Scaffolded amino acids as a close structural mimic of type-3 copper binding sites†

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We report the use of triazacyclophane (TAC)-scaffolded amino acids as a structural mimic for 3-histidine metal-binding sites in metalloproteins, especially for the mimicry of type-3 copper binding sites as are present in hemocyanin, tyrosinase and catechol oxidase.

Since the unravelling of enzyme active site structures and mechanisms,[1] scientists have felt challenged to develop appropriate mimics.[2] Although impressive results have been obtained in the field of the mimicry of metalloenzyme function by (bio)inorganic approaches,[3] the study of close structural mimics that contain virtually identical components as found in the active sites has been lagging behind.[4] This is probably due to relatively complicated synthetic strategies required for this, in addition to participation of amide nitrogen atoms and *C*- or *N*-terminal functionalities[5] in the coordination to metal ions. In this report, we present the first amino acid based close structural mimic of type-3 copper binding sites in proteins as are found in hemocyanin, tyrosinase and catechol oxidase.[6] To achieve this, a scaffold was decorated with three histidine residues and the copper(II) chelating properties were analyzed using absorption and vibrational spectroscopy. Subsequently, the oxygen-binding and activating properties of the Cu(I)-complex of this model-system has been assessed and the structure of the CuO-species analyzed by vibrational spectroscopy.

For the synthesis of the first coordination sphere of type-3 copper binding sites, we relied on the application of a suitably protected TAC-scaffold, earlier designed in our group and used previously, among others, for the construction of synthetic receptor molecules[7] (Scheme 1). The tridentate ligand containing *N*-acetylated histidine amino acids was constructed using the HO–TAC(*o*-NBS)$_3$ (*o*-NBS = *ortho*-nitrobenzenesulfonyl) scaffold in solid-phase synthesis. First, the scaffold was attached to a polystyrene resin decorated with the Rink-linker using standard peptide coupling reagents BOP and D*i*PEA. Removal of the *o*-NBS protecting groups was achieved using 2-mercaptoethanol and DBU, after which Fmoc–His(Trt)–OH was coupled to each of the liberated amines using BOP and D*i*PEA.‡ The *N*-terminal Fmoc-protected amine was acetylated after piperidine effected Fmoc-deprotection. Protection of the α-amine group of the histidine was necessary in order to mimic the peptide backbone environment of the active site and to avoid participation of the amine in the chelation of the copper(II) ion. After acidic cleavage and deprotection the tridentate ligand was purified by column chromatography.

An initial analysis of the Cu(II)-complex was performed by UV/vis absorption spectroscopy (Fig. 1). In order to be able to analyze the d–d transition bands, a 25 mM solution of the complex (a 1 : 1 mixture of the ligand and $CuSO_4$) was measured at pH values ranging from 3.4 to 7.2. A clear blue-shift in the absorption maximum of the d–d transition bands in the visible region of the spectrum is observed, indicating an increasing number of coordinating histidinyl imidazole rings upon increasing pH. The maximum shifts from 786 nm (at pH 3.4) of the octahedral $[Cu(H_2O)_6]$ complex to 650 nm (at pH 7.2), corresponding to a, probably slightly twisted, square-planar geometry in which three

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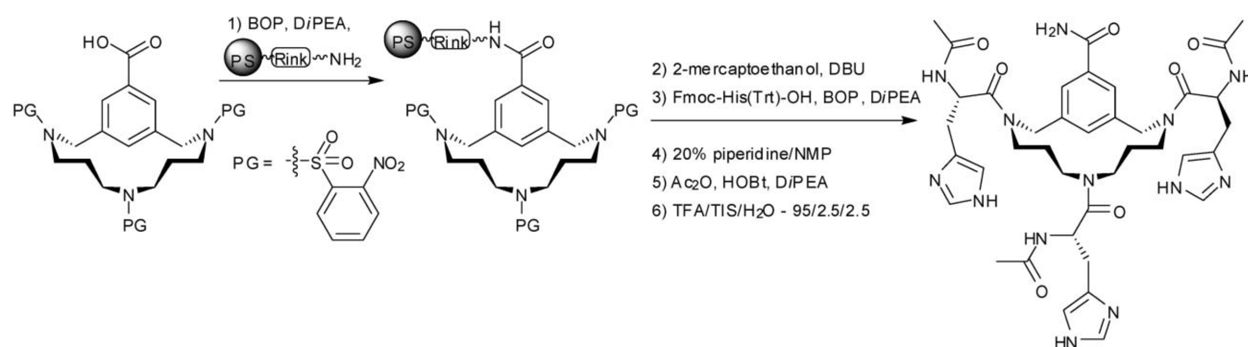

Scheme 1 Synthesis of the tridentate ligand.

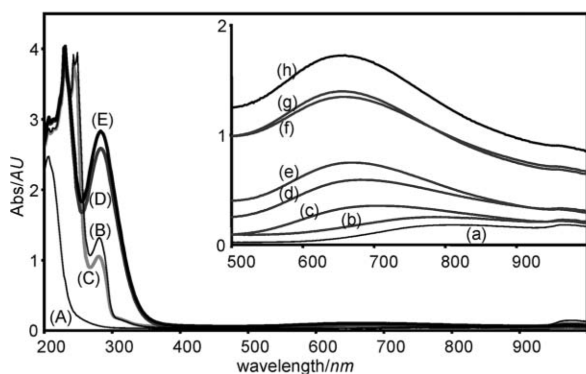

Fig. 1 Absorption spectra of the d–d transitions (inset) and of the charge-transfer bands (main) at different pH. Charge-transfer bands: (A) $CuSO_4$ (aq); (B) ligand (aq); (C) pH 4.1; (D) pH 6.6; (E) pH 8.6. d–d Transition bands: (a) $CuSO_4$ (aq); (b) pH 3.4; (c) pH 4.8; (d) pH 5.6; (e) pH 5.9; (f) pH 6.4; (g) pH 6.8; (h) pH 7.2.

positions are occupied by the imidazole rings and one position is occupied by water (for a square-planar complex the maximum should be at 629 $\pm$ 11 nm).[8] With this twisting of the square-planar geometry towards a tetrahedral geometry an increase in intensity together with a small red-shift occurs, explaining the maximum at 650 nm. This results in the following coordination reaction:

$$[Cu(H_2O)_6] + \text{ligand} \rightarrow [Cu(\text{ligand})(H_2O)] + 5H_2O$$

Concomitant coordination of peptide bond nitrogens can be ruled out since this would result in a more pronounced blue-shift. As a result of the deprotonation and simultaneous coordination of the imidazole rings, the charge transfer bands at 230–242 nm and 280 nm shift or increase, respectively. Although this analysis indicates coordination of the imidazole rings, identification of the coordinating nitrogen atoms was achieved using infrared and Raman spectroscopy.

In the IR spectrum (Fig. 2, top), obtained from lyophilized samples of ligand and complex, the stretching vibrations of the N–C=N part of the imidazole ring (1100–1205 cm^{-1}) significantly increased in intensity as a result of coordination to the cupric ion. Most importantly, the C^4=C^5 stretching vibrations at 1574 cm^{-1} (a) in the ligand (indicating prominent N^τ-tautomer) are found at 1595 cm^{-1} in the complex (slightly obscured by a water vibration), showing the shift of the N^τ–N^π-tautomeric mixture to the N^π-tautomer as a result of the complex formation. Moreover, the broad absorption at 1000 cm^{-1} (b) in the ligand moved to a single peak at 997 cm^{-1} in the complex, corresponding to the N^π-tautomer. With respect to a possible coordinating carbonyl oxygen from the peptide bond, the vibrations at 1682–1628 cm^{-1} showed no significant alteration, indicating the absence of this O–Cu coordination. However, a broad signal of medium intensity at 1595 cm^{-1} indicated the presence of coordinating water.

Raman studies were performed on a hydrated lyophilized sample (pH 7.5). From the Raman spectrum (Fig. 2, bottom) clear differences between the complex and the free ligand were observed. Firstly, a medium signal at 458 cm^{-1} (d) in the spectrum of the complex reveals a Cu–N dative bond; aditionally, a weak Cu–O bond was present at 588 cm^{-1} (c). Secondly, concerning the

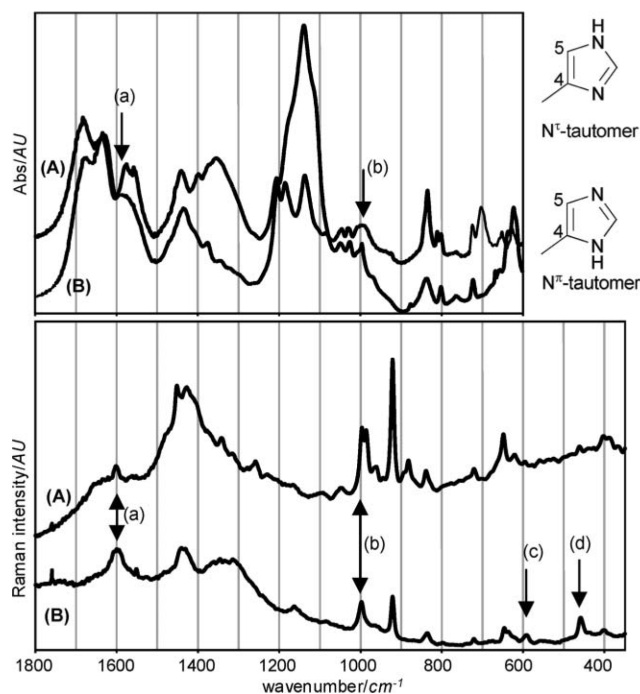

Fig. 2 Infrared (top) and Raman (bottom) spectra of the ligand (A) and the complex (B). The two histidinyl imidazole tautomeric forms and corresponding designators used are shown (upper right). Arrows indicate most important peaks, the small letters by the arrows refer to peaks mentioned in the text.

binding nitrogen atom of the imidazole ring, the signal at 997 cm^{-1} (b) clearly shows that only the N^π-tautomer is present in the complex, indicating coordination by the N^τ-nitrogen. In the ligand the presence of the two tautomeric forms is shown by the doublet at 986 and 997 cm^{-1} (b). N^τ-Coordination is further confirmed by a broad signal at 1598 cm^{-1} (a), which originates from vibrations of the C^4=C^5-bond in the N^π-tautomer of which the N^τ-nitrogen atom is bound to the copper(II)-ion.[9] These results clearly show that coordination takes place by the N^τ-nitrogen of the imidazole ring (Table 1); the remaining (fourth) coordination site of the copper(II)-ion is occupied by a water molecule.

Finally, the oxygen binding and activating capacity of the Cu(I)-complex was analyzed by mixing freshly prepared solutions of ligand and $[Cu(MeCN)_4](PF_6)$ in DMSO in a 1 : 1 ratio. A colourless solution was formed that immediately became blue and of which the intensity increased substantially during the first

Table 1 Assignment of the most important vibrations[a]

Raman		Infrared		
Ligand	Complex	Ligand	Complex	Assignment
—	—	1680–1631	—	Amide I
1600	1598	—	—	ν C^4=C^5 (N^τ)
—	—	1595	—	δ H_2O
997	997	995 (br)	997	ν =C–N= (N^π)
986	—	995 (br)	—	ν =C–N= (N^τ)
—	588	—	—	ν Cu–O
—	458	—	—	ν Cu–N

[a] Numbers refer to wavenumbers (cm^{-1}); ν = stretching; δ = bending; br = broad.

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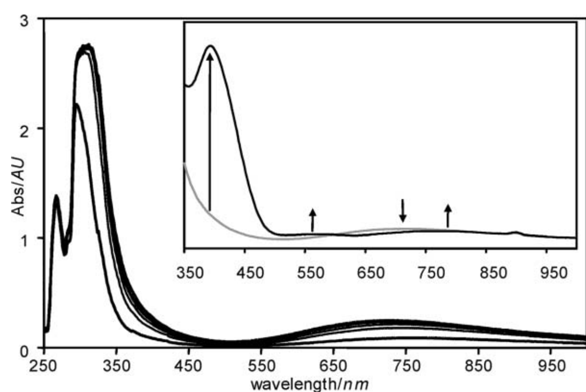

Fig. 3 Shifts in the UV/vis absorption spectra as a result of the oxygen uptake by the Cu(I)-complex during the first 2 h. The major spectral shift occurred during the first 15 min. The inset shows the spectral changes as a result of the reaction between complex (grey) and a catechol substrate (changes are indicated by the arrows).

15 min. This process was analyzed with UV/vis absorption spectroscopy. The formation of a copper(II)–oxygen-complex was evidenced by an intense charge transfer absorption band at 314 nm and a d–d transition absorption band shifting from 800 to 720 nm (Fig. 3). These bands indicate oxygen binding by the complex resulting in a dinuclear $L_2Cu_2(OH)_2$-complex[10] in which each copper(II)-ion has a square-pyramidal geometry with two nitrogen and two oxygen atoms occupying the equatorial positions and the remaining imidazole nitrogen atom of the tridentate ligand at the axial position.[11] For this reaction, the following equation can be envisioned:

$$Cu^{I} + \text{ligand} \rightarrow L\text{–}Cu^{I} + O_2 \rightarrow L\text{–}Cu^{II}\text{–}(OH)_2\text{–}Cu^{II}\text{–}L$$

Infrared and Raman spectroscopy of the CuO-species showed the presence of a bis(μ-hydroxo)dicopper complex (see ESI†). Apart from the signals that correspond to the coordinating atoms, being the N^{τ}-nitrogen atoms of the imidazole rings (1600 and 1000 cm^{-1} in the Raman spectrum) and oxygen atoms of the dicopper bridging hydroxo moieties (3400 and 957 cm^{-1} in the IR spectrum), a new intense signal is found at 740 cm^{-1} in the Raman spectrum. This corresponds with the symmetric stretching vibrations of the Cu^{II}–$(OH)_2$–Cu^{II} "diamond" core of the CuO-complex.[12] In addition, the differences of the Cu–O and Cu–N stretching vibrations of the Cu(II)- and CuO-complexes, apparent from a shift in the Raman spectrum from 588 and 458 cm^{-1} to 568 and 472 cm^{-1}, respectively, illustrate the changes in Cu–N and Cu–O bond-energies as a result of the difference in geometry.

The reactivity of this CuO-complex was illustrated by the addition of one equivalent of 3,5-di-*tert*-butylcatechol: the solution instantaneously turned yellow and the absorption band of the complex at 720 nm shifted to about 780 nm and two new bands emerged, one at 400 nm and one at 574 nm (Fig. 3, insert), corresponding to absorptions of 3,5-di-*tert*-butylbenzoquinone.[13]

In conclusion, a new mimic of type-3 copper binding sites in proteins was successfully synthesized using convenient peptide synthesis onto a TAC-scaffold attached to a resin. To the best of our knowledge, this is the first synthetic mimic of type-3 copper binding sites based on biologically relevant coordinating entities, *i.e.* histidine residues. Further investigations might shed more light on the mechanism by which tyrosinases and catechol oxidases operate and possibly lead to mimics of these enzymes.

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Notes and references

‡ Fmoc = 9-Fluorenylmethyloxycarbonyl; BOP = (Benzotriazol-1-yloxy)-tris(dimethylamino)phosphonium hexafluorophosphate; DiPEA = *N*,*N*-Diisopropylethylamine; Trt = Triphenylmethyl; DBU = 1,8-Diazabicyclo[5.4.0]undec-7-ene.

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