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Coordination compounds of tripeptides and pentapeptides containing L-histidyl residues. Studies towards structural models for the active site of copper proteins

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Abstract. Five small, completely protected, L-histidyl(N^{τ}Bzl)-containing peptides, were synthesized and characterized by NMR spectroscopy, i.e., R-Ala-His(N^{τ}-Bzl)-Ala-R′ (**1**), R-His(N^{τ}-Bzl)-Ala-Ala-Ala-Met-R′ (**2**), R-His(N^{τ}-Bzl)-Ala-Ala-Ala-His(N^{τ}-Bzl)-R′ (**3**), R-His(N^{τ}-Bzl)-Ala-His(N^{τ}-Bzl)-Ala-His(N^{τ}-Bzl)-R′ (**4**), R-His(N^{τ}-Bzl)-Ala-His(N^{τ}-Bzl)-R′ (**5**), in which: R is phenylacetyl ($PhCH_2CO$-), R′ is phenylamino (-NHPh); Bzl is benzyl ($PhCH_2$-).
It has been shown that benzylation of histidine residues takes place at the N^{τ} atom of the imidazole. The resulting peptide derivatives appear to be good ligands for Co^{II}, Zn^{II} and Cu^{I}. Titrations of zinc chloride solutions towards peptide solutions (in dmso-d_6, dimethyl sulfoxide followed by NMR spectroscopy show the formation of the two species $[Zn(N^{\pi}His)_2Cl_2]$ and $[Zn(N^{\pi}His)(dmso)Cl_2]$. Copper(I)-chloride titrations result in formation of trigonal planar and tetrahedral complexes (in dmso-d_6). Zinc, copper and cobalt trifluoromethanesulfonates were also investigated, and corresponding tetrahedral species with additional dmso ligands replacing chloride were obtained. In addition, octahedral complexes have been found, as shown for cobalt(II) using ligand-field spectroscopy.
Conductivity experiments of zinc chloride complexes in dmso and chloroform indicate non-electrolytes. Their very low conductivity in methanol indicates only slight dissociation of chloride, in agreement with a tetrahedral geometry for zinc(II). For $[Co(N^{\pi}His)_2Cl_2]$, the UV-VIS adsorption spectra in dmso and in the solid state (diffuse reflection) are comparable, indicating tetrahedral geometry, with a CoN_2Cl_2 chromophore.

Introduction

Active sites of metallo-proteins are of considerable interest[1–3] for a variety of reasons and many inorganic chemists are interested in studying such sites in copper proteins. Many structural proposals have been published, based on simple model compounds containing copper and small azole ligands[3–10]. In addition, some investigations have been reported with histidyl-containing cyclic peptides[11–13] and unprotected linear peptides[14–18]. In most cases, however, the compounds differ from natural systems. Only a few models are known that resemble the active site closely, i.e., copper dioxygen complexes such as $[(L_3Cu)_2O_2]$, of *Karlin* et al., using simple imidazoles as ligands[8] and μ-η^2:η^2-peroxo dinuclear copper complex $[[Cu(HB(3,5\text{-}iPrpz)_3)]_2O_2]$ of *Kitajima* et al.[9,10] with a Cu...Cu distance of 3.56 Å and square-pyramidal geometry around each copper atom.

An alternative approach is the use of small histidyl-containing peptides, protected at the C and N terminals to imitate the backbone of the protein, as ligands to mimic active sites in proteins. We have chosen this approach to prepare model systems for active sites of hemocyanin (a dioxygen carrier) and the so-called "blue copper" proteins (electron-transfer proteins).
The active site of deoxy-hemocyanin contains a dinuclear copper site. In hemocyanin from *Panulirus interruptus*, each copper atom is surrounded by three histidyl residues, with Cu_A–N^{τ} distances of 1.96, 1.95 and 2.76 Å and Cu_B–N^{τ} distances of 1.95, 2.10 and 2.66 Å. The Cu_A–Cu_B distance amounts to 3.54 Å. Oxy-hemocyanin contains dioxygen bridged between the two copper atoms, present as peroxo (O_2^{2-}). As recently shown[19], dioxygen binds in the μ-η^2:η^2 binding mode for the oxygenated form of *Limulus polyphemus* subunit II. All the coordinating histidyl residues originate from the strong α-helical environment of the protein[20]. Sequence comparisons of seven hemocyanin proteins show that there is a large homology of the sequences near the copper-binding units[21,22].
Several crystal structures of "blue copper" proteins are now known[23–31]. The donating groups around the copper atom are two histidyl residues (coordinated by the N^{π} atom), a cysteinyl residue and usually also a methionyl residue, comprising distorted tetrahedral geometry. In the case of *Alcaligenes denitrificans*, a very weak axial interac-

List of abbreviations
R, $PhCH_2CO$-, phenyacetyl; R′, -NHPh, phenylamino; Bzl, benzyl; ONp, *p*-nitrophenol; Z, benzyloxycarbonyl; DCC, *N,N′*-dicyclohexylcarbodiimide; DMF, *N,N*-dimethylformamide; dmso-d_6, dimethyl-d_6 sulfoxide; NaOAc, sodium acetate; HOAc, acetic acid; TEAC, tetraethylammonium chloride; HPLC, high-performance liquid chromatography; NMR, nuclear magnetic resonance.

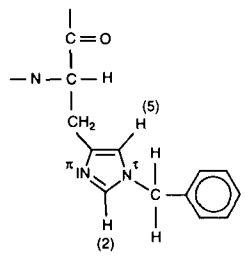


Figure 1. Structure (and labeling of the imidazole ring) of the benzylated histidyl residue.

tion of a nearby peptide carbonyl group is also present which, in fact, results in distorted trigonal pyrimidal geometry.

To mimic the active sites of the hemocyanin and azurine proteins, a number of tripeptides and pentapeptides containing histidyl residues has been selected for first investigation. Such peptides often form very stable tertiary structures by intramolecular hydrogen bonds, and these structures may be even further stabilized by coordination towards a metal ion[32-34]. The C and N termini in the selected synthetic peptides were blocked with the respective anilide and phenylacetyl groups and, as a result, two additional peptide bonds are present.

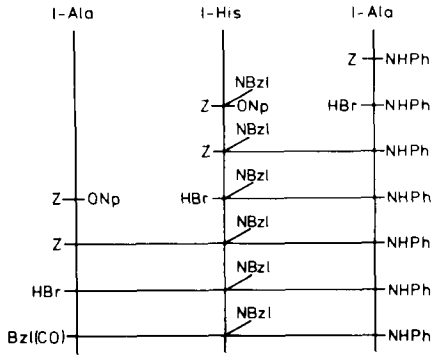
In our present investigation the following ligands were used in the protected forms: R-Ala-His(N^{τ}-Bzl)-Ala-R' (**1**), R-His(N^{τ}-Bzl)-Ala-Ala-Ala-Met-R' (**2**), R-His(N^{τ}-Bzl)-Ala-Ala-Ala-His(N^{τ}-Bzl)-R' (**3**), R-His(N^{τ}-Bzl)-Ala-His(N^{τ}-Bzl)-Ala-His(N^{τ}-Bzl)-Ala-His(N^{τ}-Bzl)-R' (**4**) and R-His(N^{τ}-Bzl)-Ala-His(N^{τ}-Bzl)-R' (**5**) [in which R is phenylacetyl ($PhCH_2CO$-) and R' in phenylamino (-NHPh)]. Coordination at the terminals of the peptides is excluded for steric reasons[14-18]. During synthesis of the peptides, it is necessary to protect the imidazole N^{τ} atom (Figure 1), and a benzyl group is usually chosen. This group is easy to introduce and appears to be very stable during the synthesis of the peptides. An additional advantage of this protection is that metal coordination can only take place at the N^{π} atom of the imidazole ring. Deprotecting to the original N-H function remains possible[35-36], allowing study of their ligand functions.

The metal salts $ZnCl_2$ and CuCl were selected because they contain diamagnetic d^{10} cations, allowing NMR investigation of complex formation. In addition, $CoCl_2$ was selected, so that UV-VIS spectroscopy could be applied to determine the geometry around the metal atoms. All three metal ions are known to prefer tetrahedral geometry in a non-aqueous environment. For NMR spectroscopy the most suitable solvent appeared to be dmso-d_6, (dimethyl sulfoxide), because the NH and α-CH resonances were separated from each other due to dipolar interaction of the sulfoxide group with the N-H protons of the peptide chain. A second advantage of dmso is the high solubility of both the peptides and their metal complexes in it.

Results and discussion

Materials and methods

The starting L-amino acids were commercial products (Sigma) and were used without further purification, as were benzyloxy carbonyl (Z) chloride, *p*-nitrophenol (ONp), *N,N'*-dicyclohexylcarbodiimide (DCC) and *N*-methylmorpholine (Janssen Chimica). Aniline (Janssen Chimica) was distilled under vacuum (15 mm Hg; 77°C) and stored in the dark under an argon atmosphere. An



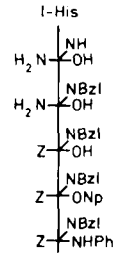
Scheme 1. The synthetic route to synthesize the tripeptide R-Ala-His(N^{τ}Bzl)-Ala-R' (1).

almost colourless 20-wt-% solution of hydrogen bromide in acetic acid was obtained by bubbling HBr through acetic acid ("Baker-analyzed" reagent) in a brown-coloured flask.

N,N-dimethylformamide, chloroform and tetrahydrofuran were dried and stored over molecular sieves (4A). Diethyl ether was dried and stored over anhydrous $MgSO_4$. Phenylacetic anhydride was prepared as described by *Autenrieth* and *Thomas*[41].

Synthesis of peptides was carried out by traditional methods[35-41], allowing the preparation of gram quantities. These syntheses are well known; therefore, only one representative example of the synthetic route, *i.e.*, preparing R-Ala-His(N^{τ}-Bzl)-Ala-R' (**1**) is presented in Scheme 1. In Scheme 2, the protection and modification of L-histidine to synthesize Z-L-His(N^{τ}Bzl)NHPh is shown as an example. This scheme has also been used to modify L-Ala and L-Met to the Z-L-AlaNHPh- and Z-L-MetNHPh-modified amino acids, respectively. The protection of the imidazole group by a benzyl (Bzl) group proceeds by using metallic sodium and benzyl chloride[35]. The protecting group for the amine terminus is a benzyloxycarbonyl (Z) group, which can easily be deprotected by HBr in anhydrous acetic acid[37]. The deprotection results in an active amino acid available for coupling reactions with an active Z–amino-acid-*p*-nitrophenyl-ester. The latter has been synthesized from a Z–amino-acid and *p*-nitrophenol using DCC in THF and, in the case, of the histidyl residue, $CHCl_3$[38,39]. The Z–amino-acid–*p*-nitrophenyl ester was converted to the Z–amino-acid-anilide[40]. At the end of the synthetic route, the HBr–peptide-aniline was converted to a phenylacetyl–peptide-anilide using phenylacetic anhydride[39]. Water was removed by azeotropic distillation using 2-propanol.

^{1}H-NMR spectra of intermediate products during the synthesis of the peptides were recorded at 200 MHz using a JEOL FX 200 spectrometer. ^{1}H-NMR spectra of the final compounds and titrations with metal chloride solutions, as well as ^{1}H–^{1}H and ^{13}C–^{1}H correlated spectra, were recorded at 300 MHz, using a Bruker WM-300



Scheme 2. Modification of L-histidine to the active amino acid to use for the coupling reactions.

Table I ^{1}H-NMR chemical shifts (ppm) in dmso-d_6 of the peptides [a].

Residue [b]	Resonance	Peptide [c]				
		1	2	3	4	5
	N-H	8.28 (d)	8.15 (d)	8.06 (d)	8.52 (d)	8.76 (d)
	α-CH	4.37 (dt)	4.40 (dt)	4.52 (dt)	4.55 (dt)	4.61 (dt)
His I	β-CH_2	2.89 (m)	2.82 (m)	2.94 (m)	2.98 (m)	2.97 (m)
	Im-ring [d]	7.68; 6.95	7.61; 6.88	7.73; 6.96	7.63; 6.92	7.67; 6.89
	ImBzl-CH_2	5.06	5.12	5.09	5.06	5.07
	ImBzl-CH	7.25–7.17	7.35–7.18	7.33–7.13	7.32–7.18	7.34–7.14
	N-H			8.16 (d)	8.54 (d)	8.21 (d)
	α-CH			4.42 (dt)	4.47 (dt)	4.44 (dt)
His II	β-CH_2			2.85 (m)	2.85 (m)	2.81 (m)
	Im-ring [d]			7.72; 6.87	7.63; 6.89	7.67; 6.82
	ImBzl-CH_2			5.09	5.06	5.07
	ImBzl-CH			7.33–7.13	7.32–7.18	8.34–7.14
	N-H				8.15 (d)	
	α-CH				4.45 (dt)	
His III	β-CH_2				2.77 (m)	
	Im-ring [d]				7.63; 6.80	
	ImBzl-CH_2				5.06	
	ImBzl-CH				7.32–7.18	
	N-H		7.89 (d)			
	α-CH		4.42 (dt)			
Met I	β-CH_2		1.99 (m)			
	γ-CH_2		2.03			
	CH_3		2.50			
	N-H	7.91 (d)	7.84 (d)	7.93 (d)	8.04 (d)	8.36 (d)
Ala I	α-CH	4.28 (dt)	4.22 (dt)	4.11 (dt)	4.05 (dt)	4.09 (dt)
	CH_3	1.16 (d)	1.24 (d)	1.16 (d)	1.06 (d)	1.10 (d)
	N-H	8.51 (d)	8.78 (d)	8.69 (d)	8.27 (d)	
Ala II	α-CH	4.18 (dt)	4.24 (dt)	4.27 (dt)	4.11 (dt)	
	CH_3	1.22 (d)	1.26 (d)	1.25 (d)	1.12 (d)	
	N-H		8.33 (d)	8.28 (d)		
Ala III	α-CH		4.14 (dt)	4.12 (dt)		
	CH_3		1.19 (d)	1.16 (d)		
anilide	N-H	9.84	9.75	9.60	9.53	9.58
	Phe-CH	7.61; 7.29	7.63; 7.06	7.60; 7.03	7.66; 7.02	7.64; 7.04
		7.04	7.35–7.18	7.33–7.13	7.32–7.18	7.34–7.14
phenyl-	Bzl-CH_2	3.50	3.44	3.41	3.40	3.37
acetyl	Phe-CH	7.25–7.17	7.35–7.18	7.33–7.13	7.32–7.18	7.34–7.14

[a] dmso as internal standard. [b] The Roman characters mean the something residue viewed from the C terminal of the peptide.): R-Ala_{II}-$His(N^{\tau}\text{-}Bzl)_I$-$Ala_I$-R′ (**1**), R-$His(N^{\tau}\text{-}Bzl)_I$-$Ala_{III}$-$Ala_{II}$-$Ala_I$-$Met_I$-R′ (**2**), R-$His(N^{\tau}\text{-}Bzl)_{II}$-$Ala_{III}$-$Ala_{II}$-$Ala_I$-$His(N^{\tau}\text{-}Bzl)_I$-R′ (**3**), R-$His(N^{\tau}\text{-}Bzl)_{III}$-$Ala_{II}$-$His(N^{\tau}\text{-}Bzl)_{II}$-$Ala_I$-$His(N^{\tau}\text{-}Bzl)_I$-R′ (**4**), R-$His(N^{\tau}\text{-}Bzl)_{II}$-$Ala_I$-$His(N^{\tau}\text{-}Bzl)_I$-R′ (**5**). [c] (d) = doublet, (dt) = doublet of triplet, (m) = multiplet. [d] Im-ring: resp H2 and H5.

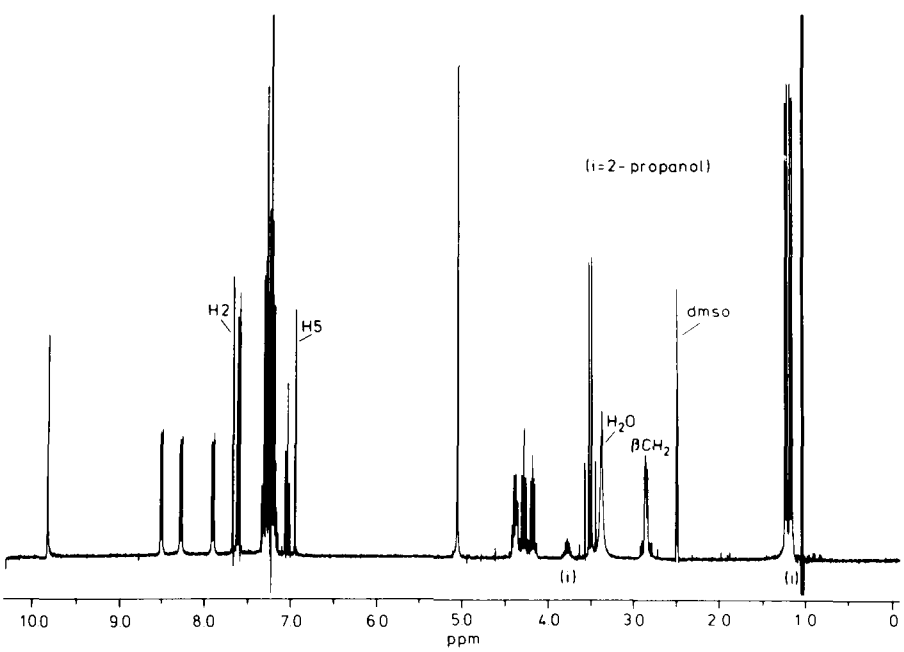


Figure 2. ^{1}H-NMR spectrum of the free ligand R-Ala-His(N^{τ}-Bzl)-Ala-R′ (1), in dmso-d_6. The hydrogens with change most upon complexation towards metal ions are indicated. For other hydrogen assignments see Table I.

spectrometer. NMR experiments were carried out in well-dried dmso-d_6, stored over molecular sieves 4Å.
The Pharmacia HPLC gradient system, with a Spherisorb 5 μm, 4 × 250 mm column, a Spectrovision FD-300 fluorescence detection system and a flow rate of 0.4 ml/min, was used to analyse the hydrolysates of R-His(N^{τ}-Bzl)-Ala-Ala-Ala-Met-R′ (**2**) and R-His(N^{τ}-Bzl)-Ala-Ala-Ala-His(N^{τ}-Bzl)-R′ (**3**). The following buffer systems were used: (A) 0.03 molar NaOAc (pH 4 by HOAc and 85 volume % of acetonitrile); (B) a 0.03 molar NaOAc (pH 7.6, 50 volume % of acetonitrile) from 100% A up to 100% B during 45 minutes and 10 minutes constant. Complete hydrolysis of peptides was obtained by refluxing the peptides in 6M hydrochloric acid for 12 h. The procedure of *Nimura* and *Kinoshita*[42] (*o*-phthalaldehyde + *N*-acetyl-L-cysteine as chiral derivatization reagent) was used.
Metal-chloride complexes were prepared by weighing the anhydrous metal chlorides and peptides in molecular compositions expected for tetrahedral geometry, *e.g.*, $[Zn(RAHAR'_2)_2Cl_2]$, $[Zn(RHAAAHR')Cl_2]$, $[Cu(RAHAR')_3Cl]$, etc. In the case of CuCl, the samples were prepared under an argon atmosphere and the NMR tubes were properly sealed.
The different molar ratios (peptide to metal chloride) for the metal titrations were obtained by adding calculated volumes of a metal chloride solution of known concentration (using a micro-syringe) directly into an NMR tube containing a known solution of the peptide (approximately 0.02 mmol).
Conductivity measurements of 10^{-3}M $[Zn(RHAAAHR')Cl_2]$ solutions in dimethyl sulfoxide, methanol, or chloroform were carried out using a Philips PW 9526 digital conductivity meter with automatic temperature compensation to 293K.
Syntheses of the solid-state complexes $[Co(RAHAR')_2Cl_2]$, $[Co(RHAAAHR')Cl_2]$ and $[Zn(RHAAAHR')Cl_2]$ were performed in methanol. Peptide **1** (0.12 mmol) (in the case of peptide **3** 0.06 mmole) was dissolved in 10 ml ethanol and added to 10-ml solution of $ZnCl_2$ (0.06 mmol) in methanol by room temperature. After one week, a solid had precipitated, which was isolated by decantation and washed three times with diethyl ether and then dried in air.
UV-VIS measurements were carried out in dimethyl sulfoxide using a Perkin–Elmer 330 spectrophotometer with a 3600 data station; the same equipment fitted with a reflectance attachment using MgO as a reference material was used for solid-state measurements.

Characterisation of peptides

All peptides were characterized using ^{1}H-NMR and correlated (2D-COSY) NMR spectroscopy. The spectral data are listed in Table I. All five peptides contained one mole

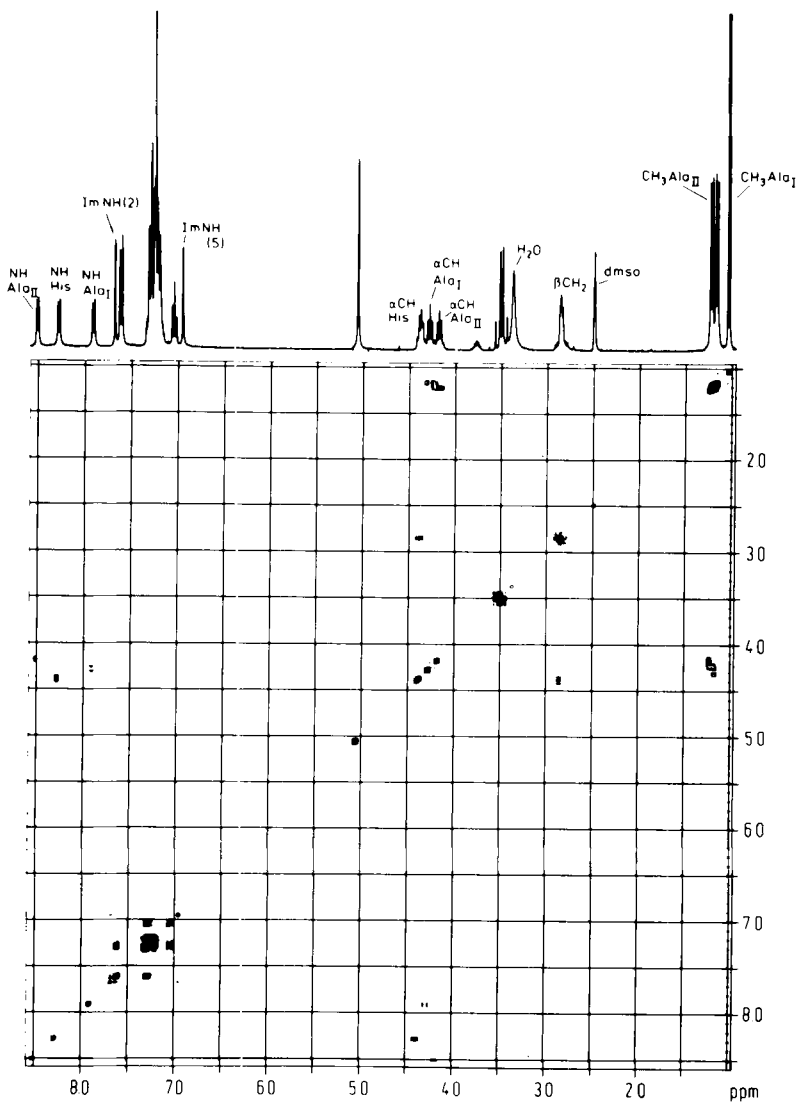


Figure 3. Correlated $^1H-^1H$ spectrum (COSY) of the free ligand R-Ala-His(N^{τ}-Bzl)-Ala-R′ (1), in DMSO-d_6.

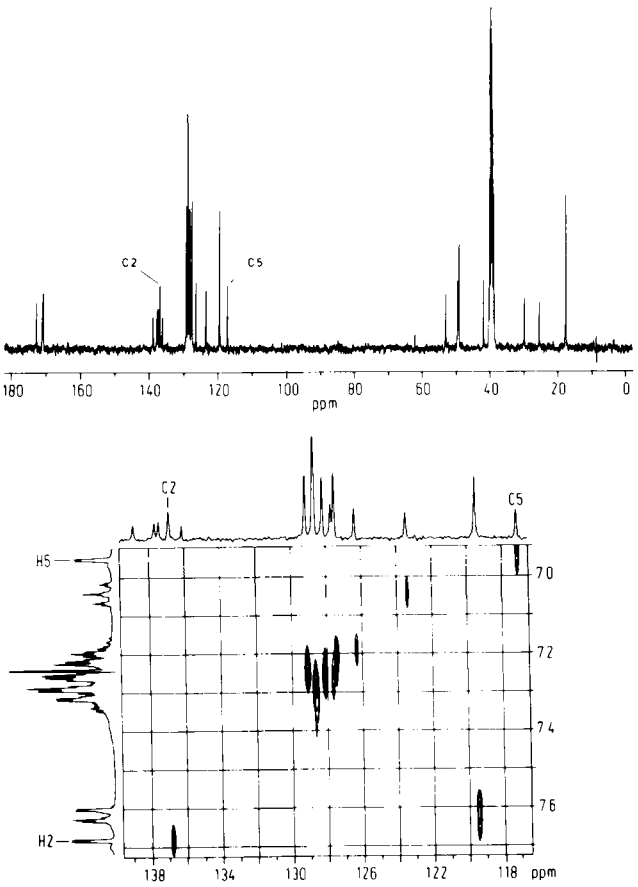


Figure 4. ^{13}C-NMR spectrum and the expanded region of the $^{13}C-^{1}H$ correlated NMR spectrum (COSY) of the free ligand R-Ala-His(N$^{\tau}$-Bzl)-Ala-R' (1) in dmso-d_6.

of crystal water per mole of peptide (observed at 3.34 ppm) and it is not possible to remove this "crystal water" by distillation of the azeotrope 2-propanol/water. In a few cases, not all 2-propanol could be removed from the peptides (Figure 2, i); fortunately, this solvent does not disturb the subsequent reactions.

As an example, the characterization of ligand R-Ala-His(N$^{\tau}$-Bzl)-Ala-R' (**1**) will be discussed. Most of the resonances can be assigned directly (Figure 2) from the ^{1}H-NMR spectra. However, for the α-CH and NH hydrogens, it is not obvious which α-CH and NH hydrogen belong to which residue and only the $^{1}H-^{1}H$-correlated spectrum provides clarity (Figure 3). All cross-peaks are well defined, except for the ring hydrogens of the imidazole. A $^{13}C-^{1}H$-correlated spectrum (Figure 4) has resolved this problem. Data have been published[43] for the imidazole ring carbons, δ(C2) 135.7 ppm and δ(C4, C5) 121.8 ppm. In ligand **1**, the resonances for C2 and C5 are found at 136 ppm and 117 ppm (Figure 4), respectively. The $^{13}C-^{1}H$-correlated spectrum clearly shows that C5 correlates with H5, and C2 correlates with H29 (Figure 4), and allows all ^{1}H resonances of ligand **1** to be assigned.In the synthesis of the ligand R-His(N$^{\tau}$-Bzl)-Ala-Ala-Ala-Met-R' (**2**) some racemization has taken place, the coupling reaction of the dipeptide Z-L-Ala-L-Ala-ONp with the active dipeptide HBr-L-Ala-L-Met-NHPh, to form the tetrapeptide Z-Ala-Ala-Ala-Met-NHPh. Partial racemisation has taken place on the central alanine.

The HPLC chromatogram of the hydrolysate of peptide R-His(N$^{\tau}$-Bzl)-Ala-Ala-Ala-Met-R' (**2**) shows a ratio of 93/7 of R-(L)-His(N$^{\tau}$-Bzl)-L-Ala-L-Ala-L-Ala-L-Met-R' and R-(L)-His(N$^{\tau}$-Bzl)-L-Ala-*D*-Ala-L-Ala-L-Met-R'. The amount of the latter isomer is too small to be observed in the ^{1}H-NMR spectra of the ligand and its metal complexes. Apparently, all resonances are observed as single signals in the ^{1}H-NMR spectra. Therefore, this racemic mixture has been used, also because only a small influence on the coordination properties towards the metal ions is expected. The synthesis of this pentapeptide in the pure L configuration is in progress to definitely confirm our expectations of its coordination properties.

In the case of the synthesis of ligand R-His(N$^{\tau}$-Bzl)-Ala-Ala-Ala-His(N$^{\tau}$-Bzl)-R' (**3**), the active mono amino acids are used and this does not result in racemization (as shown by HPLC). Investigations on the structures of the ligand R-His(N$^{\tau}$-Bzl)-Ala-Ala-Ala-His(N$^{\tau}$-Bzl)-R' in

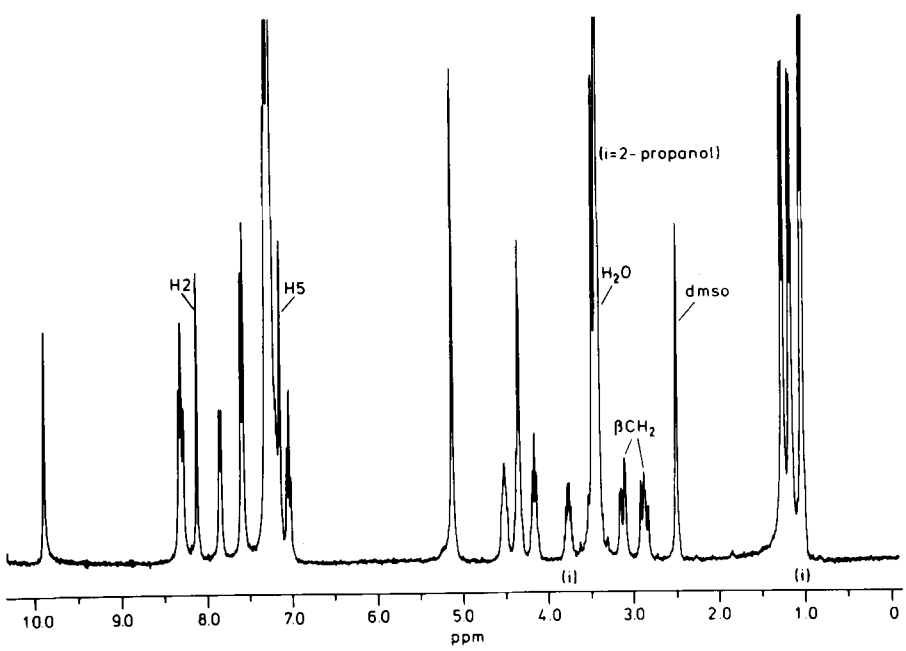


Figure 5. ^{1}H-NMR spectrum of the [Zn(R-Ala-His(N$^{\tau}$)-Ala-R')$_2$Cl$_2$] complex in dmso-d_6, in which the resonances for protons at positions of H2, H5 and the βCH_2 are indicated. Comparison with the positions of these hydrogens with those in Figure 2 (the free ligand) provides the change in chemical shift as a result of complexation towards the zinc ion.

dmso-d_6, as well as on the zinc complexes in dmso-d_6, are in progress.

Metal-binding sites

Since the publication of *Du Vigneaud* (1936), it has been unclear whether benzylation takes place at the N^{π} or at the N^{τ} atom of the imidazole ring (Figure 1)[35]. Direct evidence for benzylation on the N^{τ} atom of the imidazole in our peptides comes from the ^{1}H-NMR spectrum of the complex of ligand **1** with $ZnCl_2$, namely $[Zn(RAHAR')_2Cl_2]$. Upon complexation, relatively large changes are to be expected for those hydrogens which are close to the zinc centre. In the case of ligand **1**, the resonance for H2 shifts more downfield than the resonance for H5 (0.47 ppm and 0.15 ppm, respectively; Figure 5)[11], strongly indicating that H2 is closer to the zinc centre and that, therefore benzylation has taken place at N^{τ}.

The other peptides have also been studied as ligands towards $ZnCl_2$, $CoCl_2$ and CuCl. In general, they show the same changes in ^{1}H-NMR spectra with regard to the shift of resonances of H2 and H5, except for coordination to $CoCl_2$. As little as a few mole percent of $CoCl_2$ results in severe broadening of the resonances, especially for the H2 and H5 hydrogens. Higher percentages of $CoCl_2$ also result in broadening of the other resonances, and prevent assignments of the different resonances. On the other hand, for $ZnCl_2$ and CuCl additions, the resonances remain very sharp (at all molar ratios) and can be easily assigned.

The ^{1}H-NMR signals of the β-CH_2 group (2.89–2.82 and 2.98 ppm), split in the free ligands because they are diastereotopic, and show further splitting originating from the steric effects when the histidyl residue coordinates at the metal ion. As a result, the histidyl residue is fixed, hampering free rotation of the imidazole group. There are no indications that coordination has taken place with other possible donor atoms in the peptide chain (such as carbonyl oxygens), as no significant changes are found in positions of the hydrogens signals near these donor centres.

In general, the spectra of CuCl are similar to those of $ZnCl_2$. However, in the case of the complex with R-His(N^{τ}-Bzl)-Ala-Ala-Ala-Met-R' (**2**), interaction of the thioether of the methionyl residue with the copper centre appears to be occur. This interaction is clearly not observed for $ZnCl_2$ and is in agreement with some tetrahedrally coordinated model compounds with thio-ether-containing azole ligands, where binding to copper(I) occurs and binding to zinc(II) does not take place[44–47]. The diastereotopically split γ-CH_2 methionine resonance shifts 0.16 ppm downfield (in the case of a Cu^1/RHAAAMR' molar ratio of 6:1). The increase of the splitting in the β-CH_2 hydrogens (1.82 to 1.95 and 2.07 ppm) is also indicative of binding of the thio-ether sulfur with Cu(I).

Titrations of different peptide solutions with $ZnCl_2$ results in a change of chemical shifts, depending on the molar ratio of $ZnCl_2$ towards peptide. The largest change was observed for H2. In this case, different slopes are observed in the curves. These different slopes would suggest the possibility of formation of several complexes (Figure 6, A, B and C; I to XI). When more than one type of complex is present in solution, it is nearly impossible to calculate the complex equilibria constants from the NMR spectra only by the changes of the H2 signal[48]. The equilibria which are likely to be present for all five peptides are given in Scheme 3. These complex formations are observed in all cases. The titrations of peptides **3** and **4** will be discussed in some detail (Figure 6B). The ligand R-His(N^{τ}-Bzl)-Ala-Ala-Ala-His(N^{τ}-Bzl)-R' (**3**) shows unusual donor properties towards the zinc ion, which are possibly caused by internal hydrogen bondings. Changes in the chemical shifts of the amide-NH resonances of the residues indicates such bondings (especially those of the central alanine residue). It is clearly observed that the slope of the curve by addition of $ZnCl_2$ up to a molar ratio of 1:1 (*i.e.*, $1Zn/2N^{\pi}$) is steeper and results in a more downfield chemical shift of hydrogen H2 than in the case of ligand R-His(N^{τ}-Bzl)-Ala-His(N^{τ}-Bzl)-Ala-His(N^{τ}-Bzl)-R' (**4**). Subsequent additions of $ZnCl_2$ to the complex solution does not result in major changes in the chemical shift. Above the molar ratio 2:1, the curve is constant and independent of the used molar ratios, inferring that complex V is more stable (in dmso-d_6) than complex VI. This is easily seen, since the $\Delta\delta$ of complex V compared with complex VI is small. These changes in the chemical shifts of the NH and α-CH environments clearly suggest that a very stable complex has been formed after complexation with $ZnCl_2$ in this case. Internal hydrogen bonds of the peptide probably further stabilize the tetrahedral geometry around the zinc ion. The titration of $ZnCl_2$ to a R-His(N^{τ}-Bzl)-Ala-His(N^{τ}-Bzl)-Ala-His(N^{τ}-Bzl)-R' (**4**) solution results in a similar reaction up to a molar ratio 1.5:1 (*i.e.*, $1Zn/2N^{\pi}$, VII Figure 6B), but with a flatter slope than in the case of ligand R-His(N^{τ}-Bzl)-Ala-Ala-Ala-His(N^{τ}-Bzl)-R' (**3**). This indicates different donor properties of the peptide to the zinc ion. This titration resembles more the titration of ligand R-His(N^{τ}-Bzl)-Ala-His(N^{τ}-Bzl)-R' (**5**) (see Figure 6C) and it is likely that

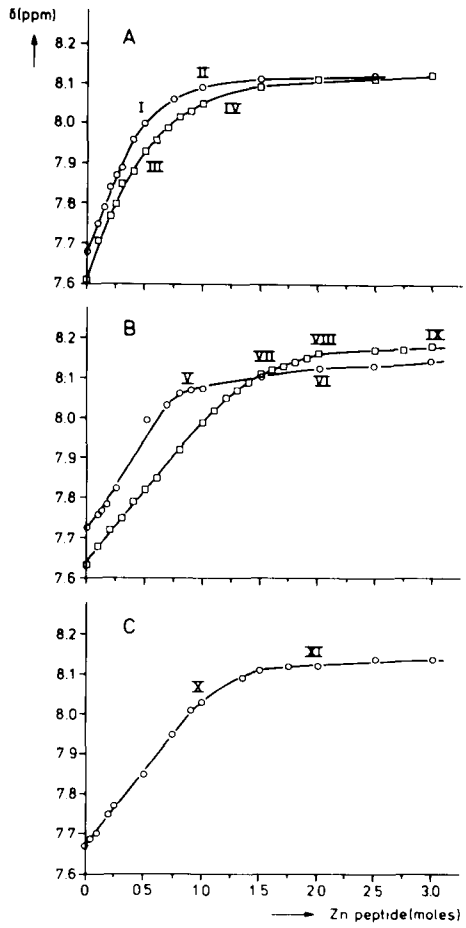


Figure 6. Titration curves of $ZnCl_2$ with the various peptides (0.02 mmole), determined by the chemical-shift changes of the resonance of hydrogen H2 (in dmso-d_6). The Roman characters indicate the different possible complexes (see text). A: (○) R-Ala-His(N^{τ}-Bzl)-Ala-R' (1) and (□) R-His(N^{τ}-Bzl)-Ala-Ala-Ala-Met-R' (2). B: (○) R-His(N^{τ}-Bzl)-Ala-Ala-Ala-His(N^{τ}-Bzl)-R' (3) and (□) R-His(N^{τ}-Bzl)-Ala-His(N^{τ}-Bzl)-Ala-His(N^{τ}-Bzl)-R' (4). C: R-His(N^{τ}-Bzl)-Ala-His(N^{τ}-Bzl)-R' (5).

(1) R_1-Ala-His(N^τ-Bzl)-Ala-R_2

$(R_1AHAR_2)_n + ZnCl_2 \rightleftharpoons [Zn(R_1A\underline{H}AR_2)_2Cl_2]$ **I** $+ (R_1AHAR_2)_{n-2}$

$[Zn(R_1A\underline{H}AR_2)_2Cl_2] + ZnCl_2 \rightleftharpoons 2[Zn(R_1A\underline{H}AR_2)(dmso)Cl_2]$ **II**

(2) R_1-His(N^τ-Bzl)-Ala-Ala-Ala-Met-R_2

$(R_1HAAAMR_2)_n + ZnCl_2 \rightleftharpoons [Zn(R_1\underline{H}AAAMR_2)_2Cl_2]$ **III** $+ (R_1HAAAMR_2)_{n-2}$

$[Zn(R_1\underline{H}AAAMR_2)_2Cl_2] + ZnCl_2 \rightleftharpoons 2[Zn(R_1\underline{H}AAAMR_2)(dmso)Cl_2]$ **IV**

(3) R_1-His(N^τ-Bzl)-Ala-Ala-Ala-His(N^τ-Bzl)-R_2

$(R_1HAAAHR_2)_n + ZnCl_2 \rightleftharpoons [Zn(R_1\underline{H}AAA\underline{H}R_2)Cl_2]$ **V** $+ (R_1HAAAHR_2)_{n-1}$

$[Zn(R1\underline{H}AAA\underline{H}R_2)Cl_2] + ZnCl_2 \rightleftharpoons [Zn_2(R_1\underline{H}AAA\underline{H}R_2)(dmso)_2Cl_4]$ **VI**

(4) R_1-His(N^τ-Bzl)-Ala-His(N^τ-Bzl)-Ala-His(N^τ-Bzl)-R_2

$(R_1HAHAHR_2)_n + ZnCl_2 \rightleftharpoons [Zn(R_1\underline{H}A\underline{H}AHR_2)Cl_2]$ **VII** $+ (R_1HAHAHR_2)_{n-1}$

$[Zn(R_1\underline{H}A\underline{H}AHR_2)Cl_2] + ZnCl_2 \rightleftharpoons [Zn_2(R_1\underline{H}A\underline{H}A\underline{H}R_2)(dmso)Cl_4]$ **VIII**

$[Zn_2(R_1\underline{H}A\underline{H}A\underline{H}R_2)(dmso)Cl_4] + ZnCl_2 \rightleftharpoons [Zn_3(R_1\underline{H}A\underline{H}A\underline{H}R_2)(dmso)_3Cl_6]$ **IX**

(5) R_1-His(N^τ-Bzl)-Ala-His(N^τ-Bzl)-R_2

$(R_1HAHR_2)_n + ZnCl_2 \rightleftharpoons [Zn(R_1\underline{H}A\underline{H}R_2)Cl_2]$ **X** $+ (R_1HAHR_2)_{n-1}$

$[Zn(R_1\underline{H}A\underline{H}R_2)Cl_2] + ZnCl_2 \rightleftharpoons [Zn_2(R_1\underline{H}A\underline{H}R_2)(dmso)_2Cl_4]$ **XI**

Scheme 3. The equilibria which are likely to be present for each of the peptides (in dmso-d_6). The coordinating histidyl residues by ImN^π groups towards Zn^{II} are underlined and the several complexes specified with the Roman characters correspond with those given in Figure 6A, B and C.

ligand R-His(N^τ-Bzl)-Ala-His(N^τ-Bzl)-Ala-His(N^τ-Bzl)-R' (**4**) reacts in the same way, thus with the central histidyl residue and the histidyl residue at the phenylacetyl side of the peptide, up to a molar ratio of 1.5 : 1 (*i.e.*, 1Zn/2N^π). However, upon addition of an excess of $ZnCl_2$ to this solution, the chemical shifts change up to a complex ratio of 2 : 1 (*i.e.* 2Zn/3N^π, VIII Figure 6B). Addition of even more $ZnCl_2$ results in complex IX near the molar ratio 3 : 1 (*i.e.*, 3Zn/3N^π, IX Figure 6B).

Titrations of CuCl to the five peptide solutions (dmso-d_6) in dioxygen-free conditions (under argon), results in a variety of complexes, varying from only one N^πHis donor group per copper ion up to three N^πHis donor groups per copper ion. Formation of trigonal planar and tetrahedral geometries are known[49] to be possible for Cu(I). No attempts were undertaken to identify all these different complexes.

Titrations of zinc trifluoromethanesulfonate to the different peptides result in a smaller change of chemical shift of the different hydrogens compared to $ZnCl_2$. In this case, H2 also shows the largest change (0.16 ppm downfield). Different slopes are observed from the curves, and octahedral complexes of $[Zn(N^\pi His)_{m-n}(dmso)_n](CF_3SO_3)_2$ ($m = 6$; $n = 0$–5) are likely to be formed. When $n > 0$, the octahedral geometry is completed by dmso ligands.

To model the active sites of the copper proteins, the non-coordinating trifluoromethanesulfonates as anion, are preferred, because of the tendency of the chlorine anion to bind the metal ion. Replacing the chloride ion by other donor atoms may be difficult. Deprotection of the imidazole (N^τBzl) group of the histidyl residues of the ligands is necessary in a next step to approach the natural systems. As a first try, deprotection of the N^τBzl group of the ligands, R-Ala-His(N^τ-Bzl)-Ala-R' (**1**) and R-His(N^τ-Bzl)-Ala-Ala-Ala-His(N^τ-Bzl)-R' (**3**) to the original ImNH function (small-scale synthesis) in liquid ammonia was carried out and did not result in any problems. A few complexes with those ligands and zinc chloride, *i.e.* $[Zn(RAHAR')_2Cl_2]$, $[Zn(RAHAR')(dmso)Cl_2]$, $[Zn(RHAAAHR')Cl_2]$ and $[Zn_2(RHAAAHR')(dmso)_2Cl_4]$, were investigated by 300-MHz NMR in dmso-d_6. The same changes (and in approximately the same proportions) in the chemical shifts were observed as with the complexes of the histidyl (N^τBzl) protected ligands, implying that zinc coordination also takes place at the N^π atom of the histidyl residue.

Optical spectra and conductivities

The UV-VIS experiments performed with $CoCl_2$ (Table II) clearly indicate a tetrahedrally based chromophore for Co^{II}. The small differences in the λ_{max} values must be caused by the slightly different modes of coordination. In the case of the peptides **1** and **2**, coordination takes place by the imidazole group of the histidyl of two separated ligands. In the case of the peptides (**3**) and (**4**), coordination most likely takes place by the imidazole groups of the histidyl residues from one peptide. Those structures are consistent with the metal titrations of the peptides followed by NMR. The molar metal-to-peptide ratios are in agreement with the different complex formations (no loss of metal).

Evidence for non-coordination of dmso (dimethyl sulfoxide) to Co^{II} is obtained from the ligand-field spectrum in

Table II UV-VIS adsorption data and some molar extinction coefficients of $CoCl_2$ and $Co(CF_3SO_3)_2$ complex solutions in dmso and in the solid state (diffuse reflectance).

	Complex	λ_{max} (10^3 cm^{-1})	ϵ ($cm^2 \cdot mmol^{-1}$)
In dmso [a]	$[CoCl_4]^{2-}$,	14.8	246
	$[Co(RAHAR')_2Cl_2]$	15.6	263
	$[Co(RHAAAMR')_2Cl_2]$	15.5	
	$[Co(RHAAAHR')Cl_2]$	15.9	n.d. [b]
	$[Co(RHAHAHR')Cl_2]$	16.0	
	$[Co(RHAHR')Cl_2]$	15.9	
	$[Co(dmso)_6](CF_3SO_3)_2$	18.6	10
	$[Co(RAHAR')(dmso)_5](CF_3SO_3)_2$	18.8	
	$[Co(RHAAMR')(dmso)_5](CF_3SO_3)_2$	19.1	n.d. [b]
	$[Co_2(RHAAAHR')(dmso)_{10}](CF_3SO_3)_4$	19.0	
	$[Co_3(RHAHAHR')(dmso)_{15}](CF_3SO_3)_6$	18.9	16
	$[Co_2(RHAHR')(dmso)_{10}](CF_3SO_3)_4$	19.2	26
	$[Co_3(RHAHAHR')(dmso)_3Cl_6](TEAC^+CF_3SO_3^-)_6$	15.9	197
	$[Co_2(RHAHR')(dmso)_2Cl_4](TEAC^+CF_3SO_3^-)_4$	15.8	166
Solid state	$[Co(RAHAR'_2)_2Cl_2]$	15.6	
	$[Co(RHAAAHR'_2)Cl_2]$	15.9	

[a] 10^{-2} M. [b] n.d.: not determined.

dmso and, in the solid state, in the case of ligand **1** and **3**. Both spectra are the same (see Table II). Therefore, an equilibrium:

$$2\ CoCl_2 + 6\ dmso \rightleftharpoons [Co(dmso)_6]^{2+}[CoCl_4]^{2-}$$

as known[50] for free $CoCl_2$, can be excluded. The blank experiment (free $CoCl_2$ in dmso) results in a different value for λ_{max} and for the molar extinction coefficient (ϵ), which are characteristic for the $[CoCl_4]^{2-}$ anion (see Table II).

UV-VIS measurements in dmso of $[Co(N^{\pi}His)_{m-n}(dmso)_n](CF_3SO_3)_2$ complexes ($m = 6$; $n = 0$–5) agree with octahedral geometries, as deduced from the much lower molar extinctions and the shifted band maxima. When $n = 0$ and $n = 2$, the adsorption is very weak and undetectable, although, in all cases, the adsorption is significantly higher than in blank experiments. When tetraethylammonium chloride (TEAC) is added to the $[Co_2(RHAHR')(dmso)_{10}](CF_3SO_3)_4$ complex solution, λ_{max} changes from $19.2 \cdot 10^3$ to $15.8 \cdot 10^3$ cm^{-1}, which means that the more stable tetrahedral geometry is stabilized by the presence of coordinated chloride ions. The molar extinction coefficient (ϵ) changes from 26 to 166 $cm^2 \cdot mmol^{-1}$ (see Table II).

Conductivity experiments of a complex solution $[Zn(RHAAAHR')Cl_2]$ (10^{-3} molar) in dimethyl sulfoxide, methanol and chloroform results in a conductivity of 7.6, 37.0 and < 0.1 $\mu S \cdot cm^{-1}$ respectively [cf. a 10^{-3}-molar $Zn(RHAAAHR')_2(CF_3SO_3)_2$ solution yields a conductivity of 52.8 $\mu S \cdot cm^{-1}$ in dimethyl sulfoxide and 130.0 $\mu S \cdot cm^{-1}$ in methanol). These results indicate very small ionisation of the chloride ions at the zinc ion. Apparently, the two chloride ions in $ZnCl_2$ remain coordinated (see also the NMR $ZnCl_2$-titration curves, Figure 6) to form tetrahedral geometry, as well as the λ_{max} comparisons from UV-VIS measurements of the solutions and solid states of the cobalt complexes.

NMR experiments of $CoCl_2$ in the presence of ligand R-His(N^{τ}-Bzl)-Ala-Ala-Ala-Met-R' (**2**) show that there is no influence on the γ-CH_2 hydrogens of the methionyl residue. Because no broadening of these resonances are observed, it is likely that the thio ether of the methionyl is not near the cobalt centre under these conditions. Even when an excess of $CoCl_2$ is used, λ_{max} does not change. This is in agreement with the ^{1}H-NMR measurements of $ZnCl_2$ in the presence of ligand R-His(N^{τ}-Bzl)-Ala-Ala-Ala-Met-R' (**2**), where no change in the chemical shift of H2 is observed upon adding excess $ZnCl_2$.

Concluding remarks

NMR investigations of all five peptides has allowed assignments of all hydrogens, and provided evidence for benzylation at the N^{τ} atom of the L-histidine amino acid. Coordination of the peptides towards metal ions results in major changes of the chemical shift of the H2 hydrogen of the histidyl residue downfield and coordination take place only at the N^{π} atom of the histidyl group. There are no other indications from NMR spectra for coordination by other possible donor atoms in the peptides, not even when excesses of metal ions are used. Only the sulfur atom of the methionyl residue in ligand R-His(N^{τ}-Bzl)-Ala-Ala-Ala-Met-R' (**2**), appears to bind to Cu^{I}, but not to Zn^{II} and Co^{II}.

Indication for intramolecular hydrogen-bond stabilisation is found for the pentapeptide R-His(N^{τ}-Bzl)-Ala-Ala-Ala-His(N^{τ}-Bzl)-R' (**3**) in the $[Zn(RHAAAHR')Cl_2]$ complex, and this already stable structure is likely to be further stabilized by the coordination.

UV-VIS measurements of the Co^{II} complexes have made clear that dimethyl sulfoxide is not a competitive ligand with the imidazoles of the peptides and that tetrahedral geometries are formed under the conditions used. Dimethyl sulfoxide does not coordinate to the free cobalt chloride to form the $[Co(dmso)_6]^{2+}$ cation and the $[CoCl_4]^{2-}$ anion. Titrations of the peptides with $ZnCl_2$ and CuCl also suggest tetrahedral geometries, whereas conductivity experiments for $ZnCl_2$ in dimethyl sulfoxide and methanol at best indicate partial ionisation of the chloride atoms.

The study of the coordination of protected histidyl-containing peptides is a first step in the synthesis of model peptides for hemocyanin and azurine as found in natural systems. Further research will be devoted to deprotection of histidyl-N^{τ}Bzl to histidyl-NH residues and coordination of these deprotected peptides towards copper ions with the non-coordinated anion trifluoromethanesulfonate. To obtain suitable model compounds mimicking the active sites of the metallo-proteins, 1:1 mixtures of the described peptides will also be required. Investigations are in progress in this area. To model the active site of hemocyanin, a combination of $CuCF_3SO_3$ and the ligands R-Ala-His(ImNH)-Ala-R' and R-His(ImNH)-Ala-Ala-Ala-His(ImNH)-R' in a molar ratio of 1:1:1 is required. For this, the $[Cu(HisImNH)_3]^+(CF_3SO_3)^-$ complex will be a starting material. Oxidation of this complex to create a copper–peroxo–copper bridge (with pure dioxygen) could provide a useful model compound. Current work is directed to finding the optimal circumstances and solvents for these reactions. Deprotection of the histidyl-N^{τ}Bzl groups to the histidyl-ImNH groups of the other ligands and reactions with copper(I) ions to model the copper proteins with the above ligands in deprotected form are also in progress.

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