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Metal-binding and redox properties of substituted linear and cyclic ATCUN motifs

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ABSTRACT

The amino-terminal copper and nickel binding (ATCUN) motif is a short peptide sequence found in human serum albumin and other proteins. Synthetic ATCUN–metal complexes have been used to oxidatively cleave proteins and DNA, cross-link proteins, and damage cancer cells. The ATCUN motif consists of a tripeptide that coordinates Cu(II) and Ni(II) ions in a square planar geometry, anchored by chelation sites at the N-terminal amine, histidine imidazole and two backbone amides. Many studies have shown that the histidine is required for tight binding and square planar geometry. Previously, we showed that macrocyclization of the ATCUN motif can lead to high-affinity binding with altered metal ion selectivity and enhanced Cu(II)/Cu(III) redox cycling (*Inorg. Chem.* **2013**, *52*, 2729–2735). In this work, we synthesize and characterize several linear and cyclic ATCUN variants to explore how substitutions at the histidine alter the metal-binding and catalytic properties. UV–visible spectroscopy, EPR spectroscopy and mass spectrometry indicate that cyclization can promote the formation of ATCUN-like complexes even in the absence of imidazole. We also report several novel ATCUN-like complexes and quantify their redox properties. These findings further demonstrate the effects of conformational constraints on short, metal-binding peptides, and also provide novel redox-active metallopeptides suitable for testing as catalysts for stereoselective or regioselective oxidation reactions.

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1. Introduction

The amino-terminal Cu- and Ni-binding (ATCUN) motif is a sequence derived from the N-termini of mammalian serum albumins and other proteins. ATCUN motifs are tripeptides with a free N-terminus and histidine in the third position, and these motifs bind several divalent transition metal ions with high affinity [1]. Cu(II) and Ni(II) complexes of ATCUN peptides have been used as oxidation catalysts for site-specific DNA cleavage and protein cleavage [1–13], protein–protein cross linking [14–16], enzyme inhibitors [17,18], and metallodrugs [19–21]. ATCUN motifs bind Cu(II) or Ni(II) in a square planar geometry using nitrogens from the N-terminal amine, imidazole, and two backbone amides. The imidazole and the amine are the major contributors to the high thermodynamic stability of ATCUN–metal complexes [22]. Amino acid substitutions at the first and second positions can affect DNA cleavage and other intermolecular processes, but have more modest effects on affinity, stoichiometry, and redox characteristics of the peptide–metal complex [11,23]. Adding additional histidines at the first [24] or second position [25] reduces the binding affinity and alters the coordination geometry of metal complexes, demonstrating that imidazole positioning within short peptides has an overriding effect on metal complexation.

Despite the importance of the imidazole, the ATCUN histidine has been substituted with other amino acids in order to design altered metal–peptide complexes. Several studies have described complexes between tripeptides and metal ions that are mediated through the N-terminal amine, amide nitrogens, backbone carbonyl oxygens, the carboxy terminal group, and/or various non-imidazole side chains. For instance, Arg-Lys-Asp binds Cu(II) and Ni(II), though only at more alkaline conditions than Gly-Gly-His [26,27]. Similar results were observed for Gly-Gly-Cys and Ala-Ala-Cys [28,29]. Spectroscopic and potentiometric studies using Gly-Gly-Met suggested that the thioether of Met is not the primary binding site for *3d* transition metal ions due to the rapid oxygenation of sulfur [22,30]. Ultimately, most of these tripeptide ligands do not form 1:1, square planar complexes with divalent metal ions (an "ATCUN-like complex"). Instead, the intrinsic flexibility of the peptide backbone and the lack of the strongly chelating imidazole group allow other binding modes to compete with the square planar, 1:1 complex characteristic of high-affinity ATCUN motifs.

While complexes between linear peptides and metals have been broadly explored, there are fewer studies on metal binding by designed cyclic peptides [22,31–37]. Macrocyclization has powerful effects on metal-binding behavior, and the design of cyclic ligands has been reported for selective metal ion recognition, ion transport, metalloenzyme modeling, catalysis, MRI contrast agents, luminescence probes, and carriers for drug delivery [38–44]. We recently reported macrocyclization of the ATCUN motif in a manner that maintains a high-affinity complex

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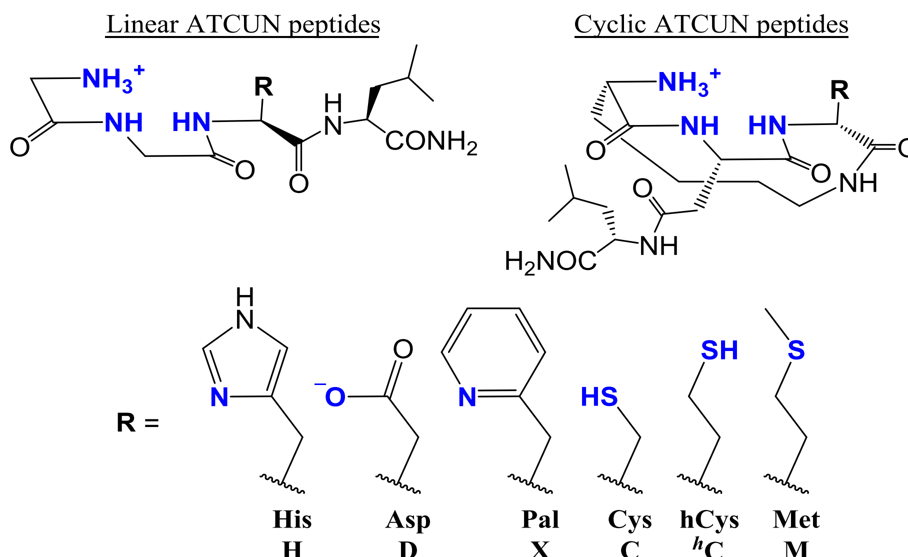

Scheme 1. Structures of linear and cyclic ATCUN peptides. Linear peptides used in this study include GGHL, GGDL, GGXL, GGCL, GG^{h}CL, and GGML, where X represents 2-pyridylalanine and ^{h}C represents homocysteine. Cyclic peptides used in this study include peptide **1** containing D-His, **1D** with D-Asp, **1X** with D-Pal, **1C** with D-Cys, **1^{h}C** with D-^{h}Cys, and **1M** with D-Met. Cyclic peptides used D-amino acids whereas linear analogs used L-amino acids at the same position. In prior work, we showed that the glycines' lack of chirality makes L- and D-amino acids interchangeable within these short, linear tetrapeptides [45]. Structures are shown as the predominant species at pH 7.0. Donor atoms are shown in blue boldface.

with Cu(II) or Ni(II) [45]. By characterizing several diastereomers and linear analogs, we demonstrated that the binding of the macrocyclic ATCUN peptide (peptide **1**, shown in Scheme 1) to Cu(II) and Ni(II) was altered due to its cyclic structure. Considering the limitations of non-imidazole-containing, linear tripeptides as metal ligands, we hypothesized that the cyclic scaffold could enforce the square planar, 1:1 complex even in the absence of the imidazole group. This would allow direct substitution of other metal-binding side chains in order to produce metallopeptides with unique metal-binding selectivities and redox properties.

Peptide **1** consists of the ATCUN motif Lys-Asp-D-His (where D-His refers to the D-enantiomer of natural L-histidine) with two modifications. First, the side-chain of Lys is linked via amide bond to the C-terminus of the D-His. Second, the side-chain of the Asp is linked via amide bond to an amide-capped Leu residue to provide for a linkage to solid-phase synthesis resin and to allow for reverse-phase HPLC purification. Peptide **1** binds Cu(II) and Ni(II) in an ATCUN-like, 1:1, square planar complex with unique metal-binding and redox properties [45]. In this article, we expand this class of macrocyclic metal ligands by synthesizing and characterizing analogs that substitute the D-His of **1** with D-aspartate (D-Asp), D-pyridylalanine (D-Pal), D-cysteine (D-Cys), D-homocysteine (D-^{h}Cys), and D-methionine (D-Met) (Scheme 1). We synthesized linear and cyclic versions of each peptide in order to compare the roles of His within linear and cyclic metallopeptides, to explore how other ligand sets bind Cu(II) and Ni(II), and to produce a diverse set of metal–peptide complexes for use in ongoing catalysis investigations.

2. Experimental

2.1. Materials

N-α-(9-fluorenyl methyloxycarbonyl) (Fmoc) protected amino acids, MBHA Rink Amide resin, N-hydroxybenzotriazole (HOBt), and Benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate (PyBop) were purchased from AnaSpec. Protected amino acids used in this work included Fmoc-Gly-OH, Fmoc-Leu-OH, Fmoc-His(Trt)-OH, Fmoc-Asp(OtBu)-OH, Fmoc-Pal-OH, Fmoc-Cys(Trt)-OH, Fmoc-Hcy(Trt)-OH, Fmoc-Met-OH, Fmoc-Asp-OAll, Boc-Lys(Fmoc)-OH, Fmoc-DHis(Trt)-OH, Fmoc-DAsp(OtBu)-OH, Fmoc-DPal-OH, Fmoc-DCys(Trt)-OH, Fmoc-DHcy(Trt)-OH, and Fmoc-DMet-OH. Diisopropylethylamine (DIPEA), trifluoroacetic acid (TFA), acetic anhydride, $Pd(PPh_3)_4$, 2′,7′-dichlorofluorescein diacetate, hydrogen peroxide, $CuCl_2$ and $NiCl_2$ were purchased from Sigma-Aldrich. N,N-dimethylformamide (DMF) and acetonitrile were purchased from VWR. DTT was purchased from Ultra Pure. All chemicals were used as received without any further purification.

2.2. Peptide synthesis and purification

Peptides were synthesized manually using 25 mL reaction vessels and wrist shakers using standard Fmoc-based protection strategies on Rink amide methylbenzhydrylamine (MBHA) resin (0.20 mmol scale) with PyBop/HOBt/DIPEA coupling conditions [46]. Linear and cyclic peptides were synthesized as previously reported [45]. For linear peptides, Fmoc was deprotected with 20% piperidine, but for cyclic peptides attached to the resin through Leu-Asp linkages, 20% piperidine was observed to promote aspartimide formation. Aspartimide formation was minimized through screening of deprotection conditions. Briefly, a small aliquot of resin was cleaved (TFA/TIPS/H_2O), ether precipitated, and analyzed by ESI-MS (electrospray ionization-mass spectrometry) and HPLC to quantitate aspartimide formation. For cyclic peptides, Fmoc removal with 5% piperazine and 0.1 M HOBt (5 min, twice at room temperature) limited aspartimide formation to 10–15%. After synthesis of the tetrapeptide, the allyl-protected C-terminus of Asp was deprotected using $[Pd(PPh_3)_4/PhSiH_3]$ (0.25:10 eq) in dry DCM for 2 h. In order to remove $Pd(PPh_3)_4$, the resin was washed with sodium *N*,*N*-diethyldithiocarbamate (0.5% w/v in DMF, 5 × 5 min) [47]. The peptides were then cyclized using PyBOP/HOBt/DIEA (5:5:10 eq) for 2 h. Peptides were cleaved off the resin using TFA/TIPS/H_2O (95:3:2) or TFA/TIPS/EDT/H_2O (92:3:3:2) for thiol-containing peptides. The volume was reduced by evaporation and peptides were ether-precipitated. Crude peptides were dissolved in 3–5 mL of 50:50 water:acetonitrile. Thiol-containing crude peptides were reduced by dithiothreitol (DTT) at this stage, prior to HPLC purification. Peptides were purified using reverse-phase HPLC on a preparative-scale C8 or C18 column (solvent A: water/0.1% TFA, solvent B: acetonitrile/0.1% TFA, linear gradient of 5–40% solvent B over 20 min was used). The fraction containing desired product was collected and lyophilized. The purities and identities of the peptides were assessed by analytical HPLC (linear gradient: 5–40% B

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