Zinc Complexes of a Helical 22-mer Peptide with Two Histidine Donors[‡]

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Dedicated to Professor Karl Wieghardt on the occasion of his 60th birthday

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A new 22-mer model peptide **P22** was designed for maximal α-helicity in nonpolar media, synthesized by solid-phase methods, and isolated in an analytically pure state. It contains histidine at positions 10 and 14 representing the *i*, *i* + 4 motif which is typical for metal ion binding. Treatment with zinc halides yielded the uncharged molecular complexes (**P22**)$ZnCl_2$ and (**P22**)ZnI_2 which are the first metal complexes of medium-sized peptides to be isolated in an analytically pure state and identified by mass spectrometry. The helicity of both the free peptide and its complexes was confirmed by IR and CD spectroscopy as well as by molecular mechanics calculations. This has allowed us to propose a molecular structure which shows that the complexes are viable models for zinc-binding sections in zinc finger proteins.

Introduction

Of the two functions of zinc in biology — structural and catalytic[2] — the latter has found much more attention in the literature even though the structural function may be the more important. It is essential for many enzymes,[3] and the ubiquity of zinc finger motifs[4] is underlined by the fact that about 1% of the human genome seem to encode zinc finger proteins.[5]

The classical zinc finger sequence is X_3-Cys-X_{2-4}-Cys-X_{12}-His-X_3-His-X_4 with X being any amino acid.[6] Its most well preserved feature is the location of two histidines in the *i* and *i* + 4 positions on a helical peptide chain. This has been found in the two X-ray structure determinations of zinc finger fragments,[7] and confirmed by chemical modifications of truncated zinc finger peptides.[8]

Based on this information the methods of chemical peptide synthesis were applied to the design of some medium-sized peptides containing pairs of cysteine and/or histidine residues at appriopriately spaced positions on the peptide chain.[9] It was found that such peptides form stable zinc complexes when the donor residues are placed at the *i* and *i* + 4 positions.[9−17] In some cases the addition of zinc and other metal ions enhanced the helicity of these peptides.[10−12,15] This proves the metal binding by the two His or Cys donors, which in the *i* and *i* + 4 positions on the α-helix point in the same direction. So far the identification of such metal-peptide complexes has rested only on spectroscopic methods, and none of them has been isolated in a pure state or subjected to mass spectrometry.

Our own contributions to the chemistry of zinc-peptide complexes[1] have focused so far on small peptides, as exemplified by the role of structural zinc in bis(cysteinyl) protein sequences[18] or in β-turn motifs,[19] and the parameterization of the donor qualities of histidine and cysteine in peptides toward zinc.[20] In continuation of this we have now turned to the complexation of zinc by histidine and cysteine donors which are constituents of helical peptides. This calls for the synthesis of medium-sized peptides and the use of amino acids which favor helix formation. This paper reports our first results in this area in the form of the preparation, isolation in the pure state, and the full characterization of a zinc complex of a 22-mer peptide with histidine in the *i* and *i* + 4 positions.

Results and Discussion

In order to enable the isolation and full characterization of both the peptide and its zinc complexes a strategy was chosen which differs from that in the previous investigations. The peptide was chosen to be hydrophobic and to contain no donor functions other than two histidine constituents. This should allow us to work in non-aqueous media and facilitate isolations. For the same reason both termini of the peptide were derivatized: the N-terminus was chosen to be *N*-acetyl glycine as the simplest terminator of a capping box,[21] and the C-terminus was protected as the

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amide. Only amino acids that are predominantly hydrophobic were considered, and among these those with the highest tendency for helix formation. This limited the choice of the amino acids for the peptide backbone to alanine, leucine, methionine and phenylalanine, which hold the top positions in the various rankings of helix formation propensity.[22−25]

The length of the peptide was chosen to be above the minimum length for helix formation (16−18)[26] but below the maximum length for clean chromatographic separation (25−30). It was decided to use a 22-mer peptide with histidine at positions 10 and 14. The amino acid sequence was optimized with the AGADIR algorithm[27] for a maximum degree of α-helicity. Among 100 randomly chosen sequences made up from the four amino acids the computation selected the one given below in the peptide **P22** as the best choice, with a computed helicity of 88%.

NAc-Gly-Phe-Leu-Met-Ala-Phe-Leu-Met-Ala-His-Phe-Leu-Met-His-Phe-Leu-Met-Ala-Phe-Leu-Met-Ala-NH_2

P22

P22 was prepared on a solid-phase synthesizer using Fmoc-protected amino acids and purified by reversed-phase HPLC. Its purity was confirmed by elemental analysis, and its identity was verified by an ESI mass spectrum which reproduced the calculated isotope pattern for $C_{128}H_{187}N_{27}O_{23}S_5$. As expected, **P22** was found to have a very low solubility in water but to be quite soluble in acetonitrile and THF. This was the first indication of the helical structure of **P22**, based on the experience that hydrophobic oligopeptides which are not helical (i.e. which don't have their hydrogen bonds in their interior) have a very low solubility in these solvents.[28]

Further support for the helical nature of **P22** was obtained by FTIR and CD spectroscopy. The intense amide-I band in the IR spectrum was observed at 1654 cm^{-1} in THF solution and at 1658 cm^{-1} in a KBr matrix, as expected for α-helices.[29] The CD spectra of **P22** and its $ZnCl_2$ complex are shown in Figure 1. The CD spectrum in acetonitrile displays a molar ellipticity Θ at 222 nm of −497000 deg·cm^2·$dmol^{-1}$. This converts to an average ellipticity per peptide function (n = 21) of −24000 deg·cm^2·$dmol^{-1}$. According to the equation

helicity [%] = $100 \cdot \Theta_{222}/[-39500 \cdot (1 - 2.57/n)]$[30]

the helicity of **P22** is thus 69%. Considering that five of the 22 amino acids in **P22** are phenylalanine, which at 222 nm has a positive ellipticity, it can be concluded that the 69% value is a lower limit and the actual helicity is considerably higher. This is confirmed by the CD spectrum in 2,2,2-trifluoroethanol, a solvent which strongly favors the formation of α-helices.[31] In this solvent the Θ_{222} value of **P22** is even slightly lower than in acetonitrile, i.e. the helicity of **P22** cannot be raised, supporting the notion that **P22** is nearly perfectly helical even in the absence of 2,2,2-trifluoroethanol.[32]

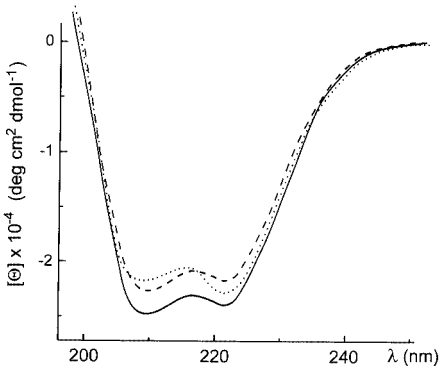

Figure 1. CD spectra of **P22** in acetonitrile (solid line) and in 2,2,2-trifluoroethanol (dashed line) and of **P22**·$ZnCl_2$ (**1**) in acetonitrile (dotted line); concentrations 0.4−0.5 mM

The NMR spectroscopic data of **P22** were not as informative as expected. As is typical for α-helical peptides,[33] the ^{1}H NMR resonances of the amide protons are spread over a range of several ppm, and the resonances of the mobile side-chains are sharper than those of the α-CH and amide groups in the peptide backbone. Unfortunately the side-chain interactions, which are very diagnostic for α-helical peptides,[34] could not be extracted from NOESY and ROESY spectra, even when taken at 500 MHz, as neither intensive NOE effects with a positive nor those with a negative phase occurred. For the same reason the resonances for the individual amino acids (alanine, phenylalanine, leucine and methionine occur 4 or 5 times each) could not be assigned. The failure to obtain structural information from NMR spectroscopic data, albeit not unprecedented for medium-sized peptides or their zinc complexes,[16,35] is a drawback of this work. It calls for a more detailed NMR study which is beyond the scope of this investigation.

As isolated by HPLC, peptide **P22** contained about five equivalents of trifluoroacetic acid. In order to remove these before the reaction with zinc salts, **P22** was treated with gaseous ammonia in THF solution. Filtration and prolonged pumping left **P22** in a water-, acid- and base-free form. In this form **P22** as an uncharged hydrophobic molecule is soluble only in nonpolar media. As such it is a chelating organic bis-nitrogen ligand, and in terms of zinc coordination chemistry it is well suited to form molecular L·ZnX_2 complexes. In contrast, we have experienced repeatedly that small bis-histidine peptides form insoluble coordination polymers with ionic zinc species such as $Zn(ClO_4)_2$.[36] For this reason we reacted **P22** with zinc salts which are themselves molecular in nonpolar media, i.e. the zinc halides.

Treatment of THF solutions of **P22** with equimolar amounts of anhydrous $ZnCl_2$ or ZnI_2 resulted in clear solutions of complexes **1** and **2**, respectively. After workup both complexes were isolated as yellow or brown powders which are analytically pure. Both **1** and **2** are quite soluble in nonpolar solvents but insoluble in water. Many attempts to obtain them as X-ray quality crystals were unsuccessful.

(**P22**)$ZnCl_2$ (**P22**)ZnI_2

1 **2**

The fact that **1** and **2** are molecular entities and not constituents of equilibrium mixtures containing **P22**, Zn^{2+} and Hal^- is not only evident from their insolubility in water, but could also be proved by their ESI mass spectra taken from acetonitrile solutions (see Exp. Sect.), which show the complex constituents but neither the free peptide nor the free zinc halide. The conservation of the peptide structure in the complexes is indicated by the amide-I IR bands which, for both **1** and **2**, are observed at 1655 cm^{-1}, i.e. almost at the same position as that of the free peptide. The CD spectra, displaying the 222 nm maxima with about the same intensities as that of free **P22** (see Figure 1), lead to the same conclusion.

The attachment of the $ZnHal_2$ units to the peptide could clearly be identified by ^1H NMR spectroscopy. While most of the proton resonances remained in place both after deprotonation with ammonia and after addition of the zinc halide (which means that the helical secondary structure is not affected), significant shifts of the histidine imidazole CH resonances were observed. Table 1 lists the four imidazole CH signals for the three states of the peptide. They were assigned by H,H-COSY NMR spectra taken in CD_3CN. Under these conditions the NH resonances could not be observed. Upon deprotonation all four CH signals move to higher field, the successive zinc complexation moves two of them further up but the two others down again. The shifts for the protonation of the uncharged peptide are larger than those for zinc binding. Altogether the NMR spectroscopic data are the clearest indication for the attachment of the $ZnHal_2$ units to both imidazole side chains of the histidine constituents in the 10 and 14 positions. In the absence of X-ray structure data they also provide the main basis for a structural assignment of the complexes.

Table 1. ^1H NMR spectroscopic data for the imidazole CH protons in Peptide **P22**

	CH^2	$CH^{2'}$	CH^4	$CH^{4'}$
protonated	8.02	8.45	7.14	7.21
deprotonated	7.64	7.89	6.58	6.70
$ZnCl_2$ complex	7.85	7.86	6.51	6.77
ZnI_2 complex	7.83	7.86	6.49	6.75

Conclusions

The approach and outcome of this work represent a conceptual difference from previous work on medium-sized peptides and their metal complexes. Both the peptide **P22** and its zinc complexes were designed to be nonpolar molecular entities. This has not only simplified their synthesis and isolation but also made them susceptible to the rules of molecular zinc chemistry. This means that **1** and **2** could be predicted to be stable and inert toward dissociation and that the chelate ligand **P22** and the halide ligands would be an ideal combination for a tetrahedral ZnN_2Hal_2 coordination. The accumulated evidence supports these predictions.

As expected and designed, the peptide **P22** was found to exist in the α-helical form. The usual criteria applied to this − solubility, IR and CD spectra − have confirmed it both for the free peptide and for its complexes with $ZnCl_2$ and ZnI_2. Although NMR spectroscopy has failed to assist in the structural assignment of the peptide, it has shown unambiguously that the $ZnHal_2$ units are attached to the two imidazole side chains of the histidines. As these are placed in the 10 and 14 positions of the peptide they are located one above the other and are separated by one turn of the helix. The identity of both the peptide and its zinc complexes was certified by mass spectra. To the best of our knowledge this is the first time that metal complexes of a medium-sized peptide have been isolated in an analytically pure form and fully characterized.

The available data allowed us to assign the peptide and complex structures with confidence by molecular modelling software. This was done by applying the force field method MM+ of the HYPERCHEM program package.[37] Figure 2 shows the energy-minimized structures of the uncharged peptide and its $ZnCl_2$ complex. It is obvious that only minimal conformational changes are necessary to convert the peptide to the complex. Thus the aims of this investigation − the design of a highly helical peptide with two preorganized binding sites for zinc − have been achieved.

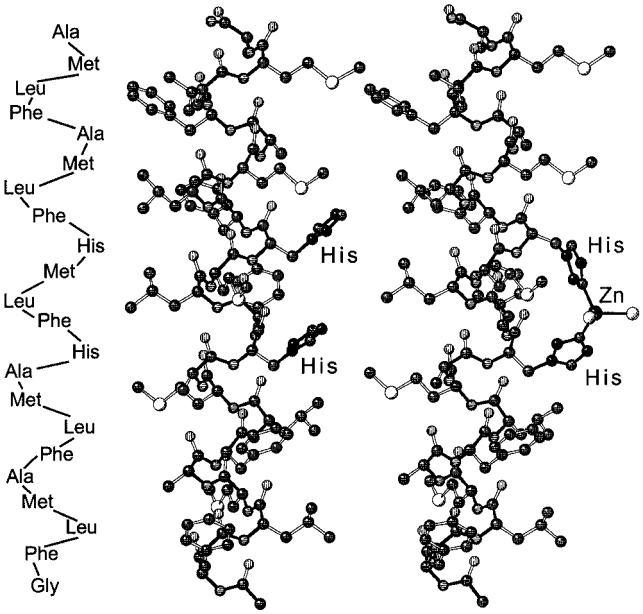

Figure 2. Computed structures[37] of peptide **P22** (left) and its $ZnCl_2$ complex (right); hydrogen atoms omitted, N-terminus at the bottom

Experimental Section

General: All manipulations were performed under an inert gas atmosphere. Starting materials were obtained commercially. IR: Bruker IFS-25 FTIR. NMR: Varian Unity 300 and Bruker Avance DPX 500. CD: Jobin-Yvon CD6. MS: Finnigan TSQ 700. Peptide synthesis: Milligan 9050 Synthesizer, PAL-PEG-PS resin, Fmoc protected L-amino acids, coupling reagent TBTU, for further details see ref.[38] Chromatography: Waters Model 4000, Waters DeltaPak C18 PrepPak reversed phase column. The figure was produced with SCHAKAL.[39]

Peptide P22: This peptide was prepared to order by the Merrifield method at the Institut für Biologie II of Freiburg University. The details of preparation and workup corresponded to the published procedures.[38] The raw product was dissolved in acetonitrile and purified by HPLC using a gradient of acetonitrile/water (75:25 → 100:0) containing 0.1% of HTFA. Retention time: 35 min. After freeze-drying 194 mg (28%) of **P22**·4.8HTFA were obtained as a colorless powder, m.p. 135 °C (dec.) whose HTFA content was determined by potentiometric titration.[16] $C_{128}H_{187}N_{27}O_{23}S_5$·$4.8C_2HF_3O_2$ (2632.38 + 547.31): calcd. C 51.98, H 6.08, N 11.89; found C 51.88, H 6.08, N 11.69. ESI-MS: correct isotope pattern for M^+ from 2630.3 to 2637.3. IR (KBr): $\tilde{\nu}$ = 3299s, 2959w, 2922w, 1658vs, 1543s, 1454w, 1203m (CF), 1174m, 833w, 800w, 701w, 628w cm^{-1}. ^1H NMR (signal ranges) in CD_3CN: δ = 0.74−2.80 (95 H, aliphatic protons), 2.80−3.40 (14 H, β-protons of Phe and His), 3.71 (t, 2 H, CH_2-Gly), 3.78−4.37 (21 H, α-protons), 7.13−7.33 (25 H, Phe-aromatic), 7.20−8.68 (26 H, NH and imidazole-CH).

Complex 1: Ammonia was bubbled for 3 min through a solution of **P22**·4.8HTFA (25 mg, 7.77 μmol) in 15 mL of dry THF. After pumping to remove excessive NH_3 and to reduce the volume to 10 mL, the mixture was filtered through a fine-porosity frit. A 0.01 M solution of anhydrous $ZnCl_2$ in THF (0.78 mL, 7.8 μmol) was then added. Slow evaporation of the solvent left a yellow oil. This was treated several times with a few millilitres of water to remove the remaining ammonia and ammonium salts. After prolonged pumping 21 mg (96%) of **1** remained as a pale yellow powder, m.p. 142 °C (dec.). $C_{128}H_{187}Cl_2N_{27}O_{23}S_5Zn$ (2768.68): calcd. C 54.53, H 6.81, N 13.66, Zn 2.36; found C 54.65, H 6.89, N 12.12, Zn 1.94. ESI-MS: *m*/*z* = 2733 (11) [M − Cl], 2698 (13) [M − 2Cl], 1349 (7) [M − 2Cl]$^{2+}$. IR (KBr): $\tilde{\nu}$ = 3300m, br, 2958w, 2924w, 1655vs, 1541s, 1453w, 1297w, 1129w, 802w, 700w cm^{-1}. ^1H NMR (CD_3CN): identical to **P22** except for the data in Table 1.

Complex 2: Prepared in a similar manner to complex **1** from 25 mg (7.77 μmol) of **P22**·4.8 HTFA and 0.78 mL (7.8 μmol) of a 0.01 M ZnI_2 solution. Yield 22 mg (96%), light brown powder, m.p. 179 °C (dec.). $C_{128}H_{187}I_2N_{27}O_{23}S_5Zn$ (2951.58): calcd. C 52.09, H 6.39, N 11.81, Zn 2.22; found C 49.58, H 6.29, N 11.72, Zn 2.66. ESI-MS: *m*/*z* = 2825 (59) [M − I], 2698 (20) [M − 2I], 1349 (45) [M − 2I]$^{2+}$. IR (KBr): $\tilde{\nu}$ = 3301m, br, 2957w, 2922w, 1655vs, 1540s, 1453w, 1296w, 1114w, 700w cm^{-1}.

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