Published in final edited form as:

J Inorg Biochem. 2014 October ; 0: 65–76. doi:10.1016/j.jinorgbio.2014.06.004.

Metal-Binding and Redox Properties of Substituted Linear and Cyclic ATCUN Motifs

Kosh P. Neupane, Amanda R. Aldous, and Joshua A. Kritzer*

Department of Chemistry, Tufts University, 62 Talbot Avenue, Medford, Massachusetts 02155, USA

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.

Keywords

ATCUN; catalysis; copper; cyclic peptides; metallopeptides; nickel

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

© 2014 Elsevier Inc. All rights reserved.

*Corresponding Author: Tel.: +1-617-627-0451, fax: +1-617-627-3443, Joshua.Kritzer@tufts.edu (J. A. Kritzer) .

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

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 have 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 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-^hCys), 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 methoxycarbonyl) (Fmoc) protected amino acids, MBHA Rink Amide resin, N-hydroxybenzotriazole (HOBt), and 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) 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(PPh₃)₄, 2', 7'-dichlorofluorescein diacetate, hydrogen peroxide, CuCl₂ and NiCl₂ were purchased from Sigma-Aldrich. N,N-Dimethylformamide (DMF) and acetonitrile were purchased from VWR. Dithiothreitol (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/H₂O), 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(PPh₃)₄/PhSiH₃] (0.25: 10 equiv) in dry DCM for 2 h. In order to remove Pd(PPh₃)₄, the resin was washed with sodium *N,N*-diethyldithiocarbamate (0.5% w/v in DMF, 5 $\times$ 5 min) [47]. The peptides were then cyclized using PyBOP/HOBt/DIEA (5:5:10 equivalents) for 2 h. Peptides were cleaved off the resin using TFA/TIPS/H₂O (95:3:2) or TFA/TIPS/EDT/H₂O (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 over 30 min) and ESI-MS. HPLC retention times and ESI-MS data for *apo*-peptides, Cu(II)-peptide complexes, and Ni(II)-peptide complexes are given in Table S1 (supporting information).

2.3. pH dependence of metal binding and metal binding stoichiometry

Each lyophilized peptide was freshly dissolved in deionized Milli-Q water ($18 \text{ M}\Omega \text{ cm}^{-1}$) purged with argon prior to metal complexation. Concentrations of thiol-containing peptides were measured by dithionitrobenzoic acid (DTNB) assay as described [48, 49]. Concentrations of other peptides were initially estimated by mass and then calculated more exactly using metal-binding titrations [50]. To measure the pH dependence of metal binding, 1.0 mM peptide solutions were prepared and 1.0 equivalent of metal ion (CuCl_2 or NiCl_2) was added to the solution. The pH of the resulting solution was lowered to roughly 2.5-3.0 using dilute HCl. UV-vis spectroscopy (Cary 100, Agilent) was used to verify that no metal complexation occurred at this low starting pH. As small aliquots of dilute KOH were slowly added to the solution, pH was measured using a microelectrode (3 mm, Mettler Toledo) and absorption spectra were recorded. *d-d* transition bands near 525 and 425 nm were observed for ATCUN-like Cu(II)-peptide and Ni(II)-peptide complexes, respectively. KOH was added until a saturation point was observed. For plotting pH dependence curves, the absorption was normalized to unity at the upper bound, and percent formation of each metalloprotein complex was plotted against pH.

For titrations at constant pH to determine metal-binding stoichiometry, 1.0 mM peptide solution was prepared in 50 mM N-ethylmorpholine (NEM) buffer at appropriate pH. Background absorption due to the peptide was normalized to zero, and 0.2 equivalents of CuCl_2 or NiCl_2 were added from a 200 mM aqueous stock solution. The samples were mixed well and absorption spectra were recorded. The titration was repeated until there was no further change in absorbance other than scattering due to formation of metal-hydroxide precipitate.

2.4. EPR spectroscopy

Fresh Cu(II)-peptide complexes (0.9 mM CuCl_2 and 1.0 mM peptide in water with 10% glycerol) were prepared at the specified pH by adding small aliquots of dilute KOH/HCl. These were transferred into capillary tubes and inserted into a quartz EPR tube, then slowly frozen in liquid nitrogen. X-band EPR data were recorded using a Bruker EMX instrument at a microwave frequency of 9.32 GHz. All spectra were recorded at -150°C (123 K) using microwave power of 0.64 mW and modulation frequency of 100 kHz. Other instrumental parameters include a sweep width of 1500 G (2250 to 3750 G) for a total of 1024 data points, time constant 655.36 ms, conversion time 163.84 ms, sweep time 167.77 s, and receiver gain 1×10^4 to 2×10^4 . All spectra were average of 5 scans.

2.5. Cyclic voltammetry

A standard three-electrode cell (glassy carbon electrode as working electrode, platinum wire as auxiliary electrode, and saturated calomel electrode as a reference electrode) was used to perform the electrochemical measurements on a CHI830 Electrochemical Workstation (CH Instruments Inc., USA). All metallopeptide samples were prepared freshly in degassed water and 200 mM KCl was added as supporting electrolyte. The pH was adjusted as required with KOH and HCl. The sample was purged with nitrogen gas for 5 min before data collection. Scan velocity was 100 mV/s for each scan. Cyclic voltammograms presented are the average of three scans that were then background-subtracted. The half-wave potential ($E_{1/2}$) was determined from the expression $E_{1/2} = (E_{ap} + E_{cp})/2$.

2.6. Fluorescence assay for detection of hydroxyl radicals

Concentrated stocks of 0.9 mM peptide-metal complexes were prepared using 1 mM peptide and 0.9 equivalents of CuCl_2 or NiCl_2 , in order to ensure complete complexation of metal ion. Thus, following dilution, assays included final concentrations of 0.9 μM metal(II)-peptide complex or metal salt with an additional 0.1 μM *apo*-peptide (as shown below, all *apo*-peptides were shown to have no effect). Assays also included 0.9 mM H_2O_2 and 100 mM NaCl in 20 mM 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid (HEPES), pH 8.5 or 9.5. Solutions were incubated at room temperature for 60 minutes, after which 10 μM 2',7'-dichlorofluorescein diacetate was added. After an additional 15 minute incubation, total fluorescence intensity was read in 96-well plates using a TECAN infinITE 200 plate reader with excitation at 485 nm and emission at 535.

3. Results and Discussion

3.1. UV-visible spectroscopy of complexes between Cu(II) and linear peptides

For these peptide ligands, formation of the metal complex involves competition between protonation and metal coordination at several donor atoms. The more stable the metal-peptide complex, the lower the pH at which it can form [51]. In the past, the pH at which 50% of metal ions are complexed (or precipitated, in cases for which free metal ions precipitate) has been used to judge the relative stabilities of related complexes [51]. In this work, we use pH_{50} (the pH at which 50% of the metal ions are complexed) to assess complex stability in a semiquantitative manner.

Peptide-metal complexes were initially characterized by UV-vis spectroscopy as pH was gradually raised from 2.5. Figure 1A shows selected UV-vis spectra and pH dependences of Cu(II) binding to linear peptides Gly-Gly-His-Leu (GGHL), Gly-Gly-Asp-Leu (GGDL), Gly-Gly-Pal-Leu (GGXL), Gly-Gly-Cys-Leu (GGCL), Gly-Gly-^hCys-Leu (GG^hCL), and Gly-Gly-Met-Leu (GGML). As pH was raised, a discrete, two-state transition between the aqua complex (λ_{max} near 800 nm, observed at low pH) and a complex with λ_{max} at 530 to 545 nm was observed for GGHL, GGXL, and GGDL (Figure 1B and Figure S1). The $d-d$ transition band at 530-545 nm is consistent with the formation of a square-planar complex with an N_4 or N_3O donor atom set, and the wavelengths, intensities, and cooperative transitions are all identical to classical ATCUN motifs [1, 27, 52-55]. This led us to conclude that GGDL and GGXL form ATCUN-like complexes with Cu(II).

For the sulfur-containing linear peptides GGCL, GG^hCL, and GGML, more complicated behavior was observed above pH 5.0 consistent with the formation of different metal-peptide complexes. Since *d-d* transition bands cannot reliably distinguish among N, O or S coordination to Cu(II), we also examined the charge transfer bands at lower wavelengths for sulfur-containing peptides. N→Cu(II) and O→Cu(II) charge transfers produce a characteristic band at 260-270 nm, while S→Cu(II) charge transfer produces a characteristic band at 290-330 nm [28, 29]. For GGCL, Cu(II) binding to the cysteine thiolate starts near pH 4.6, as indicated by a shoulder near 350 nm due to the S→Cu(II) transfer band (Figure 1C). Between pH 4.6 and 5.2 a *d-d* transition band at 570 nm (amine, thiolate, and two H₂O ligand set) appears, then gradually shifts to 540 nm (amine, thiolate, amide and H₂O ligand set) when pH is increased up to 5.5. At pH 6.5, a band centered at 517 nm is observed due to the amine, two amide and thiolate ligand set, which is the ATCUN-like configuration for this peptide. These interpretations are consistent with known absorption bands for these ligand sets [56, 57], and are supported by EPR data (see below). The *d-d* transition band centered at 517 nm appears at all pHs above 6.5. At higher pH, the S→Cu(II) charge transfer band is more pronounced and shifts from 350 nm to 320 nm (2400 M⁻¹cm⁻¹) (Figure 1C).

Cu(II) binding to GG^hCL also starts at pH > 4.5, but the S→Cu(II) charge transfer band near 325 nm only starts appearing at pH near 7.0 (Figure 1D). This shows that homocysteine is less able to coordinate Cu(II) in the context of the ATCUN-like architecture compared to cysteine. A band centered at 625 nm was observed at pH near 6.0, and this band persisted as the more characteristic 515 nm band appeared near pH 7.4. The 625 nm band gradually diminished through pH 10.0. At this high pH, both the 515 nm band and the 325 nm band intensified, indicating the dominance of a discrete N₃S-coordinated Cu(II) complex (likely the ATCUN-like, square-planar complex). For Cu(II) complexation with GGML (Figure 1E), the *d-d* transition bands and S→Cu(II) charge transfer bands are somewhat similar to those observed for GG^hCL. One important difference is that the band at 625 nm is prominent only up to a pH of 6.0, and by 7.6 the spectrum is dominated by a band at 545 nm. In agreement with the lower pH transition observed for GGML, the S→Cu(II) charge transfer band at 295 is more prominent at pH 6.0 for GGML compared to GG^hCL.

Overall, in contrast to the discrete, two-state transitions observed for GGHL, GGD_L and GGXL, all three sulfur-containing linear peptides have a variety of intermediate complexes below neutral pH and oxidation products at basic pH (Figure S2-S4). All three sulfur-containing linear peptides showed an absorption band at roughly 625-650 nm at mid-range pH values, either as a very minor contributor (GGCL) or a significant contributor to the UV-vis spectrum (GG^hCL and GGML) (Figure 1F). Based on prior results and on additional EPR data (see below), the 625-650 nm band may arise from Cu(II) coordinated to the N-terminal amine, the sulfur, one amide nitrogen, and one oxygen from a carbonyl group or water [58]. Hydroxide or chloride coordination at this fourth site also cannot be ruled out. The 545 nm band observed for GGML is most likely the ATCUN-like Cu(II) complex with the N-terminal amine, two amide nitrogens and the thioether as the ligand set, an interpretation supported by the observed charge transition band, our own EPR data (see below), and by other observations of short, methionine-containing peptides [59].

3.2. UV-vis spectroscopy of complexes between Cu(II) and cyclic peptides

We previously reported that cyclic peptide **1** binds Cu(II) using the amine, imidazole, and two amide nitrogens in a square planar geometry at neutral pH [45]. Cyclic analogs of **1** that replace the imidazole with a carboxylic acid (**1D**), pyridine (**1X**), thiol (**1C** and **1hC**) or methyl thioether group (**1M**) were each prepared, and Cu(II) complexes of these cyclic peptides were characterized by UV-vis spectroscopy. All these cyclic peptides show *d-d* transition bands (and, for sulfur-containing peptides, S→Cu(II) charge transfer bands) that indicate Cu(II) complexation between pH 4.0 and pH 11.5. However, for all cyclic peptides except **1**, several shifts among *d-d* transition bands between 800 nm (Cu(II)-aqua complex) and 500 nm are observed, indicating multiple competing complexes at mid-range pH values (Figure 2 and Figure S5-S10).

First, we examined the effects of cyclization within ATCUN peptides without sulfur. While GGD_L and GGXL showed similar Cu(II) binding characteristics as GGHL, the cyclic analogs containing Asp and Pal (**1D** and **1X**) differ greatly from the His-containing peptide **1**. **1D** and **1X** have an initial pH transition from the aqua complex (*d-d* transition band at 800 nm) to another complex with a λ_{max} at 550 nm for **1D** and 555 nm for **1X**. This band matches the typical ATCUN-Cu(II) square planar complex, and EPR data (see below) support the conclusion that this represents a square-planar complex with the Cu(II) coordinated by the N-terminal amine, two amide nitrogens, and the carboxylate (for **1D**) or pyridyl group (for **1X**). For both cyclic peptides, this ATCUN-like complex is the predominant species at pH 8.6 to 8.8, but at higher pH values a blue-shift is observed to a band near 500 nm. This is not observed for peptide **1** (Figure S5-S7).

Cyclic peptides with sulfur-containing coordination groups have similarly complicated Cu(II)-binding behavior, but for **1C**, **1hC**, and **1M** the additional information from S→Cu(II) charge transfer bands allows further insight (Figure S11). Specifically, the thiol and thioether groups are not involved in Cu(II) coordination until pH approaches 6.0, implying that the Cu(II) complexes with *d-d* transition bands at 650-700 nm observed near pH 6.0 involve coordination to N-terminal amines and the side-chain sulfurs. The transition from that complex to one with a *d-d* transition band near 550 nm is consistent with deprotonation of two amide nitrogens and formation of an ATCUN-like, square planar complex; EPR data support this conclusion (see below). At pH above 8.0, a blue-shift to a band near 500 nm is observed for Cu(II) complexed to **1C**, **1hC**, and **1M** (Figure S8-S10). Cu(II)-**1D** and Cu(II)-**1X** show similar high-pH shifts, while these shifts are not observed for Cu(II)-**1** or for any Cu(II) complexes of linear peptides that contain His at the third position [45]. This implies that these shifts are not due to sulfur oxidation, which might be expected at elevated pH, but are due to some other structural or electronic reason. At higher pH, it may be possible for coordination to exchange between the non-imidazole side chain and an amide from the macrocycle linker or the Leu outside of the macrocyclic ring. A similar switch between Cu(II) coordination by side chains and backbone amides was observed at alkaline pH in a cyclic peptide with multiple imidazoles, cyclo-(Gly-His-Gly-His-Gly-His-Gly) [52]. In a different example of the same phenomenon, a blue shift was observed for the non-ATCUN Cu(II)-GGGH complex at high pH due to the coordination of a third amide nitrogen to Cu(II) [60]. Taken together, all these data suggest that alternative ligand sets

become available at higher pH, but that strong coordination by the amino terminus and the side chain at the third position (the hallmarks of the ATCUN complex) can thermodynamically favor ATCUN-like complexes even at high pH [1, 31, 61, 62]. Since the blue shift is missing for **1**, minimal for **1X**, moderate for **1D**, and pronounced for the sulfur-containing peptides, this suggests that D-Pal and D-Asp behave as stronger anchoring groups within cyclic ATCUN peptides, but not as strong as D-His.

3.3. Stoichiometry of Cu(II) binding

The stoichiometries of the observed Cu(II)-peptide complexes were investigated using UV-vis spectroscopy and ESI-MS spectrometry. Stoichiometric titrations were performed at the lowest pH values for which maximum ATCUN-like binding was observed for each peptide-Cu(II) complex. For most peptides, ESI-MS revealed masses consistent with metal-binding complexes with 1:1 stoichiometry (see Table S1) and titrations using UV-vis spectroscopy confirmed saturation of the ATCUN-like band at 525-550 nm at 1:1 stoichiometry (see Figure S12). However, for sulfur-containing linear and cyclic peptides, the Cu(II)-binding stoichiometry was observed at less than 1:1 due to metal-catalyzed thiol oxidation; still, all UV-vis spectroscopy, mass spectrometry, and EPR spectroscopy data (see below) are consistent with the formation of 1:1 stoichiometries for these Cu(II)-peptide complexes prior to sulfur oxidation.

3.4. EPR Spectroscopy of Cu(II)-peptide complexes

EPR spectroscopy is a useful technique for studying Cu(II)-peptide complexes with various ligand donor sets in solution. EPR spectra of Cu(II)-complexes of linear and cyclic peptides at different pHs are shown in Figures 3 and 4, with measured EPR parameters summarized in Table 1. The EPR parameters obtained for all complexes follow the trend $g_{\parallel} > g_{\perp} > g_e$ suggesting that all Cu(II)-peptide complexes have a $d_{x^2-y^2}$ ground state. This is a characteristic feature of square planar (D_{4h}), square pyramidal (C_{4v}), or axially elongated tetragonal octahedral geometries with D_{4h} symmetry [63-65]. Thus, the $d-d$ transitions observed for these complexes is due to the Cu(II) $d_{x^2-y^2} \rightarrow d_{z^2}$ transition. The magnitude of $g_{\parallel}$ in four-coordinate Cu(II) complexes is dependent on the nature of the ligand atoms and the coordination geometry [66]. A detailed study by Peisach and Blumberg using a variety of ligand sets suggests that $g_{\parallel}$ decreases in the series $O_4 > N_2O_2 > N_3O > N_4 > N_2S_2 > S_4$ [67]. This reflects that the higher the covalency in the Cu(II)-ligand bond, the more the electron from $d_{x^2-y^2}$ delocalizes towards the ligand and thus the less it will interact with the Cu(II) nucleus. Also, for a given set of ligands, the $g_{\parallel}$ will increase with the degree of distortion from square planar towards tetragonal or tetrahedral geometry. According to these trends, we would expect the $g_{\parallel}$ value for the Asp-containing ligands to be highest, followed by His- and Pal-containing ligands, followed by Cys-, hCys- and Met-containing ligands. The $g_{\parallel}$ values obtained for the Cu(II) complexes of GGD L ($g_{\parallel} = 2.20$), GGXL ($g_{\parallel} = 2.19$) and GGHL ($g_{\parallel} = 2.18$) at pH 7.0 followed the Peisach and Blumberg trend (Figure 3 and Table 1). In agreement with the electronic absorption spectra, the low-temperature (123K) X-band EPR spectra of Cu(II)-peptide complexes of GGD L , GGXL, **1D**, and **1X** are all highly similar to their imidazole-containing analogs GGHL and **1** (Figure 3 and Table 1). As expected, $g_{\parallel}$ for Cu(II)-**1D** ($g_{\parallel} = 2.21$) at pH 8.5 is slightly higher than Cu(II)-**1** ($g_{\parallel} = 2.18$) and Cu(II)-**1X** ($g_{\parallel} = 2.19$). The EPR data suggest that cyclic ATCUN motifs with His and

Pal (**1** and **1X**) form square-planar Cu(II) complexes nearly identical to those formed by their linear analogs.

For sulfur-containing linear and cyclic peptides, we would expect lower $g_{\parallel}$ values compared to peptides containing His, Asp or Pal. However, higher $g_{\parallel}$ values were observed at pH 7.0 to 7.5, indicating that the complexes may be distorted from square-planar geometry, or that the thiol is oxidized, and/or that a coordination site is occupied by a water, hydroxide, or chloride.

As noted above, the electronic absorption spectra indicated a shift to a different species at elevated pH for Cu(II) complexes with **1D** and **1X**, but not with **1** or with linear analogs GGDL or GGXL. To investigate the nature of the blue-shifted species, we obtained EPR spectra for these Cu(II) complexes at pH 11.0. EPR spectra of Cu(II)-**1X** showed broadened peaks that were difficult to interpret, but EPR spectra of Cu(II)-**1D** showed two distinct signals in the $g_{\parallel}$ region (Figure S6 & S7). One signal had a $g_{\parallel} = 2.21$, and is the same as the ATCUN-like complex observed at lower pH. The second signal has a lower $g_{\parallel}$ of 2.18, and could indicate a square planar, N_4 donor set. The fourth nitrogen could arise from deprotonation of the Leu amide, linker amide, OH^- or possibly from an intermolecular interaction. We did not observe any hydrolysis or oxidation of these non-thiol-containing peptides at any pH (Figure S13).

Next, we examined EPR spectra of thiol-containing Cu(II)-peptide complexes, which showed multiple species during pH titrations. The EPR spectra of Cu(II)-peptide complexes of GGCL, **1C**, GG^hCL , **1hC**, GGML, and **1M** are overlaid in Figure 4. The EPR spectra of Cu(II) bound to each of these sulfur-containing linear and cyclic peptides are all similar except for Cu(II)-GGML, suggesting similar coordination environments. From the electronic absorption spectra, it was evident that thiols in GGCL and GG^hCL start to coordinate Cu(II) at pH 4.6, and are fully engaged in Cu(II) binding at pH 9.0. For these complexes, the $g_{\parallel}$ region of the EPR spectrum clearly shows a single species at pH 7.0 with $g_{\parallel} = 2.22$ and $A_{\parallel}$ between 179 and $182 \times 10^{-4} \text{ cm}^{-1}$. The ratio of $g_{\parallel}/A_{\parallel}$, denoted f , is a parameter that is empirically correlated with the coordination environment of Cu(II) complex, with the range of 105-120 cm for square planar, 130-150 cm for slight-to-moderate distortion from square planar, and 180-250 cm for a large distortion [66]. The f values obtained for Cu(II)-GGCL and Cu(II)- GG^hCL are 122-124 cm, which indicate that the geometry for these complexes is nearly square planar. The $g_{\parallel} = 2.21$ and $A_{\parallel} = 186 \times 10^{-4} \text{ cm}^{-1}$ values observed for Cu(II)-**1C** and Cu(II)-**1hC** at neutral pH are slightly different from the analogous linear peptide complexes. The corresponding value of $f = 119 \text{ cm}$ suggests that cyclization enforces a planar geometry for cysteine- and homocysteine-containing, ATCUN-like peptides.

When pH was raised to 8.5 or higher, an additional Cu(II) complex was observed by UV-vis spectroscopy (Table 1 and Figure S8-S10). Cu(II) and Ni(II) can catalyze sulfur oxidation in short peptides containing thiol or thioether groups, so we suspected these represented complexes between Cu(II) and oxidized peptides [68, 69]. The two broad EPR signals observed at higher pHs suggest that these oxidation products still form complexes with Cu(II), which would be expected for sulfoxo species or for disulfide-bridged dimers [70].

Analytical HPLC and ESI-MS analysis of Cu(II)-GGCL and Cu(II)-GG^hCL at pH 9.0 clearly showed species with oxygenated sulfurs (~40% sulfoxide and sulfone), with ~40% covalent dimers (Figure S13). All together, the spectroscopic and mass spectrometric data show that the thiol-containing linear and cyclic peptides form ATCUN-like complexes, but that Cu(II) can irreversibly oxidize the thiol, especially at pHs above 8.5. These findings validate the ability to switch out the imidazole for a thiol group within the cyclic scaffold, but will complicate future applications with thiol-containing cyclic peptides.

Next, we examined the EPR spectra for Cu(II)-GGML to better understand the several species observed in the electronic absorption spectra. At pH 6.0, there are at least two species of Cu(II)-GGML complex (Figures 4 and S4, Table 1). One species has EPR parameters that correlate well to values for Cu(II)-N₂O₂ complexes or highly distorted Cu(II)-NSO₂ complexes ($g_{\parallel} = 2.32$, $A_{\parallel} = 146 \times 10^{-4} \text{ cm}^{-1}$ and $f = 159$), and the other species has parameters more consistent with an ATCUN-like Cu(II)-N₃S complex ($g_{\parallel} = 2.22$, $A_{\parallel} = 197 \times 10^{-4} \text{ cm}^{-1}$, $f = 112$). These interconverting species have been previously observed for complexes of Cu(II) and methionine-containing peptides [71]. By pH 8.0 the ATCUN-like species dominates. Interestingly, the EPR spectra show that the Cu(II)-binding environment for **1M** at pH 7.5 is similar to the Cu(II)-binding environment of Cys- and ^hCys-containing linear and cyclic peptides, but not that of GGML (Figure 4). This indicates that the macrocyclic constraint pre-organizes the N₃S ligand set within **1M** into a square-planar geometry similar to those formed by thiol-containing peptides, a geometry which is not necessarily preferred by the linear analog.

3.5. UV-vis spectroscopy of complexes between Ni(II) and linear peptides

In a sharp contrast to Cu(II) binding, Ni(II) binding by the linear peptides GGCL, GG^hCL and GGML showed no evidence of multiple species at any pH. Binding behavior for all the linear peptides was consistent with a discrete, two-state transition from a Ni(II)-aqua complex to a species with a *d-d* transition band between 420 and 442 nm. As shown in Figure 5A, the pH profiles showed that Ni(II) binding is most favorable for the imidazole-containing ATCUN sequence GGHL, which has a pH₅₀ of 6.0 (50% complexation at pH 6.0). Binding of Ni(II) to GGCL, GG^hCL, and GGXL is also observed at relatively low pHs, with pH₅₀ values near 6.4 and complete binding by pH 7.2. pH₅₀ values were 7.2 for Ni(II)-GGDL and pH 8.3 for Ni(II)-GGML (Figure 5A, Table 2), and the electronic absorption bands observed for these complexes are very similar to values reported in the literature for analogous peptides [1, 28, 29, 59]. For all sulfur-containing Ni(II)-peptide complexes, electronic transition bands at 255 nm ($\epsilon = \sim 4300 \text{ M}^{-1}\text{cm}^{-1}$, $\text{N}^-, \text{S}_\sigma \rightarrow \text{Ni(II)}$) and two *d-d* transition bands at 420 to 440 nm ($d_{xy} \rightarrow d_{x^2-y^2}$) and 500 to 550 nm ($d_{zx}, d_{yz} \rightarrow d_{x^2-y^2}$) are observed (Table 2) [29]. The ligand-to-metal charge transfer band characteristic for $\text{S}_\pi \rightarrow \text{Ni(II)}$ is not very distinct for the linear peptides, probably due to the overlap with the $\text{O/N}^- \rightarrow \text{Ni(II)}$ transition. Finally, not only do all linear peptides form ATCUN-like complexes with Ni(II), but Ni(II) complexes with thiol-containing linear peptides are observed to be quite stable for hours at neutral pH, with no sulfur oxidation products as observed for Cu(II) complexes.

3.6. UV-vis spectroscopy of complexes between Ni(II) and cyclic peptides

The original, imidazole-containing cyclic peptide **1** formed an ATCUN-like Ni(II) complex with a pH_{50} of 8.8 [45]. This represents a somewhat destabilized form of the linear ATCUN complex formed by Ni(II)-GGHL, which has a pH_{50} of 5.8. Interestingly, while linear peptides GGD and GGXL show very similar Ni(II) binding properties to GGHL, cyclic peptides **1D** and **1X** do not bind Ni(II) up to pH 8.0, at which point $\text{Ni}(\text{OH})_2$ was observed to precipitate from solution. This demonstrates that the shape enforced by the macrocyclic linker can support square planar complexation of Ni(II) in the context of the imidazole ligand, but not for pyridyl or carboxyl ligands at the same position.

Not surprisingly, we observed stable complexation of Ni(II) with thiol-containing cyclic peptides, with pH_{50} values near 7.4 for Ni(II) binding to **1C** and **1hC**. This is a lower pH_{50} value than the Ni(II)-**1** complex (pH_{50} 8.8), implying greater complex stability (Figure 6A). In fact, complexation of Ni(II) to thiol-containing cyclic peptides is nearly as favorable as complexation to linear thiol-containing peptides, which have pH_{50} values of 6.4. Thus, we conclude that the cyclic architecture is compatible with high-affinity Ni(II) binding, particularly when the imidazole is switched out for a thiol group.

There are several additional interesting differences among the electronic absorption spectra of Ni(II) complexes with linear and cyclic thiol-containing peptides. Specifically, the spectra of Ni(II)-**1C** and Ni(II)-**1hC** have an extra band at 335 nm and higher overall extinction coefficients for all bands (Figure 6B, Table 2). Ni(II) complexes with thiolate ligands usually produce an intense absorption band in the 250-350 nm range. The band at 335 nm is thus attributed to a charge transfer transition from $\text{S}^- \pi \rightarrow \text{Ni}(\text{II})$ [29, 72]. The higher extinction coefficient of the band at 335 nm for Ni(II)-**1C** and Ni(II)-**1hC** may originate from one of two effects. First, more favorable ligand orientation and energy match between the sulfur π -orbitals and metal ion d -orbitals may lead to better orbital overlap and thus a higher extinction coefficient [73]. Second, the higher extinction coefficients of the 420-440 nm $d-d$ bands for Ni(II)-**1C** and Ni(II)-**1hC** suggest that the Ni(II)- N_3S plane is distorted from square planar, making the $d-d$ transition more favorable. The extinction coefficient for Ni(II)-GG^hCL is lowest, followed by Ni(II)-GGCL, then Ni(II)-**1hC**, then Ni(II)-**1C**. This is probably due to the ring strain created by 5,5,5-membered chelate rings in Ni(II)-GGCL and Ni(II)-**1C**, which would distort the planar geometry of Ni(II) center and result in higher extinction coefficients [74].

3.7. Stoichiometry of Ni binding

The stoichiometries of the observed Ni(II)-peptide complexes were investigated using UV-vis spectroscopy and ESI-MS spectrometry. Stoichiometric titrations were performed at the lowest pH values for which maximum ATCUN-like binding was observed for each peptide-Ni(II) complex. For all peptides, ESI-MS revealed masses consistent with metal-binding complexes with 1:1 stoichiometry (see Table S1) and titrations using UV-vis spectroscopy confirmed saturation of the ATCUN-like band at 425-450 nm at 1:1 stoichiometry (see Figure S12).

3.8. Cyclic voltammetry

We next examined how substitution of the imidazole group affects the redox properties of linear and cyclic ATCUN peptides. Unsurprisingly, cyclic voltammetry (CV) of thiol- and thioether- containing peptides was obscured due to the irreversible oxidation of sulfur atoms. Cyclic voltammograms for Cu(II)/Cu(III) and Ni(II)/Ni(III) couples for complexes of GGHL, GGDL, and GGXL are shown in Figure 7A-B and the electrochemical data are summarized in Table 3. All three linear metallopeptides undergo one-electron redox processes that are quasi-reversible, and the Cu(II)/Cu(III) couples for Cu(II)-GGHL and Cu(II)-GGDL are nearly reversible ($E = 0.070$ V and $I_p^{ox}/I_p^{red} = 1.07$ for Cu(II)-GGHL, $E = 0.064$ V and $I_p^{ox}/I_p^{red} = 0.954$ for Cu(II)-GGDL). Substitution of His to Asp reduces the redox potential of Cu(II)-GGDL ($E_{1/2} = 0.758$ V). This is consistent with the more electron-rich carboxylate coordinated to the metal center. This implies that these complexes are more easily oxidized to corresponding M(III) species, and thus may make more potent catalysts for M(III)-mediated transformations. By contrast, substitution of the imidazole with pyridine increases the redox potential of Cu(II)-GGXL ($E_{1/2} = 0.853$ V) and Ni(II)-GGXL ($E_{1/2} = 0.748$) relative to corresponding GGHL complexes. A similar increase in redox potential was observed when a His to Pal substitution was made in a heme protein [75, 76].

CV of Cu(II)-**1D** and Cu(II)-**1X** complexes showed similar trends as Cu(II) complexes with linear peptides. The substitution of D-His to D-Asp lowered the redox potential from 0.739 V for Cu(II)-**1** to 0.677 V for Cu(II)-**1D**, and the substitution of D-His to D-Pal raised the redox potential from 0.739 V for Cu(II)-**1** to 0.812 V for Cu(II)-**1X** (Figure 7C and Table 3). These data indicate that Asp-containing linear and cyclic peptides may have the best potential as catalysts for processes that involve a Cu(II)/Cu(III) or Ni(II)/Ni(III) redox cycle. Since the UV-vis spectra of Cu(II)-cyclic peptide complexes indicated the formation of alternate species at high pH, we measured the redox potential of Cu(II)-**1D** at pH 11.4. The lower redox potential of Cu(II)-**1D** at pH 11.4 ($E_{1/2} = 0.426$ V) indicates stronger donor ligands involved in Cu(II) coordination at this pH (Figure S16) [77]. A similar phenomenon is also observed for Cu(II)-**1X**.

Also, Cu(II)-**1X** was observed to have a particularly high E_p value, with anodic current (oxidation) higher than cathodic current (reduction). This can be attributed to one of two factors. Either the presence of a non-ATCUN, more poorly defined complex at this pH is causing slower electron transfer and a broadening of oxidation and reduction peaks, or the Cu(II)-**1X** complex must undergo a pronounced structural rearrangement between the two redox states.

3.9. Fluorescence assay for detection of hydroxyl radicals

In order to directly measure catalytic activity, we incubated metallopeptide complexes with 2',7'-dichlorofluorescein diacetate in the presence of hydrogen peroxide. This profluorescent dye reacts with hydroxyl radicals (but not peroxides or other radical oxygen species) to form 2',7'-dichlorofluorescein in solution [78]. This and similar dyes allow quantitative measurement of each complex's ability to produce hydroxyl radicals via a Fenton-like reaction [5, 45]. The assay was unable to detect hydroxyl radical formation by all peptides

tested in the absence of metal ion (Figure 8, white bars), demonstrating that the redox-active metal ion is required for producing hydroxyl radicals under these conditions. In adapting this assay for a range of linear and cyclic peptides, we found that background signal increased with increasing pH of solution. To control for these variations, all copper complexes were tested at pH 8.5 to ensure complete metal complexation for all peptides (see Figures 1 and 2). Nickel complexes were similarly tested at pH 9.2, at which all peptides are fully complexed, and also at pH 8.5, at which all peptides except for **1** were fully complexed (Figures 5 and 6). Peptides with sulfur-containing side chains are rapidly oxidized in the presence of H₂O₂, and were thus omitted from these assays.

In accordance with previous studies, Cu(II)-**1** produces diffusible hydroxyl radicals [45], though to a lesser extent than unliganded copper ions at pH 8.5. We were interested in the effects of substitution of the imidazole with a carboxylic acid or pyridyl group on catalytic activity for linear and cyclic peptides, and whether cyclization would consistently boost catalytic activity. At pH 8.5, Cu(II)-**1D** and Cu(II)-**1X** produced hydroxyl radicals to a greater extent than Cu(II)-**1**, with average intensities similar to that of unliganded copper (CuCl₂) (Figure 8). Linear analogs Cu(II)-GGDL and Cu(II)-GGXL showed no measurable formation of hydroxyl radicals, reinforcing that macrocyclization promotes Cu(II)/Cu(III) cycling. We conclude that copper complexes of cyclic ATCUN motifs containing carboxylate and pyridyl groups have potential as oxidation agents, and that this growing collection of copper-peptide complexes represents a potentially useful set of redox catalysts.

We then examined nickel-peptide complexes to evaluate their utility as redox catalysts. Nickel-peptide complexes were tested at pH 8.5 (Figure 8A) and pH 9.2 (Figure 8B). At both pHs, it is clear that Ni(II) alone has no activity above baseline and that Ni(II)-**1** has no activity as well. However, the other four complexes tested (Ni(II)-GGH, Ni(II)-GGHL, Ni(II)-GGDL, and Ni(II)-GGXL) all produce diffusible hydroxyl radicals. These data show that complexes of Ni(II) and linear ATCUN-like peptides also have potential as oxidation catalysts.

Interestingly, cyclization of the canonical, imidazole-containing ATCUN motif appears to enhance hydroxyl radical production under Fenton conditions for copper complexes, but diminishes hydroxyl radical production for nickel complexes. Also, based on $E_{1/2}$ values calculated from CV data, one might expect Cu(II)-**1D** to be the most redox-active catalyst, followed by Cu(II)-**1** and then Cu(II)-**1X**. Under Fenton conditions at pH 8.5, we observe Cu(II)-**1D** and Cu(II)-**1X** to be equally active at producing hydroxyl radicals, and Cu(II)-**1** to have less activity. The presence of very small amounts of unliganded Cu(II) at these conditions cannot be ruled out, since the Cu(II)-**1D** and Cu(II)-**1X** UV-vis spectra show some absorbance at 800 nm, the characteristic band for the Cu(II)-aqua complex (Figures S7 and S8). However, the potential amounts of unliganded copper do not account for the high extent of activity in the hydroxyl radical assay. Thus, we are currently investigating other structural and electronic causes of these observations.

4. Conclusion

Building on the recent discovery that macrocyclization can alter the properties and redox characteristics of ATCUN complexes, we sought to diversify the ligand sets within this cyclic architecture. Herein, we report several linear and cyclic analogs that replace the imidazole with carboxylate, pyridyl, thiol, and thioether groups. We found that all linear and cyclic peptides bind Cu(II) and/or Ni(II), and use data from electronic absorption spectroscopy and EPR spectroscopy to verify the formation of square planar, ATCUN-like complexes for each peptide. Overall, we found that cyclization promotes the formation of a square planar, ATCUN-like complex even in the absence of an imidazole group. Cyclic ATCUN peptides substituted with a carboxylate or pyridyl group can produce more hydroxyl radicals under Fenton conditions than the original imidazole-containing ATCUN macrocycle. We also find that linear ATCUN motifs containing a carboxylate or pyridyl group can catalyze the same chemistry using nickel instead of copper. This growing collection of linear and cyclic ATCUN motifs represents a novel source for redox catalysts which, due to their chirality and modularity, have great potential for enantioselective or regioselective transformations.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by the National Institutes of Health, award number R21AG038776. We thank Prof. Samuel Kounaves, Dr. Glen O'Neil and Andrew Weber for access and training on the cyclic voltammeter.

Abbreviations

ATCUN	Amino terminal Cu(II) and Ni(II) binding
GGHL	NH ₂ -Gly-Gly-His-Leu-CONH ₂
GGDL	NH ₂ -Gly-Gly-Asp-Leu-CONH ₂
GGXL	NH ₂ -Gly-Gly-Pal-Leu-CONH ₂
GGCL	NH ₂ -Gly-Gly-Cys-Leu-CONH ₂
GG^hCL	NH ₂ -Gly-Gly- ^h Cys-Leu-CONH ₂
GGML	NH ₂ -Gly-Gly-Cys-Leu-CONH ₂
1	<i>cyclic</i> [Lys-Asp(Leu)-DHis]
1D	<i>cyclic</i> [Lys-Asp(Leu)-DAsp]
1X	<i>cyclic</i> [Lys-Asp(Leu)-DPal]
1C	<i>cyclic</i> [Lys-Asp(Leu)-DCys]
1hC	<i>cyclic</i> [Lys-Asp(Leu)-D ^h Cys]
1M	<i>cyclic</i> [Lys-Asp(Leu)-DMet]

PyBop	Benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate
HOBt	N-Hydroxybenzotriazole
DIPEA	N,N-Diisopropylethylamine
TFA	Trifluoroacetic acid

References

- [1]. Harford C, Sarkar B. *Accounts of Chemical Research*. 1997; 30:123–130.
- [2]. Mack DP, Dervan PB. *J. Am. Chem. Soc.* 1990; 112:4604–4606.
- [3]. Mack DP, Dervan PB. *Biochemistry*. 1992; 31:9399–9405. [PubMed: 1390723]
- [4]. Jin Y, Cowan JA. *Journal of the American Chemical Society*. 2005; 127:8408–8415. [PubMed: 15941274]
- [5]. Joyner JC, Reichfield J, Cowan JA. *J. Am. Chem. Soc.* 2011; 133:15613–15626. [PubMed: 21815680]
- [6]. Cuenoud B, Tarasow TM, Schepartz A. *Tetrahedron Lett.* 1992; 33:895–898.
- [7]. Shullenberger DF, Eason PD, Long EC. *J. Am. Chem. Soc.* 1993; 115:11038–11039.
- [8]. Liang Q, Ananias DC, Long EC. *J. Am. Chem. Soc.* 1998; 120:248–257.
- [9]. Burrows CJ, Perez RJ, Muller JG, Rokita SE. *Pure Appl. Chem.* 1998; 70:275–278.
- [10]. Singh RK, Sharma NK, Prasad R, Singh UP. *Protein and Peptide Lett.* 2008; 15:13–19.
- [11]. Jin Y, Lewis MA, Gokhale NH, Long EC, Cowan JA. *J. Am. Chem. Soc.* 2007; 129:8353–8361. [PubMed: 17552522]
- [12]. Fang YY, Ray BD, Claussen CA, Lipkowitz KB, Long EC. *J. Am. Chem. Soc.* 2004; 126:5403–5412. [PubMed: 15113212]
- [13]. Huang XF, Pieczko ME, Long EC. *Biochemistry*. 1999; 38:2160–2166. [PubMed: 10026300]
- [14]. Brown KC, Yang SH, Kodadek T. *Biochemistry*. 1995; 34:4733–4739. [PubMed: 7718579]
- [15]. Brown KC, Yu Z, Burlingame AL, Craik CS. *Biochemistry*. 1998; 37:4397–4406. [PubMed: 9521759]
- [16]. Horowitz ED, Finn MG, Asokan A. *Acs Chem. Biol.* 2012; 7:1059–1066. [PubMed: 22458529]
- [17]. Gokhale NH, Cowan JA. *Chem. Comm.* 2005:5916–5918. [PubMed: 16317474]
- [18]. Gokhale NH, Bradford S, Cowan JA. *J. Am. Chem. Soc.* 2008; 130:2388–2389. [PubMed: 18251475]
- [19]. Bradford S, Cowan JA. *Chem. Comm.* 2012; 48:3118–3120. [PubMed: 22343977]
- [20]. Kimoto E, Tanaka H, Gytoku J, Morishige F, Pauling L. *Cancer Res.* 1983; 43:824–828. [PubMed: 6293704]
- [21]. Joyner JC, Cowan JA. *Braz. J. Med. Biol. Res.* 2013; 46:465–485. [PubMed: 23828584]
- [22]. Sovago I, Osz K. *Dalton Trans.* 2006:3841–3854. [PubMed: 16896443]
- [23]. Hureau C, Eury H, Guillot R, Bijani C, Sayen S, Solari P-L, Guillon E, Faller P, Dorlet P. *Eur. J. Chem.* 2011; 17:10151–10160.
- [24]. Agarwal RP, Perrin DD. *Dalton Trans.* 1975:268–272.
- [25]. Farkas E, Sovago I, Kiss T, Gergely A. *Dalton Trans.* 1984:611–614.
- [26]. Sovago I, Bertalan C, Gobi L, Schon I, Nyeki O. *J. Inorg. Biochem.* 1994; 55:67–75. [PubMed: 8046437]
- [27]. Sovago I, Kiss T, Gergely A. *Inorg. Chim. Acta.* 1984; 93:L53–L55.
- [28]. Cherifi K, Reverend BD, Varnagy K, Kiss T, Sovago I, Loucheux C, Kozlowski H. *J. Inorg. Biochem.* 1990; 38:69–80. [PubMed: 2332765]
- [29]. Kozlowski H, Révérend BD, Ficheux D, Loucheux C, Sovago I. *J. Inorg. Biochem.* 1987; 29:187–197.

- [30]. Wmagy K, Boka B, Sovago I, Sanna D, Marras P, Micera G. *Inorg. Chim. Acta*. 1998; 275-276:440–446.
- [31]. Kozlowski H, Bal W, Dyba M, Kowalik-Jankowska T. *Coord. Chem. Rev.* 1999; 184:319–346.
- [32]. Pratesi A, Zanella P, Giorgi G, Messori L, Laschi F, Casini A, Corsini M, Gabbiani C, Orfei M, Rosani C, Ginanneschi M. *Inorg. Chem.* 2007; 46:10038–10040. [PubMed: 17979273]
- [33]. Burke SK, Xu Y, Margerum DW. *Inorg. Chem.* 2003; 42:5807–5817. [PubMed: 12971748]
- [34]. Brasun J, Gabbiani C, Ginanneschi M, Messori L, Orfei M, Swiatek-Kozłowska J. *J. Inorg. Biochem.* 2004; 98:2016–2021. [PubMed: 15541490]
- [35]. Brasun J, Matera A, Oldziej S, Swiatek-Kozłowska J, Messori L, Gabbiani C, Orfei M, Ginanneschi M. *Journal of Inorganic Biochemistry*. 2007; 101:452–460. [PubMed: 17215044]
- [36]. Brasun J, Matera-Witkiewicz A, Kamysz E, Kamysz W, Oldziej S. *Polyhedron*. 2010; 29:1535–1542.
- [37]. Brasun J, Matera-Witkiewicz A, Oldziej S, Pratesi A, Ginanneschi M, Messori L. *J. Inorg. Biochem.* 2009; 103:813–817. [PubMed: 19329186]
- [38]. Aime S, Batsanov AS, Botta M, Dickins RS, Faulkner S, Foster CE, Harrison A, Howard JAK, Moloney JM, Norman TJ, Parker D, Royle L, Williams JAG. *Dalton Trans.* 1997:3623–3636.
- [39]. Aime S, Botta M, Fasano M, Terreno E. *Chemical Society Reviews*. 1998; 27:19–29.
- [40]. Cram DJ, Cram JM. *Science*. 1974; 183:803–809. [PubMed: 17780761]
- [41]. van Veggel FCJM, Verboom W, Reinhoudt DN. *Chemical Reviews*. 1994; 94:279–299.
- [42]. Galaup C, Carrie MC, Azema J, Picard C. *Tetrahedron Letters*. 1998; 39:1573–1576.
- [43]. Azema J, Galaup C, Picard C, Tisnes P, Ramos P, Juanes O, Rodriguez-Ubis JC, Brunet E. *Tetrahedron*. 2000; 56:2673–2681.
- [44]. Driggers EM, Hale SP, Lee J, Terrett NK. *Nature Rev. Drug Discov.* 2008; 7:608–624. [PubMed: 18591981]
- [45]. Neupane KP, Aldous AR, Kritzer JA. *Inorg. Chem.* 2013; 52:2729–2735. [PubMed: 23421754]
- [46]. Chan, WC.; White, PD. *Fmoc solid phase peptide synthesis: A practical approach*. Oxford University Press; USA:
- [47]. Isidro-Llobet A, Alvarez M, Albericio F. *Chem. Rev.* 2009; 109:2455–2504. [PubMed: 19364121]
- [48]. Ellman GL. *Arch. Biochem. Biophys.* 1958; 74:443–450. [PubMed: 13534673]
- [49]. Bulaj G, Kortemme T, Goldenberg DP. *Biochemistry*. 1998; 37:8965–8972. [PubMed: 9636038]
- [50]. Trapaidze A, Hureau C, Bal W, Winterhalter M, Faller P. *J. Biol. Inorg. Chem.* 2012; 17:37–47. [PubMed: 21898044]
- [51]. Irving H, Williams RJP. *Nature*. 1948; 162:746–747.
- [52]. Laussac JP, Robert A, Haran R, Sarkar B. *Inorg. Chem.* 1986; 25:2760–2765.
- [53]. Gajda T, Henry B, Aubry A, Delpuech J-J. *Inorg. Chem.* 1996; 35:586–593.
- [54]. Tesfai TM, Green BJ, Margerum DW. *Inorganic Chemistry*. 2004; 43:6726–6733. [PubMed: 15476372]
- [55]. Agarwal RP, Perrin DD. *Dalton Trans.* 1977:53–57.
- [56]. Pandiyan T, Mariappan M, Palaniandavar M. *Transition Met. Chem.* 1995; 20:440–444.
- [57]. Stibrany RT, Fikar R, Brader M, Potenza MN, Potenza JA, S. HJ. *Inorg. Chem.* 2002; 41:5203–5215. [PubMed: 12354054]
- [58]. Sigel H, Martin RB. *Chem. Rev.* 1982; 82:385–426.
- [59]. Varnagy K, Boka B, Sovago I, Sanna D, Marras P, Micera G. *Inorg. Chim. Acta*. 1998; 275-276:440–446.
- [60]. Varnagy K, Szabo J, Sovago I, Malandrinos G, Hadjiliadis N, Sanna D, Micera G. *Dalton Trans.* 2000:467–472.
- [61]. Hanaki A, Odani A. *J. Inorg. Biochem.* 2007; 101:1428–1437. [PubMed: 17643491]
- [62]. Bal W, Jezowska-Bojczuk M, Kasprzak KS. *Chem. Res. Toxicol.* 1997; 10:906–914. [PubMed: 9282840]
- [63]. Hathaway BJ, Billing DE. *Coord. Chem. Rev.* 1970; 5:143–207.

- [64]. Hathaway BJ, Tomlinson AG. *Coord. Chem. Rev.* 1970; 5:1–43.
- [65]. Lucchese B, Humphreys KJ, Lee D-H, Incarvito CD, Sommer RD, Rheingold AL, Karlin KD. *Inorganic Chemistry*. 2004; 43:5987–5998. [PubMed: 15360248]
- [66]. Addison, AW. Spectroscopic and redox trends from model copper complexes. In: Karlin, KD.; Zubieta, J., editors. *Copper Coordination Chemistry: Biochemical and Inorganic Perspectives*. 1983.
- [67]. Peisach J, Blumberg WE. *Archives of Biochemistry and Biophysics*. 1974; 165:691–708. [PubMed: 4374138]
- [68]. Cavallini D, Marco CD, Dupre S, Rotilio G. *Arch. Biochem. Biophys.* 1969; 130:354–361. [PubMed: 4305160]
- [69]. Ross SA, Burrows CJ. *Inorg. Chem.* 1998; 37:5358–5363.
- [70]. Lee Y, Lee D-H, Narducci-Sarjeant AA, Zakharov LN, Rheingold AL, Karlin KD. *Inorg. Chem.* 2006; 45:10098–10107. [PubMed: 17140215]
- [71]. Rotilio G, Calabrese L. *Arch. Biochem. Biophys.* 1971; 143:218–225. [PubMed: 4327237]
- [72]. Jenkins RM, Singleton ML, Almaraz E, Reibenspies JH, Darensbourg MY. *Inorg. Chem.* 2009; 48:7280–7293. [PubMed: 19572492]
- [73]. Brines LM, Shearer J, Fender JK, Schweitzer D, Shoner SC, Barnhart D, Kaminsky W, Lovell S, Kovacs JA. *Inorg. Chem.* 2007; 46:9267–9277. [PubMed: 17867686]
- [74]. Sanna D, Agoston CG, Micera G, Sovago I. *Polyhedron*. 2001; 20:3079–3090.
- [75]. Lu Y, Yeung N, Sieracki N, Marshall NM. *Nature*. 2009; 460:855–862. [PubMed: 19675646]
- [76]. Privett HK, Reedy CJ, Kennedy ML, Gibney BR. *J. Am. Chem. Soc.* 2002; 124:6828–6829. [PubMed: 12059195]
- [77]. Neupane KP, Shearer J. *Inorg. Chem.* 2006; 45:10552–10566. [PubMed: 17173410]
- [78]. Myhre O, Andersen JM, Aarnes H, Fonnum F. *Biochem. Pharmacol.* 2003; 65:1575–1582. [PubMed: 12754093]

Highlights

- Twelve different linear and cyclic peptides synthesized and complexed to Cu(II) and Ni(II)
- Spectroscopic and redox characterization of metal-peptide complexes
- Structural and electronic trends observed for Ni(II) and Cu(II) binding for linear and cyclic ATCUN motifs
- Enhanced redox activity under Fenton conditions for several linear and cyclic metallopeptides

Figure 1.

UV-vis spectroscopy of Cu(II) binding to linear peptides. (A) Percentage formation of Cu(II)-peptide complexes versus pH. (B) UV-vis spectra of Cu(II) complexes of linear peptides GGHL, GGD, and GGXL at pH 6.5. Inset shows a magnification of the *d-d* transition bands near 550 nm. (C) UV-vis spectra at selected pHs for GGCL. (D) UV-vis spectra at selected pHs for GG^hCL. (E) UV-vis spectra at selected pHs for GGML. For (C-E), the insets show a magnification of the *d-d* transition bands between 550 and 800 nm. (F) pH dependences of the wavelength of maximal intensity (λ_{max}) for sulfur-containing

peptides, demonstrating the presence of multiple metal-peptide complexes at intermediate pHs.

Figure 2.

UV-vis spectroscopy of Cu(II) binding to cyclic peptides. Plots show full spectra at selected pHs for **1** (A), **1D** (B), **1X** (C), **1C** (D), **1^hC** (E), and **1M** (F). Insets show the pH dependences of the wavelength of maximal intensity (λ_{max}) for Cu(II) complexes with each peptide, demonstrating the presence of multiple metal-peptide complexes at intermediate pHs. For additional spectra at a wide range of pH values, see Figures S5-S10 in the supporting information.

Figure 3.

EPR spectra of Cu(II) complexes with linear and cyclic peptides containing D-His, D-Asp or D-Pal. All spectra were recorded with 0.9 mM Cu(II)-peptide complex at 123K in water with 10% glycerol.

Figure 4.

EPR spectra of Cu(II) complexes with linear and cyclic peptides containing Cys, ^hCys or Met. The EPR spectra for Cu(II)-GGHL and Cu(II)-1 are shown for comparison. All spectra were recorded using 0.9 mM Cu(II)-peptide complex at 123K in water with 10% glycerol.

Figure 5.

pH dependence of Ni(II) binding to linear peptides (A) and UV-vis spectra of Ni(II)-linear peptide complexes (B). Percentage formation was calculated using absorption at 530 nm for GGHL, 545 nm for GDDL, 535 nm for GGXL, 517 nm for GGCL, 515 nm for GG^hCL, and 545 nm for GGML. Additional full spectra at different pHs are shown in Figure S14.

Figure 6.

pH dependence of Ni(II) binding to cyclic peptides **1**, **1C** and **1^hC** (A). UV-vis spectra of Ni(II) complexes with cyclic peptides **1** (black trace, pH 9.5), **1C** (light gray trace, pH 9.0) and **1^hC** (gray trace, pH 8.4) (B). Percentage formation was calculated using absorption at 430 nm for Ni(II)-**1**, 420 nm for Ni(II)-**1C** and 435 nm for Ni(II)-**1^hC**. A distinct charge transfer band at 335 nm was seen for Ni(II) complexes of **1C** and **1^hC** which was not observed for Ni(II)-GGCL or Ni(II)-GG^hCL. Additional full spectra at different pHs are shown in Figure S15.

Figure 7.

Cyclic voltammetry of Cu(II) and Ni(II) complexes. (A) Cu(II)-linear peptide complexes, (B) Ni(II)-linear peptide complexes, (C) Cu(II)-cyclic peptide complexes. The voltammograms were recorded using 1.0 mM peptide-metal complex in 200 mM KCl with a scan rate of 100 mV/s at pH 7.5 for Cu(II)-linear peptide complexes, pH 8.0 for Ni(II)-linear peptide complexes, and pH 8.5 for Cu(II)-cyclic peptide complexes. Each trace is average of 3 scans and is background-subtracted.

Figure 8.

(A) Production of diffusible hydroxyl radicals by unliganded metal ions, *apo*-peptides, Cu(II)-peptide complexes, and Ni(II)-peptide complexes at pH 8.5. Fluorescence arises from the reaction of diffusible hydroxyl radicals at 25 °C with 10 μ M 2',7'-dichlorofluorescein diacetate in 20 mM HEPES, 100 mM NaCl, and 0.9mM H₂O₂. (B) Experiments identical to (A) were performed at pH 9.2 with NiCl₂, *apo*-peptides and Ni(II)-peptide complexes. Metal-free assays are shown as white bars, copper-containing assays are shown as light gray bars, and nickel-containing assays are shown as dark gray bars. Error bars show standard deviations from 3 independent trials. Metal salts and metal-peptide complexes were each tested at 0.9 μ M, with *apo*-peptides tested at 1.0 μ M. **At pH 8.5, the Ni(II)-1 complex accounts for only ~10% of the Ni(II) ions present (Figure 6A).

Scheme 1.

Structures of linear and cyclic ATCUN peptides. Linear peptides used in this study include GGHL, GGD^L, GGXL, GGCL, GG^hCL, and GGML, where X represents 2-pyridylalanine and ^hC 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^hC** with D-^hCys, 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.

Table 1

EPR data of Cu(II)-linear and Cu(II)-cyclic peptide complexes. Multiple entries indicate the presence of multiple sets of peaks.

Cu(II)-Peptide	pH	g	A x10 ⁻⁴ (cm ⁻¹)	f(g /A) (cm)
GGHL	7.0	2.18	202	108
1	7.5	2.18	204	107
GGDL	7.0	2.20	204	108
1D	8.5	2.21	193	115
	11.0	2.21	194	114
		2.18	198	110
GGXL	7.0	2.19	204	107
1X	8.5	2.19	197	112
	11.0	2.18	204	107
	7.0	2.22	179	124
GGCL	8.5	2.22	179	124
		2.16	204	106
	12.0	2.22	204	124
		2.16	204	106
1C	7.0	2.21	186	119
	12.0	2.24	195	115
		2.21	186	119
		2.18	194	112
GG ^b CL	7.0	2.22	182	122
	9.5	2.22	182	122
		2.15	191	113
	12.0	2.15	191	112
1^hC	7.0	2.21	186	119
	12.0	2.24	196	115
		2.21	186	119
		2.17	201	108
GGML	6.0	2.32	146	159
		2.22	197	113
	8.0	2.32	146	159

Cu(II)-Peptide	pH	g	A x10 ⁻⁴ (cm ⁻¹)	f (g /A) (cm)
1M		2.17	201	108
	7.5	2.20	188	117
	11.0	2.21	204	108
		2.20	188	117
		2.18	196	112

Table 2

UV-vis data for Cu(II) and Ni(II) binding to linear and cyclic peptides

Name	Sequence (Linear peptides)	Cu(II) binding		Ni(II) binding	
		pH ₅₀	$\lambda_{\max}$ (ϵ , M ⁻¹ cm ⁻¹)	pH ₅₀	$\lambda_{\max}$ (ϵ , M ⁻¹ cm ⁻¹)
GGHL	Gly–Gly–His–Leu	4.5	530 (95)	6.0	425 (122)
GGDL	Gly–Gly–Asp–Leu	5.5	545 (130)	7.2	442 (220)
GGXL	Gly–Gly–Pal–Leu	4.5	535 (100)	6.4	427 (150)
GGCL	Gly–Gly–Cys–Leu	6.5	517 (200)	6.4	427 (290)
GG ^h CL	Gly–Gly– ^h Cys–Leu	7.5	515 (150)	6.4	440 (115)
GGML	Gly–Gly–Met–Leu	6.2	545 (120)	8.3	420 (175)

Name	Sequence (Cyclic peptides)	Cu(II) binding		Ni(II) binding	
		pH ₅₀	$\lambda_{\max}$ (ϵ , M ⁻¹ cm ⁻¹)	pH ₅₀	$\lambda_{\max}$ (ϵ , M ⁻¹ cm ⁻¹)
1	Lys–Asp(Leu)–D–His	6.0	525 (98)	8.8	430 (98)
1D	Lys–Asp(Leu)–D–Asp	6.6*	570 (84)	–	–
1X	Lys–Asp(Leu)–D–Pal	6.4*	555 (87)	–	–
1C	Lys–Asp(Leu)–D–Cys	6.3*	590 (47)	7.4	420 (875)
1^hC	Lys–Asp(Leu)–D– ^h Cys	6.3*	590 (47)	7.4	435 (490)
1M	Lys–Asp(Leu)–D–Met	6.5*	570 (62)	–	–

* Indicates there are at least two species present at this pH.

Table 3

Electrochemical data for Cu(II)- and Ni(II)-peptide complexes.

Peptides	Cu(II)-Peptides					Ni(II)-Peptides				
	E_p^{ox} (V)	E_p^{red} (V)	E_p (V)	I_p^{ox}/I_p^{red}	$E_{1/2}$ (V)	E_p^{ox} (V)	E_p^{red} (V)	E_p (V)	I_p^{ox}/I_p^{red}	$E_{1/2}$ (V)
GGHL	0.830	0.760	0.070	1.07	0.795	0.761	0.678	0.083	1.19	0.719
I	0.778	0.704	0.078	1.56	0.739	0.888	irrev	–	–	–
GGDL	0.790	0.726	0.064	0.954	0.758	0.689	0.605	0.084	1.21	0.647
ID	0.712	0.641	0.071	1.55	0.677	–	–	–	–	–
GGXL	0.889	0.818	0.071	1.24	0.853	0.792	0.705	0.087	1.24	0.748
IX	0.875	0.765	0.110	18.2	0.820	–	–	–	–	–

Note: **ID** and **IX** do not bind Ni(II) up to pH 8.0, above which Ni(OH)₂ precipitate was observed .