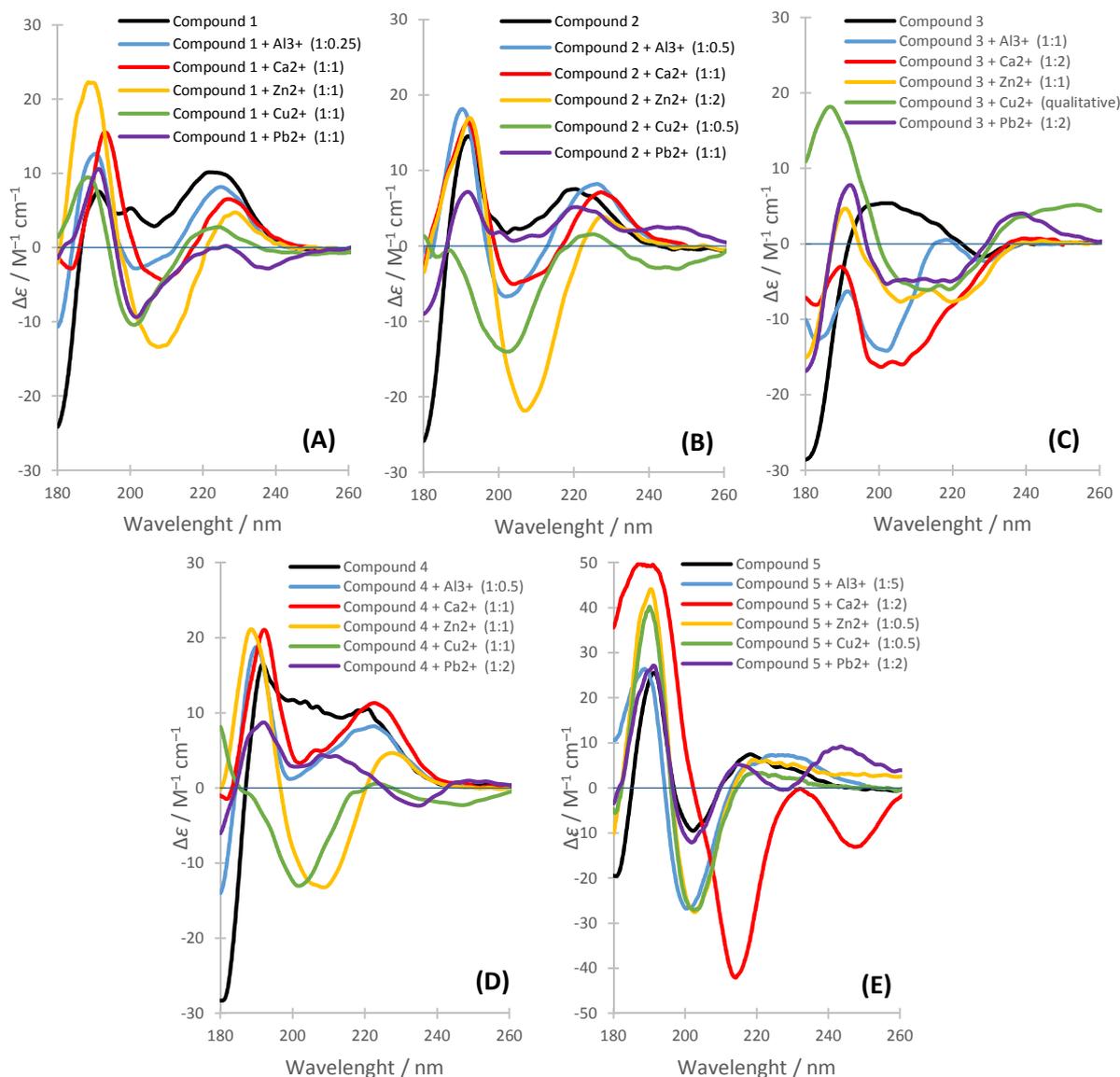
Figure 2. CD spectra of (A) Compound **1**; (B) Compound **2**; (C) Compound **3**; (D) Compound **4**; (E) Compound **5**; and their complexes with Al^{3+}, Ca^{2+}, Zn^{2+}, Cu^{2+} and Pb^{2+} ions.

can give more potential cation-binding places then the corresponding parent peptide **1**.

Leu-enkephalin **1** with the cations Pb^{2+}, Cu^{2+} and Al^{3+} shows the adoption of more folded conformers stabilized by free carboxyl and/or side chain binding at $r_{cat} \leq 1$. These ECD spectra are characterized by a negative band near 205 nm and positive bands near $\sim$ 190 nm and 230 nm, suggesting βI/III turn-type (for a classification and ECD spectroscopic features of different types of turns see ref. 38), while with Cu^{2+} an extra negative peak with weak intensity around 240 nm appeared. In the presence of Ca^{2+}

all extremes are red shifted with $\sim$ 5–10 nm (βII turn-type, Figure 2a), while with Zn^{2+} all peaks are more intense and broader indicating a lot of different turn-types.

Amadori compound **2** (Figure 2b) gave βI/III turn-type, C-like spectrum with Ca^{2+} at $r_{Ca} = 1$ as a stable complex, with two negative bands above 205 and 218 nm and a positive band at $\sim$ 195 nm.[37,38] With Zn^{2+} compound **2** shows a spectrum marked by a strong positive band at $\sim$ 195 nm, a weaker one at $\sim$ 225 nm and a strong, broad negative band centered at 208 nm. This spectral feature indicates the presence of a mixture of folded conformers,
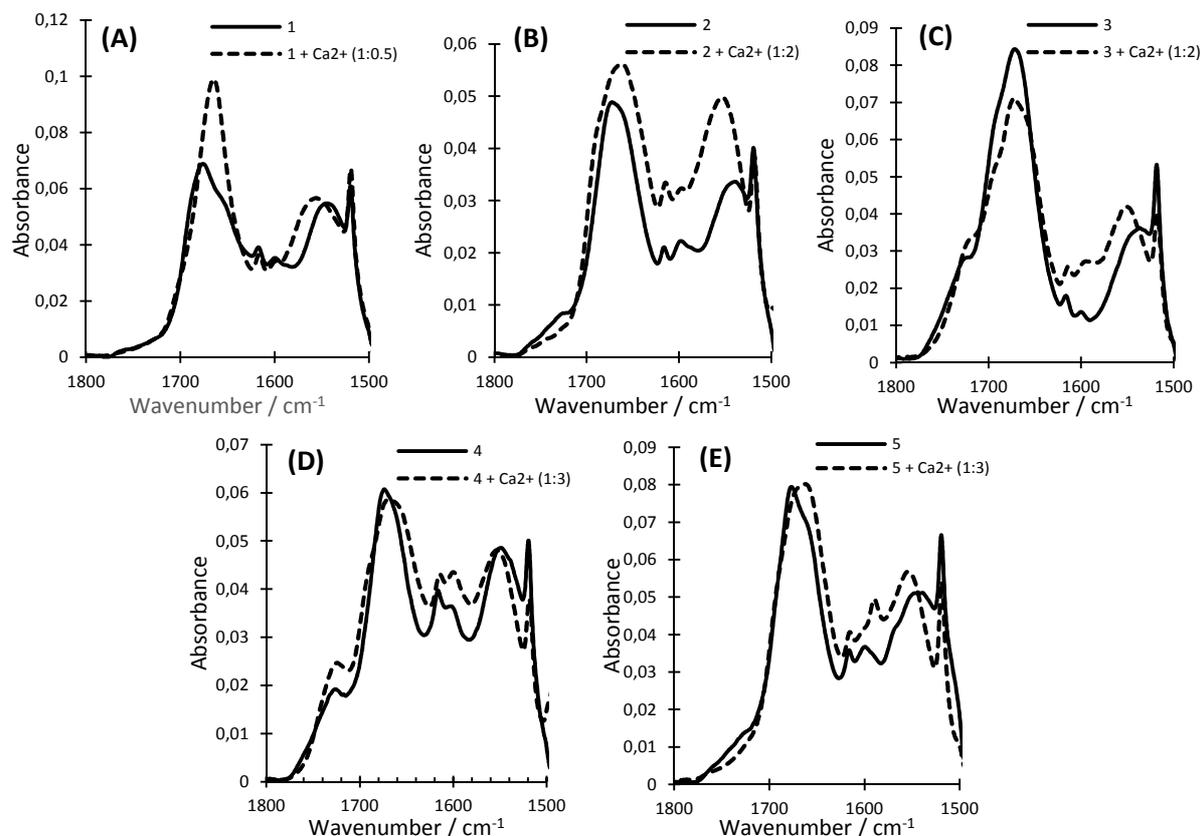
Figure 3. FTIR spectra of (A) Compound **1**; (B) Compound **2**; (C) Compound **3**; (D) Compound **4**; (E) Compound **5**; and their complexes with Ca^{2+} ion.

mainly β- and γ- turn.[38] In the presence of Cu^{2+} the ECD spectrum shows a strong, very broad negative band centered at ∼ 200 nm and a weaker one at ∼ 240 nm.

Imidazolidinone compound **3** (Figure 2c) gave more or less βI/III turn-type C-like spectra with all cations except Al^{3+}. The cation-induced ECD spectra of compound **3** (Figure 2c) are generally marked by a very broad negative band between 200–230 nm with more overlapping extremes. Al^{3+} gave an unusual ECD shape which can suggest different complexation with the various functional groups.

In Amadori compound **4** (Figure 2d) free carboxylic acid can contribute to the complex formation but only subtle spectral effect with Ca^{2+} (r_{cat} = 1), Al^{3+} (r_{cat} = 0.5) and Pb^{2+} (r_{cat} = 2) were observed. With Zn^{2+} and Cu^{2+} ions compound **4** shows the same spectral behavior as in the case of compound **2** which can be attributed to the Amadori structure but they have also similarity with compound **1**.

In the solution of 3-hydroxypyridinium derivative **5** (Figure 2e), all cations, except Ca^{2+}, induce the same spectral character and the observed β-turn-type C-like spectra differ only in the relative band intensities which means that there is no complex formation with backbone

amide carbonyl groups so there are no significant conformational changes. The only exception is Ca^{2+} which with **5** gave a broad and strong positive band at 190 nm, a strong negative band at ∼ 215 nm and an additional weaker one at ∼ 248 nm. This spectrum shows β-sheet like features and it is not a typical "Ca-induced spectrum", characteristic for many peptide-Ca^{2+} complexes, where the main features would be a weak positive band above 210 nm and a strong negative one at ∼ 195 nm. [39]

FTIR Spectroscopic Characterization of Compounds and Their Metal Complexes

The infrared spectra of compounds **1–5** (Figure 3) were taken in TFE in the 1800–1500 cm^{-1} spectral region, which is mainly composed of bands due to $v_{C=O}$(COOH), amide I, amide II and v_{as}(COO$^-$) vibrations. Absorption by the aromatic Tyr and Phe side chains also give contribution in this spectral region. The sharp component bends near 1519, 1600 and 1616 cm^{-1} in the infrared spectra of all analyzed compounds are assigned to the ring vibrations of Tyr and Phe. Since FTIR measurements require much higher sample concentrations than ECD, which may induce

precipitation in the presence of certain metal ions (especially Al^{3+}), a systematic study with all the metal ions was not possible. Based on its high biological relevance and considering the characteristic changes observed in the ECD spectrum, the FTIR based complexation studies were restricted to the Ca^{2+} ion. The infrared spectra of compounds **1–5** with Ca were taken at peptide-metal ratios for which the stable complexes were observed (Figure 3) according to the ECD spectra. Main difference observed in complex spectra compared to compounds alone were shifts from 1542 cm^{-1} to 1552 cm^{-1} in the amide II spectral region of compounds **1–3** and **5**, while compound **4** does not show shift of this extreme which is in agreement with ECD spectrum of compound **4** with Ca^{2+} where no significant changes of spectrum shape was observed. The Ca^{2+}-induced changes observed in the FTIR spectra of compounds **1–5** are more subtle than those in ECD and do not provide sufficient information for a more detailed conformational interpretation in terms of possible types of folded structures.

CONCLUSION

In the case of short chain peptides containing aromatic amino acid residue, as Leu-enkephalin, ECD spectroscopy can give a good diagnostic of conformational changes (*e.g.* cations, solvent, temperature), but cannot discriminate well the aromatic contribution from the conformational effect.[40,41] Binding of the cation to the free carboxylic group or OH-group of side chain of Tyr can stabilize the backbone conformation of the peptide, but cation-bindings to the backbone amide carbonyl groups can have higher influence to the conformational changes. It is known that the hard metals such as Ca^{2+} and Al^{3+} ions bind to the carboxyl group, phenolic side chain of Tyr and backbone amide carbonyl group. In TFE the cation-binding involves deprotonation of the C-terminus carboxyl group or hydroxyl Tyr side chain, or sugar hydroxyl group. The zwitter-ion formation enables this reaction. Appearance of the stronger spectral feature of $\pi \rightarrow \pi^*$ at 195 nm may be attributed to folded structures stabilized by the cation.

The slight changes of the ECD shapes are similar in the case of Ca^{2+} and Al^{3+} with compounds **1**, **2**, and **4** at the same ratio. We suppose that these changes are only due to the modified distribution of the conformers. The ECD curves of compounds **3** and **5** with Ca^{2+} ion show characteristic conformational changes toward ordered structures. Complexation of compound **3** with Al^{3+} and Ca^{2+} results in ECD changes which might be interpreted as binding of both cations to the side chains and also to amide carbonyl(s) of the peptide backbone which results in appearance of different types of turns. Compound **5** with Al^{3+} shows only difference in the intensity of ECD spectra at

higher Al^{3+} (1 : 5) concentration, while with Ca^{2+} ion (1 : 1) the ECD spectrum shows a typical β-sheet-like spectrum (conformational changes involving backbone amide carbonyls) with an additional band at 248 nm.

Compounds **1**, **2** and **4** gave the same ECD shape but at different peptide / Zn^{2+} ratios. Primarily binding of Zn^{2+} to the OH-group of side chains (Tyr) and C-terminus carboxyl group is responsible for stabilizing the structure, leading to higher distribution of ordered structures. The C-like spectrum in the case of compound **3** can reflect a mixture of the different turn types, *e.g.* βI/III.

ECD spectra of compounds **2**, **4** and **5** show no changes during titration with Pb^{2+}. The ECD spectrum of compound **1** with Pb^{2+} shows conformational changes, mainly formation of βI/III type turns and also a positive band at $\sim$ 240 nm. Compound **3** with Pb^{2+} gave βI/III turn-type ECD shape, similar to that with Zn^{2+}.

Complexation of compounds **1** and **5** with Cu^{2+} results in an increase of the population of ordered structures. In the case of compounds **2** and **4** the changes in the ECD spectra reflect the increase of the ordered structures and appearance of the band at $\sim$ 245 nm. The measured ECD spectra of compound **3**-Cu^{2+} complexes show typical features of a mixture of βI/III- and γ-turns which correlate with the changes produced by Pb^{2+} and Zn^{2+} upon complexation with compound **3**.

The conformational changes of compounds **1–5** can include self-aggregation and consequently alterations in physicochemical properties and biostability of natural compounds having interesting biological activities from the medicinal chemistry viewpoint.

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