Zn^{2+}-Complexation by a β-Peptidic Helix and Hairpin Containing β^3hCys and β^3hHis Building Blocks: Evidence from CD Measurements

Preliminary Communication

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Dedicated to Professor *Wolfgang Steglich* on the occasion of his 70th birthday

Two new β^3-homohistidine- and β^3-homocysteine-containing β-peptides have been prepared by solid-phase synthesis. A β-octapeptide (**2**) contains seven β^3-amino acids and one β^2-amino acid. The β^2/β^3 segment has been placed in the middle of this peptide, which contains β^3-amino acids of alternating configuration, to induce the formation of a hairpin secondary structure. A β-decapeptide (**3**) has been designed to fold to a 3_{14}-helical secondary structure with neighboring His side chains in 6- and 9-positions. Circular-dichroism (CD) measurements show the capability of both peptides to bind Zn^{2+} ions in aqueous solution. In the case of the β-octapeptide, binding of Zn^{2+} causes a dramatic change of the CD spectrum, indicating a change or a stabilization of its secondary structure. Zn^{2+} Ions clearly stabilize the 3_{14}-helix of the β-decapeptide, in neutral and basic solution. For the construction of the two new β-peptides, we needed to have a supply of the β-amino acid derivatives Fmoc-β^3hCys(Trt)-OH and Fmoc-β^3hHis(Trt)-OH, the preparation of which is described herein.

During the last decades, chemists and biochemists have spent many efforts to gain deeper understanding of the factors governing the stabilization of different secondary structures of peptides and proteins, with the aim to understand the rules of molecular recognition and to design new and effective peptidomimetic compounds [1]. Among the numerous possible approaches to induce and reinforce intramolecular interactions, the use of a metal ion capable of binding certain functional groups in a peptide is interesting for at least two reasons: *a*) ion complexation is commonly used in nature to stabilize protein structures, and *b*) it takes the correct positioning of only a few amino acids in order to reinforce secondary structures such as a helix or a turn by metal-ion binding. In the world of α-peptides, two histidines in positions i and $i+4$, at the C-terminus as well as in the middle of a short peptide, can induce nucleation and stabilization of a helical conformation upon addition of metal ions such as Zn^{2+}, Cd^{2+}, Cu^{2+}, and Ni^{2+} [2–4].

A similar approach can be used to stabilize turn secondary structures. *Searle* and co-workers [5] have synthesized short α-peptides with a proline to induce a reverse turn, and two or three histidines for metal binding on both strands near the N- and C-termini. They found a substantial stabilizing effect on the hairpin structure by complexation

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between Zn^{2+} ions and the histidine residues. *Imperiali* and co-workers [6] have inserted unnatural multidentate amino acids, which bear bipyridyl and phenanthrolyl groups as side chains, in order to tune the affinity towards different metal ions. However, it looks as if metal coordination alone is not sufficient for the folding of the peptide; there must be additional effects; for instance, steric and conformational ones, to stabilize the folded structure [7].

To the best of our knowledge, no example of a β-peptidic secondary structure, stabilized by metal-ion complexation is known until now. It has been established that 3_{14} helices of β-peptides can be stabilized by disulfide bridges [8] or by salt bridges between ornithine, arginine, or lysine, and glutamic acid side chains [9][10], or by macrodipole–charge interactions [11]. Less well-explored is the world of hairpin structures of β-peptides. *Balaram* and co-workers [12][13] have prepared mixed α/β-peptides in which a D-proline and a glycine induce a so-called reverse turn of two peptide strands; *Gellman* and co-workers [14] have used a template formed by two nipecotic acid units to enforce the formation of a hairpin secondary structure in a β-tetrapeptide; and our group has shown [15][16] that insertion of a β^2/β^3-amino acid dimer segment in the middle of a β-hexapeptide (**1**) with two sheet-inducing disubstituted β-amino acid residues of *unlike* configuration on each *terminus* causes folding to a hairpin in MeOH (NMR analysis) and probably also in aqueous solution (CD analysis) (*Fig. 1*). This β-peptidic turn is characterized by a ten-membered H-bonded ring at the site of the turn and a *trans*-strand 14-membered H-bonded ring, with the C- and N-termini poorly ordered.

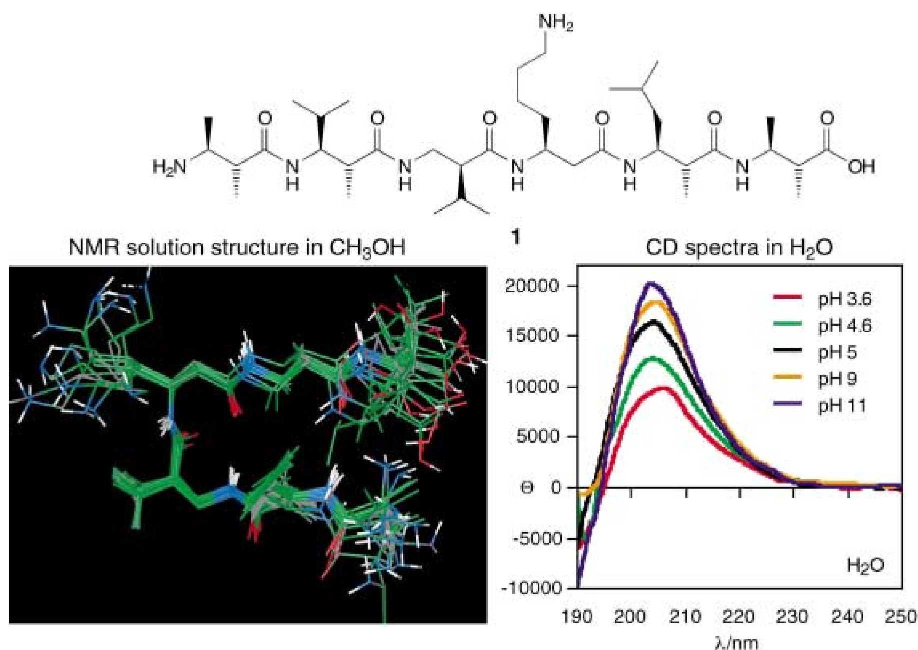

Fig. 1. *Turn-forming β-peptide* **1**. Molecular formula, bundle of 15 lowest-energy conformers in MeOH [15][23], and CD spectra of the β-peptide **1** in H_2O at different pH values [23][27].

We have now studied the influence of a metal ion in stabilizing β-peptidic secondary structures in H_2O, a solvent in which unfolding may occur [10][17]. This is especially important for the turn structure that has been demonstrated [18] to be capable of mimicking α-peptidic turns; this fact, together with the high stability of β-peptides towards proteolytic enzymes [19][20], has opened the door for development of novel pharmacologically promising peptidomimetics [18][21][22]. Thus, the β^3-homocysteine- and β^3-homohistidine-containing β-peptides **2** and **3** were designed and synthesized in solid phase[3]) with the aim to induce or strengthen their folding to a hairpin and a 3_{14} helix, respectively, by complexation with Zn^{2+} ions (*Fig. 2*).

The design of the sequence to fold to a helix was simple: the 1*H*-imidazol-4-yl groups of β^3hHis residues had to be placed in i and $i+3$ position for the Zn^{2+} complexation, and proper side chains for salt-bridge formation (CO_2^-/imidazolium$^+$ and CO_2^-/NH_3^+) were added in an all-β^3-peptide (**3**). To avoid the elaborate preparation of *u*-$\beta^{2,3}$-amino acids (*cf.* **1**), we chose a new strategy for the design of the sequence to fold to a turn: besides the central β^2/β^3-segment (*cf.* **1**), we used β^3-amino acids of alternating absolute configuration[4]) for the desired antiparallel sheet part of the turn structure (**2**). The β-peptides **2** and **3** (*Fig. 2*) were assembled by manual solid-phase synthesis on a *Wang* resin according to the Fmoc strategy. They were purified by preparative RP-HPLC and identified by high-resolution mass spectrometry. Compound **2** with the terminal β^3hCys was somewhat sensitive to air oxidation leading to the formation of a disulfide.

Preparation of the required β-amino-acid derivatives Boc-β^3hHis(Trt)-OH, and Fmoc-β^3hHis(Trt)-OH and Fmoc-β^3hCys(Trt)-OH *via Arndt–Eistert* homologation, starting from Boc-His(Tos)-OH and Fmoc-Cys(R)-OH (R = Acm, PMB), respectively, is outlined in the *Scheme*.

For the binding experiments, $ZnCl_2$ was chosen because Zn^{2+} is known to have an expecially high affinity for the 'softer' ligands such as S^- of cysteine and N of histidine. Thus, Zn^{2+} is expected not to bind competitively to the carboxylates and amino groups present in the peptides; also, Zn^{2+} is not an oxidizing cation that would cause disulfide formation from the β^3hCys-containing peptide **2**; finally Zn^{2+} is one of the most-abundant divalent metal ions in living organisms (*ca.* 0.003% of the weight of a person) and one of the least toxic ones, it is a 'cofactor' of many metabolic enzymes and transcription factors, and it is often found to play a structural role in stabilizing proteins such as zinc fingers.

In the absence of an NMR analysis of the new β-peptides **2** and **3**, we had to resort to CD spectroscopy at this stage[5]). CD Measurements in MeOH solution of the β-

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[3]) All the information about the new β-amino acids and about the β-peptide synthesis, as well as their characterizations and structure elucidations, will be provided in a forthcoming full paper.

[4]) β-Peptidic sequences of this type have been prepared by us before [23][24]. They cannot possibly fold to a 3_{14} helix, because every other side chain would wind up in the 'forbidden' *axial* position of such a helix. Previous attempts to determine the NMR-solution structure of a β^3-peptide with alternating absolute configuration of the building blocks have been futile [23].

[5]) Extensive 2D-NMR measurement of the β-peptides **2** and **3** in MeOH, in H_2O, and in the absence and presence of Zn^{2+} ions are being undertaken; they will be followed by cumbersome and time-consuming analyses of the data, which might or might not lead to NMR-solution structure(s). It may be several months before we can possibly report the results of this NMR analysis.

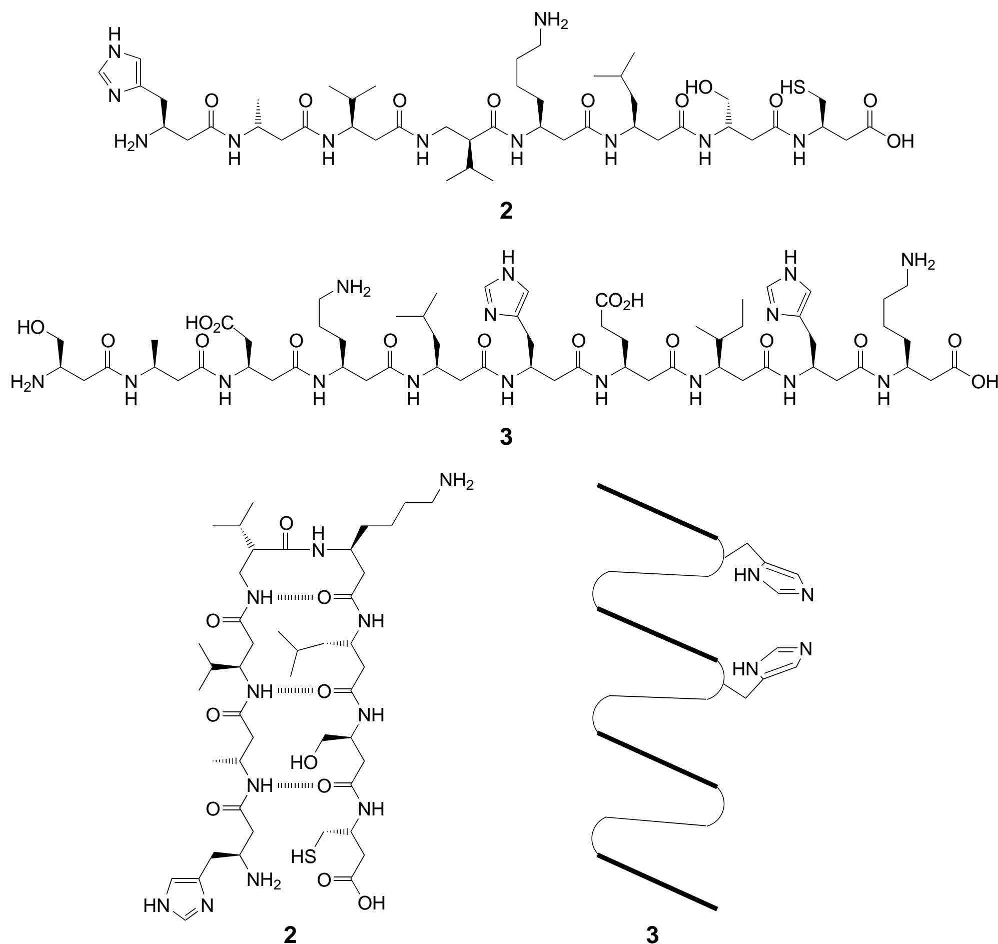
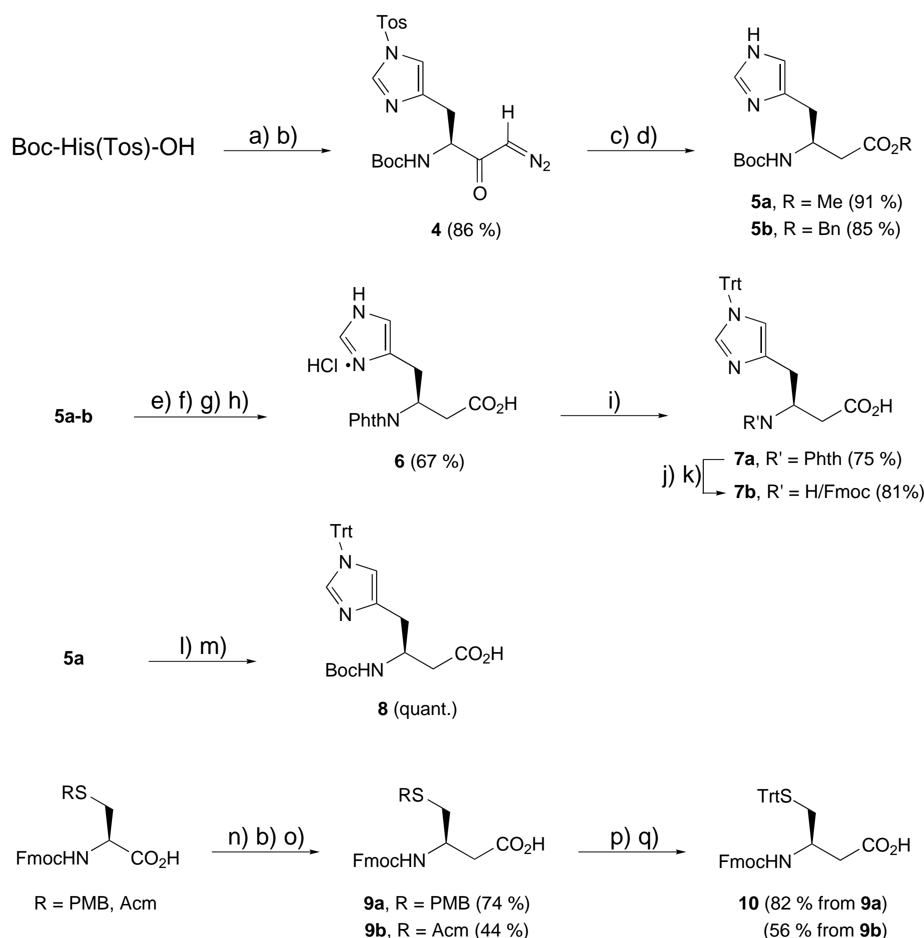


Fig. 2. *Formulae and schematic presentations of the β-peptides* **2** *and* **3**. β-Octapeptide **2** in a possible hairpin conformation with three *trans*-strand H-bonds, and β^3hCys and β^3hHis in the right position for metal-ion binding. β-Decapeptide **3** in a 3_{14}-helical conformation where two β^3hHis, as well as β^3hOrn, β^3hLys and β^3hGlu are placed in i and $i+3$ positions, respectively. The two 1*H*-imidazol-4-yl groups in juxtaposition on the helix should complex Zn^{2+}. At slightly acidic pH, two salt bridges should stabilize the helix.

octapeptide **2** show a single positive *Cotton* effect and *no* zero-crossing, with a maximum of molar ellipticity of *ca.* 10000 (10 deg cm^2 mol^{-1}, normalized) near 202 nm. From previous work of our group, this kind of CD spectrum is common to both the β-peptidic hairpin and the *10/12*-helix [25–27]. From the design of this β-peptide, as described above, we may assume that the CD spectrum signals the presence of a hairpin structure [27]. On the other hand, the MeOH solution of β-peptide **3** shows a negative *Cotton* effect at *ca.* 215 nm (mean molar ellipticity of *ca.* −6000, 10 deg cm^2 mol^{-1}, normalized), a zero crossing at *ca.* 207 nm and a positive *Cotton* effect near 200 nm, a pattern that is associated with a 3_{14} helix [9][24][28–30] (*Fig. 3*). Interestingly, no

Scheme



a) *N*-Methylmorpholine (NMM), $ClCO_2(^iBu)$, THF. b) CH_2N_2, Et_2O. c) NEt_3, CF_3CO_2Ag, MeOH or BnOH/THF. d) Ac_2O, Py. e) Trifluoroacetic acid (TFA)/CH_2Cl_2. f) NaOH or Pd/C, H_2, MeOH. g) *N*-(Ethoxycarbonyl)phthalimide ($PhthNCO_2Et$), Na_2CO_3. h) HCl aq. i) Tritylbromide (TrtBr), NEt_3, $CHCl_3$/DMF. j) N_2H_4, EtOH. k) FmocOSu, Na_2CO_3, H_2O/acetone. l) Tritylchloride (TrtCl), NEt_3, $CHCl_3$. m) LiOH, n) NMM, $ClCO_2Et$, THF. o) For R = 4-methoxybenzyl (PMB): $PhCO_2Ag$, H_2O/THF, sonication. For R = (acetamido)-methyl (Acm): *hν* (Hg), CH_3CN. p) For R = PMB: $Hg(OAc)_2$, TFA, anisole. For R = Acm: $Hg(OAc)_2$, THF/H_2O/AcOH. q) TrtCl, NMM, $CHCl_3$.

appreciable change of the CD spectrum is observed for either β-peptide upon addition of 1 equiv. of $ZnCl_2$ to their MeOH solution. One plausible explanation is that **2** and **3** cannot bind Zn^{2+} under these conditions because the peptides have been purified and isolated by preparative HPLC and lyophilized as trifluoroacetate salts.

Structural changes or, rather, loss of predominant secondary structure(s) of the β-peptides **2** and **3** are expected, when going from MeOH to H_2O solution [9][17][31]. For β-peptide **3**, a destabilization of the helix should be counteracted by salt-bridge

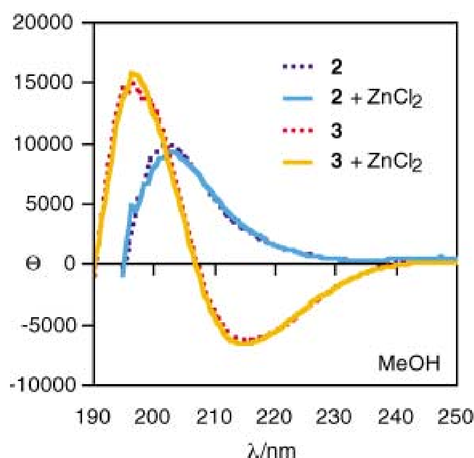


Fig. 3. *Normalized CD spectra of β-octapeptide* **2** *and β-decapeptide* **3** *(as their tris- and pentakis(trifluoroacetate) salts) in MeOH, 0.2* mM. The CD spectra of **2** (blue, dotted line) could be attributed to a hairpin conformation, while a 3_{14} helix can be derived from the CD pattern of **3** (red, dotted line). No appreciable changes are observed upon addition of $ZnCl_2$ (blue and orange, solid lines).

formation (*Fig. 4*) at pH 5.6, when the positively charged ornithine and the negatively charged glutamate side chains attract each other. Indeed, the CD spectrum still shows the characteristic pattern associated with a 3_{14} helix under these conditions. As the pH is increased, the salt bridge disappears due to loss of the positive charge ($RNH_3^+ \rightarrow RNH_2$), and this is compatible with a decrease in the *Cotton* effect. Interestingly, addition of Zn^{2+} ions leads to increased intensity of the CD band at higher pH (*Fig. 4*).

The CD spectra of β-peptide **2** in H_2O and in MeOH look similar: the maximum is shifted to shorter wavelengths by a few nm, and the intensity decreases from 10000 to 7800 (10 deg cm^2 mol^{-1}, normalized) when we switch to H_2O. On the other hand, a dramatic change occurs upon addition of 1 equiv. of Zn^{2+} at different pH values (*Fig. 4*): in neutral and basic media, the maximum shifts from 202 to 210 nm, and the molar ellipticity falls to half the value (from *ca.* 7800 to 3700 10 deg cm^2 mol^{-1}, normalized). This behavior is not observed under acidic conditions (pH 5), probably because the donor 1*H*-imidazol-4-yl group of **2** is protonated and no longer available for coordination to the metal. This observation is compatible with the assumption that the secondary structure is stabilized by complexation with Zn^{2+} (similar behavior has been reported for α-peptides, *vide supra*).

We have also performed a titration of a solution of **2** at pH 7.6 with $ZnCl_2$, varying the relative concentration of Zn^{2+} ions and keeping the concentration of the β-peptide constant. The results are shown in *Fig. 5,a:* the position of the *Cotton* effect shifts to longer wavelengths and decreases in intensity as the Zn^{2+} concentration approaches an equimolar amount, and no changes are observed upon addition of more than 1 equiv. of Zn^{2+}, even when we go to 10 equiv.!

From this titration, it is possible to derive the stoichiometry of the complex by plotting the molar ellipticity, observed at 202 nm, *vs.* the number of equiv. of Zn^{2+}: a strong decrease in molar ellipticity is observed between 0 and 1.0 equiv., then it remains

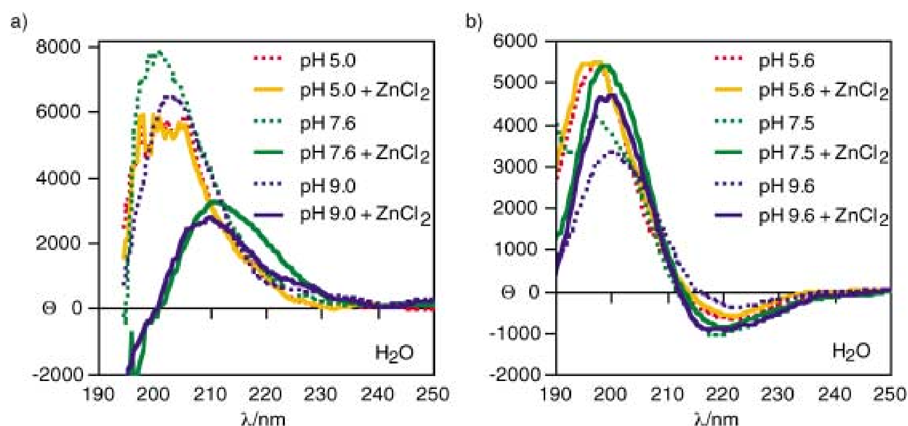


Fig. 4. *Normalized CD spectra of β-octapeptide* **2** *and β-decapeptide* **3** *in H_2O at different pH values, with and without $ZnCl_2$. a*) The CD spectrum of *β*-peptide **2** at pH 7.6 looks similar to the one in MeOH (green, dotted line). Upon addition of Zn^{2+} ions, the λ_{max} is shifted by *ca.* 10 nm, and the intensity of the CD band decreases to half the value (green, solid line). The same behavior is observed at pH 9 (blue, dotted and solid lines, resp.), but not at pH 5.0 (red and orange, dotted and solid lines, resp.), probably because the donor 1*H*-imidazol-4-yl group of **2** is protonated and no longer available for coordination to the metal (peptide 0.2 mM in 50 – 10 mM *Tris* and $NaHCO_3$ buffers). *b*) The CD spectra of **3** in H_2O can be attributed to a 3_{14}-helical conformation; the highest molar-ellipticity value is observed at pH 5.6 (red, dotted line), while it decreases as the pH is raised to 7.5 (green, dotted line) and 9.6 (blue, dotted lines), due to the disappearance of the salt bridges. Interestingly, the addition of Zn^{2+} ions restores the intensity of the CD band that reaches at pH 7.5 (green, solid line) the highest value observed at pH 5.6 (peptide 0.2 mM in 10 mM AcOK, Tris, and $NaHCO_3$ buffers).

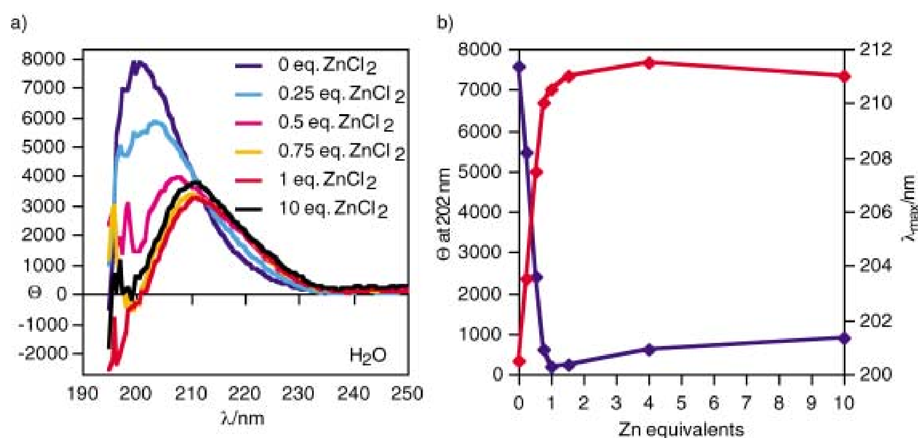


Fig. 5. *Titration of β-peptide* **2** *with $ZnCl_2$ and determination of the complex stoichiometry. a*) The intensity and the wavelength of the CD band change rapidly when addition of $ZnCl_2$ approaches equimolar amounts (red curves). Upon addition of excess $ZnCl_2$ (up to 10 equiv.), the CD curve remains unchanged (black curve). *b*) Plotting the molar ellipticity, observed at 202 nm (blue), and the shift of the wavelength of the maximum of each curve (red) *vs.* the number of equiv. Zn^{2+}, it is possible to conclude that the complex stoichiometry is 1:1 (peptide 0.2 mM in 50 mm *Tris* buffer).

roughly constant above 1 (*Fig. 5,b*). Similar behavior is seen when the shift of the wavelength of the maximum of each curve is plotted *vs.* the number of equiv. of Zn^{2+}: a shift of *ca.* 10 nm occurs between 0 and 1.0 equiv., and no more change above 1 equiv. of Zn^{2+}. According to the molar-ratio method [32], these two plots are evidence for the existence of a 1:1 complex between the β-peptide **2** and the Zn^{2+} ion.

We have prepared for the first time β-peptides containing β^3hCys *and* β^3hHis building blocks. The CD spectrum of the β-peptide **3** can be attributed to a 3_{14}-helical conformation, both in MeOH and in H_2O solution, and the pH dependence of the CD spectrum is compatible with salt-bridge stabilization; also, Zn^{2+} complexation appears to stabilize this helix. We cannot draw any conclusions about the nature of the prevailing secondary structure(s) of **2** from the CD measurements. It is clear that a conformational change occurs upon complexation with Zn^{2+}, and that a 1:1 complex is formed.

It is important to point out that CD spectra can give hints about the secondary structures that these β-peptides may predominantly have in solution [17][27][31][33]; only after the detailed structure analysis by the high-resolution techniques that NMR spectroscopy[5]) or X-ray diffraction can provide will it be possible to confirm or dismiss the conclusions we have drawn from CD measurements [34] about the predominant conformations of the β-peptides **2** and **3**.

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