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PAPER

Triazacyclophane (TAC)-scaffolded histidine and aspartic acid residues as mimics of non-heme metalloenzyme active sites†

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We describe the synthesis and coordination behaviour to copper(II) of two close structural triazacyclophane-based mimics of two often encountered aspartic acid and histidine containing metalloenzyme active sites. Coordination of these mimics to copper(I) and their reaction with molecular oxygen leads to the formation of dimeric bis(μ-hydroxo) dicopper(II) complexes.

Introduction

It has been estimated that metalloenzymes comprise 40% of all enzymes known to date.[1] They can catalyze reactions like the hydrolysis of (bio)molecules or the oxidation of organic substrates using activated small molecules like water or oxygen, respectively.[2] In general, their scope of catalyzed reactions is less limited than that of non-metalloenzymes. For immobilization of the catalytically active metal ion(s) in a special site in the protein, tetrapyrroles like heme and/or amino acid side-chain functionalities such as histidyl imidazole, aspartyl and/or glutamyl carboxylate, or cystinyl thiolate groups are frequently utilized.[1] Whereas immobilization of metal ions by tetrapyrrole has been successfully mimicked by synthetic porphyrins, mimicry of the immobilization by amino acid functionalities has been mainly limited to the use of small synthetic organic molecules that lack resemblance with natural amino acid counterparts.[3] Nevertheless, many of these small unnatural models have been very useful to study important features of the mimicked enzymes,[4] even leading to the identification of active site intermediates before their presence in the enzyme was revealed.[3] However, the application of true biomimetic models has been restricted to large peptidic constructs[5] and the behaviour of biologically relevant amino acid based coordinating groups in small constructs lagged behind.

To bridge this gap, we recently described the mimicry of copper-coordinating tris-histidine active sites, as in tyrosinase and hemocyanin, for instance, by attaching these histidine residues to a synthetic triazacyclophane (TAC)-scaffold.[6] In order to complete this small series of true biomimetic ligands, we have now added two mimics of metalloenzymes using aspartic acid (Asp, D) and histidine (His, H) residues attached to the TAC-scaffold. With this addition, three TAC-based structural models of prominent metalloenzyme active sites have now been constructed.

Results and discussion

Synthesis of the mimics

For the preparation of the mimics, a TAC-scaffold was prepared containing Fmoc groups on the benzylic amines and an Alloc group on the middle aliphatic amine group (see ESI for synthesis†). Using this scaffold, the two mimics could be prepared by straightforward solid-phase synthesis procedures (Scheme 1). First, the Fmoc groups of resin-bound scaffold **1** were removed and Fmoc-His(Trt)-OH or Fmoc-Asp(O*t*Bu)-OH were coupled using BOP and D*i*PEA. After replacement of the Fmoc groups with acetyl groups—in order to mimic the backbone of the metalloenzyme and to prevent coordination of terminal amine groups with metal ions—the middle position was functionalized. For this, the Alloc group was removed using Pd^0 followed by attachment of Fmoc-Asp(O*t*Bu)-OH or Fmoc-His(Trt)-OH using BOP and D*i*PEA. After N-terminal acetylation, side-chain deprotection and cleavage of resin-bound constructs resulted in two mimics: one with two histidine and one aspartic acid residues (the "2-His-1-Asp" or HDH-mimic (**2**)) and one with one histidine and two aspartic acid residues (the "1-His-2-Asp" or DHD-mimic (**3**)).

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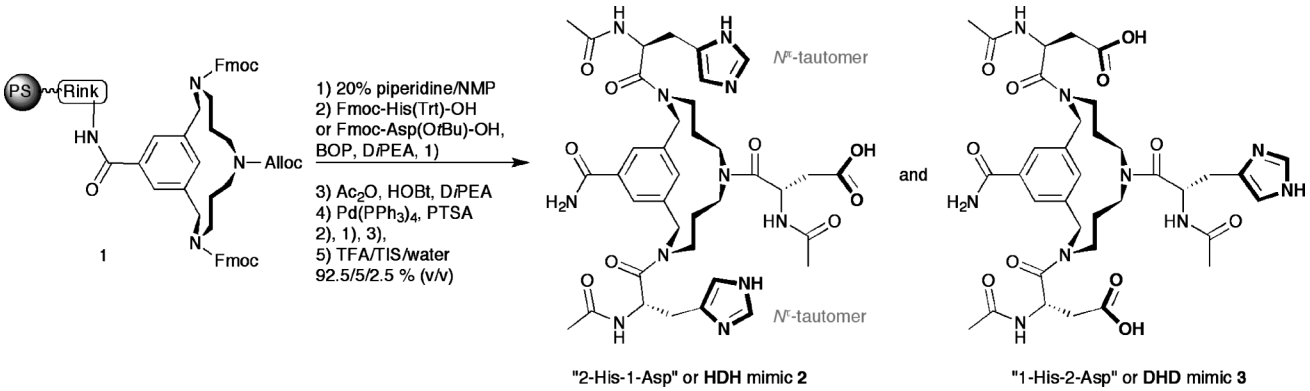

Scheme 1 Solid-phase synthesis of the tridentate mimics.

Coordination of the mimics to copper(II)

The coordination chemistry of these mimics was assessed by mixing stoichiometric ratios of these ligands with $CuSO_4$ in water at low pH. As the pH value of the mixture was raised by the addition of base, the carboxylate functionality from aspartic acid residues and the imidazole ring from the histidine residues became available for coordination to Cu(II), which was evident from the blue-shift and increase of the absorption maxima (Fig. 1). At pH 7.2, an absorption maximum was observed at 672 nm for the HDH-mimic (**2**), whereas for the DHD-mimic (**3**) a maximum was found at 688 nm. For the HDH- this value corresponds roughly to a square-planar geometry with two imidazole rings and one carboxylate functionality. A similar geometry with two carboxylate functionalities and one imidazole ring can be envisioned for the DHD-mimic.[7] The remaining position of the square-plane could be occupied by either the sulfate ion or a water molecule. Additionally, the isotope pattern found in mass spectrometric analyses of these two complexes at pH 7.2 corresponds with the presence of predominantly 1 : 1 complexes (see ESI†).

After this, lyophilized samples from the UV-vis studies were subjected to infrared and Raman spectroscopic analysis in order to determine changes in functional group vibrations as result of their coordination to Cu(II). From this it became clear that coordination indeed occurred by histidyl imidazole ring(s) as well as the carboxylate group(s) of the aspartic acid residue(s) and that coordination of amide–bond oxygen atoms to Cu(II) was absent. Coordination of histidyl imidazole rings was especially clear from an increase in intensity in the bands between 1200 and 1100 cm^{-1} in the IR spectra, going from the mimics to the complexes (Fig. 2). These bands originated from breathing vibrations of the imidazole rings and donation of electron density of the ring towards the Cu(II) ion resulted in a significant increase in intensity.[6] In addition, bands around 1600 cm^{-1} in the Raman spectra indicated coordination by the N^{τ}-nitrogen atom of the ring (see ESI†). Additional marker bands for this coordinating tautomer, usually observed at 997 cm^{-1}, were obscured by breathing vibrations of the aromatic ring of the TAC-scaffold. Additionally, coordination of the carboxylate groups to Cu(II) seemed to be monodentate due to a ν_{as} at 1593 and 1594 cm^{-1}, and a ν_s at 1397 and 1398 cm^{-1} (Fig. 2), for carboxylate groups in the HDH and DHD complexes, respectively. The resulting Δ ($\nu_{as} - \nu_s$) of 196 cm^{-1} is a hallmark for monodentate coordination of carboxylate groups to transition

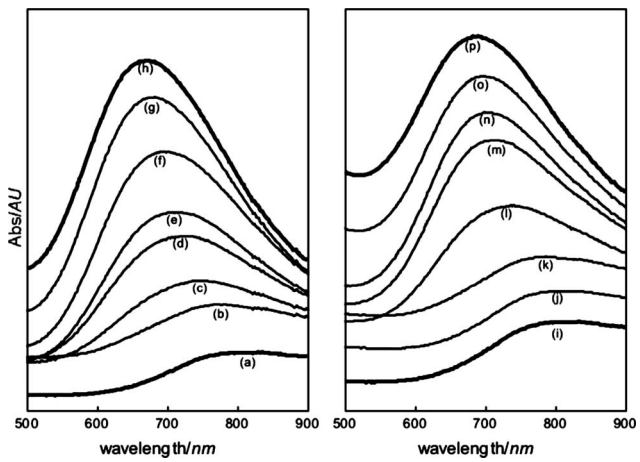

Fig. 1 Absorption bands of d–d transitions at different pH values. *Left*: HDH-Cu(II) complex: (a) pH 1.1; (b) pH 3.4; (c) pH 3.8; (d) pH 4.2; (e) pH 4.4; (f) pH 5.3; (g) pH 6.8; (h) pH 7.2. *Right*: DHD-Cu(II) complex: (i) pH 1.2; (j) pH 2.2; (k) pH 3.0; (l) pH 3.7; (m) pH 4.5; (n) pH 5.9; (o) pH 6.5; (p) pH 7.2.

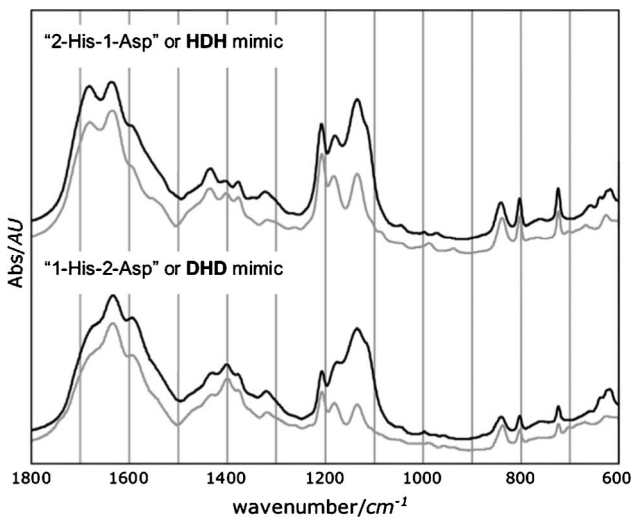

Fig. 2 Infrared spectra of the two ligands (grey traces) and their Cu(II) complexes (black traces).

metal ions.[8] Next to these functional group vibrations, Cu–N and Cu–O bonds were also observed in the Raman spectra, at 466 and around 400 cm^{-1}, respectively (see ESI†).

Coordination to copper(I) and reaction with O_2

After this initial mapping of the coordination chemistry of these mimics to Cu(II) ions, which showed close structural mimicry of their biological counterparts, we studied their performance in a biologically relevant reaction, namely the immobilization of molecular oxygen by copper(I) complexes. Although the natural ligand environments for this reaction consist solely of histidine residues, we envisioned that the application of the current mimics in this chemistry would be highly instructive to study the potential application of these mimics in biologically relevant reactions. Unfortunately, these mimics based on biologically relevant coordinating moieties did not dissolve in the aprotic solvents that are usually required for these studies.§ Therefore, the following studies were performed in the aprotic but very hygroscopic DMSO at room temperature. First, recrystallized $[Cu(MeCN)_4]PF_6$ and the sodium salts of either the DHD- or the HDH-mimic was mixed in stoichiometric ratios. Initially, only a vague blue colour was formed even under exclusion of air, most likely caused by oxidized Cu(I) due to traces of oxygen and water present in DMSO. However, when air was bubbled through the solution at set intervals a strong blue coloration of the mixture was observed, corresponding to the generation of Cu(II) species. UV-vis measurements of this solution at regular time intervals indeed revealed an increase in absorption intensity and a small shift in the position of the absorption maxima (λ_{max}): for the HDH-mimic a shift to shorter wavelengths was observed whereas for the DHD-mimic a shift to longer wavelengths was observed (ESI†). Interestingly, oxygenation of the Cu(I) complex was complete within 90 min for the HDH-mimic and in 60 min for the DHD-mimic (ESI†). Considering the conditions used formation of dimeric bis(μ-hydroxo) dicopper(II) complexes was most likely.[9] Indeed, an absorption maximum for the HDH-based complex at 720 nm corresponded with a square-pyramidal complex in which the equatorial positions were occupied by the bridging hydroxo groups, one of the two imidazole rings and the carboxylate group and the remaining imidazole ring of the mimic was coordinating axialy.[7] Similarly, the DHD-mimic resulted in a complex with a λ_{max} of 740 nm, also in agreement with a square-pyramidal complex with two hydroxo-groups and the two carboxylate groups of the mimic positioned in the equatorial and the remaining imidazole ring in one of the axial positions.[7] In addition, both mimics showed hydroxo-to-copper(II) charge-transfer bands around 425 nm (ESI†). A similar complex was previously uncovered for a tris-histidine containing TAC-based mimic.[6]

The samples of these Cu(I)–O_2 studies were then condensed under high vacuum and analyzed by infrared and Raman spectroscopy. From these measurements it was clear that indeed dimeric bis(μ-hydroxo) complexes were formed in which the bis(μ-hydroxo) dicopper(II) core was surrounded by either the HDH- or the DHD-mimic. For instance, the infrared spectra showed absorptions at 953 and 950 cm^{-1} of the O–H bending vibrations of the $Cu_2(OH)_2$-core for both complexes (see ESI†). In addition, Cu–(μ-$OH)_2$–Cu diamond core breathing vibrations were observed by intensities at 739 cm^{-1} in the Raman spectra of both mimics (arrow (a), Fig. 3). Even though, Cu–N and Cu–O bonds can also be observed using Raman spectroscopy, only a weak Cu–N stretching vibration was observed for the complex of the HDH-mimic (arrow (b), Fig. 3). The absence of this band in the complex of the DHD-mimic can be explained by a weaker axial coordination of the imidazole ring in that complex. Alternatively, one of the imidazole rings of the HDH-mimic is weakly coordinating to an axial position and the other one is more strongly coordinating to an equatorial position, which is apparent from a weak intensity at 470 cm^{-1}. In addition, both mimics showed Cu–O bond vibrations by peaks just below 400 cm^{-1} (arrows (c), Fig. 3). Concerning the coordinating functional groups, histidyl imidazole rings appeared to be coordinating by their N^{τ}-nitrogen atoms, as was concluded from a band at 1600 cm^{-1} in the Raman spectra (Fig. 3).[10] A slightly more complicated picture was obtained for coordination of the carboxylate functionalities. Although the mononuclear Cu(II)-mimic complexes mostly showed monodentate coordination, these dinuclear complexes could also have some bidentate bridging coordination, as was inferred from absorbances between 1541 and 1548 cm^{-1} in the IR spectra (see ESI†).[8] Apart from bidentate coordination to the copper(II) ions, another possibility could be hydrogen bond formation with a proximate hydroxo group originating from the $Cu_2(OH)_2$ core. A combination of both mono- and bidentate coordination is also possible.

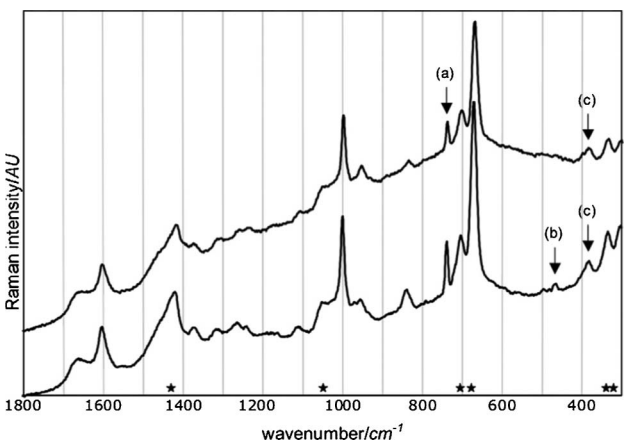

Fig. 3 Raman spectra of the bis(μ-hydroxo) dicopper(II) complexes based on the HDH- (top) or DHD-mimics (bottom). Arrows indicate the most prominent features mentioned in the text. Asterisks mark positions of peaks from residual DMSO.

Based on these spectroscopic findings, two bis(μ-hydroxo) dicopper(II) complexes in which each copper centre is surrounded by one of the two mimics can be postulated (Fig. 4). That these two complexes and the previously described "3-His" complex[6] can be useful to study metalloenzymes, can already be inferred from the different rates observed for the reaction between Cu(I) and molecular oxygen. The slower kinetics of oxygen binding of the "3-His" variant when compared to the other two that are currently described—*i.e.* 100 min for the HHH-mimic, 90 min for the HDH-mimic and 60 min for the DHD-mimic—is likely connected to the presence of only "3-His" sites in metalloenzymes like the oxygen carrier hemocyanin and to the substrate converting

§ Cu(I)–O_2 chemistry is usually performed in aprotic solvents and at low temperatures (typically around −80 °C). See ref. 9

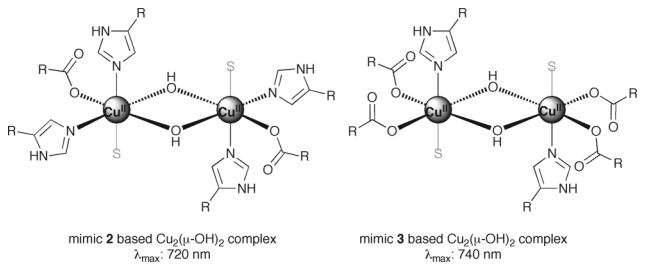

Fig. 4 Proposed structures of the two bis(μ-hydroxo) dicopper(II) complexes based on the two mimics. R represents the rest of the mimic; S is an optional solvent molecule. Distortions from the perfect square-pyramidal geometry as depicted above are likely to be present in the actual compounds.

enzymes tyrosinase or catechol oxidase. Although this faster binding by especially the DHD-mimic could be expected based on general hard–soft acid–base principles, *i.e.* that the harder Cu(II) will coordinate stronger to carboxylates and thereby pull the oxygenation reaction, such subtle differences can now be determined more precisely using these mimics.

Conclusions

Two TAC-based mimics of frequently observed biological non-heme metal-binding sites have been synthesized and their coordination to copper ions has been characterized. Although none of these complexes could be crystallized, UV-vis, ESI-MS, and especially infrared and Raman spectroscopic analysis revealed unambiguously that the coordination chemistry of these mimics to Cu(II) and their behaviour in a biological relevant Cu(I)–O_2 reaction closely mimic their counterparts found in nature. Together with a tris-histidine containing TAC-based mimic previously described by us,[6] these three mimics represent three of the most prominent non-heme metal-binding sites found in nature. We anticipate that these and similar mimics will find useful synthetic applications in the near future and will help the elucidation of mechanisms in the often complicated enzymatic reactions performed by non-heme metalloenzymes.

Experimental

General remarks concerning the syntheses as well as detailed description of procedures not described below can be found in the supporting information†.

Synthesis of "2-His-1-Asp" (2) and "1-His-2-Asp" mimic (3)

For the synthesis of these two mimics, the scaffold described in the previous synthesis was coupled to 500 mg of PS-S RAM-Fmoc resin (loading: 0.78 mmol g^{-1}) using 1 equiv of the scaffold. After overnight coupling, the remaining amine functionalities were protected with the acetyl group using the capping reagent. For coupling of the amino acids, 8 equiv were used compared to the initial loading of the resin. After removal of the Fmoc groups, the scaffold containing resin was divided in two equal portions: one to construct mimic **2** and one for mimic **3**. For mimic **2** Fmoc-Asp(O*t*Bu)-OH and for mimic **3** Fmoc-His(Trt)-OH were coupled. After coupling, the N-terminal Fmoc protecting group was removed and replaced by an acetyl group. For the functionalization of the middle position, the Alloc groups on the resin-bound precursors were removed using Pd^0 and a scavenger. To the liberated amine groups, Fmoc-His(Trt)-OH (for mimic **2**) and Fmoc-Asp(O*t*Bu)-OH (for mimic **3**) were coupled. Acetylation of the N-terminal amine functionality was performed after Fmoc removal. Resin-bound protected mimics were cleaved and deprotected using the cleavage cocktail mentioned and the released mimics were precipitated in cold MTBE and hexanes (1 : 1, at −20 °C). Lastly, the mimics were purified by column chromatography (eluent: $CHCl_3$/MeOH/25% NH_4OH 8 : 4 : 1.5 (v/v)). Pure fractions were concentrated and lyophilized from water with pH 7. Yields: 174 mg for **2** (226 μmol, 58%) and 185 mg for **3** (234 μmol, 63%). R_f 0.33 for **2** and 0.58 for **3** and (eluent: $CHCl_3$/MeOH/25% NH_4OH 60 : 45 : 20 (v/v)). RP-HPLC (C_8): t_R mimic **2** = 13.58 min; t_R mimic **3** = 13.94 min. HRMS: mimic **2** = 792.3824 (calc. 792.3793 for $[M + H]^+$); mimic **3** = 770.3450 (calc. 770.3473 for $[M + H]^+$).

Coordination complexes with Cu(II) ions

For each of the ligands, stoichiometric mixtures of both ligand and $CuSO_4$ were prepared and the pH was adjusted to ~1.2 using 1 N HCl. After recording the first UV-vis spectrum, the pH was stepwise increased using 0.5–5% $NaHCO_3$ solutions and at each significant difference in pH value an absorption spectrum was measured. For the d–d transition absorptions, 25 mM solutions of the complexes were prepared by mixing equal amounts of 50 mM solutions of both mimic and $CuSO_4$. The charge-transfer spectra were obtained using the above procedure with 5 mM solutions of the mimic-Cu(II) complexes. Spectra were recorded on a 96-well microtiterplate suitable for UV measurements using a microtiterplate reader measuring from 200–998 nm.

Infrared and Raman spectra

Lyophilized Cu(II)-mimic complexes and vacuum dried $Cu(II)_2(\mu\text{-}OH)_2$-mimic complexes obtained after UV-vis experiments were used for measuring infrared and Raman spectra. For measuring the Raman spectra of both Cu(II)-mimic complexes a drop of water had to be applied to the lyophilized powder in order to prevent incineration of the powder by the laser beam. Reference spectra of the sodium salts of the mimics were obtained using freshly lyophilized samples of solutions of the mimics of which the pH was set at ~7.5 using 1 N NaOH (aq).

Reaction of Cu(I)-mimic complexes with molecular oxygen

A solution of one of the mimics in DMSO (50 mM, 150 μL) was added to a freshly prepared solution of $[Cu(MeCN)_4](PF_6)$ in DMSO (50 mM, 150 μL). Then, 300 μL of DMSO was added and the resulting 12.5 mM solution was immediately analyzed. After this, air was bubbled through the solution at regular intervals, resulting in an increasing intensity of the blue colour.

Abbreviations

Alloc = allyloxycarbonyl; BOP = (benzotriazol-1-yloxy)-tris-(dimethyl-amino)phosphonium hexafluorophosphate; D*i*PEA = *N*,*N*-diisopropyl-ethylamine; Fmoc = 9-fluorenylmethyloxycarbonyl; *t*Bu = *tert*-butyl; PTSA = *para*-toluenesulfinate anilinium;

Trt = triphenylmethyl; TFA = trifluoroacetic acid; HOBt = 1-hydroxybenzotriazole; TIS = triisopropylsilane.

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